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The role of temperature variation on macrophage physiology and function in vitro in mice, chicken, and fish

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The role of temperature variation on macrophage physiology and function *in vitro* in mice, chicken, and fish

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Abstract

Fever is an essential component of the immune response. Baseline body temperatures vary in different species, and fever represents increased temperature over baseline. Fever initiates and enhances immune responses as well as creates an environment in which the body has advantages over pathogens. Macrophages are often the first line of cells that come in contact with pathogens, as

they reside in and traffic through tissues. They are important for their engulfment of pathogens that results in the digestion of the pathogen, and they also produce nitric oxide and cytokines that contribute to immune responses in a variety of ways, including initiating adaptive immunity and directing the production and activity of other immune cells. On this context, we have studied the role of temperature variation on macrophage physiology and function *in vitro* in mice, chickens and fish. During exposure to a variety of temperatures and stimuli and immunomodulators such as lipopolysaccharide (LPS), poly I:C, and peptidoglycan, the metabolism and activity was measured, in both macrophage cell lines and primary cells, at various times using protein synthesis, production of nitric oxide, and cytokine mRNA production.

In mice, with a macrophage cell line, RAW264.7, we find that the incubation at fever temperature of 39°C induced a much faster reprogramming from mitochondria-based oxidative phosphorylation to aerobic glycolysis in response to macrophage stimulation. High temperature also increased protein synthesis in general and specifically iNOS and its product NO, IL-6, IL-1α, IL-1β, IFNα, IFNβ and IL-12, but not TNFα or IL34, in LPS-stimulated RAW264.7 cells. Testing primary macrophages from both young and aged mice, we find that peritoneal resident

macrophages show similar outcomes with fever temperature. Cells from young mice have larger enhancement in protein synthesis and IL-34 responses than cells from aged mice. For many responses, baseline levels are already very high in cells from aged mice. In mouse bone marrow-derived macrophage (BMDM) cultures, we find that increasing the temperature shows some increases in both iNOS mRNA and NO production. Again, the responses are greater in cells from aged mice, males and females, than from young mice. While protein synthesis is enhanced by fever temperature for stimulated BMDMs from both young and aged mice, there was no temperature effect on MHC class II expression levels.

Based on the tests of the effect of fever temperature ranges for 3 species -mice, chicken, and rainbow trout, - via NO production by stimulated macrophages, we found increases of 2 degrees for mice and 1 degree for chickens was optimal, while fish showed increasing responses over a 7-degree range. Above these temperatures, cells from all three species showed substantial drops in responses. In chickens, primary splenic cells as well as the chicken macrophage cell line HTC showed increases in NO production at 42°C than 41°C. Interestingly, substantial increases in protein synthesis were also observed at 42°C alone, with further increases with stimulation with LPS.

Both mice and chickens are homeotherms thus, and it was interesting to examine the fever responses of macrophages from a poikilothermic species. The RTS11 cell line derived from the spleen of rainbow trout, and primary cells from rainbow trout head kidney were utilized here for studies on the influence of varying temperature on fish macrophage functions. Even without stimulation, cells from both sources showed increases from 16°C to 19°C in baseline nitric oxide

production and protein synthesis. Cytokine mRNA production responses peaked at later times in fish cells than in mouse or chicken cells. TNFα showed some increase with LPS and temperature, IL-1B1 showed highest production at 19°C with all stimuli, Il-1B2 only increased with LPS and high temperature, IL-12 showed a strong temperature effect alone and with polI:C at 2h stimulation.

Collectively, our results indicate that initiating fever temperatures has major positive effects on macrophage function increasing both speed and magnitude of the responses. However, this thermomodulation of macrophage responses is wired differently in different species in terms of the actual temperature levels detected. Further, macrophages from a poikilothermic species show a relatively more variable responses to temperature-mediated functional modulation than cells from homeothermic species do. Our data make future work on the molecular evolution of the regulatory circuits involved of great interest.

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ABBREVIATIONS

BMDM Bone marrow derived macrophage
BV421 Brilliant violate 421 color flow cytometric
$CD8^+$ T cell CD8-positive T cells
DMSO Dimethyl sulfoxide
EDTA ethylenediaminetetraacetic acid
ELISA= Enzyme-linked immunosorbent assay
HPG Homopropargylglycine
IFN-α Interferon-alpha
IFN-γ Interferon-gamma
IL-12 Interleukin-12
IL-1α interleukin 1 alpha
IL-1β interleukin-1 beta
IL-6 interleukin-6
iNOS Inducible Nitric oxide synthase
LPS lipopolysaccharide
M-CSF macrophage colony-stimulating factor
MHC II Major histocompatibility complex class II
mRNA messenger ribonucleic acid
NF-κB nuclear factor-kappa B
NK cell natural killer cell
NK Natural killer cell
NO Nitric Oxide
OCR Oxygen consumption rate
PBS Phosphate buffered saline
PER Proton efflux rate
PGE2 prostaglandin E2
PGN Peptidoglycan
Poly I:C Polyinosinic: polycytidylic acid
PRC Peritoneal resident macrophage
PRR pattern recognition receptor
ROS reactive oxygen species
RT-qPCR Real time Quantitative polymerase chain reaction
STAT signal transducers and activators of transcription
TLR Toll like receptor
TLR4 Toll like receptor 4
TNF tumor necrosis factor

TABLE OF CONTENTS

LIST OF FIUGERS

LIST OF TABLES

Introduction:

Fever is generated by the release of pyrogenic cytokines IL-1, IL-6, TNFα, and IFNγ. These cytokines move via the bloodstream and enter the brain. Interaction is via specific receptors in the hypothalamus which increases synthesis of prostaglandin E2 (PGE2), which alters the firing rates of thermoregulatory neurons, resulting in an increased thermal set-point (Roth & Blatteis)and de Souza, G. E. P. 2002). This in turn regulates both the generation of heat through shivering of muscles and a decrease in heat loss through constriction of blood vessels. Fever has a role in activating both (de Souza et al., 2002) the innate and the adaptive immune system. Fever stimulates both innate and adaptive immune responses and is known to be beneficial for the following reasons: bacterial and viral pathogens replicate less efficiently, adaptive immunity is more potent, and human cells are more resistant to the toxic effects of cytokines at higher temperatures. Fevers also induce lethargy to conserve energy, allowing the body to invest a greater amount of energy in immune responses. However, the use of antipyretic therapy, aggressively attempting to regulate febrile temperatures, is commonly used in medicine today despite the known benefits of increased temperature in an immune response (Schulman et al.) 2005). In a study conducted in an intensive care unit, patients receiving antipyretic therapy had a higher mortality rate than patients whose febrile temperatures were only treated when they rose above 40°C (Schulman, et al.2005). Clearly it is relevant and very important to the advancement of modern medicine that we better understand the role and mechanisms of fever in immune responses. Some form of fever has been highly conserved by natural selection among cold blooded and warm-blooded vertebrates for over 600 million years of evolution (Evans, et al). Fever temperatures thus clearly provide a significant survival advantage to organisms. There have been some changes recently in what is considered normal core body temperature for

humans and for chickens and what is observed as fever for both with increasing age. This is summarized in the table below:

Table 1. The changing notions of normal and fever temperature in humans, chickens, and rainbow trout.

animal (subject)	Normal temperature (°C)	Fever temperarure (°C)
Human (accepted) Human (current, (Protsiv, Ley, Lankester, Hastie, & Parsonnet, 2020) Human (with aging, (Protsiv et al., 2020) Age 20- 40 40-60 60-80 Male [36.7] [36.6] [36.5] Female [36.8] [36.7] [36.6]	37 36.2 −0.003°C males and −0.0043°C females per year of age	39 and above
Chicken (accepted) UK college of Agriculture Food and environment https://afs.ca.uky.edu/poultry/chapter-7-ventilation-principles	40.6 - 41.7	42. and above
Chicken (22 week old, (Meyer M Fau - Bobeck, Bobeck E Fau - Sato, Sato, & El-Gazzar)	41.6 ±0.55	42.2± 1.6
Chicken(96 week old (Meyer M Fau - Bobeck et al.)	41.3±0.5	41.1 – 41.6 ±1.5
Rainbow trout (stream conditions) North America Journal of Fisheries management in 2010	4 - 20 * *Metabolisms stops at 2	19

In humans, the aged no longer mount fever responses to many

observations in chickens that also refine our idea of normal core and fever responses. Old laying hens did not mount the same level of fever during the same infection as the young lying hens [(Meyer M, 2021) Fever in birds is over an even narrower temperature range than in mice and humans. There are finely tuned distinctions between normal and. fever in birds.

While mice and humans have similar normal body temperatures, it is also useful to think about how macrophages function at different fever temperatures. Two economically important models, which are in use in UARK research groups, chickens, and rainbow trout fish, were thus obvious choices. Fish live wider temperature range and have tolerance to environment that poikilotherms live in.

Inflammation is energetically costly ((Fei, Lee, McCarry, & Bowdish), and each 1° increase in temperature needs a 10-12.5% increase in metabolic rate. This increase is about equivalent of going from sleep state to waking state energy use levels. Fever is often blunted in elderly. (Norman & Yoshikawa), thus, appearing interesting to examine and compare the responses of macrophages from young versus aged animals.

For practical reasons, experiments were begun with NO assays using the Griess reagent, and with cell lines, moving on to primary cells from different sources to assure our ability to do the basic experimental manipulations and run optimizations for kinetics of responses and concentration of

stimuli and in handle cells without impairing or killing them, and then to use mRNA quantification for stimulation-mediated gene expression in these macrophages.

While similar cytokines and signaling molecules are used, ectotherms (cold blooded vertebrates) employ a different method of fever regulation than endotherms. Ectotherms including reptiles, fish, and insects raise their core temperature during infection through behavioral regulation, in which animals seek warmer environments when they are ill despite the risk of predation (Covert & Reynolds, 1977) Behavioral regulation has been shown to be very effective in ectothermic immune responses. Previous studies of desert iguanas showed that survival was reduced by 75% when the iguana was prevented from seeking warmer environments (Evans, et al). Interestingly, antipyretic drugs prevented the desert iguana from participating in this behavior (Evans, et al). Therefore, it is likely that similar biochemical pathways are involved in fever production and regulation in endotherms and ectotherms. Fish have not yet been extensively tested, but it is known that fish like many other ectotherms choose to go to warmer areas of their environments when they have infections (Covert and Reynolds). Initial research with fish showed that fish that maintained a higher febrile temperature had a higher survival rate when injected with a pathogen (Reynolds WW, 1977

) A study of sockeye salmon demonstrated that higher temperatures do not kill the virus, but instead enhance the immune response of the salmon (Covert and Reynolds). Therefore, we were curious about the direct effects that temperature has on the immune response in fish. Previous research defines a complex cytokine and chemokine network in fish that functions to regulate

physiological and immunological processes, making rainbow trout a suitable model organism (Mackenzie, Planas, & Goetz, 2003) The RTS11 cell line used in this research was derived from rainbow trout spleen. RTS11 cells are predominantly non-adherent, but include a population of mature, adherent cells that we utilize in our functional studies. The non-adherent cells are likely a precursor cell at an earlier stage of macrophage development (Ganssin, 1998). Therefore, this cell line can be used to study the effect of fever temperature on rainbow trout macrophage activity.

Resident macrophages are frequently the first immune system cells to encounter pathogens due to their residence in tissues throughout the body. In addition to their role in phagocytosing pathogens at the initial stages of an immune response, macrophages are also responsible for releasing a variety of signaling proteins called cytokines that are important in inducing the adaptive immune response. Specifically, the cytokines, including interleukins (IL), interferons (IFN) and tumor necrosis factors (TNF), made by macrophages have many functions on their own and in conjunction with other signaling molecules. Some of these critical immune functions of major cytokines are outlined below.

IL-1α is an acute phase cytokine made early during innate responses to infection. It promotes inflammation [heat, redness and swelling which is the result of fluid and cellular infiltration/accumulation from circulation]. It also plays a role in promoting fever synergistically with TNF-α and IL-6. IL-1β is similar to IL-1α with regards to fever and has important additional functions in modulation of blood supply to sites of infection. IL-1β is key in responses of inflammatory fibrosis and stimulates gamma/delta T cells in conjunction with IL-23. IL-1 isoforms are produced as inactive precursors and then processed into active forms. IL-6, another

acute phase cytokine, is also critical for inducing fever. It has been implicated in age related pathologies (Kang S 2019,(Tyrrell & Goldstein, 2021). IL-12 is a key activator of NK cells and T cells and thus bridges the gap between (Kang, Tanaka, Narazaki, & Kishimoto, 2019) innate and adaptive immunity. IL-12 is known to be upregulated in dendritic cells by fever temperatures [Sharon S. Evans,2001, Edward et al, 2016] and as dendritic cells are closely related to macrophages, similar regulation by temperature could be expected in macrophages. Further, dendritic cells [unlike T cells] are not responsive to capsaicin suggesting a diversity of pathways involved in fever responses (Evans, Repasky, & Fisher, 2015), (Umar et al., 2020). IFNα is important in stimulating macrophages and NK cells to generate anti-viral responses, is a potent pyrogen in the hypothalamus where it binds via opioid receptors and causes release of prostaglandin E2 (PGE2), causing fever. IFNβ stimulates anti-viral activity from macrophages and NK cells and can modulate the functions of angiogenesis and growth factors.

TNFα is another major acute phase cytokine that promotes both local inflammation and systemic fever. In work reported with a mouse macrophage line, TNF-α and IL-1 were produced over a wide range of temperatures, but at higher concentrations at 31°C than at 37°C, while IL-6 was produced in higher concentrations at temperatures greater than 37°C (Kirkley, et al .2003). IL-34 is a cytokine that promotes myeloid lineage differentiation, proliferation, and survival and it also induces secretion of IL-6, IL-8 and CCL2 in human blood leukocytes (Lin et al.).2007, Lin H, 2014, (Masteller & Wong, 2014), making it of interest to studies if temperature affects its production.

NO is a free radical that can kill pathogens, with a direct antimicrobial property against many pathogens including bacteria, yeasts, protozoa, helminths, and viruses (De Groote & Fang).

Activated macrophages induce the expression of inducible nitric oxide synthase (iNOS), which mediates NO production. Numerous studies, mostly conducted using mouse macrophages, suggest that NO is free to diffuse across membranes and therefore has both intracellular and extracellular antimicrobial effects (De Groote and Fang). In the context of the present studies, NO is thought to be a key modulator of the shift from mitochondrial redox oxidative metabolism to aerobic glycolysis in microbially stimulated macrophages. NO has been reported to cause a second, late, increase in glycolysis in responding mouse macrophages (Kam, Swain, & Dranka, 2017). For inducing substantial NO concentrations via iNOS expression, a combination of a microbial signal as well as an NK or T-cell produced cytokine, IFN-g, appears to be essential (Kam, Swain, & Dranka, 2017). It has been argued that NO increases rates of glycolysis in activated macrophages (Li et al., 2017), in turn enhancing macrophage function by providing more metabolic energy, freeing the mitochondria to make other free radicals, so as to control infections. Of course, NO is a gaseous signaling molecule in many other places in the body as well.

We have chosen to look at both cell lines and primary cells. The purity of cell lines, that is having large collections of genetically identical 'clonal' cells, is very attractive for consistency and predictability from experiment to experiment. However, cell lines are transformed and immortalized, which means that some of their properties may be modulated by transformation and continuous growth rather than reflecting normal physiology. We therefore also worked on primary cells to validate our findings and to study the effects of aging on macrophages. The downside with primary cells is that their sources do not always yield pure macrophage populations, but rather provide macrophage-enriched cell mixtures, since they come from tissues with multiple cell types, and these contaminating cell types can influence the response readouts.

However, for studies of the effects of aging, primary cells are essential, as tissue culture cells are timeless in many respects.

The aged are at a severe disadvantage when it comes to mounting effective immune responses to new pathogens. There is a 60% increase in bacterial sepsis in the aged. Death from viral infections like viral pneumonias and COVID-19 is much more likely with being older than 80, being worse than being over 65, and worse in aged males than in aged females. [See interactive data from the CDC https://data.cdc.gov/NCHS/Provisional-COVID-19-Deaths-by-Sex-and-Age/9bhg-hcku; same site for pneumonia deaths]. Macrophages from aged animals (mostly mice or humans) have been compared with those from young animals for cytokine and metabolic responses post-activation, but invariably at non-fever temperatures. At baseline temperature, they have been reported to show substantial changes in both their cytokine and metabolic responses, although there is variability in the reports so far (Fei et al.; Fix et al.) 2021). It is important to study both males and females as differences due to sex have been particularly noted with aging (Menees et al., 2021)), as others have seen with phagocytosis (Eimear Linehan 2014). This is consistent with the general pattern that the aged responses involve greater baseline inflammation (Fei et al. 2016-, (Stout & Suttles, 2005) but muted activation levels, a combination that does not appear to result in greater protection or specific immunity.

In vitro studies of primary cells from male and female young and aged mice give clues as to the different capacities of the young and aged by sex. There are known differences between the sexes with BMDMs from young males showing greater ROS, TNF-a and IL-1b induction than BMDMs from females, while the resident macrophage cells in the peritoneum and lung show greater numbers of macrophages in females with higher cell-surface levels of microbial receptors such as toll-like receptors (TLRs), greater phagocytosis and ROS-mediated microbial killing

(Dunston & Griffiths, 2010). Accumulation of resident macrophages in aged liver is also seen [ref]. Aging is associated with chronic, sub-acute inflammation with increases in TNF-a and IL-6. Current interpretations for some of this points to reduced autophagy in the aged and increases in a pro-oxidative state (Bloomer & Moyer). While in vitro studies such as ours are limited by the fact that the cells are no longer in their in vivo environments, a substantial advantage is that it is possible to manipulate and test the cells from a single population in multiple conditions. This facilitates comparisons under many conditions which provide predictions and are an economical preamble to in vivo experiments.

Materials and methods

Ethics statement

All experiments were performed in accordance with Institutional Animal care recommendations under the university of Arkansas, Fayetteville, IACUC. Utilization protocols approved from the animal welfare committee (approval #18100. We had mice that had been born and raised in our colony. About Jun 2018 date to our current experiments, mice C57BL/6 young (2-6 mo.) and old (18-38 mo.) were procured from the Jackson Laboratory on arrival mice are isolated for one week. Mice are housed under conventional conditions with food and water as desired, and then quarantined for at least a week because of their high heat sensitivity. An immunosuppressive virus was discovered by health screening and the entire universities rodent population killed to prevent further spread. The mouse colony was probably infected by pathogen squirrels carried infiltrating the CLAF during construction on the floor above. Resident mice are kept in smaller numbers and for a shorter period because of the general danger of infection, including the infiltration of local rodents or the transmission of illnesses from them to

humans. Males are housed separately since they will fight to the death. Both males and females have, and enhanced environments are the usual practice.

Materials

Cell culture media and supplements

Product	Company
RPMI 1640 medium	Corning
RPM1 1640 medium (Without methionine)	Thermo-Fisher scientific
RPM1 1640 medium (Without phenol red)	Thermo-Fisher scientific
Penicillin/streptomycin	Thermo Fisher scientific
Leibovitz's L-15 media	Hyclone
Fetal bovine serum FBS	Nero Mice
Glutamine	Thermo Fisher scientific
Seahorse XF RPMI Medium (without phenol red)**	Agilent
Seahorse XF Calibrant Solution	Agilent
Pyruvate	Agilent
Glucose	Agilent
Glutamine	Agilent
SuperScript III H- MMLV reverse transcriptase	Promega
RNasin	Promega
Deoxynucleotide DNTP	Biosciences
Trypsin	Thermo Fisher scientific
M-CSF	SHENANDOAH

Kit	Company
Click-iT HPG Alexa Fluor 488 protein synthesis kit	Thermo Fisher scientific
Click-iT OPP Alexa Fluor 647 488 protein synthesis kit	Thermo Fisher scientific
DNase 1	AMPD1-1KT-Sigma
F4/80	Biolegend
BV421	BD Bioscience
Mouse anti chicken macrophage Alexa 488	Bioleged

Chemical	
Lipopolysaccharides LPS	Sigma Aldrich
Dimethyl sulfoxide (DMSO)	Sigma Aldrich
Chloroform	Fisher scientific
Isopropyl	IBI SCIENTIFIC
Trypan blue solution	Sigma Aldrich
Phosphate-buffered saline (PBS)	Growcell.com
Ethanol	KOPTEC
Sulfanilamide	Sigma Aldrich
N-1-naphthylethylenediamine dihydrochloride NED)	Sigma Aldrich
Trizol	Thermo Fisher scientific
RNA Bee	amsbio
Ethylenediaminetetraacetic acid (EDTA)	Sigma Aldrich
phosphoric acid	Sigma Aldrich
Triton 100x	Sigma Aldrich
Ficoll	Sigma
Formaldehyde 3.7%	Fisher scientific
Cell dissociations	Sigma Aldrich
ACK lysing buffer	Gibco
DPBS(1X) Dulbecco's Phosphate buffer saline	Gibco
Percoll density 1.130 g/ml	GE Healthcare Bio- science AB, Uppsala, Sweden
α – Dithiotheritol DTT	Sigma
CT23V	Integrated DNA Technologies IDT
Syber Green	BIOTIUM
Sulfanilamide	Sigma
KOAc	
Syncaine® (MS 222)	fish anesthetic, Ferndale, Washington
Fico/Lite Lympho H	Atlanta Biologicals; density 1.077)
Sodium dodecyl sulfate SDS	EMID
BSA Fraction V	EMD
Tris hydrochloride ultra-pure HCL	MP Biomedical
Mgcl2	Fisher
Agarose	VWR
Sodium Nitrite	Fisher
Phenol	BTC
Trypan Blue dye Con >60%	Alfc Aescr

methods

Stimulation.

In PBS, a stock of LPS (1 mg/ml) was prepared and kept at -20°C. cells were stimulated with 0.1 ng/ml LPS, PGN and poly I: C.

Cell isolation and enrichment of macrophages:

Primary bone marrow, spleen and peritoneal cells from mice were collected and purified. Briefly, the mouse bone marrow cells were isolated from the femur of mice kill by cervical dislocation then collect using incomplete media RPMI1460 with fleshing a 25-G needle from 1 ml syringe. Bone marrow cells were cultured for 7 days into 15 cm Petri dishes with complete medium (RPMI 1640 plus 10% heat inactivated FBS supplemented with 2 mM L-glutamine and (penicillin 100unite /ml, streptomycin 100 µg/µl) and 10 ng/µl recombinant mouse M-CSF (Yrlid et al 2000) at 37°C in a humidified atmosphere with 5% CO2. 5 ml fresh cell culture medium with M-CSF was added on day 3 on. day 7 the adherent BMDM cells were harvested from the petri dishes. Cell counting and viability determined by trypan blue exclusion on a countess was consistently between 85 and 90 %.

For Spleen Resident Macrophages, single cell suspensions were prepared by pressing the spleen between frosted glass slides on small fragments. Pass 10 mL of staining buffer (1×PBS plus 5%FBS) through the cell strainer and centrifuge the cells at 1000 rpm × 8 min. Discarded the supernatant and re-suspend the cell pellet in 3 mL ACK lysis buffer incubate on ice for 5 min to lyse red blood cells. Then washed with 30 mL of 1×PBS. Centrifuged for 5 min at 800-1000

rpm and discard the supernatant. Cells counted and viability determined by trypan blue exclusion on the countess. Mix 10 µL of the cell suspension with 10 µL Trypan blue and fill the hemocytometer by 10 µl using pipette. Place the pipette at the edge of the hemocytometer and slowly released allowing the chamber to fill. The countess ™ has two chambers labeled by A and B. We count both chambers to get the average. Samples are kept on ice until ready to proceed.

Preparation of Single-Cell suspension from peritoneum cavity

A small incision in the skin with the scissors is made. Separated skin from the peritoneum to expose the body wall and ribs. A 10 mL syringe with 21G needle filled with 10 ml cell disassociation free enzyme insert into cavity. Transfer solution into a 15 mL test tube. Centrifuge for 8 min at 1500 rpm and discard the supernatant

(Ray & Dittel). If blood is visible, resuspend the pellet with a vortex in 300 µL of red blood cell lysis buffer for 3 min. Then wash with 10 mL of 1X PBS Centrifuge for 5 min at 1000 rpm and discard the supernatant. Samples kept on ice until ready to proceed to.

Counting cells

Cell suspensions were mixed (1:1 v/v) with a trypan blue solution in disposable Countess® chamber (InvitroGene). Trypan blue. Cells counted and viability determined by trypan blue exclusion on the countess. Mix 10 µL of the cell suspension with 10 µL Trypan blue and fill the hemocytometer by 10 µl using pipette. Place the pipette at the edge of the hemocytometer and slowly released allowing the chamber to fill. The countess ™ has two chambers labeled by A and B. We count both chambers to get the average.

Nitrite concentration or measurement

For NO experiments, cells were plated into 96-well plates at a plating density of $5x10^{\wedge 5}$ per well and then treated with different stimulates for 20-24 h at different incubation temperatures. The production of NO was determined by the microplate reader at 570 nm by (BIO –TeK) measured accumulation of nitrite $NO^{-2\ in}$ medium. 100 μl supernatant of the cell culture medium with 50μl of each one of Griess reagent [0.1% sulfanilamide and 0.01% N-(1 -naphthyl) ethylenediamine dihydrochloride in 2.5% phosphoric acid] as described (Stuehr and Nathan, 1989; Dileepan et al., 1993). Mixed should incubated in dark for 10 min at room temperature. -Then the absorbance of the reaction mixture is measured at 570 nm. Sodium nitrite (NaN02) was used as a standard.

L-HPG Labeling of Cultured Cells and Detection by flowcytometry.

Cells were grown in 24 well plates in RPMI 1640 without methionine supplemented with 10% fetal bovine with serum, penicillin, and streptomycin. supplemented with 10 μM Homopropargylglycine (HPG). HPG is a methionine analog that contains a modification in an alkyne moiety that can be fed to cultured cells incorporates into newly synthesized protein and then made fluorescent with Alexa 488 -click it chemistry. Alternatively, cultures in complete medium with OPP, a puromycin analog, can be used since it which incorporates into growing polypeptides and then stained with Alexa 647 via Click-it chemistry. After incubation for 4 hours at different incubation temperatures with different LPS concentration, the cells were washed with phosphate buffer saline (1×PBS) and then fixed with 3.7% formaldehyde in PBS for 15 min at room temperature. The cells were washed twice with 3% bovine serum albumin (BSA) in 1×PBS, permeabilized with 0.5% Triton® X-100 in PBS for 20 min at room temperature, and then washed with 3% BSA in PBS again. A cocktail of copper(I)-catalyzed click chemistry reaction

between azide-alkyne cycloaddition (CuAAC) bearing of fluorescent Alexa Fluor® 488 detection of HPG incorporated into nascent protein was performed and added to the fixed cells for 30 min in the dark at room temperature. After staining, cells were washed once. Cells Performing antibody surface labeling. Staining analysis of cells by flow cytometry was carried out using FLOWJO software. Mouse macrophages were identified based on F4/80 cell surface marker expression. Viable cells were gated based on FSC vs. SSC.

Flow cytometry

Specific BD FACS instrument, and FlowJo software was used for analysis (BD Biosciences). Forward scatter FSC and side scatter SSC signals were used to exclude dead cells typically 100.000 cells were collected foe analysis and debris as well as doublets and clumps of cells from the analysis gate as shown in the examples below. Prepare each antibody cocktail in staining medium (100 µl per sample). Add appropriate antibodies to sample. The volume of each antibody needed to stain 1 million cells. This volume must be multiplied by the total number of cells to be stained. Add 100 µl of staining medium with antibody cocktail to the appropriate samples. Add 100 µl of single antibodies directly to each of the single antibody staining controls, incubate for 45–60 min at 4°C, protected from light.

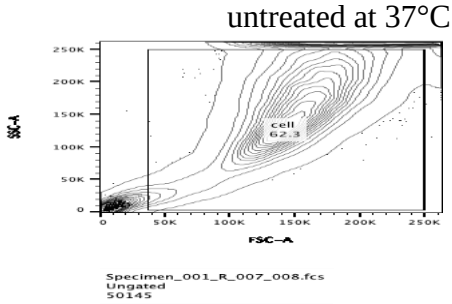


Figure 1: Forward scatter FSC and side scatter SSC signals were used to exclude dead cells

RAW264.7 cells line

RAW264.7 macrophage cell line. The murine macrophage RAW264.7 cell line was purchased from the American Type Culture Collection (ATCC). RAW264.7 cells were derived from the ascites of a tumor generated in a male BALB/c mouse by injecting the Abelson Leukemia Virus intraperitoneally (15). The cells were kept in RPMI1640 Medium (RPMI 1640, Corning, USA) with 10 % fetal bovine serum (FBS, Neuromice, USA)), 2 mM L-glutamine and (penicillin 100unite /ml, streptomycin 100 µg/µl) (Fisher scientific, USA). The cells were kept in a humidified incubator 5% CO2 at 37°C.

RTS11 a rainbow trout macrophage cell line.

RTS11, grow out from a long-term spleen culture (Ganassin and Bols, 1998). The line kept at 16°C in 25 cm2 tissue-culture-treated flasks (Thermo Fisher Scientific) in Leibovitz's L-15 media (Hyclone) supplemented with 1% penicillin/streptomycin and 15% fetal (FBS). Approximately monthly, the confluent flasks were passaged by dividing the cells and continuing medium into two separate flasks and replacing with an equal volume of fresh media. RTS11 was generous get from the WHO DR. AND (University of Waterloo, Ontario. Canada).

HTC cell line

The is a spontaneously converted chicken monocytic cell line derived from a single colony of cells (Rath et al.2003). These cells, known as HTC, grow effectively in culture medium with either 5% FBS or 2% chicken serum at 37 and 41 degrees Celsius. HTC was generous get from Dr. Rath University of Arkansas Poultry science).

Glycolysis and oxidates phosphorylation.

Using seahorse XFp Analyzers, the energy metabolism profiles of cells can be generated to estimate glycolysis measured by proton efflux rate (PER) and mitochondrial oxidative phosphorylation as measured by oxygen consumption rate (OCR) (Agilent, Santa Clara, CA). RAW 264. 7 cells were cultured at near confluence in RPMI 1640 complete medium, the cells were allowed to seed for 24 hours at 37°C . XFp sensor cartridges were hydrated with XF Calibrant media for 24 hours at 37°C. On the day of the experiment the media was switched from RPMI to XF Assay Media and allowed cells to settle in a non-CO2 incubator at 37°C. The Seahorse XFp analyzer run using the glycolytic rate program at 37°C and 39°C. We noticed that the calibration period for the 39°C is 45 minutes, rather than the 15 that it takes at 37°C. PER indicates the protons secreted by cells over time. PER is calculated when the experiment is completed and provides an estimate of extracellular acidification as it adjusts for the change in media PH. In Wave software (Agilent the following equation is used to determine PER: PER (pmol H+/min) = ECAR (mpH/min) × buffer factor (mol/L/pH) × Geometric Volume (μL) × Kvol.

RNA isolation

After removing the medium from each the cell culture well one ml Trizol (Ambion,Life Technologies, USA) reagent was used to extract the RNA by pipetting up and down to lyse and homogenize the cells. The solution was moved to a 1.5 ml tube and stored at 80 until further isolation. Chloroform (200 μl) was added with shaking for 2-3 minutes at room temperature then centrifuged for 15 minutes at 4°C at 12.000 rpm. Three layers were formed; one lower red containing phenol-chloroform, one interphase and one higher aqueous phase. The aqueous layer was removed into a fresh tube containing 0.5 ml isopropanol and shaken intermittently for 10

minutes on ice. Centrifuged at 12,000 rpm for 15 minutes at 4°C. The bottom of the tube is covered with a white gel-like pellet, which is the precipitated total RNA. Supernatant is discarded and 5 M KOAc pH ~5 ($1/10^{th}$) and one ml 95%ethanol were added for one hours or overnight. The samples were then washed by adding 75% cold ethanol centrifuged at 7.500 rpm for 5 minutes and the ethanol layer was removed. The pellet was dried using a vacuum centrifuge Savant Speed Vac and SC110 for 5 minutes after. 50 µl of Te (10 mM TrisCl pH 7.5, 0.1 mM EDTA) was added to dissolve the pellet. The total RNA concentration was determined using a Nanodrop 2000 spectrophotometer (Thermo scientific, USA) to measure total RNA concentration at a wavelength of 260 nm. Agarose gel electrophoresis was used to check the integrity and quality of the RNA. Due to substantial DNA seen in the wells of the gel DNase I (Sigma Aldrich) treatment was performed following manufacturers' instructions. After precipitation and resuspension, RNA was quantified on the Nano Vue spectrophotometer.

cDNA synthesis

A mixed consisting of the following ingredients for one sample: 20 µl of 5x S buffer (Promega), 1 µl of 20 mM dNTP, 2 µl of 30 µM first strand primer CT23V, 5 µl of 0.1 M DTT, and 57 µl of H2O for each sample (1-10µg/ml RNA). After that, the samples were placed in a 40°C water bath for 5 minutes. Then 0.5 µl of RNasin (Promega) and 3 µl SuperScript III H- MMLV reverse transcriptase was added. After thoroughly mixing the samples, they were incubated at 40°C for 1 hour. To each sample 100 µl of stop solution (TE 5 µl of 10% SDS, and5 µl of 250 mM EDTA) was added and mixed. These samples were extracted with 100 µl of phenol-chloroform-isoamyl alcohol (50:48:2) by inversion several times. Sample were then centrifuged at 12K RPM for 15 minutes at 4°C and the upper aqueous layer placed into new 1.5 ml tubes. To these samples 100 µl chloroform-isoamyl alcohol (24:1) was added, centrifuged at 12.000 rpm for 15 minutes at 4°C. About 300 µl will be in the aqueous layer to which is added to that is $1/10^{th}$ volume 5 M

KOAc (pH ~5) and 2.5 volumes of 95% ethanol for precipitation for one hour to overnight. After centrifugation 12.000 rpm for 15 minutes at 4°C, the supernatant discarded and the pellet was washed with 70% EtOH to remove the residual salts and dried using a vacuum centrifuge (Savant Speed Vac SC110) for 5 minutes and redissolved in 100 µl of Te. This cDNA sample it was used for RT-qPCR.

Quantitative real-time PCR (RT-qPCR).

Using primer-blast (www.ncbi.nlm.nih.gov/tools/primer-blast/) to build RT-qPCR primers that cross exon junctions when primers were not available in the literature. The RT-qPCR analysis was performed for each reaction. Cocktail was made that include a mixture of 50mM of each primer, 2µL of diluted cDNA templates, and 1µL of 20x SYBR® green in a 20µL total volume of the reaction on Bio-Rad CFX-96 Real-Time PCR system (Bio-Rad, USA). The thermal cycling conditions were as follows: 90 °C for 30 s, followed by 42 cycles of 95 °C for 15 s and 60 °C for 60 s. Melting curve analysis was performed after 42 cycles to verify the reaction specificity. Each RT-qPCR sample had three replicates. The qPCR threshold (Ct) was used to determine threshold values. The Bio-Rad CFX-96 Manager software calculated the Ct value for each amplification curve (Bio-Rad, USA). The ΔCt values were calculated by subtracting the Ct value of gene of interest from each replicate from the Ct of the average of βactin as run housekeeping gene. ΔΔCt was calculated by ΔΔCt (ΔCt from each experimental sample subtracted from the ΔCt of the unstimulated control at 37°C). The fold change was calculated by 2^ΔΔCt of gene expression best on 37°C.

Table 2: contains a complete list of primers used in this experiment.

Primer	Sequence -F	Sequence -R	Adjusted TM	Annealing TM	Reference
β-Actin	CCTCTATGCCAACACAGTGC	ACATCTGCTGGAAGGTGGAC	62	54	(Junqiang Ye, 2011)
IL-6	CAAAGCCAGAGTCCTTCAGAG	GCCACTCCTTCTGTGACTCC	62	54	IH- Designed
IL12	CAGCACCAGCTTCTTCATCA	GCTGACCTCCACCTGTGAGT	60-63	52-56	IH- Designed
IFN β	CCCTATGGAGATGACGGAGA	CTGTCTGCTGGTGGAGTTCA	61.8	54	(Junqiang Ye, , 2011)
IFN- α	AAAGAAATGTAAGAAAGCTTTTGAGA	TACACTTTGGCTCAGGACTCA TTT	58- 63	50-55	Fung, M. C.et al,2004
IL-1β	GCCTCGTGCTGTCGGACCCAT	TTGAGGCCCAAGGCCACAGGTA	68	60	(Rokade, Kishore, & Madan, 2017)
IL-1 α	TCTCAGATTCACAACTGTTCGTG	AGAAAATGAGGTCGGTCTCACTA	61	54	(Daniels, 2017)
IL-34	TTGCTGTAAACAAAGCCCCAT	CCGAGACAAAGGGTACACATTT	59- 61	50-53	(Yukihiro et al., 2019)
TNF-α	CCCTCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG	61.		Yu Wang,
iNOS	CAG CTG GGC TGT ACA AAC CTT	CAT TGG AAG TGA AGC GGT TCG	63 -65	55 -57	Kara L Cumming designed by Vector NTI
iNOS	GATCAGGAACCTGAAGCCCC	CCTATGGGGCAAAAAAGGGC	62-63	56	(Tsujino et al.)(discounted)
iNOS	ACGAGACGGATAGGCAGAGA	GTGGGGTTGTTGCTGAACTT	60-61	54	IH- Designed is not good

Table 3: contains a complete list of primers used Rainbow trout and RTS11 experiments.

Primer	Sequence -F	Sequence -R	Adjusted TM	Annealing TM	REFRENCE
IL-1β1	CCTGGAGCATCATGGCGTG	GCTGGAGAGTGCTGTGGAAGAACATATAG	63-69	55- 61	(Husain et al,2012)
IL-1β2	GAGCGCAGTGGAAGTGTTGG	AGACAGGTTCAAATGCACTTTATGGT	60	54	(Husain et al,2012)
B-Actin	ACTGGGACGACATGGAGAAG	GTATCGTCATGGACTCCGGT	62	54	IH-Designed
IL-6	GTCAGGAGCATCACTGGACA	TAACTAACACCACGAGCGCC	62	54	IH-Designed
IL-12	TGGGGGTTTAAACTGCTCGG	TGGTCAACGGAACACCACAT	60-61	54	IH-Designed
TNF-α1	TGTGTGGGGTCCTCTTAATAGCAGGTC	CCTCAATTTCATCCTGCATCGTTGA	64- 69	61	(Hong, 2013)
TNF-α2	CTGTGTGGCGTTCTCTTAATAGCAGCTT	CATTCCGTCCTGCATCGTTGC	64-67	60	AJ401377 Hong
TNF-α3	GCTGCACTCTTCTTTACCAAGAAACAAG	GCTGCACTCTTCTTTACCAAGAAACAAG	66	59	HE798544 Hong
IFN-α	CCTGCCATGAAACCTTGAGAAGA	TTTCCTGATGAGCTCCCATGC	62-63	55	(Donald ,2015)
IL-34	AGGCAGAAGACGTAACATGAAACACA	CCACCCTCGCCCTCAGCTT	64	57	(Tiehui , 2012)
iNOS	TTACCGTCTTTCCTCCGAGA	CTGCAGGACTAGTGGCAACA	60-61.8	54	IH- Designed

Table 4: contains a complete list of primers used in HTC and chicken tissue experiments.

Primer	Sequence -F	Sequence -R	Adjusted TM	Annealing TM	REFRENCE
β-Actin	CTCCCTGATGGTCAGGTCAT	ATGCCAGGGTACATTGTGGT	60.0- 62	54	IH- Designed
IL-12b	ACCAGCCGACTGAGATGTTC	TTGAGGTGGGTCTGGCTTTA	60.0- 62	54	IH- Designed
IL-6	GCTACAGCACAAAGCACCTG	AAGTGGTCATCCCAGACTCG	62	54	IH- Designed
IFN- α	GACAGCCAACGCCAAAGC	GTCGCTGCTGTCCAAGCATT	60- 61	54	(Lian , 2012)
iNOS	AGGCCAAACATCCTGGAGGTC	TCATAGAGACGCTGCTGCCAG	64	56	(Rath, 2003)
iNOS	ATTTCCGTGTTTGGAACAGC	GAAATATGGCCGCTTTGATG	58	50	IH- designed
IL-1B	TGCCTGCAGAAG AAGCCTCG	CTCCGCAGCAGTTTGGTCAT	64	57	(Truong et al., 2018)
IL-34	CGGAATTCATGCACCAGGGCTGCGCGGC	CCAAGCTTAGCGGAGTCCCACCGACAGTG	75	68	Truong et al, 2018
IFN-b	CTTGCCCACAACAAGACGTG	GTGTTTTGGAGTGTGTGGGC	62	54	Truong et al, 2018
TNF-α	AGTTCAGCTTGCCTCGCTAA	TACAGAAGGAGATGTCGGGG	61	54	IH- Designed

Preparation of single cell suspension and isolation of mononuclear cells from chicken spleen by Ficoll density gradient separation.

Each spleen from recently euthanized birds was placed in 50 ml tube containing 5 ml of ice-cold RPMI 1640 medium. With scissors the tissue was cut into 2cm pieces and pushed through the metal mesh with the plunger from a 10 ml syringe. Ice cold 1x PBS was used to flush the tissue through the mesh. Cells were washed in PBS at (300 × g for 8 min at 4°C) 1400 rpm in a Sorvall RT 6000D centrifuge and resuspended in 5ml of Ix PBS at ambient temperature. Equal volumes of suspended cells were layered above the same volume of Ficoll. Typically 5 ml cells were layered on top of a 5ml Ficoll layer (Fico/Lite Lympho H, Atlanta Biologicals; density 1.077) in 15 ml conical tube, centrifuged at room temperature (500 x g 1800 rpm for 30 min) 1800 rpm, and the interface ficoll layer was retrieved and washed twice with PBS (pH 7.4) by spinning the cells at 300 × g 1400 rpm for 8 min at 4°C) 1400 rpm Viable cells were counted by trypan blue exclusion. Cell resuspended in RPMI were cultured in 100 X15 mm one million cells in each plate. We cultured from 1 to 2h, plates swirled and nonadherent cells discarded, the adherent cells were released by incubation in PBS without Ca^{+2} and Mg^{+2} on ice for at least 10 min followed by scraping. Cells were spun, counted and used for experiments.

Isolation and culture of head kidney macrophages

Rainbow trout (Oncorhynchus mykiss) weighting from 300-500g were utilized Before physical euthanasia, chemical anesthesia was applied. Fish were euthanized after anesthetic (200 mg of Syncaine® (MS 222) fish anesthetic, Ferndale, Washington) for 10 minutes followed by rapidly cutting the spinal cord beneath the scull and subsequently pithing the brain stem. The

membranes covering the head kidney were cut with a sterile scalpel blade and gently pulled away with fine forceps. A spatula was then used to scoop out all the head kidney tissue. If the tissue was collected at fishery (Norfork national fish hatchery) was transported on ice to the laboratory. The dissected head kidney was placed in a 60mm petri plate in L-15 medium. Cold medium was used to adjust the volume of the cell suspension to 24 ml, and the solution was split into six 4 ml aliquots. Each aliquot was placed in a 15 mL conical tube over 4.2 mL isotonic Percoll. To make Percoll isotonic 9 volumes of Percoll stock (IPS) (density 1.130 g/ml, GE Healthcare Bio- science AB, Uppsala, Sweden) l were mixed with 1 volume of sterile 1.5 M NaCl. We made batches of 100 ml of stock isotonic Percoll on a regular basis to keep the majority of the product undiluted in its original bottle. The 15ml tubes were then centrifuged for 25 minutes at 500 x g at 4°C 1800 rpm and the middle brown band and the third band of head kidney cells collected for enrichment of adherent cells. The upper band contains dead cells and clumps. The second band from the top contains melanocytes and the third band contains macrophages. Cells from the bands were washed twice with Leibovitz's L-15 supplemented with 2 mM glutamine, penicillin (100 iu ml) as well as streptomycin (100 g ml) and 10% FBS Fetal bovine serum and placed in culture for three days following which plates were swirled with media and non-adherent cells removed. Swirling with fresh medium was repeated until the brown color of the melanocytes was removed (GarduÑO & Kay, 1994) . The adherent cells were used for the experiments. As attachment was loser then mouse or chicken cells, the adherent cells were removed by scraping.

Isolation and culture rainbow trout splenocytes.

Phosphate buffered saline was used to make cell suspensions (PBS.) Spleen in a 10 cm petri plate was cut into small pieces using sterile scalpel and scissors and pressed through a 40-mesh cell strainer using the plunger of a 1 mL syringe. The cell suspension was centrifuged and then

resuspended in growth medium to adjust the volume of the cell suspension to 24 ml, and the solution was split into six 4 ml aliquots. Each aliquot was placed in a 15 mL conical tube over 4.2 mL isotonic Percoll. To make Percoll isotonic 9 volumes of Percoll stock (IPS) (density 1.130 g/ml, GE Healthcare Bio- science AB, Uppsala, Sweden) l were mixed with 1 volume of sterile 1.5 M NaCl and centrifuged at 400 g for 30 minutes at 4°.C The red blood cells and debris are found at the bottom of the centrifuge tube and leukocytes are concentrated in a ring shape on Percoll interface surface. Leukocytes were collected and diluted in Leibovitz's L-15 culture medium and rinsed once (400 g centrifugation for 10 minutes). After the supernatant was discarded, the cells were resuspended in 1 mL complete Leibovitz's L-15. Cell was placed on a 10 cm petri plate and after 1-2 days the petri plate surface was rinsed with medium, non-adherent cells were removed and adherent cells cultured in fresh medium and harvested for experiments.

Statistical analysis

Data were tested for statistical significance by Excel analysis or two-way ANOVA and the Tukey Multiple Comparisons Test using Prism (Version 8; GraphPad). $P < 0.05$ was taken as the level of significance. Data are representatives of at least three independent experiments.

Results and discussion

The overall goal of this study was to see how fever temperature alters macrophage functions. By measuring multiple parameters of metabolism such as glycolysis, oxidative phosphorylation, and protein synthesis, and comparing those with specifically induced functions of macrophages including nitric oxide, MHC class II expression, inflammatory cytokine and growth factor production, an understanding of the importance of fever on both global cellular properties and critical functions of these crucial cells of the innate immune system is sought.

