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**PIVOTAL ROLE OF INTERLEUKIN-10 ON MICRORNA-155 EXPRESSION IN
REGULATION OF THE MONOCYTE RESPONSE IN HYPOTHERMIA**

By

Adrian Theophil Billeter M.D.

University of Zurich 2009

A Dissertation

Submitted to the Faculty of the
School of Medicine of the University of Louisville

In Partial Fulfillment of the Requirements

For the Degree of

Doctor of Philosophy

Department of Physiology and Biophysics

University of Louisville

Louisville, Kentucky

August 2012

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ACKNOWLEDGMENTS

This dissertation is dedicated to my parents, Maja and Theophil, for their strong support and for believing in me, and my mentor Hiram C. Polk Jr., M.D. I learned more from Dr. Polk than I could ever imagine and more than many will learn in a lifetime. His dedication to clinical work, research and mentoring of young surgeons like me has become a personal standard to which I try to aspire.

I would like to express my deepest gratitude to Matthias Turina, M.D. Ph.D. He introduced me to research at the first place and made it possible that I have this great opportunity to be part of this program. He is a good friend and is always willing to help and give his best suggestions.

I am grateful to Dr. Susan Galandiuk for the opportunity to work at the Price Institute of Surgical Research and for contributing her thoughts and ideas throughout my studies. Ms. Sarah A. Gardner and Mr. Devin Druen also deserve my gratitude. Without their dedicated work, I would not have been able to succeed in my studies. Furthermore, Dr. Ziad M. Kanaan and Mr. Robert Eichenberger deserve my gratitude for their guidance and introduction to the world of microRNAs. In addition, I am grateful to all my committee members Dr. Irving Joshua, Dr. Gary Anderson and Dr. Stanley E. D'Souza from the Department of Physiology and Biophysics for their help and advice.

I would like to thank Dr. Aruni Bhatnagar for his guidance as committee member but especially for the opportunity to conduct animal experiments in his laboratory. Without the guidance, advice and support of Dr. Matthew R. Spite and Dr. Jason Hellmann, conducting these promising animal experiments would not have been possible. I am look forward to the final results of our experiments and further collaboration.

Lastly, I wish to thank the Royal College of Surgeons of Edinburgh (RCSEd) and the James and Emmeline Ferguson Research Fellowship Trust without whose generosity my work would not have been possible. A special thank you goes to Mr. and Mrs. R. Bruce Bass for their on-going generosity to their family trust, the Price Institute itself.

ABSTRACT

PIVOTAL ROLE OF INTERLEUKIN-10 ON MICRORNA-155 EXPRESSION IN REGULATION OF THE MONOCYTE RESPONSE IN HYPOTHERMIA

Adrian Theophil Billeter

July 26, 2012

This project investigated the effect of hypothermia on the monocyte response with the goal of understanding, which intracellular processes are affected by hypothermia leading to differences in cytokine secretion. A better understanding of the effects of hypothermia on the regulation of monocyte responses would allow targeted interventions and may reduce complications and death in hypothermic surgical patients. We found the following results:

1. The three major pro-inflammatory signaling pathways, Nuclear Factor $\kappa\beta$, p38 and c-Jun N-terminal-Kinase (JNK) of the Mitogen Activated Protein Kinases pathway, have increased and prolonged activation with hypothermia (32°C). The extracellular signal-related kinase (Erk) pathway shows increased activation at 15 minutes at 39°C.

2. The prolonged and increased activation of the pro-inflammatory signaling pathways results in a prolonged and increased expression of TNF- α messenger RNA (mRNA) and protein and microRNA-155 at 32°C.
3. Increased activation of Erk at 39°C leads to induction of Interleukin-10 mRNA and production of IL-10 protein.
4. The high IL-10 protein levels at 39°C result in suppression of the microRNA-155 expression, whereas the lack of IL-10 at 32°C prolongs microRNA-155 expression.
5. The increased and prolonged expression of microRNA-155 results in increased and prolonged TNF- α production at 32°C.

The findings of our research demonstrate the importance of regulatory feedback loops in order to achieve a balanced immune response. The lack of the inhibitory IL-10 at 32°C results in a prolonged pro-inflammatory response, which may have detrimental effects on host defense with a subsequently increased susceptibility to infections and organ dysfunction. The improved understanding of the intracellular mechanisms involved in the regulation of the monocyte response may result in targeted interventions to ameliorate the detrimental effects of hypothermia.

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CHAPTER 1

GENERAL INTRODUCTION

Hypothermia is a strong independent predictor of complications and mortality in surgical patients (1-10). Hypothermic trauma patients are at an especially high risk for death (1,4-6,8,9). In severely injured patients, hypothermia very often occurs together with acidosis and coagulopathy in patients with hemorrhagic shock, the so-called "triad of death". The detrimental effects of hypothermia have also been described in elective surgery with increased rates of surgical site infections and increased blood loss (2,3,7,11). Therefore, prevention of hypothermia, but especially aggressive re-warming in already hypothermic patients are important steps during every surgical procedure and an integral part of the care of the injured patient (12-14).

The reason for the increased rate of surgical site infection and multiple organ dysfunction (MODS) in hypothermic patients is largely unclear. Early work demonstrated decreased formation of Reactive Oxygen Species (ROS) in phagocytes of hypothermic patients. These results have been reproduced in cooled blood of healthy volunteers (15,16). Other investigators found impaired dendritic cell function in hypothermia, a cell with a pivotal role in the initiation of the immune response (17). Differences in pro- and anti-inflammatory cytokine

production between hypo- and normothermic conditions have been reported by several groups, although some of these results are contradictory (18-22).

As of yet, the intracellular mechanisms leading to these observed differences in cytokine production and cell function have not been thoroughly investigated. It has been shown that hypothermia affects activation of central inflammatory signaling pathways and that the gene expression of monocytic cell lines, when were exposed to hypothermia, are profoundly dysregulated (19,23-25). The interactions of the different activation of signaling pathways, gene expression and other regulators of cell function such as microRNAs have not been investigated.

The purpose of this work is to investigate which mechanisms lead to the observed differences in cytokine secretion, in order to understand the regulation of the monocyte immune response and, identify areas for potential therapeutic interventions that may restore monocyte function.

CHAPTER II

BACKGROUND

CLINICAL IMPORTANCE OF HYPOTHERMIA

The care of the surgical patient has progressed significantly over the last century. The mortality of elective colon resection continuously decreased from above 30% in 1914 to 1.5% currently (26,27). The reasons for this development are manifold. The introduction of blood banks in the 1940's allowed surgeons to operate on more patients and reduced the immediate post-operative mortality (26). Prophylactic antibiotics use decreased surgical site infections (SSI) subsequently reducing postoperative morbidity and mortality (28). Several decades later, among many other interventions, adequate blood glucose control was found to further reduce morbidity and mortality in surgical patients (29).

In order to assure safe and high quality surgical care throughout the United States of America, the Surgical Care Improvement Project (SCIP) was started with the goal of reducing complications and mortality of surgical patients. Practice guidelines based on clinical trials were issued, which aim to improve surgical safety and outcome. SCIP includes one process measure to reduce cardiac complications, two process measures with regard to venous

thromboembolism prophylaxis and seven process measures, which aim to reduce surgical site

infection. Three of these seven measures deal with the appropriate choice, timely administration and discontinuation of antibiotics within 24 hours post operatively.

The next three measures recommend appropriate hair removal, blood glucose control and early removal of urinary catheters. The last SCIP measure aiming to reduce surgical site infection is prevention of hypothermia. In contrast to hypothermia, fever is believed to be beneficial for host defense. Kluger demonstrated that fever is protective and that infected, ectothermic animals increase their body temperature by exposing themselves to higher environmental temperatures (30).

Hypothermia has been shown to increase wound infections in several studies. The early work of Kurz et al. in 1996 demonstrated in a prospective and randomized study that prevention of hypothermia reduces surgical site infections (7). Besides reduction in wound infections, the authors also found a significantly shorter time to food intake and a shortened length of hospital stay. These results were confirmed by other groups. Flores-Maldonado found a six-fold increased risk for wound infections in patients who became hypothermic during an elective cholecystectomy (31). Seamon et al. also found increased wound infections in trauma patients who became hypothermic during the initial operations, but were not hypothermic at admission (4). In non-abdominal operations, hypothermia was also found to increase blood loss and post-operative complications (32). Taking these results a step further, Melling et al. demonstrated that warming of the site

of the surgical incision or the whole patient before the operation, is able to reduce the rate of surgical site infections (33).

Other studies challenged these results. Barone et al. could not find an association between intraoperative hypothermia and wound infection in a retrospective chart review (34). Lehtinen et al. and Smith et al. found no impact of hypothermia on wound infection (35,36). Walz et al. even found a decrease in wound infection in hypothermic patients, although the body temperature difference was clinically not significant between the two groups (37).

In addition to the effect on the rate of operative wound infections, hypothermia has also been associated with increased mortality. The main body of this evidence originates from the trauma literature. Several publications found a highly significant association between admission temperature and death (1,5,8-10,14). Beilman et al. found increased rates of organ failure but no impact on mortality in hypothermic trauma patients (6). Two recent studies challenge the direct effect of hypothermia on mortality. The authors of these studies propose that hypothermia is a marker for severely injured patients rather than the cause for the increased mortality (38,39). Of note, Mahid et al. found that hypothermia in elective surgical patients is associated with a four-fold increase in mortality (2). Importantly, the cause of death in hypothermic patient is not necessarily related to infectious complications. Frank et al. found a significant increase in cardiac morbidity in elective surgical patients with perioperative hypothermia (40).

In septic patients, the development of hypothermia is very detrimental or at least a sign of failing host defenses. Septic patients, which present with or develop hypothermia, have a two-fold increase in mortality compared to febrile septic patients (41-44). Furthermore, a very recent study proposes that the use of anti-pyretic agents in septic patients may be detrimental and should be avoided (45). These results suggest that fever has a beneficial effect on the host defense and the lack of fever, especially when hypothermia develops, is a marker of the failure of host defense.

In summary, the majority of the available literature demonstrates that hypothermia has detrimental effects on outcomes in both surgical and medical patients. In surgical patients, hypothermia often occurs during or even before the operation and subsequently leads to increased rates of complications and death. In medical patients with sepsis, hypothermia is more a consequence of a failure of the immune system to mount an appropriate response and, consequently, is strongly associated with death in these patients.

The mechanisms by which the body temperature is regulated and the roles of cytokines in this process are very complex and not very well understood. Interleukin-1 (IL-1) and IL-6 have mainly pro-pyretic functions, whereas Tumor Necrosis Factor- α (TNF- α) is believed to induce hypothermia (46). The organization of the immune response, the involved cells, their secreted cytokines as well as the impact of hypothermia on these processes will be discussed next.

OVERVIEW OF THE IMMUNE RESPONSE

The purpose of the immune system is to defend the host against invading pathogens such as bacteria, fungi, and viruses. It is also responsible for removal and clearing of dead cells and plays an important role in inducing repair mechanisms after injury (47). While executing all these tasks, the immune system must not attack healthy host cells and the development of autoimmunity must be avoided. For this purpose, the immune system evolved in two different systems, which are separated into the innate system, which is considered the older of these two systems, and the adaptive system. The innate system is the primary defense and reacts immediately, but not specific to any kind of trauma or infection with a reproducible response. This type of immune response can be found in all vertebrates as well as some plants, fungi and primitive multicellular organisms (47). The adaptive system mounts a much slower response, it takes several days to react, but is much more specific and targeted than the innate system. Once the adaptive system has been exposed to a certain pathogen, the second exposure to this same pathogen results in an immediate, stronger response, often preventing the actual infection. However, these two systems cannot be separated as clearly as described here and there are multiple important interactions between cells of both systems. In fact, the adaptive system relies on activation signals from the innate system. We focus our studies on the monocyte/macrophage, a cell of the innate immune system, which is the principle activator of the adaptive system.

The innate immune system consists of two major components: passive defense mechanisms such as epithelial barrier, mucous fluids and secreted proteins, which inhibit the growth of pathogens. The second and active defense system consists of several different types of immune cells. The largest numbers of these innate immune cells are granulocytes of which the majority are neutrophil granulocytes (neutrophils). The main role of these neutrophils is the phagocytosis and destruction of pathogens, mainly bacteria and fungi. The eosinophil granulocytes are involved in the destruction of parasites, whereas the role of the basophil neutrophils is not very clear. Mast cells are found in tissues and the abdominal cavity. These cells are loaded with vesicles filled with histamine and other vasoactive and immune-active substances, which can be released via an antibody-dependent pathway or by direct activation of the mast cell. Mast cells have been implicated in development of hypothermia in endotoxemic shock (48).

Monocytes, Macrophages, Dendritic Cells and Their Relationship

Monocytes, macrophages and dendritic cells are important cells of the innate immune system. These three cell types are related: the macrophages and dendritic cells originate from the monocytes, which is the precursor found in blood and bone marrow (49,50). Macrophages and dendritic cells are found in all tissues. Tissue macrophages have different names based on the tissue they were first described such as Kupffer cells for the liver or Langerhans cell for the

skin because they were described in histologic preparations before their role and common origin had been elucidated. Macrophages and dendritic cells patrol the tissue in order to detect invading pathogens, but also to remove necrotic cell debris. After tissue injury or the detection of pathogens, macrophages release a host of cytokines and chemokines leading to the attraction of neutrophils and other immune cells, which act to eliminate the infection and repair of the tissue injury (51). Macrophages and especially dendritic cells have another, very important role which is to induce the adaptive immune response. Macrophages present antigens with the Major Histocompatibility Complex II (MHC II) from the phagocytized pathogen to T- and B-cells of the adaptive system resulting in the induction of the adaptive immune response. This function of antigen-presentation is an essential role of these cells. One of the major MHC II receptors is HLA-DR (Human Leukocyte Antigen-DR). Expression of HLA-DR on monocytes has been demonstrated to be a reliable marker for outcome after trauma and burns (52-59). Low expression of HLA-DR and especially the lack of up-regulation of HLA-DR after ex-vivo stimulation with LPS, correlates with increased complications and death. The restoration of the antigen presenting capability of peripheral blood monocytes can be achieved by administration of immune-modulatory drugs such as Interferon- γ (IFN- γ) or the Granulocyte-macrophage colony-stimulating factor (GM-CSF) (56,57,60,61). However, thus far a clinically relevant improvement in survival rates parallel with increased HLA-DR expression has not been demonstrated. Hypothermia reduced HLA-DR expression whereas fever increases HLA-DR (62,63).

