# Sulforaphane and its Target Macrophage Migration Inhibitory Factor in Acute and Chronic Inflammation

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**Abstract**

Sulforaphane (SFN) is a naturally occurring chemopreventive agent with putative anti-inflammatory capabilities. SFN binds to and covalently inhibits the tautomerase domain of the pro-inflammatory protein Macrophage Migration Inhibitory Factor (MIF). MIF is a protein expressed by most cells that can function in intracellular signaling pathways, has chemokine-like functions, and induces cytokine production in immune cells. There is no known *in vivo* substrate for MIF tautomerase; however, studies suggest that this enzymatic activity is still biologically relevant. Upregulation of MIF is a characteristic seen in many diseases, including cancer, obesity, and rheumatoid arthritis. Given the role of MIF in such a wide variety of diseases with immunological components, we set out to examine the effects of MIF in both acute and chronic disease models with the intent of evaluating the effect of SFN as an inhibitor of MIF in these models.

We hypothesized that in the context of the acute inflammatory response, SFN administration would inhibit the tautomerase activity of MIF and thereby reduce the pro-inflammatory cellular response to an acute inflammatory stimulus. *In vitro* experiments demonstrated the ability of SFN to inhibit pro-inflammatory signaling through reduction of TNFα secretion, NF-κB activation, and NO production. SFN also blocked both MIF-dependent and CSF-1-dependent migration of bone marrow-derived macrophages (BMDM) in a Transwell assay. Examination of SFN treatment in the murine air pouch model of acute inflammation showed that while SFN does inhibit MIF tautomerase activity in the pouch lining, it does not decrease the number of infiltrating immune cells as hypothesized. We did observe a shift in

immune cell populations in the SFN pre-treated mice compared to LPS-induced mice, such that the percentage of $CD11b^{int}GR1^{lo}$ cells decreased with SFN pre-treatment while the percentage of $CD11b^{hi}GR1^{hi}$ cells increased. The absolute numbers of these populations in the SFN pre-treated group were not significantly different from the LPS alone group and, in fact, trended toward inducing more cells than LPS alone. The F4/80+ macrophage population was not significantly altered. These data suggest that SFN does not have the anticipated anti-inflammatory activity *in vivo*, and may actually have a pro-inflammatory function in the murine air pouch model of acute inflammation.

We concurrently studied the effects of MIF on the immune response to LPS in the air pouch model. We hypothesized that addition of rmMIF, but not tautomerase-dead rmMIF, into the air pouch would rescue the immune response to LPS in $MIF^{-/-}$ mice. However, in our hands, $MIF^{-/-}$ mice unexpectedly did not have a less robust cellular response to LPS compared to WT, as had been previously published. We observed equivalent numbers of infiltrating immune cells in both genotypes in response to LPS. This complication with the model prevented us from moving forward with examination of the role of MIF tautomerase activity in acute inflammation.

Finally, we investigated the role of MIF in the chronic immune response to obesity. We hypothesized that genetic ablation of MIF would result in fewer visceral white adipose tissue (vWAT) infiltrating immune cells and concurrent protection from the development of insulin resistance. Using a high fat diet-induced model of obesity in $MIF^{-/-}$ and MIF WT mice, we demonstrated that that absence of MIF does

not result in differences in weight gain or visceral white adipose tissue mass. Immune cell infiltration into vWAT was not dependent upon MIF. Finally, endpoint analysis of metabolic outcomes in obese $MIF^{-/-}$ mice showed that the absence of MIF did not have an effect on blood glucose or blood insulin levels, nor did it change the course of the development of insulin resistance compared to WT controls. Had there been a demonstrable MIF-dependent difference in this model, we would have followed up with studies examining the effect of SFN treatment of obese MIF WT mice. However, our data led us to conclude that global MIF expression is not a critical factor for the development of obesity and related metabolic complications in this model.

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**List of Abbreviations**

ANOVA: Analysis of Variance

AP-1: Activator Protein 1

BAEC: Bovine Aortic Endothelial Cell

BMDM: Bone Marrow-Derived Macrophage

BMI: Body Mass Index

CCL-2: CC-motif Ligand 2, aka MCP-1

COX-2: Cyclooxygenase-2

CSF-1: Colony Stimulating Factor 1

ERK: Extracellular-signal-regulated Kinase

DDT: D-Dopachrome Tautomerase

DHICA: 5,6-dihydroxyindole-2-carboxylic acid

DIO: Diet-induced Obesity

gMDSC: granulocytic Myeloid Derived Suppressor Cell

H&E: Hematoxylin and Eosin

HED: 2-hydroxyethyldisulfide

HFD: High Fat Diet

ICAM-1: Intracellular Adhesion Molecule 1

IFNY: Interferon gamma

iNOS: inducible Nitric Oxide Synthase

IP: Intraperitoneal

IPGTT: Intraperitoneal Glucose Tolerance Test

ITC: Isothiocyanate

JAB1: c-Jun activation domain-binding protein 1

JNK: c-Jun N-terminal Kinase

KC: aka CXCL1, neutrophil chemoattractant

KO: Knock-out

LDLR: Low Density Lipoprotein Receptor

LPS: Lipopolysaccharide

MAPK: Mitogen Activated Protein Kinase

MCP-1: Monocyte Chemotactic Protein 1

MDSC: Myeloid Derived Suppressor Cell

mMDSC: monocytic Myeloid Derived Suppressor Cell

MIF: Macrophage Migration Inhibitory Factor

MIP-2: Macrophage Inflammatory Protein 2

NF-κB: Nuclear Factor kappa B

NO: Nitric Oxide

$PGE_2$: Prostaglandin $E_2$

PMA: Phorbol 12-Myristate 13- Acetate

PMN: Polymorphonuclear leukocyte, aka neutrophil

RA: Rheumatoid Arthritis

rmMIF: recombinant mouse Macrophage Migration Inhibitory Factor

ROS: Reactive Oxygen Species

SEB: *Staphylococcus aureus* Enterotoxin B

SEM: Standard Error of the Mean

SFN: Sulforaphane

STZ: Streptozotocin

SVF: Stromal-vascular Fraction

sWAT: subcutaneous White Adipose Tissue

T2D: Type 2 Diabetes

TAM: Tumor-associated Macrophage

TCR: T cell receptor

TLR4: Toll-like Receptor 4

TNFα: Tumor Necrosis Factor alpha

VCAM-1: Vascular Cell Adhesion Molecule 1

vWAT: visceral White Adipose Tissue

WAT: White Adipose Tissue

WT: Wildtype

# Chapter 1: Introduction

## The Immune System

The immune system serves as the body's natural defense against pathogens and disease. It is composed of a complex network of cell types and soluble mediators that work in concert to balance one another and ensure the proper response to any identified danger to the organism [1]. The immune system can be classified into two primary types of responses to pathogens: acute and chronic. Acute immune responses are designed to act rapidly to quickly eliminate dangerous pathogens that enter the body. The acute response is critical for clearing problems such as bacterial and viral infections and initiating the healing response. Chronic responses are ones that occur over longer periods of time and tend to result in tissue damage due to failure to resolve the inflammatory stimuli [2, 3]. Our approach to the study of the immune system was to examine the effect of a naturally occurring compound, sulforaphane (SFN), that is known to inhibit the tautomerase domain of the pro-inflammatory protein macrophage migration inhibitory factor (MIF) to determine if it could be used to inhibit inflammation in either the acute response, in an LPS model of acute inflammation, or in the context of the chronic inflammation that occurs during obesity.

## Sulforaphane as an Inflammatory Mediator

Sulforaphane has recently come on the radar within the biomedical community as a compound with potential to be used as an anti-inflammatory mediator. It has been well characterized as a cancer chemopreventive agent [4-7], and it has been suggested that some if its anti-tumor activities may be due to its

ability to block pro-inflammatory signals such as NF-κB activation, TNFα secretion, and iNOS production [8, 9]. It is also known to protect cells from oxidant and electrophile damage through induction of Phase II genes [10, 11]. Phase II genes produce essential enzymes that are able to convert potential carcinogens into harmless metabolites [6]. SFN induces Phase II genes through activation of the transcription factor Nrf2 [5].

