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Elevated temperature alters cytokine output and c-Jun N-terminal kinase signaling downstream of toll-like receptor activation

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Elevated temperature alters cytokine output and c-Jun N-terminal kinase signaling downstream of toll-like receptor activation

by

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Thesis

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Dedication

To my Parents, Younger Brother, and Fiancé, for being involved in my journey every step of the way.

Acknowledgements

First and foremost, I would like to thank Dr. Rolf Konig for his continuous support and guidance. I would like to thank Dr. Endsley and Dr. Sun for being on my supervisory committee. I would also like to thank Dr. Rockx and Mardelle for their help with preparation of my thesis. Additionally, I would like thank my lab mates, Alexander McAuley and Anjana Kalita, for their help with experiments and in editing this manuscript.

Elevated temperature alters cytokine output and c-Jun N-terminal kinase signaling downstream of toll-like receptor activation

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Fever is a fundamental and important response to infection. Previous studies have shown that alterations in temperature can alter the phenotype of innate immune cells, such as phagocytic ability, but the effects of elevated temperature on the molecular mechanisms that underlie these differences remain poorly understood. Here, we describe alterations in cytokine production following stimulation with lipopolysaccharide or polyinosinic:polycytidylic acid when U937 cells, a human monocyte cell line, are incubated at elevated temperature. The observed responses differ depending on the stimulus, suggesting that they are programmed responses to different stimuli rather than a non-specific response to temperature. We also show that signaling pathways may be 'rewired' during hyperthermia to signal through alternative pathways. These findings demonstrate the critical importance of considering temperature as a variable when studying immune responses and host-defense mechanisms in the context of infectious diseases that cause fever.

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List of Abbreviations

AP-1	Activator Protein 1
Cdk	Cyclin-dependent kinase
HSP	Heat-Shock Protein
MAPK	Mitogen-Activated Protein Kinase
PAMP	Pathogen-Associated Molecular Pattern
PolyI:C,	Polyinosinic:polycytidylic acid
LPS	Lipopolysaccharide
TLR	Toll-Like Receptor.

Chapter 1: Introduction

Fever is a common response to infection, which has been shown to confer reduced morbidity and mortality to a number of infectious diseases[1,2]. Many infectious diseases have optimal temperature ranges for pathogen propagation from 35□C to 40□C[3,4], and elevation of host temperature can provide an effective, non-specific means of inhibiting replication of invading microorganisms. Some immune functions are altered by elevated temperature, but these altered functional responses can be highly specific. Generally, macrophage phagocytosis at 40□ is enhanced by 40% over a physiological temperature[5]. Specifically, phagocytosis of E.coli, S. typhimurium, and L. monocytogenes by polymorphonuclear leukocytes is enhanced at increased temperature; however, this was not seen for S. aureus[6]. These previous studies indicate that immune function is altered by changes in temperature, and that these changes can be pathogen-specific. Therefore, when investigating immune cell function in the context of anti-pathogen host defenses that result in a febrile response, it is important to consider temperature as a critical variable.

Fever is induced via the infection of host cells, starting with recognition of pathogens via Toll Like Receptors (TLRs) that recognize conserved motifs known as Pathogen-Associated Molecular Patterns (PAMPs)[7]. This results in the induction of a signaling cascade, leading to a cytokine-induced rise in temperature and an inflammatory response[8,9]. The TLR4 pathway targets lipopolysaccharide (LPS), an endotoxin specific to the gram-negative bacterial outer membrane. It causes a strong immunogenic

response in macrophages through the MyD88 dependent pathways[7]. TLR3 targets double-stranded RNA, which is specific to viral particles, and signals through the TRIF pathway[7]. An analog of dsRNA, polyinosinic:polycytidylic acid (polyI:C), can also activate this receptor[7]. Both LPS and polyI:C are capable of inducing a febrile response in vivo[10,11].

Experiments by Jiang et al. have shown that stimulation with LPS at 40□C causes a significant increase in the cytokines TNFα, at 1 hour post-stimulation, and IL-1β and IL-6, at 5 hours post-stimulation[12,13]. Additionally, neutrophils in suspension are not able to activate the mitogen-activated protein kinases (MAPKs) p38 and ERK, the non-MAPK serine/threonine-specific kinase Akt, or the transcription factor NF-□B in response to TNF-□ stimulation at 42□C, whereas these pathways are activated at 37□C[14]. These results illustrate the ability of elevated temperatures to alter intracellular signaling pathways downstream of a particular receptor.

This finding led us to hypothesize that at increased temperatures, cell signaling networks may be altered. Further, such alterations may occur because signaling pathways considered 'canonical' at 37□C prove to be less important in signaling than other pathways not normally considered as having a role in driving cellular responses to a given stimulus, although it is also possible that the latter pathways possibly augment the canonical pathways.

Both LPS and polyI:C stimulation of U937 human monocyte-like cells maintained at 37□C or 39.5□C were used to investigate the effects of elevated temperature on cytokine production and transcription factor activation and activity. We show that increased temperature leads to differential alterations in cytokine production downstream

of LPS or polyI:C stimulation, and that, at increased temperature, JNK signaling plays an increased role in signaling upstream of specific cellular responses. As ‘systems immunology’ continues to emerge as a paradigm, considering how immunological signaling networks are differentially regulated by temperature will be critical in the understanding of how these host defenses are regulated in disease[15–19].