At least two different subtypes of peripheral blood monocytes have been described. The “classical” monocyte expresses a high level of CD14, an essential surface receptor for recognition of LPS, a medium level of HLA-DR and no CD16 (CD14++HLA-DR+CD16-) (49,64-67). Over 80% of the peripheral blood monocytes are “classical” monocytes; the remaining are so-called “inflammatory” monocytes. In addition, an intermediate between the “classic” and “inflammatory” monocyte may exist. The “inflammatory” monocyte expresses a low level of CD14 but high levels of HLA-DR and CD16 (CD14+HLA-DR++CD16+). These “inflammatory” monocytes produce high levels of TNF- α , but low or no IL-10 (64,67,68). In addition, stimulation of T-cells is stronger and more efficient. In inflammatory states, a shift to a higher percentage of “inflammatory” monocytes can be observed (64,69). It is currently believed that these “inflammatory” monocytes develop into strongly pro-inflammatory dendritic cells, the so-called “tip-DCs” (50,70). In addition to the differences in cytokine secretion, “inflammatory” monocytes (CD16⁺) are able to leave but also re-enter the blood stream or lymphoid tissue, whereas “classic” monocytes are only able to leave the blood stream but cannot re-enter (50,65,67,70,71). These “classical” monocytes replenish the pool of tissue macrophages and support the tissue macrophages during infections.

In summary, monocytes are the precursor of the macrophages and dendritic cells and play an important role in the immune response. Clinical evidence suggests that the dysfunction of the monocyte/macrophage systems is a major contributor for infectious complications such as sepsis. We and others

believe that hypothermia affects the monocyte function, as indicated by reduced HLA-DR expression and changes in cytokine secretion, and this impaired function contributes to the higher risk of infectious complications in hypothermic patients (17).

Sequence of the Immune Response

An outline of the initial phase of the immune response and the role of the monocyte/macrophage is shown in Figure 1. An infection with bacteria leads to activation of monocytes or macrophages by two routes. Monocytes recognize pathogen associated molecular patterns (PAMPs) such as components of cell walls of bacteria such as Lipopolysaccharide (LPS) from gram-negative bacteria or Lipoteichoic-acid from gram-positive bacteria (72). In addition, the infection leads also to tissue destruction, which results in the release of endogenous proteins from these cells such as heat shock proteins (HSP) or the High-Mobility Group Protein B1 (HMGB1), which are referred to as alarmins (73-76). PAMPs and alarmins together are referred to as danger associated molecular patterns (DAMPs) (77,78). The bacterial components as well as the endogenous proteins are recognized by a variety of receptors on monocytes. These receptors recognizing certain distinct patterns of pathogens are termed Pathogen Recognition Receptors (PRR's). The activation of these PRR's results in the activation of intracellular signaling pathways and subsequent secretion of cytokines and chemokines (51,79,80). The cytokines activate other immune cells

in the same tissue but also in the whole body if they are released systemically. Furthermore, cytokines such as TNF- α , IL-6 and IL-1 β are responsible for the systemic effects of an infection such as fever, increased cardiac output, vasodilatation and the release of more immune cells from the bone marrow (72). The chemokines attract other immune cells to the site of infection. The main incoming cells are neutrophils, which phagocytize and destroy the pathogens. After clearance of the bacteria, lipid-mediators such as Protectins and Resolvins are secreted attracting more monocytes/macrophages to the site of infection. These lipid mediators increase the phagocytic activity of the monocytes/macrophages without stimulating the release of pro-inflammatory cytokines (resolution of inflammation) (81,82). The non-inflammatory macrophages remove the debris and remainders of the apoptotic or necrotic neutrophils. Furthermore, tissue repair is stimulated and fibroblasts induce the healing of the wound and usually form a scar which matures over time.

Role of Cytokines in the Immune Response

The secreted cytokines serve as messengers of the immune system and coordinate the local response by attraction of different leukocyte subtypes, as well as by inducing a whole body response, which aims to prepare the host to fight the infection in the best possible way (83-86). The attraction of additional leukocytes is supported by the local secretion of chemokines. Leukocytes follow a gradient of chemokines to the site of infection and are activated by cytokines in

the same environment. In addition, the cytokines activate the endothelium of the local vasculature, which leads to the expression of several adhesion molecules, facilitating the extravasation of the leukocytes at the site of infection and increasing the capillary permeability (87). The increased capillary permeability allows also the extravasation of complement proteins and coagulation factors, which support the cellular defense by opsonizing bacteria. Moreover, cytokines also have vasodilating effects resulting in an overall local increase in blood flow, but also to decrease in flow speed in each vessel, which again facilitates attachment and extravasation of leukocytes. Another result of the endothelium activation is the activation of the coagulation cascade, which leads to clotting of some of the outflowing vessels and therefore helps containing the infection locally (88). Clinically, these local changes can be appreciated by swelling (tumor), redness (rubor), heat (calor) and pain (dolor).

Besides these very important local effects, cytokines induce systemic effects known as the acute phase response (83,84,89,90). The acute phase response consists of fever, increased permeability of the vasculature, changes in metabolism and the increased or decreased production of certain proteins by the liver. The main effectors of the acute phase response are TNF- α , IL-1 β , and IL-6.

In summary, the secretion of these pro-inflammatory cytokines, TNF- α , IL-1 β and IL-6 is the first and very important step of the monocytes response. These cytokines then trigger a cascade of events, which optimize the local host defense but also prepare the rest of the body to support the host defense process. Consequently, an increased or reduced secretion of these cytokines can have

Figure 1: Overview of the Inflammatory Response

Legend to Figure 1

Infection with a pathogen (E. coli) is recognized by monocytes/macrophages. Activation of monocytes and macrophages result in the secretion of pro-inflammatory cytokines. These cytokines attract other immune cells such as Neutrophils to the site of infection but also initiate a systemic whole body response with fever and other effects which support the defense of the host. After phagocytosis of the pathogens and their clearance, the resolution of the inflammation is initiated by secretion of lipid mediators such as Resolvins and Protectins. These mediators attract non-inflammatory macrophages, which phagocytize necrotic Neutrophils without secretion of pro-inflammatory cytokines. At the same time tissue repair mechanisms are initiated.

detrimental effects to the host. Over-expression of TNF- α results in a lethal shock, whereas an inadequately weak immune response is also detrimental (91-96).

Monocytes and macrophages produce also IL-10. IL-10 is a cytokine with anti-inflammatory properties, which has in general opposing effects to the pro-inflammatory cytokines (97-100). It suppresses the further secretion of the pro-inflammatory cytokines by inhibiting their production in monocytes and macrophages. This property of IL-10 led to the original name of IL-10: human cytokine synthesis inhibitory factor. The strong anti-inflammatory properties of IL-10 have been demonstrated in models of lethal endotoxemic shock, which result in an exaggerated secretion of pro-inflammatory cytokines, mainly TNF- α . In these models, injection of IL-10 reversed the shock symptoms and increased the survival of the animals. An appropriate anti-inflammatory response is an important step to limit systemic and local inflammation and prevent excessive damage to the host organism.

These data reveal a tightly regulated and ordinarily balanced system of pro- and anti-inflammatory mediators. The balanced secretion of first pro-inflammatory followed by anti-inflammatory mediators is believed to be the key for an adequate host defense. Inadequate secretion of pro-inflammatory cytokines can result in lethal shock (75,91). Blockade of these cytokines can increase survival (93,97,98,101). However, an inappropriate suppression of the immune system makes the host susceptible to over-whelming systemic infections (94-96,102,103). Unfortunately, neither reduction of the pro-inflammatory

response nor stimulation of the immune system were successful in clinical trials (56,57,61,104-107). In retrospect, many of these substances were crude sledgehammers and the test situations clinically unlikely to respond significantly to a single addition to an array of therapies already in place. Changes in this tightly controlled system have profound consequences on the immune response and therefore survival of the host. In our research, we focus on the role of the monocyte/macrophage in the initiation of the immune response. We believe that hypothermia results in dysfunction of the monocyte cytokine secretion and thereby impairs the host defense.

ROLE AND REGULATION OF THE MONOCYTE

The principle role of monocytes is the recognition of pathogens, initiation of the immune response and presentation of antigens from phagocytized pathogens to cells of the adaptive immune system (51,72,79). The secretion of pro-inflammatory cytokines is the cornerstone of the initiation of the immune response. Besides these pro-inflammatory cytokines, monocytes produce also IL-10, which is an inhibitory cytokine and limits the immune response in order to prevent excessive damage to the host. The secretion of pro- and anti-inflammatory cytokines is tightly controlled on several levels with negative feedback loops within the cell as well as from outside the monocyte.

Pathogen Recognition and Intracellular Signaling

The first step in the immune response is the recognition of the pathogen (72,79,80). For this purpose, monocytes are equipped with a variety of extra- and intracellular PRR's of which Toll-Like Receptor 4 (TLR-4) is one of the best described. TLR-4 can recognize LPS but also several endogenous proteins such as heat shock proteins and HMGB1 (79,108-110). Besides TLR-4, at least twelve other Toll-like receptors have been described. Some of these Toll-like receptors are localized within the cell in phagosomes and allow recognition of phagocytized pathogens. Other extracellular receptors are the Receptor for Advanced Glycation Endproducts (RAGE), which recognizes endogenous proteins and glycated proteins, which occur in poorly controlled diabetic patients. The Scavenger receptor is another extracellular receptor, which recognizes mainly bacterial products but also oxidized lipoproteins. This receptor is believed to contribute to the formation of arterial plaques (111). Another large group of PRR's are the Nucleotide Oligomerization Domain receptors (NOD-like receptors) (112-114). These receptors are mainly located intracellularly. Intracellular receptors often respond to bacterial or viral RNA and DNA fragments which are released from digested bacteria or viruses, which infect the cell. The detection of bacterial RNA is believed to be an important assessment point for the immune system in order to determine the virulence of the infection (115). Detection of bacterial RNA indicates the presence of viable, growing bacteria and results in a more vicious and prolonged inflammatory response compared with stimulation with LPS alone.

LPS is bound by the LPS-binding protein, which in turn binds the CD14 surface receptor on monocytes (116). The complex of CD14, LPS and LPS-binding protein associates with TLR-4, resulting in the activation of the TLR-4 signaling cascade. TLR-4 may interact with several down-stream signaling proteins. However, the seemingly most important signaling protein is Myeloid differentiation primary response gene 88 (MyD88), which in turn interacts with the Interleukin-1 Receptor Associated Kinase 1 and 2 (IRAK 1 and 2) (108,117). IRAK 1 and 2 activate then the TNF-Receptor Associated Factor 6 (TRAF 6), which is able to activate two central, stress response pathways, the Nuclear Factor κ Beta (NF- κ B) and the Mitogen Activated Protein Kinase Pathway (MAPK). After activation of the TLR-4 signaling pathway, inhibitory proteins are expressed and inactivate the TLR-4 pathway by targeting the central complex consisting of IRAK 1/2, and TRAF-6. These inhibitory proteins are phosphatases, which cleave off the activating phosphate on these proteins. Two of these upstream inhibitors are the Suppressor of Cytokine Signaling 1 (SOCS-1) and the SH2 domain-containing inositol 5'-phosphatase 1 (SHIP-1) (118-120).

NF- κ B and MAPK-Pathway in the Monocyte Response

Intracellular signaling pathways are crucial to the capability of cells to cope with changes in their environment, process and integrate incoming signals from several receptors and to respond in an appropriate manner (121-124). Two pathways are essential for the monocyte response: the NF- κ B and MAPK-

pathway. Both are activated by a variety of surface and intracellular receptors. Importantly, there is a broad cross-activation between the NF- κ B and the MAPK-pathway. An outline of these two pathways is shown in Figure 2.

The activation of TRAF 6 by IRAK 1 or 2 results in the activation of the Inhibitory K-Kinase (IKK), of which three subtypes, exist. IKK then phosphorylates the Inhibitory κ protein Beta (I κ B), which is bound to the NF- κ B complex consisting of the p65 and p50 subunit. The complex of p65/p50 and I κ B is localized in the cytoplasm of the monocyte. Phosphorylation of I κ B results in its degradation and the release of the p65/p50 complex (109,123-125). The NF- κ B complex is now able to migrate into the nucleus and binds promoter sites of cytokine genes and other stress response genes resulting in their transcription. The inactivation of NF- κ B occurs by constitutional re-expression of I κ B, which binds the p65/p50 subunit resulting in its inactivation. The importance of this activation mechanism is that NF- κ B itself is a transcription factor and does not depend on the activation of other transcription factors or their production.

The MAPK-pathway is also activated by TRAF 6. In contrast to the NF- κ B pathway, the MAPK-pathway is organized in three arms and several levels of protein kinases. The three arms are p38, the extracellular signal-regulated kinases (Erk 1 and 2), and the c-Jun N-terminal kinases (JNK 1-3). These three arms can induce expression of a variety of similar or closely related genes such as pro-inflammatory cytokines (109,121,122,126). However, the Erk-pathway seems to hold a pivotal role in the induction of the IL-10 production, whereas the p38 and JNK-arm appear not to be mandatory for IL-10 production (99). Over-

simplified, the p38 and JNK-arms have mainly pro- inflammatory properties, whereas the Erk-arm also has anti-inflammatory properties, mainly by induction of IL-10 expression.

The MAPK-pathway is organized in several layers, in which the top layer activates the subsequent level, which in turn activates the next level. There are a total of at least three levels, MAPK1 – 3 in which MAPK3 is the first level of activation and MAPK1 is the level of p38, Erk 1/2, and JNK1-3. TRAF 6 activates the MAPK3 level by phosphorylation. The MAPK1 proteins, i.e. p38, Erk and JNK, phosphorylate transcription factors such as the Activated Transcription Factor 1 (ATF1), c-Jun or c-Fos. The purpose of organization in to these several levels is that an incoming signal can be amplified resulting in a strong, reliable activation of the down-stream targets. In contrast to the NF- κ B-pathway, the inactivation of the MAPK-pathway is an active process, which is tightly regulated and may result in selective inhibition of a certain arm of the MAPK-pathway. The inactivation occurs mainly on the MAPK1 level by specific so-called Dual Specificity Phosphatases (DUSP's) or MAPK-phosphatases (MKP's) (127-130). Most of these DUSP's are specific for one or two of the three arms of the MAPK-pathway. DUSP 1 for example inactivates the p38 and JNK arm but not the Erk arm. This organization offers the opportunity to fine tune the activation of each of the three arms. There are at least 30 proteins described, which contain the typical phosphatase domain. However, only eleven of these also contain the MAPK-binding site. The physiologic role of these DUSP's without MAPK-binding

Figure 2: Overview of the Inflammatory Signaling Pathways

Legend Figure 2

The NF- κ B and Mitogen-Activated Protein Kinases Pathway (MAPK) are intracellular signaling pathways which are activated by stress signals and induce a stress response. TLR-4 recognizes LPS and activates a cascade of signaling proteins, which results in activation of NF- κ B and MAPK. The key activator of NF- κ B is the Inhibitory K Kinase (IKK), which phosphorylates the Inhibitory κ Protein β resulting in its degradation. The NF- κ B complex consisting of the p65 and p50 subunit translocates into the nucleus and activates gene transcription.

The MAPK-pathway is activated by a variety of proteins. This pathway consists of three arms, JNK, Erk and p38. There are several layers of kinases, which are activated subsequently starting at the MAPK3K level. This arrangement leads to an amplification of the incoming signal. JNK, Erk and p38 activate several transcription factors, which then induce transcription of the target genes.