Much of the interest in SFN as both a chemopreventive and anti-inflammatory agent is due to the fact that it is a naturally occurring compound. It is found most commonly in cruciferous vegetable such as broccoli, cauliflower, and watercress [12]. It is part of a family of compounds known as isothiocyanates (ITCs) that are broken down from glycosinolates by myrosinases to yield the more reactive ITC. This process occurs naturally in plants, following cellular damage. However, humans lack myrosinases; therefore, this enzymatic reaction is dependent on the gut flora of each individual and can vary dramatically between individuals [7]. Physiologically relevant levels of SFN have been achieved in humans through consumption of large quantities of broccoli [13, 14]; however, it has a relatively short systemic half-life. Intraperitoneal treatments of SFN in mice resulted in a peak in circulating levels at around 2 hours post-injection and begin returning rapidly back to low concentrations around 4 hours post-injection [15]. The half-life of SFN in rat studies is approximately 2.2 hours [16].

Cell signaling and protein secretion studies in the literature support the idea that SFN may act as an anti-inflammatory compound. Heiss *et al*. performed a study showing that SFN treatment of RAW264.7 macrophages resulted in inhibition of NF-

κB DNA binding [9]. NF-κB is one of the primary transcription factors for proteins of the immune system and inhibition of NF-κB significantly reduces pro-inflammatory gene expression. Treatment of LPS-stimulated RAW264.7 cells with SFN results in decreased production of nitric oxide (NO), $PGE_2$, and TNFα. Intracellular levels of COX2 are also reduced by SFN [9]. Similar results have been seen in peritoneal macrophages, and studies in human keratinocytes showed that SFN inhibits AP-1 activation, suggesting that these effects are not unique to immortalized cells [17, 18]. Finally, SFN has been shown to block TLR4 oligomerization, implying that SFN may be able to inhibit activation of TLR4-dependent immune responses [19].

SFN has been shown to exert effects on the function of inflammatory cells. Dias *et al.* demonstrated that SFN inhibits PMA-induced ROS production in human neutrophils. They used both the cell line HL60 and primary human neutrophils to show this effect [20]. Thejass and Kuttan studied the effect of SFN on peritoneal macrophages and showed that mice that had been given five IP treatments of SFN had enhanced phagocytic ability in an *in vitro* assay [21]. The same study showed that SFN is able to inhibit LPS-induced TNFα secretion by peritoneal macrophages, suggesting that SFN is also able to regulate cytokine secretion of immune cells [21].

SFN may be able to regulate immune cell trafficking. It has been shown that SFN acts on endothelial cells to affect both chemokine secretion and expression of adhesion molecules. Liu *et al*. pre-treated bovine aortic endothelial cells (BAECs) with SFN prior to LPS stimulation and used an *in vitro* adhesion assay with co-cultured THP-1 monocytes to show that SFN significantly reduced monocyte

adhesion. Quantitative real-time PCR indicated that this effect was due to a decrease in ICAM-1 expression [22]. Another study demonstrated that SFN is able to inhibit endothelial cell secretion of the chemokines MCP-1 and IL-8 [23]. The effects that SFN exerts on endothelial cells could have a significant impact on the chemotactic gradients and intravasation of immune cells in *in vivo* models; however, those effects remain largely untested at present.

A few *in vivo* studies support an anti-inflammatory mechanism for SFN within the immune system. In a collagen-induced model of arthritis, Kong *et al.* demonstrated that IP administration of SFN reduced the severity of the disease as measured by mean arthritis and histological scores [15]. Circulating antibodies to collagen were reduced in SFN treated mice. Both lymph node and spleen cells from arthritic mice treated with SFN produced less TNFα, IFNγ, IL-6, and IL-17 *in vitro* [15]. A second study, in the spontaneously hypertensive stroke-prone rat, showed that oral administration of broccoli sprouts (high in the precursor to SFN) for 14 weeks resulted in decreased oxidative stress in the cardiovascular system and kidney, and fewer macrophages within the aorta, carotid artery, and endometrium [24]. Finally, Youn *et al.* showed that oral administration of SFN reduced the local thickness and edema in the ears of mice had been given intradermal treatments of LPS [25]. Altogether, the studies of the roles of SFN in the immune system support a complex and potentially important regulatory function for SFN in many different aspects of the immune response.

**SFN is an Inhibitor of MIF**

Our primary interest in SFN originates from our discovery that it functions as a covalent inhibitor of the protein Macrophage Migration Inhibitory Factor (MIF) [26]. MIF is a pro-inflammatory protein that is upregulated in diseases such as cancer and obesity [27, 28]. We have previously shown that SFN binds covalently to the N-terminal proline of MIF [26]. This results in an irreversible inhibition of the tautomerase activity of the protein [29]. The goal in using SFN as a MIF inhibitor is to demonstrate the importance of the tautomerase activity of MIF in its biological activities and to show that SFN, as a MIF inhibitor, can be used as an immune modulator. Binding of SFN to the N-terminus of MIF will leave the remainder of the protein open to perform other functions, such as its oxidoreductase activity or receptor binding. Other synthetic MIF inhibitory compounds, such as ISO-1, are able to bind to the same region on MIF, but there is particular interest as to whether SFN may be a safer way to manipulate MIF and its tautomerase activity because it is a naturally occurring compound that can be administered orally as a dietary supplement [30].

**The History of MIF**

Macrophage Migration Inhibitory Factor (MIF) was identified as the first cytokine in 1966 [31, 32]. Its name stems from the original study that showed that MIF secreted from T cells is able to halt the random migration of macrophages *in vitro* [32]. The name is somewhat misleading because MIF was later shown to be a macrophage chemoattractant, and people speculate that in the original study once

the macrophages came in contact with MIF they stopped moving because they had effectively moved toward a chemotactic signal [33]. MIF is a 12.5kDa protein that functions as a homotrimer and is highly conserved across many species [34]. MIF has a relatively recently identified homologue known as D-dopachrome tautomerase (DDT) that shares the tautomerase function of MIF. Neither MIF nor DDT have identified *in vivo* substrates; however, this enzymatic activity is conserved all the way back to *C. elegans* [35].

Most cell types secrete MIF, including those in the immune system, brain, endocrine system, lung, skin, and gastrointestinal system [27]. MIF has been shown to have an effect on hormone secretion, glucocorticoid activity, metabolism, intracellular signaling pathways such as ERK, and immune cell function [27, 36-40]. MIF also promotes the production of several key pro-inflammatory cytokines, such as TNFα, IL-1β, and nitric oxide (NO) [27, 36, 41]. Many human diseases have been shown to have upregulated levels of MIF, leading researchers to examine the role of this protein in pathological models such as rheumatoid arthritis, cancer, inflammatory bowel disease, and pneumonia [27, 42, 43].

**The Enzymatic Domains of MIF**

MIF has two different enzymatic domains. It can function as both a keto-enol tautomerase and as a thiol oxidoreductase. The tautomerase activity of MIF is located within the N-terminal region of the protein and the proline at position 2 is known to be required for maintenance of that enzymatic function [44, 45]. As a tautomerase, MIF has the ability to bind to a substrate and move double bonds

within a molecule, resulting in a keto-to-enol transition. No *in vivo* substrate has yet been identified; however, MIF does tautomerize the synthetic compound D-dopachrome into 5,6-dihydroxyindole-2-carboxylic acid (DHICA) *in vitro* [44]. This has allowed for the development of *in vitro* assays to measure the kinetics of the tautomerase activity [46]. It is still unclear whether the tautomerase activity has physiological relevance; however, the tautomerase activity has been shown to attenuate the effects of *Pseudomonas aeruginosa* infection in a model of cystic fibrosis suggesting that MIF tautomerase does have a role in disease [47].

In 1998, Kleeman *et al*. discovered that MIF is a thiol oxidoreductase. As with the tautomerase activity, there is not yet a known substrate for this activity. However, it is known that there is a highly conserved CXXC motif within MIF that is required for maintenance of the oxidoreductase activity as quantified by *in vitro* assay that measures the ability of MIF to reduce both insulin and 2-hydroxyethyldisulfide (HED). This study also demonstrated that, despite the lack of identified physiological substrate, mutation of the CXXC motif leads to a reduction in the ability of MIF to promote macrophage killing in a well-characterized assay using *Leishmania* [48].