The Activator Protein 1 (AP-1) pathway is a canonical pathway that plays an important role in growth and inflammation [20]. The AP-1 transcription factor is formed primarily by homodimers of c-Jun or heterodimers of c-Jun and c-Fos, allowing for a variety of functions and specific regulation[21]. This pathway is a great model for studying signaling changes, as it is well characterized and has a quantifiable effect on cellular functioning[20]. In particular, the c-Jun protein is a key transcriptional activator that has a direct effect on cytokine production. Dysregulation of the Jun proteins can lead to cytopathic effects, including, but not limited to, the development of cancer[21]. Additionally, multiple signaling pathways mediate the activation of c-Jun[22]. We hypothesize that these pathways are affected and rewired by elevated temperatures, as seen through the phosphorylation state of c-jun in combination with various pathway inhibitors. LPS and polyI:C signal through the TLR pathways to activate members of the Mitogen-Activated Protein Kinase (MAPK) family, including ERK, p38, and JNK[20,22]. These proteins are also activated by stressors, possibly including elevated temperature[21], and phosphorylate the c-Jun protein. Changes in these signaling pathways alter c-jun phosphorylation levels. The effects of these MAPKs are additive and represent a summation of all inputs[21,22]. However, at different temperatures the input level and weighting may be modulated.

Knowing the difference between cellular signaling at normal and febrile temperatures is valuable in understanding the role of the febrile response. Here, we show that there are significant differences between the normal and febrile signaling pathways that activate the c-Jun protein.

Chapter 2: Materials

2.1 Cells and Reagents

U937 human monocyte-like cells were a gift from Dr. Janice Endsley, UTMB, and were cultured in RPMI (Life Technologies, Carlsbad, CA). RAW 264.7 murine macrophages were a gift from Dr. Lynn Soong, UTMB, and were cultured in complete RPMI. Cells were maintained at 37°C with 5% CO2. For elevated temperature experiments, cells were split and maintained at 37°C for 8 hours, a time by which RAW cells were predominantly adherent. After this initial incubation, cells were transferred to 39.5°C for overnight incubation where appropriate, prior to stimulation. ERK, JNK, p38 and Akt inhibitors were purchased from EMD Calbiochem (San Diego, CA) and used at a final concentration of twice the listed IC50. Following overnight incubation, cells were treated with inhibitors for 1 hour prior to stimulation. No visible cytotoxicity was observed for the period of the inhibitor treatment. Cytokines were purchased from eBioscience (San Diego, CA). Where indicated, cells were stimulated with either 100 ng/ml LPS or 10 □g/ml polyI:C (Sigma, St. Louis, MO).

2.2 Cell proliferation assays

Equal numbers of U937 human monocyte-like cells were split into four wells each of two six-well plates (2ml per well) and incubated at 37°C or 39.5°C with 5% CO2. Cell number, size and viability were monitored for 96 hours. Every 12 hours, a 20-□l aliquot was harvested and mixed 1:1 with trypan blue (Sigma-Aldrich, St. Louis, MO), and samples were assayed using a Cellometer Auto T4 (Nexcelom, Lawrence, MA).

2.3 LUCIFERASE REPORTER ASSAYS

Stable AP-1 luciferase reporter cells were purchased from Panomics [Affymetrix] (Santa Clara, CA), and cultures were established and maintained following the manufacturer's instructions. Following treatment and/or stimulation, cells were maintained for 12 hours and cell extracts prepared and luciferase substrate added by using the luciferase substrate assay system (Promega, Madison, WI) following the manufacturer's instructions. Luciferase activity was measured by using a Tecan Infinite M200 plate reader (Tecan, Männedorf, Switzerland).

2.4 MULTIPLEX CYTOKINE ELISA

Following culture at either 37°C or 39.5°C, cells were treated and stimulated where appropriate in triplicate. Medium was harvested and cytokine levels assayed by using the human inflammatory cytokines panel ELISA kit (SA Biosciences, Valencia, CA), following the manufacturer's instructions.

2.5 C-JUN PHOSPHORYLATION ELISA

Phosphorylation status of c-Jun was assayed by using the c-Jun FACE assay kit (Active Motif, Carlsbad, CA). Experiments were performed following the manufacturer's instructions and as per our previous use of this assay[23]. Cell number was controlled by normalizing to crystal violet staining.

2.6 SDS-PAGE AND IMMUNOBLOTTING

Whole-cell extracts were prepared by lysis in sample loading buffer (Invitrogen, Carlsbad, CA). SDS-PAGE was performed by using NuPAGE® Novex® Bis-Tris Mini Gels and the XCell SureLock™ Mini-Cell (Invitrogen, Carlsbad, CA). Following

transfer, immunoblotting was performed by using the WesternBreeze® Chemiluminescent Western Blot Immunodetection Kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions.

2.7 KINASE ACTIVITY

Raw 264.7 cells were treated for one hour with 100 ng/ml LPS at both normal and elevated temperatures. Controls were not treated with LPS. Cell lysates were obtained and subjected to SDS-PAGE. As a loading control, GAPDH was measured by Western blotting using a rabbit antibody. HSP 60 was measured by using a specific rabbit antibody. Phosphorylation of kinase substrates was analyzed by using rabbit antibodies specific for phosphorylated sequences: Akt kinase activity was measured with an antibody against the phospho-consensus sequence (RXXS*/T*) of Akt substrates, and phosphorylation of MAPK/Cdk substrates was performed using antibodies against the phospho-peptide sequences (P PXS*P or S*PXR/K) of MAPK/Cdk substrates.

2.8 STATISTICAL ANALYSIS

Prism version 5 (GraphPad, La Jolla, CA) was used to perform statistical analyses. One-way analysis of variance and Student's t-test were used where appropriate to determine statistical significance. A p-value of < 0.05 was considered significant.