Fever temperature both enhances and accelerates a cellular metabolic shift crucial for cell survival and function in a mouse macrophage cell line, RAW 264.7.

To investigate glycolysis and oxidative metabolism, RAW 264.7 cells, a mouse macrophage cell line originating in the peritoneum of an Abelson murine leukemia virus infected BALC/c mouse, was utilized. Using a Seahorse analyzer, we monitored both the oxygen consumption rate [OCR, Figures 1 and 3] and the proton efflux rate [PER, Figures 2 and 4] as indicators of the mitochondrial energy metabolism and redox status.

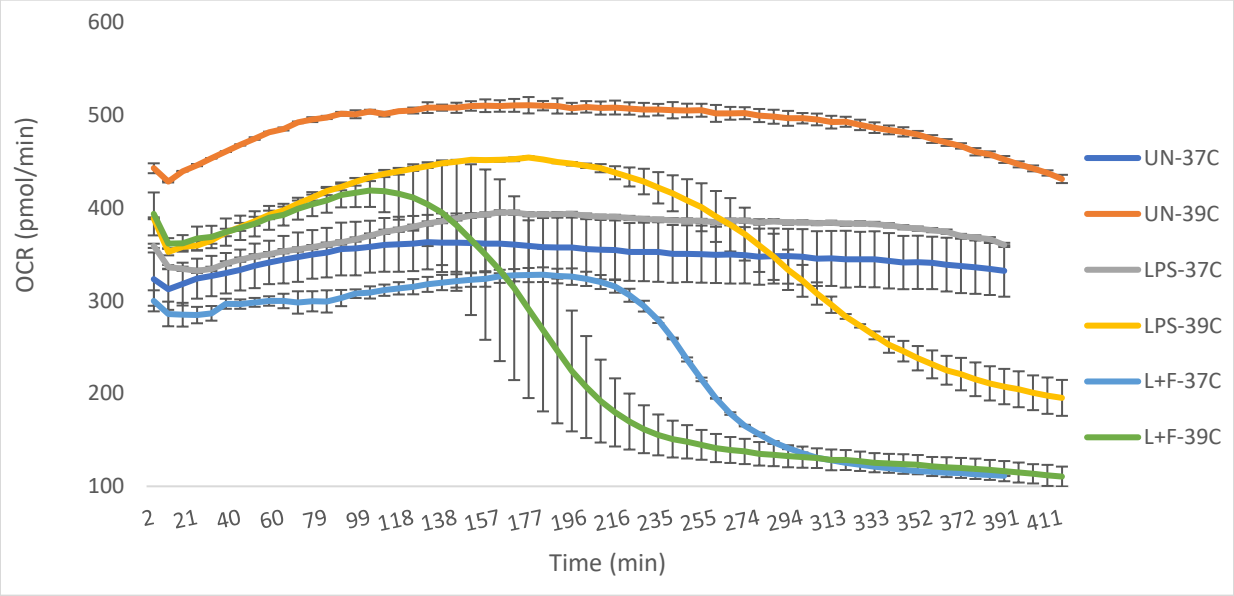


Figure 1. Effect of fever and stimulation on mitochondrial respiratory activities of RAW264.7 cells. Oxygen consumption rate (OCR) measurement of RAW264.7 cells ($2x10^5$ cells/well), either unstimulated (UN) or stimulated with LPS [0.1 µg/ml] or LPS plus IFNγ [0.1 µg/ml] at either 37°C or 39°C. Duplicates for each condition were monitored for their OCR at 7-minute intervals for 6 hours in an Agilent Seahorse XFp 8-well plate analyzer as detailed in the Materials and Methods. The dark blue line represents unstimulated RAW264.7 cells at 37°C and the orange line represents unstimulated cells at 39°C. The light blue line represents stimulated cells with LPS and IFNγ at 37° and the green line represents stimulated cells at 39°C. The gray line represents LPS stimulation of cells at 37°C and the yellow line at 39°C.

The treatments were initiated prior to loading into the instrument; thus, the OCR values differences are observed from time zero of measurement. The OCR is higher at 39°C than at 37°C even in unstimulated cells. All the treatments initially increase the OCR further to a modest extent. This is expected of and consistent with cells becoming 'activated' and reconfiguring their metabolism for increases in energy production, initially through mitochondrial energy production associated with increased oxygen consumption [Fig 1]. However, the data show that, with strong stimulation (LPS plus IFNg) and/or at fever temperature, mitochondrial metabolism eventually drops sharply, and this drop occurs much faster with the addition of IFNg, and at 39°C than at 37°C (Fig. 1).

In Figure 1, we see OCR for the first group, the unstimulated cells at 37°C, stayed relatively constant (323 to 360 pmol/min) over the time points tested. Unstimulated cells at 39°C showed a similarly constant but higher OCR (428 to 510 pmol/min). Cells stimulated with LPS at 37°C started with a low OCR (323 pmol/min) which rose over 160 minutes [395 pmol/min] and then remained flat for of rest of the time tested. Cells stimulated with LPS at 39°C had a lower OCR at the start of the experiment (359 pmol/min) which rose over 160 minutes [452 pmol/min], after which the OCR gradually declined. Cells stimulated with LPS and IFNγ at 37 °C showed a level OCR for the first approximately 200 minutes at which time a steep decline in OCR was observed. At 39 °C a similar pattern was seen but the decline began much earlier at 105 minutes.

In the absence of mitochondrial respiration, cellular energy needs will have to be met by glycolysis. The proton efflux rate (PER), or the extracellular acidification rate (ECAR), an indirect measure of glycolysis, starts out at higher levels in the stimulated groups and at the

fever temperature [Fig 2]. These higher initial levels represent a first increase in glycolysis. The PER measurements tend to fall over time in this experiment since there is a high number of cells in the medium, leading to progressive acidification of the culture medium. However even on that background, glycolysis is clearly rapidly enhanced in a second peak in response to strong activation and/or at fever temperature (Fig. 2) starting at about the time point at which mitochondrial respiration rates begin to drop (Fig. 1). That is, it can be seen that in every case the OCR begins to decline prior to the increase in PER. A more subtle but notable fever temperature effect is seen in the LPS-only treatment group at 39°C, where the PER at later times diverts upwards from the 37°C LPS-only group which it had been running parallel with until then. This is the first time an increase in glycolysis has been seen in LPS-only stimulated macrophages, and it should be noted that previous reports have all tested this issue at 37°C. Thus, it appears that fever temperature is relaxing the requirement for a second signal such as IFN-g for inducing an increase in glycolysis, or that fever temperature itself acts as the second signal.

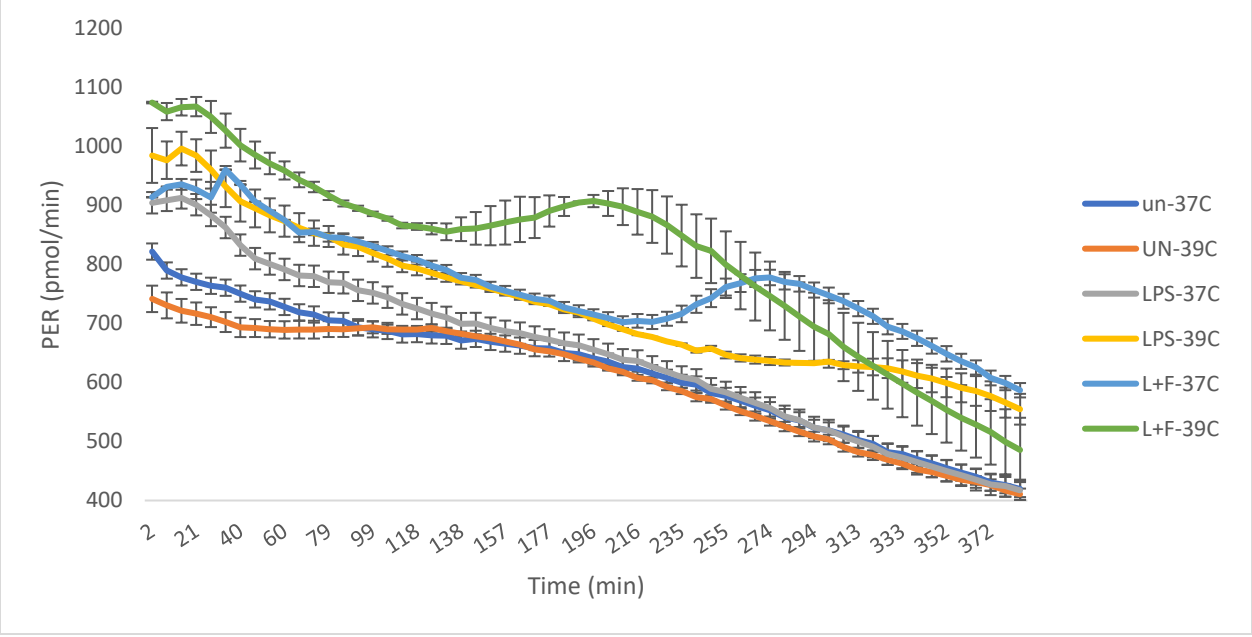


Figure 2. Effect of fever and activation on the proton efflux rate [PER] in a macrophage cell line. RAW264.7 cells were stimulated as in figure 1 above and the PER monitored for 6 hours in a XFp 8-well plate analyzer. RAW264.7 cells ($2x10^5$ cells) were stimulated with LPS [0.1µg/ml] and LPS plus IFNγ [0.1µg/ml] or nothing at 37°C and 39°C. Cells were monitored for 6 hours for PER in a XFp 8 well plate analyzer. The dark blue line represents unstimulated RAW264.7 cells at 37°C and the orange line represents unstimulated cells at 39°C. The light blue line represents stimulated cells with LPS and IFNγ at 37° and the green line represents stimulated cells at 39°C. The gray line represents LPS stimulation of cells at 37°C and the yellow line at 39°C. The graph displays the mean ±SEM of two technical replicates from the same run as Figure 1.

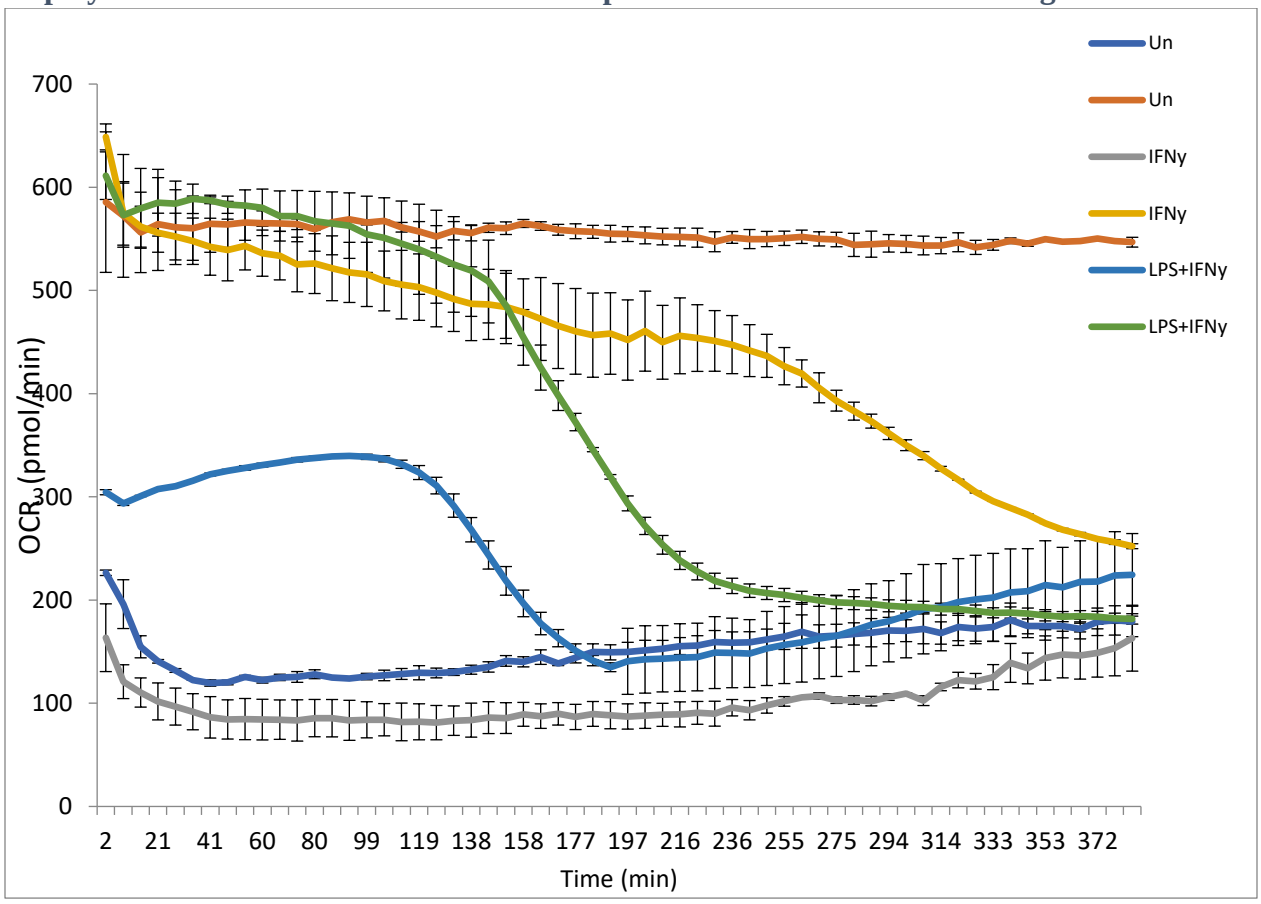
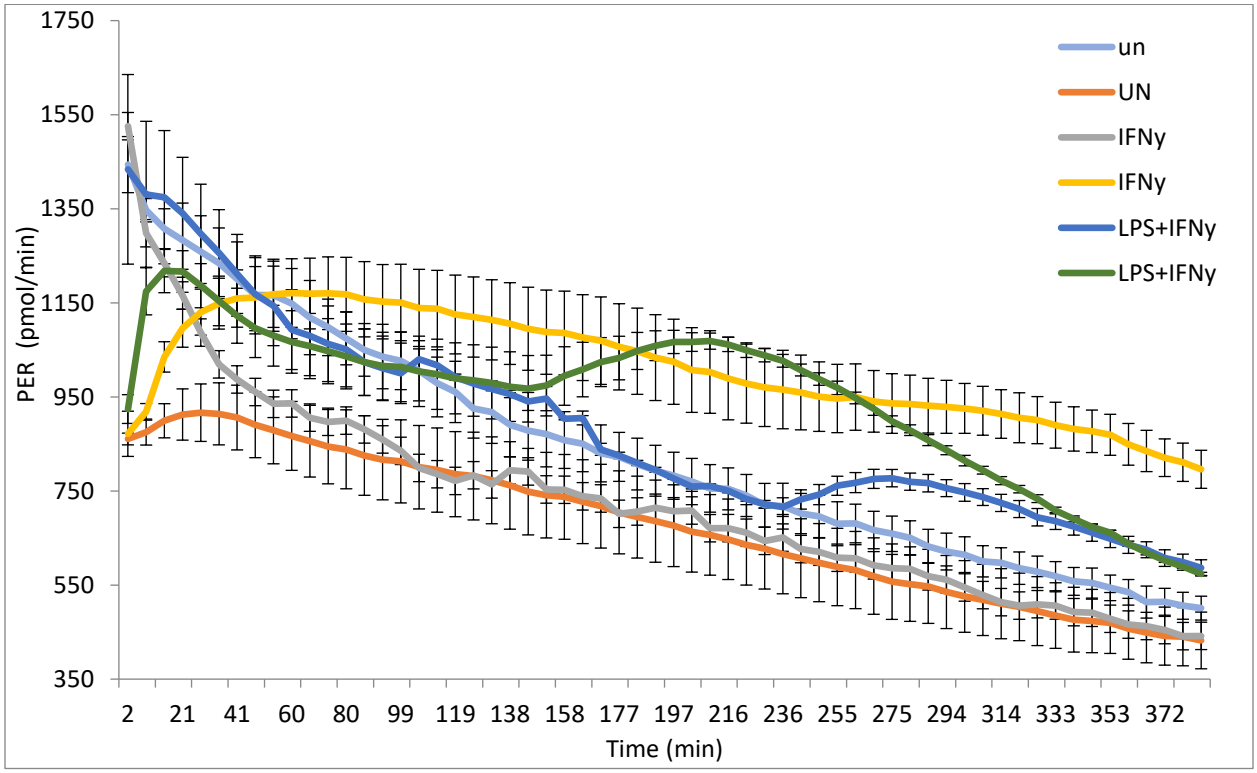


Figure 3. a] Effect of fever and stimulation on mitochondrial respiratory activities of RAW264.7 cells. Oxygen consumption measurement rate (OCR) of RAW264.7 cells ($2x10^5$ cells) stimulated with 0.1µg/ml IFNy or .1µg/ml LPS plus IFNγ or nothing at 39°C. Cells were monitored for 6 hours for OCR in a XFp 8 well plate analyzer. The dark blue line represents unstimulated cells at 37°C, the orange line represents unstimulated cells at 39°C. The blue line represents the OCR of cells stimulated LPS and IFNγ at 37°C, the green line represents stimulated cells with LPS and IFNγ at 39°C. The grey line represents the OCR of cells stimulated with IFN-g and the yellow line represents the OCR of IFN-g stimulated cells at 39°C. The graph displays the mean ±SEM of two technical replicates. b] Effect of fever and stimulation on the glycolytic activities of

RAW264.7 cells. The same color scheme is utilized to present the data on the PER of the same groups.

As the XFp8 model of the Seahorse analyzer only allows 6 experimental wells at a time, we did a second set of runs looking at IFN-g alone along with unstimulated and the LPS and IFN-g combination. For OCR, fever temperature led to initial higher oxygen consumption for all groups [Figure 3a], followed later by steep reductions for the IFNg at 39^0C and for LPS+IFN-g at both temperatures. For PER, fever temperature led to a faster increase in LPS+IFN-g-stimulated cells. In the absence of efficient oxygen consumption (Fig. 1 and 3), this shift to glycolysis is likely to be essential to provide cellular metabolic energy needed to continue to make the activated immune responses. One particularly complex immune function of activated macrophages, which can affect cellular metabolism as well, is the production of NO via the induction of iNOS protein expression.



. Figure 3 B: Effect of fever on PER with macrophage activation. RAW264.7 cells ($2x10^5$ cells) were stimulated with IFNy [0.1µg/ml] and LPS plus IFNγ [0.1µg/ml] or nothing 39°C. Cells were monitored for 6 hours for PER in a XFp8 well plate analyzer. The light

blue line represents stimulated cells with LPS and IFNγ at 37° and the green line represents stimulated cells at 39°C. The gray line represents LPS stimulation of cells at 37°C and the yellow line at 39°C. The graph displays the mean ±SEM of two technical replicates

We chose to use confluent cells to maximize the experimental sensitivity for NO production. High cell numbers are known to result in some decrease in OCR and in PER over time, and high local extracellular concentrations of NO have been reported to enhance the second increase in glycolysis in strongly stimulated macrophages at 37°C (Kam et al., 2017). Experiments with lower cell densities will be needed to titrate this effect. Adding more glucose to the medium at the beginning is inadvisable since cells are quite sensitive to high glucose [Pavlou 2018 BMC Immunology, s12863], although it may be possible to feed more glucose at a midway point during the 6 hour assay. More evidence for NO being important comes from the observation that culture supernatants from cultures making NO shift the metabolism of otherwise unstimulated cells, and an iNOS inhibitor [1400 W] blocks secondary metabolic response from activated RAW264.7 macrophages (Kam et al., 2017). Another possible confounding effect of the presence of NO has been reported, namely that extracellular NO can lower the OCR [ref], and NO decreases mitochondrial complex IV activity [Paolo Sarti et al 2003]. However, exogenous NO has no effect on the PER/ECAR so that the correspondence with the change to glycolysis in the present context would remain important to watch for as part of our insight into fever-mediated modulation of metabolic reprogramming of activated macrophages.

There is some evidence that the combination of LPS and IFN-y would result in an increase in glycolysis and an NO-driven decrease in oxidative phosphorylation in activated macrophages

(Kam et al., 2017), allowing mitochondria to produce free radicals. Figures 1, 2, and Table 1 show that fever temperature at 39°C hastens these processes. Further, LPS alone at 37°C does not induce these changes, while the combination of LPS and increased temperature both increases glycolysis and causes a dramatic reduction oxygen consumption.

Because of this complex role of iNOS-NO in cellular energy metabolism in activated macrophages, the results from these cellular metabolism studies need to be interpreted in conjunction with our studies examining the production of iNOS and NO.

Nonetheless, it is evident that, in the absence of efficient oxygen consumption (Fig. 1 and 3), the shift to glycolysis is likely to be essential to provide cellular metabolic energy needed not simply to continue to make the activated immune responses, but also to maintain cellular viability. Thus, it would be plausible to predict that fever-mediated alterations in the trajectory of cellular energy metabolism is accompanied by enhanced production of nitric oxide and inflammatory cytokines. A necessary precondition for that production is the global ability of the cell to undertake protein synthesis, which is what we examined next.

Protein synthesis as another general measure of metabolic activity.

We examined protein synthesis in RAW 264.7 cells at normal and fever temperatures before and after stimulation. Protein synthesis by RAW264.7 cells with or without LPS stimulation at 37 C or 39 C was determined by flow cytometry after a 4-h pulse with Click-It-HPG followed by Alexa 488-based detection. We observed a trend toward higher protein synthesis at 39°C compared to 37°C.

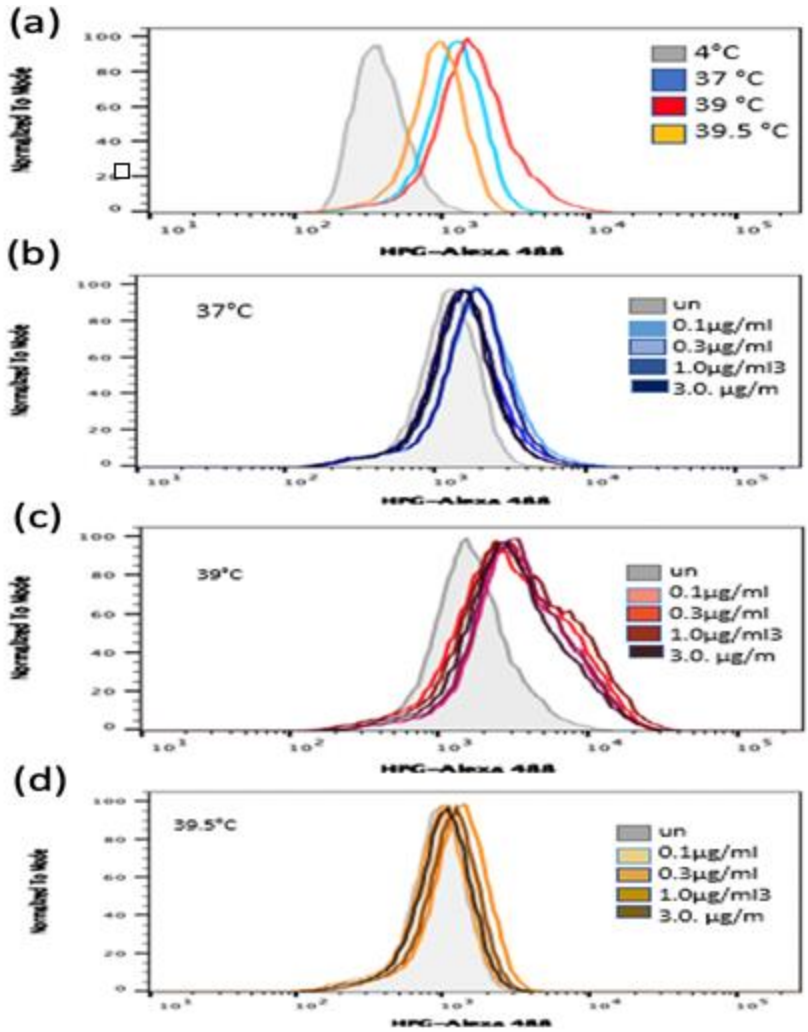


Figure 5: Protein synthesis of RAW 264.7 cells at different temperatures with and without LPS stimulation. Triplicate cultures on one million cells for each treatment were incubated for 4 hours in methionine-free medium with HPG, pooled, and then made fluorescent via Click-it chemistry and analyzed by flow cytometry. The number of events collected for each sample was 100,000. A gate for size was used to remove small debris and large aggregates of cells (a) HPG incorporation of RAW264.7 cells at different temperatures [4, 37,39 and 39.5°C]. The light gray filled histogram in (a) represents the normalized fluorescent of distribution unstimulated cells at 4°C. The blue line protein synthesis at 37°C, the light red at 39°C and the orange line at 39.5°C. (b) histogram of HPG incorporation of RAW264.7cells at 37°C with different LPS concentrations. The gray filled histogram unstimulated cells at 37°C overlaid the cells treated with different LPS concentrations [0.1, 0.3, 1.0 and 3.0 and 10µg/ml] shown from light blue to dark blue. (c) Flow cytometry pattern of RAW264.7 cells at 39°C with different LPS concentrations. The gray filled histogram represent unstimulated cells at 39°C. LPS stimulations range from light red to dark red. (d) The gray filled histogram unstimulated at 39.5°C. LPS stimulations range from light orange to dark orange.

Protein synthesis studies Figure 5 show an increase at 39°C red compared to 37°C blue in unstimulated RAW264.7 cells. A further increase in temperature to 39.5 °C reduced levels below cells at 37°C but well above those kept at 4°C (Figure 5a). Figure 5b and d depict the effect of LPS on protein synthesis showing small shifts to the right in the histogram at 37°C and 39.5°C compared to the unstimulated cells shown a filled gray histogram and a major increase at 39°C (Figure 6c).

Effect of temperature on Nitric oxide production in LPS, IFN- **γ** and LPS plus IFN- **γ** stimulated RAW264.7 mouse cells for 24 h.

A comparison the effects of temperature and stimulation on NO production between 37 °C and 39°C are presented in Figure 6. RAW264.7 cells were exposed to different concentrations of LPS ranging from 0.1 to 10 µg/ml, IFN- γ 3.0 µg/ml and the combination for 24h, then the production of NO was detected by the Griess assay. Background level of NO are detectable at both temperatures. RAW264.7 cells showed a much higher response at fever temperature compared to the control 37°C for all treatments. The combination further enhanced NO production.

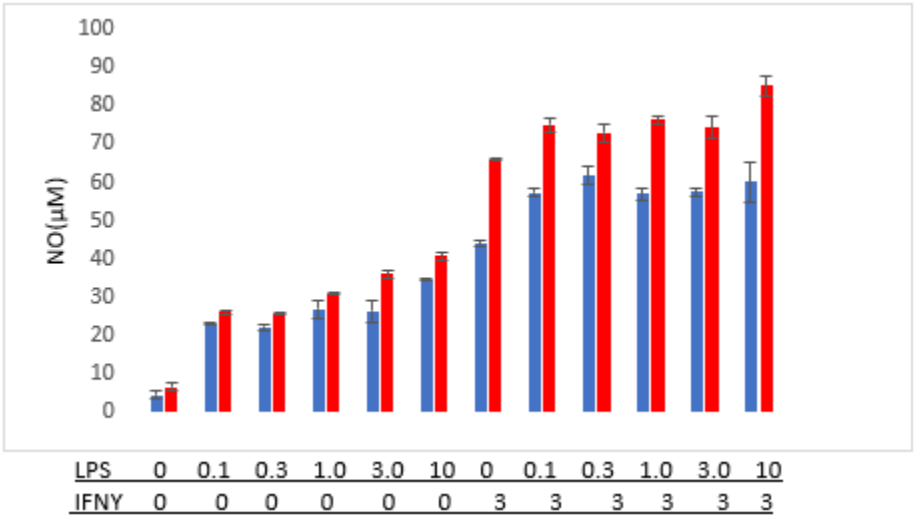


Figure 6: RAW 264.7 cells cultured in 96 well plate (5x105in each well) was stimulated with different treatments in triplicate for 24 h. Supernatant collected and tested for NO

(µM). LPS ranged from 0.1 to 10 µg/ml, IFN- **γ** 3.0 µg/ml and in combination LPS and IFN- **γ**. The results shown are the averages from one experiment ±SE. Blue bars represent cell cultured at 37°Cand the red bars cell cultured at 39°C, (UN). Results are representative of 4 experiments

RAW 264.7 cells response to LPS activation at four different temperatures.

Figure 6 compares the effects of temperature and stimulation on NO generation between 37°C ,38°C, 39°C and 39.5°C. RAW264.7 cells were treated with increasing dosages of LPS (0.1-6µg/ml) Fig 6a for 24 hours and (0.1-10µg/ml) Fig 6b, and NO production was measured using the Griess assay. RAW264. 7 cells responded substantially better to 39°C than to 37°C for all treatments. However, the NO response at 38°C was less or equal to 37°C. for the fever effect, it is critical to be above 38°C.

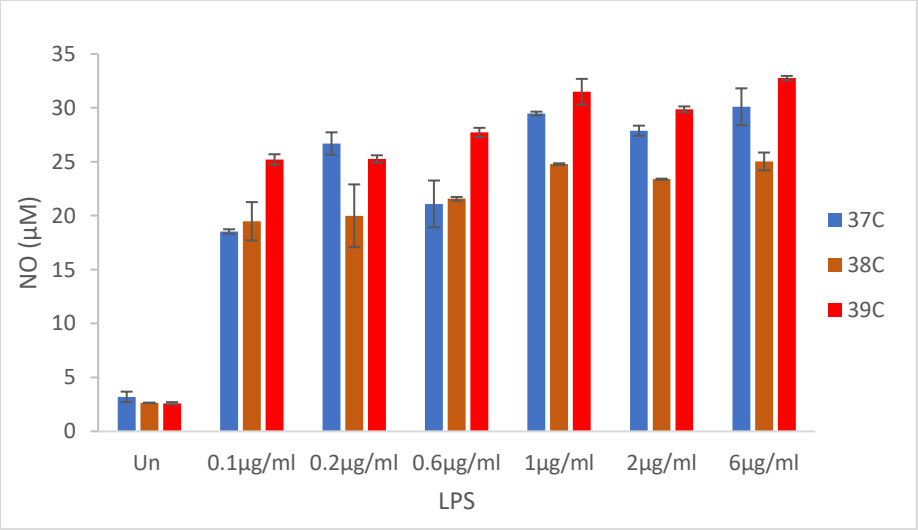


Figure 6a: shows a comparison of the effects of temperature and stimulation on NO generation between 37 °C ,38 and 39°C For 24 hours. RAW 264.7 cells cultured in 96 well plates ($5x10^5$in each well) were stimulated with different treatments in triplicate for 24h at 37°C, 38°C and 39°C cultured for 24h until harvest. Supernatant was collected and tested for NO (µM). LPS ranged from 0.1 to 6µg/ml. The blue bars represent cells cultured at 37°C, the orange bars those cultured 38 °C, the red those cultured at 39°C. The results shown are the averages from experiment ±SE.

Figure 6b shows a comparison of the effects of incubation at different temperatures on NO production. RAW264.7 cells were stimulated with increasing dosages of LPS (0.1-10µg/ml) at 37, 39 and 39.5°C for 24 h, and NO production was measured using the Griess assay. RAW264. 7 cells responded substantially better to 39°C than to 37°C for all treatments. However, the NO response at 39.5°C dropped down dramatically indicating is detrimental and that. a 0.5 °C difference in temperature is perceived. These 2 experiments establishes that two degrees above the core temperature is optimal for fever response we used 39°C all further mouse studies. for

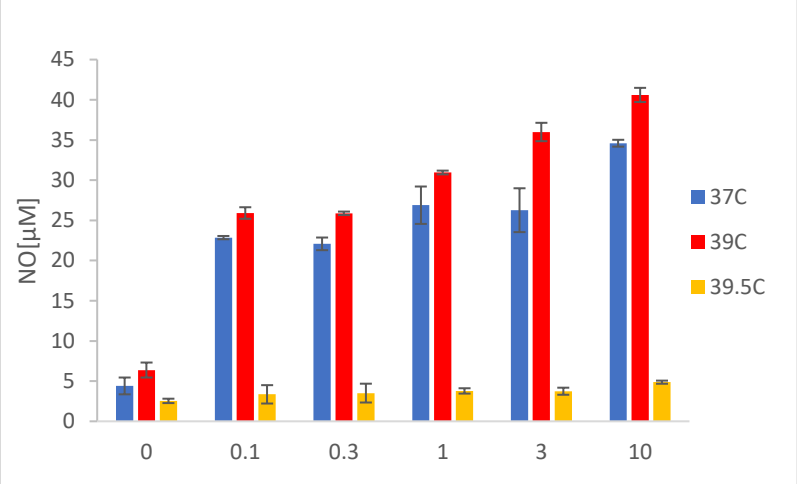


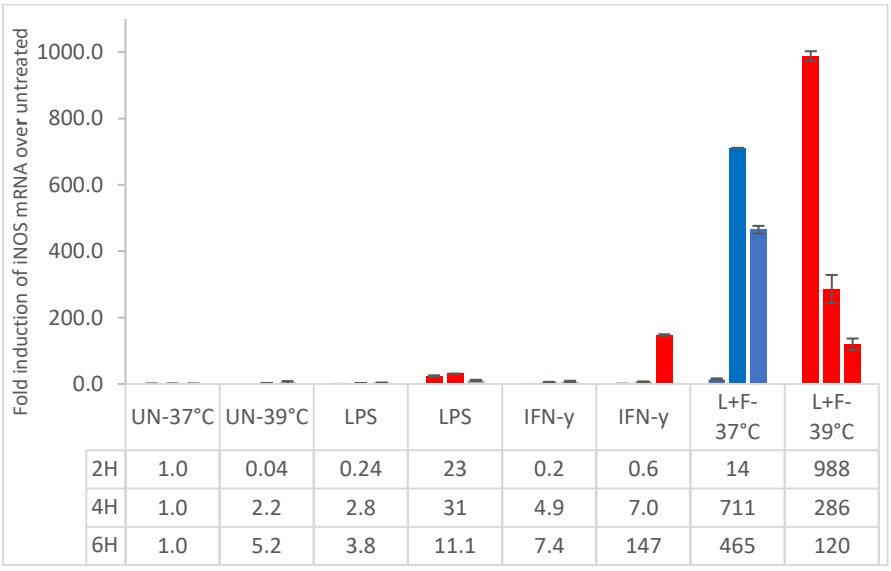
Figure 6b: Treatments at 37°C, 39°C and 39.5°C. The blue bars represent cell cultured at 37°C and the red bars at 39°C and yellow bars 39.5°C for 24h. The results shown are the averages from triplicates ±SE and representative of 3 experiments.

Effect of fever temperature on iNOS production.

Nitric oxide (NO) is a highly reactive free radical produced in cells by nitric oxide synthase (NOS) from arginine and oxygen [5]. Large amounts of nitric oxide (NO) are cytotoxic to pathogens and protective the host. It also regulates blood flow modulation, smooth muscle

relaxation, and neurotransmission. Two NOS isoforms are expressed constitutively in neurons and epithelial cells. Macrophages make inducible NOS (iNOS) which is triggered by inflammatory cytokines, hypoxia, and oxidative stress in a Ca2+-independent manner. TNFα, IFN-γ and LPS are the most common induction of NO. Investigation of whether fever temperature directly altered NO and iNOS expression NO by Griess reagent, iNOS mRNA levels by quantitative real-time PCR (RT-qPCR).

A fever temperature of 39°C had a poster effect on iNOS mRNA production at every level. Unstimulated cells were up 2-5-fold at 2 and 6 respectively. (Figure 7). LPS showed a group peak at an earlier time than the 37°C and with a 10 fold greater response. IFNγ peaked at 6h with a > 10 fold increase. The weaken stimuli were greater enhanced by fever. The combination of LPS and IFN-γ is a potent stimulation of iNOS at 37°C with a peak at 4h, stimulation at 39°Cincreasess the speed of induction as well as the magnitude of the response.



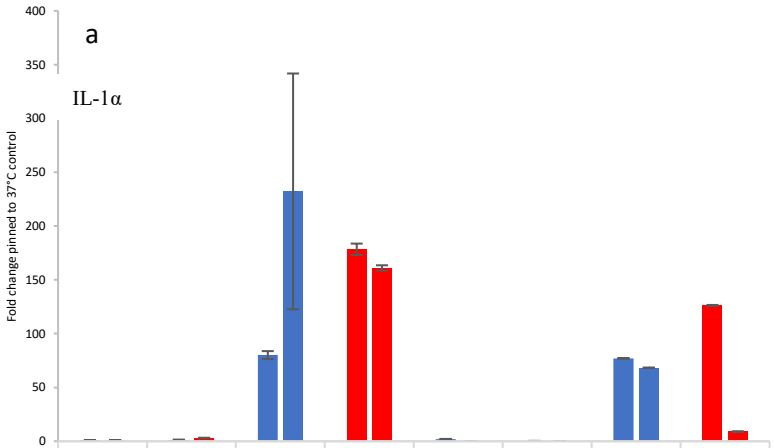
	UN-37°C	UN-39°C	LPS	LPS	IFN-γ	IFN-γ	L+F-37°C	L+F-39°C
2H	1.0	0.04	0.24	23	0.2	0.6	14	988
4H	1.0	2.2	2.8	31	4.9	7.0	711	286
6H	1.0	5.2	3.8	11.1	7.4	147	465	120

Figure 7: The effect of fever on iNOS induction over 6 hours in RAW264.7 cells. Duplicate samples of in RAW264.7 cells (3x106in each well) were stimulated with LPS [100ng/ml], IFNγ [100 ng/ml] or LPS plus IFNγ at 37 and 39°C for 2h, 4h and 6h. Duplicated were pooled,

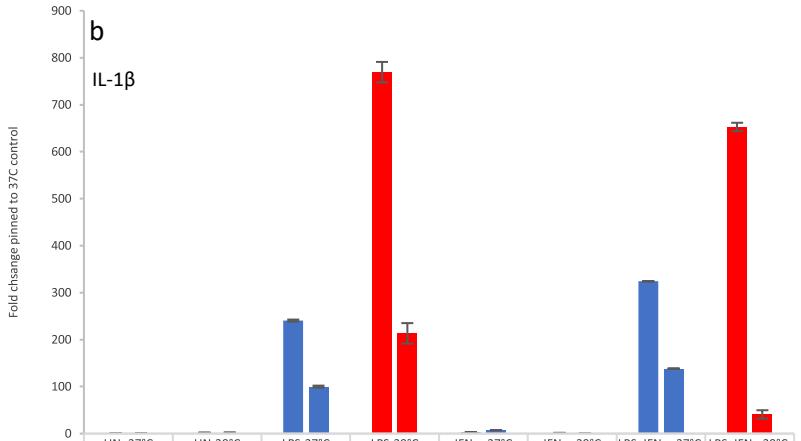
RNA purified and used as templates for cDNA. cDNA samples were run in triplicate for RT-qPCR. 37°C is (blue bars) and 39 °C (red bars) Relative fold change expression levels normalized to the B-actin.

Effect of fever temperature on proinflammatory cytokine expression

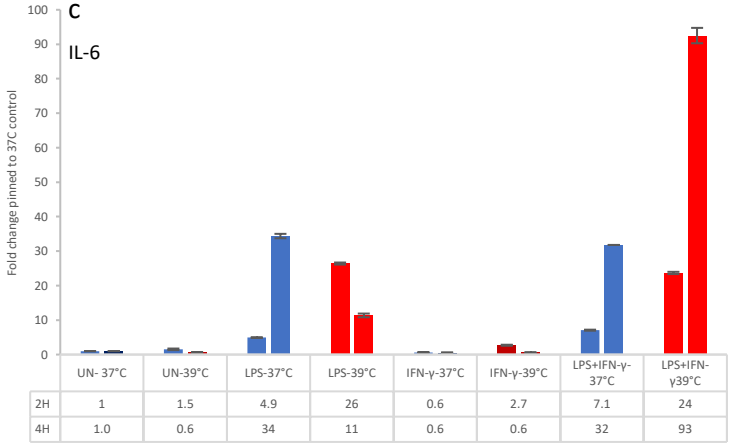
Effect of fever on proinflammatory with LPS Stimulation on the expression of proinflammation cytokine expression by RAW264.7 cells. All the proinflammation cytokines tested showed an increase at some stimulation and time companied with the 37°C control in all 7 cells cases the combination of LPS and IFN-γ. treatment and in 5 cases LPS alone an increased the proinflammatory cytokine response. The unusual combinations were IFNα and TNF αwhich only showed fever off do with combination and IL-12 which showed an increase with temperature alone (without stimulation) as well as with LPS and the combination.



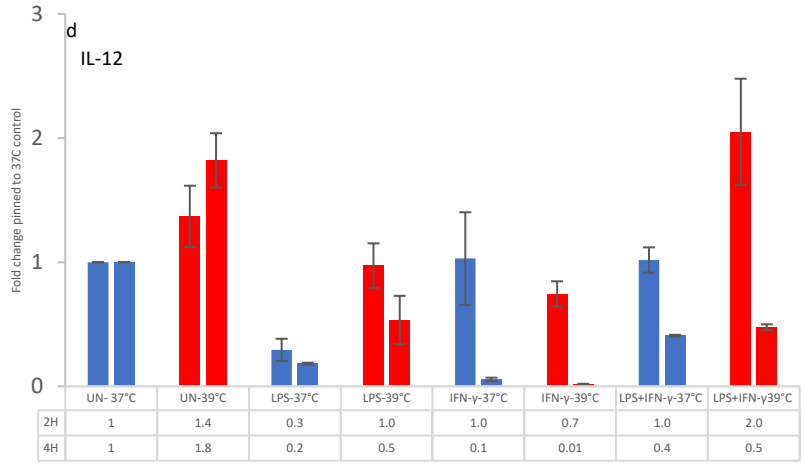
	UN- 37°C	UN-39°C	LPS-37°C	LPS-39°C	IFN-γ-37°C	IFN-γ-39°C	LPS+IFN-γ-37°C	LPS+IFN-γ39°C
2H	1	1.4	80	178	2	0.3	77	126
4H	1	3	232	161	0	0	68	9



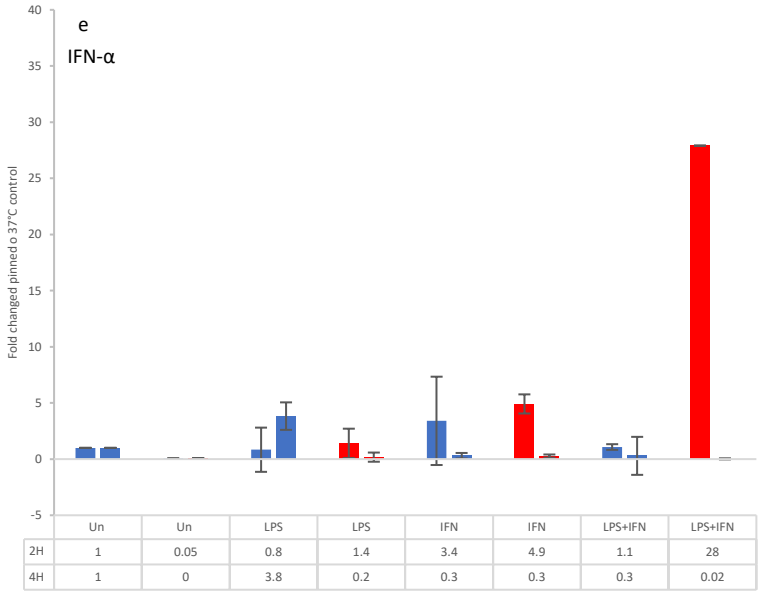
	UN- 37°C	UN-39°C	LPS-37°C	LPS-39°C	IFN-γ-37°C	IFN-γ-39°C	LPS+IFN-γ-37°C	LPS+IFN-γ39°C
2H	1	1.8	241	769	2.7	1.5	324	653
4H	1	2.0	100	213	7.2	0.01	138	41



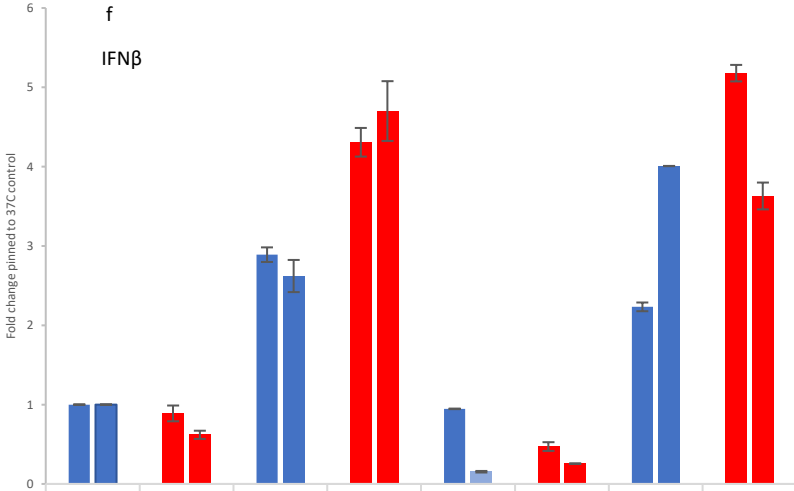
	UN- 37°C	UN-39°C	LPS-37°C	LPS-39°C	IFN-γ-37°C	IFN-γ-39°C	LPS+IFN-γ-37°C	LPS+IFN-γ39°C
2H	1	1.5	4.9	26	0.6	2.7	7.1	24
4H	1.0	0.6	34	11	0.6	0.6	32	93



	UN- 37°C	UN-39°C	LPS-37°C	LPS-39°C	IFN-γ-37°C	IFN-γ-39°C	LPS+IFN-γ-37°C	LPS+IFN-γ39°C
2H	1	1.4	0.3	1.0	1.0	0.7	1.0	2.0
4H	1	1.8	0.2	0.5	0.1	0.01	0.4	0.5

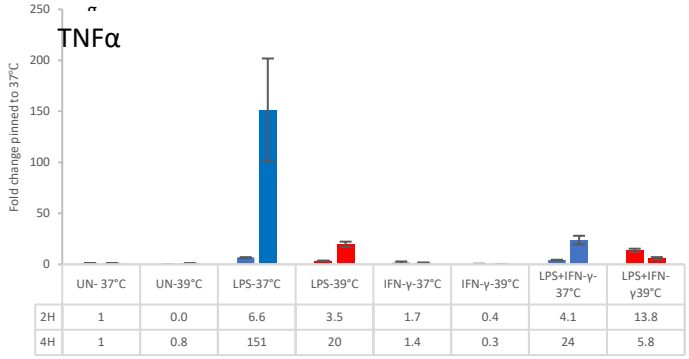


	Un	Un	LPS	LPS	IFN	IFN	LPS+IFN	LPS+IFN
2H	1	0.05	0.8	1.4	3.4	4.9	1.1	28
4H	1	0	3.8	0.2	0.3	0.3	0.3	0.02



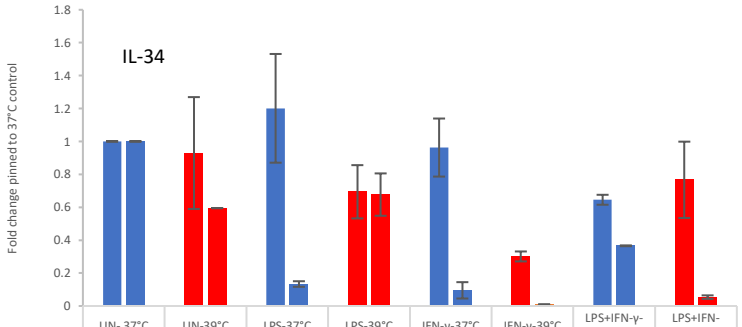
	UN- 37°C	UN-39°C	LPS-37°C	LPS-39°C	IFN-γ-37°C	IFN-γ-39°C	LPS+IFN-γ-37°C	LPS+IFN-γ39°C
2H	1	0.9	2.9	4.3	0.9	0.5	2.2	5.2
4H	1	0.6	2.6	4.7	0.2	0.3	4.0	3.6

we examined its role for the febrile response on IL-12 or IL-1 α and IL-34. IL-12 contributes to cell-mediated immune responses by promoting differentiation of naïve T cells and IFN-γ production. Unstimulated RAW264.7 macrophages produce more IL-12 in response to fever. The only treatment further increases the combination of LPS and IFN-γ at 39°C. Next, we examined whether fever and LPS effect on IL-34 expression in RAW264.7 cells which are essential for on macrophage polarization into M1 and M2 macrophage. Co-stimulation with LPS and IFN-γ did not induced higher levels of expression than either LPS or IFN-γ at 39°C.