**MIF and Intracellular Signaling**

MIF has been implicated in several critical intracellular signaling cascades. Binding of MIF to the CD74-CD44 receptor complex results in rapid phosphorylation of ERK and consequent activation of the ERK signaling cascade that is sustained for several hours [27, 38]. This activity is protein kinase A-dependent and results in

increased proliferation of NIH/3T3 cells. A yeast two-hybrid screen followed by immunoprecipitation and pull-down experiments demonstrated that MIF is able to co-localize with cytoplasmic JAB1. This association results in inhibition of the pathway leading to JNK activation and consequent inhibition of AP-1 transcriptional activation [27, 37]. MIF has also been identified in cDNA screens as an inhibitor of p53 activity. Functional assays confirmed that MIF is able to reverse p53-induced growth arrest, transcriptional activation, and apoptosis *in vitro* [49, 50].

**MIF Receptors**

MIF has been reported to bind to four receptors. CD74 was the first identified receptor for MIF in a study showing that binding to CD74 was required for the ability of MIF to activate ERK signaling, induce cell proliferation, and upregulate $PGE_2$ production [51]. There was some initial controversy over this discovery because CD74 lacks an intracellular signaling domain. It was later shown that CD74 bound to MIF co-localizes with CD44, which acts as a co-receptor with an intracellular signaling domain [52].

MIF is also a non-cognate ligand for the chemokine receptors CXCR2, CXCR4, and CXCR7. MIF is very similar in tertiary structure to the natural chemokine ligands for these receptors, and is therefore able to compete with the natural chemokine ligands for binding to CXCR2, CXCR4, and CXCR7. Bernhagen *et al*. performed the first competition and internalization studies in HEK293 cells that established the ability of MIF to bind to both CXCR2 and CXCR4 [53]. The same report demonstrated that surface-bound MIF is able to arrest the migration of both

monocytes and T cells via CXCR2 and CXCR4, respectively, and that MIF works through these receptors to induce monocyte and T cells migration in a transwell assay. Another group demonstrated binding of MIF to CXCR4 and CXCR7 in human rhabdomyosarcoma (RMS) cells [54].

**MIF and Immune Cell Trafficking**

MIF is a chemoattractant for several types of immune cells. As mentioned above, MIF works through CXCR2 to regulate monocyte trafficking and through CXCR4 to induce migration of T cells [53]. MIF was first identified as a factor secreted by T cells that is able to halt the random *in vitro* migration of macrophages from peritoneal exudate [31, 32]. Since then, it has been determined that MIF is a potent macrophage chemoattractant [33]. Our lab has shown that MIF is important for regulating the prevalence of Myeloid Derived Suppressor Cells (MDSCs) in the murine 4T1 breast tumor model [55]. We have not yet demonstrated that MIF is a chemoattractant for MDSCs in this system; however, ongoing experiments have not yet ruled out that hypothesis.

MIF can also regulate immune cell trafficking by indirect mechanisms. An example of this is a study performed in the murine K/BxN serum transfer model of arthritis in which $MIF^{-/-}$ mice were unable to muster the same level of neutrophil recruitment in response to KC, as seen by intravital microscopy. Mechanistic experiments in this study suggested that MIF deficiency resulted in decreased MAPK activation in neutrophils, thereby impairing their response to KC [56]. Blocking antibody studies have shown that MIF is important in the recruitment of neutrophils

into the lung in an LPS model, potentially by upregulating MIP-2 protein levels in the lung [57]. MIF is also known to be important in promoting cell adhesion and rolling on endothelial cells through increasing expression of P-selectin, E-selectin, ICAM-1, and VCAM-1 [58, 59].

**MIF and the Acute Inflammatory Response**

MIF has been shown to be a critical regulator of the acute inflammatory response. The first major report in support of this function of MIF was published in 1993 by Bernhagen *et al*. demonstrating that MIF was secreted from the pituitary in response to LPS and that blocking antibodies against MIF conferred protection from endotoxemia [60]. A second report by Bozza *et al.* in 1999 showed that MIF knockout mice are protected from lethality induced by either high doses of LPS or *Staphylococcus aureus* enterotoxin B (SEB) in comparison to the high level of lethality induced in MIF WT mice [61]. Calandra *et al.* quickly followed that report by conducting a study in which they utilized the cecal ligation and puncture (CLP) model of septic shock in mice to demonstrate that administration of an anti-MIF antibody protected TNF-α knockout mice from lethal peritonitis. In addition, application of rmMIF increased lethality in this model [62]. It was on these reports that we based our initial hypothesis of the importance of MIF as a target of SFN in the air pouch model of acute inflammation.

It is critical to note that there was a follow up publication by a Japanese group in 2000 that contradicted the report described above by Bozza *et al*. [63]. Honma *et al*. demonstrated that, repeating the initial study of the effect of LPS in

MIF$^{-/-}$ mice, they were unable to see any difference in response compared to MIF WT mice. Both groups of mice developed equivalent levels of septic shock. This controversy is of importance in light of the results of our studies of the LPS-induced immune response in the MIF$^{-/-}$ mice.

**The Murine Air Pouch Model**

The rodent air pouch model was first described by Edwards *et al.* in 1981 [64]. That report detailed that subcutaneous injection of sterile air under the dorsal skin of mice and rats resulted in a space that looked remarkably similar to the synovium of the joint. Microscopic and histologic studies suggested that the pouch formed a very homogenous lining made primarily of fibroblasts, with a notable macrophage population. Following this initial description of the air pouch, investigators began using this method primarily as a model for arthritis and later as a model for inflammatory studies [65, 66]. It provides a very easily accessible space for administration of a stimulus and easy harvest of infiltrating cells and surrounding tissues [67].

**Obesity, Inflammation, and MIF**

Obesity is one of the fastest growing health problems in the world. In the U.S. alone, there was a dramatic increase of 2.4 million people who became clinically obese between 2007 and 2009 [68]. It is estimated that about 2.8 million people die each year from obesity-related co-morbidities [69]. Obesity promotes the development of many serious health issues, such as cardiovascular disease, Type 2

diabetes (T2D), and cancer [70-72]. This leads to a huge economical burden on patients and governments alike. The cost of health care for obese patients is estimated to be about $150 billion dollars in the U.S each year [73].

Obesity is characterized by a gross increase in adipose tissue mass and overall weight gain. People are considered to be clinically obese if their body mass index (BMI) is 30 or greater [74]. In healthy individuals, dietary fat is predominantly stored in the subcutaneous white adipose tissue (sWAT) as triglycerides [75]. When an individual consumes too much fat, the adipocytes of the sWAT reach the limit of their storage capacity and begin sending fat to the visceral adipose tissue (vWAT) for storage. If dietary fat consumption is still too high for the vWAT to handle in conjunction with the sWAT, then free fatty acids begin traveling through the circulation where they can be deposited in organs like the liver and pancreas [76]. Fat deposition in peripheral organs most often leads to cellular damage that promotes the development of secondary diseases such as fatty liver disease or Type 2 diabetes [75, 77].

One of the first tissues to be affected by the onset of obesity is the white adipose tissue (WAT). Researchers typically classify the cells in the WAT in two different groups: the adipocyte fraction and the stromal-vascular fraction (SVF) [70, 75]. During obesity, the adipocytes become stressed with the pressure of trying to store an excess of dietary fat. This stress often leads to adipocyte death that triggers signals of damage and cell death to be sent out to the immune system, alerting the body of the problem [78, 79]. Immune cells are one cell type of the SVF. Certain immune cells, such as Th2 T cells and M2 macrophages, are present in healthy

adipose tissue as maintenance cells. However, once the WAT begins to expand and adipocytes send out death signals, the immune system starts to switch to a proinflammatory Th1/M1 type response because of the damage that is occurring [80]. Macrophages are the primary component of the immune response to obesity and can constitute up to half of the cells in the WAT during obesity. Thus far their main roles have been identified to be clearance of dead adipocytes and secretion of cytokines such as TNFα, IL1β, and MCP-1 [81, 82]. This is typically a much larger cellular response than the maintenance populations and is maintained throughout the course of obesity as chronic inflammation, promoting damage within the adipose tissue [83].