Chapter 3: Results

3.1 Monocyte Proliferation

We hypothesized that exposing a monocyte cell line to elevated temperatures *in vitro* would alter basic growth kinetics and prime cells to better respond to exogenous immunological stimuli. To test this hypothesis, we incubated the human monoblast cell line U937 cells at 37°C or at 39.5°C, a temperature occurring during a high fever. Cells grown at 37°C proliferated faster than those grown at 39.5°C, with a concomitant increase in mean cell diameter (Figure 1a). To induce differentiation, we incubated U937 cells with GM-CSF. When GM-CSF was added to the growth medium, U937 proliferation rates did not differ at the two temperatures (Figure 1b).

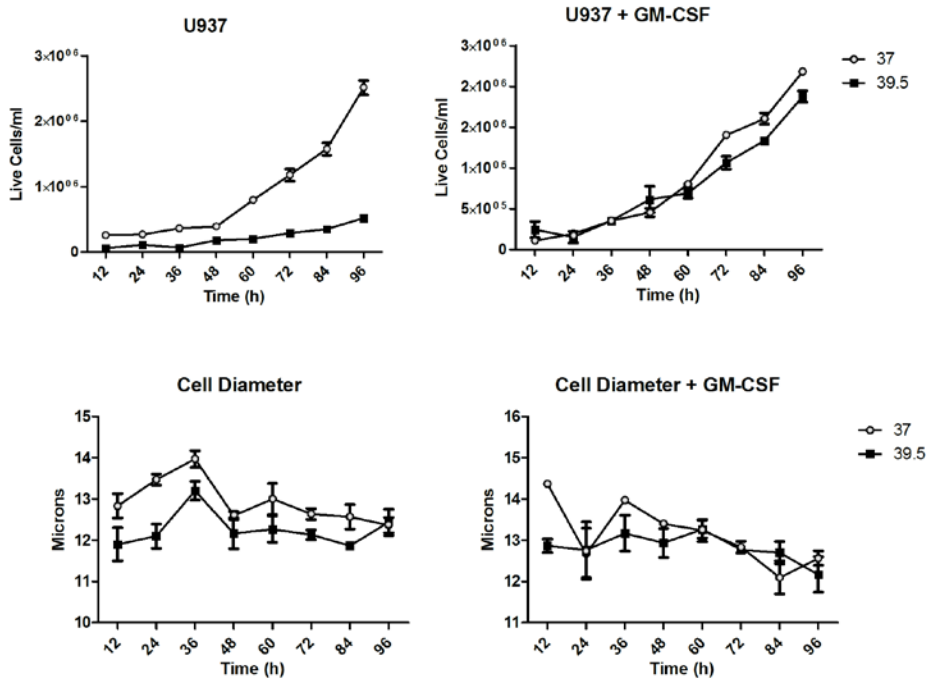

Figure 1: Effect of elevated temperature on growth of U937 cells.

However, while GM-CSF had no effect on growth rates of U937 at 37°C, it significantly increased growth rates at 39.5°C (Figure 2). This finding demonstrates that the normal response to fever reduces monocyte proliferation. However, in the media with GM-CSF, the number of live monocytes during fever is similar to those at normal temperatures.

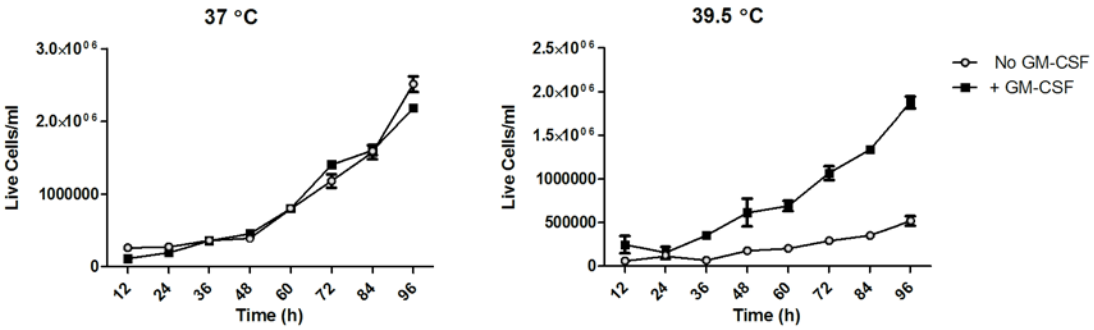

Figure 2: Proliferation of U937 cells in response to GM-CSF

3.2 CYTOKINE PROFILES

We next investigated the effect of differential temperature on the ability of U937 cells to produce cytokines in response to stimulation via toll-like receptors. Cells were treated with LPS to activate signaling through TLR 4, or polyI:C to activate TLR3[7]. Stimulation with LPS led to the production of a number of cytokines (Figure 3a). Most showed similar levels of production between the two temperatures. However, a significant decrease was observed in GM-CSF production. There was also a possible trend towards increased production of IL-1β and IL-10 at 39.5°C, but these differences were not statistically significant (p = 0.0649 and 0.0815, respectively). IL-1β is a regulator of inflammation and has effects on cellular proliferation and apoptosis[12,24],