	UN- 37°C	UN-39°C	LPS-37°C	LPS-39°C	IFN-γ-37°C	IFN-γ-39°C	LPS+IFN-γ-37°C	LPS+IFN-γ39°C
2H	1	0.0	6.6	3.5	1.7	0.4	4.1	13.8
4H	1	0.8	151	20	1.4	0.3	24	5.8

Figure 8: Effect of fever on pro-inflammatory cytokine expression, IL1α (panel a), IL1β (panel b), IL6 (panel c), IL-12 (panel d) , IFNα (panel e) IFNβ (panel f), TNFα (panel g) measured at 2 and 4 hours, by qRT-PCR relative to β-actin with normalization to the unstimulated 37 C group, in RAW264.7 cells stimulated at 37 C (blue bars) or 39 C (red bars).



	UN- 37°C	UN-39°C	LPS-37°C	LPS-39°C	IFN-γ-37°C	IFN-γ-39°C	LPS+IFN-γ-37°C	LPS+IFN-γ39°C
2H	1	1	1	1	1	0.3	1	1
4H	1	0.6	0.1	0.7	0.1	0.0	0.4	0.1

Figure 9: Effect of fever and activation on expression of IL-34. Unstimulated or LPS [100ng/ml], IFNγ [100 ng/ml] or LPS combination of RAW 264.7 cells were carried out for 2or 4h cDNA from RNA sample from these were tested by RT-qPCR for IL-34 expression. Blue bars 37°C Red bars 39°C.

We were interested in IL-34 because reported to increase expression at high temperature. For RAW246.7 cells this was not the case. Treatment resulted in expression level less than the control group.

Limitations of interpretation of RT-qPCR. Each PCR run as only internally valid for comparisons, repeats often show large variation in absolute fold change but show similar patterns - not between runs- wide differences between repeats. Variations in b-actin -others have tried different solutions, running 3 housekeeping genes but we know that temperature increases metabolism— and at some point, is it hard for cells to make proteins –so those they do make get exaggerated by our computations. On the other hand, fish appear to increase their b-Actin with LPS stimulation, and this makes the same level of increase in the cytokines appear as nothing in our calculations. Therefore, we have included our primary data and are figuring ways to graphically show the B-actin and the genes of interest to watch for coordinated changes and be able to note stability in cytokine expression or not!

More general limitations of RT-qPCR assays:

Using methods that were applicable to all three species meant we did not test for cytokine secretion but instead for level of mRNA production as primers were easily achieved for all three species whereas antibodies and ELISAs or multiplexes were not. Looking at only transcriptional control means that we did not measure post-translational effects on cytokine levels or effects of precursor proteins that need further maturation to be active as IL1-β requires.

Table 5: The cytokine expression to various stimuli over a 2 and 4h period. Increases in 7 of the 9 gene of interest cytokines (iNOS, IL-6, IL-1α, IL-1β, 1L-12, IFN-α, IFNβ, TNF-α and IL-34, at 39°C compared to 37°C baseline was observed. Table R presents cytokine levels at the various study time points at 37 and 39°C with information about the expression of cytokine gene mRNA at RAW264.7 at 37 and 39°C.

Treatment	Temperature [°C]	RUN	Time		
		2H B-actin ± SD	2H iNOS	4H B-actin ± SD	4H iNOS
UN	37	21.2± 0.1	20.6± 3.4	27.8± 1.0	26.8± 0.5
UN	39	17.5± 0.3	23.5±0.5	27.2± 0.9	25.0±0.3
LPS	37	16.1± 2.0	20.3± 0.5	23.9± 0.7	21.6± 0.3
LPS	39	20.9± 0.3	18.2± 0.3	23.2± 0.2	17.1± 0.0
IFN-γ	37	14.3± 0.7	19.3± 0.7	24.3± 0.1	21.0± 0.4
IFN-γ	39	19.1± 0.4	21.6± 0.1	23.2± 0.1	19.4± 0.4
LPS+IFN-γ	37	18.8± 0.7	16.7± 0.3	26.2± 0.3	15.7± 0.3
LPS+IFN-γ	39	23.5± 0.2	15.4± 0.0	24.0± 0.2	14.8± 0.2
		B-actin -IL-6	2H IL-6	4H B-actin ± SD	4H IL-6
UN	37	5.6± 0.1	21.3± 0.1	9.5± 0.0	23.2± 0.7
UN	39	7.6± 0.3	22.6±0.2	9.9± 0.0	24.5±0.3
LPS	37	5.7± 0.1	19.0± 0.0	7.3± 0.2	16.1± 0.0
LPS	39	5.9± 0.2	16.8± 0.0	5.7± 0.1	16.0± 0.1
IFN-γ	37	5.0± 0.2	21.4± 0.2	8.3± 0.0	23.0± 0.3
IFN-γ	39	8.9± 0.2	23.1± 0.2	7.7± 0.0	22.3± 0.5
LPS+IFN-γ	37	6.0± 0.1	18.8± 0.1	6.9± 0.0	15.7± 0.1
LPS+IFN-γ	39	8.1± 0.1	19.2± 0.3	8.2± 0.5	15.6± 0.0
		2H B-actin IL-12	2H IL-12	4H B-actin ± SD	4H IL-12
UN	37	7.0± 0.0	25.2± 0.4	8..4± 0.0	25.7± 0.4
UN	39	6.4± 0.1	24.1±0.4	8.7± 0.1	24.1±0.3
LPS	37	5.9± 0.1	25.8± 0.6	3.8± 0.1	23.4± 0.1
LPS	39	5.1± 0.1	23.3± 0.3	6.9± 0.1	24.9± 0.5
IFN-γ	37	6.1± 0.1	24.2± 0.4	2.6± 0.1	23.8± 0.5
IFN-γ	39	4.6± 0.1	23.2± 0.2	4.1± 0.1	27.4± 0.5
LPS+IFN-γ	37	5.9± 0.1	24.1± 0.1	5.5± 0.0	23.9± 0.4
LPS+IFN-γ	39	8.2± 0.1	25.4± 0.3	3.4± 1.3	22.1± 0.1
		B-actin IFNβ	2H IFNβ	4H B-actin ± SD	4H IFNβ
UN	37	6.7± 0.1	20.3± 0.2	8.1± 0.0	26.6±0.5
UN	39	6.4± 0.1	20.1.0±0.5	8.6± 0.0	22.8±0.1
LPS	37	5.8± 0.1	16.2± 2.3	3.9± 0.1	16.1±0.1
LPS	39	4.9± 0.1	16.3± 0.1	7.1± 0.1	18.3±0.1
IFN-γ	37	6.0± 0.1	19.7± 0.1	2.2± 0.1	18.4±0.1
IFN-γ	39	4.3± 0.1	19.3± 0.2	4.1± 0.1	19.6±0.1
LPS+IFN-γ	37	5.9± 0.1	18.3± 0.1	5.4± 0.0	16.9±0.1
LPS+IFN-γ	39	7.9± 0.1	19.2± 0.0	2.2± 0.3	13.8±0.1
		2H B-actin ± SD	2H IFNα	4H B-actin ± SD	4H IFNα
UN	37	7.2± 0.1	34.7± 4.3	10.4± 0.0	31.0±4.3
UN	39	6.6± 0.1	35.2± 5.0	10.8± 0.0	40.3±0.0
LPS	37	5.9± 0.1	35.5± 3.3	5.7± 0.2	27.8±0.7
LPS	39	5.5± 0.0	31.5± 4.2	9.2± 0.0	32.8±6.4
IFN-γ	37	6.3± 0.1	34.9± 0.8	5.1± 0.1	26.3±0.8
IFN-γ	39	4.2± 0.2	35.6± 5.1	6.0± 0.3	33.1±2.2
LPS+IFN-γ	37	6.3± 0.2	30.1± 0.4	7.6± 0.1	33.5±4.1
LPS+IFN-γ	39	8.4± 0.1	40.3± 1.6	4.6± 0.5	28.5±0.2

		2H B-actin IL-1β	2H **IL-1β**	4H B-actin ± SD	4H **IL-1β**
UN	37	7.2± 0.1	22.9± 0.3	8.0± 0.1	22.3±0.2
UN	39	6.6± 0.1	201.5±0.2	8.2± 0.0	21.4±0.0
LPS	37	5.9± 0.1	13.7± 0.0	3.1± 0.1	10.6±0.3
LPS	39	5.5± 0.0	11.1± 0.0	6.6± 0.1	13.0±0.1
IFN-γ	37	6.3± 0.1	20.4± 0.0	8.3± 0.0	19.6±0.2
IFN-γ	39	4.2± 0.2	19.7± 0.2	3.3± 0.2	24.8±0.2
LPS+IFN-γ	37	6.3± 0.2	13.5± 0.1	4.8± 0.1	11.8±0.1
LPS+IFN-γ	39	8.4± 0.1	14.7± 0.0	3.3± 0.2	12.1±0.1
		2H B-actin IL-1α	2H IL-1α	4H B-actin ± SD	4H IL-1α
UN	37	7.2± 0.1	24.0±0.2	10.4± 0.0	23.5±0.2
UN	39	6.6± 0.1	23.1±0.5	10.8± 0.0	22.3±0.1
LPS	37	5.9± 0.1	16.5±0.1	5.7± 0.2	11.9±0.9
LPS	39	5.5± 0.0	15.0±0.0	9.2± 0.0	15.1±0.0
IFN-γ	37	6.3± 0.1	22.2±0.1	5.1± 0.1	21.2±0.1
IFN-γ	39	4.2± 0.2	22.6±0.2	6.0± 0.3	24.3±0.2
LPS+IFN-γ	37	6.3± 0.2	16.9±0.1	7.6± 0.1	14.7±0.1
LPS+IFN-γ	39	8.4± 0.1	18.3±0.0	4.6± 0.5	14.6±0.3
		B-actin TNF-α	2H TNF-α	4H B-actin ± SD	4H TNF-α
UN	37	6.7± 0.1	16.5±0.9	8.1± 0.0	19.1±0.1
UN	39	6.4± 0.1	20.1±0.1	8.6± 0.0	20.0±0.9
LPS	37	5.8± 0.1	12.2±0.1	3.9± 0.1	9.0±1.8
LPS	39	4.9± 0.1	12.2±0.0	7.1± 0.1	13.8±0.2
IFN-γ	37	6.0± 0.1	14.5±0.8	2.2± 0.1	12.8±0.5
IFN-γ	39	4.3± 0.1	14.7±0.5	4.1± 0.1	17.1±0.5
LPS+IFN-γ	37	5.9± 0.1	12.3±1.0	5.4± 0.0	11.8±0.2
LPS+IFN-γ	39	7.9± 0.1	14.9±2.2	2.2± 0.3	10.7±0.3
		B-actin - IL-34	2H IL-34	4H B-actin ± SD	4H IL-34
UN	37	7.2± 0.1	26.3±0.3	10.4± 0.0	28.0±1.4
UN	39	6.6± 0.1	26.1±0.8	10.8± 0.0	29.2±0.6
LPS	37	5.9± 0.1	25.3±0.5	5.7± 0.2	26.2±0.2
LPS	39	5.5± 0.0	25.4±0.3	9.2± 0.0	27.5±0.4
IFN-γ	37	6.3± 0.1	25.8±0.3	5.1± 0.1	25.4±0.6
IFN-γ	39	4.2± 0.2	25.3±0.1	6.0± 0.3	30.9±0.5
LPS+IFN-γ	37	6.3± 0.2	26.3±0.6	7.6± 0.1	26.7±0.3
		8.4± 0.1	30.0±2.6	4.6± 0.5	26.4±0.0

Priming mouse cell studies.

Age-dependent changes in body weight in male and female C57BL/6J mice

There are some differences in young and aged that at documented here prior to into our functioned metabolic and proinflammatory response experiments as they influenced our data

To determine the effect of age on body weights, young and aged male and female C57BL/6J mice body weights were measured prior to each experiment. An age-associated increase in body weight was observed across both sexes. As shown in (Fig.1a) the average of young male and female body weight was 26.2 ±0.7g and 22.5± 0.2 g, respectively. Young males were on average 3.7 g heavier than females. Aged males gained an average 6.7g to weigh 33.0 ± 0.8 g while aged females gained an average 12.5 g to weigh 34.9 0± 0.7g. Fig 1b plot the differences between individual young and aged male and female mice body weight (g) by age weight is known to may play a role in age-related disorders and diseases.

Priming mouse cell studies.

Having worked out obtained assay condition for our model mouse cell line we moved on to examining the role of fever on priming cultures of mouse macrophage from variers source, differentiated BMDM, PRM, Spleen resident macrophage and even lung resident macrophages. Of were priming macrophage from aged mice as the decline in immune function over time is responsible for much and death, we know that the aged tend to make poor fever response so we wondered if adjusted fever temperature might show a more youthful pattern.

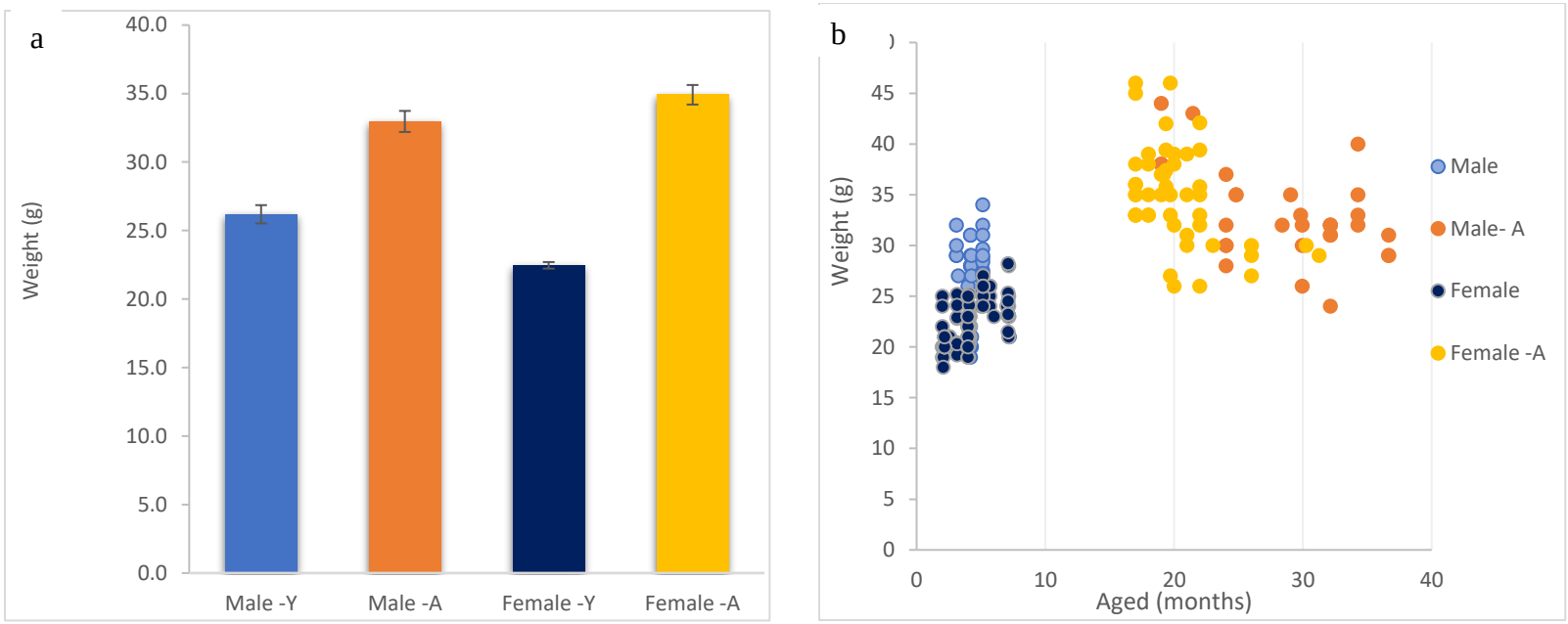


Figure 10: Age-dependent changes in body weight in male and female C57BL/6J mice. Graph a represents body weight in g.Light and dark blue bars represent young males and females respectively, and orange bars represent aged males and yellow bars females. Young males (M-Y, n=39) ranging from 8 to 23 weeks old and aged males (M-A, n=34) ranging from 48 to 116weeks old; young females (F-Y, n=53) ranging from 8 to 22 weeks old and aged females (F-A, n=48) ranging from 75 to 116 weeks old. Data represent mean ± SE. Fig 10b graph the most recent experiments mice from individual young and aged male and female mice weight by age. X axis x is age in months and the y axis shows mice body weight in g. Light blue and dark blue dots represent young males and females respectively, and the orange dots represent aged males and yellow represent aged females.

Bone marrow cell recoveries

The ratio of total cells age to young counted before culture *in vitro* (Fig.11) for age males was 1.77 compared to 1.29 for aged females. The ratio after culture for 7 days to yield bone marrow derived macrophage from progenitor cells (Fig.11b) was 1.21 for aged males compared to 1.93 for aged female.

The efficiency of adherent BMDM a output from total bone marrow (a/b) was 49%for young and 34% for age males and 71% for young females compared with 81% for aged females. Thus, there to a major bias in female to having more cellular bone marrow and those cells are niches in cells responding to M-CSF.

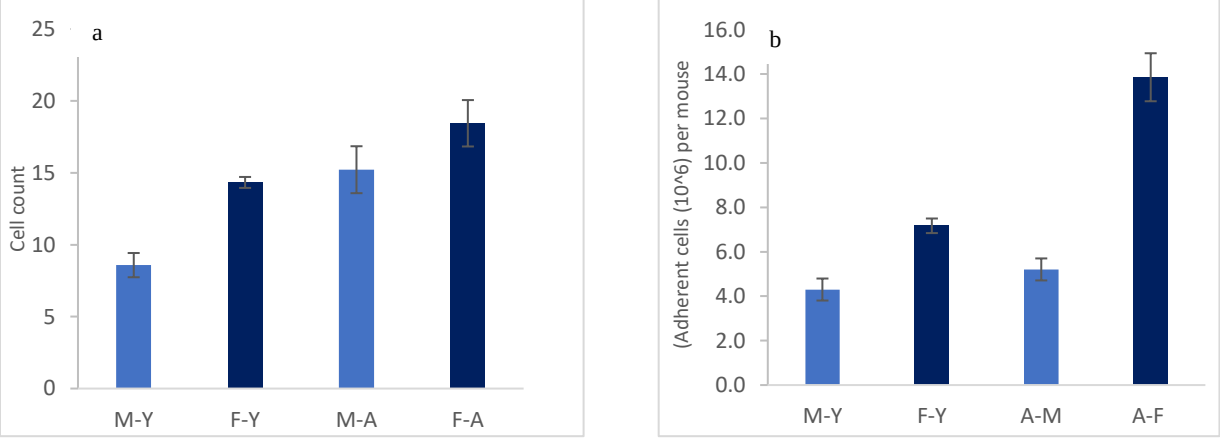


Figure 11: Initial total bone marrow cell recoveries per mouse **are shown in (a).** For young males (M-Y, n=35) ranging from 8 to 23 weeks old and aged males (M-A, n=23) ranging from 48 to 116 weeks old. Young females (F-Y, n=53) ranging from 15 to 22 weeks old and aged females (F-A, n=23) ranging from 75 to 97 weeks old. The average from 6 experiments as well as is shown as the mean ±SEM for males and females.

Flow cytometry was used to examine protein synthesis and MHC class II induction in young and aged BMDM.

The effect of effect of temperature on macrophage protein synthesis bone marrow cells were differentiated into macrophages and then cultured with proinflammatory stimulation for 4h.

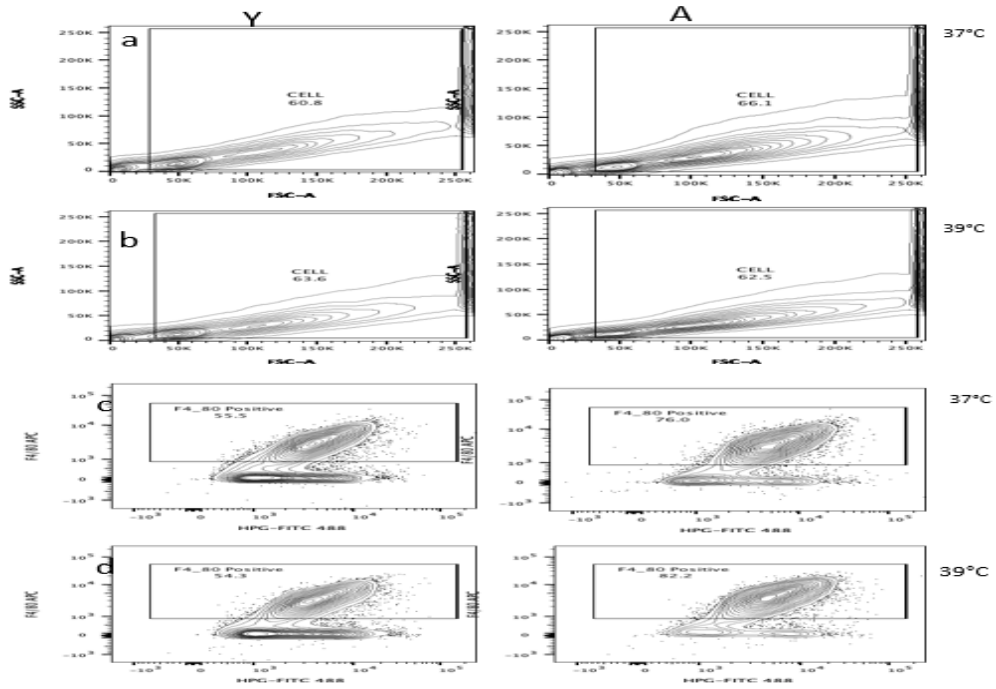


Figure 12: Gating strategy used to identify young and aged female BMDM F4-80 positive cells. Cells were first selected for by utilizing forward scatter (FSC) on the x axis and side scatter (SSC)on the y axis signals to exclude debris and clumps of cells from the analysis. The gate for cells is shown in (a) at 37°C and b at 39°C for unstimulated samples. The plots show young BMDM on the left and aged on the right. We identified macrophages by selecting the F4/80 - APC positive cells (y axis) as shown in c and d with FSC on the x axis. The numbers are the percentage F4/80 positive cells compared to total cells. Plots for 37°C are shown in b and for 39°C in a &c and b& d

After selecting these gates, we examined all samples and found that (data not shown). The level of expression of F4/80 on individual cells was constant regardless of temperature or treatment after 4h of stimulation. However, the percentage of F4/80 positive cells averaging from 65.7 to 66.0 at 37°C and 39°C respectively for the young and from 79.2 to 82.9 for the aged (Table 6) showed some variation. Overall aged BMDM cells show a greater number of F4/80 positive cells of (82.9%) compared to the young (66.0%) averaged over all the treatment conditions. Overall, the recoveries of young and aged cells were roughly equivalent after 4h stimulation. The most prominent pattern was the increase in F4/80 positive cells in the aged cells at the higher

temperature. For the aged LPS had the most positive effect on MHCII expression independently and in the combination with IFN-y. There was little to no increase in the young in 4h in MHC class II. The LPS treatment showed increase in protein synthesis at 39°C for the young and a temperature for the aged while the IFN-y alone and combination treatments did not show protein synthesis increase (figure 13). For BMDM the two parameters varied independently.

Table 6: The percentage of F4/80 positive cells for young and aged BMDM after 4h of stimulation at 37°C or 39°C

			LPS					
	°C	Un	0.05	0.1	1.0	3	0.1 IFN-γ	LPS+ IFN-γ
Young	37 39	59.6 58.1	73.1 70.3	67.4 65.0	59.6 66.1	60.4 63.7	70.8 68.7	68.7 70.3
Aged	37 39	79.4 85.8	83.0 87.2	83.6 85.8	76.5 80.1	73.0 79.3	79.0 82.5	80.1 79.5

Raising the temperature had no effect on the background protein synthesis of young or aged cells. The only treatment condition that increases protein synthesis was LPS which showed a greater increase for the young then the aged.

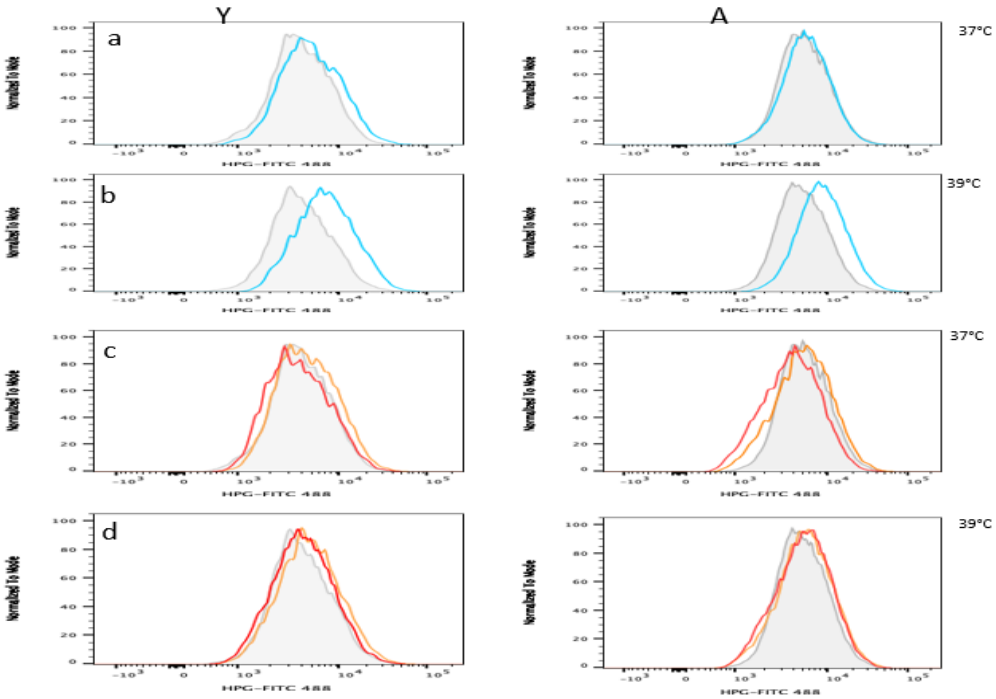


Figure (13) Effect of temperature in protein synthesis from young and aged BMDM cells gated from being (Figure 12). F4-80 positive macrophages were analyzed for protein synthesis with increasing HPG incorporation on the x axis. The peaked show young on left and aged on the right, 37°C for a and c, 39°C for b and d. The normalized histogram shows untreated background histogram in gray, 0.1 µg/ml LPS in blue (a and b), 0.1 µg/ml IFN-y shown in orange and combination of LPS and IFNy shown in red (c and d).

Unstimulated young BMDM show a greater level of MHC class II expression then the aged but no temperature differences in expression. Young BMDM show small shift to the right that is with all LPS treatments at both temperatures with the exception of highest dose (3µg/ml) which

becoming toxic to young cells. For the aged with all LPS treatments including the highest dose and combination treatments at 37°C and at 39°C IFN-y alone more dramatically also increase MHCII expression. The levels of induced MHCII expression in the aged are higher at 37°C than at 39°C.

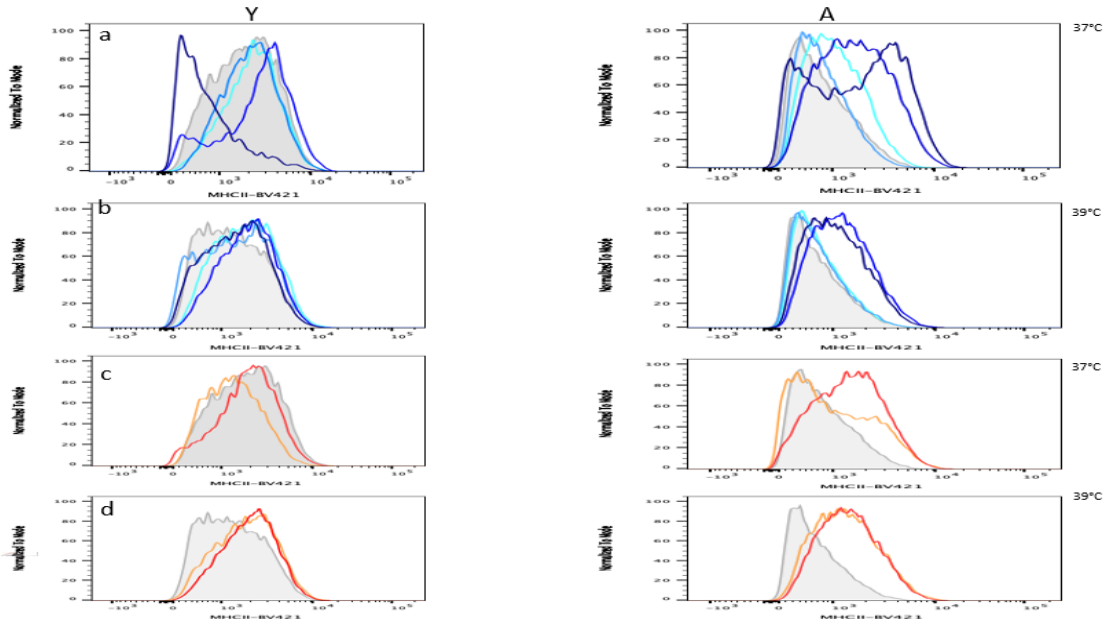


Figure (14) Effect of temperature on MHCII expression from young and aged BMDM F4-80 positive cells as gated in Figure 12 were analyzed for MHC class II expression for level of anti-mouse I-Ab -APC staining. **The histograms show young on the left and aged on the right, 37°C for a and c, 39°C for b and d. Untreated background is shown in gray, 0.05 -3 µg/ml LPS shown in light to dark blue (a and b), 0.1 µg/ml IFN-y shown in orange and combination of LPS and IFNy shown in red (c and d).**

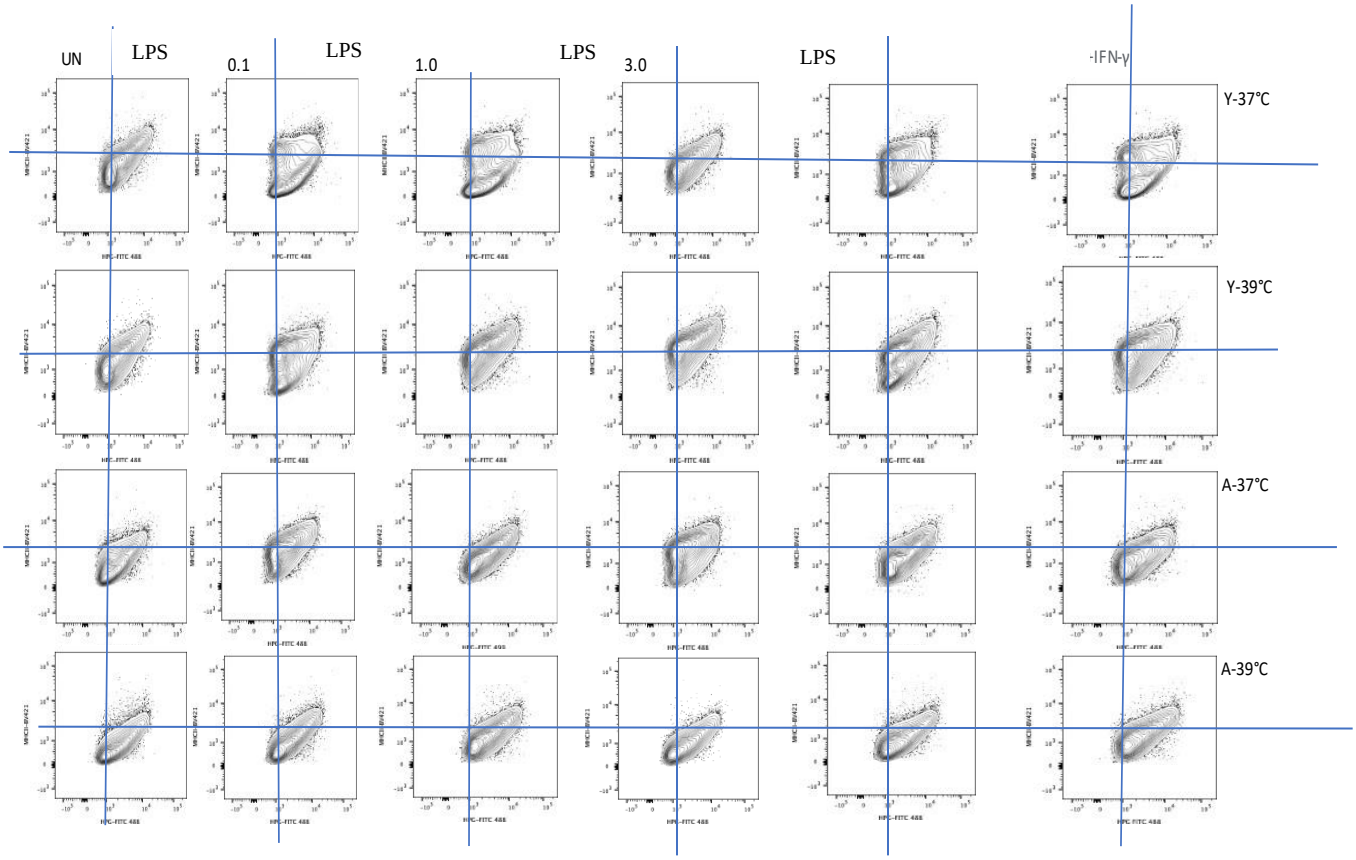


Figure (15) Flow cytometric analysis of protein synthesis and MHC class II expression for young and aged female mice. BMDM cells from young (ranging from 15 to 19 weeks old) and aged females (from 75 to 88 weeks old) were pooled, respectively) were utilized. Bone marrow from both young and aged C57BL/10 mice was extracted and cultured in M-CSF for one week with feeding on d3. Adherent BMDM were harvested cultured at $1x10^5$ cells in each well in stimulated with or without LPS at different concentrations (0.05 to 3µg/ml) or IFN-γ (0.1µg/ml) or combination as described in the Methods section for 4h at 37°C and 39°C. MHC class II expression was determined using mouse anti mouse I -A[b] – BV421 color flow cytometric analysis. In both young and aged BMDM cells forward scatter FSC and side scatter SSC signals were used to exclude debris and clumps of cells from the analysis gate (Fig Ba). F4-80 positive cells were gated (Fig B bc) and then analyzed for changes in MHCII expression and protein synthesis. F4/80 positive cells ranged from 58% to 70% for young and 79.5% to 87% for aged. First row: Young untreated BMDM cell (Un) with 4 different LPS concentrations (0.05 to 3µg/ml) or IFN-γ or the combination LPS and IFN-γ at 37°C; second row: young at 39°C; third row: aged at 37°C and the four row: aged at 39C °

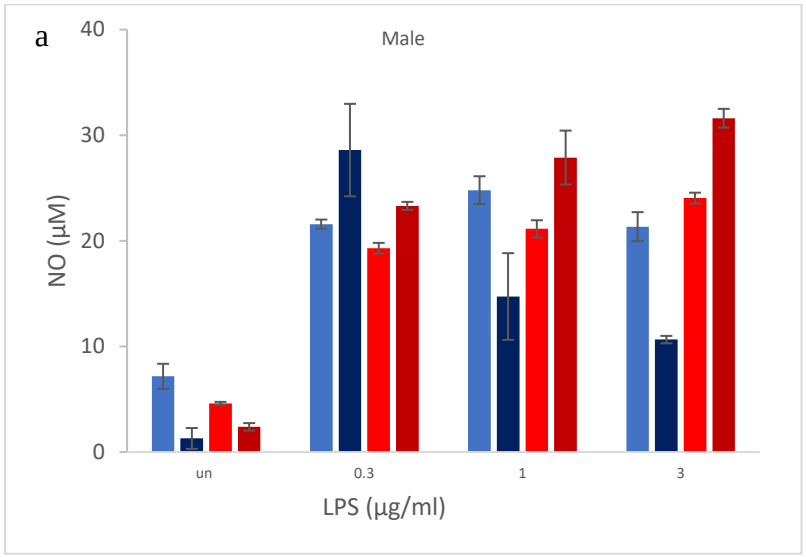
Temperature effect on LPS and IFN-γ -stimulated NO production in bone marrow derived macrophage for young and aged mice.

NO production in unstimulated BMDM cells from young and aged male and female mice at 37 and 39°C was low (figure 6a+b). On a per cell basis young male adherent bone marrow cells showed no temperature effect on NO production at 39°C Fig 6. Until the highest LPS dosage (3.0 µg/µl) where the increase is small With LPS concentrations (1.0 and 3.0 µg/µl) aged male bone marrow adherent cells show higher in NO production at 39°C compared to 37°C. Since per mouse basis aged male adherent cell recoveries are 1.7 times higher than those from young males and an enhanced recovery of 1.2 times the young male macrophages after culture. On a per mouse basis the NO response is equivalent or greater in aged males than the young males.

With stimulations incubation at 39°C consistently induced higher in NO production in both young and aged cells from females. On a per cell basis the aged female showed responses equivalent or better than young female cells in all conditions Fig 16b. On a per mouse basis given an increased cell yield of 1.5 times the young from aged female mice and an enhanced recovery of 1.8 times the young from aged female mice after a week in M-CSF, the NO response is more than 2.7 times greater in the aged. When we differentiate macrophages from young and aged bone marrow, we see no deficit in production of macrophages or in ability to make NO. The greater numbers of macrophages progenitors on per mouse basis indicate a likelihood greater overall inflammation in the aged specially if stimulates.

While it is tempting to interpret the lower male responses on a per cell basis as important, there are reasons that may only be technical for these results. Over time we changed our methods of harvesting adherent cells early on we only used mechanical scraping, we used 0.05% trypsin with examination under the microscope to determine the length of time of exposure to the enzyme and we settled on Ca^{+2} and Mg^{+2} free medium and scraping for removal. Also, variations in the batch of FBS + LPS can change the overall responsiveness of cells. Further we were not testing males and females at the same time because it is too labor-intensive adherent cell harvesting. he male BMDM in figure 16a from an earlier experiment.

Over three experiments (see supplementary data Fig BM 1 and 2) using a variety of LPS concentrations both young and aged female BM showed increase at 39°C compared to 37°C in 15 of 15 total trails. Over the same three experiments, IFN- y stimulation showed increases at 39°C in 7 of 7 trails for the young and aged female cells. For the combination of LPS and IFN-y a temperature dependent increase was seen in the one trail. All three experiments showed high NO production with the maximum for these experiments ranging from 100 to 120 μM NO.



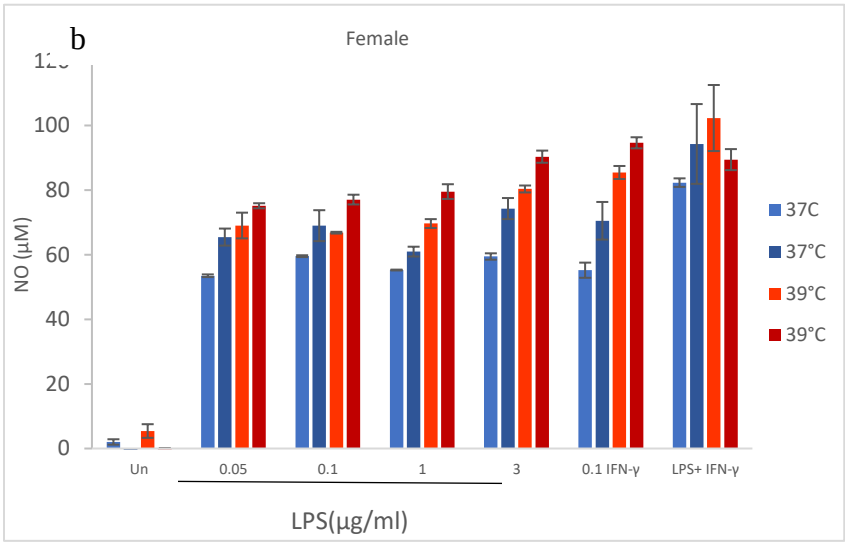
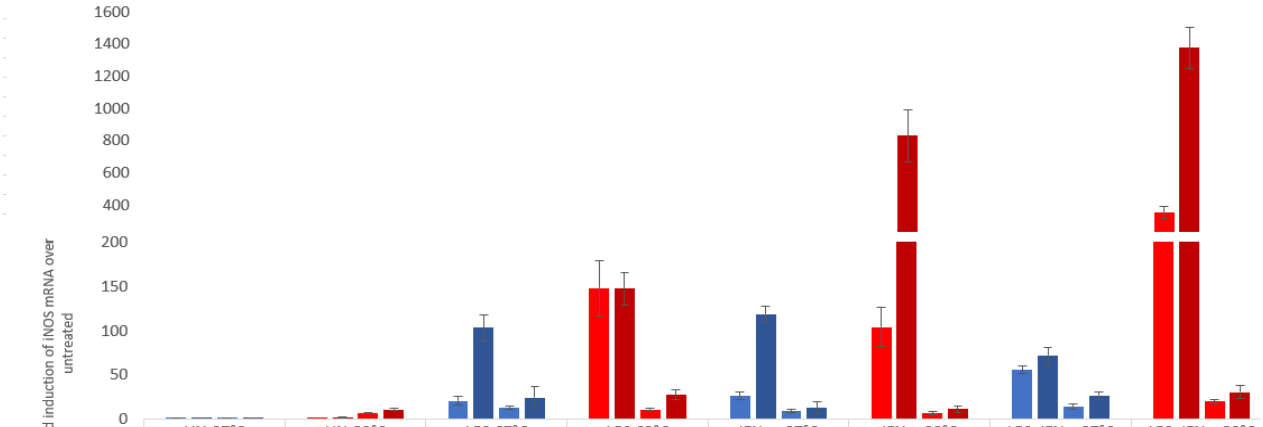


Figure16: Temperature effect on LPS and IFN-γ stimulated NO production in bone marrow derived macrophage for young and aged mice. BMDM from young and aged males (16 weeks old and 138 weeks old, respectively) and young and aged females (ranging from 15 to 22 weeks old and from 75 to 97 weeks old, respectively) were utilized. In (a) Male BMDM were cultured at 5x105in each well in stimulated with or without LPS at different concentrations (0.3 to 3µg/ml. In (b) female BMDM were stimulated with and without LPS at different concentrations (0.05 to 10 µg/ml), IFN-y (0.1 µg/ml) or a combination of 0.1 µg/ml of both for 24 h at 37°C and 39°C and supernatant tested by Griess reagent for NO. The light blue bar represents young BMDM at 37°C. The dark blue bar represents aged BMDM at 37°C. The light red bar represents young BMDM at 39°C and the dark red bar aged BMDM at 39°C. Data are mean ± SE of triplicate determinations. The results shown are representative of 3 different experiments for females (maximum 100, 120 and 120 µM/ml NO) and one experiment for males (maximum 40 µM/ml NO).

Effect of fever temperature on iNOS production by BMDM.