MIF has come to light as a protein of potential interest in obesity research. Human studies have demonstrated that circulating MIF levels positively correlate with increasing BMI [28, 84]. Interestingly, MIF mRNA levels have also been shown to be increased in the vWAT of obese patients [85]. These correlations have led clinicians and researchers to ask if the increase in MIF in obese patients is exerting physiological effects that impact the course of the disease, and if yes, could it be a potential anti-obesity drug target?

There are some studies that suggest that MIF could impact the metabolic aspects of obesity in concert with its known role as a pro-inflammatory protein. Verschuren *et al*. performed experiments in $LDLR^{-/-}$ mice and demonstrated that mice that are simultaneously deficient in MIF are protected from glucose intolerance compared to MIF expressing mice on this atherosclerotic background. That study showed that the absence of MIF resulted in inhibition of macrophage

deposition in the vWAT as well [86]. It has also been shown that MIF promotes insulin secretion [87] and that MIF deficiency protects mice from development of STZ-induced diabetes [88, 89] and age-induced glucose intolerance [90].

**Myeloid Derived Suppressor Cells**

Myeloid derived suppressor cells (MDSCs) are an immune cell subset that is thought to expand almost exclusively during disease [91]. The current dogma is that MDSCs are a subset of immature myeloid cells that have been arrested in their differentiation and have developed a suppressive phenotype that enables them to inhibit T cell function [92]. Under normal, healthy conditions, myeloid cells exit the bone marrow and terminally differentiate into cells such as macrophages, dendritic cells, and neutrophils as they enter peripheral organs [93]. A poorly understood process associated with disease onset has been suggested to interrupt this normal myeloid differentiation process, giving rise to an expansion of MDSCs.

MDSCs are identified in mice by CD11b+ and GR-1+ cell surface markers [94]. These markers are also found on neutrophils; therefore, true identification of MDSCs is based on a functional assay in which putative MDSCs are shown to be able to suppress T cell proliferation and cytokine secretion (IFNγ) of CD4+ or CD8+ T cells [95, 96]. There are two known subsets of MDSCs: monocytic and granulocytic [97]. Monocytic MDSCs (mMDSCs) are defined by $Ly6G^{low}$ $Ly6C^{hi}$ cell surface staining. Granulocytic MDSCs (gMDSCs) are defined by $Ly6G^{hi}$ $Ly6C^{low\text{-}int}$ cell surface staining [98]. MDSCs have been shown to express markers that are typically used to differentiate M1 and M2 macrophages [99, 100]. The appropriate *in vitro* culture

conditions have been able to differentiate MDSCs into terminally differentiated myeloid cells [101, 102]. It is unknown whether MDSCs have this plasticity *in vivo*.

The primary known function of MDSCs is to suppress T cells [95]. MDSCs suppress T cells through production of mediators such as iNOS, arginase 1, ROS, and peroxynitrite [92, 103]. It has been suggested that MDSCs may nitrosylate the T cell receptor (TCR) and MHC molecules, resulting in inhibition of T cell proliferation and activity [104]. Both classes of MDSCs have been shown to have suppressive activity; however, the mMDSC subset is typically considered to be more strongly suppressive than the gMDSCs. This difference in suppressive ability may be due to the fact that mMDSCs tend to produce more iNOS, while gMDSCs tend to produce more ROS [92, 103]. Additionally, gMDSCs have been shown to promote angiogenesis and support the epithelial-mesenchymal transition to enhance the metastasis of tumor cells [105, 106].

The fact that MDSCs are detectable in animals only during disease suggests that they are critical to disease processes. Thus far, MDSCs have primarily been studied in tumor models [92, 96]. MDSCs are generally thought to promote tumorigenesis due to their ability to block the T cells that attack tumors [96]. More recently, a study was published demonstrating the presence of MDSCs in obese adipose tissue in a murine model of obesity [99]. This report showed that MDSCs are more common in obese than lean WAT. It also demonstrated that the presence of MDSCs promotes insulin sensitivity in obese mice and is therefore protective against some of the metabolic dysregulation that occurs during weight gain. However, others have shown that MDSCs arise in the liver of obese mice and that

their presence is detrimental to preservation of liver function [107]. These potentially conflicting reports of the role of MDSCs in obesity communicate to the scientific community that there is much work yet to be done to fully understand the role of MDSCs in various tissues during the chronic immune response to obesity. The goal of our obesity study was to understand the roles of MIF and MDSCs during obesity.

**The Murine Model of High Fat Diet-Induced Obesity**

The murine model of High Fat Diet (HFD) feeding is commonly used to mimic the pathogenesis seen in humans who become obese from over-eating. Not all strains of mice respond with an equivalent increase in mass due to the HFD; therefore, C57Bl/6-based strains are the most prevalent in the field because they rapidly gain weight over the course of the diet [108]. Mice are typically started on a HFD regimen at 4-6 weeks of age for a period of an average of 8-16 weeks [109]. Parameters such as change in weight, metabolic function, and circulating protein levels are commonly monitored over the course of the model. End point analysis can include any examinations associated with murine models, such as evaluation of immune cell populations within organs or protein analysis in circulation or organs [83, 110]. Our focus in this study was on the examination of the role of MIF in the inflammation that results from HFD-induced obesity in this model.

## Chapter 2: SFN Does Not Inhibit Inflammatory Cell Infiltration in the Acute Inflammatory Response In Vivo

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**Abstract**

Sulforaphane (SFN) is a well-characterized naturally occurring cancer chemopreventive agent with proposed anti-inflammatory activities. SFN is known to induce phase two gene expression and to covalently inhibit the inflammatory protein macrophage migration inhibitory factor (MIF). SFN has been shown *in vitro* to inhibit NF-κB activation and production of pro-inflammatory signaling molecules TNFα and NO. The purpose of this study was to further characterize the anti-inflammatory activities of SFN *in vitro* and in an *in vivo* model of acute inflammation. SFN inhibited LPS-induced TNFα and NO production as well as NF-κB activation in RAW264.7 macrophages *in vitro*. SFN also reduced bone marrow-derived macrophage (BMDM) migration in a Transwell migration assay. However, when examined in a murine air pouch model of the acute inflammatory response *in vivo,* SFN pre-treatment prior to LPS stimulation did not significantly change the number of infiltrating cells when compared to LPS alone. Instead we observed that SFN shifted the ratio of LPS-induced cells such that the percentage of $CD11b^{int}GR1^{lo}$ cells decreased with pre-treatment while the percentage of $CD11b^{hi}GR1^{hi}$ cells increased. F4/80 macrophage numbers were not significantly altered. The absolute numbers of these populations in the SFN pre-treated group were not significantly different from the LPS alone group and trended toward inducing more cells than LPS alone. These data suggest that SFN does not have the anticipated anti-inflammatory response to LPS *in vivo*, and may actually exhibit pro-inflammatory activity in the murine air pouch model of acute inflammation.

## Introduction

Sulforaphane (SFN) is a well-recognized and potent inhibitor of cancer with a number of proposed mechanisms of action [5, 6]. SFN is a member of the isothiocyanate (ITC) class of compounds, which are abundant in cruciferous vegetables such as broccoli, brussel sprouts, and watercress. ITCs originate as inactive precursor glucosinolates in plant cells and are broken down by myrosinases to yield the more active ITCs [7]. This process occurs spontaneously in plants in response to damage and in humans can be mediated by the breakdown of ingested gluconsinolates by enzymes in the gut flora [111]. ITCs induce phase two gene expression [10, 111], which is invoked as explanation for their cancer prevention activity, as the products of these genes are thought to promote detoxification and excretion of carcinogens thereby protecting from damage. In addition, we [26] and others [112, 113] have shown that ITCs covalently modify the proinflammatory protein macrophage migration inhibitory factor (MIF).