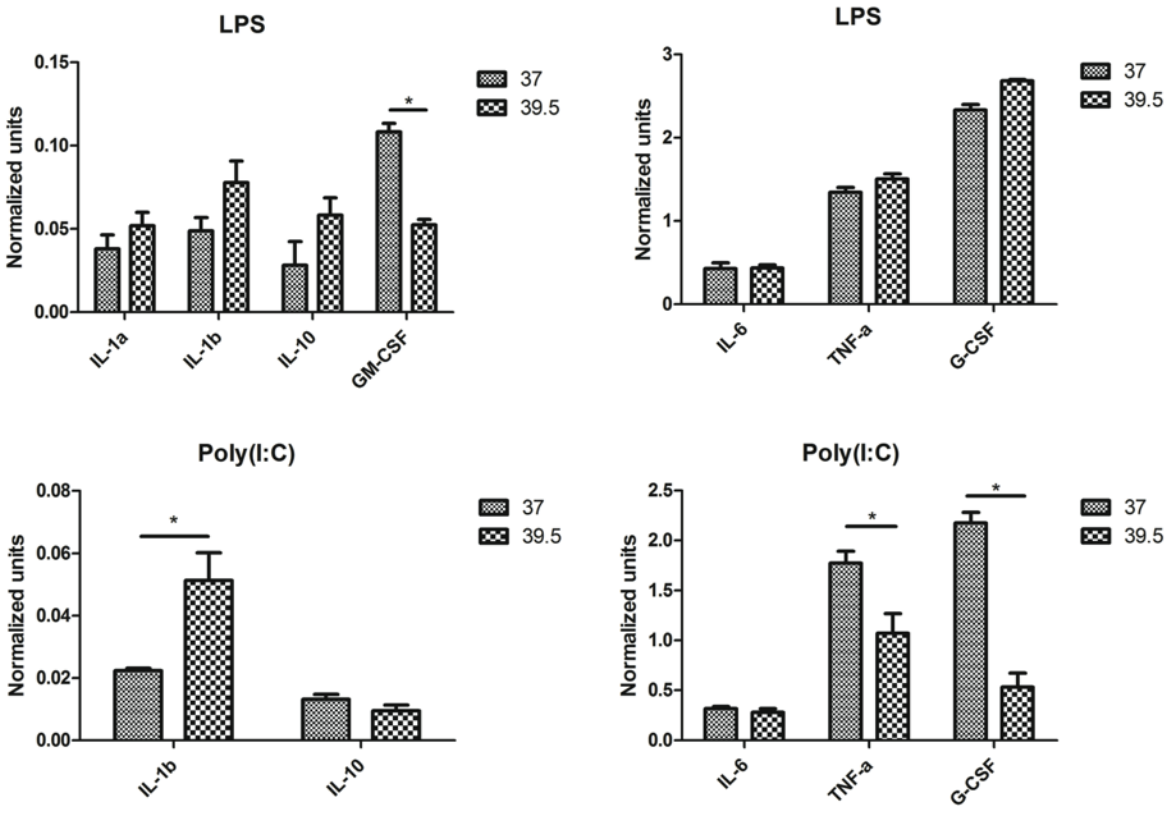

Figure 3: Increased temperature alters cytokine outputs following TLR stimulation

while IL-10 is an anti-inflammatory cytokine that regulates the action of various pro-inflammatory cytokines, including GM-CSF. Monocytes stimulated with GM-CSF typically develop into macrophages; however, when monocytes are cultured with both GM-CSF and IL-10, they undergo apoptosis. IL-10 has the ability to inhibit the signaling changes induced by GM-CSF only if it is added within 48 hours of GM-CSF stimulation[25]. Thus, this trend towards IL-10 increase may help explain the observed decrease in monocyte growth at febrile temperatures (Figure 1).

When cells were treated with polyI:C to activate stimulation via TLR3 signaling, different patterns of cytokine production was observed (Figure 3b). Interestingly,

following polyI:C treatment, IL-1β secretion was increased, while TNF-α and G-CSF secretion was decreased at 39.5°C when compared to secretion levels in cells incubated at 37°C. TNF-α is a pro-inflammatory cytokine, while G-CSF stimulates the production of granulocytes[13,26]. These findings suggested that elevated temperature leads to differential responses in monocytes dependent on the stimulus.

3.3 Basal AP-1 Levels

Given that elevated temperature affected both proliferation and inflammatory cytokine production, we focused on the role of AP-1 because this transcription factor influences both growth and inflammation

Previous studies on febrile temperatures have looked at the MAPK proteins that influence AP-1[14]. We investigated the basal levels of AP-1 transactivating activity, without any stimulation, by using a stable reporter cell line expressing the luciferase gene under the control of an AP-1 promoter. At the basal level, cells incubated at 39.5°C showed a significantly lower level, almost one half of AP-1 transactivation activity than cells at 37°C (Figure 4). This finding is consistent with the observed reduced rate of proliferation at higher temperature and may mean that the reduced rate of proliferation is influenced by reduced AP-1 activity. [20].

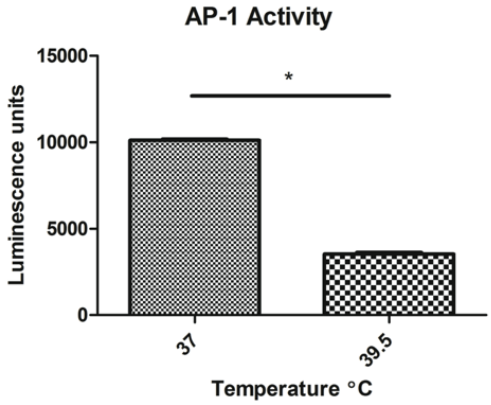

Figure 4: Effect of signaling inhibition on AP-1 activity at normal and elevated temperature

3.4 CHARACTERIZATION OF THE AP-1 PATHWAY

To attempt to define the upstream pathways responsible for regulating AP-1 activity, we treated cells with inhibitors of ERK, JNK, p38 and Akt. The MAPK proteins ERK, JNK, and p38 were chosen because they influence c-Jun activity[22]. Akt was selected because it is serine/threonine-specific protein kinase that does not belong to the MAPK family of proteins, plays a central role in mediating critical cellular responses, including cell growth and survival, and transcriptional regulation, and can be used to quantify effects of non-MAPKs on AP-1[20]. At both temperatures, the effect of inhibition showed a similar pattern, albeit with lower luciferase activity levels in cells incubated at 39.5°C, with ERK inhibition displaying the most significant reduction of AP-1 activity (Figure 5). Interestingly, at 37°C, p38 and Akt inhibition reduced AP-1 activity compared to JNK inhibition;, however, at 39.5°C, these differences were no longer observed. This finding indicates that JNK may play a more significant role in controlling AP-1 activity at higher temperatures. The variation in these activity profiles

may mean that alteration of the pathways occurs at different temperatures. The influence of each upstream input upon AP-1 activity is changed during febrile temperatures.