There is a very high degree of redundancy in activation of these pathways.

domain is unclear. It appears also that there is a certain tissue specificity of the DUSP's and only a few of them are expressed in all tissues and cell types.

These DUSP's are newly produced after activation of the MAPK-pathway within 30min to 1 hour. The degree of up-regulation and also the subtype of the DUSP, which is up-regulated, can therefore lead to a modification of the MAPK-pathway activation and shift the response of the cell. The result of activation of both, the NF- κ B and MAPK pathway, is the production and secretion of cytokines as well as other proteins, for example heat shock proteins, which increase the resilience of the cell.

MICRORNAS REGULATE THE MONOCYTE RESPONSE

In the last few years, microRNAs have been recognized to be potent regulators of cell function (131-134). MicroRNAs are short, single-stranded RNA fragments with a length of ~22 nucleotides (nt). The main role of microRNAs is the interference of protein production, mainly through a reduction in protein expression, although an increased protein production has been observed in starved cells (135).

The biogenesis of microRNAs is outlined in Figure 3. MicroRNAs are transcribed from DNA like normal genes. These pri-microRNAs are then processed by Drosha, an enzyme which cleaves RNA-stands, to pre-microRNAs. The pre-microRNA is transported into the cytoplasm by a specific transporter, Exportin-5. In the cytoplasm, the microRNA is processed by Dicer, a protein

similar to Drosha but located in the cytoplasm, into the active, mature microRNA and is integrated into a protein complex, the RNA-induced silencing complex (RISC). Depending on the complementarity of the microRNA-seed sequence with the mRNA sequence, the mRNA is either degraded or the binding of the translation machinery inhibited. A 100% complementarity of the microRNA seed-sequence with the mRNA results in degradation of the microRNA, whereas an 80% match results in inhibition of the protein translation. Both processes result in reduced protein production. Importantly, a change in mRNA-levels is only evident when the mRNA is degraded. The catalytic active protein, which is able to degrade mRNA, is the Argonaut 2 protein (Ago2). As of yet, the main function of microRNAs in a broad sense appears to be the inhibition of protein production rather than degradation of the mRNA-strand.

MicroRNAs Described in Monocytes

Several microRNAs have been investigated and found to be important in the monocyte response (136-146). Figure 4 shows some of these microRNAs and their proposed targets. Among these microRNAs, miRNA-155, -146a, -101, and -21 are among the best described. We will focus on microRNA-155 in our research. Depending on the target protein, the microRNAs can either have a pro-inflammatory or anti-inflammatory action (139,140,145,147,148). MiRNA-155 and -101 both target inhibitors of the TLR-4 signaling pathway. Since microRNAs inhibit protein production, the inhibition of an inhibitor results in a prolonged

Figure 3: Biogenesis of microRNAs

MicroRNAs are transcribed from DNA into pri-microRNA and are then processed by Drosha into the pre-microRNA. The pre-microRNA is transported into the cytoplasm by Exportin-5 and processed by Dicer and integrated into the RNA-induced silencing complex (RISC). The complementarity of the microRNA-seed sequence determines the effect on the target mRNA: a 100% match results in the degradation of the mRNA whereas an 80% match inhibits protein production. Drosha and Dicer are RNA-processing enzyme located in the nucleus or cytoplasm, respectively.

Figure 4: MicroRNAs involved in the Toll-like Receptor Signaling Pathway

Several microRNAs target the TLR-4 pathway. Depending on the target, the microRNAs can either have pro- or anti-inflammatory properties. The fact that multiple microRNAs target several proteins on all levels of the TLR-4 pathway demonstrates a high redundancy and tight control of the activation of the pathway. Adapted and modified from (137,139,144,146,149).

Figure 5: Action of miRNA-155 and -101 in the TLR-4 Pathway

MiRNA-155 decreases production of SHIP-1/SOCS-1 and increases activation of both NF- κ B and MAPK-pathway. MiRNA-101 reduces production of DUSP-1 increasing activation of p38 and JNK. The synergistic action of these two microRNAs increases the production of pro-inflammatory cytokines.

activation of the TLR-4 signaling pathway. MiRNA-155 targets SOCS-1 and SHIP-1 both are upstream inhibitors of the TLR-4 signaling pathway and inhibit IRAK-1/2 and TRAF-6 (139,145,149). Up-regulation of miRNA-155 inhibits the expression of these inhibitors and results in a prolonged activation of the TLR-4 signaling prolonging the secretion of pro-inflammatory cytokines such as TNF- α , IL-1 β and IL-6 (Figure 5). Because SHIP-1/SOCS-1 are upstream inhibitors, only a slight reduction of their expression level can have profound impact on the activation of the NF- κ B and MAPK-pathway. Besides the pro-inflammatory actions, miRNA-155 can also have anti-inflammatory actions. Suppression of MyD88 or TAB2 production by miRNA-155 results in a reduced signaling through the TLR-4 pathway and reduced secretion of TNF- α (140,148). The pro- or anti-inflammatory effect of miRNA-155 is time-dependent and depends on the targeted protein and cell type. MiRNA-101 has pro-inflammatory properties (Figure 5). MiRNA-101 inhibits DUSP-1 production prolonging the activation of the p38 and JNK-arm of the MAPK pathway, which also results in an increased secretion of TNF- α (146). Because miRNA-155 and -101 both target inhibitors of the same pathway, they have synergistic function.

In contrast to miRNA-155 and -101, miRNA-146a targets IRAK-1 and TRAF-6 to reduce the expression of these two proteins, resulting in a decrease in the TLR-4 signaling (Figure 4). Therefore, miRNA-146a has anti-inflammatory properties (136,137,141,150,151).

MiRNA-21 is a very well described microRNA in cancer development because many of its targets are proteins of important signaling pathways (152-

159). One of these target proteins is the Programmed Cell Death Protein 4 (PDCD-4). The exact function of PDCD-4 is unclear, but it has been proposed that it is involved in the induction of apoptosis. Therefore, up-regulation of miRNA-21 results in a decrease of PDCD-4 and consequently reduces apoptosis promoting cancer development. In the monocyte response, Sheedy et al. have proposed another regulatory loop (143). In this complex feedback loop, PDCD-4 inhibits the transcription of IL-10 mRNA. Up-regulation of miRNA-21 by activation of the TLR-4 signaling pathway results in a reduction of PDCD-4 expression and subsequently an increase of IL-10 mRNA, which then will be translated into IL-10 protein. The IL-10 protein will be secreted and acts as the above described inhibitor of the monocyte response.

Considerations of Target Protein and Timing for Action of MicroRNAs

In order to determine the effects of microRNAs on the immune response, it is important to consider the expression level of the targeted protein. MicroRNAs can only inhibit the production of proteins. Therefore, already existing proteins are not affected immediately by newly expressed microRNAs. This is the case for miRNA-146a, which targets IRAK-1 and TRAF-6, but also for miRNA-155 when the target is MyD88 or TAB2. All these proteins are existing proteins and part of the TLR-4 signaling pathway. These proteins are essential for proper function of the TLR-4 pathway and must respond immediately if a pathogen is detected. Up-regulation of miRNA-146a or miRNA-155 will not affect these existing proteins.

Only the re-expression of these proteins will be affected and a reduction in downstream signaling will only result in a delayed manner. Consequently, the inhibitory effect of miRNA-146a and miRNA-155 up-regulation will be delayed.

In contrast, the effect of miRNA-155 is immediate when SHIP-1/SOCS-1 are targeted; both proteins are expressed after activation of the TLR-4 pathway and act as a negative feedback loop. Up-regulation of miRNA-155 will inhibit the production of these two inhibitors of the TLR-4 pathway immediately. Consequently, up-regulation of miRNA-155 results in increased downstream signaling. Up-regulation of miRNA-101 has the same effect. DUSP-1 is also newly expressed after stimulation of the TLR-4 pathway. The up-regulation of miRNA-101 inhibits the production of DUSP-1 immediately and will also increase the activation of the p38 and JNK-arm of the MAPK-pathway. However, the Erk-arm will not be affected.

EFFECT OF TEMPERATURE ON MONOCYTE RESPONSE

The effect of temperature on the monocyte response has been investigated in order to identify potential mechanisms, which may explain, why hypothermic patients experience such detrimental outcomes. Some of our own and other published data are conflicting. One of the most consistent results is the effect of hypothermia on cytokine expression in human monocytes or peripheral blood mononuclear cells (PBMCs) cultures. Hypothermia reduces the initial secretion of early, pro-inflammatory cytokines compared to 37°C, but results in

an increased and prolonged secretion of TNF- α , IL-1 β and IL-6 24h or longer after initial stimulation (18,20,23,62,160-163). In contrast to the pro-inflammatory cytokines, the expression of IL-10 was found to be reduced under hypothermic conditions in the same studies and some others (21,164). HLA-DR was also found to be reduced in hypothermia. Furthermore, rewarming seems to reduce the detrimental effects of hypothermia on HLA-DR expression (165).

The only study to our knowledge, which investigates cytokine levels in hypothermic patients without cardiovascular operation, was conducted in patients, who were deliberately cooled after successful out-of-hospital reanimation after cardiac arrest. In this study, IL-6 was found to be higher in the hypothermic group, whereas TNF- α was not different (166). Supporting the hypothesis that hypothermia impairs the immune response, the rate of bacterial colonization was higher in hypothermic patients although no difference in infectious complications was found. Another study in which therapeutic hypothermia after cardiac arrest was applied, found a significantly increased rate of early onset pneumonia in hypothermic patients (167). Both studies report a higher survival rate in patients with therapeutic hypothermia, which is likely due to the beneficial effect of hypothermia on the recovery of the hypoxic brain injury. In a study with brain injury patients, IL-6 was found to be elevated for a longer period in the jugular vein of normothermic patients and a much more rapid decrease in jugular IL-6 levels in hypothermic patients (168). Since the difference in IL-6 was only found in the jugular vein, IL-6 may rather mirror the cytokine levels in the brain than the systemic response to hypothermia.

Animal models are of limited value in investigating the effects of hypothermia. In rodents, which are almost exclusively used for these experiments, the development of hypothermia is a physiologic process in sepsis and therefore probably not detrimental as it is for humans (169). Consequently, the data are conflicting. Some authors found a detrimental effect of hypothermia, whereas others proposed a beneficial effect. Torossian et al. found detrimental effects of hypothermia on survival in a bacterial peritonitis model in rats (170,171). Huet et al. and Taniguchi et al. found a beneficial effect of hypothermia (172,173). Importantly, these two studies used LPS as challenge and not live bacteria as Torossian et al. in their studies, which may contribute to the opposing results. All studies found an increase of IL-10 in hypothermic rodents, which is in strong contrast to the available *in vitro* data from human cells. In line with the human data, restoration of normothermia was found to increase survival in a bacterial peritonitis model (174). The suitability of mice for investigation of temperature regulation and effects of temperature has recently been questioned both scientifically and teleologically (175).

Besides the investigation of cytokines, some studies have examined the effect of hypothermia on gene expression and pathway activation. Fairchild et al. found in a series of studies that hypothermia affects the NF- κ B pathway (19,23). These investigators found increased activation of IKK, delayed re-expression of I κ B and prolonged nuclear localization of the NF- κ B complex. These findings would explain the prolonged secretion of pro-inflammatory cytokines (19,23). Arai et al. confirmed the prolonged NF- κ B activation in hypothermia (164). In line with

a profound impact of hypothermia on cell homeostasis, Sonna et al. found also a strong impact of hypothermia on the whole genome expression in the human monocytic cell line THP-1 and liver cells (24,176). Interestingly, genes involved in the immune response and signaling pathways were particularly strongly affected by hypothermia. To date, there is no data on effects of hypothermia on the MAPK-pathway in monocytes or macrophages. The existing data in Human Umbilical Vein Endothelial Cells (HUVEC) and astrocytes indicates that these pathways are also affected by hypothermia (25,177,178). Of particular interest is the finding of Yang et al, that the reduced JNK-activation in hypothermia in their model of ischemia/reperfusion injury is due to an increased expression of DUSP-1 and not due to an increased up-stream signaling (177).

In summary, hypothermia was found to increase and prolong the secretion of pro-inflammatory cytokines and to reduce the expression of IL-10 in human cell cultures. As a possible underlying mechanism, hypothermia prolongs NF- κ B activation in monocytes.

CHAPTER III

HYPOTHESIS, SPECIFIC AIMS AND EXPERIMENTAL PLAN

A) Key Objective

To investigate the effects of clinically-relevant altered temperatures on primary monocyte function such as cytokine production and to identify mechanisms likely to be responsible for increased rate of infectious complications and death in hypothermic surgical patients.

B) Hypothesis

Hypothermia dysregulates elementary cell functions such as the activation of inflammatory signaling pathways and gene expression. We hypothesize that hypothermia increases the activation of the NF KB and MAPK-pathway. This increased pathway activation leads to increased expression of pro-inflammatory genes and miRNA-155 resulting in a prolonged secretion of pro-inflammatory cytokines in hypothermia.

C) Specific Aims

- a. **Specific Aim 1:** Investigation of NF- κ B and MAPK pathway activation under hypothermic and hyperthermic conditions in isolated monocytes.
- b. **Specific Aim 2:** Analysis of expression of miRNAs miR-155 at 32°C to 39°C.
- c. **Specific Aim 3:** Verification of the effect of the dysregulated miRNA-155 on the cellular response through the use of miRNA mimics and antagomirs on the cytokine production.
- d. **Specific Aim 4:** Investigation of the suppressive role of IL-10 on miRNA-155 expression and the prolonged TNF- α production at 32°C and 39°C.

D) Experimental Plan

The purpose of this project is to investigate how hypothermia modulates the monocyte response on the cellular level and which processes lead to the observed differences in cytokine secretion. We analyze each step of the monocyte response, starting with the activation of the signaling pathways NF- κ B and MAPK. If we observe differences in the activation of these pathways, a subsequent difference in gene expression can be expected. Gene expression in our understanding includes mRNA and microRNAs, mainly miRNA-155 for our purposes. Identification of differential expression of miRNA-155 in hypothermia

should result in differences in secretion of pro-inflammatory cytokines. To confirm the role of miRNA-155, we will up-regulate or suppress the expression of miRNA-155 and demonstrate its effect on TNF- α . The last purpose of our research is to determine, which role negative feedback loops play in the regulation of the monocyte response. Modulation of the action of IL-10 allows determining its effect on cytokine gene expression and cytokine secretion as well as miRNA-155 expression. An outline of the research plan is showed in Figure 6.

Figure 6: Research Plan and Investigated Steps of the Monocyte Response

We investigate the monocyte response stepwise and determine the effect of hypothermia on each of these steps. The first step is the activation of the NF- κ B and MAPK-pathway followed by the expression of cytokine mRNA and miRNA-155. The role of miRNA-155 on TNF- α secretion and the impact of the negative feedback loop of IL-10 on miRNA-155 is also analyzed.