Our Griess assay initiated a temperature effect is observed on NO production over 24h period and that iNOS must be induced to further investigate RT- qPCR analysis of iNOS mRNA levels was used to see if fever temperature directly affected iNOS expression (RT-qPCR) for young and aged male mice BMDM cells at 2 +4h. Fever temperatures increased the expression and speed of iNOS gene expression in macrophages as shown in Figure 17. In comparison to 37°C, unstimulated BMDM from aged mice at 39°C enhances iNOS mRNA synthesis slightly mor than young at 39°C with all treatment show increased iNOS mRNA with a peak at 2h production compared to 37°. C



	UN-37°C	UN-39°C	LPS-37°C	LPS-39°C	IFN-γ- 37°C	IFN-γ-39°C	LPS+IFN-γ 37°C	LPS+IFN-γ 39°C
2H-Y	1	0.3	20	148	26	104	56	355
2H-A	1	1.5	103	147	119	829	72	1375
4H -Y	1	6.4	13	11	8.9	6.3	14	20
4H-A	1	11	24	27	13	11	26	31

Figure 17: The effect of fever on iNOS mRNA production by BMDM in young and aged male mice. Duplicate samples of BMDM cells (2x106 cells in each well) were stimulated with LPS [0.1µg/ml], IFN-γ [0.1µg/ml] and LPS plus IFN-γ at 37 and 39°C for 2h and 4h. Samples were pooled, RNA purified and used for templates for cDNA, and run-in triplicate for RT- qPCR. Light and dark blue bar represents 15-week-old young and aged 75-week-old male mice, respectively. Light and dark blue bar represents 4h incubation time. For each treatment group in the Fig 16 the 2h samples are shown on the left and the 4h pair on the right. Light and dark red bars represent young and aged BMDM. Relative fold change in expression levels is normalized to the 37°C unstimulated control.

When compared to individual treatments, IFN-y alone at 39°C gave responses above LPS alone

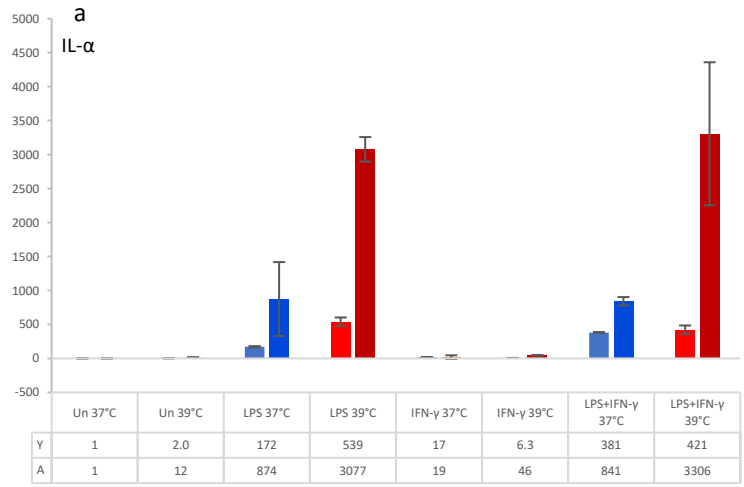
at 2h, the combination of LPS and IFN-g produced the most iNOS mRNA. We saw a similar

increase with IFN-y alone at 6h at 39°C when examining RAW 264.7 cells Figure (7) however with BMDM the kinetics of expression was much faster peaking at 2h. The peak for LPS only treated young BMDM lagged behind at 4h.

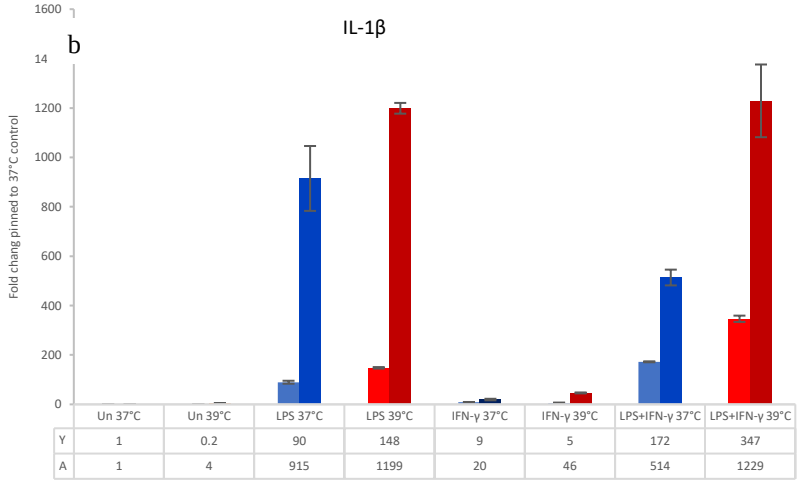
The effect of fever temperature on proinflammatory cytokine expression by young and aged BMDM

To determine if temperature exhibits widespread upregulation of proinflammatory cytokine mRNA in BMDM we tested young and aged BMDM by RT-qPCR at 2 hr. In young untreated BMDM the levels of IL-1α, IL-1β, IL-6 are unchanged at 39°C compared to 37°C [Figure 18]. However, in the aged there is a major increase in IFN-α expression and there are small increases seen at 39°C in the expression of IL-1a, IL-1b, IL-6, IL-12, and IFN-β. Fever increases in LPS, or the combination treatment were observed for IL-1a, IL-1b, IL-6, IL-12 and IFN-b for both young and aged with aged showing overall higher responses. TNF-a was unusual in that the greatest responses were seen in the young with LPS. It is possible that under fever conditions.

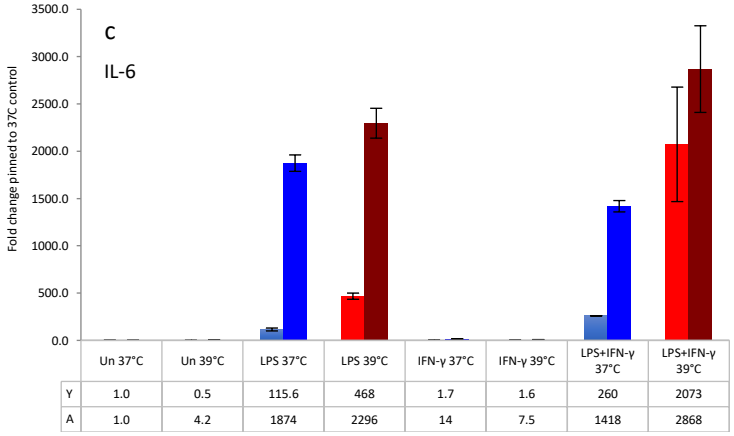
and with the combination treatment that the response peaked before the 2h assay point. Therefore, at this point, the data points to widespread upregulation of proinflammatory cytokine mRNA with fever temperatures.



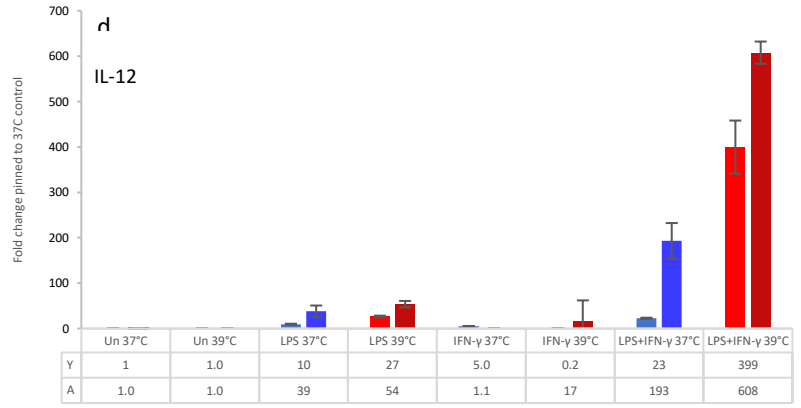
	Un 37°C	Un 39°C	LPS 37°C	LPS 39°C	IFN-γ 37°C	IFN-γ 39°C	LPS+IFN-γ 37°C	LPS+IFN-γ 39°C
Y	1	2.0	172	539	17	6.3	381	421
A	1	12	874	3077	19	46	841	3306



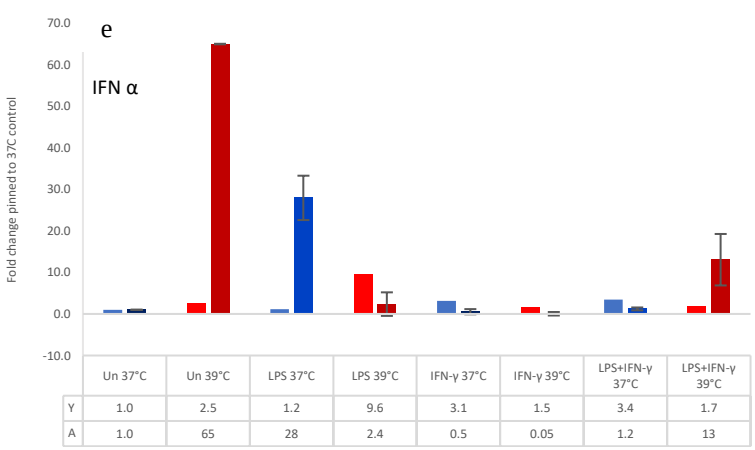
	Un 37°C	Un 39°C	LPS 37°C	LPS 39°C	IFN-γ 37°C	IFN-γ 39°C	LPS+IFN-γ 37°C	LPS+IFN-γ 39°C
Y	1	0.2	90	148	9	5	172	347
A	1	4	915	1199	20	46	514	1229



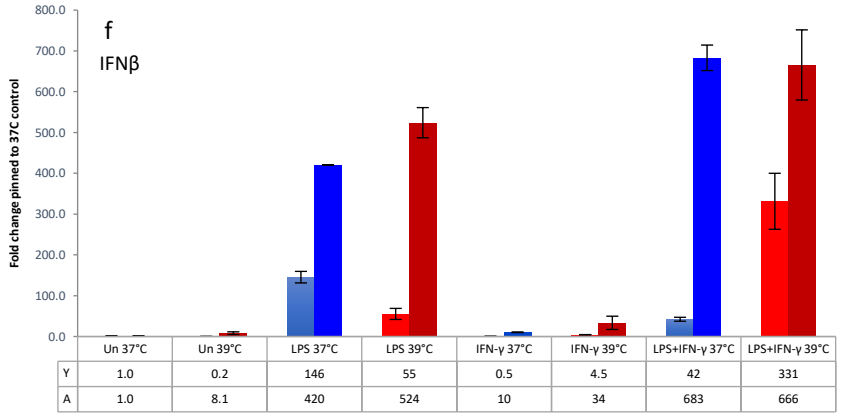
	Un 37°C	Un 39°C	LPS 37°C	LPS 39°C	IFN-γ 37°C	IFN-γ 39°C	LPS+IFN-γ 37°C	LPS+IFN-γ 39°C
Y	1.0	0.5	115.6	468	1.7	1.6	260	2073
A	1.0	4.2	1874	2296	14	7.5	1418	2868



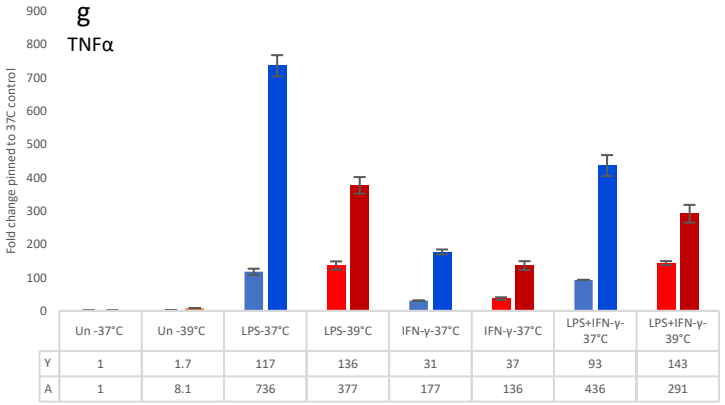
	Un 37°C	Un 39°C	LPS 37°C	LPS 39°C	IFN-γ 37°C	IFN-γ 39°C	LPS+IFN-γ 37°C	LPS+IFN-γ 39°C
Y	1	1.0	10	27	5.0	0.2	23	399
A	1.0	1.0	39	54	1.1	17	193	608



	Un 37°C	Un 39°C	LPS 37°C	LPS 39°C	IFN-γ 37°C	IFN-γ 39°C	LPS+IFN-γ 37°C	LPS+IFN-γ 39°C
Y	1.0	2.5	1.2	9.6	3.1	1.5	3.4	1.7
A	1.0	65	28	2.4	0.5	0.05	1.2	13



	Un 37°C	Un 39°C	LPS 37°C	LPS 39°C	IFN-γ 37°C	IFN-γ 39°C	LPS+IFN-γ 37°C	LPS+IFN-γ 39°C
Y	1.0	0.2	146	55	0.5	4.5	42	331
A	1.0	8.1	420	524	10	34	683	666



	Un -37°C	Un -39°C	LPS-37°C	LPS-39°C	IFN-γ-37°C	IFN-γ-37°C	LPS+IFN-γ-37°C	LPS+IFN-γ-39°C
Y	1	1.7	117	136	31	37	93	143
A	1	8.1	736	377	177	136	436	291

Figure 18. Effect of fever and activation on expression of pro-inflammatory cytokine genes. Duplicate samples of BMDM cells (2x106 cells in each well) were stimulated with LPS [0.1µg/ml], IFN-γ [0.1µg/ml] and LPS plus IFN-γ at 37 and 39°C for 2h. Samples were pooled, RNA purified and used for templates for cDNA, and run-in triplicate for RT- qPCR. Light and dark blue bars represent 15-week-old young and aged at 75-week-old male mice at 37°C and light and dark red bars for young and aged at 39°C, respectively. All cytokines measured at 2 for BMDM of both young and aged mice, by RT-qPCR relative to beta-actin with normalization to the unstimulated 37°C group. IL- α showed in (panel a), IL-1β (panel b), IL-12 (panel c) IFN-α (panel d) IFN-β (Panel e) , IL-12 (panel f) and TNF α (Panel g).

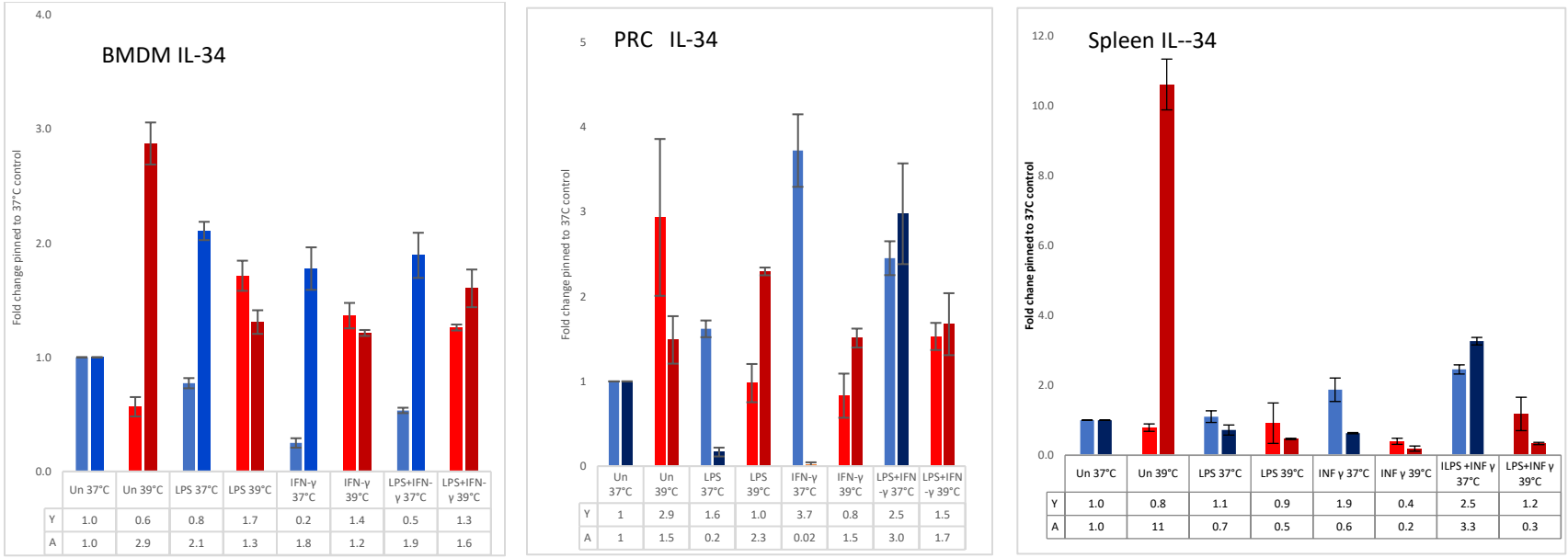


Figure 19. Effect of fever and activation on IL34 genes expression.Duplicate samples of BMDM, PRC and spleen cells (2x106 cells in each well) were Unstimulated or stimulated with LPS [0.1µg/ml] or IFNγ [0.1µg/ml] or the combination at 37 and 39°C for 2h Samples were pooled, RNA purified and used for templates for cDNA, and run-in triplicate for RT- qPCR. Light and dark blue bars represent young and aged mice at 37°C and light and dark red bars for young and aged at 39°C, respectively.

Aged vs young.

Numbers of macrophages are up in aged animals in BM and spleen [Steven (Bloomer & Moyer) and PRC. In terms of function BMDM show milder changes than tissue resident macrophages from peritoneum or spleen. For example, phagocytosis of young and aged BMDM is roughly equal and there is a decrease in Il-1b with LPS stimulation whereas aged PRC and spleen have decreased cytokine expression (Jackaman et al.) 2013, (Mahbub, Deburghgraeve Cr Fau - Kovacs, & Kovacs) 2012) and phagocytosis (Eimear Linehan 2014)) responses are increased to some stimuli amongst the aged. For example, GM-CSF increases NO while LPS response are unchanged (Dimitrijević et al.) 2016) and some responses seem to have relaxed or even mixed up their usual outcome, such as IL-4 inducing IL-6 production in the aged (Dimitrijević et al, 2016). Jackaman called the aged PRC hypersensitive to stimuli (Jackaman et al.) 2013)The pathways for these

As these aged mice have a greater weight especially the females, let's consider obesity and glucose levels. The increases in weight even in female mice are not sufficient for them to be considered obese (Pettersson,et al, 2012) Our animals are lower in weight than all the experimental models that examine effects of obesity. This doesn't mean our findings are not influenced by or even an effect of fat. It just means that our investigations are on animals that are considered healthy regarding their weight and our animals are healthy -as they are not showing signs of illness. Similarly, it is known that short term increases in glucose results in high IL-1b and TNFa with decreases in IL-12 and NO and long-term increases in glucose increase macrophage cytokines but decreases phagocytosis. Metabolically high glucose decreases the reprograming to glycolysis. (Pavlou, 2018).

Treatment	Temperature [°C]	Y		A	
		B-actin ±SD	iNOS±SD	B-actin ±SD	iNOS±SD
UN	37	10.6 ± 0.0	25.0 ± 0.9	10.6 ± 0.0	27.3 ± 0.6
UN	39	10.4± 0.1	26.8± 0.6	10.7± 0.0	26.8± 0.5
LPS	37	15.1± 0.1	25.2± 0.3	14.0± 0.0	24.1± 0.2
LPS	39	14.8± 0.1	22.1± 0.3	11.9± 0.1	21.4± 0.2
IFN-γ	37	12.0± 0.2	21.8± 0.2	12.4± 0.0	22.4± 0.2
IFN-γ	39	15.6± 0.3	23.3± 0.3	15.2± 0.0	22.2± 0.3
LPS+IFN-γ	37	11.5 ± 0.3	20.1 ± 0.1	9.6 ± 0.1	20.2 ± 0.2
LPS+IFN-γ	39	14.6± 0.3	20.1± 0.1	12.9± 0.0	19.2± 0.1
		B-actin ±SD	IL-6 ±SD	B-actin ±SD	IL-6 ±SD
UN	37	4.6 ± 0.3	17.0 ± 0.0	5.3 ± 0.1	18.7 ± 1.2
UN	39	4.2± 0.2	17.7± 1.7	6.4± 0.1	17.9± 0.1
LPS	37	2.9± 0.7	8.5± 0.2	5.2± 0.3	7.7± 0.1
LPS	39	3.1± 0.8	6.7± 0.1	4.2± 0.2	6.5± 0.1
IFN-γ	37	2.4± 0.1	14.2± 0.1	4.9± 0.2	14.5± 0.1
IFN-γ	39	3.5± 1.5	15.3± 0.2	5.1± 0.4	15.6± 0.2
LPS+IFN-γ	37	2.4 ± 0.1	6.8 ± 0.0	4.3 ± 0.1	7.2 ± 01
LPS+IFN-γ	39	4.4± 0.8	5.8± 0.4	3.8± 0.8	5.9± 0.3
		B-actin ±SD	IL-1α±SD	B-actin ±SE	IL-1α±SD
UN	37	12.5 ± 0.6	25.2 ± 0.2	11.5 ± 0.5	26.3 ± 0.9
UN	39	10.2± 0.1	21.9± 1.4	10.0± 0.5	21.5± 0.2
LPS	37	10.7± 0.1	15.3± 0.9	12.3± 0.3	16.9± 0.6
LPS	39	10.4± 0.3	14.1± 0.2	7.2± 0.2	10.4± 0.1
IFN-γ	37	11.5± 0.0	20.1± 0.2	10.5± 0.3	20.4± 0.9
IFN-γ	39	10.9± 0.2	21.0± 0.2	6.6± 0.2	15.9± 0.3
LPS+IFN-γ	37	11.1 ± 0.4	15.2 ± 1.0	9.5 ± 0.2	14.7 ± 0.1
LPS+IFN-γ	39	8.3± 0.2	11.6± 0.9	8.4± 0.2	11.3± 0.4
		B-actin ±SD	IL-1β±SD	B-actin ±SD	IL-1β±SD
UN	37	12.5 ± 0.6	24.3 ± 0.2	11.5 ± 0.5	24.8 ± 0.3
UN	39	10.2± 0.1	24.3± 0.1	10.0± 0.5	21.0± 0.1
LPS	37	10.7± 0.1	16.1± 0.1	12.3± 0.3	16.0± 0.5
LPS	39	10.4± 0.3	15.0± 0.0	7.2± 0.2	10.2± 0.1
IFN-γ	37	11.5± 0.0	20.2± 0.1	10.5± 0.3	18.5± 1.4
IFN-γ	39	10.9± 0.2	20.3± 0.4	6.6± 0.2	14.3± 0.1
LPS+IFN-γ	37	11.1 ± 0.4	15.5 ± 1.7	9.5 ± 0.2	13.8 ± 0.1
LPS+IFN-γ	39	8.3± 0.2	11.6± 0.1	8.4± 0.2	11.4± 0.2
		B-actin ±SD	IFNα±SD	B-actin ±SD	IFNα±SD
	37	11.5 ± 0.4	36.0 ± 0.4	10.9 ± 0.3	35.5 ± 02.8
UN	39	9.8± 0.4	23.9± 0.7	15.7± 0.3	34.0± 0.0
LPS	37	9.5± 0.2	33.8± 0.2	12.1± 0.4	31.7± 0.3
LPS	39	12.5± 0.3	33.8± 0.4	10.2± 0.1	33.3± 2.0
IFN-γ	37	10.5± 0.2	33.3± 0.5	9.8± 0.2	35.0± 1.7
IFN-γ	39	9.4± 0.2	33.3± 0.7	9.6± 0.2	38.4± 3.0
LPS+IFN-γ	37	10.1 ± 0.2	32.7 ± 0.5	10.1 ± 0.1	34.2 ± 0.4
LPS+IFN-γ	39	7.1± 0.2	30.7±2.2	11.7± 0.3	32.4± 0.7
		B-actin ±SD	IFN β±SD	B-actin ±SD	IFNα±SD
UN	37	4.6 ± 0.3	21.1 ± 0.0	5.3 ± 0.1	23.2 ± 0.2
UN	39	4.2± 0.2	22.6± 0.5	6.4± 0.1	21.5± 0.5
LPS	37	2.9± 0.7	12.2± 0.1	5.2± 0.3	11.3± 1.0
LPS	39	3.1± 0.8	12.5± 0.3	4.2± 0.2	12.2± 1.3
IFN-γ	37	2.4± 0.1	20.1± 0.1	4.9± 0.2	19.5± 0.1
IFN-γ	39	3.5± 1.5	17.8± 0.2	5.1± 0.4	17.8± 0.8
LPS+IFN-γ	37	2.4 ± 0.1	11.5 ± 0.2	4.3 ± 0.1	12.9 ± 01
LPS+IFN-γ	39	4.4± 0.8	12.5± 0.4	3.8± 0.8	13.2± 0.5

		B-actin ±SD	IL-12±SD	B-actin ±SD	IL-12±SD
UN	37	10.3 ± 0.6	28.9 ± 0.2	11.9 ± 0.8	21.0 ± 0.9
UN	39	10.1± 0.1	37.0± 0.1	17.5± 0.3	26.7± 0.3
LPS	37	11.4± 0.1	26.2± 0.2	17.2± 1.2	21.4± 0.5
LPS	39	12.3± 0.3	25.7± 0.1	18.0± 3.7	21.6± 0.2
IFN-γ	37	14.0± 0.0	29.6± 0.2	12.7± 0.1	22.1± 0.1
IFN-γ	39	6.8± 0.2	27.7± 0.3	19.1± 0.9	24.5± 1.9
LPS+IFN-γ	37	10.4 ± 0.4	24.1 ± 0.1	14.9 ± 0.2	16.8 ± 0.3
LPS+IFN-γ	39	10.9± 0.2	20.4± 0.2	13.9± 0.3	14.5± 0.5
		B-actin ±SD	IL-34±SD	B-actin ±SD	IL-34±SD
UN	37	4.6 ± 0.3	19.8 ± 0.1	5.3 ± 0.1	21.6 ± 0.1
UN	39	4.2± 0.2	20.3± 0.2	6.5± 0.1	21.4± 0.1
LPS	37	3.5± 0.1	19.1± 0.1	5.3± 0.3	20.6± 0.1
LPS	39	4.1± 0.3	18.6± 0.1	4.4± 0.2	20.4± 0.2
IFN-γ	37	2.5± 0.0	19.7± 0.2	4.9± 0.2	20.6± 0.2
IFN-γ	39	3.5± 1.5	18.3± 0.3	4.9± 0.4	20.0± 0.0
LPS+IFN-γ	37	2.4 ± 0.1	18.5 ± 0.1	4.3 ± 0.1	19.8 ± 0.1
LPS+IFN-γ	39	4.0± 2.2	18.9± 0.0	4.9± 0.2	20.6± 0.1
		B-actin ±SD	TNFα ±SD	B-actin ±SD	TNFα ±SD
UN	37	4.9 ± 0.2	15.0 ± 1.0	5.2 ± 0.1	16.7 ± 0.2
UN	39	4.2± 0.2	13.5± 0.0	6.5± 0.1	14.9± 0.1
LPS	37	5.1± 0.1	8.3± 0.1	5.3± 0.3	7.2± 0.1
LPS	39	4.1± 0.3	7.2± 0.1	4.4± 0.2	7.3± 0.2
IFN-γ	37	4.5± 0.1	9.6± 0.0	4.9± 0.2	8.9± 0.1
IFN-γ	39	3.5± 1.5	8.5± 0.2	4.9± 0.4	9.2± 0.1
LPS+IFN-γ	37	3.8 ± 0.0	7.4 ± 0.1	4.3 ± 0.1	7.0 ± 0.1
LPS+IFN-γ	39	5.2± 0.1	8.1± 0.0	4.9± 0.2	7.6± 0.1

Table 7: RT-qPCR Cytokine expressions analysis of stimulated BMDM isolated from young and aged mice. Incubation at fever or control temperatures was substantially for 2 h. Data are the values shows CT ± Standard Deviation. This data is included as the b-actin varies between sample with both temperature and treatment showing difference.

Temperature effect on LPS-stimulated Nitric oxide (NO) production in peritoneal resident macrophage (PRC) for young and aged mice.

Cell recoveries

Peritoneal resident cells from 6 or 7 young and 3 or 4 aged mice were harvested pooled and viable cells counted. Result for 6 independent experiments is shown in (Fig 20 a and b) The aged had a 4 to 5- fold increase in cell number in the peritoneum compared with young mice. the number of adherent cells was also increased (panel b) but this does not necessarily mean these are macrophage. We tested bacterial growth from supernatant and directly from peritoneal cavity on three different kinds of agar plates tryptic soy (TS) Agar, MacConkey agar and Luria Bertani (LB) agar,and detected no growth. This makes less likely acute infection as the cause the increase in cell number. However, only small number of bacterial species are culturable by these methods. Our result may be explained by the findings (Thevaranjan et al., 2017) who demonstrate age related microbiota changes drive intestinal permeability resulting in leakage of microbes into the peritoneal cavity. Thus, we expect to see age associated inflammation in this tissue which would result in the influx of immune cells. An increase of macrophage responses in absence of overt additional stimulation may be seen and possibly a decrease in macrophage functions may be seen after this possible prolonged and chronic activation (Conley et al., 2016) PRC with aging have continuous exposure to both pro-and anti-inflammatory cytokines. With aging there are increases in serum IL-1b , IL-6 and TNFa but also IL-10 (Alvarez-Rodríguez, López-Hoyos M Fau - Muñoz-Cacho, Muñoz-Cacho P Fau - Martínez-Taboada, & Martínez-Taboada) 2012) this will entail shifts in homeostasis and starting point for macrophage responses. PRC numbers are thought to be controlled by both self-renewals driven by TNF-a (Covarrubias et al., 2020) (Loukov, Naidoo, Puchta, Marin, & Bowdish) 2016, (Kale, Sharma, Stolzing, Desprez, & Campisi, 2020; Kim et al., 2020). of the yolk sac cells that initially seeded

the peritoneum (Gomez Perdiguero et al., 2015)]and by gains from cell recruitment over time from circulating macrophages (Yona et al.),2013

(Lai et al.), 2018 , (Beerman et al., 2010)] This is similar for liver which also gains in macrophage numbers as aging occurs (Bloomer & Moyer). This could be an equilibrium issue with the renewal outpacing the losses or via recruitment.

Recent investigations have questioned monocytes' role in basal peritoneal macrophage maintenance (Yona et al., 2013). During homoeostasis, local proliferation may sustain peritoneal macrophages. Long-term exposure to substances in aged mice's peritoneum may shape these self-renewing cells' phenotype. Stout and Suttles proposed that age-associated macrophage dysfunction is due to functional adaptation to age-related tissue changes (Stout & Suttles, 2005).

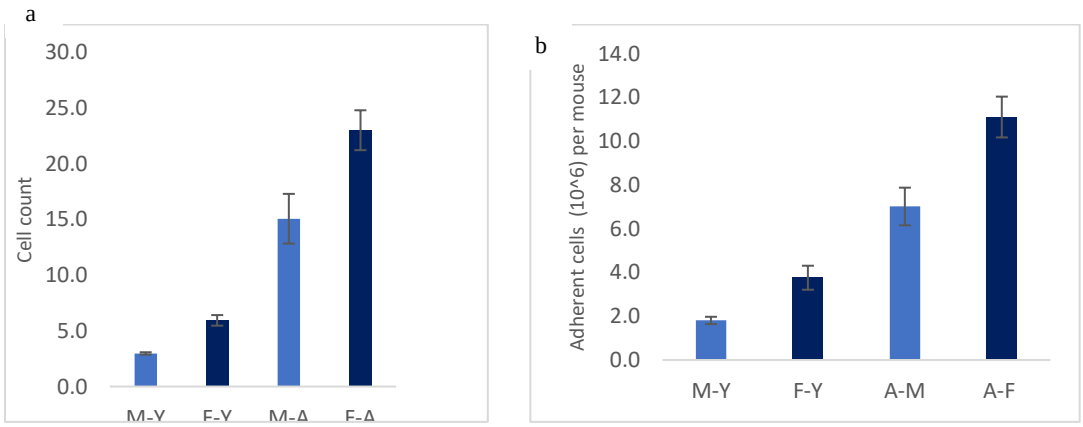


Figure 20. Peritoneal resident cells recovery from young and aged male and female mice. Total peritoneal resident cells were harvested from young and aged mice as described in the methods section. Initial total peritoneal resident cell recoveries per mouse are shown in (a). Young males (M-Y, n=35) ranging from 8 to 23 weeks old and aged males (M-A, n=23) ranging from 48 to 116 weeks old. Young female (F-Y, n=53) ranging from 15 to 22 weeks old and aged females (F-A, n=23) ranging from 75 to 97 weeks old. The average from 6 experiments for males and females is shown as the mean ±SE for individual males and females. The ratio of cell yield of aged males was 5.07 times that of young males. The cell yield of aged females was 3.87 times that of young females. Shown in (b) are the adherent cell recoveries per mouse as the mean ±SE. after a 2h incubation on petri dish at 37°Cper mouse the ratio of aged male adherent cells was 3.88 times that of young males while the ratio of adherent aged female cells was 2.95 of that of young female.

Males vs females.

Males are known to be much more likely to suffer from sepsis and post-surgical complications from infection than females. The causes for this may in part be differences in macrophage number and activity for example in the PRC females have more cells than males and those are more efficient at phagocytosis, ROS production and express more TLR receptors [Scotland., 2011) Not only are macrophage numbers increased, there are more T and B cells present as well. On the other hand, BMDM from males show greater production of ROS, TNFa and IL-1b than females (Barcena et al.) 2021).

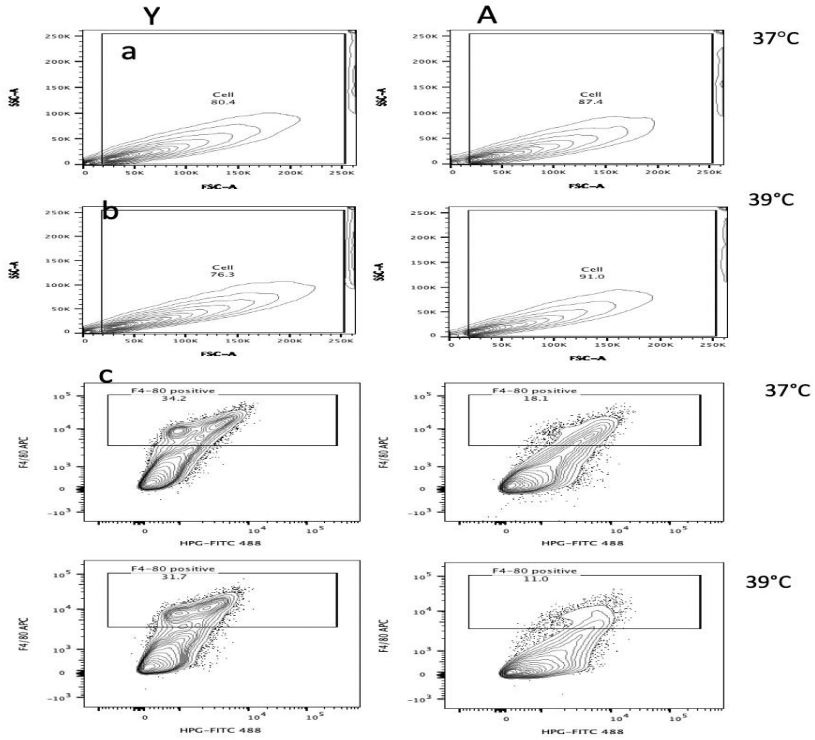


Figure 21: flow cytometry gating for measurement of PRC macrophage analysis of protein synthesis and MHC class II expression. Strategy used to gate young and aged PRC F4-80 positive cells. Adherent PRC were harvested after 2h incubation in petri plates at 37°C. Cells were first selected for by utilizing forward scatter (FSC) and side scatter (SSC) signals to exclude debris and clumps of cells from the analysis. The gate for cells is shown in (a) for unstimulated samples at 37°C and (b) at 39°C. The plots show young PRC on the left and aged on the right. We identified macrophages by selecting the F4/80 - APC positive cells as shown in c and d. The numbers are the percentage F4/80 positive cells compared to total cells. Plots for 37°C are shown in b and for 39°C in c.

In the untreated aged gates shown in Figure P c and d it is clear that there are female F4/80 positive cells in the aged. We analyzed this for all our treatment groups. At 4h incubation adherent unstimulated PRC from young mice showed 34.2 % F4/80 positive cells at 37°C dropping to 31.7 at 39°C and about half that number from aged mice (18.1. % at 37°C and 11.0% at 39°C) only 6.2% are present at 37°c and 2.5% at 39°C such losses are not seen with young. As shown in Figure P and table p. The most drastic drop in the percentage of F4/80 positive cells is with combination treatment for the aged where the F4/80 positive cells. In general, the aged showed much greater variability in the percentage of F4/80 positive cells Table p. The proportion of F4/80 positive cells decreased for aged PRC, except for dose of (3µg/ml) LPS. This decrease is unique to aged macrophages as those differentiated from aged bone marrow precursors are equivalent or better than the young (Table B) Again because the recovery of aged PRC is so much greater than the young averaging 11.1 million cells ±0.9 for aged and 3.9±0.5 for young mice there is not likely to be a deficit of macrophages per mouse (as defined here as F4/80 positive cells) but on a per cell basis there less production of macrophage related products (NO, TNF-alpha) from the aged in our in our invitro tests. . In PRC unlike BMDM there is a positive correlation between MHCII expression and protein synthesis. That is they increased together with all treatment and the combination showing increases most pronounced in the aged at 39°C.

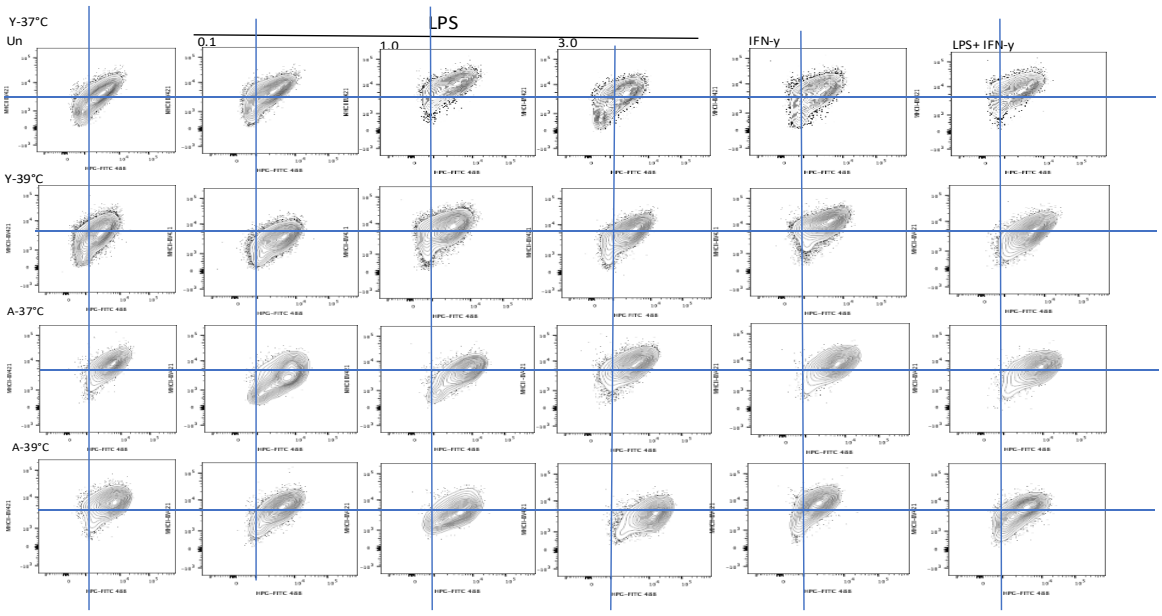


Figure 22: PRC flow cytometric data of protein synthesis test for young and aged female mice.

PRC from young and aged females (ranging from 15 to 19 weeks old and from 75 to 88 weeks old, respectively) adherent PRC were harvested after 2h incubation on petri plates at 37°C. PRC were cultured at $1x10^5$in each well in stimulated with or without LPS at different concentrations (0.1 to 3µg/ml) or IFNy (0.1µg/ml) or combination incubated with Click-iT® HPG Alexa Fluor® 488 Protein Synthesis Assay Kit as described in the Methods section for 4h at 37°C and 39°C. MHC class II expression was determined using mouse anti $Ia^{b\,--}$ BV421 color flow cytometric analysis. F4/80 positive cells were gated and then analyzed for changes protein synthesis in Figure 2 ^{hd} MHCII expression F4/80 positive cells ranged from 28 to 35%% to 51.5% for young and 2.5 to 42% for. aged. First row Figure 21: cell s gated as in Figure P are shown as contour plate of HPG us MHC class II. Cross lines are provided to allow comparison between the shifting for both parameters. In general, as cells population shift both parameter measure. Young untreated PRC (Un) with 3 different LPS concentrations (0.1 to 3µg/ml) or IFN-y or the combination LPS and IFN-Y at 37°C; second row: young at 39°C; third row: aged at 37°C and the four row: aged at 39C °.

Table 8: The percentage of F4/80 positive cells for young and aged PRC after 4h of stimulation at 37°C or 39°C.

			LPS				
	°C	Un	0.1	1.0	3.0	IFN-γ	LPS+ IFN-γ
Young	37	34.2	33.5	29.5	26.2	30.9	35.3
	39	31.7	27.6	30.7	34.2	31.1	29.8
Age	37	18.1	5.3	10.8	41.9	12.3	6.2
	39	11.0	18.9	11.3	16.6	24.8	2.5

Raising the temperature had no effect on the background protein synthesis of young or aged cells. The treatment condition that increased protein synthesis for the young was the combination at 39°C while the aged increased with both LPS and the combination at 39°C.

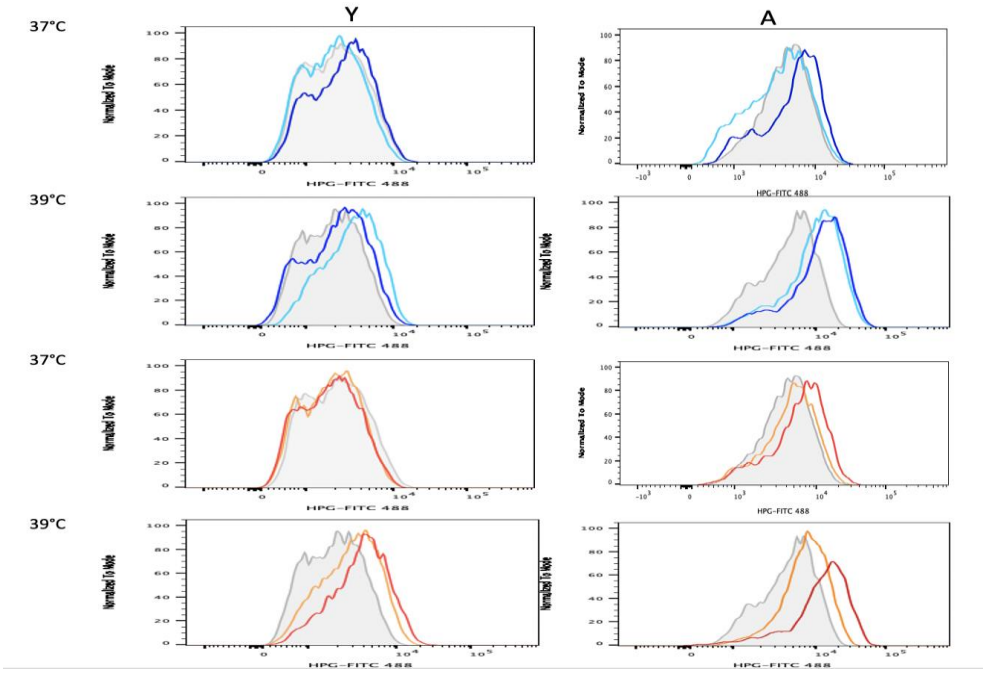


Figure 23: Effect of temperature in protein synthesis from young and age mice PRC. F4-80 positive macrophages were analyzed for protein synthesis. The histograms show young on left and aged on the right, 37°C for a and c 39°C for b and d. Untreated background is shown in gray, 0.1 and 1.0 µg/ml LPS are shown in light and dark blue respectively a and b, 0.1 µg/ml IFN-y is shown in orange and the combination of LPS and IFNy is shown in red.

Young PRC show more MHCII dull cells then the aged (gray filled unstimulated histogram). For the young MHCII expression is increased at 37°C by 1 µg/ml LPS and by IFN-y alone and combination. At 39°C MHCII expression is increased by 1 and 3 µg/ml LPS and by IFN-y alone. Aged PRC show initial unstimulated levels of MHCII that are equivalent to highest levels shown after stimulation by the young PRC. No further increases in aged PRC MHCII expression are observed with stimulation.

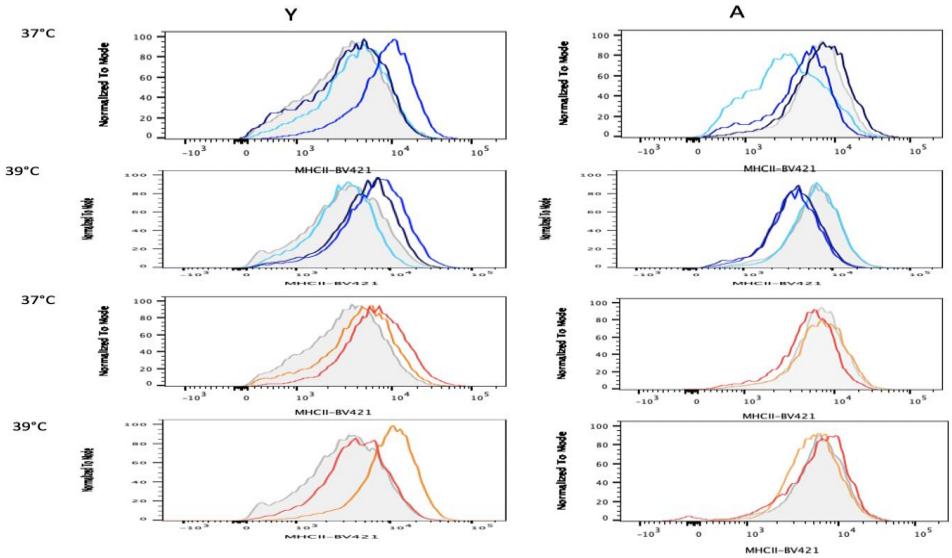


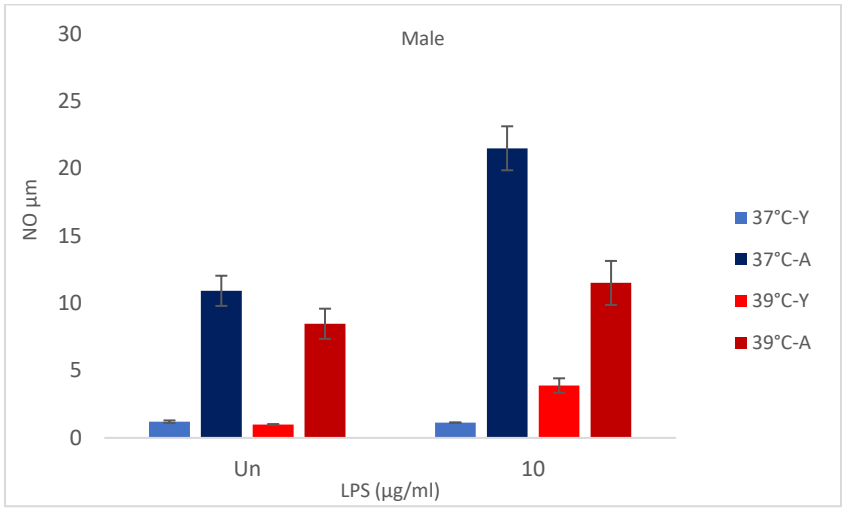
Figure 24: Effect of temperature on MHCII expression from young and aged PRC. The histograms show young on left and aged on the right, 37°C for a and c, 39°C for b and d. Untreated background is shown in gray filled histogram, 0.1 -3 µg/ml LPS shown in light to dark blue (a and b), 0.1 µg/ml IFN-γ shown in orange and combination of LPS and IFN-γ shown in red (c and d).