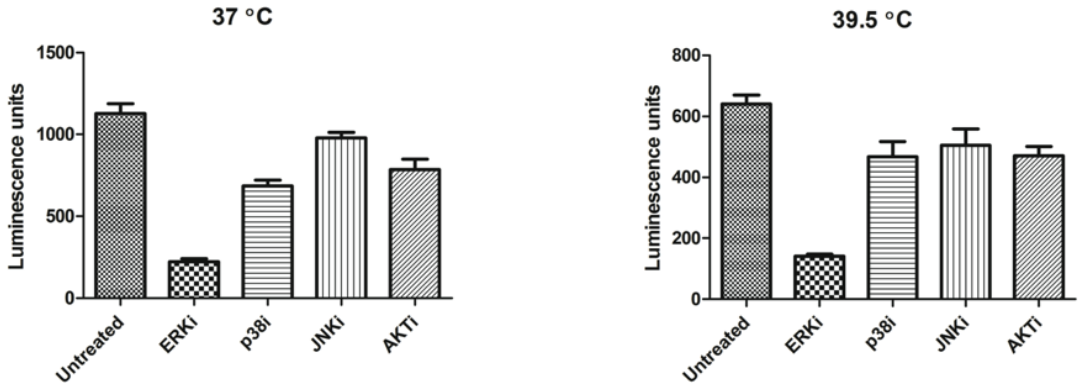

Figure 5: Effect of temperature on AP-1 activity.

3.5 AP-1 Pathway Response to LPS Stimulus

We next investigated the effect of temperature and LPS stimulation on phosphorylation of the AP-1 family member c-Jun, again using inhibitors to block specific upstream pathways. Interestingly, following LPS stimulation at 39.5°C, c-Jun phosphorylation was increased (Figure 6). At the lower temperature of 37°C, a trend to increased c-Jun phosphorylation was observed, however it was not statistically significant. JNK inhibition significantly reduced phosphorylation of c-Jun at 39.5°C, but not at 37°C (p = 0.0585). ERK and Akt inhibitors significantly affected c-Jun phosphorylation at both temperatures. The data on c-Jun phosphorylation corroborate the data on AP-1 activity shown in Figure 5, again indicating a rewiring of pathways.