E) Methods

E1) Study Subjects

Approval of the study protocol was obtained from the University of Louisville Institutional Review Board prior to enlisting any study subjects (HSPPO 08.0018). Written informed consent was obtained from all participants. Volunteers with any signs of acute sickness, history of chronic conditions such as diabetes mellitus or immune-suppressive disorder and subjects with chronic medication use were excluded. We enrolled a total of 16 healthy donors, although the majority of experiments were conducted with seven donors per experiments. The age of the participants ranged from 19 – 49 years.

E2) Monocyte Isolation

Venous blood was collected in EDTA Vacutainers (Becton Dickinson, Franklin Lakes, NJ). Primary human monocytes were isolated using the magnetic cell sorting technique according to manufacturer's instruction. The collected whole blood was incubated with Human CD14 MicroBeads (Miltenyi Biotec, Auburn, CA) for 15min in an incubator at 37°C. After washing, the blood was resuspended in the original volume with MACS Separation Buffer (Miltenyi Biotec, Auburn, CA) and run through Whole Blood Columns (Miltenyi Biotec, Auburn, CA). After isolation, the columns were washed three times and the monocytes eluted from the columns with MACS Elution Buffer (Miltenyi Biotec, Auburn, CA).

The cells were washed twice with Phosphate Buffered Saline (PBS) and counted. The purity of the isolated monocytes was >95% as determined by Flow Cytometry.

The primary monocytes were cultured in 1640 RPMI medium (MP Biomedicals, Solon, OH) supplemented with 10% heat-inactivated defined Fetal Bovine Serum, 2mM L-Glutamine and 100IU/ml penicillin, 100µg/ml streptomycin and 250ng/ml amphotericin B (all items purchased from Thermo Scientific, Waltham, MA). Cells were cultured in a concentration of 0.25×10^6 cells/ml in a humidified incubator with 5% CO₂. The temperature varied according to the experimental conditions. In general, the monocytes were cultured at 32°C or 39°C for the different experiments. 39°C was chosen to avoid the strong induction of a heat shock response (179-181). Preliminary experiments with 34°C and 37°C demonstrated higher TNF-α levels and lower IL-10 levels in 34°C compared to 37°C. However, the high inter-subject variation of the cytokine response ameliorated some of the temperature differences. We therefore decided to use 32°C and 39°C in order to enhance the temperature induced differences. If the cells were rested or stored, the culture temperature was 37°C. The monocytes were cultured in 50ml polypropylene tubes as cell suspension. Lipopolysaccharide (LPS), 100ng per 0.25×10^6 cells (E. coli 0111:B4; Sigma-Aldrich Co., St. Louis, MO) was used for stimulation. After incubation for the indicated time-periods, the monocytes were centrifuged, the supernatant was collected and stored at -80°C until measurement. The

cell pellet was washed with PBS and transferred to 1.5ml centrifugation tubes and centrifuged for 3min at 10,000rpm (Eppendorf MiniSpin).

E3) Phosflow Pathway Analysis

For the pathway analysis, the freshly isolated monocytes were rested at 37°C for 24h until further use. After 24h, cells were washed and resuspended in fresh medium (10% FBS supplemented 1640 RPMI). Cells were stimulated with LPS (100ng per 0.25×10^6 cells) and then incubated for the indicated time points at either 32°C or 39°C. There was no pre-incubation at the corresponding temperature. For definition of baseline pathway activation, unstimulated monocytes cultured at 37°C for the duration of the experiment were used (=0h time point).

In preliminary experiments, we also investigated the activation of NFκB at 37°C. Table 1 shows the Mean Channel Fluorescence (MCF) at 15min and 20min after LPS at 32°C, 37°C and 39°C. NFκB activation at 37°C lies between 32°C and 39°C.

After the indicated time, the monocytes were immediately fixed for 10min at 37°C with a pre-warmed Formaldehyde solution (final concentration 3.2%) in order to preserve the phosphorylation status of the pathways. Cells were then washed twice with wash Buffer containing FBS (FBS wash Buffer, BD Bioscience, San Jose, CA). The monocytes were permeabilized with pre-chilled 100% methanol at -20°C for 30min in order to allow the antibodies access to the pathway epitopes. After three

washes, cells were resuspended in 100µl FBS-wash buffer and separated into two tubes. One tube was used for analysis of NF-κB and p38 activation; the second tube was used for analysis of Erk and JNK activation. Samples were stained for 1h in the dark at room temperature. The following antibodies were used (all BD Bioscience Phosflow antibodies, San Jose, CA): phospho-p38 MAPK (pT180/pY182), Alexa Fluor® 488; phospho-ERK1/2 (pT202/pY204), Alexa Fluor® 488; phospho-JNK (pT183/pY185), Alexa Fluor® 647; NF-κB p65 (pS529), Alexa Fluor® 647.

After staining, cells were washed twice and resuspended in 250µl FBS-wash buffer and immediately analyzed. A FACS Calibur (BD Bioscience, San Jose, CA) was used to acquire the samples. We counted 10,000 events for each sample. The freely available online-software cytobank.org, which was specifically developed for analysis of Phosflow samples, was used for analysis. Example histograms for NF-κB activation are shown in Figure 7.

E4) RNA Isolation

Total RNA was isolated from the monocyte pellet using the RNeasy Mini Kit (Qiagen, Valencia, CA). Concentrations and purity of the RNA were determined with the Nanodrop N-1000 (Agilent Biosystems, Santa Clara, CA). All samples fulfilled the quality criteria (A260/A260 ratio between 1.9 and 2.1).

Table 1: NFκB Peak Activation at 32°C, 37°C and 39°C

Temperature	15min post LPS (MCF)	20min post LPS (MCF)
32°C	1148.31	1061.81
37°C	1099.83	975.83
39°C	975.23	772.36

Comparison of peak activation between 32°C, 37°C and 39°C of the NFκB pathway in one preliminary experiment. The highest activation is recorded at 32°C and remains elevated. The lowest activation is measured at 39°C while 37°C is in between.

MCF: mean channel fluorescence

Figure 7: Example of NF- κ B Activation at 32° and 39°C

Example of histograms demonstrating the activation of the NF- κ B pathway at 32°C and 39°C. The x-axis reflects the degree of activation (mean channel intensity in a logarithmic scale). In addition, the degree of activation is color-coded with brighter colors indicating a higher degree of activation. The different levels of histograms in the y-axis indicate the different time points. The 2h time point at 39°C reaches almost baseline levels, whereas 32°C remains clearly activated. In addition, the peak level of activation at 32°C is higher than at 39°C.

E5) Cytokine Measurements

TNF- α , IL-6, and IL-10 levels were determined by enzyme-linked immunosorbent assays (ELISA) (eBioscience, San Diego, CA) in 96-well plates according to manufacturer's protocol. All samples were analyzed in duplicates; cytokine levels of samples were determined by a standard curve using recombinant human TNF- α , IL-6, or IL-10.

E6) MicroRNA and MessengerRNA Expression

For expression analysis of miRNA-155 TaqMan single microRNA assays were used according to manufacturer's instruction (Life Technologies, Foster City, CA). The housekeeping gene U6 was used for normalization. An exemplary amplification plot is shown in Figure 8.

For expression of TNF- α and IL-10 mRNA, complementary DNA (cDNA) was generated using the High Capacity cDNA Reverse Transcription Kit (Life Technology, Foster City, CA). TaqMan single gene assays for human TNF- α and IL-10 (Life Technologies, Foster City, CA) were then used for assessment of gene expression. 18s was chosen as internal control.

The Polymerase Chain Reactions (PCR) were run on a StepOne Plus RealTime-PCR-System using Fast Advanced Master Mix and the fast protocol (all products purchased from Life Technologies, Foster City, CA). Fold changes were calculated using the $\Delta\Delta CT$ -method (182).

Figure 8: Exemplary Amplification Plot for miRNA-155

The x-axis indicates the cycle threshold (C_t), which is used to determine the ΔC_t -value. The y-axis is logarithmic and shows the level of fluorescence. Amplification of a gene increases the fluorescence exponentially. The difference in C_t -values between the housekeeping gene U6 and miRNA-155 is used to calculate the ΔC_t . The difference between the control sample and the sample of interest allows the determination of the $\Delta \Delta C_t$, which can be transformed into Fold Change.

E7) MicroRNA-Transfection

In order to demonstrate the effect of miRNA-155 on the prolonged expression of pro-inflammatory cytokines, we transfected the monocytes with miRNA-155 mimics and antagomirs. MicroRNA-mimics are pre-microRNAs, which are processed by Dicer and become part of the RISC and act in the same way as endogenous microRNAs. MicroRNA-antagomirs are the anti-sense strand of the mature microRNA of interest. The binding of the antagomir with the target microRNA leads to the degradation of the microRNA by the same process as described for silencing RNAs (siRNA) (183). Negative controls are RNA-strands, which have the same hairpin structure like a natural pre-microRNA and are also processed by Dicer. However, the seed-sequence of the negative controls is not complementary to any known gene and has therefore no biologic action.

For this experiment, monocytes were isolated as described and then immediately transfected with either miRNA-155 mimic, negative control or miRNA-155 antagomir. We used the N-TER nanoparticle transfection system for this purpose according to the manufacturer's instructions (Sigma-Aldrich Co., St. Louis, MO). The miRNA-155 mimic and negative control were incubated in a concentration of 40nM, whereas the miRNA-155 was incubated in a concentration of 80nM. MicroRNA-mimics, antagomirs and negative control were purchased from Ambion (Life Technologies, Foster City, CA).

After 24h of transfection, the cells were washed and resuspended in fresh RPMI-media with 10% FBS. The cells were then stimulated with LPS for another 24h at the appropriate temperature, 32°C or 39°C. After 24h of LPS-stimulation, the supernatant was collected for cytokine measurements and the total RNA was isolated for analysis of microRNA expression. Cell viability at all time points was >90% as determined by flow cytometry and Trypan Blue.

E8) IL-10 Addition / Anti-IL-10-Receptor Blockade

In order to elucidate the role of the IL-10 feedback loop, we added recombinant human IL-10 (rhIL-10, Sigma-Aldrich Co, St. Louis, MO) or blocked the action of IL10 by blocking the IL-10 receptor. For the IL-10 addition experiment, monocytes were isolated and stimulated with LPS at 32°C or 39°C as outlined above. After 4h of LPS-stimulation, 20ng of rhIL-10 for a final concentration of 5ng/ml were added to the cell culture tubes in both temperatures for the remaining time of 12h and 24h. The 4h time point at which IL-10 was added was chosen based on preliminary experiments. At this time point, IL-10 is being secreted by monocytes after LPS stimulation.

In order to block the action of IL-10 the isolated monocytes were incubated for 1h at 37°C with 10µg of anti-human IL-10-receptor antibody (α-subunit, Sigma-Aldrich, St. Louis, MO). We performed a dilution series

in order to determine the amount of anti-IL-10-receptor antibody, which can block the action of IL-10 (Figure 9). After incubation with the antibody, cells were stimulated with LPS and incubated for the indicated time point at 32°C and 39°C. These experiments were conducted with the same donor at the same time with both treatments in both temperatures. In addition, untreated samples cultured at 32°C or 39°C were used as controls. At the indicated time points, supernatant was collected and total RNA isolated until further analysis.

E9) Statistical Analysis

For all experiments, monocytes from the same person were used and the different temperatures or treatments were compared against each other within the same person. Therefore, a related sample test was used. For two comparisons, the Wilcoxon ranked sign-test was used; for multiple comparisons, Friedman's Two-Way Analysis of Variance by Ranks was used. Data are presented as mean $\pm$ SEM. SigmaPlot 11.0 was used for presentation of the data (Systat Software Inc., San Jose, CA). Statistical analysis was performed with SPSS 18.0 (PASW Statistics 18, IBM, Chicago, IL). Results were considered significant at $p < 0.05$.

Figure 9: Effect of Different Amounts of Anti-IL-10 Receptor Antibody on TNF- α and miRNA-155

Blockade of the IL-10-Receptor results in a dose-dependent increase in TNF- α and miRNA-155.

CHAPTER IV

EFFECT OF TEMPERATURE ON CYTOKINE EXPRESSION

The secretion of cytokines by cells of the immune system serves several important purposes. Cytokines serve as messengers among different immune cells and coordinate the immune response in order to contain infections (47,51). The first step in a successful engagement of the immune system is the signal that an invading pathogen has been discovered. This signal attracts other leukocytes in relatively large numbers with the goal to clear the infection. Tissue macrophages and dendritic cells serve exactly this cause. These cells patrol through the tissues and phagocytize foreign and endogenous material. Pathogens such as bacteria are recognized by a variety of receptors like the TLR-4 receptor (72,79,80). These receptors signal through signaling pathways and induce secretion of cytokines. The first response is the secretion of preformed cytokines, which are stored in vesicles. Secretion of these preformed cytokines allows an immediate response to any signs of danger. These pro-inflammatory cytokines activate the local endothelial cells and attract other immune cells such as neutrophils and monocytes from the blood. These cells evade at the site of the infection the blood stream and engage in the immune defense replenishing and supporting the local cells.

The second purpose of cytokines, after activation of the initial immune response, is to limit the host response to the infected tissue and avoid damage to the host. An uncontrolled release of pro-inflammatory cytokines may lead to severe, detrimental effects such as generalized vasodilation resulting in shock or systemic activation of the coagulation system known as disseminated intravascular coagulation (DIC) (75,91). To prevent the systemic spread of the immune response, anti-inflammatory feedback loops are automatically activated after the first detection of LPS by a local tissue macrophage (56,97-100).

IL-10 was the first described anti-inflammatory cytokine. Its original name human cytokine synthesis inhibitory factor (CSIF) describes clearly what the function of IL-10 is. IL-10 suppresses the production of pro-inflammatory cytokines directly in any immune cell. It also stimulates its own production and the production of other, anti-inflammatory cytokines such as Transforming Growth Factor- β (TGF- β). Furthermore, IL-10 induces the development of a certain subtype of T-helper cells such as T_H2 T-cells and regulatory T-cell (T_{regs}). T_H2 T-cells stimulate wound healing and the switch of B-cells to the production IgG-antibodies (47). This is in contrast to the T_H1 T-cell, which produces pro-inflammatory cytokines and stimulate the activity of macrophages and cytotoxic T-cells (CD8+). T_{regs} are another subtype of T-cells, which suppress the immune system. The exact mechanism how these cells function remains unclear. It has been suggested that they secrete IL-10 and suppress the function of pro-inflammatory immune cells by transferring anti-inflammatory microRNAs such as miRNA-146a into T-cells (184,185).