Temperature effect on LPS and IFN -γ -stimulated NO production in peritoneal resident macrophage for young and aged mice.

To examine how macrophage function is altered by temperature increase by cells that had differentiated in young and aged environments we harvested peritoneal resident cells from young and aged male and female mice. In untreated PRC cultures from young and aged male mice, in some experiment decreased. untreated cultures showed detectibly more NO production in the aged compared to young but not in others (Fig 25) and some of the experiments shown in the supplementary data). Stimulation resulted in an increase in NO production our background levels from young and aged at both 37 and 39°C. On the entire peritoneal cavity per cell basis only young female PRC showed an increase at 39°C while young male, aged female and aged male PRC did not. When considering, on a per mouse basis aged cell recoveries are (Figure 20b) higher. Factoring this in makes NO production 3 to 4 times greater in the aged mouse peritoneal cavity. It is novel that no temperature increase is observed.

While we note that on a per cell basis between experiments that male PRC do not perform as well as female. PRC, with a max of 25µm NO for males compared with 65µm NO for female, reasons for this may be due to harvesting conditions as early on we included heparin in the media injected in the cavity and later we used cell dissociation medium which is Ca^{+2} and Mg^{+2} free. The PRC were enriched in macrophages by adherence, but it is possible that there are different non-macrophage subpopulations that are different between males and females and finally these are not the results from simultaneous comparisons. It is clear that aged female does not response to fever with an increase as readily as young females. Summarizing three experiments with young female PRC, we observed an increase at 39°C in 13 of 15 total trials with LPS (see supplementary data Fig PRC 1 and 2) using a variety of doses. Aged mice only showed an

increase in two of 15 trials at (0.3 µg/ml LPS). IFN-y stimulation increased NO production at 39°C in 9 of 10 trials for the young and only 1 of 10 trials for the aged.



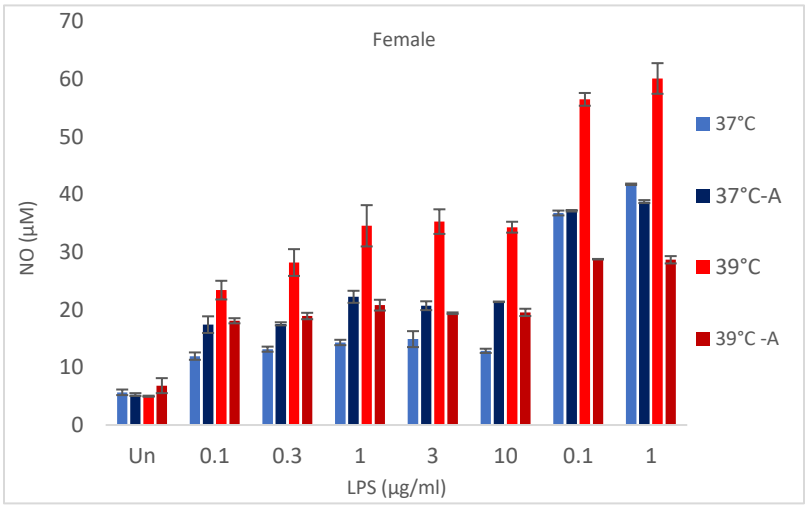


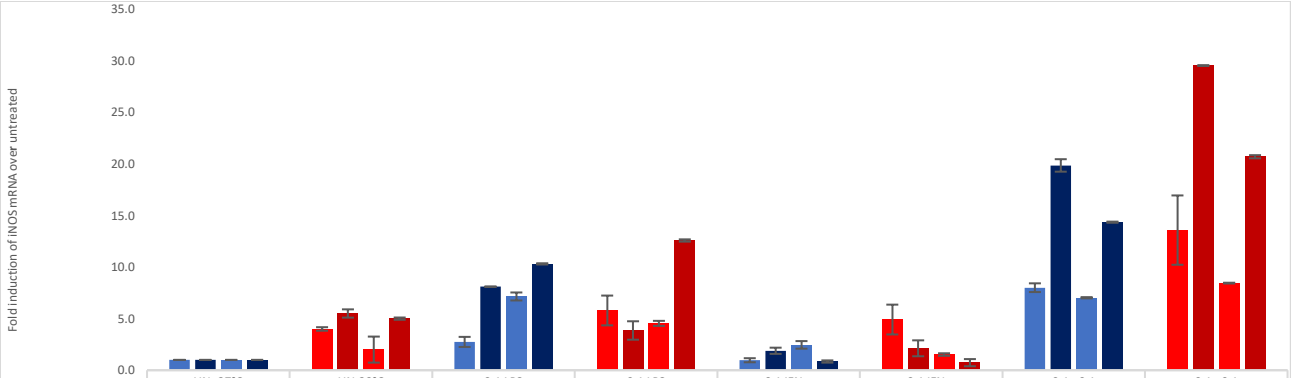
Figure 25. Temperature effect on LPS and IFN-γ stimulated NO production in peritoneal resident cells for young and aged mice. PRC from young and aged males (19 weeks old and 123 weeks old, respectively) and young and aged females (ranging from 15 to 22 weeks old and from 75 to 97 weeks old, respectively) were utilized. In Fig a male PRC were cultured at 5x105 cells in each well in stimulated with or without LPS at 10 µg/ml concentrations In Fig b 5x105 female PRC were stimulated with and without LPS at different concentrations (0.1 to 10 µg/ml), IFN-y (0.1 or 1.0 µg/ml) for 24 h at 37°C and 39°C and supernatant tested by Griess reagent for NO. The light blue bars represent young PRC at 37°C. The dark blue bars represent aged cells at 37°C. The light red bars represent young PRC at 39°C and the red dark bars aged PRC at 39°C. Data aremean ± SE of triplicate determinations. The results shown are representative of 3 different experiments for females (Maximum 50, 60 and 70 µM/ml NO shown in the supplementary data) and one experiment for males (maximum 25 µM/ml NO).

We observed comparable responses between BMDMs from young and aged while spleen showed differences (Mahbub et al., 2012). Similarly, Chen et al. discovered increased NO generation in peritoneal macrophages from aged mice in response to LPS, but not in BMDMs (Chen, Pace, Russell, & Morrison, 1996) Adherent cells from aged mouse PRC were greater in number from both sexes than young but this does not necessarily mean these are macrophages as eosinophils, NK cells, or CD8+ T cells have been observed (Schleicher, Hesse A Fau - Bogdan, & Bogdan) *et al*., 2005 (Misharin, Saber, & Perlman, 2012)

Effect of fever temperature on iNOS production from PRC of young and aged mice.

A RT-qPCR analysis of iNOS mRNA levels was used to see if incubation at fever temperature affected iNOS expression for young and aged adherent PRC. For the aged PRC the response at 39°C increases at 2h for the combination and shows a more sustained response at 4h see figure T and the aged PRC response is greater than the young in all cases of LPS and LPS plus IFN-y.

Young PRC showed increased at 39°C for all groups at 2h, by 4h response was decrease compared to 2h. For the aged PRC, the response at 39°C an increase for the combination treatment at 2h and show a more sustained response at 4h compared with young PRC.

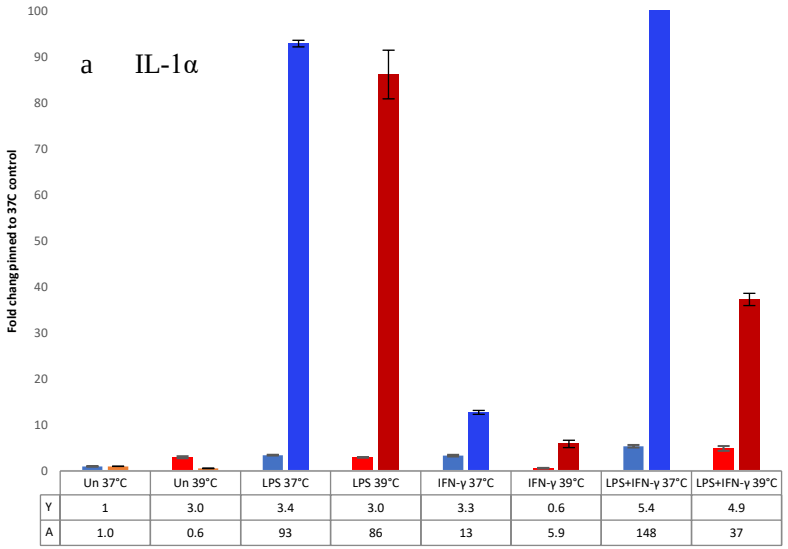


	UN- 37°C	UN-39°C	0.1 LPS	0.1 LPS	0.1 IFN-γ	0.1 IFN-γ	0.1+ 0.1	0.1+ 0.1
2H-Y	1.0	4.0	2.7	5.8	1.0	4.9	8.0	13.6
2H-A	1.0	5.5	8.1	3.9	1.9	2.1	19.8	29.5
4H-Y	1.0	2.0	7.1	4.5	2.5	1.5	7.0	8.4
4H-A	1.0	5.0	10.3	12.6	0.8	0.7	14.3	20.7

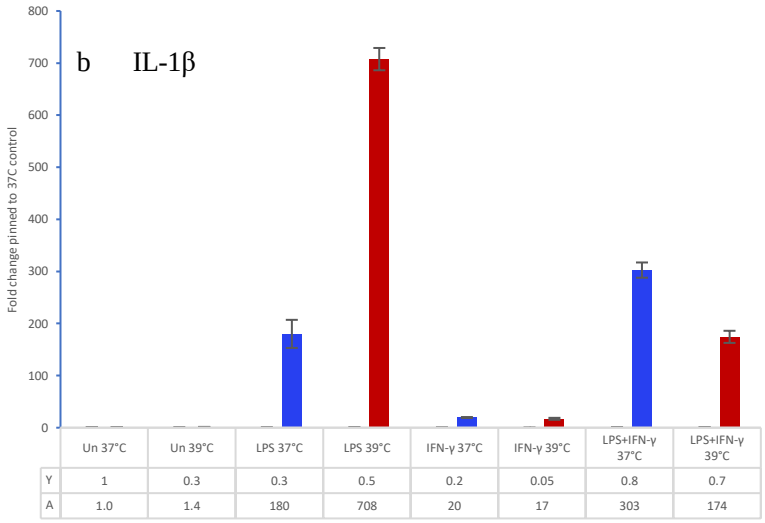
Figure 26: The effect of fever on iNOS mRNA production in young and aged PRC. Duplicate samples of PRC cells (2x106 cells in each well) were stimulated with LPS [0.1µg/ml], IFNγ [0.1µg/ml] and LPS plus IFNγ at 37 and 39°C for 2h and 4h. Samples were pooled, RNA purified and used for templates for cDNA, and run-in triplicate for RT-qPCR. Light and dark blue bar represents 15-week-old young and aged 75-week-old male mice, respectively. 4h Light and dark blue bar represents 15-week-old young and aged 75-week-old female PRC, respectively for each treatment group in the Fig P the 2h samples are shown on theleft and the 4h pair on the right. Light and dark red bars represent young and aged PRC at 37°C. At 39°C, the relative fold change in expression levels is normalized to the 37°C unstimulated control.

The effect of fever temperature on proinflammatory cytokine expression by young and aged PRC

The inflammatory cytokine profiles for young PRC showed much lower level of expression compared to the aged with the exception of IFNβ where the aged did not respond. Aged PRC showed highest responses for the combination treatment for IL-6, IL-12 and TNF -α, and highest for LPS for IL-1α and IL-β1



	Un 37°C	Un 39°C	LPS 37°C	LPS 39°C	IFN-γ 37°C	IFN-γ 39°C	LPS+IFN-γ 37°C	LPS+IFN-γ 39°C
Y	1	3.0	3.4	3.0	3.3	0.6	5.4	4.9
A	1.0	0.6	93	86	13	5.9	148	37



	Un 37°C	Un 39°C	LPS 37°C	LPS 39°C	IFN-γ 37°C	IFN-γ 39°C	LPS+IFN-γ 37°C	LPS+IFN-γ 39°C
Y	1	0.3	0.3	0.5	0.2	0.05	0.8	0.7
A	1.0	1.4	180	708	20	17	303	174

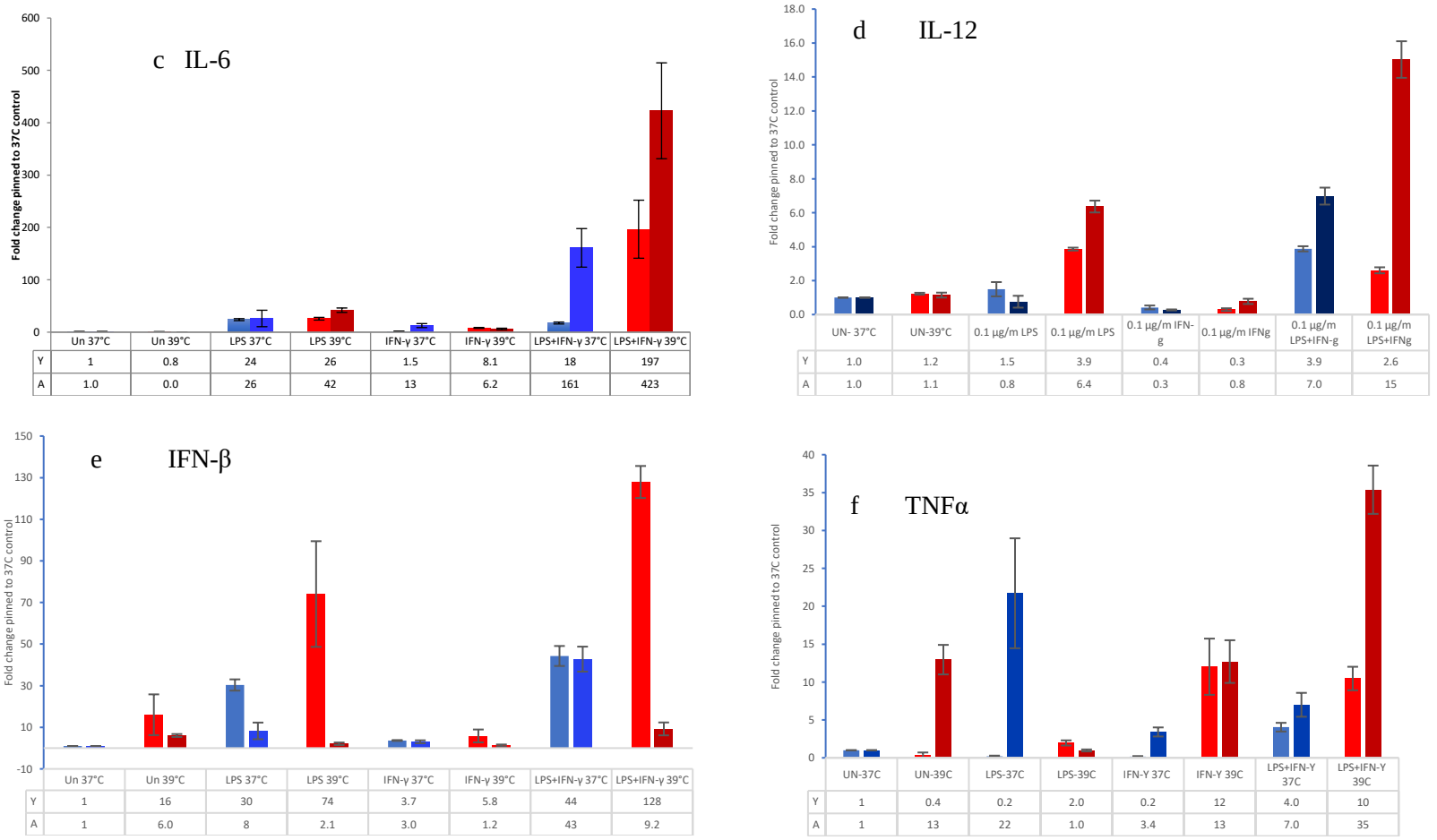


Figure 27: Effect of fever and activation on expression of pro-inflammatory cytokine genes. Duplicate samples of PRC cells (2x106 cells in each well) were stimulated with LPS [0.1µg/ml], IFNγ [0.1µg/ml] and LPS plus IFNγ at 37 and 39°C for 2h. Samples were pooled, RNA purified and used as templates for making cDNA which was run in triplicate for RT- qPCR. Lightand dark blue bars represent both 15 to 23-week-old young and aged at 75 to 83 -week-old male mice at 37°C and light and dark red bars for young and aged at 39°C, respectively. All cytokines were measured at 2h for PRC of both young and aged mice, by qRT-PCR relative to beta-actin with normalization to the unstimulated 37°C group. IL- α showed in (panel a), IL-1β (panel b), IL-6 (panel c), IL-12 (Panel d), IFNβ (Panel e) abd TNF α(Panel f).

Treatment	Temperature [°C]	Y		A	
		B-actin ±SE	iNOS±SE	B-actin ±SE	iNOS±SE
UN	37	12.1 ± 0.1	15.6 ± 0.2	9.5 ± 0.1	20.8 ± 0.1
UN	39	16.2± 1.5	18.3± 0.9	11.3± 0.3	21.3± 0.2
LPS	37	15.1± 0.1	17.5± 0.2	8.8± 0.1	18.6± 0.3
LPS	39	14.3± 0.1	15.3± 0.4	11.3± 0.6	20.5± 0.4
IFN-γ	37	18.3± 0.2	16.0± 0.3	8.9± 0.1	19.4± 0.3
IFN-γ	39	14.4± 0.3	15.6± 0.3	13.4± 0.1	23.6± 0.5
LPS+IFN-γ	37	14.4 ± 0.3	16.0 ± 0.2	11.2 ± 0.6	18.4 ± 0.4
LPS+IFN-γ	39	17.6± 0.3	17.4± 0.4	13.4± 0.1	19.6± 0.2
		B-actin ±SE	IL-6 ±SE	B-actin ±SE	IL-6 ±SE
UN	37	13.7 ± 0.1	20.1 ± 0.0	8.5 ± 0.2	19.2 ± 0.1
UN	39	17.9± 0.4	24.7± 0.2	8.0± 0.0	37.8± 1.3
LPS	37	14.5± 0.4	16.3± 0.1	15.6± 0.3	21.5± 1.0
LPS	39	15.2± 0.1	16.9± 0.1	12.1± 0.2	17.3± 0.1
IFN-γ	37	14.3± 0.3	20.1± 0.2	14.5± 0.4	21.4± 0.5
IFN-γ	39	18.3± 0.2	21.7± 0.2	11.9± 0.1	19.9± 0.3
LPS+IFN-γ	37	14.6 ± 0.1	16.9 ± 0.2	12.3 ± 0.2	15.6 ± 0.0
LPS+IFN-γ	39	17.0± 0.2	15.8± 0.4	13.1± 0.2	15.0± 0.3
		B-actin ±SE	IL-1α±SE	B-actin ±SE	IL-1α±SE
UN	37	13.2 ± 01	14.9 ± 0.1	12.0 ± 0.1	18.3 ± 0.0
UN	39	14.8± 0.1	14.9± 0.1	10.6± 0.1	17.7± 0.2
LPS	37	13.2± 0.1	13.1± 0.0	11.5± 0.1	11.2± 0.0
LPS	39	13.1± 0.0	13.2± 0.0	11.0± 0.0	10.8± 0.1
IFN-γ	37	13.8± 0.2	13.8± 0.1	10.8± 0.2	13.4± 0.0
IFN-γ	39	13.5± 0.1	15.9± 0.2	10.8± 0.1	14.5± 0.2
LPS+IFN-γ	37	13.2 ± 0.1	12.5 ± 0.1	12.9 ± 0.1	12.0 ± 0.0
LPS+IFN-γ	39	12.8± 0.1	12.2± 0.2	11.7± 0.1	12.8± 0.1
		B-actin ±SE	IL-1β±SE	B-actin ±SE	IL-1β±SE
UN	37	10.3 ± 0.2	10.3 ± 0.0	8.6 ± 0.1	19.8 ± 0.1
UN	39	12.2± 0.1	14.0± 0.0	8.0± 0.0	18.8± 0.1
LPS	37	10.8± 0.1	12.6± 0.1	7.5± 0.3	11.2± 0.2
LPS	39	10.4± 0.1	11.4± 0.3	9.0± 0.1	10.7± 0.0
IFN-γ	37	11.2± 01	13.5± 0.1	8.9± 0.2	15.0± 0.0
IFN-γ	39	10.0± 0.3	14.5± 0.0	9.4± 0.2	16.5± 0.2
LPS+IFN-γ	37	10.8 ± 0.1	11.2 ± 0.1	9.2 ± 0.3	12.2 ± 0.2
LPS+IFN-γ	39	10.8± 0.9	11.4± 0.2	9.4± 0.8	13.2± 0.1
		B-actin ±SE	IFN β±SE	B-actin ±SE	IFN β±SE
UN	37	13.0 ± 0.0	29.4 ± 0.4	11.1 ± 0.1	27.8 ± 0.3
UN	39	15.8± 0.1	27.5± 1.2	13.7± 0.1	27.8± 0.2
LPS	37	11.2± 2.1	22.7± 0.1	13.3± 0.1	26.9± 0.4
LPS	39	14.0± 0.1	24.2± 0.5	11.7± 0.1	27.4± 0.4
IFN-γ	37	12.4± 0.0	27.0± 0.1	12.6± 0.2	28.0± 07
IFN-γ	39	14.6± 0.0	28.4± 0.8	12.0± 0.0	28.4± 0.6
LPS+IFN-γ	37	12.3 ± 0.0	23.2 ± 0.2	12.2 ± 0.1	23.5 ± 0.2
LPS+IFN-γ	39	13.9± 0.1	23.3± 0.1	14.6± 0.0	28.8± 0.4
		B-actin ±SE	IL-12±SE	B-actin ±SE	IL-12±SE
UN	37	10.3 ± 0.2	21.3 ± 0.2	8.6 ± 0.1	21.1 ± 0.4
UN	39	12.2± 0.1	23.0± 0.1	8.0± 0.0	20.3± 0.2
LPS	37	10.8± 0.1	21.2± 0.4	7.5± 0.3	20.3± 0.2
LPS	39	10.4± 0.1	19.5± 0.0	9.0± 0.1	18.8± 0.1
IFN-γ	37	11.2± 01	23.4± 0.4	8.9± 0.2	23.2± 0.2
IFN-γ	39	10.0± 0.3	22.8± 0.4	9.4± 0.2	22.2± 0.3
LPS+IFN-γ	37	10.8 ± 0.1	19.9 ± 0.1	9.2 ± 0.3	18.9 ± 0.1

LPS+IFN-γ	39	10.8± 0.9	20.4± 0.1	9.4± 0.8	17.9± 0.1
		B-actin ±SE	IL-34±SE	B-actin ±SE	IL-34±SE
UN	37	10.3 ± 0.2	24.2 ± 0.2	8.6 ± 0.1	23.0 ± 0.5
UN	39	12.2± 0.1	24.7± 0.4	8.0± 0.0	21.9± 0.3
LPS	37	10.8± 0.1	24.1± 0.1	7.5± 0.3	24.5± 0.5
LPS	39	10.4± 0.1	24.4± 0.3	9.0± 0.1	22.2± 0.0
IFN-γ	37	11.2± 01	23.2± 0.2	8.9± 0.2	29.0± 1.2
IFN-γ	39	10.0± 0.3	24.2± 0.5	9.4± 0.2	23.2± 0.1
LPS+IFN-γ	37	10.8 ± 0.1	23.5 ± 0.1	9.2 ± 0.3	22.1 ± 0.3
LPS+IFN-γ	39	24.1± 02	18.9± 0.0	9.4± 0.8	23.1± 0.1
		B-actin ±SE	TNFα ±SE	B-actin ±SE	TNFα ±SE
UN	37	15.1 ± 01	24.1 ± 0.6	12.3 ± 0.3	23.9 ± 1.3
UN	39	16.4± 0.2	27.2± 1.2	19.1± 0.1	26.4± 1.0
LPS	37	14.7± 0.1	26.4± 06	18.4± 0.0	25.6± 0.4
LPS	39	15.3± 0.1	23.7± 0.2	12.7± 0.0	24.4± 0.2
IFN-γ	37	17.9± 0.1	26.2± 0.6	12.4± 0.4	22.3± 0.3
IFN-γ	39	13.2 ± 0.1	23.7 ± 0.4	24.5 ± 0.2	31.6 ± 1.4
LPS+IFN-γ	37	15.1± 0.1	22.5± 0.2	12.5± 0.0	21.7± 0.4
LPS+IFN-γ	39	17.0 ± 01	23.1 ± 0.2	18.9 ± 0.2	26.6 ± 1.7

Table 9: RT-qPCR analysis of stimulated PRC isolated from young and aged mice incubation was for 2h. Data are Ct values ± SD. This data is included as the b-actin varies substantially between sample with both temperature and treated showing difference.

Spleen cell recoveries

Splenic macrophages are important in sampling blood borne pathogens. Spleen cells from young and aged mice were harvested and viable cells counted. For each experiment the splenocyte cell numbers Fig 28a are pooled spleens from 6 or 7 young mice and 3 or 4 aged mice 6 independent experiments total spleen count are show in Fig 28a. (Fig 28 the average spleen cell recovery obtained from aged male mice shows no difference in the cell number compared with young male. Aged female mice show 1.6 times the total spleen cell number of the young before selecting adherent cells. After 2h at 37°C on petri dish adherent cells were harvested and counted (Fig b), the yield of aged male adherent cells was less then young while the yield of aged female adherent cells was greater than young.

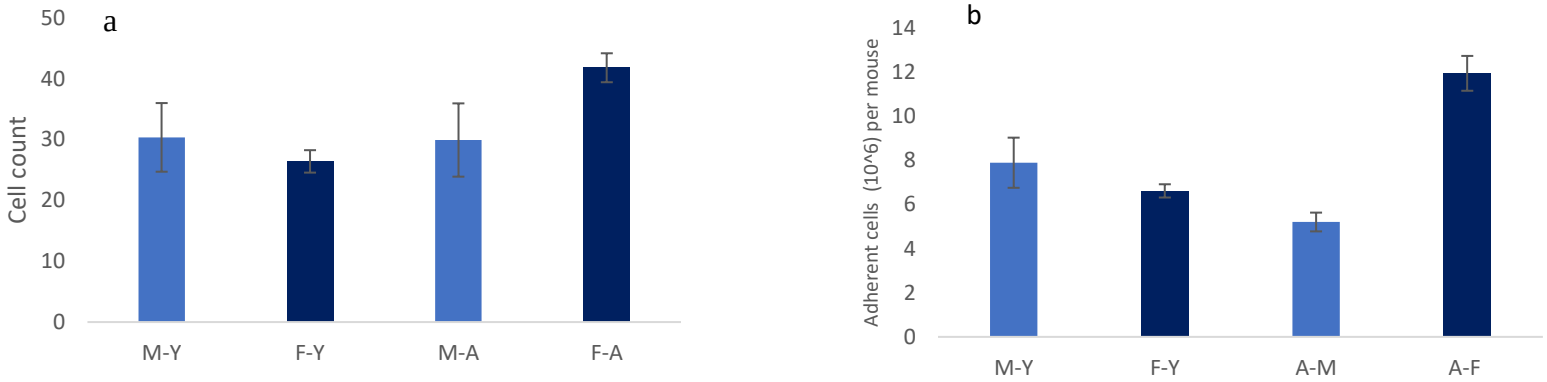


Figure 28: Splenocyte recovery from young and aged male and female mice. Spleen were harvested from young and aged mice as described in the Methods section. Total splenocyte recoveries per mouse are shown in (a). Young males (M-Y, n=35) ranging from 8 to 23 weeks old and aged males (M-A, n=23) ranging from 48 to 163 weeks old. Young female (F-Y, n=53) ranging from 15 to 22 weeks old and aged females (F-A, n=23) ranging from 75 to 97 weeks old. The average number per spleen from 6 experiments ±SE for males and females. Fig Sa shows the cell yield of aged males was about equal to that of young males. The cell yield of aged females was 1.6 times that of young females. Shown in (b) are the adherent cell recoveries per mouse after 2h incubation cells on petri plate at 37°C. cells per young males was 26% as and young female mice was 25%. The adherent cell number was 17% of the total input of aged male mice and 29% for aged females

Figure 28a shows Initial splenocyte cell recoveries for young males is about the same as that of young females. For aged males the cell recovery was about equal to that of young males. However, aged females total spleen cell number were 1.6 times that of young females. Fig 28b shows the yield after 2h of culture to enrich for macrophages by selecting for adherent cells. (Figure 28b) aged female yielded the most adherent by young males, young female and then aged males . Taken together these results indicate the number of adherent cells in aged male spleen is significantly lower than aged female spleen.

Temperature effect on LPS and IFN-y stimulated NO production in splenocyte-derived adherent cells (SP) for young and aged mice.

Unstimulated SP cells from young and aged mice produced comparable NO levels at 39°C and 37°C. With LPS stimulation young male SP had a significantly higher NO level at all concentration from 0.1-10µg/ml at 39°C versus 37°C. lower LPS concentrations aged males show no significant increase in NO production at 39°C but do show a significant increase in NO production at 3 and 30 µg/µl LPS compared to 37°C (Fig 29a). In Fig 29b there was no difference in the small amount of NO production seen by unstimulated SP cells from young and aged female mice at 37°C and 39°C. On a per cell basis. The trend for young females is increased at 39°C compared to 37°C and whereas for aged female adherent splenocytes there is generally or decrease at 39°C. A similar pattern is seen for IFN-y stimulation alone. However, with the combination of LPS and IFN-y young female mice produce more NO compared to aged females at both temperatures. Given the increased cell number in the aged females and the

enhanced adherent cell recovery after culture, the NO response is equal or greater in the aged female.

Over three experiments (see supplementary data Fig Sp 1 and 2) using a variety of LPS concentrations young female Sp showed increases at 39°C in 7 of 15 total trails while aged mice also showed an increase in 7 of 15 trials. The difference was the young NO increases were at lower doses and the aged increased at the higher concentrations. Over the same three experiments, IFN- y stimulation increased at 39°C in 2 of 6 trails for the young and in 3 of 6 trails in the aged. For the combination of LPS and IFN-y a temperature dependent increase was seen in the one trail. All three experiments showed rather low NO production with maximum for these experiments being 10, 15 and 30 µM NO.

Adherent cells from mouse spleen, in general, had lower responses than the other tissues. This is probably a reflection of the fact that there are more cells even after our selection for adherent cells in our mixture that are not macrophages. Further, spleen cells showed more complicated patterns in comparing aged and young which may hinge on the level of age dependent contamination with other cell types affecting our overall results. Thus, the results from spleen cells are not weighed as heavily in our interpretation of our results.

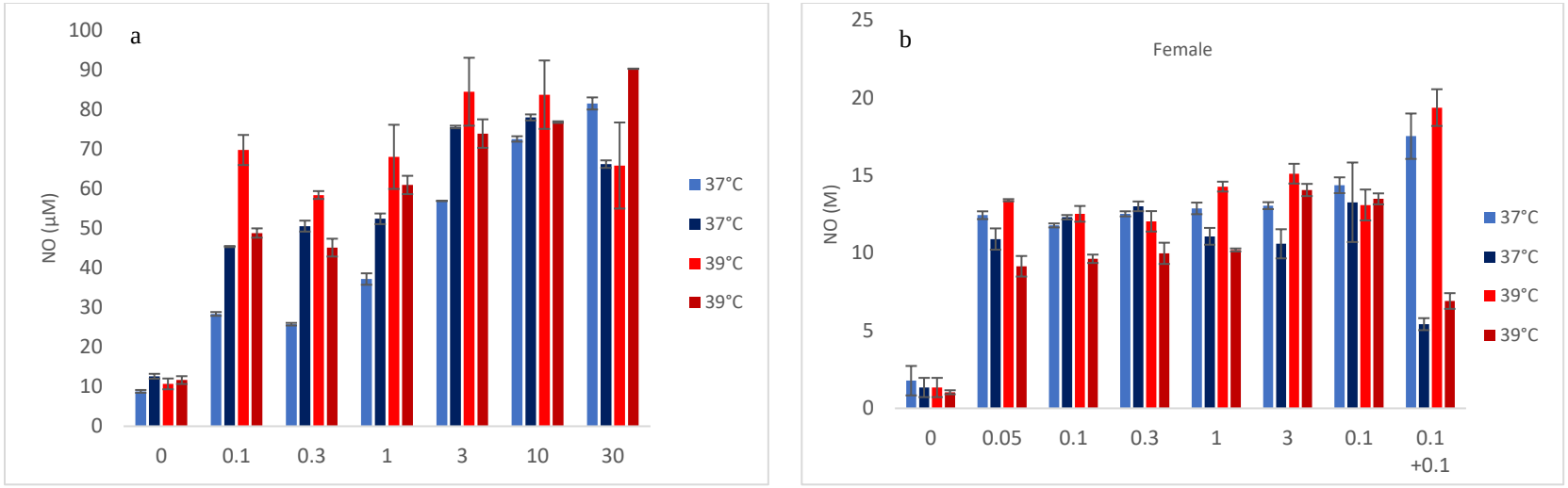
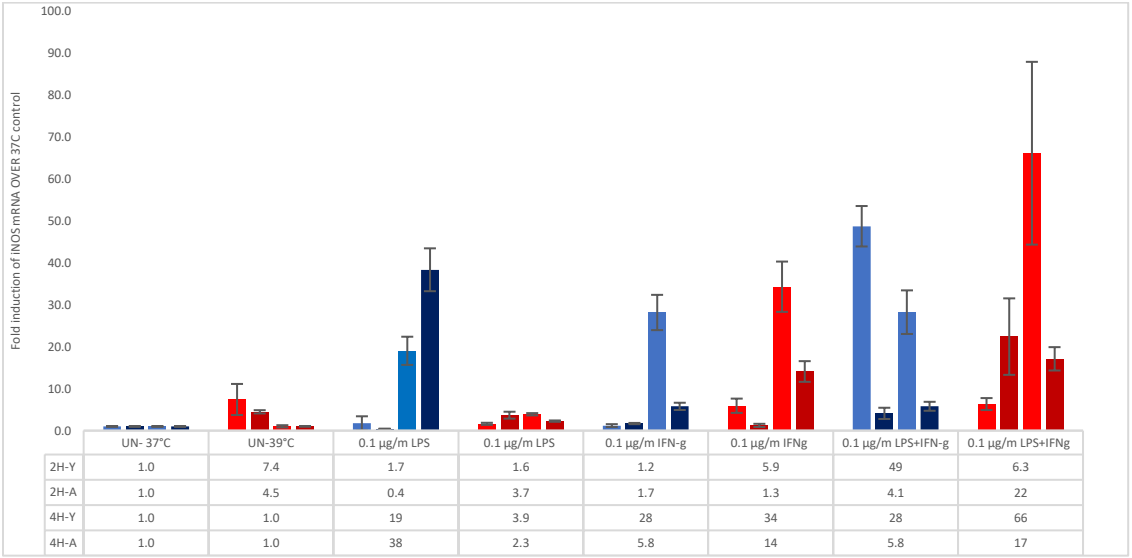


Figure 29. Temperature effect on LPS and IFN-y stimulated NO production in splenocyte-derived adherent cells (SP) for young and aged mice adherent spleen cells (Sp) from young and aged male (11 and 142 weeks old, respectively) and young and aged females (ranging from 15 to 22 weeks old and aged mice ranging from 75 to 97 weeks old, respectively). In Fig a male SP cells were cultured $5x10^5$in each well in 96-well stimulated with or without LPS at different concentrations (0.1 or 30 µg/ml) and In Fig b female SP were stimulated with and without LPS at different concentrations (0.1 or 10 µg/ml) or IFN-y (0.1 µg/ml) or a combination of 0.1 µg/ml of both for 24 h at 37°C and 39°C. Supernatant tested **by Griess reagent for NO. The light blue bar represents young bone marrow cells at 37°C. The dark blue bar represents aged cells at 37°C. The light red bar represents young SP cells at 39°C and the dark red bar at 39°C. Data are mean ± SE of triplicate determinations. The results shown are representative** of 3 different experiments for females (maximum 50, 25 and 12 µM/ml NO shown in the supplementary data) and one experiment for males (maximum 90 **µM/ NO) although no direct comparisons between male and female were done.**

Effect of fever temperature on iNOS production by splenic adherent cells

An RT- qPCR analysis of iNOS mRNA levels was used to see if fever temperature affected iNOS expression for young and aged male mice Sp cells. With LPS stimulation young SP cells showed a peak at 4h with no increase at 39°C compared to 37°C and aged cells showed same lack of LPS enhancement at 39°C. With IFN-y the peak was at 4h for young and aged with a modest increase in iNOS expression at 39°C. With the combination of LPS and IFN-y the young at 37°C peaked at 2h but at 4h at 39°C, the aged showed increases at both 2h and 4h at 39°C fever temperatures with no clear peak.



	UN- 37°C	UN-39°C	0.1 µg/m LPS	0.1 µg/m LPS	0.1 µg/m IFN-g	0.1 µg/m IFNg	0.1 µg/m LPS+IFN-g	0.1 µg/m LPS+IFNg
2H-Y	1.0	7.4	1.7	1.6	1.2	5.9	49	6.3
2H-A	1.0	4.5	0.4	3.7	1.7	1.3	4.1	22
4H-Y	1.0	1.0	19	3.9	28	34	28	66
4H-A	1.0	1.0	38	2.3	5.8	14	5.8	17

Figure 30: The effect of fever on iNOS mRNA production in young and aged Sp. Duplicate samples of Sp cells (2x106 cells in each well) were stimulated with LPS [0.1µg/ml], IFNγ [0.1µg/ml] and LPS plus IFNγ at 37 and 39°C for 2h and 4h. Samples were pooled, RNA purified and used for templates for cDNA, and run-in triplicate for RT- qPCR. Light and dark blue bars represent 15-week-old young and aged 75-week-old male mice at 37°C for 2h and 4h, respectively. Light and dark red bars represent young and aged Sp mice cell. For each treatment group in the Fig 30 the 2h samples are shown on the left and the 4h pair on the right. The relative fold change in expression levels was normalized to the 37°C young or aged unstimulated control.

Compare between RAW264.7 cell line and primary cells.

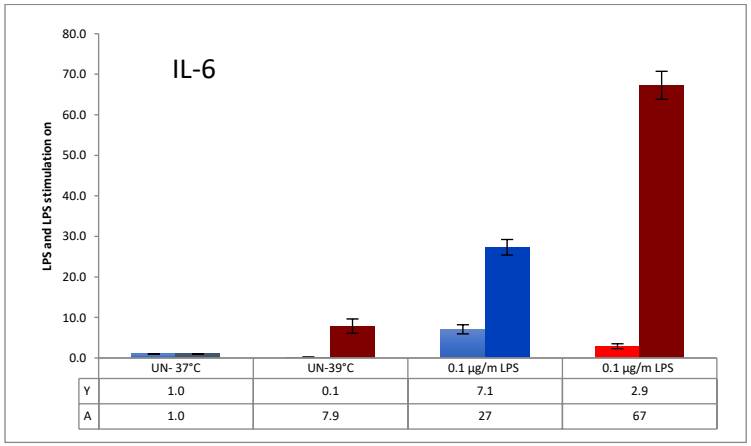
Inflammatory cytokines show increases with increased temperature

In summary, we note that for iNOS and all the inflammatory cytokines that there is an increase in the magnitude of expression at the mRNA level at fever temperatures. We scored our qPCR results as positive if any of the stimuli gave a temperature dependent increase. Specifically, the enzyme iNOS shows an increased response to fever temperatures for all tested tissues which lead to increased levels of NO during stimulation and fever temperature response. IL-6 mRNA increases for all the tested cell types [RAW 264.7, BMDM, S and PRC from young and aged mice with the exception of lung macrophages from the young. IL-1α shows a temperature response for RAW 264.7 cells and BMDM from Y and A but no increase from resident macrophages of spleen and PRC, IL-1β shows the same pattern but with an increase with the combination stimuli for Y S. IFNα shows fever temperature increases in the tested samples of RAW264.7 cells and Y and A BMDM. IFNβ is different. IL-12 shows increases for RAW264.7, Y and A BMDM and PRC. TNFα is increased for Y BMDM and Y and A PRC. The exception where no increases are observed for the A BMDM for TNFα may be our assay-- aged responses are so high we may have missed the peak by taking our first point at 2 h rather than earlier. In testing iNOS and proinflammatory cytokines aged BMDM and PRC give highest response then the young. With spleen proinflammatory cytokine expression where is more variability of purity we see mixed result

A non-inflammatory cytokine tested was IL-34. We were interested in testing it because of its function as a growth factor and because there are reports that it increases expression at 39°C. While the control increased with temperature as expected with stimulation the responses went down rather than up.

Effect of temperature and LPS stimulation in lung macrophage gene expression.

IL-6 and IFN-β showed grater response at 39°C aged mice compared to young. However, IL-6 was greater than IFNβ expression.



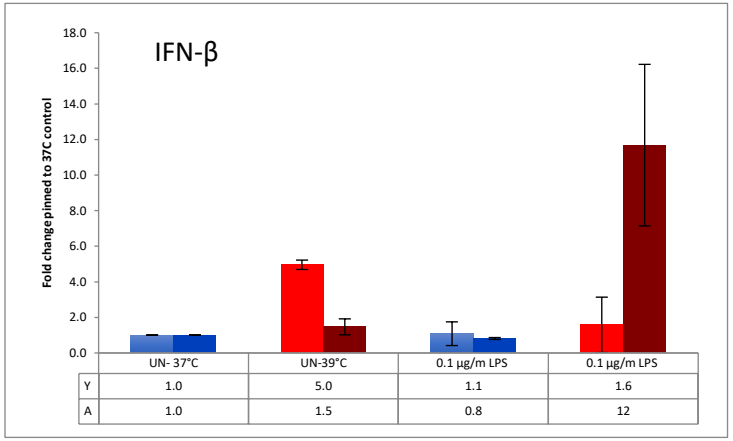


Figure 31: Effect of fever and activation on Lung macrophage expression of pro-inflammatory cytokine genes for young and aged mice. Triplicate samples of adherent splenocyte (2x106 cells in each well) were stimulated with LPS (0.1µg/ml) at 37 and 39 for 2h. Samples were pooled, RNA purified and used for templates for cDNA, and run-in triplicate for RT- qPCR. Blue bars represent the incubation time at 37°C and red bars at 39°C All cytokines measured by qRT-PCR relative to beta-actin with normalization to the unstimulated 41°C. IL-6 (Panel a) and IFNβ showed in (panel b).

Effect of temperature on functional activity of macrophages in three different species.

Macrophages were isolated from mice, chicken, or rainbow trout primary tissue or from macrophage cell lines and were activated. With lipopolysaccharide (LPS) or IFN-γ. For mice, both Raw264.7 and primary cells, nitric oxide (NO) production was similar at 35°C and 37°C but dropped dramatically at temperatures below 35°C. At fever temperature (39°C), NO release increased in response to LPS. Young bone marrow derived macrophage (BMDM) and peritoneal resident macrophage (PRC) showed increased protein synthesis at 39°C compared to 37°C. Chicken splenic Macrophages (CSM) showed NO responses that were similar at 37, 39, and at 41°C (normal for avian). A fever of 42°C had a large stimulatory effect on NO production compared to 41°C. A chicken liver derived macrophage cell line, HTC, showed the same pattern. They also showed higher protein synthesis at 42°C compared with (41°C) after LPS stimulation. Trout head-kidney macrophages (FHK) showed the highest NO responses at 19°C when compared to more typical stream temperatures of 13, 15, and 16°C. They still showed some response at 28 and 37°C. Thus, their macrophages respond at higher

temperatures than the fish can tolerate. Stimulated HTC cells with LPS at 19°C showed increased protein synthesis compared to 13 and 16°C. We can conclude from our experiments that fish macrophages had a much broader range of temperatures at which they could respond by NO generation and protein synthesis compared to mice and chickens, and all had increased responses at fever temperatures.

The overall goal of this part of my research was to investigate how fever impacts macrophage activity in chickens, and fish.

Fever responses have been part of vertebrates for 600 million years. Studies demonstrate that a fever response increases pathogen clearance and infection survival (Evans et al., 2015). The effect of temperature on immune system cell metabolism is unknown. Macrophages create and orchestrate limited immune responses at infection sites, preventing infections from spreading. Macrophages influence innate immunity pathways at infection sites. Macrophages are body-wide myeloid leukocytes. Macrophages come from pluripotent hematopoietic stem cells in bone marrow (Parham, 2009). Monocytes are blood-borne macrophage precursor cells that transit the circulatory system before maturing into resident macrophages. Macrophages phagocytose pathogens and destroy them. They are the first cells to encounter foreign chemicals in infected tissues, triggering and directing a local innate immune response (Parham, 2009, 2014) Cold-blooded vertebrates (ectotherms) regulate fever differently than endotherms, but share many of the same cytokines and signaling molecules. Reptiles, fish, and insects use behavioral regulation to elevate their core temperature during infection, choosing warmer sites despite predation danger (Covert and Reynolds). Behavioral modulation is beneficial in ectothermic immune responses. Fish, like many other ectotherms, travel to warmer parts of their ecosystems

when unwell. (Reynolds, Covert) . Boosting the temperature does not killing the virus, rather than boosts sockeye salmon's immunological response (Covert and Reynolds).