More recently, sulforaphane has been characterized as a potential anti-inflammatory compound based predominantly on its effects on a number of cell signaling pathways central to inflammation [4, 7, 9]. The anti-inflammatory activities of SFN have been described in multiple *in vitro* model systems. For example, treatment with SFN reduced the expression of NO, PGE2, COX2, and TNFα in LPS-stimulated RAW264.7 and peritoneal macrophages [9, 17]. SFN inhibits LPS induced expression of inflammatory genes by blocking NF-κB activation [9, 114] and has also been demonstrated to reduce monocyte adhesion by inhibition of ICAM-1 expression [115]. Finally, we have shown that SFN suppresses production of

prostaglandin E2 (PGE2), an inflammatory mediator with provocative connections to cancer [29].

In contrast to numerous in vitro studies, only a few studies have examined the anti-inflammatory effects of SFN in *in vivo* models. SFN treatment has been shown to reduce the inflammatory symptoms of collagen II-induced rheumatoid arthritis (RA) [15]. SFN can inhibit both the accumulation and TNFα secretion of murine peritoneal macrophages in healthy Balb/c mice and in the brains of herpes encephalitis-infected mice [21, 116]. It has also been shown to be highly protective of the inflammatory damage that is caused in rodent models of stroke leading to protection from central nervous system degeneration and cardiovascular disease [24, 117]. In a mouse model of LPS-induced acute inflammation, SFN was shown to inhibit inflammation as measured by a reduction in edema following intradermal injection of LPS in the ear [25].

The molecular mechanisms by which SFN acts are not yet well understood. For example, although SFN inhibits NF-κB activation in response to numerous stimuli [25], the specific mechanisms mediating NF-κB inhibition remain unclear. However, inhibition of thioredoxin reductase by SFN has been implicated as a mechanism of NF-κB inhibition [118]. The isothiocyanate cancer preventives, including sulforaphane, are reactive chemicals with the potential to covalently modify protein targets. We have pursued identification of these targets in an effort to explain the effects of these agents on cell signaling. We have demonstrated that a related isothiocyanate, PEITC, covalently modifies and inhibits the protein kinase MEKK1, leading to downstream inhibition of SAPK/JNK signaling [119], and have

also characterized the inflammatory cytokine, MIF, as a target for covalent inhibition by SFN [26]. Others have shown that SFN can modify TLR4 and impair its oligomerization, thereby blocking a primary method of activating the acute inflammatory response [19].

The putative anti-inflammatory function of SFN described in the literature led us to investigate whether SFN would inhibit the acute inflammatory response in the murine dorsal air pouch model. We hypothesized that SFN pre-treatment of the air pouch would inhibit macrophage infiltration into the pouch in response to LPS. We confirmed that SFN inhibits several pro-inflammatory signaling responses in *in vitro* systems. Moreover, we show that SFN blocks CSF-1-induced BMDM migration in a transwell system. Using the murine air pouch model, we examined the effect of SFN pretreatment on the LPS induced F4/80 macrophage response, demonstrating that this population was not impacted by SFN pre-treatment. Instead, we observed that SFN pre-treatment increased the percent of $CD11b^{hi}GR1^{hi}$ cells and decreased the percent of $CD11b^{int}GR1^{lo}$ cells that infiltrated the pouch in response to LPS. Taken together, these results suggest that SFN does not act as an anti-inflammatory compound in this *in vivo* model of acute inflammation.

## Methods

### Cell Lines, Reagents, and Antibodies

RAW264.7 cells were cultured at 37°C and 5% $CO_2$ in DMEM (Gibco) supplemented with 10% FBS (HyClone) and 1% penicillin/streptomycin (Sigma). Bone marrow-derived macrophages were cultured in in αMEM (Gibco), supplemented with10%

heat-inactivated FBS (HyClone), 10% CMG supernatant (supernatant from cells that secrete high levels of CSF-1), and 1% Penicillin/Streptomycin (Sigma). Antibodies for p65 and IkBα were ordered from Santa Cruz and phospho-p65 and phospho-IkBα were ordered from Cell Signaling Technologies. Sulforaphane was obtained from LKT labs, LPS from Sigma, and Greiss reagents from Cayman Chemicals.

**Bone Marrow-Derived Macrophage Preparation**

Femurs were harvested from mice and sterilized in 70% ethanol for 2 minutes. Bone marrow was isolated by lavage of the bone cavity with PBS. Cells were red cell lysed and plated at two million cells per 10cm plate. Media was replaced on day 3 and cells were collected for use on day 7.

**Measurement of TNFα Secretion**

RAW264.7 cells were seeded in 24-well plates at 250,000 cells/well and incubated overnight. BMDM were plated at 400,000 cells in a 24-well plate. Cells were pre-treated with 10μM SFN for 30 minutes prior to stimulation with 100ng/mL LPS overnight. TNFα was quantified by ELISA per manufacturers protocols (R&D).

**Quantitative real-time PCR**

One million RAW264.7 cells or BMDM were plated per well in 6-well plates and incubated overnight. Cells were pre-treated with 10μM SFN (or vehicle control) for 30 minutes prior to stimulation with 50ng/mL LPS. Cells were incubated for 4

hours and then lysed in Trizol (Invitrogen) and processed for RNA extraction, per manufacturer's instructions. cDNA was prepared using an iScript kit (BioRad). Quantitative real-time PCR was performed using primers for TNF $\alpha$, IL6, IL12p35, normalized to actin control, and analyzed using the $\Delta$Ct method. Primer sequences are as follows. TNFα F: GGCAGGTCTACTTTGGAGTCATTGC, R: ACATTCGAGGCTCCAGTGAATTCGG [120]. IL-6 F: GAGGATACCACTCCCAACAGACC, R: AAGTGCATCATCGTTGTTCATACA [121]. IL12p35 F: AAATGAAGCTCTGCATCCTGC, R: TCACCCTGTTGATGGTCACG [122]. Actin F: CCCAGAGCAAGAGAGGTATC, R: AGAGCATAGCCCTCGTAGAT [123].

**Western Blotting**

Six-centimeter plates were seeded with 250,000 RAW264.7 cells and treated with vehicle control, 500ng/mL LPS, 20μM SFN, or 20μM SFN for 30 minutes prior to 500ng/mL LPS stimulation. All treatments were for 30 minutes (a total of one hour in the case of the SFN pre-treated samples). Normalized protein samples were loaded onto 7.5% gels for SDS/PAGE, transferred onto PVDF membranes (Millipore), blocked with 5% milk, and probed with antibodies to p65, phosho-p65, IkBα, and phospho-IkBα. Primary antibodies were incubated overnight at 4°C. Blots were detected using an anti-rabbit-HRP antibody was incubated with the blots for two hours at room temperature. Images of the chemiluminescent detection were developed using the Alpha Innotech Imager system.

**Nitrate Secretion Assay**

$4x10^5$ Raw 264.7 cells were seeded into the wells of a 24 well plate in 500μL media (DMEM, 10% FBS, with penicillin and streptomycin). The following day 18 wells were treated with 100ng/mL LPS. Triplicate LPS treated wells were co-treated with 0.625, 1.25, 2.5, 5, or 10μM Sulforaphane. Three wells were left untreated as a negative control.

After 24 hours treatment 100μL media was transferred from wells into a 96 well plate. DMEM was used as a blank. A nitric oxide assay was performed by adding 50μL Griess reagent R1 and R2 to wells (R1 Cayman Chemicals # 170018, R2 Cayman Chemicals # 170020). The plate was incubated for 20 minutes at room temperature. The absorbance was read at 540nm in a BioRad Benchmark Plus plate reader.

**Migration Assay**

Cell culture transwell inserts (Falcon, 8μm pore size, 12 well format) were coated with 90μL of 100 μg/mL Type I collagen and allowed to dry completely. 100,000 bone marrow macrophages in 660μL of serum free αMEM were placed in the cell culture insert. For SFN treated samples, cells were pre-incubated with 5μM SFN for 10 minutes prior to plating. 1mL of media with the indicated chemoattractants was placed in the bottom chamber. Plates were incubated at 37°C at 5% $CO_2$ for 24 hours, after which media was aspirated from the inserts. The inserts were stained with Crystal Violet and washed with $dH_2O$. Non-migrating cells were removed from the interior of the insert with a KimWipe (Kimberly Clark

Professional). Migrated cells were viewed at 10x magnification and cells in 5 randomly selected fields were counted for each insert.