Taking together, these results suggest that the key to an appropriate immune response is the balanced production of first pro-inflammatory cytokines followed by the production of anti-inflammatory cytokines. It has been suggested that a cause for the post-traumatic multiple organ dysfunction syndrome (MODS) without sepsis is prolonged and unbalanced secretion of pro-inflammatory cytokines (186,187). In this concept, the increased and prolonged secretion of pro-inflammatory cytokines is followed by a profound and prolonged suppression of the immune system, also described as immune-paralysis or Compensatory Anti-Inflammatory Response Syndrome (CARS).

Hypothermia dysregulates the secretion of cytokines and this effect may contribute to the detrimental effect of hypothermia. The available data in human *in vitro* cultures indicate that hypothermia results in a delayed, but increased and prolonged secretion of pro-inflammatory cytokines after LPS stimulation (18,20,161,163). In contrast, hypothermia decreases the production of anti-inflammatory cytokines such as IL-10 (21,22,163).

The purpose of the first experiment was to determine, how hypothermia affects the production of cytokines in our model and if we can reproduce the published results.

RESULTS

Monocyte Purity and Viability

In order to achieve reliable results from our investigations, we first determined the purity of our monocyte culture. We confirmed a purity >95% after the isolation process for every donor. An example of a flow scatter plot is shown in Figure 10. Next, we monitored the viability of the monocytes over time. Figure 10 also demonstrates a scatter plot of a sample of the same donor after 36h of LPS stimulation at 39°C. There is a slight increase in size and granularity of the monocytes as indicated by a shift of the population to the right (size) and upwards (granularity). These changes are explained by the stimulation with LPS and the subsequent response of monocytes with production of cytokines and phagosomes. Importantly, there is no significant change of cells outside the gate (R1), which would indicate disintegration and necrosis or apoptosis of the monocytes.

Figure 10: Flow Scatter Plot of Freshly Isolated Monocytes and 36h after LPS Stimulation

One distinct cell population can be determined in both graphs. The size (forward scatter – FSC) and granularity (side scatter – SSC) are typical for monocytes. Events outside the left side of gate R1 are considered cell debris such as partial lysed red cells or platelets, which remain in a low amount after the isolation process. The monocyte population remains at the same location after 36h of LPS stimulation. However, there is a slight increase in size (shift to the right on the forward scatter – FSC) and granularity (upwards shift on the side scatter – SSC). This is a typical finding of activated monocytes. There is an insignificant increase of cells in the left side of gate R1. These cells are intact and express all receptors but are becoming necrotic or apoptotic.

Secretion of Pro-Inflammatory Cytokines

Tumor Necrosis Factor Alpha – TNF- α

Figure 11 shows TNF- α levels over 36h at 32°C and 39°C, respectively. The TNF- α levels rose quickly after LPS-stimulation. Six hours after LPS-stimulation TNF- α levels were significantly higher at 39°C than at 32°C ($p < 0.001$). The TNF- α levels at 39°C peak at 12h and decay rapidly over the next 24h. In contrast, the rise of TNF- α at 32°C is delayed but peaks at 24h with only little decrease at 36h. The differences in TNF- α are significant at 24h and 36h between 32°C and 39°C ($p < 0.05$).

Interleukin-6 – IL-6

Changes in IL-6 were comparable to TNF- α . Similar to TNF- α , IL-6 increases rapidly at 39°C at 6h and is significantly higher than at 32°C (Figure 11). This initial response was very uniform among the different donors as indicated by the small error bars. At 12h and 24h, there was no difference between the two temperatures but the IL-6 levels at 32°C increased until the 36h time point whereas the IL-6 levels at 39°C peak at 24h and begin to decrease afterwards. The prolonged and increased secretion of IL-6 at 32°C led to significantly different IL-6 levels at 36h. Of note, there was considerable variation of IL-6 levels among the different subjects as indicated by the large error bars. Because every donor showed a similar influence of temperature on IL-6, the differences at 36h are statistically significant.

Secretion of Anti-Inflammatory Cytokine

Interleukin-10 – IL-10

Changes in the levels of IL-10 were reversed in comparison with TNF- α and IL-6 (Figure 11). IL-10 started to rise with very low levels at 6h at 39°C whereas essentially no IL-10 was detectable at 32°C. After 6h, the IL-10 levels rose notably at 39°C until they reached a peak at 24h followed by a slight decrease. In clear contrast to 39°C, IL-10 rose slowly at 32°C and never reached comparable levels. The IL-10 differences between the two temperatures are statistically highly significant at every time point (all $p < 0.001$).

Figure 11: TNF- α , IL-6 and IL-10 Over 36h at 32°C and 39°C

Legend Figure 11

Both pro-inflammatory cytokines show a comparable influence of temperature. At 6h, the levels of TNF- α and IL-6 are higher at 39°C. However, at the prolonged time points 24h and 36h, TNF- α and IL-6 are higher at 32°C.

In contrast, IL-10 is higher at 39°C at all time points. The IL-10 levels at 32°C never reach comparable levels to IL-10 at 39°C.

** indicates $p < 0.05$; data are presented as mean $\pm$ SEM*

DISCUSSION

Cytokines are important players in the immune response because they are the first response after recognition of a pathogen or tissue injury and this initial response is also the trigger of a sequence of events, which should result in clearance of the infection without damaging the host. Therefore, an inappropriate secretion of cytokines may result in detrimental effects on the host. An unbalanced, exaggerating immune response can induce tissue damage, shock, multiple organ dysfunction and ultimately death (75,186). An insufficient immune response is not able to contain the infection and would result in a first local than systemic spread of the pathogens resulting in an overwhelming infection.

Hypothermia has been shown to affect the secretion of cytokines (18,20,161). The available data of human cell cultures indicates that hypothermia diminishes the early response but results in a prolonged and increased production of pro-inflammatory cytokines. There is an on-going debate, which part of the response is clinically more important. Depending on the focus of the authors, some reports underline the diminished pro-inflammatory response in the early phase (20,178). Others believe that the prolonged and sustained secretion of pro-inflammatory cytokines is more important and will have long lasting effect on the ability of the host to fight infections. The data on anti-inflammatory cytokines is sparse. *In vitro* models demonstrate in general a decrease in IL-10 production in hypothermia (20,21).

I believe that the prolonged and increased production of pro-inflammatory cytokines such as TNF- α and IL-6 is more important for the long-term than the

initial reduction in pro-inflammatory cytokines in hypothermia. Exaggerated production of TNF- α can induce multiple organ failure and death (91). The more important effect of hypothermia in our results seems to be on the counter-regulatory part of the immune response, the anti-inflammatory phase. Administration of IL-10 can reduce almost all of the detrimental effects of TNF- α (97,98). We found that IL-10 was consistently higher at 39°C than at 32°C and believe that this result may be of greater importance and the lack of IL-10 at 32°C may influence the prolonged pro-inflammatory response. The value of an appropriate anti-inflammatory immune response has been demonstrated in several models, in which the exaggerated secretion of pro-inflammatory cytokines is the main pathophysiologic mechanism of shock and administration of IL-10 can rescue these animals (97,98). Others have found that IL-10 is the primary inhibitor of leukocyte influx in the peritoneum and that macrophages are the principle source, also indicating the importance of counter-regulatory mechanisms (188).

Taking together, these results suggest that hypothermia shifts the overall response pattern of the monocytes into a pro-inflammatory response with a prolonged and increased secretion of pro-inflammatory cytokines. At the same time, the natural negative feedback loop with IL-10 seems to be suppressed in hypothermia. In contrast, 39°C results in a reduced and shortened immune response with a strong induction of the anti-inflammatory IL-10.

CHAPTER V

ANALYSIS OF THE NF-K-B AND MAPK SIGNALING PATHWAYS AT DIFFERENT TEMPERATURES

Intracellular signaling pathways control the response of the cell by integrating signals from surface receptors as well as other information on cell homeostasis (109,121,123). Detection of pathogen associated molecules, the so-called PAMPs, by surface and intracellular PRR's results in a strong activation of stress response pathways. Two major stress response pathways are the NF- κ B and the MAPK-pathway. Both pathways activate the production of proteins, which increase the stress resistance of the cell such as heat shock proteins. The expression of these proteins is essential; otherwise the cell would not survive the insult (180,181,189). In addition to supporting the survival of the cell, heat shock proteins also serve as endogenous danger signals for the immune system (alarmins) amplifying the immune response (77). The second goal of the activation of the signaling pathways is to orchestrate the response of the whole body in order to eliminate the invading pathogen. Pro-inflammatory cytokines have local effects, which result in the extravasation of immune cells but also systemic effects such as fever and the initiation of the acute phase response. All of these measures enhance the ability of the host to defend it.

Modulation of the activation of these pathways has therefore a profound effect on the response of the cell. Temperature has been shown to affect pathway activation profoundly (23,160,177). In human monocytic cell lines, NF- κ B activation was found to be increased and prolonged in hypothermia (19,23). In human endothelial cells, JNK activation was decreased in hypothermia leading to decreased apoptosis (177). The data on endothelial cells cannot necessarily be directly adapted to monocytes because the stimuli for the cells are different and also so is the response pattern to stress. To our knowledge, there is no data available on pathway activation under hypothermic conditions in primary human monocytes.

Based on the previous results with highly distinct cytokine secretion between 32°C and 39°C, we hypothesize that the activation of the NF- κ B and MAPK-pathway is different. A prolonged activation of the pro-inflammatory pathways, NF- κ B, p38 and JNK would explain the prolonged TNF- α secretion at 32°C. The strong production of IL-10 at 39°C might also be explained by differences in pathway activation. The mechanism, which lead to the production of IL-10 are poorly understood and vary greatly among different cell types (99). However, the available data suggests that the Erk-pathway is pivotal for the induction of IL-10 in macrophages (99).

The purpose of this experiment was to assess the activation of both, the NF- κ B and MAPK pathway under hypothermic conditions (32°C) in the same cell at the same time and compare it to 39°C. Measuring the activation of these

pathways in the same experiment and same primary cells reduces the confounding factor of repetitive experiments and the associated variation.

RESULTS

Activation of the NF- κ B pathway

NF- κ B was rapidly activated peaking 15min after LPS stimulation in both temperatures (Figure 12). After 15min, there was a decrease in NF- κ B activation until 2h at both temperatures. The degree of activation of NF- κ B at 32°C was significantly higher at 15min than at 39°C. This difference in activation remained over the whole investigated period. NF- κ B was increased and its activation was sustained at 32°C at every measured time point. Of note, NF- κ B remained activated at 32°C at 2h, whereas at 39°C, the degree of activation reached baseline values.

Activation of p38 of the MAPK-pathway

Similar to NF- κ B, p38 was also maximally activated after 15min followed by a decrease in activation until 2h (Figure 12). P38 reached baseline levels at 2h at 39°C but remained slightly elevated in hypothermia. Hypothermia also increased and prolonged the activation of p38. There was no effect of temperature on 15min; but at 30min, 45min, 1h and 2h, p38 was stronger and longer activated at 32°C than at 39°C. The activation of p38 reached baseline

levels at 39°C after 45min, whereas p38 remained active at the 2h time point at 32°C.

Activation of JNK of the MAPK-pathway

Figure 12 outlines the activation of JNK over time. at 39°C, JNK activation peaked at 15min followed by decay in activation and the baseline was reached after 45min, very comparable to p38. This is in contrast to JNK-activation at 32°C. The peak activation was recorded at 30min instead of 15min resulting again in an increased and prolonged activation in hypothermia. Similarly to p38, there was no effect of temperature on the activation at 15min and at 2h. JNK remained slightly activated at 2h in hypothermia.

Activation of Erk of the MAPK-pathway

While the activation of NF- κ B, p38 and JNK of the MAPK-pathway was comparable with increased and prolonged activation under hypothermic conditions, Erk-activation is different (Figure 13). The peak activation at 39°C was at 15min followed by a gradual decrease. The baseline was reached after 1h. The peak activation of Erk at 32°C never reached the same level as at 39°C. There was a clear activation at 15min, but it plateaued after 30min and remained elevated in comparison with 39°C at 30min, 45min and 1h. The baseline was reached after 2h in both temperatures.

Figure 12: Activation of the NF- κ B, p38 and JNK

Legend Figure 12

Hypothermia has the strongest effect on NF- κ B activation. The increase in activation at 32°C is five-fold and remains 2.2-fold increased 2h after initial stimulation.

The peak activation is not influenced by temperature. Hypothermia prolongs the activation of p38.

JNK is the only pathway reaching peak activation at 30min at 32°C but is also prolonged and increased activated.

MCF: Mean Channel Fluorescence

** indicates $p < 0.05$; data are presented as mean $\pm$ SEM*

Figure 13: Activation of Erk of the MAPK-pathway

Erk is the only pathway which was activated to a higher level at 39°C than under hypothermic conditions.

** indicates $p < 0.05$; data are presented as mean $\pm$ SEM*

DISCUSSION

The activation of inflammatory signaling pathways is an important step in monocyte response and in the initiation and orchestration of the immune response. Our data demonstrate that hypothermia increases and prolongs the activation of pro-inflammatory signaling pathways, namely NF- κ B, p38 and JNK, over the whole 2h investigation period. This is in line with results from Fairchild et al. (19,23). In contrast, the degree of activation of Erk is higher at 39°C at 15min and this level of activation is not reached at 32°C. However, Erk activation remains prolonged and increased after 30min – 1h in hypothermia as it is the case with the three other pathways.

The exact biologic effect of these differences in pathway activation is difficult to assess. All of these pathways induce the production of pro-inflammatory cytokines such as TNF- α , IL-6 and others (109,126,190). NF- κ B binds promoter sites of several pro-inflammatory cytokines resulting in their production. In addition, NF- κ B increases cell survival and proliferation. P38 and JNK also induce the production of pro-inflammatory cytokines, but they can induce apoptosis as well as growth and differentiation of the cell. The Erk-pathway is proposed to be more involved in control of growth and differentiation of cells than production of inflammatory cytokines. However, Erk contributes significantly to the production of pro-inflammatory cytokines in monocytes (126,190). Other data suggests that the Erk-pathway is pivotal in the initiation and also the amount of IL-10 production (99). The regulation of IL-10 is a complex process and the ability to produce as well as the mechanisms leading to

IL-10 production varies even among closely related cells such as monocytes, macrophages and dendritic cells. The available data so far suggest that Erk is a central regulator of IL-10 production. However, NF- κ B is also able to initiate the production of IL-10 but only after stimulation of the TLR-receptor.

Some of the actions are obviously contradictory and the end result of the pathway activation depends upon complex interactions between different pathways and also the transcription factors they activate and the genes they express. These data also suggest that the production of pro-inflammatory cytokines and therefore the ability to respond to trauma and inflammation is highly redundant. The knock-out of some of the MAPK-proteins does not result in reduced production of pro-inflammatory cytokines whereas knock-out mice for p38 or NF- κ B were not viable (191).

Applying this information to our results, the increased and prolonged activation of NF- κ B, p38 and JNK in hypothermia would explain the prolonged secretion of TNF- α and IL-6 in our model. The more distinct difference in IL-10 production is more difficult to explain. We believe that the increased activation of Erk at 15min at 39°C is responsible or contributes significantly to the production of IL-10. The fact that Erk-activation at 32°C never reaches comparable levels than at 39°C would fit well into our explanation because Kaiser et al. demonstrated that the degree of Erk activation correlates with the degree of IL-10 production (192).