Effect of fever on HTC cell line.

HTC was used as model for this study. These macrophage-like cells display class I and II major histocompatibility complexes and have been shown to release NO and a variety of cytokines when exposed to the LPS (Rath et al., 2003) HTC cells grow quickly and equally well at both mammalian and avian normal core body temperatures (37 and 41°C) and show traces of infection with both avian leukosis and Marek's disease viruses (Rath et al. 2003). As a result, they serve as a model for researching macrophage cellular physiology.

Nitric oxide assays.

Effects of temperature and different treatments on the Nitric oxide production

The role of nitric oxide in chicken macrophage defense against bacterial invasion is well understood (Crippen; Sun et al., 2021) In our experiments with HTC, we found the IL-6 increased at temperatures below 40°C and realized that others had similarly found in mice that IL-6 upregulates to try to get temperatures back up to normal core temperatures [Ryan's thesis]. IL-6 expression is higher at sub-physiological temperatures after two hours, but higher with LPS. This data shows that after 4 hours, expression reduces when temperature is changed from normal. Early IL-6 expression may be the body's attempt to raise core temperature. IL-6 doesn't respond to pathogens after two hours at normal and fever temperatures but does at sub-physiological temperatures. This suggests that temperature controls cytokine expression. IL-6

expression is steady for 2 to 4 hours at fever temperature while a pathogen is present, suggesting a positive feedback loop.

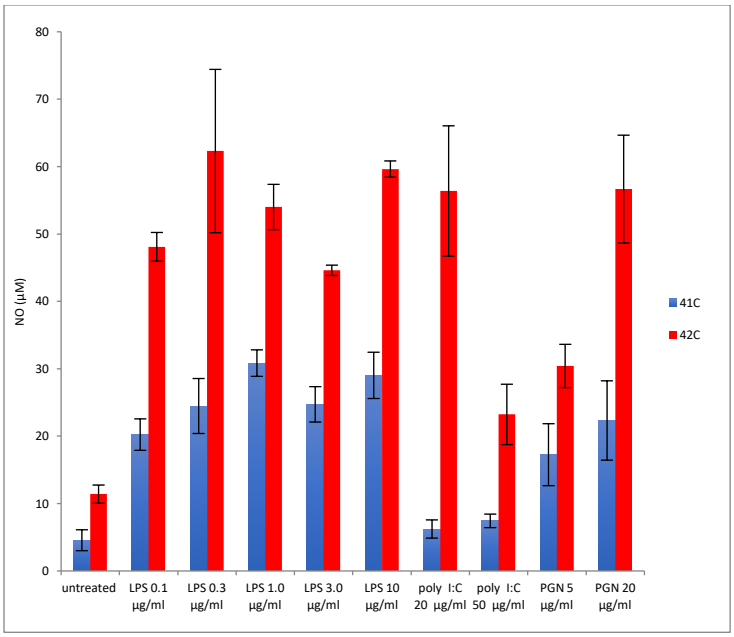


Figure 32: Effects of fever temperature on HTC production of NO different treatments on the Nitric oxide production HTC (5x105in each well) were cultured in a 96 well plate with different treatments in quadruplicate. After 20-24h supernatant was collected and tested for NO (µM). LPS concentrations ranged from 0.1 to 10 µg/ml, poly I:C from 20 to 50 µg/ml and PGN from 5 to 20 µg/ml. The results shown are representative of 3 different experiments. Blue bars are the averages ± SE at 41°C and the red bars at 42°C.

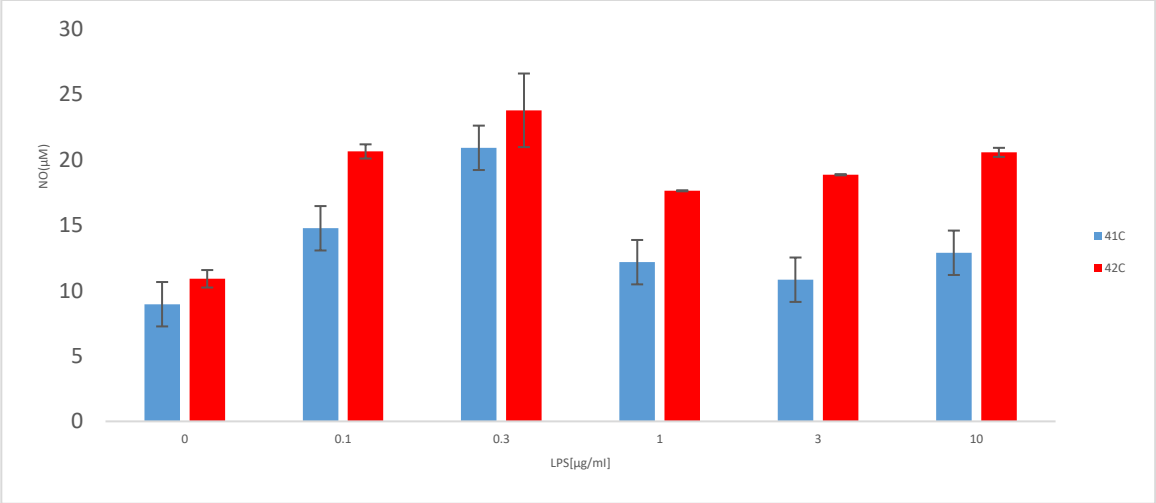


Figure 33: Effect of fever on chicken spleen adherent cell production of NO. Single cell suspensions from 6-wk-old broiler chicken spleen were made as described in the Materials and methods and mononuclear cells isolated by Ficol density (1.077) centrifugation. After washing cells were cultured in petri plates for 2h and adherent cells harvested by release in PBS without Ca^{+2} and $Mg^{+2}$and scraping. Adherent cells were seeded at $5x10^5$in each well in a 96 well plate with different LPS concentrations in triplicate for 20-24h. Supernatant was collected and tested for NO (μM). The results shown are representative of 6 experiments. The blue bars show NO ± SE at 41°C and the red bars at 42°C.

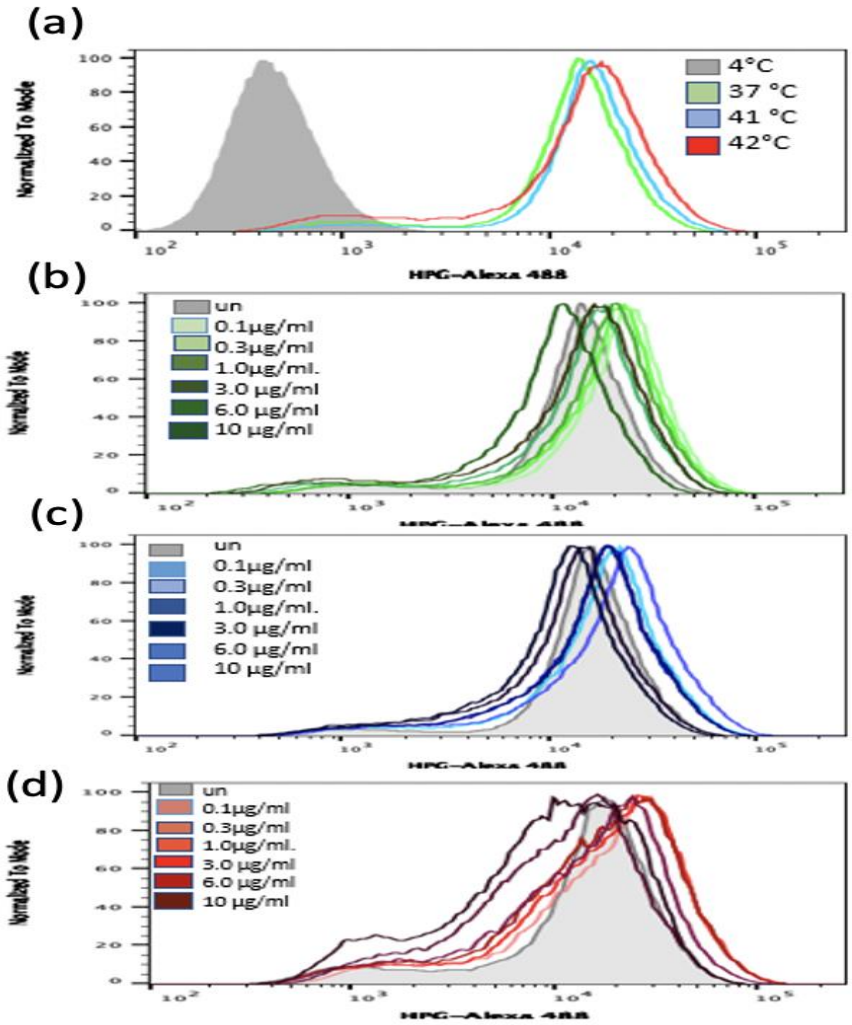


Figure (34) Protein synthesis of HTC cells at different temperatures with and without LPS stimulation. Triplicate cultures of one million cells for each treatment were incubated for 4 hours in methionine-free medium and then stained via Click-it chemistry and analyzed by flow cytometry. The number of events collected for each pooled sample was 100,000. A gate for size was used to remove small debris and large aggregates of cells. HPG incorporation of HTC cells at different temperatures the light gray filled histogram in (a) represents unstimulated cells at 4°C. The green line protein synthesis at 37°C, the blue line at 41°C and the red line at 42°C. (b) The flow cytometry pattern of HTC cells at 37°C with different LPS concentrations. The gray filled histogram depict unstimulated cells at 37°C overlaid with different LPS concentrations (.1, 0.3, 1.0 and 3.0, 6 and 10µg/ml]) shown from light green to dark green. (c) The flow cytometry pattern of HTC cells at 41°C with differentLPS concentrations. The gray filled histogram represents unstimulated cells at 41°C. LPS stimulations range from light blue to dark blue. (d) The gray filled histogram shows unstimulated cell at 42°C. LPS stimulations range from light red to dark red.

Protein synthesis in HTC and chicken macrophage.

Gobal protein synthesis show a slight increase in protein synthesis in unstimulated HTC cultures at 42°C compared to 37 and 41°C (Figure 34a) Increases in protein synthesis (Figure 34b, c and d) were seen with doses of LPS stimulation from (0.1-3µg/ml) at all temperatures with the great increase at 42°C. At the higher dosages of 6 and 10 µg/ml LPS a decrease in protein synthesis was seen at all temperatures (Figure 34b, c and d).

Effect of fever on Chicken Spleen protein synthesis.

Adherent Ficoll purified macrophage makes positive cells were Unstimulated chicken show a higher level of protein production at 42°C then at 41°C. LPS stimulated cells showed an increase at 41 with the dosage of LPS. This global increase in protein synthesis was quite dose dependent as 0.05 and 0.3 doses of LPS decreased overall protein synthesis compared to background of both 41 and 42°C.

Unlike the HTC results the highest increase in protein synthesis is observed with 1µg/ml LPS at 41°C with the control highest being the 42°C LPS which overlaps with the unstimulated at 42°C. Thus, temperature alone has a stimulation effect on protein synthesis on chicken spleen resident macrophage.

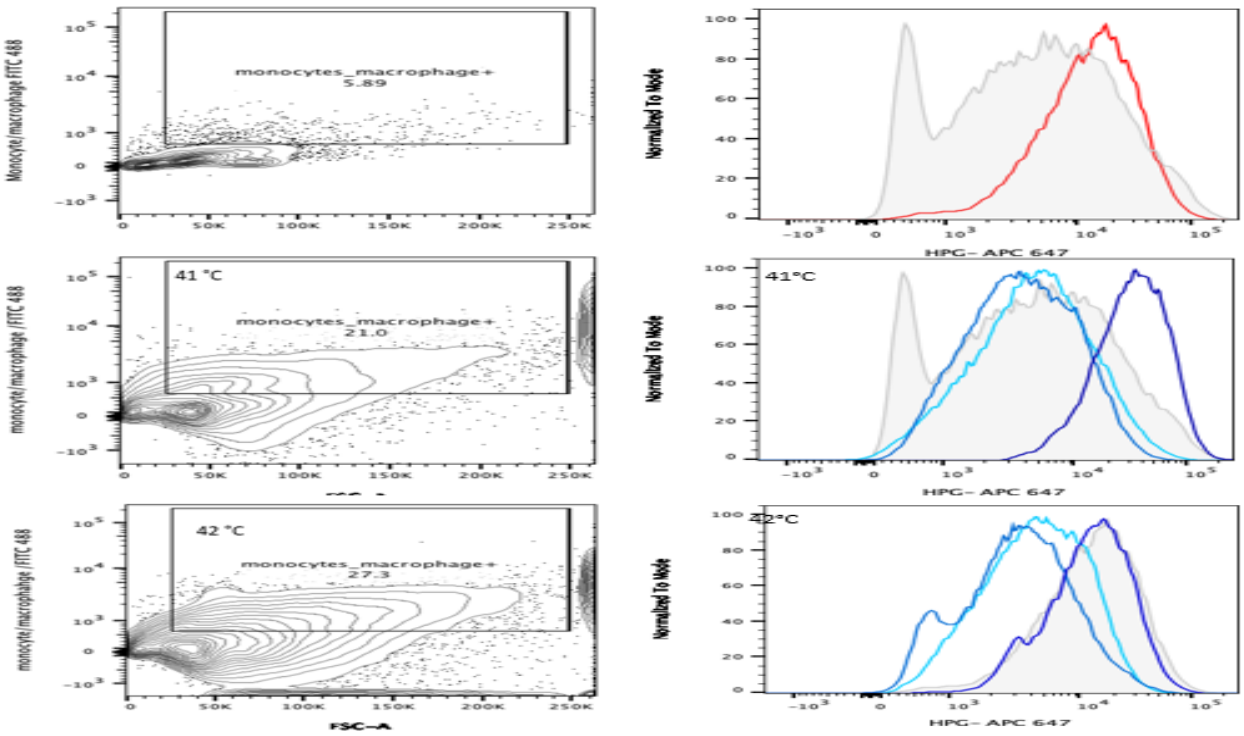
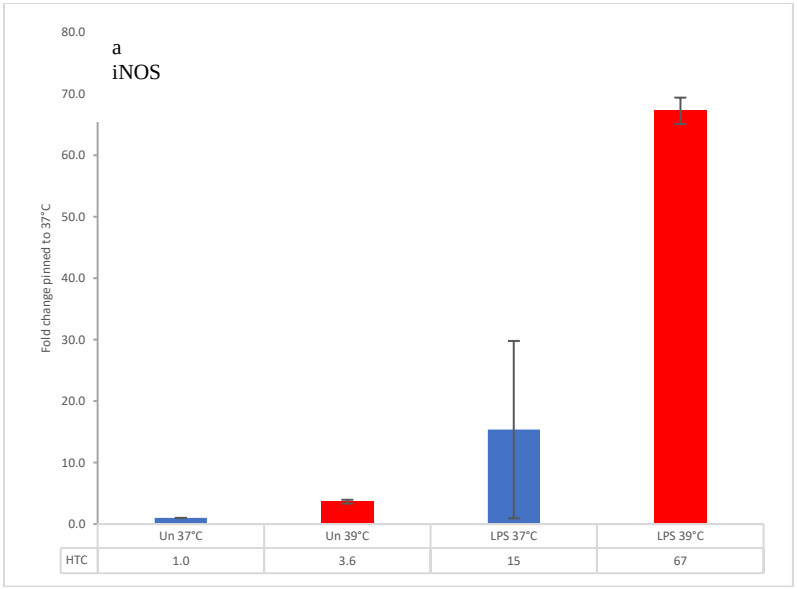
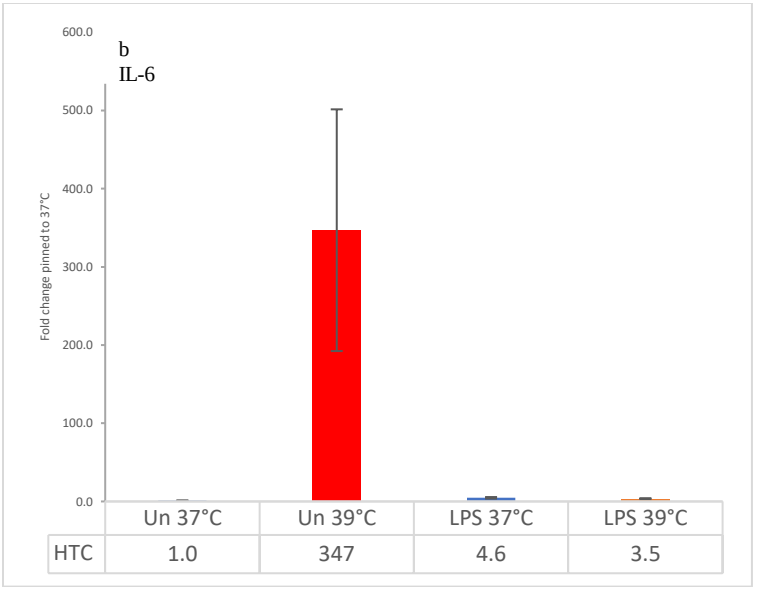


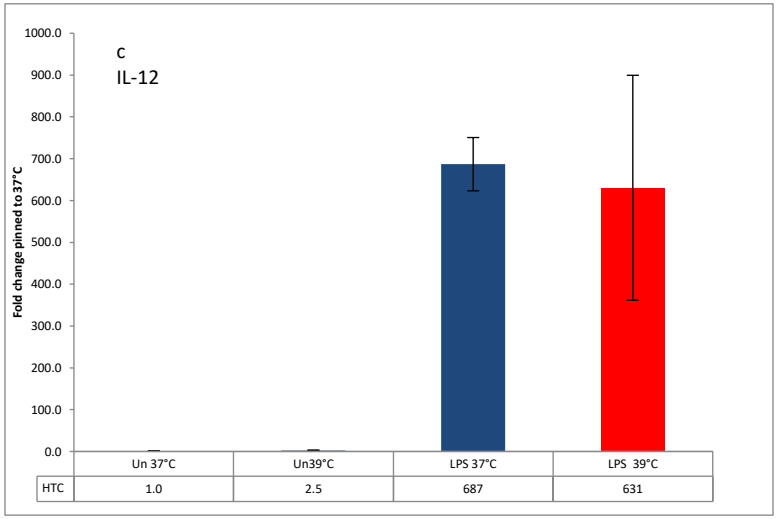
Figure 35: Protein synthesis of chicken macrophages from spleen scells at different temperatures with and without LPS stimulation. Adherent spleen cells were harvested and triplicate cultures of one million cells at either 41 or 42°C for 4h in complete medium with added OPP, a puromycin analog, which incorporates into growing polypeptides and then stained with Alexa 647 via Click-it chemistry. To identify the macrophages further gated was selected cells were also stained with mouse anti- chicken monocyte /macrophage marker-Alexa 488. Analysis by flow cytometry of 100.000 events was further gated to select for macrophage/ monocyte positive cells as shown in the left panels. On the right the top panel showed a gray filled histogram of unstimulated cells at 41°C and the red line protein synthesis at 42°C. The middle panel compares LPS stimulated cells (0.05, 0.3 and 1.0µg/ml) in light to dark blue) to unstimulated cells (gray filled histogram) at 41°C. The bottom panel compares LPS and stimulated cells control at 42°C.

The effect of fever temperature on proinflammatory cytokine expression by HTC cell.

The inflammatory cytokine profiles for stimulated HTC showed much higher level of expression for iNOS compared to IL-6 responses was much lower at 39° compared to 37°C. However, with LPS stimulation at 42°C iNOS were expression greater than IL-6 at 41 and 42 °C. While there was small modest effect for both INF -α, and IL-34 expression.


a
iNOS
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Fold change pinned to 37°C
Un 37°C
Un 39°C
LPS 37°C
LPS 39°C
HTC
1.0
3.6
15
67


b
IL-6
600.0
500.0
400.0
300.0
200.0
100.0
0.0
Fold change pinned to 37°C
Un 37°C
Un 39°C
LPS 37°C
LPS 39°C
HTC
1.0
347
4.6
3.5


c
IL-12
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900.0
800.0
700.0
600.0
500.0
400.0
300.0
200.0
100.0
0.0
Fold change pinned to 37°C
Un 37°C
Un39°C
LPS 37°C
LPS 39°C
HTC
1.0
2.5
687
631

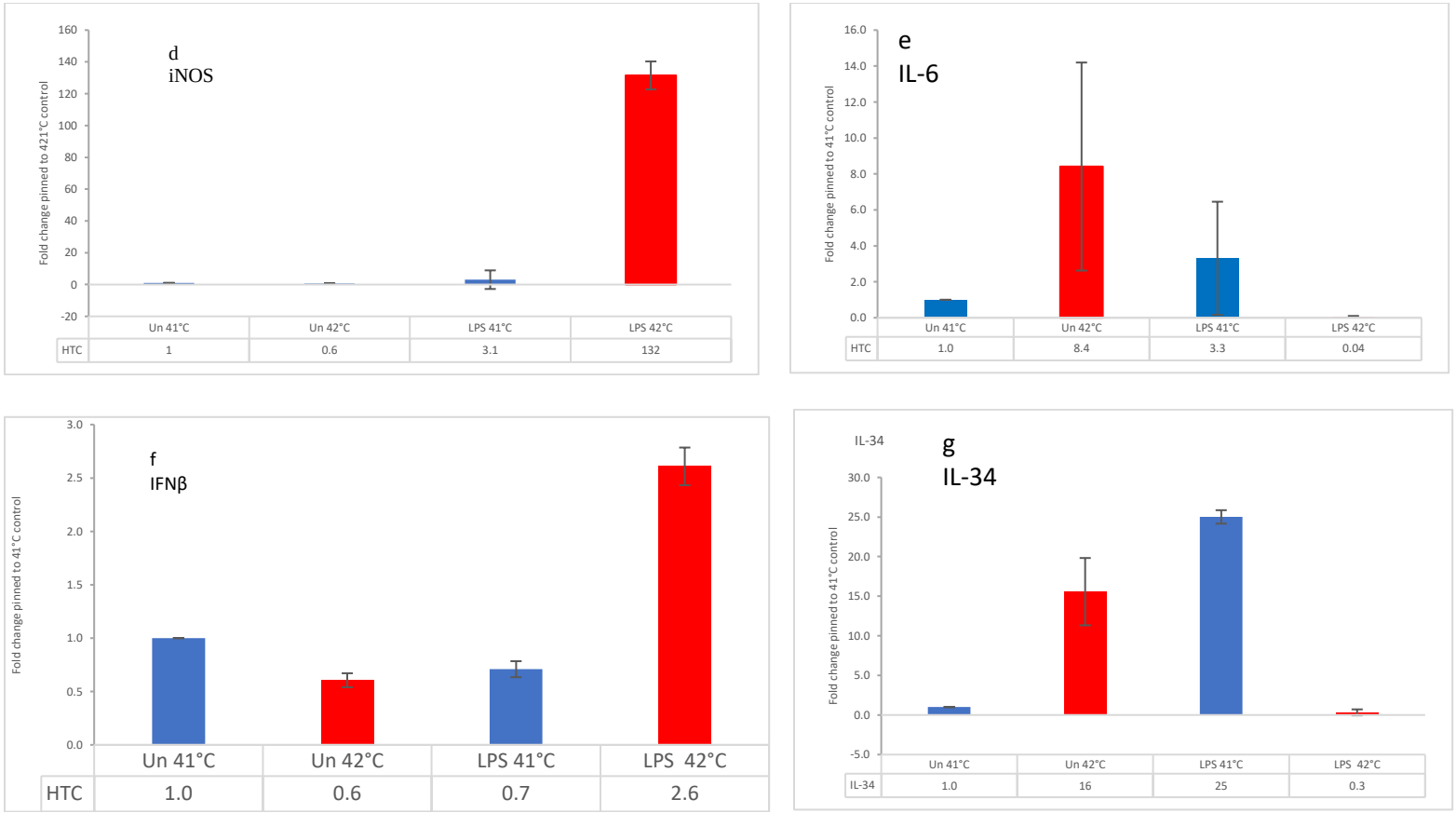


Figure 36: Effect of fever and activation on expression of pro-inflammatory cytokine genes. Triplicate samples of adherent splenocyte (2x106 cells in each well) were stimulated with LPS (0.1µg/ml) at 37, 39, 41 and 42°C for 2 and 4h respectively. Samples were pooled, RNA purified and used for templates for cDNA, and run-in triplicate for RT- qPCR. Blue bars represent the incubation time 2and 4 at 41°C and red bars at 42°C, respectively. All cytokines measured at 2 and 4, by qRT-PCR relative to beta-actin with normalization to the unstimulated 41°C. iNOS (Panel a) IL6 showed in (panel b), IL-12 (panel c), iNOS(Panel d) IL-6 (panel e) IFNβ (panel f) and (panel g) IL-34.

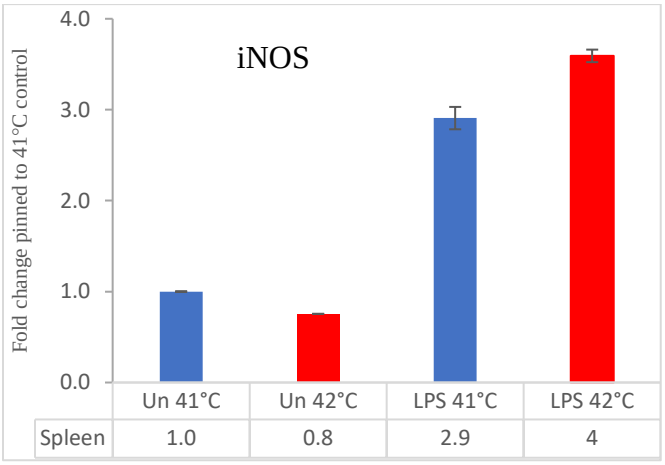
Table 10: RT-qPCR analysis of stimulated HTC cells incubation for 4h. Data are Ct values ± SD. This data is included as the b-actin varies substantially between sample with both temperature and treated showing difference.

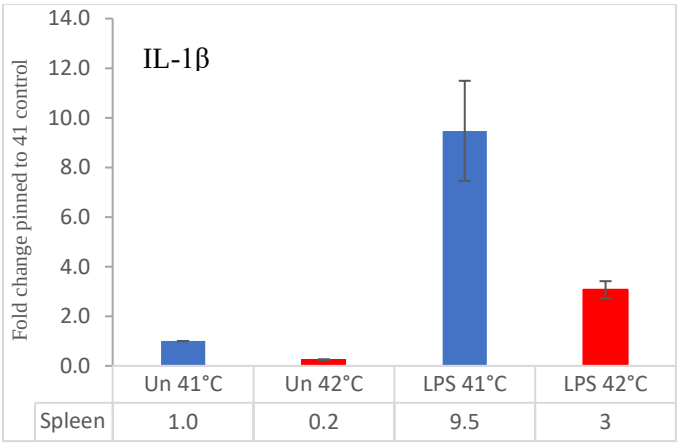
Treatment	Temperature [°C]	RUN	
		4H B-actin ± SD	
UN	37	10.4± 0.1	26.8± 0.5
UN	39	10.9± 0.5	25.0±0.3
LPS	37	9.7± 0.1	21.6± 0.3
LPS	39	11.6± 0.1	17.1± 0.0
		4H B-actin ± SD	4H IL-6
UN	37	20.4± 0.0	28.8± 0.1
UN	39	30.7± 0.4	30.7±0.6
LPS	37	20.8± 0.2	27.0± 0.3
LPS	39	20.7± 0.1	27.3± 0.3
		4H B-actin ± SD	4H IL-12
UN	37	13.8± 0.8	25.7± 0.1
UN	39	14.6± 0.2	25.1±0.5
LPS	37	16.1± 0.4	22.8± 0.1
LPS	39	30.8± 1.1	29.3± 0.5

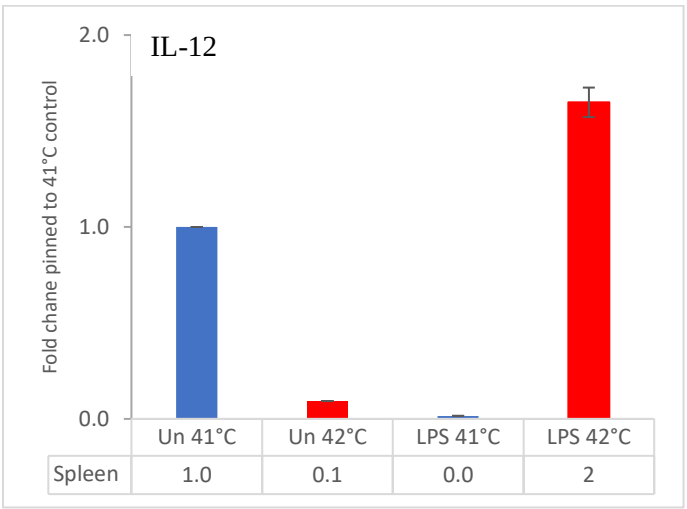
Table 11: RT-qPCR analysis of stimulated HTC cells incubation for 2 and 4h at 37, 39, 40 and 42°C. Data are Ct values ± SD. This data is included as the b-actin varies substantially between sample with both temperature and treated showing difference.

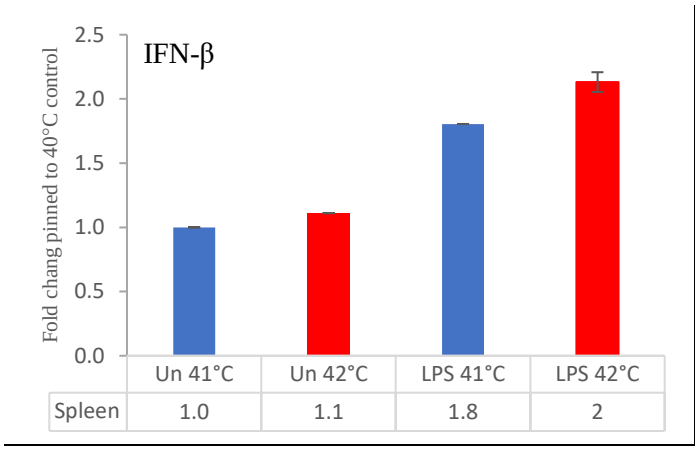
Treatment	Temperature [°C]	RUN	
		4H B-actin ± SD	iNOS
UN	40	8..6± 0.2	12.2.8± 0.1
UN	42	10.7± 0.3	15.0±0.2
LPS	40	9.9± 0.2	9.7± 0.1
LPS	42	13.6± 0.1	10.2± 0.1
		4H B-actin ± SD	4H IL-6
UN	40	8..6± 0.2	31.4± 2.2
UN	42	10.7± 0.3	33.4±5.7
LPS	40	9.9± 0.2	33..9± 5.4
LPS	42	13.6± 0.1	39.9± 2.1
		4H B-actin ± SD	4H IFN-β
UN	40	6.4± 3.3	17.5± 0.0
UN	42	6.5± 0.4	18.4±0.2
LPS	40	6.5± 0.2	18.1± 0.2
LPS	42	8.2± 0.0	18.0± 0.1
		4H B-actin ± SD	4H IL-34
UN	40	6.4± 3.3	32.2±0.4
UN	42	6.5± 0.4	27.9±0.5
LPS	40	6.5± 0.2	27.7±0.1
LPS	42	8.2± 0.0	35.6±1.4

Effect of fever and activation on expression of pro-inflammatory cytokine genes chicken spleen cell.









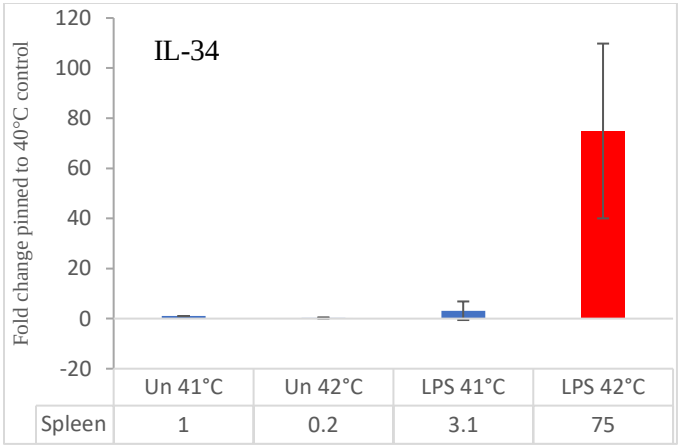


Figure 37: Effect of fever and activation on expression of pro-inflammatory cytokine genes chicken spleen cell. Triplicate samples of adherent splenocyte (2x106 cells in each well) were stimulated with LPS (0.1µg/ml) at 41 and 42°C for 4h. Samples were pooled, RNA purified and used for templates for cDNA, and run-in triplicate for RT- qPCR. Blue bars represent the incubation time at 41°C and red bars at 42°C. All cytokines measured at 4, by qRT-PCR relative to beta-actin with normalization to the unstimulated 41°C. iNOS (Panel a) IL-1β showed in (panel b), IL-12 (panel c), IFNβ (panel d) and (panel e) IL-34.

Table 12: RT-qPCR analysis of stimulated spleen cells incubation for 4h at 40 and 42°C. Data are Ct values ± SD. This data is included b-actin varies with both temperature and treated showing difference.

Treatment	Temperature [°C]	RUN	
		4H B-actin ± SD	4H iNOS
UN	40	13.8± 1.2	10.3. ± 2.1
UN	42	13.3± 0.6	10.8±0.4
LPS	40	19.0± 0.3	15.0± 0.1
LPS	42	16.8± 0.1	12.4± 0.0
		4H B-actin ± SD	IL-6
UN	40	13.8± 1.2	32.3. ± 1.0
UN	42	13.3± 0.6	>42
LPS	40	19.0± 0.3	25.1± 4.6
LPS	42	16.8± 0.1	31.8± 1.1
		4H B-actin ± SD	4H IL-12
UN	40	13.8± 1.2	15.9± 0.5
UN	42	13.3± 0.6	18.7±0.4
LPS	40	19.0± 0.3	27.1± 0.1
LPS	42	16.8± 0.1	18.2± 0.1
		4H B-actin ± SD	4H IFN-β
UN	40	13.8± 1.2	12.3± 0.5
UN	42	13.3± 0.6	11.4±0.3
LPS	40	19.0± 0.3	16.6± 0.0
LPS	42	16.8± 0.1	14.1± 0.0
		4H B-actin ± SD	4H IL-1β
UN	40	13.8± 1.2	19.0± 0.3
UN	42	13.3± 0.6	20.7±0.1
LPS	40	19.0± 0.3	21.2± 0.3
LPS	42	16.8± 0.1	20.6± 0.2
		4H B-actin ± SD	4H TNF-α
UN	40	13.8± 1.2	35.5± 2.3
UN	42	13.3± 0.6	32.7±0.8
LPS	40	19.0± 0.3	27.9± 0.3
LPS	42	16.8± 0.1	27.4 0.3
		4H B-actin ± SD	4H IL-34
UN	40	13.8± 1.2	30.0.2±1.5
UN	42	13.3± 0.6	30.7±1.1
LPS	40	19.0± 0.3	31.0±2.8
LPS	42	16.8± 0.1	26.0±0.7

Effect of behavioral fever temperature and stimulation on nitric oxide production by RTS11 macrophages.

The RTS11 cell line originate from rainbow trout spleen, was used to investigate the effect of fever temperature on fish macrophage functions. Protein synthesis and NO generation and cytokine expression were used to quantify macrophage metabolism and activity during exposure to various temperatures. In RTS11 macrophages, a temperature at 19°C with simulations can boost NO generation and protein synthesis. Lipopolysaccharide, poly I:C, and peptidoglycan, mimics of or genuine components found in viruses or bacteria known to enhance macrophage activity in mammals, birds, and fish, were used to stimulate macrophage activity. The effects of stimulations on NO generation and protein synthesis differ depending on whether the stimulation is viral or bacterial, with viral stimulations consistently increasing nitric oxide production and protein synthesis while bacterial stimulations cause more varied responses. These findings suggest that traveling to a warmer portion of the stream, which considered behavioral fever, has a significant impact on macrophage activity and, as a result, on the immune response.

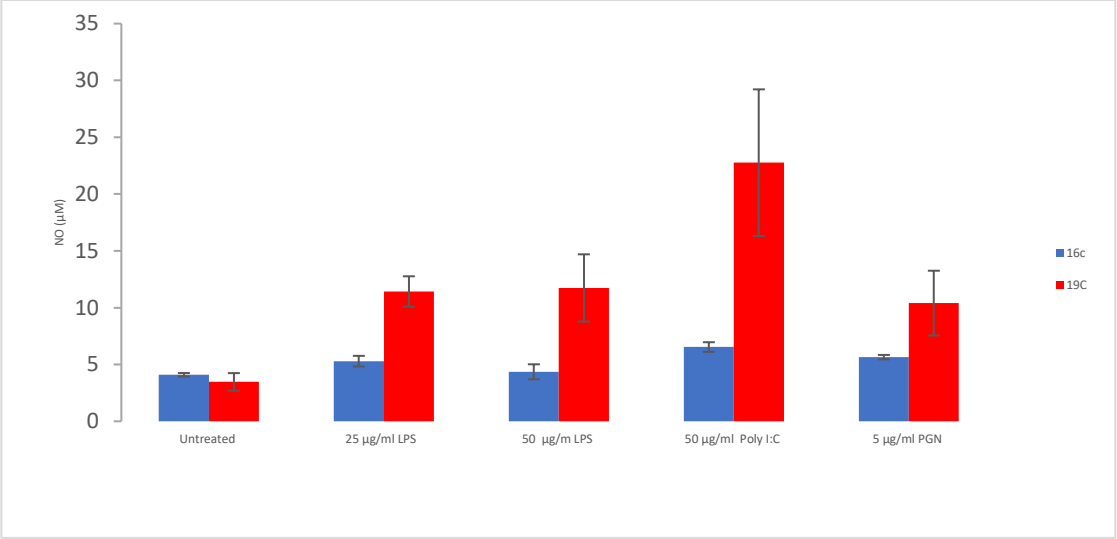


Figure 38: Effect of behavioral fever temperature and stimulation on nitric oxide production by RTS11 macrophages. Cells were incubated at 16°C and 19°C for 24- 48 hours.200,00 cells were treated 25- 50 µg/ml LPS., 50u µg/ml poly I:C and µg/ml PGN 5. NO Concentration **(µM) measured by Griess reagent This is one of 3 experiments with similar results.**

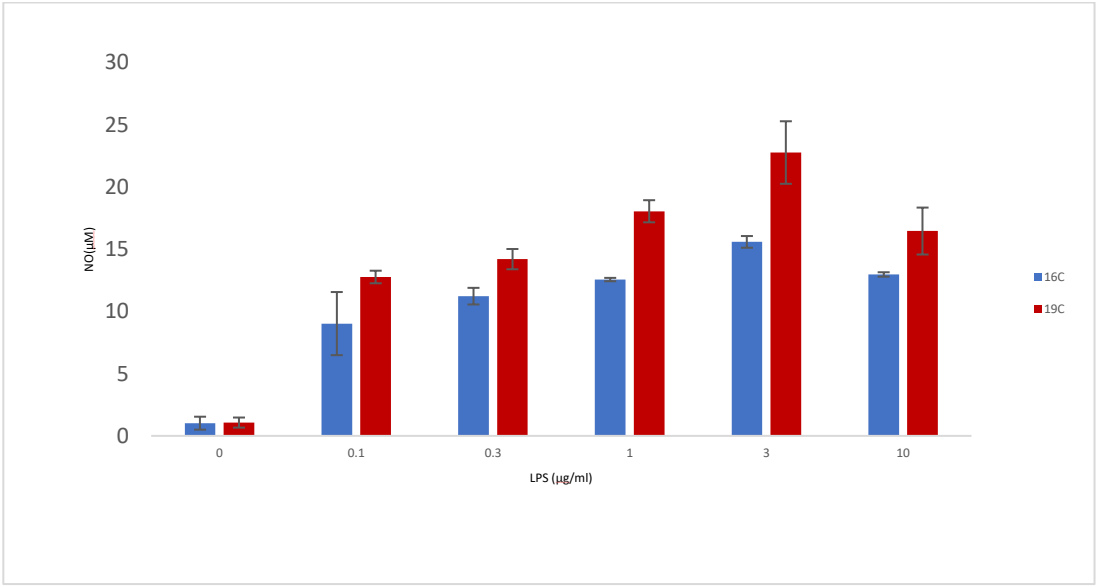
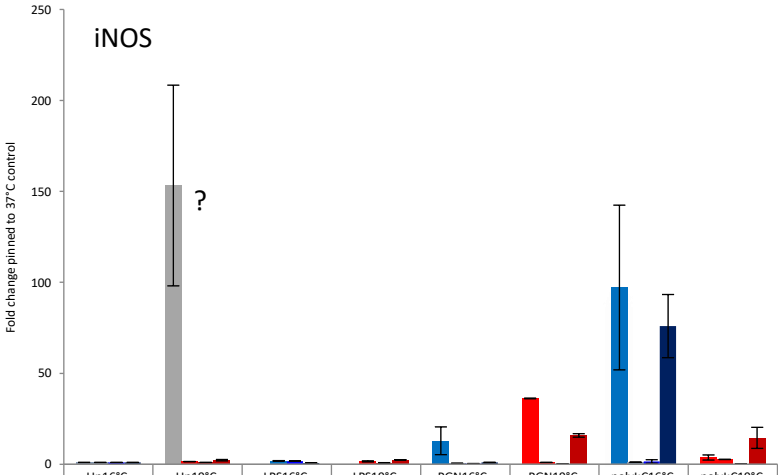


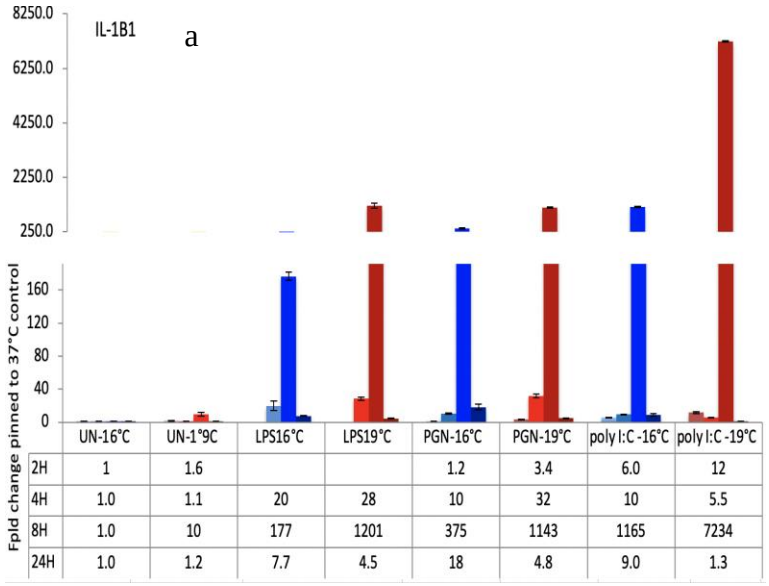
Figure 39: The production of NO by FHK cells. The amount of NO in the sample's supernatant 24 hours after treatment was measured. **Cells stimulated with different LPS concentrations (0.1-10 µg/ml) cells WERE incubated for 24 hours at 16 and 19°C was compared.** Blue bars represent cell cultured at 16°C and the red bars at 19°C.

Effect of **behavioral fever temperature** on proinflammatory cytokine expression

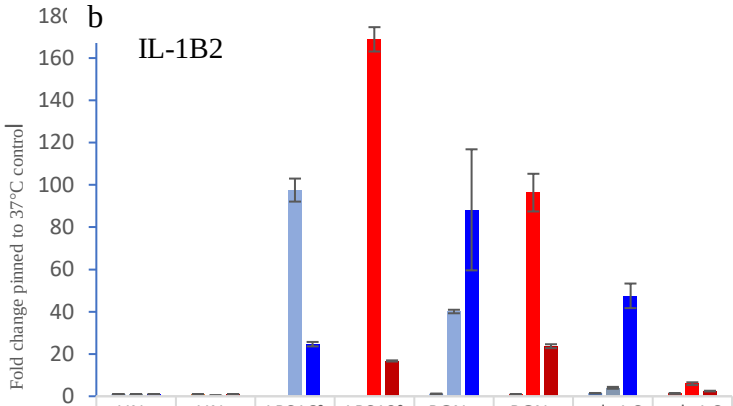
The effect of temperature with LPS, PGN and poly I:C stimulation on proinflammatory cytokine mRNA expression in RTS11 cells. In unstimulated cultures different cytokines were detectable at different times. IL-6, TNF-α1, TNF-α2, IL-β1 show a small increase at high temperatures at 4 and 8h while IL-12 and TNFα3 show a modest increase at 19°C at 8 and 24h respectively. By qRT-PCR, LPS significantly upregulated the expression of IL-6 and IFNα2 at 19°C compared to 16°C at 4h of incubation (Fig. 40). Next, we investigated whether PGN stimulation is influenced by temperature. RTS11 cells show a positive effect on IL-6 at 8h and a small increase in IL-β1, IFNα1 and IFNα3 during 4 and 8h of incubation. The effects with polyI:C on proinflammatory cytokine expression were examined on IL-12 at 2 and 24h, IL-β1at 8h and TNFα3 increase at 8h, at 2, 8 and 24h incubation time increase was observed for IL-12 (Fig.40). However, for the other cytokines (iNOS, IL-6, IL-β2, TNF-α1 and I TNF-α2) the expression was slightly reduced or equal at 19°C compared to 16°C at 2,4,8 and 24h.