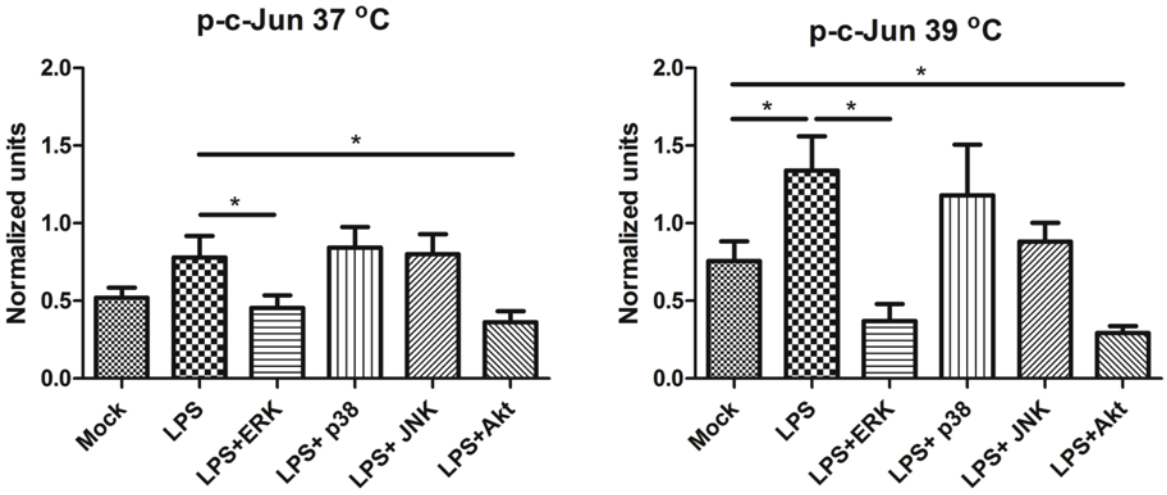

Figure 6 : Hyperthermia alters cell-signaling pathways to c-Jun phosphorylation

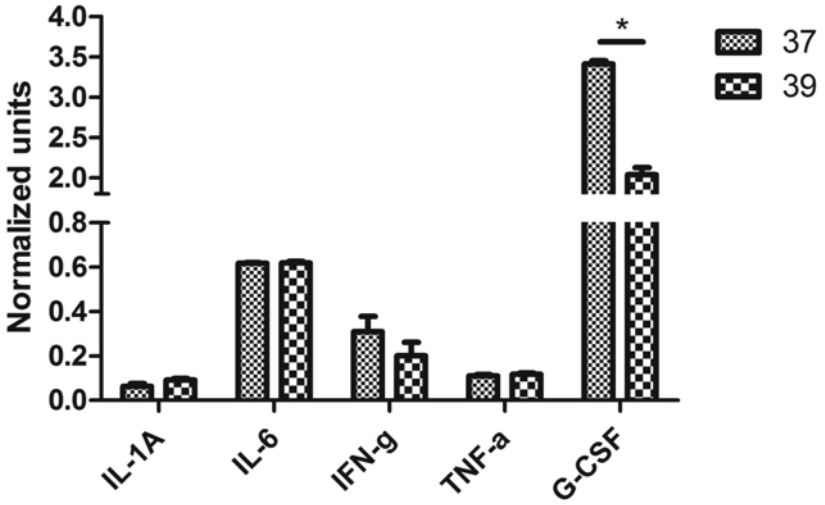

Figure 7: Inhibition of JNK reduces G-CSF production at increased temperature

3.6 JNK INHIBITION AT ELEVATED TEMPERATURES

As the activity of AP1 is controlled by a large number of upstream pathways and mediates transcription in conjunction with many other transcription factors, we hypothesized that the small differences seen in the role of JNK signaling may be due to significant crosstalk and/or redundancy between signaling pathways and transcription factors. To assay the effect of JNK inhibition in a more specific way, we incubated cells with JNK inhibitor at 37°C and 39.5°C prior to stimulation with LPS and assayed culture medium for cytokines (Figure 7). As can be seen, JNK inhibition did not alter the production of IL-1α, IL-6, IFN-γ or TNF-α, but significantly reduced G-CSF production (p = 0.0001), a difference not seen when JNK inhibition was not present (Figure 3). Taken together, these results indicate that signaling through JNK plays a more important role during hyperthermia, than at normal, non-febrile, physiological temperature.

3.7 INFLUENCE OF ELEVATED TEMPERATURE ON HEAT SHOCK PROTEIN (HSP) ACTIVATION

Generally, the expression of heat-shock proteins (HSPs) increases upon exposure to elevated temperatures or other stress conditions[27]. To determine the effects of febrile temperatures on HSPs, we stimulated Raw 264.7 cells with LPS for one hour at both normal and elevated temperatures. Protein loading was normalized by probing the cell lysate with GAPDH antibody (Figure 8a). Induced expression of HSP 60 was detected at 37 °C (Figure 8b, lanes 1 and 2). The band intensity of HSP60 doubled when incubated at 39.5 °C temperature. This increase was independent of LPS stimulation (Figure 8b, lane 3 and 4). In contrast, the effect of temperature was not prominent on related HSPs (data not

shown), possibly meaning that only HSP60 was activated in Raw 264.7 cells at febrile temperature.

3.8 KINOME PROFILING OF LPS INDUCED SIGNAL TRANSDUCTION AT ELEVATED TEMPERATURE

We measured a kinome-wide view of cellular phosphorylation in whole cell lysates, detecting serine, threonine, and tyrosine phosphorylation mediated by diverse kinase families. To test activation of a wide range of kinases in whole cell lysates, Raw 264.7 cells were stimulated with LPS at 37°C or 39.5 °C for one hour. Comparison of Akt activities in cell lysates from Raw 264.7 treated with LPS at both temperatures showed enhanced phosphorylation of various substrates of Akt following LPS stimulation (Figure 8c, lanes 2 and 4).. We also measured activities of the MAPK and cyclin-dependent kinase (Cdk) families of serine/threonine protein kinases. Kinome analysis revealed LPS-dependent increased phosphorylation of substrates specific for the MAPK/Cdk families of protein kinases at 37°C (Figure 8d, lanes 1 and 2) and a more pronounced effect at 39.5 °C (Figure 8d, lanes 3 and 4).

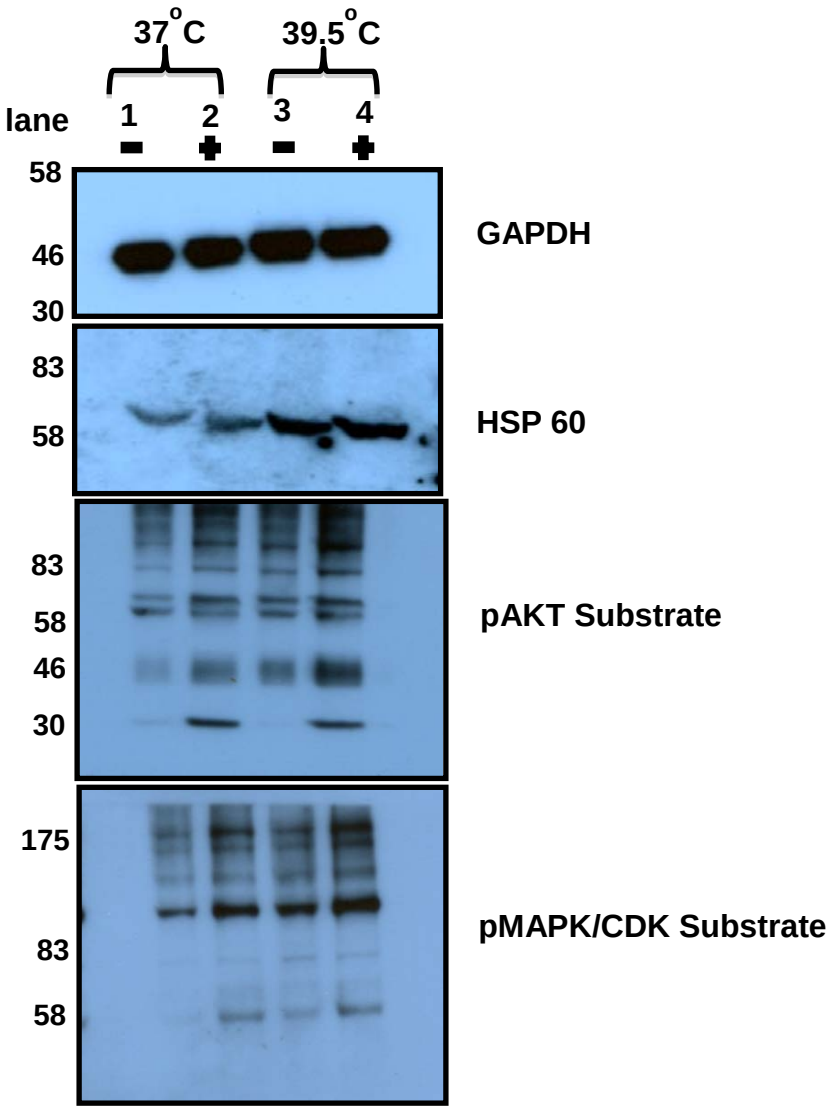

Figure 8: Western blot analysis of Raw 264.7 cell lysate untreated or treated with LPS at 37oC and 39.5oC.