**Air pouch model of acute inflammation**

Wildtype C57Bl/6J mice bred in our colony at the University of Virginia or ordered from Charles River were used for air pouch experiments. Mice were sedated with isoflurane (Butler Schein) and 5mL of sterile filtered air was injected subcutaneously to form a dorsal pouch [124]. Three days following initial installation, pouches were re-inflated with 3mL sterile air. On day 7, the mice were pretreated with 100μg SFN (LKT Labs) in 500μl saline (or saline vehicle) by injection directly into the pouch. Following the 30 minute pre-treatment, acute inflammation was induced by injection of 10μg LPS in sterile saline, or saline vehicle into the pouch. Twenty-four hours later, cell infiltrates were harvested from the pouches by lavage using PBS (2x1mL), filtered through a 70uM pore filter to remove tissue debris, and immunophenotyped using flow cytometry. All animal experiments were done in compliance with guidelines instated by the University of Virginia Animal Care and Use Committee.

**Flow cytometry**

Flow cytometry was used to characterize the cellular infiltrates in the air pouch model. Cells were stained with a panel of markers including: LIVE/DEAD Fixable Red Dead Stain (Invitrogen), CD45 PerCP (clone 30-F11, BD Biosciences), CD11b Pacific Blue (M1/70.15, Invitrogen), F4/80 APC-eFluor780 (BM8,

eBioscience), and Gr1 Alexa Fluor-488 (RB6-8C5, Biolegend). CountBright counting beads (Invitrogen) were added to each sample to quantify total cell numbers for comparison. Populations were initially gated as live, CD45+, CD11b+ and were then defined as macrophages (F4/80+), monocytes ($CD11b^{int}GR1^{lo}$), or neutrophils ($CD11b^{hi}GR1^{hi}$ with gates set using fluorescence minus-one (FMO) controls (For example gating strategy, please see Figure 2.5). Data was collected on a CyAN ADP LX 9 Color Flow Cytometer (Beckman Coulter) and analyzed using FlowJo software (Treestar, Ashland, OR).

**Statistics**

Data were analyzed using one-way ANOVA. Significance was defined as $P<0.05$.

## Results

**Sulforaphane inhibits LPS-induced inflammatory activity *in vitro***

SFN has been shown to inhibit a number of pro-inflammatory cell signaling pathways in multiple systems *in vitro*. To confirm these studies, we examined the effects of SFN on both a macrophage cell line (Raw264.7) and on primary murine bone marrow derived macrophages (BMDMs). SFN pre-treatment of RAW264.7 cells inhibited LPS-induced TNFα induction at both the protein and mRNA level (Figure 2.1 A and B). Surprisingly, TNF-alpha mRNA and protein levels were increased by SFN pretreatment of BMDMs, suggesting post-transcriptional suppression of TNF $\alpha$ protein in this cell type (Figure 2.1C and D). Having seen this

discordant effect of SFN on BMDMs, increasing TNFα mRNA while reducing TNFα protein, we measured the effect of SFN on two other pro-inflammatory transcripts known to be targeted by SFN (Figure 2.1E and F). We found that SFN inhibited LPS-induced transcripts of both IL-6 and IL-12p35 in the BMDMs, as expected [25, 125, 126].

SFN has been shown to inhibit NF-κB activation and NO production [9]. To test this, we treated RAW264.7 cells with LPS and immunoblotted for components of the NF-κB pathway. LPS induced a dramatic increase in phosphorylation of both IκB-α and NF-κB p65 when compared to untreated controls (Figure 2.2A). Consistent with the published findings, SFN pre-treatment inhibited phosphorylation of both IκB-α and NF-κB p65. Treatment of RAW264.7 cells with SFN prior to LPS stimulation also dose-dependently decreased NO production as measured by Griess reaction (Figure 2.2B). Taken together, these data confirm that *in vitro* treatment with SFN inhibits signaling pathways that are important for inflammation.

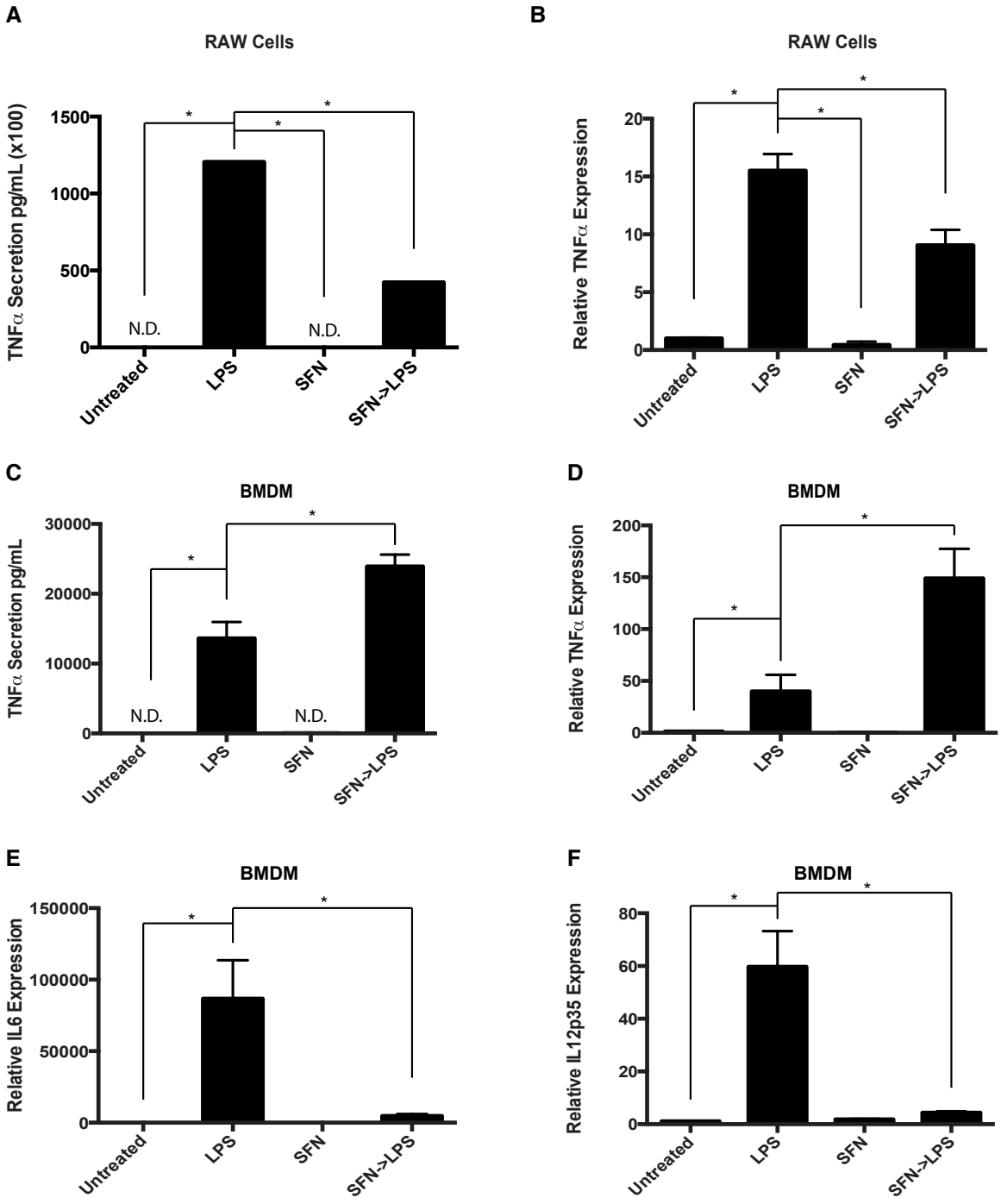


**Figure 2.1. SFN reduces LPS-induced cytokine production in RAW264.7 cells and BMDM.**

A and B) RAW264.7 cells were treated with SFN, LPS, or vehicle control alone or pre-treated with SFN for 30 minutes prior to LPS stimulation. Cell were incubated

either overnight followed by harvest of supernatants for detection of TNFα production (A) or for 4 hours for qRT-PCR (B). The LPS alone treated cells showed significant induction of TNFα compared to SFN or vehicle alone. SFN pre-treatment prior to LPS significantly reduced TNFα production. TNFα transcript levels were measured by quantitative real-time PCR and normalized to actin (B). C-F) Bone Marrow-Derived Macrophages were treated as in A and B above. SFN pre-treatment inhibited TNFα secretion (C). Quantitative RT-PCR showed a paradoxical increase in TNF $\alpha$ transcript after SFN pre-treatment, relative to LPS stimulation alone (D); however, IL-6 (E) and IL-12p35 (F) were inhibited by SFN pre-treatment. N= 3 per group. Results are displayed as the mean ± SEM. Significance is indicated by an asterisk and is defined as $p<0.05$.