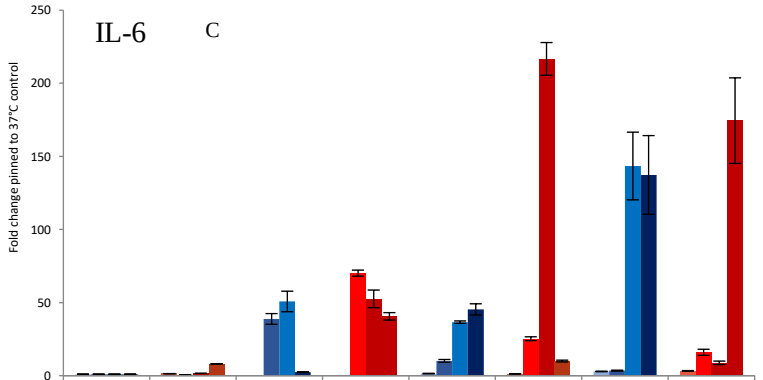
	Un16°C	Un19°C	LPS16°C	LPS19°C	PGN16°C	PGN19°C	polyI:C16°C	polyI:C19°C
2H	1	153			13	36	97	3.7
4H	1.0	1.5	1.9	1.6	0.5	1.0	1.1	2.7
8H	1.0	1.0	1.7	0.8	0.4	0.2	1.6	0.3
24H	1.0	2.3	0.7	2.4	1.0	16	76	15



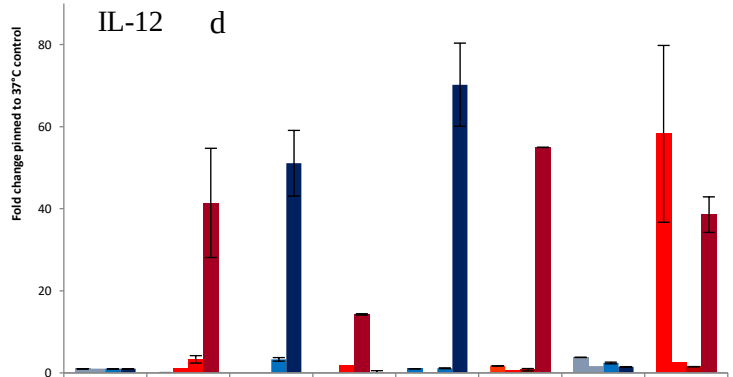
	UN-16°C	UN-1°9C	LPS16°C	LPS19°C	PGN-16°C	PGN-19°C	poly I:C -16°C	poly I:C -19°C
2H	1	1.6			1.2	3.4	6.0	12
4H	1.0	1.1	20	28	10	32	10	5.5
8H	1.0	10	177	1201	375	1143	1165	7234
24H	1.0	1.2	7.7	4.5	18	4.8	9.0	1.3



	UN-16°C	UN-1°9C	LPS16°C	LPS19°C	PGN-16°C	PGN-19°C	poly I:C -16°C	poly I:C -19°C
2h	1	0.8			1	1	1	1
4h	1.0	0.3	98	169	40	96	4.0	6.0
24h	1.0	1.0	25	17	88	24	48	2.5



	Un16°C	Un19°C	LPS16°C	LPS19°C	PGN16°C	PGN19°C	polyI:C16°C	polyI:C19°C
2H	1	1.4			1.4	1.3	2.9	3.3
4H	1.0	0.5	39	70	10	25	3.5	16
8H	1.0	1.6	51	53	37	217	143	8.8
24H	1.0	8.1	2.4	41	45	10	137	174



	Un19°C	Un16°C	LPS16°C	LPS19°C	PGN16°C	PGN19°C	polyI:C 19°C	polyI:C 16°C
2H	1	0.1			1.0	1.7	3.8	58
4H	1.0	1.1	0.1	1.8	0.2	0.6	1.6	2.5
8H	1.0	3.3	3.3	14	1.1	0.8	2.4	1.5
24H	1.0	41	51	0.1	70	55	1.5	39

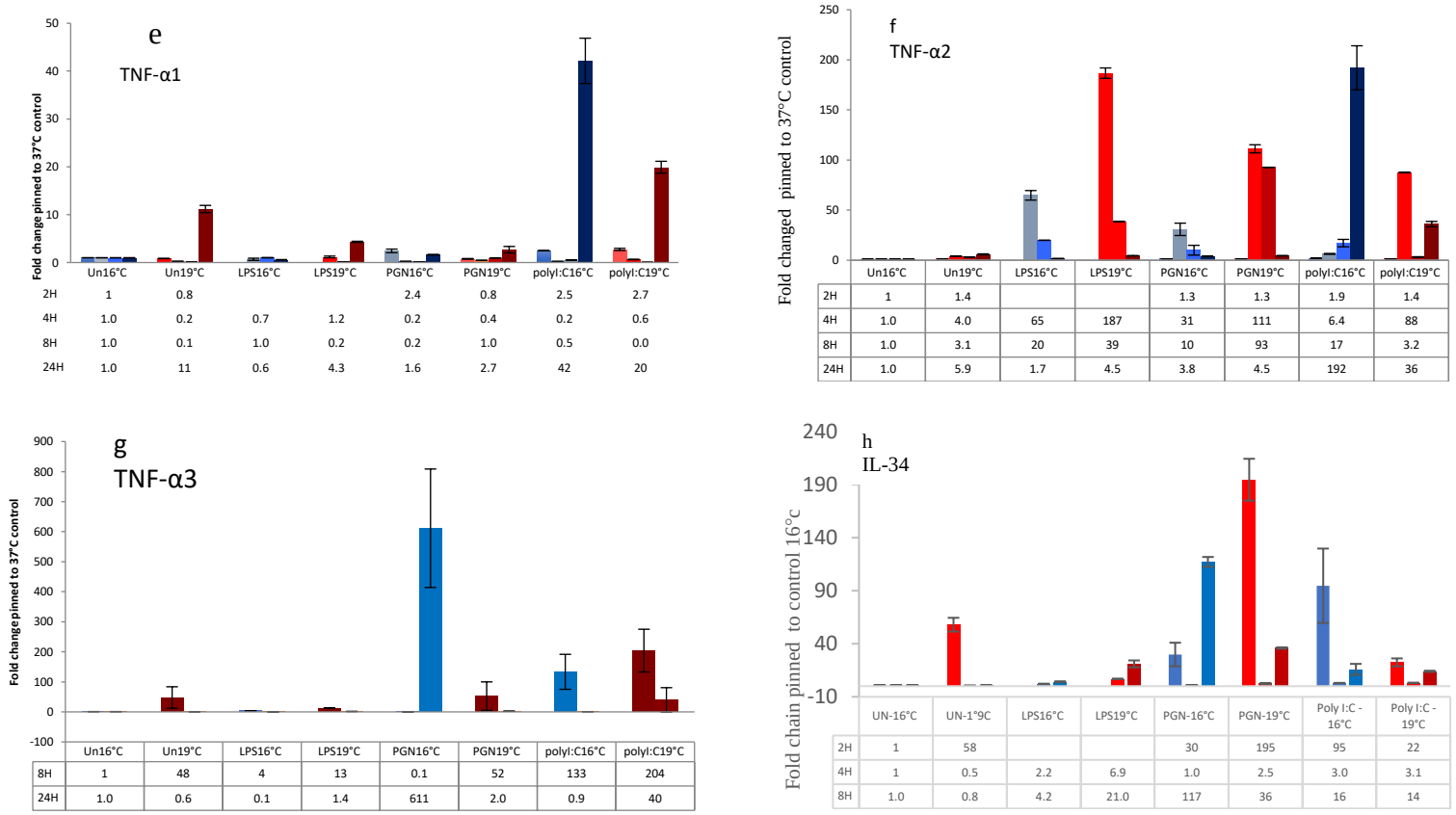


Figure 40. Effect of behavioral fever temperature and activation on expression of pro-inflammatory cytokine genes. Triplicate samples of RTS11 cells ($2x10^6$ cells in each well) were stimulated with LPS (50µg/ml), PGN (5µg/ml) and (50µg/ml) of Poly I:C at 16 and 19°C for 2,4,8and 24h. Samples were pooled, RNA purified and used for templates for cDNA, and run-in triplicate for RT- qPCR. Light and dark blue bars represent the incubation time 2,4,8 and 24h at 16°C and light and dark red bars at 19°C, respectively. ΔΔCt scored for cytokines measured at 2 ,4,8 and 24h for RTS11are shown beta-actin with reference to the unstimulated 16°C. (Panel a) IL-β1 (panel a), IL-β2 (panel b) IL6 showed in (panel c), IL-12 (Panel d), TFN-α1 (panel e), TFN-α2(Panel f) and TFN-α3 (panel g).

Table 13: The cytokine expression to various stimuli over a 2,4,8 and 24h period is presented in Table 13. Increases in 7 of the 8 gene of interest cytokines (IL-6, 1L-12, IL-1b, IL-1b 2, TNF∝1, TNF∝1, and iNOS or IL-34, at 19 compared to 16°C baseline was observed. Table F presents cytokine levels at the various study time points at 16 and 19°C with information about the expression of cytokine gene mRNA at RTS11 at 16 and 19.

Treatment	Temperature[°C]	RUN	Time[hours]						
		2H B-actin ± SD iNOS	2H iNOS	4H B-actin ± SD	4H iNOS	8H B-actin ± SD	8H iNOS	24H B-actin ± SD	24H iNOS
UN	16	13.4± 0.1	32.5± 0.3	11.6± 0.4	24.6±1.1	15.4± 0.1	20. 8±0.8	14.5± 0.2	25.4±0. 6
UN	19	9.6± 0.5	27.0±0.5	9.1± 1.3	22.0±0.0	14.4± 2.2	19.4±0.1	15.0± 0.2	24.2±0.9
LPS	16			10.7± 0.6	23.2±0.3	16.0± 0.5	20.6±0.8	13.6± 0.2	25.5±0.8
LPS	19			10.9± 1.0	24.1±0.6	15.3± 4.8	20.4±0.1	15.1± 2.6	25. 1±0.1
PGN	16	13.4± 0.4	34.4± 0.8	8.5± 0.5	22.9±0.1	13.8± 0.8	20.6±0.4	13.8± 0.8	25.1±0.3
PGN	19	10.7± 0.1	30.2± 0.0	8.6± 0.5	22.1±0.0	13.5± 1.2	20.3±0.7	16.7± 1.4	24.4±0.6
Poly I:C	16	10.8± 0.2	28.8± 0.6	10.5± 0.2	23.9±0.2	15.9± 1.4	20.2±0.7	18.8± 1.0	23.8±1.2
Poly I:C	19	10.8± 0.7	33.5± 0.5	10.3± 0. 5	23.0±0.4	13.9± 0. 7	20.6±0.2	16.7± 1.3	24.1±0.5
		B-actin IL-6	2H IL-6	4H B-actin ± SD	4H IL-6	8H B-actin ± SD	8H IL-6	24H B-actin ± SD	24H IL-6
UN	16	15.4±0.7	19.3±0.2	11.9± 0.2	20.0±0.2	16.1±0.2	21.5±0.2	20.2±1.5	20.1±0.3
UN	19	13.1±0.1	16.9±0.2	9.0± 0.3	17.9±0.1	13.9±2.2	18.8±0.3	22.8±1.3	19.7±0.0
LPS	16			11.2± 0.7	14.0±0.1	16.1±0.7	15.8±0.1	17.0±0.2	15.6±0.1
LPS	19			11.2± 0.9	13.2±0.0	14.0±1.8	13.7±0.2	22.1±0.3	16.7±0.1
PGN	16	16.2±0.3	19.9±0.2	9.6± 0.2	14.3±0.1	13.1±0.2	13.4±0.2	18.7±0.3	13.1±0.1
PGN	19	13.4±0.2	17.2±0.1	8.0± 0.5	11.4±0.1	15.6±3.7	13.3±0.1	20.7±1.1	17.3±0.1
Poly I:C	16	13.2±0.3	15.8±0.1	9.4± 0.1	15.7±0.1	15.4±0.7	13.6±0.2	23.0±0.6	15.8±0.3
Poly I:C	19	13.1±0.3	15.5±0.1	10.7± 0. 5	14.8±0.2	11.7±0.2	13.9±0.2	23.6±0.2	16.0±0.2
		B-actin IL-12	4H IL-12	4H B-actin ± SD	4H IL-12	8H B-actin ± SD	8H IL-12	24H B-actin ± SD	24H IL-12
UN	16	13.4± 0.1	42.00±0.00	11.6± 0.4	29.5±0.8	15.4± 0.1	27.7±1.2	14.5± 0.2	35.0±0.0
UN	19	9.6± 0.5	42.00±0.00	9.1± 1.3	27.2±0.1	14.4± 2.2	25.0 ±0.2	15.0± 0.2	30.1±0.5
LPS	16			10.7± 0.6	31.7±1.2	16.0± 0.5	26.7±0.1	13.6± 0.2	28.5±0.2
LPS	19			10.9± 1.0	27.4±0.4	15.3± 4.8	25.2±0.0	15.1± 2.6	38.5±2.5
PGN	16	13.4± 0.4	41.9±0.1	8.5± 0.5	29.0±0.2	13.8± 0.8	25.5±0.2	13.8± 0.8	28.2±0.2
PGN	19	10.7± 0.1	38.6±1.2	8.6± 0.5	27.3±0.2	13.5± 1.2	26.2±0.5	16.7± 1.4	30.2±1.3
Poly I:C	16	10.8± 0.2	27.3±0.1	10.5± 0.2	27.6±0.3	15.9± 1.4	26.9±0.1	18.8± 1.0	38.8±0.0
Poly I:C	19	10.8± 0.7	33.5±0.5	10.3± 0. 5	26.8±0.2	13.9± 0. 7	25.0±0.3	16.7± 1.3	32.2±2.2
		B-actin - TNFα1	2H TNFα1	4H B-actin ± SD	4H TNFα1	8H B-actin ± SD	8H TNFα1	24H B-actin ± SD	24H TNFα1
UN		15.4±0.7	24.2±0.6	11.9± 0.2	20.2±0.3	16.1±0.2	17.5±0.5	20.2±1.5	20.3±0.0
UN		13.1±0.1	22.1±0.1	9.0± 0.3	19.7±0.2	13.9±2.2	19.0±1.0	22.8±1.3	19.4±0.4
LPS				11.2± 0.7	20.8±0.8	16.1±0.7	17.8±0.1	17.0±0.2	17.8±0.1
LPS				11.2± 0.9	19.6±0.2	14.0±1.8	17.2±0.1	22.1±0.3	18.0±0.1
PGN		16.2±0.3	24.3±0.9	9.6± 0.2	20.2±0.1	13.1±0.2	17.5±0.0	18.7±0.3	16.4±0.1
PGN		13.4±0.2	22.6±0.1	8.0± 0.5	18.2±0.7	15.6±3.7	17.4±0.1	20.7±1.1	19.3±0.4
Poly I:C		13.2±0.1	20.8±0.1	9.4± 0.1	20.1±0.3	15.4±0.7	17.7±0.1	23.0±0.6	19.4±0.2
Poly I:C		13.1±0.3	20.5±0.1	10.7± 0. 5	20.3±0.6	11.7±0.2	17.8±0.9	23.6±0.2	19.3±0.1
		B-actin- TNFα2	2H TNFα2	4H B-actin ± SD	4H TNFα2	8H B-actin ± SD	8H TNFα2	24H B-actin ± SD	24H TNFα2
UN	16	15.4±0.7	22.4±0.2	11.9± 0.2	24.3±0.9	16.1±0.2	23.8±0.6	20.2±1.5	23.3±0.1
UN	19	13.1±0.1	19.9±0.1	9.0± 0.3	19.4±0.1	13.9±2.2	20.0±0.1	22.8±1.3	20.4±0.1
LPS	16			11.2± 0.7	17.6±0.1	16.1±0.7	19.5±0.2	17.0±0.2	16.4±0.0
LPS	19			11.2± 0.9	16.1±0.0	14.0±1.8	16.4±0.3	22.1±0.3	17.9±0.0
PGN	16	16.2±0.3	23.1±0.2	9.6± 0.2	17.5±0.1	13.1±0.2	17.4±0.1	18.7±0.3	15.2±0.0
PGN	19	13.4±0.2	20.6±0.4	8.0± 0.5	13.7±0.1	15.6±3.7	16.8±0.1	20.7±1.1	18.6±0.0
Poly I:C	16	13.2±0.1	19.5±0.1	9.4± 0.1	19.2±0.1	15.4±0.7	18.9±0.1	23.0±0.6	17.3±0.2
Poly I:C	19	13.1±0.3	19.9±0.1	10.7± 0. 5	16.7±0.0	11.7±0.2	17.7±0.0	23.6±0.2	18.5±0.1

		B-actin - TNFα3	2H TNFα3	4H B-actin ± SD	4H TNFα3	8H B-actin ± SD	8H TNFα3	24H B-actin ± SD	24H TNFα3
UN	16	26.1±0.2	>42	19.5±0.1	>42	15.4± 0.1	39.9± 0.0	14.5± 0.2	37.7± 0.1
UN	19	26.6±0.6		17.1±0.0	39.9±0.4	14.4± 2.2	33.3± 1.0	15.0± 0.2	42± >
LPS	16			17.7±0.0	34.2±0.0	16.0± 0.5	38.4± 0.0	13.6± 0.2	42± >
LPS	19			18.3±0.1	>42	15.3± 4.8	35.4± 2.2	15.1± 2.6	39.9± 0.0
PGN	16	20.5±0.5	>42	16.9±0.1	>42	13.8± 0.8	40.8± 1.1	13.8± 0.8	28.6± 0.5
PGN	19	24.5±0.2	>42	16.4±0.3	>42	13.5± 1.2	31.5± 2.0	16.7± 1.4	42.0± >
Poly I:C	16	21.7±0.1	>42	18.5±0.1	>42	15.9± 1.4	33.4± 0.6	18.8± 1.0	42.7± 0.0
Poly I:C	19	22.0 ±0.6	>42	17.6±0.4	>42	13.9± 0. 7	30.3± 0.5	16.7± 1.3	34.6± 1.3
		B-actin - IL-1β1	4H IL-1β1	4H B-actin ± SD	4H IL-1β1	8H B-actin ± SD	8H IL-1β1	24H B-actin ± SD	24H IL-1β1
UN	16	13.5±0.1	19.9±0.3	16.4±0.1	17.1±0.2	17.8±0.3	28.8±1.9	16.4±0.1	15.5±0.1
UN	19	10.8 ±0.4	16.5±0.1	13.9±0.0	14.5±0.1	14.7±0.0	22.5±0.5	15.94±0.1	12.1±0.1
LPS	16			15.3±0.2	11.5±0.3	12.8±0.1	16.4±0.0	14.8±0.8	9.7±0.3
LPS	19			15.8±0.0	11.7±0.1	15.0±0.0	15.8±0.1	19.12±0.2	15.1±0.3
PGN	16	13.8±0.2	19.7±0.2	13.9±0.0	10.6±0.9	15.9±0.2	18.5±0.1	16.6±0.7	15.5±0.1
PGN	19	12.1±0.2	16.7±0.2	13.6±0.1	9.4±0.1	14.3±0.1	15.2±0.0	16.6±0.1	13.6±0.1
Poly I:C	16	11.3±0.3	14.9±0.2	15.7±0.1	13.2±0.0	14.6±0.1	15.5±0.0	17.4±0.1	14.2±0.2
Poly I:C	19	11.5±0.6	14.3±0.1	14.0±0.6	12.3±0.2	14.0±0.6	12.3±0.2	16.0±0.1	14.8±0.2
		B-actin IL1β2	4H IL-1β2	4H B-actin ± SD	4H IL-1β2	8H B-actin ± SD	8H IL-1β2	24H B-actin ± SD	24H IL-1β2
UN	16	13.5±0.1	20.6±0.1	16.4±0.1	24.3±1.1	21.1±0.2	25.7±0.4	16.4±0.1	22.9±0.1
UN	19	10.8 ±0.4	18.2±0.1	13.9±0.0	23.4±0.4	18.4±0.1	22.7±0.4	15.9±0.1	17.8±0.1
LPS	16			15.3±0.2	16.6±0.1	22.1±0.2	18.6±0.4	14.8±0.8	14.9±0.4
LPS	19			15.8±0.0	16.3±0.0	14.8±0.2	15.0±0.2	19.1±0.2	20.0±0.2
PGN	16	13.8±0.2	20.7±0.1	13.9±0.0	16.5±0.0	18.4±0.1	15.6±0.1	16.6±0.7	23.1±0.2
PGN	19	12.1±0.2	19.2±0.0	13.6±0.1	14.9±0.1	18.2±0.1	15.6±0.1	16.6±0.1	19.1±0.0
Poly I:C	16	11.3±0.3	17.9±0.1	15.7±0.1	21.6±0.2	19.7±0.1	18.8±0.2	17.4±0.1	19.3±0.1
Poly I:C	19	11.5±0.6	18.2±0.1	14.0±0.6	19.4±0.2	16.8±0.2	178.3±0.2	16.0±0.1	21.2±0.1
		B-actin - IL34	2H IL-34	4H B-actin ± SD	4H IL-34	8H B-actin ± SD	8H IL-34	24H B-actin ± SD	24H IL-34
UN	16	26.1±0.2	27.0±0.0	16.4±0.1	18.2 ±0.0	17.8±0.3	21.4±0.3	16.4±0.1	18.5±0.2
UN	19	26.6±0.6	21.7±0.1	13.9±0.0	16.6±0.0	14.7±0.0	18.8±0.1	15.94±0.1	15.4±0.1
LPS	16			15.3±0.2	15.9±0.0	12.8±0.1	14.5±0.2	14.8±0.8	14.1±0.1
LPS	19			15.8±0.0	14.8±0.1	15.0±0.0	14.3±0.2	19.12±0.2	15.4±0.1
PGN	16	20.5±0.5	16.5±0.0	13.9±0.0	15.7±0.1	15.9±0.2	12.7±0.1	16.6±0.7	18.5±0.2
PGN	19	24.5±0.2	14.9±0.1	13.6±0.1	14.1±0.3	14.3±0.1	12.8±0.0	16.6±0.1	18.3±0.2
Poly I:C	16	21.7±0.1	21.6±0.2	15.7±0.1	15.9±0.0	14.6±0.1	14.3±0.3	17.4±0.1	17.4±0.1
Poly I:C	19	22.0 ±0.6	19.4±0.2	14.0±0.6	14.2±0.1	14.0±0.6	13.9±0.1	16.0±0.1	15.6±0.1

Compare RAW264.7 cells with RTS11rainbow trout cell line.

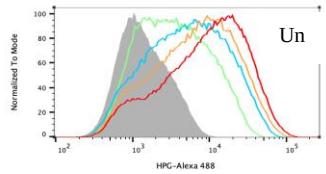
This study evaluated the effect of temperatures on fish cell line, RTS11 and primary adherent cells from rainbow trout fish head kidney and spleen. As we lacked rainbow trout IFN-γ, we utilized a viral mimic, poly I:C and PGN, a polypeptide from gram negative bacteria and LPS to explore fish macrophage temperature responses. We scored a sample as positive if it was higher in mRNA expression at any of four time points over 24 h. We observed that fever temperature enhances LPS, PGN and polyI:C stimulated iNOS, IL-6, IL-1β1, IL-1β2, IL-12, 3 TNF-∝

isoforms and IL-34 expression in RTS11. PGN gave the swiftest responses. For untreated RTS11 cells increases at 19 °C were seen for iNOS, IL-6, TNFα2, TNFα3, IL-1β1, and IL-12 but not for IL-1β2, TNFα3 and IL-34. Our results agree with previous work of (Suhee Hong, 2013) showing LPS is an inducer of *TNF-α1 and 3 isoforms* in RTS-11 cells. The time course for induction of these molecules was distinct from mouse and chicken very few inflammatory cytokines peaked by 2h, most peaked by 4h and some only by 24h.

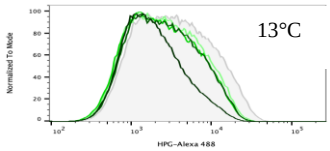
Effect behavioral fever temperature on protein synthesis of RTS11.

Protein synthesis increases with temperature over the entire range tested which was 7°C (Figure 41a). Additional treatment with LPS caused no further increase in protein synthesis (Figure 41b-e) with the exception of 0.1 µg/ml LPS at 19°C (Figure 41d) that showed a small shift to the right in the histogram indicating an increase in protein synthesis.

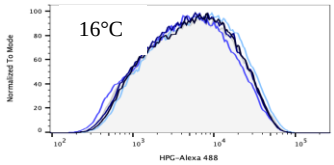
(a)



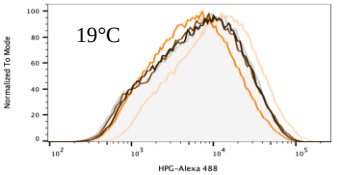
(b)



(c)



(d)



(e)

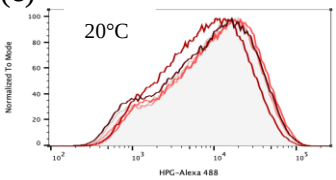


Figure 41: Protein synthesis of RTS11 cells at different temperatures with and without LPS stimulation. (a) HPG incorporation of RTS11 cells at different temperatures [4, 13,16,19 and 20°C]. Triplicate cultures on one million cells for each treatment were incubated for 4 hours in methionine-free medium supplemented with HPG and then stained via Click-it chemistry and analyzed by flow cytometry. The number of events collected for each pooled sample was 100,000. A gate for size was used to remove small debris and large aggregates of cells. The light gray filled histogram in (a) represents unstimulated cells at 4°C. The green line protein synthesis at 13°C, the light blue at 16°C and the orange at 19°C and the red line at 20°C. (b) Flow cytometry pattern of RTS11 cells at 13°C with different LPS concentrations. The gray filled histogram unstimulated at 13°C overlaid with different LPS concentrations (0.1, 0.3, 1.0 and 3.0 µg/ml) shown from lighter green to darker green. (c) Flow cytometry pattern of RTS11 cells at 16°C with different LPS concentrations. The gray filled histogram represent unstimulated cells at 16°C. LPS stimulations range from light blue to dark blue. (d) The gray filled histogram unstimulated at 19°C. LPS stimulations range from light orange to dark orange. (e) The gray filled histogram shows unstimulated at 20°C. LPS stimulations range from light red to dark red.

Effect of behavioral fever temperature in Fish head kidney

Adherent cells from rainbow trout head kidney were cultured at 4, 13, 16,19,21 and 23°C for 4h to test protein synthesis levels. The order of the levels of protein synthesis of untreated cells from the highest to the lowest temperature is 19 and 21°C, 23 16,13 and 4° C (Figure 42) left panel. When rainbow trout head kidney macrophages were stimulated with 0.3 µg/ml LPS increases compared to untreated at same temperature were observed at 19 and 21°C. Interestingly at 16°C, head kidney macrophages stimulated with a higher dosage of LPS (10 µg/ml) increase protein synthesis to the maximal levels seen at lower dosages at the higher temperatures.

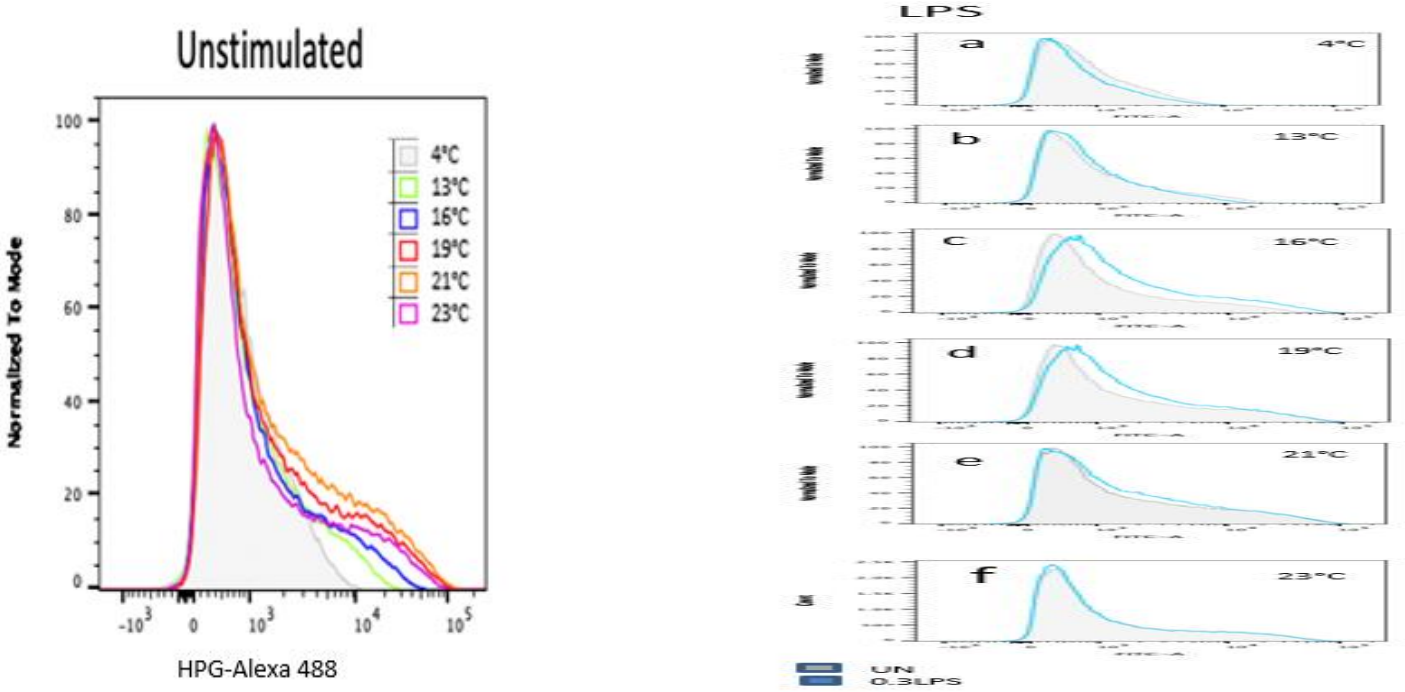


Figure 42: Protein synthesis of trout head kidney macrophage cells at different temperatures with and without LPS stimulation. (a) HPG incorporation of trout head kidney macrophage cells at different temperatures 4, 13,16,19 ,21and 23°C. Triplicate cultures on one million cells for each treatment were incubated for 4 hours in methionine-free medium containing HPG and then stained via Click-it chemistry and analyzed by flow cytometry. The number of events collected for each pooled sample 100,000. A gate for size was used to remove small debris and large aggregates of cells. Unstimulated shown in the left and LPS stimulated at the right. The light gray filled histogram in represents unstimulated cells (a) at 4°C, (b) at 13°C, (c) at 16°C, (d) at 19°C, (e) at 21°C and (f) at 23°C. The blue line represents stimulated sample with 0.3 µg/ml LPS concentrations at 4, 13, 16, 19, 21and 23 °C (a through f).

Effect of behavioral fever temperature on protein synthesis by spleen rainbow trout cell Rainbow trout adherent spleen cells were cultured at 4, 13, 16,19 and 21 °C for 4h to test protein synthesis levels. Unstimulated cells show a higher level of protein production at 19°C and 21°C than at 4, 13 and 16°C. Cell stimulated cells with LPS show a small increase 10 µg/ml in protein synthesis at 0.3 LPS indicating strong stimulation can compensate. Interestingly at 16, 19, and 21°C, cells stimulated with a higher dosage of LPS (10 µg/ml) increased protein synthesis.

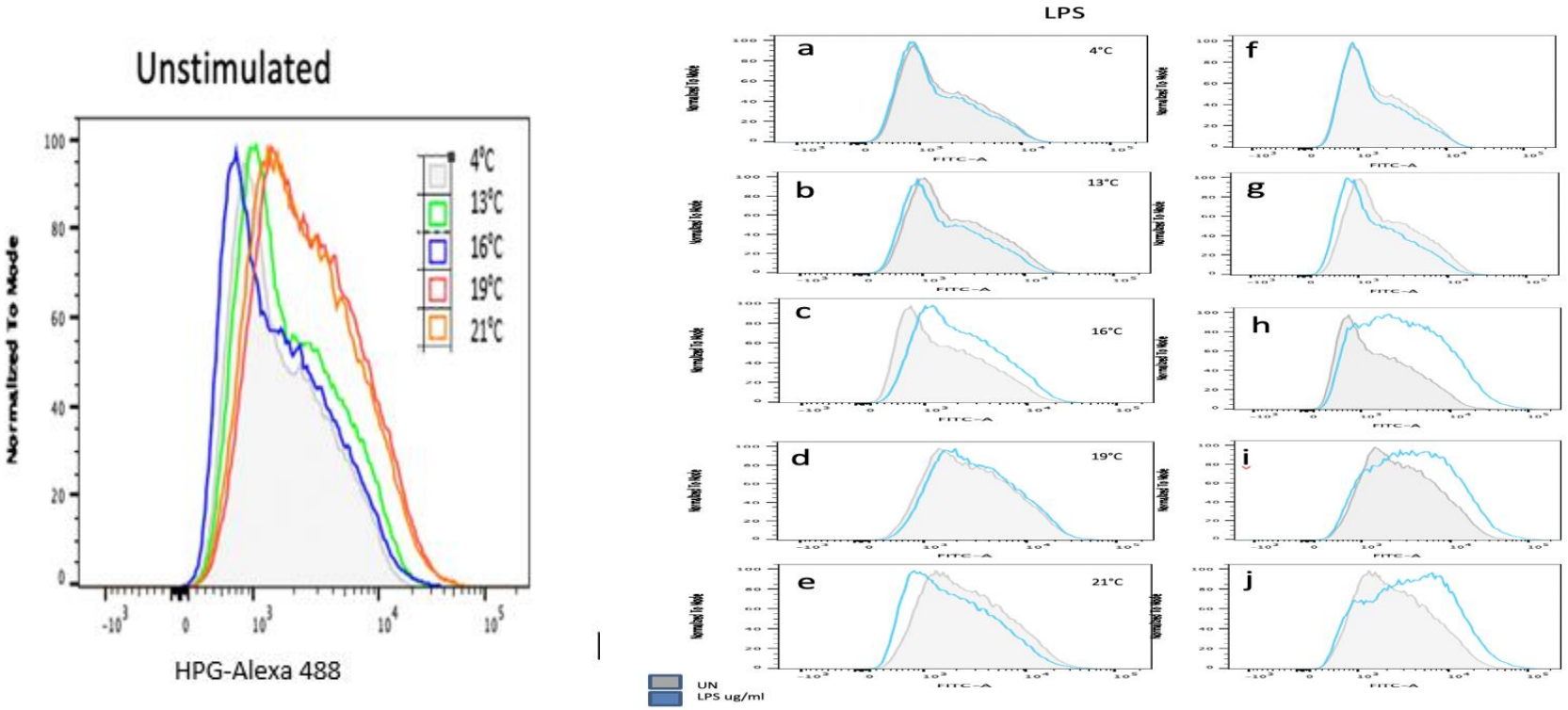


Figure 43: Protein synthesis of rainbow trout adherent splenocytes cells at different temperatures with and without LPS stimulation.(a)HPG incorporation of trout splenocyte at different temperatures 4, 13,16,19 ,21and 23°C. Triplicate cultures on one million cells for each treatment were incubated for 4 h in methionine-free medium supplemented with HPG and then stained via Click-it chemistry and analyzed by flow cytometry. The number of events collected for each pooled sample was 100,000. A gate for size was used to remove small debris and large aggregates of cells. In the left panel left light gray filled histogram in represents unstimulated cells a at 4°C, green at 13°C (b), blue at 16°C (c) show histogram for sample stimulated, red at 19°C (d), orange at 21°C (e). The blue line represents sample stimulated with 0.3 µg/ml LPS at 4 (a), 13(b), 16, (c) 19 (d)and 21°C (e) respectively. (f) represent stimulated sample with 10 µg/ml LPS concentrations at 4°C, (g) at 13°C, (h) at 16°C, (i) at 19°C and (j) at 21°C.

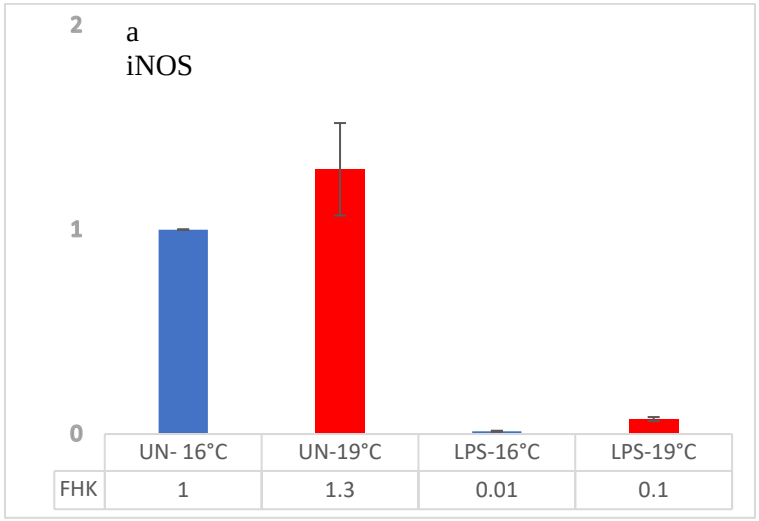
Comparing RAW264.7 cells with RTS11rainbow trout cell line.

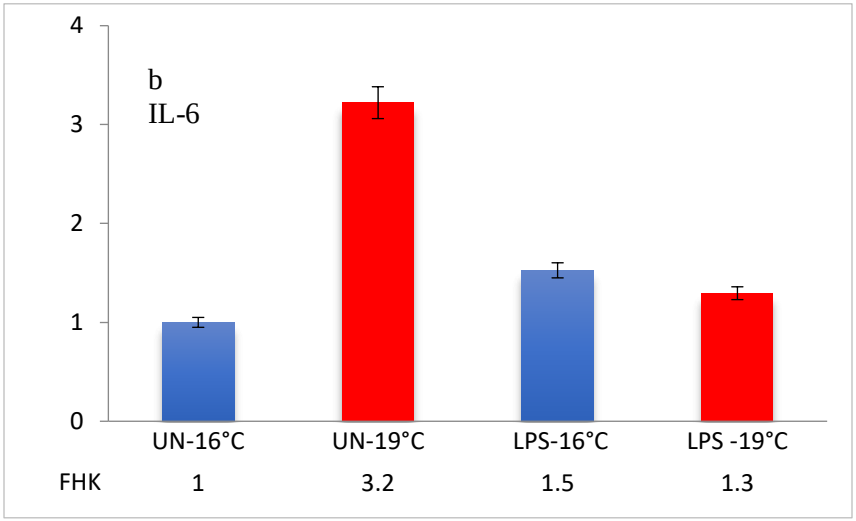
This study evaluated the effect of temperatures on rainbow trout fish head kidney and spleen adherent cells and compared that with mouse and chicken cells. We observed that fever temperature enhances LPS-stimulated IL-6, IL1β1, 1β2, IL-12, TNF-∝1 and IL34 expression in RTS11. The expression of IL-6 was higher at 19°C with LPS. As we leaked rainbow trout IFN-g we utilize other TLRs or viral mimics, PGN and poly I:C, to explore fish macrophage temperature responses.

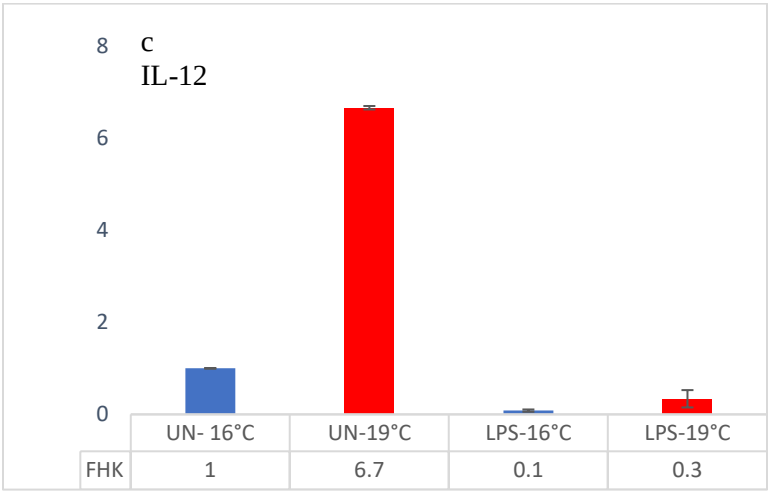
To evaluate the roles of iNOS, IL-6, TNFα1-3, IL1β1-2, IL-12 and IL34 in RTS11 cell line. Untreated of iNOS, IL-6, TNFα2 and L1β1, expression was only induced or equal to control at (2 or 24 hours) with increase in transcription observed in the presence of LPS, PGN and poly I: C.

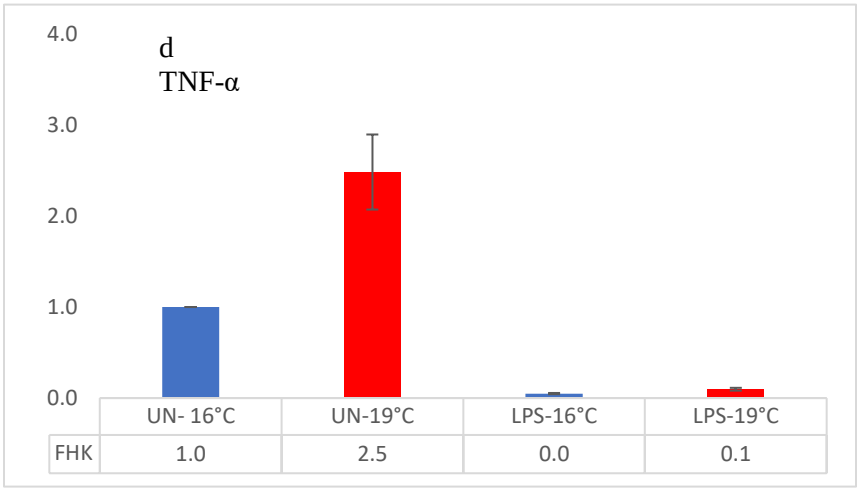
Effect of behavioral fever temperature on Fish head kidney cytokine expression

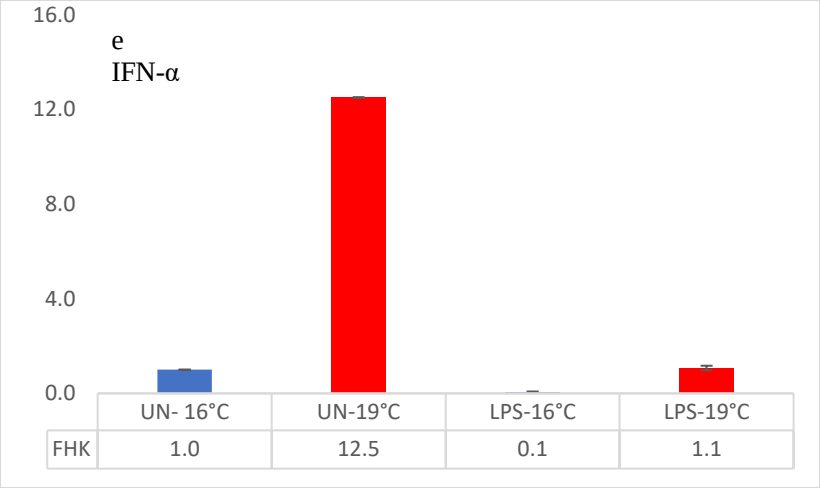
Unstimulated FHK iNOS, IL-6, IL-12, TNF-∝1 isoform, IFN-α and IL-34 expression gave a rapid response. at 19 °C were seen but not for stimulated cells. Our results agree with previous work of (Johnson et al. 1982). the work emphasizes that temperature is an important abiotic parameter for developing immunity in ectothermic vertebrates, such as rainbow trout, the time course for induction of these molecules was distinct from mouse and chicken very few inflammatory cytokines IL-12 and IFN-α peaked by 4h.


a
iNOS
2
1
0
UN- 16°C
UN-19°C
LPS-16°C
LPS-19°C
FHK
1
1.3
0.01
0.1


b
IL-6
4
3
2
1
0
UN-16°C
UN-19°C
LPS-16°C
LPS -19°C
FHK
1
3.2
1.5
1.3


c
IL-12
8
6
4
2
0
UN- 16°C
UN-19°C
LPS-16°C
LPS-19°C
FHK
1
6.7
0.1
0.3


d
TNF-α
4.0
3.0
2.0
1.0
0.0
UN- 16°C
UN-19°C
LPS-16°C
LPS-19°C
FHK
1.0
2.5
0.0
0.1



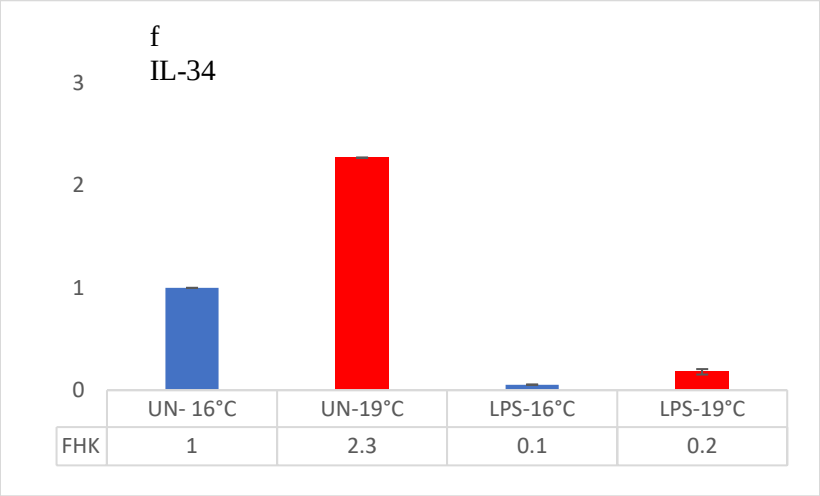


Figure 44. Effect of behavioral fever temperature and activation on expression of pro-inflammatory cytokine genes.Triplicate samples of FHK cells ($2x10^6$ cells in each well) were stimulated with LPS (0.1µg/ml) at 16 and 19°C for 4h. Samples were pooled, RNA purified and used for templates for cDNA, and run-in triplicate for RT- qPCR. Blue bars represent the incubation time at 16°C and Red bars at 19°C, respectively. ΔΔCt scored for cytokines measured at 4h for FHK are shown beta-actin with reference to the unstimulated 16°C. (Panel a) iNOS , IL6 showed in (panel b), IL-12 (panel c)) TNF-α (Panel d), IFN-α (panel e), and IL-34 (panel f).