Chapter 4: Discussion

The majority of studies that investigate the cellular response to infection or stimulation, such as through pathogen recognition receptors, are performed at 37°C. In this study, we elevated the incubation temperature to represent a febrile condition and found subsequent alterations in response to stimulation. We have expanded on previous findings, which have shown alterations in macrophages at the functional level. Culturing macrophages at 39.5°C inhibited proliferation, but responses to GM-CSF treatment were more effective than in cells grown at 37°C. Interestingly, the alteration in cytokine secretion by temperature elevation was different following stimulation by different TLRs, and may mean that the various signaling pathways are 'rewired' in different ways following an elevation in temperature.

As we continue to move into the era of systems biology, it is increasingly critical to define the effects of immunologically important variables, such as temperature, and consider these when the signaling pathway 'wiring diagrams' are constructed (Figure 9). Certain pathways may be more important for mediating specific effects at 37°C, whereas others may play central roles in controlling these effects during fever. Therefore, defining the roles of these pathways may have important implications for therapeutic design and development. For example, immune-modulators designed to treat 'cytokine-storm' type effects may be functional at 37°C, but could be less effective during a febrile response.

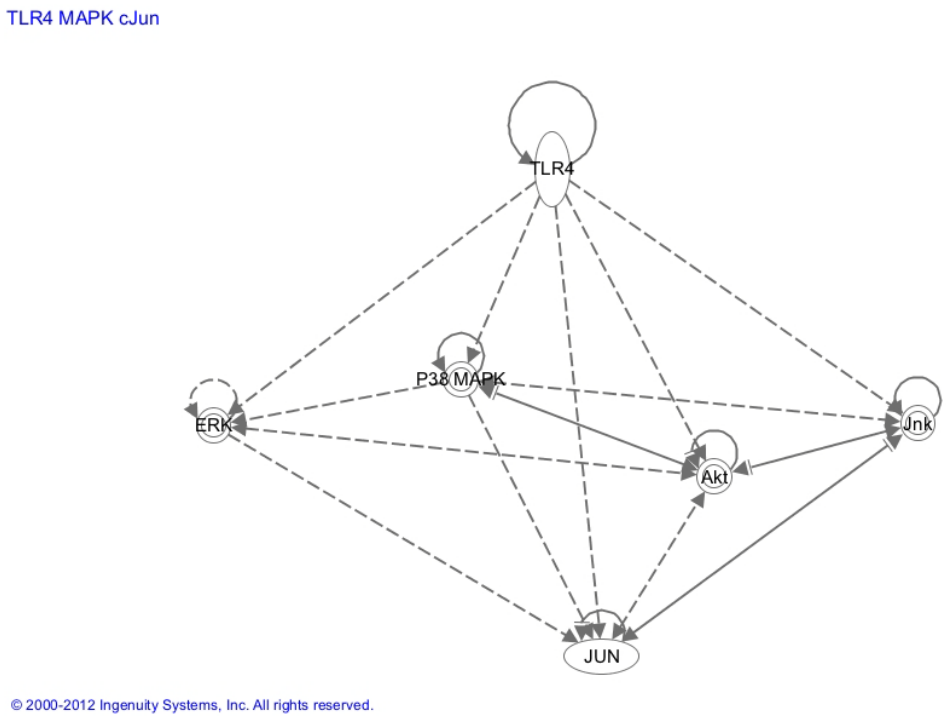

Figure 9: Wiring diagram for the TLR4-MAPK-cJun signaling pathway.

Anti-inflammatory drugs such as acetaminophen (paracetamol) are sometimes recommended during vaccination to alleviate fever or febrile seizure. It has recently been shown that the administration of acetaminophen at the time of vaccination can lower the resultant antibody titer in a number of vaccines[28], possibly meaning that the development of a fever is critical for the immune response leading to strong and protective immunity. However, the molecular mechanisms underlying this response remain largely undefined. The current public opinion is that fevers are a symptom of infection and as such require treatment. Thus, anti-pyretics are routinely administered at the first sign of a febrile response. However, the pattern of viewing fever negatively is most likely incorrect. For example, treatment of low-grade fever may hamper the immune response, leading to a longer recovery period or reduced adaptive immunity, causing low antibody titers and the possibility of re-infection. It may also decrease the effectiveness of

vaccines, reduce herd immunity, and lead to infection of patients despite their having received the vaccine. Clearly, the febrile response serves a purpose in establishing immunity to infection. It has been previously shown that some pathogens prefer normal body temperatures and that the febrile response hampers their functioning[4]. While the general effects have been known, an understanding of the molecular responses to febrile temperature has not been well characterized.

In this paper, we have demonstrated differences in function and expression of specific molecular responses between normal and elevated temperatures. We showed that GM-CSF caused proliferation of macrophages during fever (Figures 1 and 2), a response not seen at normal temperatures. However, during the response to LPS stimulation, GM-CSF was down-regulated (Figure 3), which indicates that during febrile temperatures, the number of macrophages was decreased. Thus, GM-CSF could be considered as a therapy for pathogenic infections that present with fever.