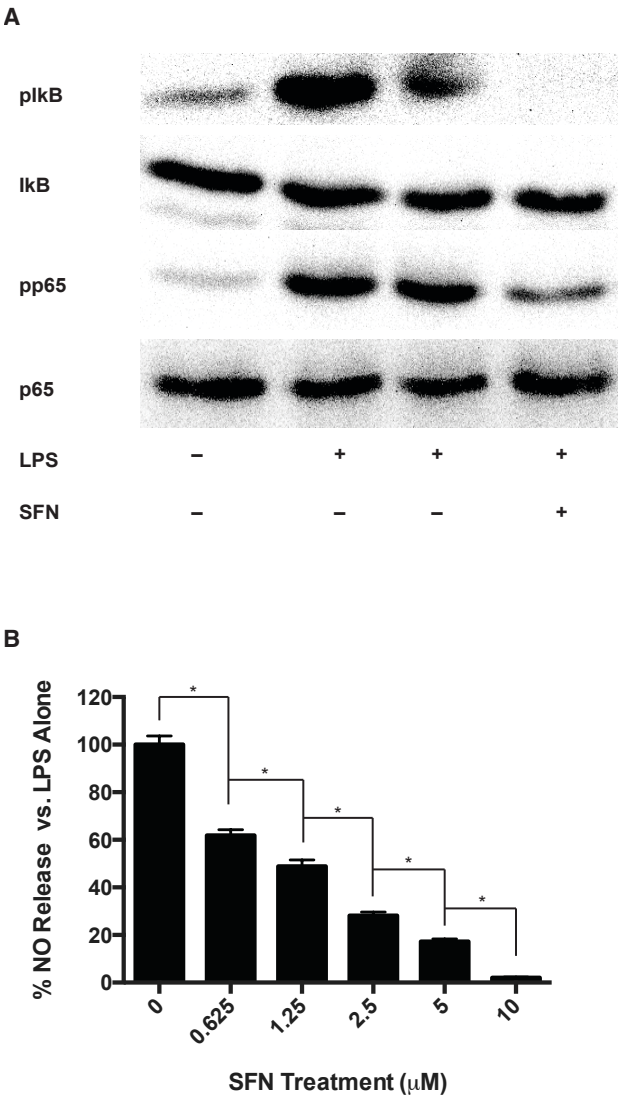


**Figure 2.2. SFN inhibits LPS-induced NF-κB signaling and NO production in RAW264.7 cells.**

A) RAW264.7 cells were treated with with SFN for 30 minutes followed by a 30 minute LPS stimulation. Lysates were probed for phospho-IκB, IκB, phosho-p65, and p65. SFN inhibited phosphorylation of p65 IκB. B) RAW264.7 cells were pre-treated with SFN for 30 minutes, and then incubated with LPS overnight. Nitrite concentration within the supernatants was determined by the Greiss reaction. SFN

pre-treatment dose-dependently abrogated LPS-stimulated NO production. Blots shown are representative of 3 separate experiments. N= 3 per group in the NO experiment. Results are displayed as the mean ± SEM. Significance is indicated by an asterisk and is defined as $p<0.05$.

**Sulforaphane inhibits macrophage migration *in vitro***

To test the potential effect of SFN on macrophage migration, we examined the migration of primary murine bone marrow-derived macrophages in a transwell chamber assay. CSF-1 (colony stimulating factor 1; M-CSF) significantly induced macrophage migration compared to media and SFN alone controls (Figure 2.3). Pre-treatment of BMDMs with SFN prior to initiating the migration assay resulted in a significant inhibition of migration toward CSF-1 (Figure 2.3), suggesting that SFN inhibits macrophage migration in response to CSF-1.

**Effect of Sulforaphane on LPS induced immune cell infiltrates in an *in vivo* model of acute inflammation**

Having demonstrated that SFN is able to effectively inhibit multiple pro-inflammatory signaling responses, as well as CSF-1 induced migration *in vitro* (Figures 2.1-2.3), we set out to test the effects of SFN on the acute inflammatory response *in vivo*. Specifically, we tested the impact of SFN on LPS-induced immune cell infiltration in the murine dorsal air pouch model. This model has been used in many studies to mimic endogenous inflammatory responses, for example to mimic the joint synovium, to study inflammatory joint diseases, and for evaluation of drug delivery [64, 124, 127]. We chose this model since it provides a simple approach to evaluate the effects of SFN on the number and composition of the immune cells that respond to an inflammatory stimulus.

Dorsal air pouches were introduced and allowed to mature, and then were pre-treated with either SFN or a vehicle control for 30 minutes, following which

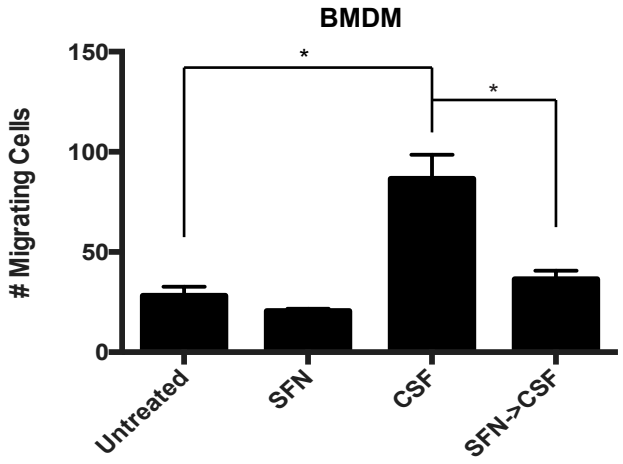


**Figure 2.3. SFN inhibits BMDM migration *in vitro*.**

Untreated or SFN-treated BMDM were seeded in transwells migrating toward media alone or CSF-1 and incubated for 24 hours. Transwells were then stained with Crystal Violet and migrated cells were counted in 5 fields per well. CSF-1 induced significant migration of untreated cells compared media alone. Pre-treatment of cells with SFN significantly decrease in the number of cells that migrated toward CSF-1. N=3 per condition. Results are displayed as the mean ± SEM and are representative of 2 independent experiments performed in triplicate. Significance is indicated by an asterisk and is defined as $p<0.05$.

inflammation was induced with LPS. Twenty-four hours later, we harvested cellular infiltrates from the air pouch by saline lavage (Figure 2.4A and B). The recovered infiltrating cells were counted and then stained for flow cytometry analysis.

LPS induced a significant increase in the number of cells recovered from the dorsal air pouch (Figure 2.4C). Unexpectedly, SFN did not reduce the number of inflammatory cells from LPS-stimulated air pouches. In fact, while not reaching statistical significance, pre-treatment with SFN somewhat increased the number of cells that were recovered in the pouch lavage following LPS induction. SFN treatment in the absence of LPS did not significantly increase or reduce the number of infiltrating cells compared to vehicle control (Figure 2.9G).

Given the effects of SFN pre-treatment that we observed on signaling and migration of macrophages using our *in vitro* assays, we examined the myeloid lineage within the pouch infiltrate by immunophenotype analysis. To this end, cells were stained with a panel of markers including CD45, a pan leukocyte marker, followed by CD11b, GR1 and F4/80 to allow enumeration of myeloid cell subpopulations. The gating strategy employed for these assays is shown in Figure 2.5.