The next step after identification of these significant differences in pathway activation is to determine if the increased and prolonged activation of the pro-inflammatory signaling pathways result in differences in gene expression. We propose that the prolonged activation of NF- κ B, p38 and JNK results in a prolonged production of pro-inflammatory cytokine genes as well as pro-inflammatory microRNAs. The higher degree of Erk-activation should result in a high degree of IL-10 gene expression.

CHAPTER VI

INFLUENCE OF TEMPERATURE ON EXPRESSION OF CYTOKINE GENES AND MICRORNA – 155

Activated signaling pathways activate transcription factors which initiate the transcription of genes with the appropriate promoter sequence. The three different arms of the MAPK-pathway are able to activate several different transcription factor or different subunits, which then associate with each other in order to induce gene transcription. NF- κ B itself is a transcription factor and induces gene expression independently without the use of other transcription factors. In the previous experiment we found a profound impact of temperature on activation of both the NF- κ B and MAPK-pathway. Hypothermia increased and prolonged the activation of NF- κ B, p38 and JNK, whereas the peak activation of Erk was diminished but still prolonged. Consequently, we determined if these differences result in increased expression of mRNA of target genes of these pathways such as TNF- α and IL-10 and if temperature demonstrates the expected effect.

MicroRNAs are also transcribed like cytokine genes from DNA to RNA and also depend upon the activation of transcription factors. MiRNA-155 expression is induced by NF- κ B (149,193). Furthermore, the MAPK-pathway has also been implicated in the induction of miRNA-155 (194-196).

Therefore, the increased and prolonged activation of both signaling pathways should also have an effect on the expression of miRNA-155. We hypothesize that miRNA-155 is increased and has prolonged expression in hypothermia. Furthermore, a significant effect of temperature on miRNA-155 may also result in expression changes of the miRNA-155 target, SHIP-1.

RESULTS

Effect of Temperature on Cytokine-Gene Expression

Expression of mRNA precedes the production and secretion of proteins. Therefore, we chose to investigate earlier time points for this experiment than for the protein secretion. We found that the induction of TNF- α mRNA occurred quickly within 3h after LPS stimulation at both temperatures (Figure 14). The mRNA-expression of TNF- α peaked at 39°C at 6h with a 22-fold increase followed by a steep drop to a 2.5 fold increase compared with baseline. Unlike 39°C, TNF- α mRNA expression increased over the whole observation period in hypothermia reaching comparable levels of fold change.

Our observation show that the induction of IL-10 mRNA begins at 3h and peaks between 12h and 24h in both temperatures (Figure 14). The fold-change of IL-10 mRNA in general was much higher compared to TNF- α , but was again higher at 39°C than at 32°C. The peak fold change at 39°C was >1100 fold at 12h, whereas 32°C reached 133-fold at the same time. The peak induction in

hypothermia appeared to be at 24h. The differences in IL-10 mRNA expression are significant between the two temperatures at 3h, 6h and 12h.

These changes in mRNA-expression in TNF- α and IL-10 are likely to be responsible for the observed differences in secreted TNF- α and IL-10. The prolonged up-regulation of TNF- α mRNA at 12h and 24h results in an increased production of TNF- α protein at 32°C at 24h and 36h (Figure 11). Conversely, the strong up-regulation of IL-10 mRNA starting at 3h is translated into IL-10 protein and secreted starting at 6h at 39°C. Although the up-regulation of IL-10 mRNA at 32°C is less pronounced than at 39°C, the difference in secreted IL-10 protein is much larger than the difference in gene expression would suggest. This discrepancy may be explained by additional mechanisms such as translational inhibition or an inhibition of IL-10 protein secretion.

Effect of Temperature on miRNA-155 Expression

The expression of miRNA-155 over time is shown in Figure 15. Similar to the cytokines, miRNA-155 expression was induced early and a 10-fold up-regulation can be observed at 6h in both temperatures. The peak expression in both temperatures occurred at 12h. MiRNA-155 remains up-regulated on a higher level, about 20-fold, in hypothermia at 24h and 36h, whereas the expression decreased to levels below 10-fold at 39°C. The expression difference at 24h and 36h was significant between 32°C and 39°C.

Effect of Temperature and miRNA-155 on SHIP-1 Expression

The expression of SHIP-1 is shown in Figure 16. SHIP-1 mRNA is down-regulated over the 36h of the study period. At 6h and 12h, SHIP-1 mRNA is stronger down-regulated at 39°C. At the later time points, at 24h and 36h, SHIP-1 mRNA remains down-regulated at 32°C the amount of down-regulations decreases at 39°C. The differences between the two temperatures are significant at all time points except at 12h.

Figure 14: Expression of TNF- α and IL-10 mRNA at 32°C and 39°C

The induction of TNF- α mRNA was more rapid but less pronounced at 39°C; the 32°C TNF- α mRNA is consistently and increasingly up-regulated within the first 24h. IL-10 mRNA is strongly induced within 3h at 39°C. The IL-10 up-regulation in hypothermia is delayed but ultimately reaches comparable levels at 24h. Of note, the degree of IL-10 mRNA up-regulation was high in both temperatures.

* indicates $p < 0.05$; data are presented as mean $\pm$ SEM

Figure 15: Expression of miRNA-155 at 32°C and 39°C

The expression changes in miRNA-155 parallel changes in TNF- α mRNA. The impact of temperature occurred at later time points (24h and 36h).

** indicates $p < 0.05$; data are presented as mean $\pm$ SEM*

Figure 16: Expression of SHIP-1 mRNA at 32°C and 39°C

SHIP-1 mRNA is initially stronger down-regulation at 39°C followed by an up-regulation compared to 32°C. The down-regulation of SHIP-1 allows a stronger activation of the TLR-pathway leading to an increased secretion of pro-inflammatory cytokines. These expression changes explain the initially higher levels of TNF- α and IL-6 at 39°C but also the prolonged and increased secretion of TNF- α /IL-6 at 32°C. Furthermore, these results are concurrent with the expression changes of miRNA-155 of which SHIP-1 is a target. The higher miRNA-155 levels in hypothermia at 24h and 36h correlate with a stronger suppression of SHIP-1 mRNA at 32°C at these two time points.

** indicates $p < 0.05$; data are presented as mean $\pm$ SEM*

DISCUSSION

The induction of gene expression is the presumed next step in the monocyte response to an infection after activation of the signaling pathways. Gene expression serves several purposes. First, after the secretion of preformed cytokines during the immediate response, new cytokines have to be produced in order to replenish the secreted cytokines but also to maintain the communication between immune cells and orchestration of the immune response. Second, the inhibition and modulation of the initial pathway activation has to be induced. For this purpose, phosphatases are induced, which de-phosphorylate the activated proteins of the TLR-signaling complex such as IRAK-1/2 and TRAF-6. SHIP-1 and SOCS-1 are two such examples of these up-stream inhibitors of the TLR-signaling pathway (119,120,145). In addition, the down-stream signaling pathways are also inhibited by phosphates. Especially the MAPK-pathway is highly regulated by inhibitors such as the DUSP's (127-129). Besides the inhibition of the signaling process, fine-tuned production of inhibitors allows also the modulation of the response by selective inactivation of one or several arms of the MAPK-pathway. Lastly, the pathway proteins are also reproduced and the expression of some of these pathways can change upon cell activation in order to maintain or diminish the response to the infection.

We investigated the expression of cytokine genes in order to determine if the reported differences in pathway activation result in differences in gene expression. We found that TNF- α is increased and has prolonged expression at 32°C; this can be explained by the prolonged activation of the pro-inflammatory

signaling pathways. Moreover, the sustained up-regulation of TNF- α mRNA at 12h and 24h correlates with the prolonged secretion of TNF- α protein at 24h and 36h. at 39°C, the induction of IL-10 mRNA is stronger and higher than under hypothermic conditions. We believe that the higher degree of Erk activation is responsible or contributes strongly to this observation. The IL-10 mRNA up-regulation correlates with the high IL-10 levels measured at 39°C. Although the IL-10 mRNA induction is not as pronounced in hypothermia as at 39°C, there is still a much stronger up-regulation of IL-10 mRNA (>800-fold) at 32°C compared to TNF- α (20-fold) indicating inhibition of IL-10 protein production and/or modulation of IL-10 protein secretion.

Besides the cytokine mRNA, miRNA-155 is also prolonged expressed in hypothermia. Since the expression of miRNA-155 is controlled by NF- κ B and probably also by p38 and JNK, the prolonged activation of these pathways is the likely cause of the increased and prolonged expression of miRNA-155 in hypothermia. Furthermore, the temperature related differences in miRNA-155 expression may explain the observed increased and prolonged secretion of TNF- α protein at 32°C. MiRNA-155 was one of the first microRNAs, which was shown to respond to inflammatory stimuli (196). Early on, an association with TNF- α was observed. The causal link between increased miRNA-155 and increased TNF- α was subsequently demonstrated by several groups (139,145,147). However, miRNA-155 has also been shown to have anti-inflammatory properties by inhibiting the expression of MyD88, the central signaling protein of TLR-4, and TAB2, an important signaling molecule of the NF- κ B and MAPK-pathway

(140,148). While the pro-inflammatory effect of miRNA-155 with an increase in TNF- α production can be observed within 24h, the suppressive effect on MyD88 requires at least 48h – 72h to be effective. Therefore, the timing of miRNA-155 expression is pivotal in order to determine the biologic effect.

SHIP-1 is one of the main targets by which miRNA-155 increases the production of pro-inflammatory cytokines (139,145,197). As expected, SHIP-1 mRNA is strongly down-regulated upon activation of the TLR-pathway. At 6h, the down-regulation of SHIP-1 is stronger at 39°C correlating with increased secretion of TNF- α and IL-6; the prolonged and strong down-regulation of SHIP-1 mRNA at 32°C at 24h and 36h explains also the prolonged and increased levels of TNF- α and IL-6 in hypothermia. Furthermore, the differences in SHIP-1 mRNA between 32°C and 39°C correlate with the expression changes of miRNA-155 indicative of a miRNA-155 induced degradation of SHIP-1 mRNA. The increased expression of miRNA-155 at 32°C lead to the degradation of SHIP-1 mRNA, whereas the decreased levels of miRNA-155 allow up-regulation of SHIP-1 mRNA at 39°C.

Based on these reports, we propose that the prolonged up-regulation of miRNA-155 is responsible for the prolonged TNF- α production in hypothermia (Figure 17). Increased and prolonged activation of NF- κ B, p38 and JNK induces a strong expression of miRNA-155. The reduced expression of the two TLR-pathway inhibitors SHIP-1 and SOCS-1 at 32°C increases pathway activation and results in prolonged production of TNF- α mRNA, which is then translated into TNF- α protein and secreted.

Figure 17: The Increased and Prolonged miRNA-155 Expression Contributes to the Prolonged and Increased TNF- α -Production

A prolonged up-regulation of the miRNA-155 results in a reduced expression of two TLR-4 inhibitors (SHIP-1/SOCS-1), which in turn increases and prolongs the activation of the TLR-4 signaling pathway including NF- κ B and the MAPK-pathway. The increased signaling of the TLR-4 pathway increases the production of pro-inflammatory cytokines such as TNF- α and IL-6, which then are translated into cytokine proteins.

CHAPTER VII

MiRNA-155 IS RESPONSIBLE FOR THE PROLONGED TNF- α SECRETION

In previous experiments, we found prolonged elevated levels of miRNA-155 in hypothermic conditions, which correlated with the prolonged expression of TNF- α mRNA and protein. Based on available literature we propose that the increased and prolonged expression of miRNA-155 is responsible for the sustained TNF- α production at 32°C (Figure 17). Two main targets of miRNA-155, SHIP-1 and SOCS-1 are negative regulators of the TLR-4 signaling pathway (139,145,149). An inhibition of the translation of SHIP-1 and SOCS-1 by miRNA-155 increases the signaling of the TLR-4 pathway and results in a prolonged expression of TNF- α mRNA, which is subsequently translated into TNF- α protein and secreted. Our results show an expression change of SHIP-1 mRNA concurrent with an inhibitory effect of miRNA-155 at 32°C at 24h and 36h.

However, miRNA-155 has also been shown to target other proteins such as MyD88 or TAB2, which results in an anti-inflammatory function for miRNA-155 (140,148). The time course of our data suggests that miRNA-155 has probably pro-inflammatory functions in our results because the time needed for miRNA-155 to develop anti-inflammatory properties is usually more than 24h – 48h. This can be explained by the targeted proteins: SHIP-1 and SOCS-1, the two proteins we propose as targets of miRNA-155 in our model, are newly expressed upon

stimulation of the TLR-4 pathway, whereas MyD88 and TAB2 are already expressed. MicroRNAs can only inhibit the production of proteins and do not have any effect on already expressed proteins. Therefore, up-regulation of miRNA-155 will immediately inhibit the production of SHIP-1 and SOCS-1 and will also consequently have an immediate effect on the TLR-4 pathway, e.g. prolong its activation. This is in contrast to MyD88 and TAB2. Both proteins are pre-existing in the cell and without their presence, the activation of the TLR-4 pathway would be impaired. These pre-existing proteins are not immediately affected by up-regulation of miRNA-155. However, the re-expression of the proteins will be affected resulting in a slow decrease of MyD88 and TAB2. Ultimately, the reduced amount of these two proteins will diminish the signaling through the TLR-4 pathway. Taken these two actions together, it appears that miRNA-155 has a dual role; first as a pro-inflammatory microRNA but at the same time, it acts as a delayed inhibitor of the inflammatory response.

The purpose of the next experiment was to confirm the pro-inflammatory role of miRNA-155 in our experiments and that the prolonged expression of miRNA-155 in hypothermia is responsible for the prolonged TNF- α production. Furthermore, successful modulation of TNF- α secretion by manipulation of miRNA-155 expression might also offer potential therapeutic applications. For this purpose, we modulated the expression of miRNA-155 in the primary monocytes with microRNA-mimics and microRNA-antagomirs.

RESULTS

Over-expression and Suppression of miRNA-155

The transfection of the primary monocytes was successfully accomplished. Figure 18 shows the miRNA-155 expression levels. The mimics strongly over-expressed miRNA-155 whereas the antagomirs suppressed miRNA-155 compared to the with negative control transfected monocytes. In average, the mimics resulted in a >100-fold up-regulation of miRNA-155 and the antagomirs reduced the expression about 3-fold. All of these changes were statistically significant.

Effect of miRNA-155 on TNF- α at 32°C and 39°C

The effect of miRNA-155 on TNF- α is outlined in Figure 19. Independent of the temperature, over-expression of miRNA-155 resulted in significantly increased levels of TNF- α . The effect of miRNA-155 up-regulation seems to be more pronounced at 32°C than at 39°C. Transfection with miRNA-155-mimics at 39°C increased TNF- α to higher levels as in the negative control transfected cells at 32°C. It is therefore possible to make “39°C look like 32°C”.