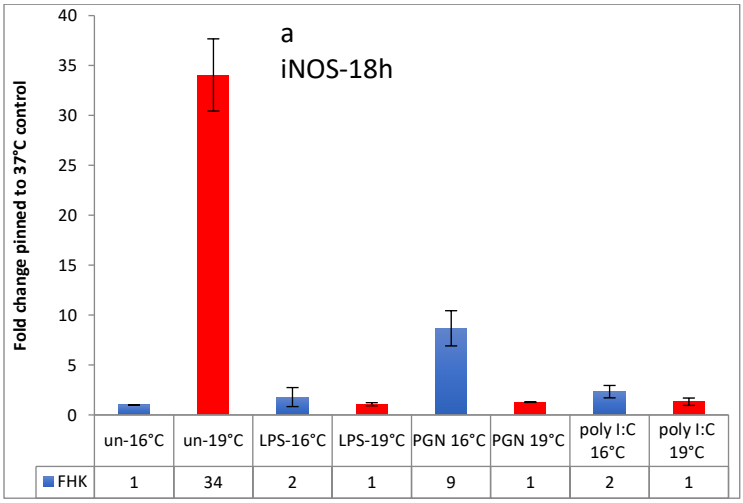
Table 14: Cytokine The cytokine expression to various stimuli over a 4H period is presented in Table FHK.

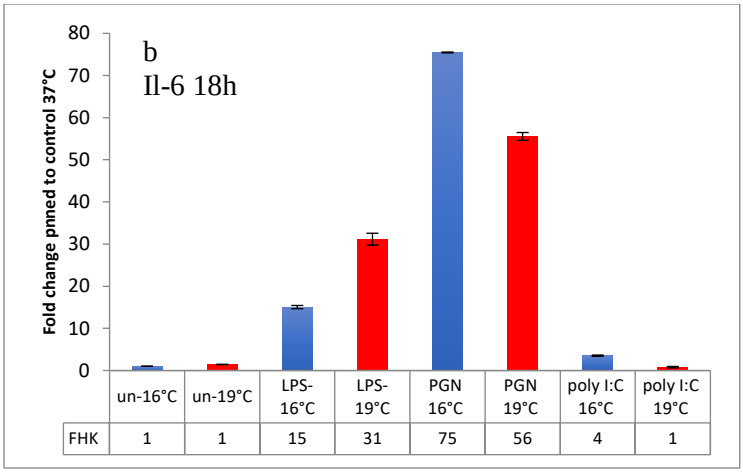
Treatment	Temperature [°C]	4H	
		B-actin ±SD	iNOS ±SD
UN	16	15.6 ± 0.3	20.0 ± 1.2
UN	19	17.2± 0.2	21.2± 0.5
LPS	16	10.3± 0.2	20.8± 0.3
LPS	19	12.6± 0.1	20.8± 0.2
		B-actin ±SD	IL-6 ±SD
UN	16	14.5 ± 0.1	15.2 ± 0.0
UN	19	19.5± 0.2	18..6± 1.7
LPS	16	18.4± 0.0	18.6± 0.2
LPS	19	25.8± 0.3	26.2± 0.1
		B-actin ±SD	IL-12±SD
UN	16	15.6 ± 0.3	27.1 ± 0.9
UN	19	17.2± 0.2	26.0± 0.7
LPS	16	10.3± 0.2	25.9± 0.4
LPS	19	12.6± 0.1	25.7± 0.7
		B-actin ±SD	TNF-α±SD
UN	16	15.6 ± 0.3	14.6 ± 0.2
UN	19	17.2± 0.2	14.9± 0.3
LPS	16	10.3± 0.2	13.8± 0.2
LPS	19	12.6± 0.1	15.0± 0.2
		B-actin ±SD	IFNβ-α±SD
UN	16	15.6 ± 0.3	16.6 ± 0.3
UN	19	17.2± 0.2	14.6± 0.1
LPS	16	10.3± 0.2	14.6± 0.7
LPS	19	12.6± 0.1	13.5± 0.1
		B-actin ±SD	IL-34±SD
UN	37	15.6 ± 0.3	14.2 ± 0.4
UN	39	17.2± 0.2	14.5± 0.8
LPS	37	10.3± 0.2	13.1± 0.0
LPS	39	12.6± 0.1	13.6± 0.1

Increases in 6 gene of interest cytokines (iNOS, IL-6, 1L-12, TNF-α, IFN-β and IL-34 at 19 compared to 16°C baseline was observed. Table F presents cytokine levels at the various study time points at 16 and 19°C with information about the expression of cytokine gene mRNA at FHK at 16 and 19.

Effect of fever and activation on expression of pro-inflammatory cytokine genes over a 18H.

Unstimulated FHK at long period of time 18h incubation showed increase in iNOS and IL-6 while there was no temperature effect with no stimulation compared with LPD treatment LPS gave increase expression for IL-6 compared with PGN at 19°C.





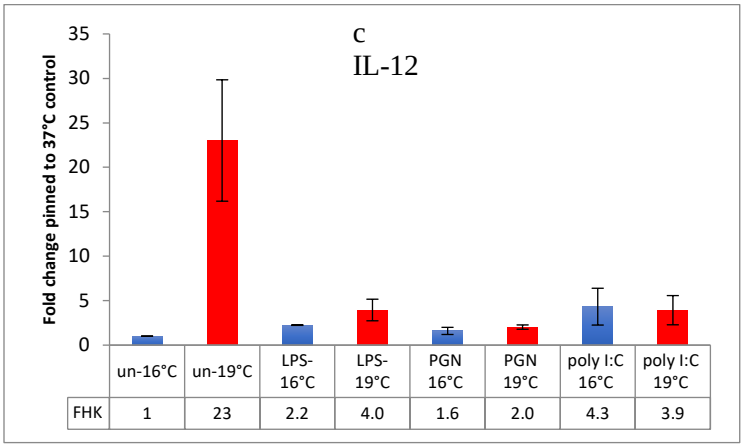


Figure 45: Effect of fever and activation on expression of pro-inflammatory cytokine genes. Triplicate samples of adherent fish head kidney cells ($2x10^6$ cells in each well) were stimulated with LPS (50µg/ml), PGN (5µg/ml) and (50µg/ml) of Poly I:C at 16 and 19°C for 18h. Samples were pooled, RNA purified and used for templates for cDNA, and run-in triplicate for RT- qPCR. The blue bars represent the incubation time 18h at 16°C and the dark red bars at 19°C, respectively. All cytokines measured at 18h, by qRT-PCR relative to beta-actin with normalization to the unstimulated 16°C. iNOS (Panel a) IL6 showed in (panel b) and IL-12 (Panel c).

Table 15: Fish head kidney cytokine expression to various stimuli over a 18H period is presented in Table 15: Increases in 6 gene of interest cytokines (iNOS, IL-6 and 1L-12, at 19 compared to 16°C baseline was observed. Table F presents cytokine levels at the various study time points at 16 and 19°C with information about the expression of cytokine gene mRNA at FHK at 16 and

Treatment	Temperature [°C]	Time 18H	
		iNOS B-actin ± SD	iNOS± SD
UN	16	7.1± 0.3	23.0± 1.5
UN	19	12.3± 0.6	22.8±1.4
LPS	16	8.4± 0.2	24.3± 0.6
LPS	19	7.8± 0.8	24.3±0.2
PGN	16	8.4± 0.2	21.4± 0.7
PGN	19	7.7± 0.4	24.1± 0.2
Poly I:C	16	7.9± 0.3	23.0± 0.6
Poly I:C	19	8.2± 0.7	25.0± 1.0
		B-actin ± SD	IL-6 ± SD
UN	16	7.1± 0.3	18.1±0.0
UN	19	12.3± 0.6	22.7±0.2
LPS	16	8.4± 0.2	15.4± 0.0
LPS	19	7.8± 0.8	13.7± 0.1
PGN	16	8.4± 0.2	13.1±0.1
PGN	19	7.7± 0.4	12.9±0.2
Poly I:C	16	7.9± 0.3	17.1±0.0
Poly I:C	19	8.2± 0.7	19.4±0.3
		B-actin ± SD	IL-12 ± SD
UN	16	7.1± 0.3	30.9±2.1
UN	19	12.3± 0.6	30.8±1.8
LPS	16	8.4± 0.2	30.5±0.8
LPS	19	7.8± 0.8	29.9±0.6
PGN	16	8.4± 0.2	32.6±1.6
PGN	19	7.7± 0.4	29.3±1.5
Poly I:C	16	7.9± 0.3	31.1±2.1
Poly I:C	19	8.2± 0.7	30.0±0.6

Fever increases the amount and speed of production of NO and its magnitude. This triggers a change in the metabolism of macrophages so they quickly to switch to glycolysis. We find several other instances of increasing magnitude or duration of responsiveness by cytokines as the

result of adding fever temperatures to the stimuli that initiate innate and adaptive immune responses

Supplementary:

The effect of fever temperature on the glycolytic and redox rates.

To investigate glycolysis and oxidative phosphorylation, RAW246.7 cells, a cell line which originate from Abelson leukemia virus transformation of BALB/c mice were used. Glycolysis and oxidative phosphorylation were monitored at normal and febrile temperatures by measuring proton efflux rate (OCR, Figure 1) and oxygen consumption rate (PER, Figure 2) in control, LPS, IFN and LPS plus IFN-g at 37°C and 39°C. Treatments were added just prior to beginning incubation. At 39°C increases were immediately observed in the treated samples in PER compared to 37°C. Focusing just on the first hour the peak PER and the time of that peak are shown in Table 1.

Experiment	Treatment	Temperature[°C]	Peak time [min]	PER [pmol/min]±SE [a]	Difference from control at 37°C
Figure 3	Untreated	37	2	821±64	0
		39	2	741±22	-80
	LPS	37	14	912±51	91
		39	14	955±28	134
	LPS+ IFN-γ	37	34	960±1	139
		39	2	1073±1	252
Figure 4	Untreated	37	15	1308±42	0
		39	28	916±61	-392
	IFN-γ	37	21	1166+195	-142
		39	60	1171±73	-137
	LPS + IFN-γ	37	2	1381±155	73
		39	21	1217±63	-91

Table 1: Peak PER measurements from the Seahorse XFp Analyzer within the first one hour. (a) Peak PER taken from the data shown in Figure 3&4.

Experiment	Treatment	Temperature (°C)	Time OCR begins to decrease [b] (min)	Time PER begins to increase relative to control slope [a] (min)
Figure 1&3	LPS+ INF-y	37	196	216
		39	105	131
	LPS	37	..	..
		39	177	255
Figure 2 &4	LPS+ INF-y	37	119	242
		39	158	164
	INF-y	37	..	..
		39	255	--

Table 2: Comparison of the time at which PER and OCR change in response to treatment and temperature by RAW 264.7 cells, and B: Comparison of the time at which PER and OCR change in response to treatment and temperature from Figures 3 B and 4B. The data in figures 1 and 2 was examined for the first point when an increase in PER was observed (a)compared to the slope of the control and the first point when a decrease (b) in OCR was noted.

(a) **(b)**

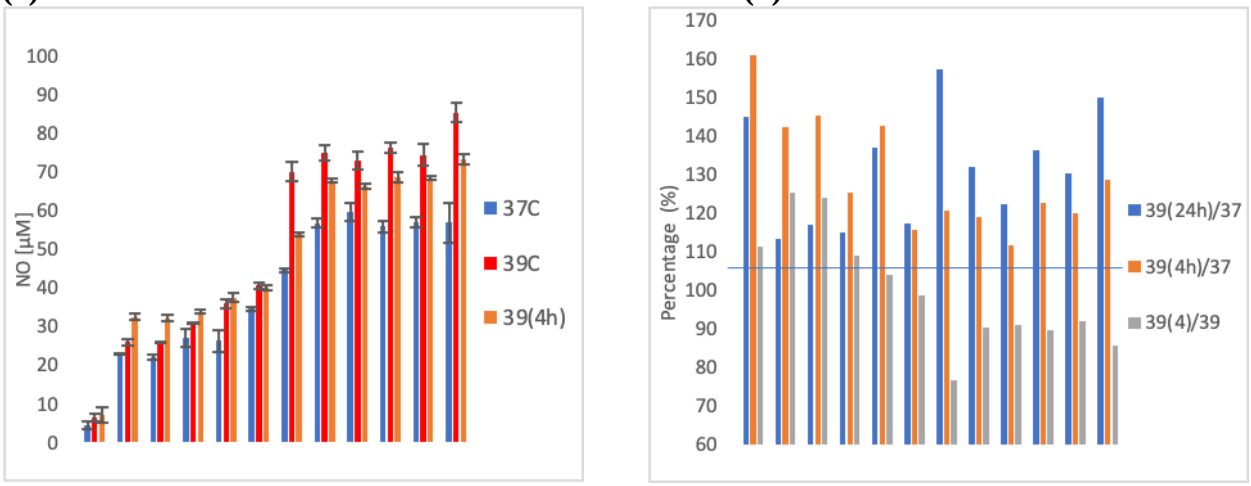
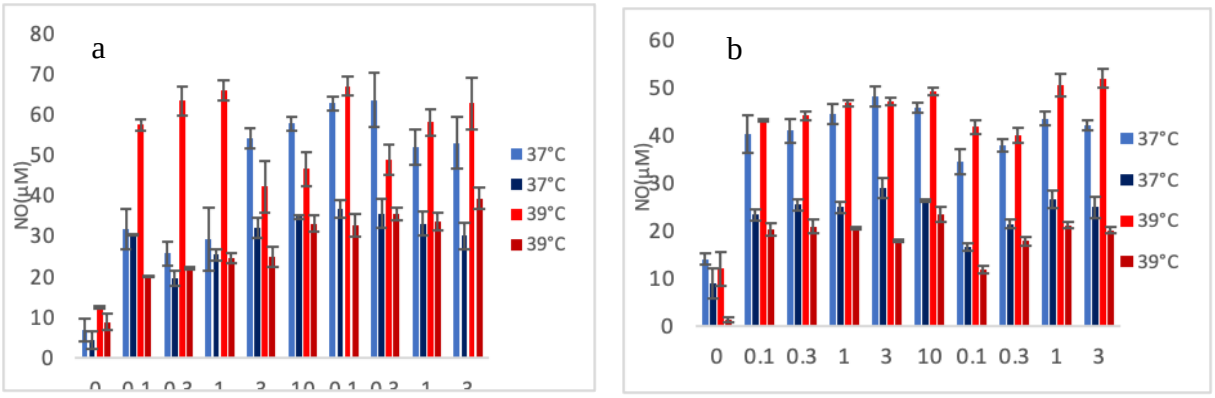


Figure R: Comparison of the effect of 4h versus 24h at fever temperature on NO levels in LPS, IFN- **γ** or LPS plus IFN- **γ** stimulated RAW264.7 mouse cells. (a) RAW 264.7 cells cultured in 96 well plates ($5x10^5$in each well) were stimulated with different treatments in triplicate for 24h at 37°C and 39°C or cultured for 4h at 39°C and shifted to 37°C until harvest. Supernatant was collected and tested for NO (µM). LPS ranged from 0.1 to 10 µg/ml, IFN- **γ** 3.0 µg/ml and in combination LPS plus IFN- **γ**. The results shown are the averages from (a) experiment ±SE. Blue bars represent cultured at 37°C and the red bars at 39°C and orange bars 39°C for 4h. (b)

The results shown are the percentages from data shown in (a) experiment. Blue bars represent the enhancement in the NO level at 39°C compared to 37°C. The orange bars represent the NO percentages level for a 4h treatment at 39°C compared to 37°C. The gray bars represent the NO percentages for a 4h stimulation at 39°C compared to a constant incubation at 39°C.

As shown in (Figure Ra) RAW264.7 cells showed a much higher response at fever temperature compared to the control 37°C for all treatments. Stimulation with IFN- γ alone and the combination further enhanced NO production at 39°C compared to 37°C. However, 4h stimulation at 39°C show higher level of NO enhancement with LPS stimulation only compared for being in 39 entire time. However, with IFN- γ alone and the combination 4h of stimulation at 39°C showed an intermediate level of NO enhancement between 37 and 39°C. (Figure Rb) shows these results as a percentage of NO enhancement. Comparing the percentage at 39 to 37 °C for 24h incubation time showed increases from 110 to 160%. However, 4h stimulation at 39°C showed a higher level of NO enhancement with LPS stimulation compared to being at 39°C the entire time the advance of LPS stimulation is about 110-125%. However, when compared to 39°C for the entire time, IFN- γ alone was 120.8 % and the combination of LPS and IFN- γ ranged from 76.8 to 85.7% with lower doses of LPS showing higher increases.

PRC



PRC: Temperature effect on LPS and IFN-***γ*** stimulated NO production in PRC for young and aged female mice.

PRC from young and aged females (15 - 17 weeks old and 74 -77 weeks old, respectively) were utilized. PRC were cultured at $5x10^5$ cells in each well in stimulated with or without LPS at different concentrations (0.1 to 10 µg/ml), IFN-y (0.1, 0.3, 1.0 and 3.0 µg/ml) for 24 h at 37°C and 39°C and supernatant tested by Griess reagent for NO. The light blue bars represent young PRC at 37°C. The dark blue bars represent aged cells at 37°C. The light red bars represent young PRC at 39°C and the red dark bars aged PRC at 39°C. Data are mean ± SE of triplicate determinations.

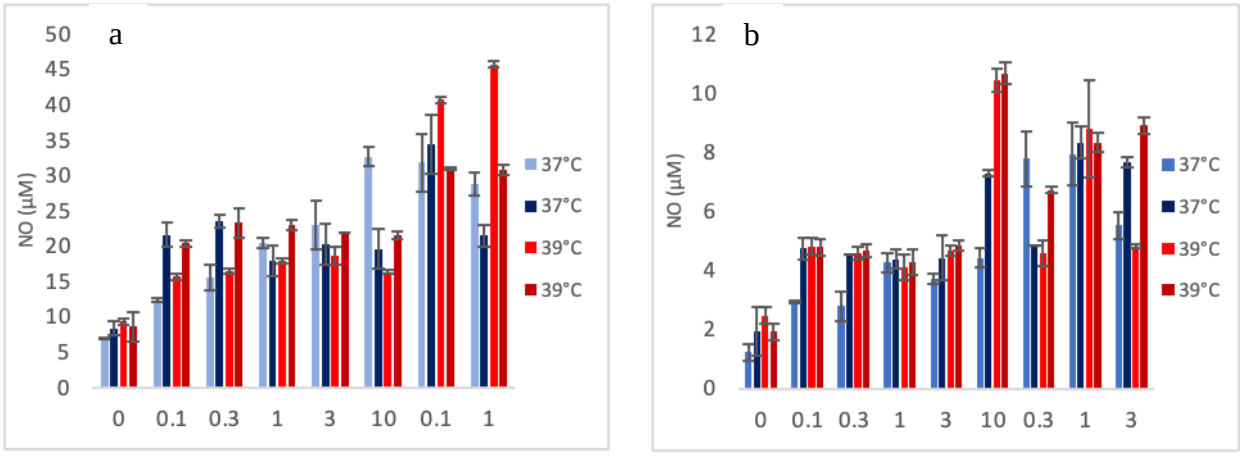


Figure S. Temperature effect on LPS and IFN-y stimulated NO production in splenocyte-derived adherent cells (SP) for young and aged mice.

Splenocyte adherent cells (SP) from young and aged females 15 Fig a – 17 Fig b weeks old and aged mice 75 Fig a to 77 Fig b weeks old, respectively). SP cells were cultured $5x10^5$ cells in each well of a 96 well plate stimulated with or without LPS at different concentrations (0.1 or 10 µg/ml), IFN-y (0.3, 1.0 and 3.0 µg/ml) for 24 h at 37°C and 39°C and supernatant tested by Griess reagent for NO. The light blue bar represents young bone marrow cells at 37°C. The dark blue bar represents aged cells at 37°C. The light red bar represents young bone marrow cells at 39°C and the dark red bar at 39°C. Data are mean ± SE of triplicate determinations.

BM

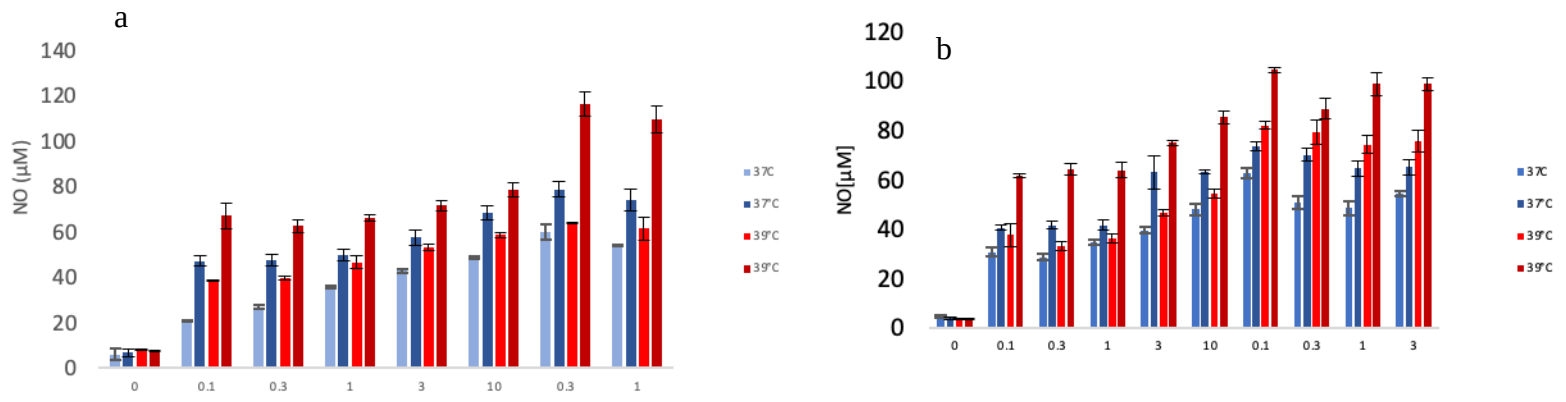


Figure B: Temperature effect on LPS and IFN-γ stimulated NO production in BMDM for young and aged female mice.
BMDM from young and aged females 15 to 17 weeks old and from 75 to 77 weeks old, respectively) were utilized. BMDM were cultured at 5x10^5 cells in each well in stimulated with or without LPS at different concentrations (0.1 to 10 µg/ml), IFN-y (0.1, 0.3,1.0 and 3 µg/ml) for 24 h at 37°C and 39°C and supernatant tested by Griess reagent for NO. The light blue bar represents young BMDM at 37°C. The dark blue bar represents aged BMDM at 37°C. The light red bar represents young BMDM at 39°C and the dark red bar aged BMDM at 39°C. Data are mean ± SE of triplicate determinations.

BMDM protein synthesis

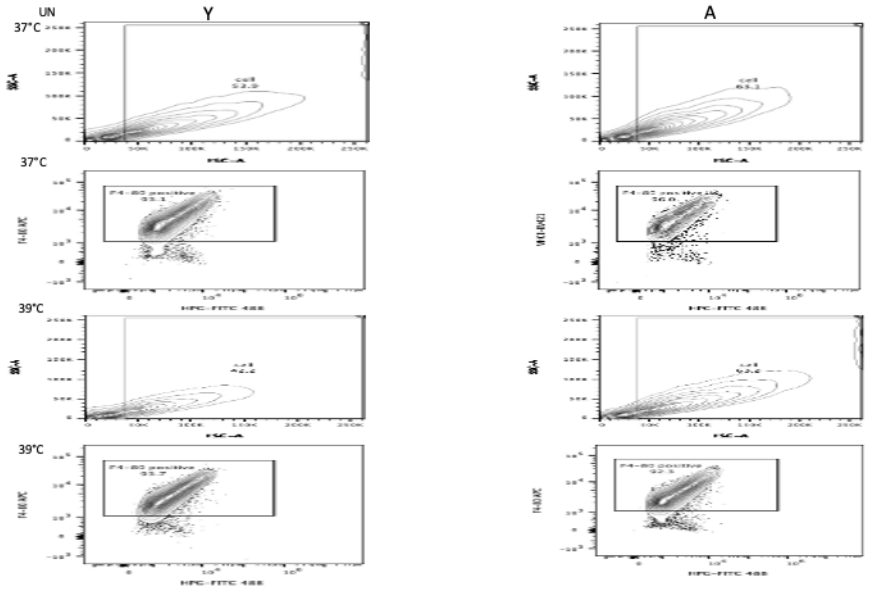


Figure Bs- Gating strategy used to identify young and aged BMDM F4-80 positive cells.

Cells were first selected for by utilizing forward scatter (FSC) and side scatter (SSC) signals to exclude debris and clumps of cells from the analysis. The gate for cells is shown in a and b for unstimulated samples. The plots show young BMDM on the left and aged on the right. We identified macrophages by selecting the F4/80 - APC positive cells as shown in c and d. The numbers are the percentage F4/80 positive cells compared to total cells. Plots for 37°C are shown in b and for 39°C in c.

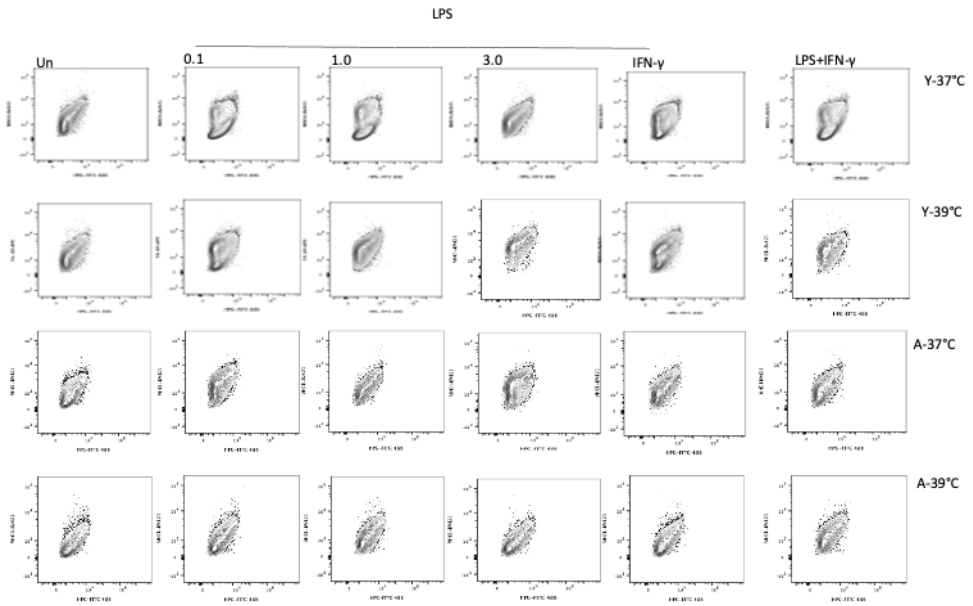


Figure (BM-1) BMDM flow cytometric analysis of protein synthesis test for young and aged female mice.

BMDM cells from young and aged females (BM ranging from 15 to 19 weeks old were pooled and from 75 to 88 weeks old were pooled, respectively) were utilized. Specifically, bone marrow from both young and aged C57BL/6 mice was extracted and cultured in M-CSF for one week with feeding with 5 ml fresh medium of 10ng/ml M-CSF .BMDM were cultured at 1x106in each well in stimulated with or without LPS at different concentrations (0.05 to 3µg/ml) or IFN-γ (0.1µg/ml) or combination incubated with Click-iT® HPG Alexa Fluor® 488 Protein Synthesis Assay Kit as described in the Methods section for 4h at 37°C and 39°C. MHC class II expression was determined using mouse anti mouse I -A[b] – BV421 color flow cytometric analysis. In both young and aged BMDM cells forward scatter FSC and side scatter SSC signals were used to exclude debris and clumps of cells from the analysis gate (Fig Ba). F4-80 positive cells were gated (Fig B bc) and then analyzed for changes in MHCII expression and protein synthesis. F4/80 positive cells ranged 51.3- 66.5 percent for young and 66.5- 82.8% for aged see table B . First row: Young untreated BMDM cell (Un) with 4 different LPS concentrations (0.1 to 3µg/ml) or IFN-γ or the combination LPS and IFN-γ at 37°C; second row: young at 39°C; third row: aged at 37°C and the four row: aged at 39C °.

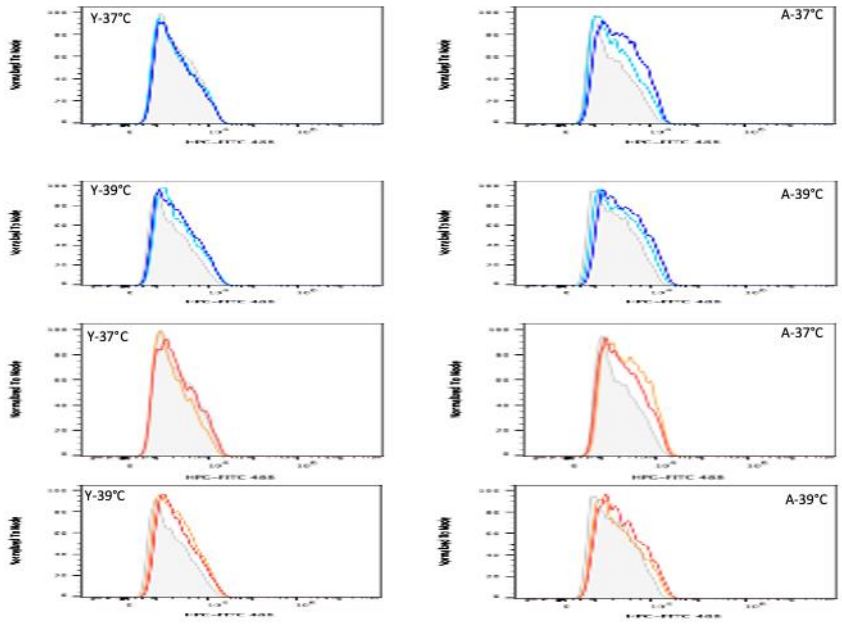


Figure (BMS-) Effect of temperature in protein synthesis from young and aged BMDM. **F4-80 positive macrophages were analyzed for protein synthesis. The histograms show young on left and aged on the right, 37°C for a and c, 39°C for b and d. Untreated background is shown in gray, 0.1 µg/ml LPS shown in blue (a and b), 0.1 µg/ml IFN-y shown in orange and combination of LPS and IFNy shown in red (c and d).**

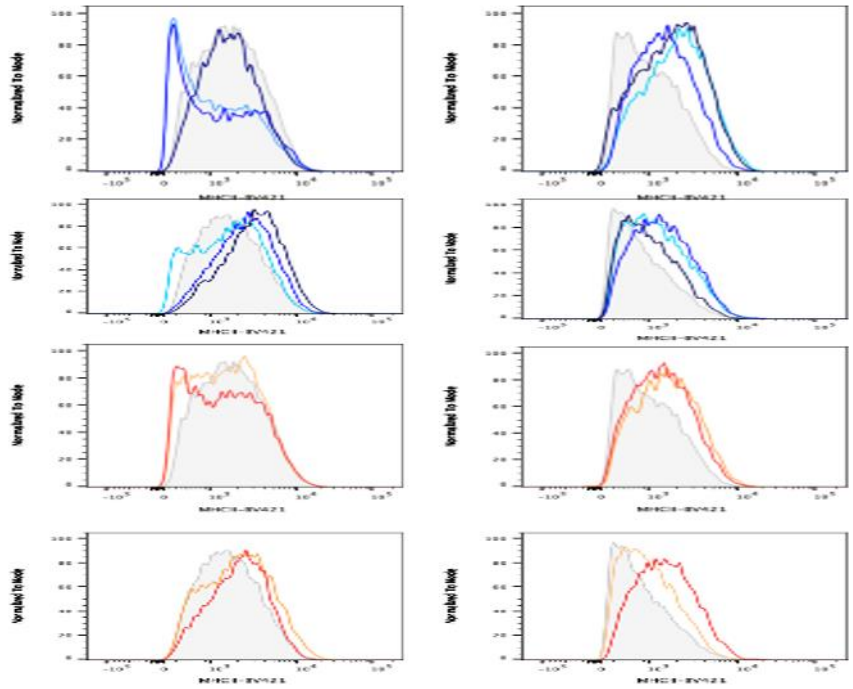
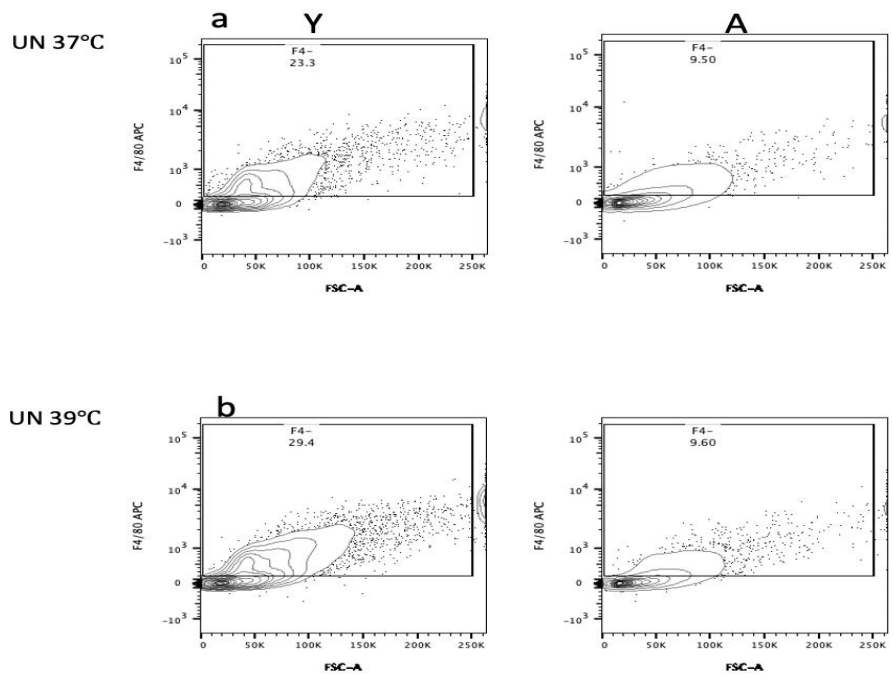


Figure (BMS-) Effect of temperature on MHCII expression from young and aged BMDM. **The histograms show young on left and aged on the right, 37°C for a and c, 39°C for b and d. Untreated background is shown in gray, 0.1 -3 µg/ml LPS shown in light to dark blue (a and b), 0.1 µg/ml IFN-y shown in orange and combination of LPS and IFN-y shown in red (c and d).**

			LPS					
	°C	Un	0.05	0.1	1.0	3	0.1 IFN-γ	LPS+ IFN-γ
Young	37 39	55.5 54.3	66.5 65.7	59.5 58.4	51.3 59.2	52.4 57.0	63.2 62.1	60.0 62.4
Aged	37 39	76.0 82.2	78.2 82.8	78.6 80.7	69.5 72.4	66.5 73.0	73.0 75.6	72.1 71.0

Table B The percentage of F4/80 positive cells for young and aged PRC after 4h of stimulation at 37°C or 39°C

Peritoneal resident macrophage (PRC).



Effect of fever temperature on protein synthesis production from PRC of young and aged mice. **Figure P Strategy used to gate young and aged PRC F4-80 positive cells. Adherent PRC were harvested after 2h incubation in petri plates at 37°C. Cells were gate for unstimulated samples. The plots show young PRC on the left and aged on the right. We identified macrophages by selecting the F4/80 - APC positive cells as shown in b and. The numbers are the percentage F4/80 positive cells compared to total cells. Plots for 37°C are shown in b and for 39°C in.**

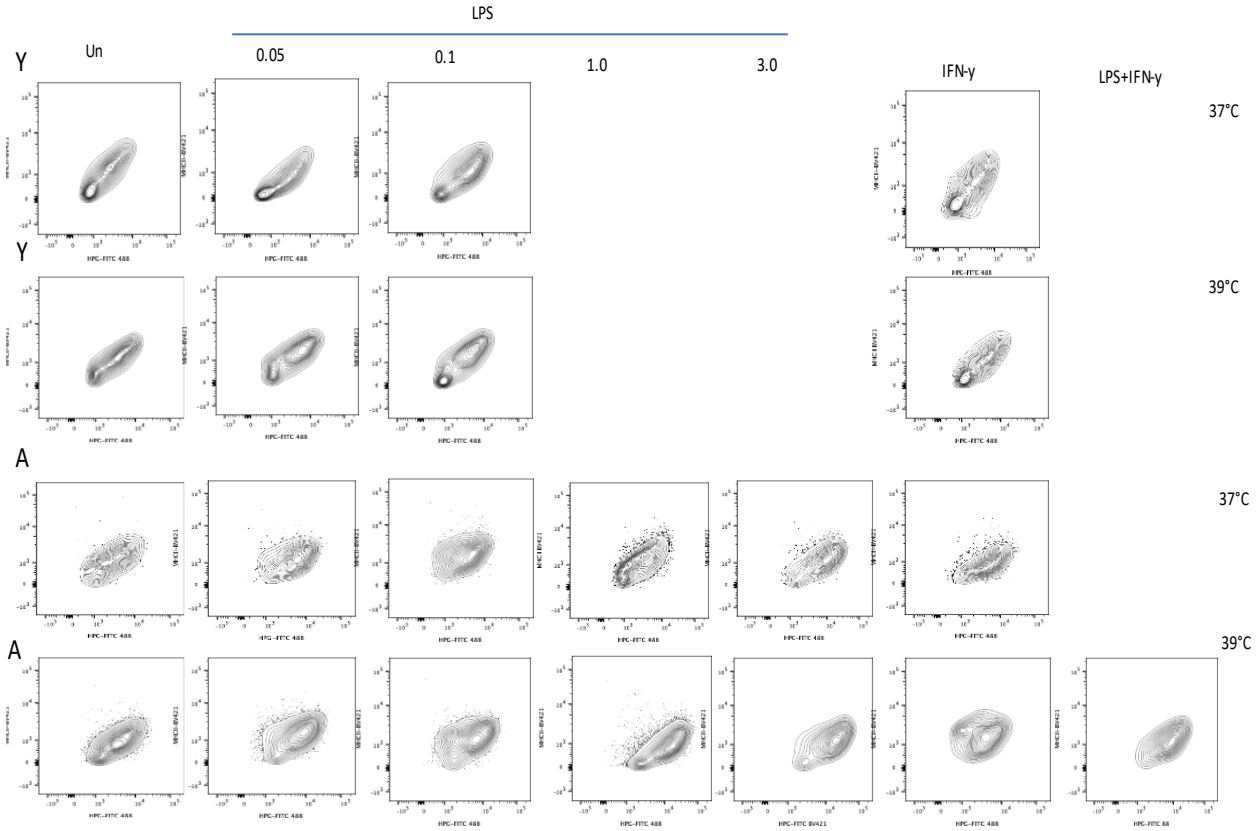


Figure PRC: Flow cytometric data of protein synthesis test for young and aged female mice. **PRC from young and aged females (ranging from 15 to 19 weeks old and from 75 to 88 weeks old, respectively) adherent PRC were harvested after 2h incubation in petri plate at 37°C. PRC were cultured at 1x105in each well in stimulated with or without LPS at different concentrations (0.1 to 3µg/ml) or IFNy (0.1µg/ml) or combination incubated with Click-iT® HPG Alexa Fluor® 488 Protein Synthesis Assay Kit as described in the Methods section for 4h at 37°C and 39°C. MHC class II expression was determined using BV421 color flow cytometric analysis. F4-80 positive cells were gated and then analyzed for changes in MHCII expression and protein synthesis. F4/80 positive cells ranged 3.0- 27.2 % for young and for aged 8.8-14.5% see table P2. First row: Young untreated PRC (Un) with 3 different LPS concentrations (0.05 to 3µg/ml) or IFN-γ or the combination LPS and IFN-Y at 37°C; second row: young at 39°C; third row: aged at 37°C and the four row: aged at 39C ° .**

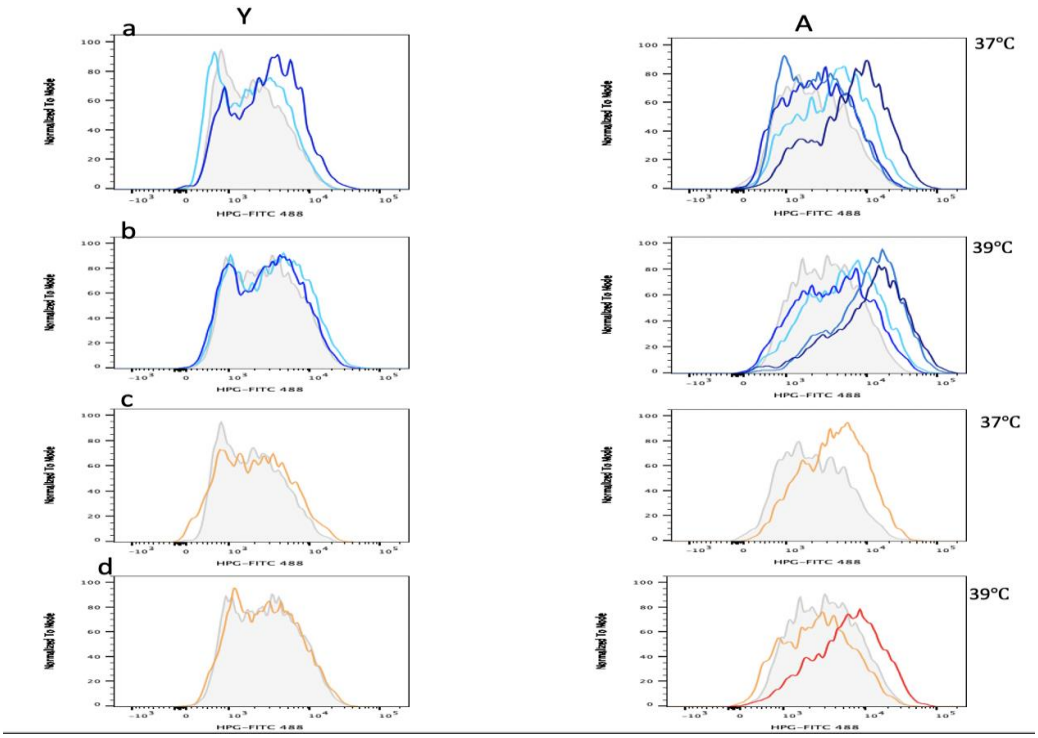


Figure P Effect of temperature in protein synthesis from young and age mice PRC.
F4-80 positive macrophages were analyzed for protein synthesis. The histograms show young on left and aged on the right, 37°C for a and c 39°C for b and d. Untreated background for young mice is shown in gray, 0.05 and 0.1 µg/ml LPS shown in light and dark blue respectively a and for aged mice two more concentration 1.0 and 3.0 µg/ml LPS. 0.1 IFN-y shown in orange and combination of LPS, and IFN-y shown in red.

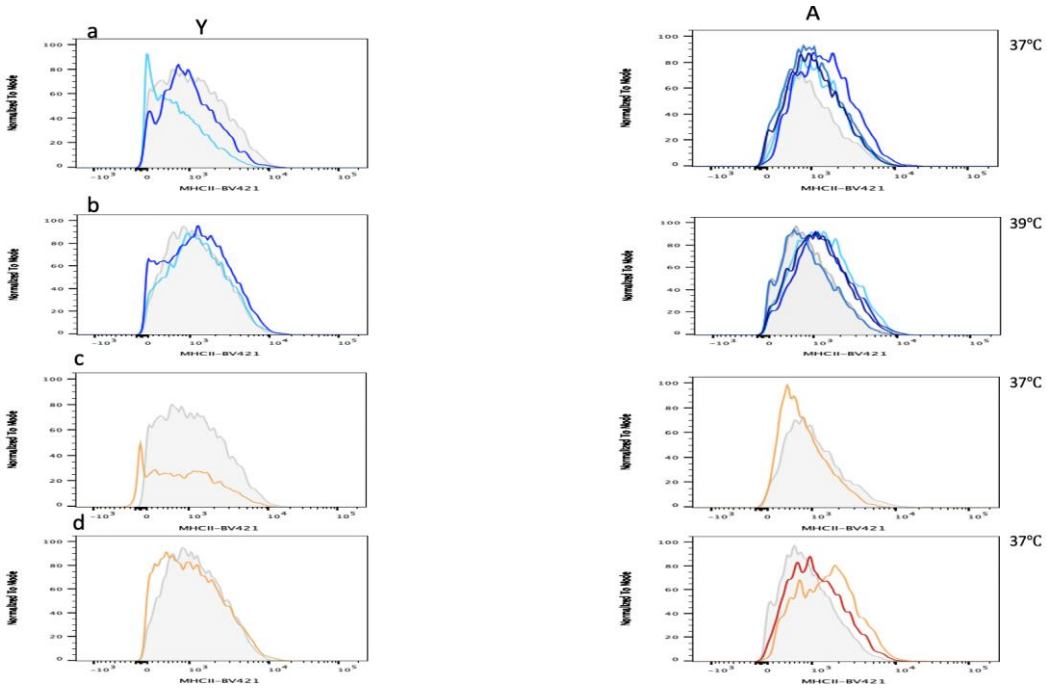


Figure (PR-2) Effect of temperature on MHCII expression from young and aged PRC.
The histograms show young on left and aged on the right, 37°C for a and c, 39°C for b and d. Untreated background is shown in gray, 0.05 -3 µg/ml LPS shown in light to dark blue (a and b), 0.1 µg/ml IFN-γ shown in orange and combination of LPS and IFN-γ shown in red (c and d).

Table P2 The percentage of F4/80 positive cells for young and aged PRC after 4h of stimulation at 37°C or 39°C

			LPS					
	°C	Un	0.05	0.1	1.0	3	0.1 IFN-γ	LPS+ IFN-γ
Young	37 39	23.3 28.4	22.2 24.7	8.14 27.2	-	-	3 [a] 24.9	- -
Aged	37 39	8.8 9.0	12.8 10.2	11.7 10.3	14.5 11.1	10.8 11.8	9.5 10.6	- 10.8

a: Possible omission of F4/80 of this sample

REFRENCE

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