HSP60 is usually localized to the mitochondria, but during periods of stress it may be redistributed throughout the cell and even expressed upon the cell surface[29]. It has unique non-specific, pro-inflammatory effect upon innate immune cells, which would account for the LPS-independent increase in expression, and the difference in expression from related HSPs (Figure 8b). Additionally, it can independently activate monocytes and increase their survival[29], however, this conflicts with our results that indicated that U937 proliferation was decreased at elevated temperatures (Figure 1). HSP60 is also known to increase phosphorylation of p38 and ERK[30]. This suggests an extremely

complex and inter-dependent relationship between temperature elevation and innate immune cell activation.

Kinomic profiling indicated up-regulation of Cdks at febrile temperatures (Figure 8d). Cdks regulate cell cycle progression by partnering with cyclins to form an active kinase. Cdk levels are normally constant throughout the cell life cycle. Overexpression of Cdks leads to dysregulation of the cell cycle by allowing the cell to push through regulatory checkpoints. This means that replication can occur even in the presence of DNA damage, and this circumvention of replication checkpoints contributes to the development of cancer. Additionally, DNA damage can trigger apoptosis. This pathway could explain how up-regulation of Cdks can occur while numbers of macrophages are decreased at febrile temperatures.

Inhibition of AP-1 signaling at elevated temperature (Figures 4 and 5) indicates the need for further research into the effect of febrile temperatures on signaling pathways. It is critical to understand how these pathways are rewired during fever. Our data indicate that the canonical knowledge may not apply in the disease state. This paper shows the effects of LPS on the AP-1 pathway during fever; however data presented in Figure 3 demonstrate that the host reaction to polyI:C is different from the response to LPS. The next steps would be to determine the reaction of host cells to polyI:C at febrile temperatures as a model of viral infection.

Chapter 5: Conclusion

Temperature-related changes in cellular signaling and cytokine levels could be significant factors in disease course. Sustaining a febrile response may decrease (or increase) morbidity and mortality for certain diseases. A detailed knowledge of innate immune responses at febrile temperatures is necessary to facilitate the development of novel vaccines and treatments for infectious diseases. Treatments that modulate host responses and return cellular signaling towards the “normal profile” could provide the required impetus to the immune system for effectively clearing pathogens. Internal temperature of a patient may be a vital component of such a signaling profile. Thus, a detailed understanding of cellular responses to model pathogens at febrile temperatures is required for combating infections and generating more effective vaccination protocols.

Appendix A: Figure Legends

Figure 1: Effect of elevated temperature on growth of U937 cells. Quadruplicate cultures of U937 cells were incubated at 37°C or 39.5°C, (a) alone or (b) with the addition of GM-CSF. Cultures were sampled every 12 hours and assayed for cell number and mean size.

Figure 2: Proliferation of U937 cells in response to GM-CSF. Data from Figure 1 were re-plotted to more clearly show the effect of GM-CSF on cell proliferation at a given temperature.

Figure 3: Increased temperature alters cytokine outputs following TLR stimulation. Triplicate cultures of U937 cells were cultured at 37°C or 39.5°C prior to stimulation with LPS or poly(I:C). At 24 hours following stimulation, culture media were harvested and cytokine levels assayed by ELISA.

Figure 4: Effect of signaling inhibition on AP-1 activity at normal and elevated temperature. AP-1 reporter 293 cells were incubated at 37°C or 39.5°C in the presence of signaling inhibitors where appropriate. At 24 h post-treatment, cell extracts were prepared for luciferase assay.

Figure 5: Effect of temperature on AP-1 activity. AP-1 reporter 293 cells were incubated at 37°C or 39.5°C. Following 24 h of incubation, cell extracts were prepared for luciferase assay.

Figure 6: Hyperthermia alters cell-signaling pathways to c-Jun phosphorylation. Triplicate cultures of RAW cells were cultured at 37°C or 39.5°C prior to LPS treatment with or without 1-hour pretreatment with small molecule pathway inhibitors. At 1 hr post stimulation cells were fixed and c-Jun phosphorylation assayed by cell-based ELISA.

Figure 7: Inhibition of JNK reduces G-CSF production at increased temperature. Triplicate cultures of U937 cells were cultured at 37°C or 39.5°C prior to stimulation with LPS. Then, 1 h prior to LPS stimulation, cells were treated with Jnk inhibitor where appropriate. At 24 h post-infection, culture media were harvested and assayed for cytokine production.

Figure 8: Western blot analysis of Raw 264.7 cell lysate untreated or treated with LPS at 37^{o}C and 39.5^{o}C. The cell lysates for SDS-PAGE of Raw 264.7 cells stimulated for 1 hour with LPS or untreated cells were obtained in parallel and investigated by using a standard Western blotting protocol and probed with phospho-specific antibodies (a) GAPDH Rabbit mAb (b) HSP60 Ab . Molecular weight was determined by using Colour Plus prestained marker (7-175KDa). **KinomeView Profiling Kit includes antibodies** (c) Phospho-Akt Substrate (RXXS*/T*) (110B7E) rabbit mAb (d) Phospho-MAPK/CDK Substrate (PXS*P or S*PXR/K) (34B2) rabbit.

Figure 9: Wiring diagram for the TLR4-MAPK-cJun signaling pathway. The Ingenuity Pathway Knowledge Database (IPKD) and Ingenuity Pathway Analysis software (Ingenuity, Mountain View, CA) was used to construct a model of the signaling pathway affected during the febrile response. Solid lines indicate direct interactions between connected proteins and broken lines indicate indirect interactions documented by published original literature reports in the IPKD. An arrow pointing from node A to node B (e.g., TLR4 and c-Jun) signifies that A acts on B, whereas a connecting edge from A ending in a perpendicular line before the arrow-head pointing to B signifies that A inhibits and acts on B (e.g., c-Jun inhibits and acts on JNK).

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Vita

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