The suppression of miRNA-155 expression had only a significant effect on TNF- α levels at 32°C. Suppression of miRNA-155 expression reduced TNF- α to slightly lower levels than the negative control at 39°C. Thus, suppression of

miRNA-155 “makes 32°C look like 39°C”. A further knock-down of miRNA-155 expression at 39°C did not translate into significantly lower TNF- α levels.

Effect of miRNA-155 on IL-6

The effects of modulation of the expression of miRNA-155 on IL-6 are shown in Figure 20. The changes of IL-6 expression changes were similar to the one observed in TNF- α . The over-expression as well as the suppression of miRNA-155 in the monocytes in both temperatures had a significant effect on IL-6 expression, similarly to the effects observed in TNF- α . In addition, suppression of miRNA-155 expression had significant effect on IL-6.

Effect of miRNA-155 on IL-10

Manipulation of miRNA-155 expression did not affect the IL-10 levels consistently (Figure 21). Suppression of miRNA-155 at 39°C reduced IL-10 levels significantly. However, at 32°C, there was a trend to higher IL-10 levels in the antagomir-transfected monocytes. However, these effects were not consistent among the different donors.

Effect of miRNA-155 on SHIP-1 mRNA

Over-expression of miRNA-155 significantly reduced the expression of SHIP-1 mRNA whereas suppression of miRNA-155 expression increased SHIP-1 mRNA (Figure 22).

Figure 18: Successful Over-expression and Suppression of miRNA-155

Transfection with miRNA-155-mimic led to a significant over-expression of miRNA-155 in both temperatures. Similarly, the use of antagomirs suppressed the expression of miRNA-155 in both temperatures. Note the logarithmic scale of the fold change.

** indicates $p < 0.05$; data are presented as mean $\pm$ SEM*

Figure 19: Effect of miRNA-155 on TNF- α

Manipulation of miRNA-155 expression showed the expected results. Over-expression of miRNA-155 increased TNF- α at 32°C and 39°C whereas the suppression only significantly reduced the expression of TNF- α at 32°C.

** indicates $p < 0.05$; data are presented as mean $\pm$ SEM*

Figure 20: Effect of miRNA-155 on IL-6

Modulation of miRNA-155 expression had a significant effect on IL-6 in both temperatures similarly to TNF- α . In addition, suppression of miRNA-155 at 39°C reduced IL-6 expression significantly.

** indicates $p < 0.05$; data are presented as mean $\pm$ SEM*

Figure 21: Effect of miRNA-155 on IL-10

Effect of MiRNA-155 on Interleukin-10

Only suppression of miRNA-155 at 39°C had a significant effect on IL-10.

** indicates $p < 0.05$; data are presented as mean $\pm$ SEM*

Figure 22: Effect of miRNA-155 on SHIP-1 mRNA

Over-expression of miRNA-155 suppresses SHIP-1 mRNA expression. A reduction of miRNA-155 expression increases the expression of SHIP-1 mRNA.

* indicates $p < 0.05$; data are presented as mean $\pm$ SEM

DISCUSSION

MiRNA-155 can have both pro- and anti-inflammatory effects during the monocyte response depending on the target and the time since the stimulation. Initially, miRNA-155 has pro-inflammatory properties and increases the activation of the TLR-4 pathway including the NF- κ B and MAPK pathways. This effect is due to the inhibition of production of SHIP-1 and SOCS-1; both proteins inhibit the TLR-4 signaling complex upstream. We found expression changes of SHIP-1 mRNA which correlate with an inhibitory effect of miRNA-155 at 24h and 36h. At a later time point, miRNA-155 reduces the signaling through the TLR-pathway by inhibition of re-expression of MyD88, the central signaling protein of TLR-4 and most other TLR's, and reduction of TAB2, a protein, which is an essential co-factor in the activation of TAK1. TAK1 is able to activate both the NF- κ B and MAPK-pathway.

We proposed that TNF- α secretion is prolonged in hypothermia because of increased and prolonged expression of miRNA-155. We show here that miRNA-155 is indeed responsible for the prolonged TNF- α secretion. Over-expression of miRNA-155 at 39°C increased TNF- α to slightly higher levels than those of monocytes transfected with negative controls at 32°C making "39°C look like 32°C". Of note, over-expression of miRNA-155 in hypothermia increased TNF- α further. The converse was also found to be true: suppression of miRNA-155 at 32°C reduced TNF- α to slightly lower levels than in the negative control of 39°C; thus, making "32°C look like 39°C". Interestingly, a further suppression of miRNA-155 at 39°C did not translate into significantly lower TNF- α levels, which

suggests that miRNA-155 is expressed at a very low level and probably inactive. In summary, modulation of miRNA-155 expression is able to abolish the effects of temperature on cytokine secretion. We confirmed the pro-inflammatory action of miRNA-155 by analyzing IL-6 production. Similar to TNF- α , over-expression of miRNA-155 increased IL-6 in both temperatures, whereas suppression of miRNA-155 reduced IL-6 expression in both temperatures. In addition, we show that these effects of miRNA-155 are likely to be mediated by SHIP-1, a well described target of miRNA-155. Over-expression of miRNA-155 suppresses SHIP-1 mRNA expression at both temperatures; reduction of miRNA-155 expression with miRNA-155 antagomirs increases the expression of SHIP-1 mRNA. These observations are in line with results of Kurowska-Stelarska et al. (145).

The effects of miRNA-155 on IL IL-10 are less clear. The effect of miRNA-155 manipulation on IL-10 was not consistent. Suppression of miRNA-155 at 39°C reduced IL-10 significantly. In contrast, there was a slight trend for higher IL-10 levels in miRNA-155 antagomir transfected cells at 32°C. Thus suggesting that temperature may influence the effect of microRNAs on an additional level independent of miRNA-155. This result contrasts with the report by Kurowska-Stelarska et al., who found that over-expression of miRNA-155 reduces IL-10 (145). The reasons for these differences remain unclear. Both experiments were conducted with primary human monocytes, which were freshly isolated by the same technique. In addition, the transfection reagent was also the same; the only difference was the source of the microRNA mimics, antagomirs and negative

controls. Further research should be directed to answer this question. We hypothesize that temperature may influence other pathways which results in these inconsistent effect of miRNA-155 on IL-10. Alternatively, the production of other microRNAs, which were not investigated in our research or other mechanisms directing the production of IL-10, may be strongly affected by temperature.

In summary, we demonstrate here that miRNA-155 is instrumental in the prolonged expression of TNF- α and that effect is likely to be mediated by suppression of SHIP-1. Furthermore, modulation of miRNA-155 expression is able to reverse the production of pro-inflammatory cytokines. These observations offer possibilities for therapeutic interventions. However, the effect of changes in miRNA-155 expression of IL-10 secretion and the influence of temperature on this process warrant further investigations.

CHAPTER VIII

IL-10 SUPPRESSES MIRNA-155 AND IS AN IMPORTANT NEGATIVE FEEDBACK LOOP

We have shown in the previous experiment that miRNA-155 is responsible for the prolonged up-regulation of TNF- α under hypothermic conditions. However, the regulation of miRNA-155 expression is not entirely elucidated. The transcription of miRNA-155 is initiated by NF- κ B and the MAPK-pathway upon stimulation of the monocytes with LPS (149,193,194,196). As Figure 15 indicates, there is no difference in miRNA-155 induction between 32°C and 39°C in the first 12h. After 12h miRNA-155 expression declines at 39°C and remains at a lower level over the remaining study period. This difference in miRNA-155 could be explained by the prolonged activation of NF- κ B, p38 and JNK in hypothermia. Since the expression is not different in the first 12h, it might as well be that other factors contribute or are mainly responsible for the observed difference in miRNA-155 expression after 12h. It has been shown that the expression of miRNA-155 is not only induced by ligands of the TLR-system but also by TNF- α (196). This is not surprising considering that the TLR-4 and the TNF-receptor share several of the intracellular signaling proteins such as IRAK-1/2 and TRAF-6. Also, the TNF- α receptors utilizes the NF- κ B and MAPK pathway. IL-10 has well described anti-inflammatory properties by suppressing

the production of TNF- α and other pro-inflammatory cytokines directly (100). McCoy et al. demonstrated that IL-10 also suppresses miRNA-155 (198). We found in our experiment that the difference in IL-10 secretion is one of the most pronounced differences between 32°C and 39°C, especially at the 12h time point (Figures 11). Based on the literature and our own data, we hypothesized that IL-10 may be the principle regulator of miRNA-155 expression as part of a negative feedback loop. In this feedback loop, IL-10 would not only reduce the production of pro-inflammatory cytokines directly, it would also reduce the signaling through the TLR-pathway by increasing the inhibitors SHIP-1 and SOCS-1 and reduce the expression of miRNA-155; thereby reinforcing the anti-inflammatory effect.

To delineate the role of IL-10 on miRNA-155 and subsequently TNF- α production we altered IL-10 action in the monocyte culture. For this purpose, we either added additional IL-10 or blocked the IL-10-Receptor. Adding additional IL-10 to the 32°C culture would imitate conditions found at 39°C. Blocking the IL-10-receptor at 39°C abolishes the action of IL-10 and would imitate conditions found at 32°C. The demonstration of the biologic relevance of this feedback loop would indicate that microRNA's are not only regulators of protein production they are also controlled by proteins. A better understanding of such feedback loops may allow the targeted modulation of the cell response.

RESULTS

Addition of IL-10 to the Monocyte Culture at 32°C

Addition of IL-10 decreased the TNF- α production significantly at 12h and 24h to half the TNF- α concentration of the untreated 32°C samples (Figure 23). Additional IL-10 was also able to suppress the expression of miRNA-155 at 32°C at 12h and 24h although only the difference at 24h was statistically significant (Figure 23). We chose to add IL-10 4h after the initial stimulus with LPS because IL-10 is not produced immediately. The 4h time point was chosen based on preliminary findings that IL-10 starts rising around 4h.

Blockade of IL-10-Receptor at 39°C

Blockade of the IL-10-Receptor with a specific antibody to the α subunit of the IL-10-Receptor increased the TNF- α protein levels at 39°C at 12h and 24h significantly (Figure 24). In line with these results, blockade of the IL-10-Receptor did increase miRNA-155 expression at 12h and 24h (Figure 24). As with the addition of IL-10 experiment, there was only a statistically significant effect on miRNA-155 expression at 24h.

Figure 23: Effect of Additional IL-10 on TNF- α and miRNA-155 at 32°C

Addition of rhIL-10 effectively suppresses TNF- α protein secretion and miRNA-155 production. In contrast to TNF- α , the suppressive effect of IL-10 occurs only after 24h but probably lasts due to the prolonged action of miRNA-155 also longer. RhIL-10 was added 4h after initial stimulation with LPS.

* indicates $p < 0.05$; data are presented as mean $\pm$ SEM

Figure 24: Effect of IL-10 Receptor Blockade on TNF- α and miRNA-155 at 39°C

Blockade of the IL-10-Receptor with anti-IL-10-Receptor antibodies effectively blocked the action of IL-10 and increased TNF- α protein secretion and miRNA-155 expression at 24h. Comparable to the addition of IL-10 at 32°C, the effect of the IL-10-Receptor blockade on miRNA-155 takes more time but will probably also last longer due to the action of miRNA-155.

* indicates $p < 0.05$; data are presented as mean $\pm$ SEM

DISCUSSION

A tight regulation of the inflammatory response is pivotal for the host in order to mount an appropriate response to defend the host but also important in order to prevent excessive damage with potential fatal complications. The understanding of the various regulatory mechanisms would potentially allow for targeted interventions. IL-10 is a potent and well described negative regulator of the immune system (97,98,100). It has been shown that administration of IL-10 in mice with endotoxemic shock and peritonitis abolishes the shock symptoms and increases survival (97,98). One of the mechanisms by which IL-10 executes its anti-inflammatory properties is by blocking the transcription of pro-inflammatory cytokines such as TNF- α (100). The suppressive effect of IL-10 is not limited to cytokine genes; IL-10 also reduces the expression of miRNA-155 as we and Sheedy et al. have shown (198).

We showed here that IL-10, which is the most pronounced difference between 32°C and 39°C, suppresses miRNA-155, resulting in an increased expression of SHIP-1 and SOCS-1 and therefore an inhibition of the TLR-4 signaling pathway, ultimately reducing TNF- α levels at 39°C. We believe that we have demonstrated the causality of IL-10 by blocking the action of IL-10 at 39°C, which resulted in an increase of miRNA-155 and TNF- α , thus, “making 39°C look like 32°C”. The opposite was also shown: administration of IL-10 after 4h of LPS stimulation at 32°C suppressed miRNA-155 and TNF- α . IL-10 administration makes “32°C look like 39°C”. We believe that we show here convincingly that IL-10 is part of an important negative feedback loop in

regulation of the monocyte response. In this negative feedback loop, IL-10 suppresses miRNA-155 and the reduced expression of this microRNA results in an increased inhibition of the TLR-4 signaling pathway followed by a subsequent decrease of TNF- α at 39°C. However, we do not believe that the reduced miRNA-155 expression is exclusively responsible for the decrease in TNF- α . The direct suppression of IL-10 on the transcription of TNF- α is an important feature of the negative feedback loop. Our data indicate that reduced expression of miRNA-155 becomes effective at the 24h time point but has long lasting effects. The addition of IL-10 as well as the block of the IL-10-Receptor had only effects on miRNA-155 at 24h, whereas TNF- α was suppressed already at 12h time point (Figures 23 and 24). These findings suggest that the suppression of miRNA-155 is responsible for long lasting anti-inflammatory effects whereas direct suppression of TNF- α production is immediately effective.

Based on our results and the available literature, it seems that IL-10 acts on several levels of the immune response. It reduces the secretion and production of pro-inflammatory cytokines such as TNF- α but also immune-modulatory cytokines, which enhance phagocytosis and antigen-presenting capability of macrophages such as IFN- γ and GM-CSF. The reduced expression of pro-inflammatory cytokines reduces the shock symptoms and the acute phase response. Furthermore, suppression of the antigen presentation reduces the activation of the adaptive immune system, which also produces pro-inflammatory cytokines. In addition, IL-10 modifies also microRNA-expression in monocytes. This action is important because microRNAs have long-lasting effects within the

cells by modulation of pathways, which will alter the response of the cell when a subsequent pathogen is encountered. The mechanism, by which IL-10 impairs the antigen presentation process are not completely elucidated. However, it would not be surprising, if microRNAs are involved in this process and that these microRNAs are regulated by IL-10.

In summary, the actions of IL-10 are not limited to one site of action; IL-10 effectively shuts down several pivotal points of the immune response: reduction of pro-inflammatory cytokines, reduced stimulation of cells of the adaptive immune system and modulation of intracellular signaling pathways by microRNAs. We propose here that the high levels of IL-10 at 39°C contribute significantly to the reduced expression of miRNA-155 resulting in reduced TNF- α (Figure 25).

Figure 25: Proposed Role of IL-10 in Suppression of miRNA-155 Expression and Subsequent Effect on TNF- α

IL-10 suppresses TNF- α directly at the 12h time point. In addition, IL-10 suppresses miRNA-155, which increases the production and secretion of TNF- α . The reduced expression of miRNA-155 would explain the reduced TNF- α production at 39°C. The lack of IL-10 at 32°C results in sustained miRNA-155 expression and subsequently prolonged TNF- α levels.

CHAPTER IX

CONCLUSIONS AND OVERVIEW

Hypothermia is a major risk factor for complications and mortality in elective and emergency surgery. Hypothermic patients are at an increased risk for wound infections and have a 3-fold higher mortality than non-hypothermic patients (2). Especially infection associated complications are more frequent in hypothermic patients but also cardio-vascular complications are increased (7,31,167).

Previous work has shown that monocytes play an important role in host defense as the first line by recognizing invading pathogens and orchestrating the immune response (54,62). It has also been demonstrated that there is prolonged secretion of pro-inflammatory cytokines and reduced IL-10 production in hypothermic monocytes (18-21,23,161). The purpose of this project was to investigate the underlying cellular mechanisms, which may explain these changes in cytokines secretion. A summary of our results are outlined in Figure 26. We found that hypothermia prolongs the activation of pro-inflammatory signaling pathways including NF- κ B, p38 and JNK. In contrast, Erk, a pathway which is pivotal for the production of IL-10, has increased activation at 39°C. These changes in pathway activation translate to an increased and prolonged up-regulation of TNF- α mRNA at 32°C. At 39°C, IL-10 mRNA

production is highly induced and is translated into IL-10 protein. The IL-10 levels in the hypothermic monocyte culture are very low. MiRNA-155 is also up-regulated in both temperatures, but miRNA-155 decreases at 39°C after 12h, whereas it remains up-regulated in hypothermia. MiRNA-155 inhibits the production of two negative regulators of the TLR-4 signaling pathway SHIP-1 and SOCS-1 (139,145,149). The reduced expression of these two inhibitors prolongs the activation of the TLR-4 signaling and is responsible for the prolonged expression of TNF- α at 32°C. The expression changes of SHIP-1 mRNA in both temperatures correlate well with this proposed role of miRNA-155. We demonstrate by modulation of miRNA-155 expression with mimics and antagomirs that miRNA-155 is directly responsible for the prolonged expression of TNF- α by changing the expression of SHIP-1. The reason for the reduced miRNA-155 expression at 39°C is the suppressive effect of IL-10. Blockade of the IL-10-Receptor in the 39°C monocyte culture increased both miRNA-155 and TNF- α , thus supporting the notion that IL-10 provides a pivotal negative feedback loop in order to limit the production of pro-inflammatory cytokines. Therefore, the failure of IL-10 production at 32°C results in a sequence of events, which prolongs the production of pro-inflammatory cytokines such as TNF- α and IL-6 and may ultimately be detrimental to the host.

Animal experiments showed that a prolonged expression of TNF- α leads to multiple organ failure and death (91,199). We believe that the prolonged secretion of pro-inflammatory cytokines contributes to the detrimental effects of hypothermia in surgical patients. Administration of IL-10 abolishes the

detrimental effects of high levels of pro-inflammatory cytokines as they can be found in endotoxemic shock or bacterial peritonitis (97,98). It has been shown that IL-10 suppresses the release and production of TNF- α on the transcription level (100). We and others demonstrate additionally that IL-10 also suppresses miRNA-155 (198). Therefore, IL-10 not only suppresses TNF- α production directly, it also inactivates the pro-inflammatory signaling pathways by increasing the inhibitors of the TLR-4-pathway, which provides an additional and especially long-lasting inhibition of the pro-inflammatory response. Thus, IL-10 acts on several levels and in a sequential manner in order to limit the pro-inflammatory response.

These results are in line with a common hypothesis of the cause of multiple organ dysfunction. In his theory, a prolonged inflammatory response results in a prolonged suppressive counter-inflammatory response, which subsequently increases the susceptibility of the host organisms to sepsis (186,187). In this case, our model mimics a systemically exaggerated inflammatory response. Dampening the increased immune response with administration of IL-10 would reduce the pro-inflammatory response and therefore also limit the counter-inflammatory response, which may result in an earlier restoration of immune function and subsequently reducing the risk for sepsis. Further investigation of this hypothesis should be undertaken by analysis of miRNA-155 expression or of other pro-inflammatory microRNAs such as miRNA-101 in blood or serum of patients with sepsis or after trauma. If up-regulation of miRNA-155 or another pro-inflammatory microRNA correlates with

poor outcome, it may be used to monitor immune function but also to guide immune modulatory treatments of these patients as discussed later.

Alternatively, our results could also mimic local conditions in a surgical incision or local infection. Hypothermia has been shown to reduce the ability of neutrophils and monocytes to produce ROS in hypothermia (16,62,200). A prolonged secretion of pro-inflammatory cytokines may try to compensate for the failure of neutrophils to kill bacteria by attraction of more leukocytes and increased stimulation of the present phagocytes. Further investigation of this theory should focus on miRNA-155 expression in tissue macrophages such as Langerhans cells or wound healing macrophages. Associations between miRNA-155 and other pro-inflammatory microRNA expression should be correlated with occurrence of wound infection. An association of miRNA-155 or other pro-inflammatory microRNA expression with surgical site infection could be used for risk stratification for infectious complications. In patients with a high risk for wound infection, additional measures such as prolonged local and systemic antibiotic treatment may be indicated.

Based on these experiments, miRNA-155 alone or possibly in combination with other pro-inflammatory microRNAs may be used to monitor the immune response and to guide immune-modulatory interventions. Patients with sepsis, after trauma or who are at a high risk for infectious complications may benefit from a functional evaluation of the immune system as indicated by high or low expression of pro-inflammatory microRNAs such as miRNA-155 followed by targeted interventions. Patients with high expression of miRNA-155 or other pro-

inflammatory microRNAs may benefit from immune-suppressive treatments whereas immune-stimulatory medications such as IFN- γ or GM-CSF support the recovery of patients with low miRNA-155 expression. Several clinical trials with immune-modulatory drugs have been undertaken and all of them failed. IFN- γ was used in trauma patients with the goal to ameliorate post-traumatic immune-suppression. However, there was no difference in outcome between the placebo and IFN- γ group (61, 107). Trials with the goal to reduce the inflammatory response in sepsis such as the use of anti-TNF- α antibodies failed as well and even had detrimental outcomes (104-107). One of the reasons for the failure of immune-modulatory therapy in patients is that it is not possible to functionally assess the immune system thus far. Treatment of endotoxin shock in animals with either anti-TNF- α antibodies or IL-10 increases survival whereas immune-stimulation in animals with a reduced immune response has been shown to increase survival (94,95,97,98). However, it is not possible at the moment to assess the functional state of a patient's immune system and if this particular patient suffers from an exaggerated immune response, such as in an endotoxemic shock model, and likely would benefit from an anti-inflammatory or suffers from immune-suppression possibly benefiting from an immune-stimulatory treatment. The first goal therefore should be to identify reliable markers for the functionality of a patient's immune system and then plan clinical trials based on the functional assessment of the immune system. MicroRNAs may provide this information because of their role as central regulators of cell function. Furthermore, patients receive several therapies such as mechanical

ventilation, antibiotics, transfusions and many others, all of which may interfere with an additional immune-modulatory therapy. In addition, it is unlikely that one additional therapy among so many already used would have a massive impact on survival or other outcome parameters.

However, if it is possible to stratify the functional status of a patient's immune system, for example by using miRNA-155 or other pro-inflammatory microRNAs, targeted interventions, which alter the expression of miRNA-155 or any of these other microRNAs may be beneficial and improve outcome. Especially if the microRNA is causally involved in the disease process, such as miRNA-155, which interacts with the TLR-4 pathway, a therapy directed to alter the expression of this particular microRNA subsequently influencing the cell response may be successful. Systemic or local administration of miRNA-155 antagomirs may reduce TNF- α and limit the tissue damage and subsequent organ dysfunction in patients with an exaggerated immune-response. Alternatively, administration of IL-10 in patients with high miRNA-155 expression in blood or serum may also be considered and would also inhibit TNF- α production directly, but in addition, also suppresses the expression of miRNA-155 in circulating monocytes and local macrophages but also other leukocyte subsets. These two effects act synergistically and may be able to restore the monocyte function in patients. Immune-modulatory drugs such as IFN- γ or GM-CSF may influence miRNA-155 expression and subsequently be able to restore monocyte function and enhance immune function. Furthermore, the effect of rewarming to 37°C or even hyper-warming to 39°C on the reversibility of the

TNF- α production should be investigated. Rewarming is the cornerstone of treatment of hypothermia in patients. If rewarming is not able to reverse the hypothermia induced changes in monocytes function, other strategies to restore monocyte function should be investigated.

There are several limitations for this work. Despite our attempts to use a gentle isolation process, the monocytes may be activated during this process and the monocyte response may be altered by this prior activation. The majority of the isolation process was conducted at 37°C. However, some steps have to be performed at room temperature and expose therefore the monocytes to a hypothermic environment. Our data indicate that hypothermia profoundly alters the cell response, especially signaling pathways, resulting in strongly reduced IL-10 production at 32°C resulting in prolonged TNF- α production. A potential contributor for these effects of hypothermia may also be a so far unidentified, temperature-sensitive protein or channel, which affects the monocyte response to LPS. Transient receptor potential cation channel subfamily V member 1 (TRPV1) are sensitive to temperature changes and play an important role in temperature regulation of the body (201,202). TRPV1 channels are also expressed on immune cells and the knock-out of TRPV1 results in a prolonged and increased pro-inflammatory response (203,204). Closing of these channels by hypothermia may contribute to the increased secretion of pro-inflammatory cytokines in our model. Further research of the role of TRPV1 on the inflammatory response is warranted.

In conclusion, we found that hypothermia increases and prolongs TNF- α and IL-6 production by monocytes. The lack of IL-10 secretion under hypothermic conditions results in a reduced suppression of miRNA-155 expression resulting in a prolonged and increased TNF- α secretion. We therefore propose that hypothermia impairs an important feedback loop based on IL-10. The reduced activation of Erk at 32°C possibly contributes to the lack of IL-10. Further research should be focused on investigation of additional mechanisms, which are influenced by hypothermia such as TRPV1 membrane channels. In addition, the effect of rewarming, the standard therapy for hypothermic patients, on the reversibility of TNF- α secretion should be examined.

Figure 26: Summary of Our Results

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APPENDIX: ABBREVIATIONS

General Abbreviations

CARS Compensatory Anti-inflammatory Response Syndrome

MODS Multiple Organ Dysfunction Syndrome

SCIP Surgical Care Improvement Project

SIRS Systemic Inflammatory Response Syndrome

SSI Surgical Site Infection

Cell Biology

Ago2 Argonaute 2; cleaves mRNA as part of the RISC

HUVEC Human Umbilical Vein Endothelial Cell

miRNA microRNA: short, single stranded RNA

mRNA messenger RNA: longer, single stranded RNA; serves as template for
protein translation

PBMC	Peripheral Blood Mononuclear Cells
PDCD4	Programmed Cell Death Protein 4; target of miRNA-21
RISC	RNA-induced silencing complex
siRNA	silencing RNA
SHIP-1	SH2-domain containing inositol 5' phosphatase 1; target of miRNA-155 and inhibitor of the TLR-4 pathway
SOCS-1	Suppressor of Cytokine Signaling 1; target of miRNA-155 and inhibitor of the TLR-4 and JAK/STAT
THP-1	Human monocytic cell line
TRPV1	Transient receptor potential cation channel subfamily V member 1; a temperature sensitive plasma membrane channel, which may contribute to the effects of hypothermia

Immunology

Alarmins	Endogenous proteins, which indicate tissue destruction or danger such as HSP or HMGB1
CD14	Cluster of Differentiation 14; marker for monocytes

CD16	Cluster of Differentiation 16; low affinity Fc γ -Receptor Marker for a subtype of inflammatory monocytes
DAMP	Danger Associated Molecular Pattern; includes Alarmins and PAMPs
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HLA-DR	Human Leukocyte Antigen DR; subtype of MHC II correlates with outcome in surgical patients
HMGB1	High-Mobility Group Protein B1; an alarmin
HSP	Heat Shock Proteins; serve as alarmins
IFN- γ	Interferon- γ ; immune stimulatory cytokine
IL-6	Interleukin-6; pro-inflammatory cytokine
IL-10	Interleukin-10; anti-inflammatory cytokine
LPS	Lipopolysaccharide; part of the outer membrane of gram-negative bacteria; is a DAMP
MHC II	Major Histocompatibility Complex II; expressed on Antigen Presenting Cells and is necessary for stimulation of T- and B-cells
NOD	Nucleotide Oligomerization Domain Receptor, a PRR
PAMP	Pathogen Associated Molecular Pattern; bacterial or viral

Products, which are recognized by the immune system

- PRR Pathogen Recognition Receptor; receptors, which recognizes DAMPs
such as TLR-4 and NODs
- RAGE Receptor for Advanced Glycation End Products, a PRR
- ROS Reactive Oxygen Species; produce by immune cells in order to destroy
bacteria
- TLR-4 Toll-Like Receptor 4; well described PRR, which recognizes LPS,
HMGB1 and HSP
- TNF- α Tumor Necrosis Factor α ; pro-inflammatory cytokine

Pathways

- ATF-1 Activated Transcription Factor 1; produces pro-inflammatory cytokines
- DUSP-1 Dual Specificity Phosphatase 1; target of miRNA-101 and
inactivator of p38 and JNK of the MAPK
- Erk Extracellular Signal Related Kinase pathway; part of the MAPK and
important for IL-10 production
- IKK Inhibitory Kappa Kinase; activator of NF κ B I κ β Inhibitory kappa protein
Beta; inactivates NF κ B

IRAK 1 / 2	Interleukin-1 Receptor associated Kinase 1 / 2; central signaling protein of the TLR-4 pathway
JNK	c-Jun N-terminal Kinase pathway, part of the MAPK pathway
MAPK	Mitogen Activated Protein Kinases pathway; central stress response pathway and part of the TLR-4 signaling; consisting of p38, JNK and Erk
MyD88	Myeloid differentiation primary response gene 88; central signaling protein of TLR-4
NFkB	Nuclear Factor Kappa Beta; a central stress response pathway and part of the TLR-4 signaling; consisting of the p65 and p50 subunits
p38	part of the MAPK pathway
TAB2	TGF-beta activated kinase 1/MAP3K7 binding protein 2; target of miRNA-155 and part of the MAPK pathway
TAK1	TGF-beta activated kinase 1; part of the MAPK pathway
TRAF6	TNF-Receptor Associated Factor; central signaling protein of the TLR-4 pathway

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