We first examined the effect of SFN pretreatment on LPS-induced infiltration of F4/80+ macrophages into the pouch. Flow plots of CD11b+F4/80+ cells showed very similar populations from the LPS treated animals whether pre-treated with SFN or not (Figure 2.6A). Quantification of the relative percentage of infiltrating CD11b+F4/80+ macrophages confirmed that the SFN-pretreatment had no significant effect on the percent of infiltrating macrophages compared to LPS alone

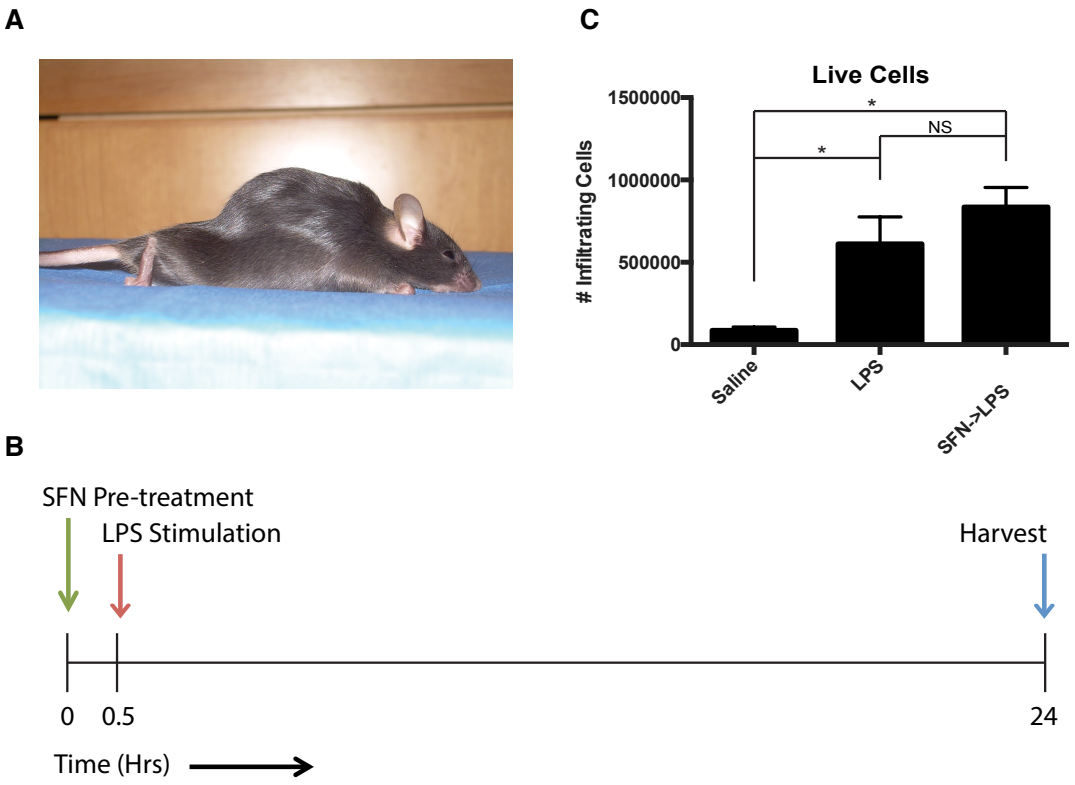


**Figure 2.4. SFN pre-treatment prior to LPS stimulation in the air pouch model does not impact the total number of infiltrating cells.**

Air pouches were installed in C57Bl/6 mice (A) and pre-treated with SFN or vehicle control for 30 minutes prior to 24 hours of LPS stimulation (B). Infiltrating immune cells were collected from the pouches and quantified during FACS analysis using counting beads and a LIVE/DEAD stain (C). LPS induced a large cellular infiltrate when compared to saline control; however, SFN pre-treatment did not significantly change the cell number compared to LPS alone. N=10-13 per treatment, pooled from 3 independent experiments. Results are displayed as the mean ± SEM. Significance is indicated by an asterisk and is defined as $p<0.05$.

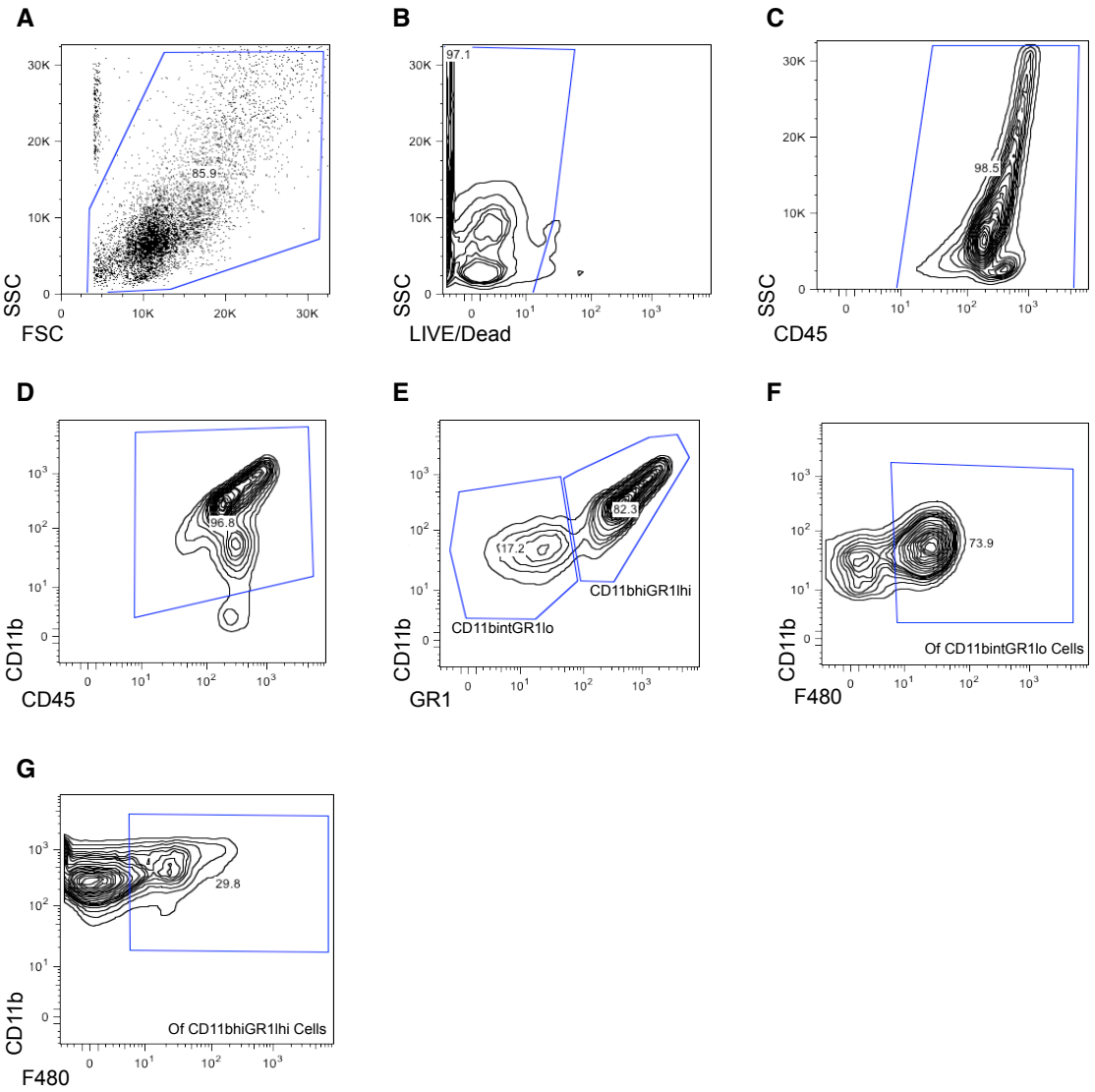


**Figure 2.5. Flow cytometry gating strategy.**

Cells from air pouch lavages were stained with the markers LIVE/DEAD Fixable Red Dead Stain, CD45, CD11b, F4/80, and Gr1. A leukocyte gate was drawn in the forward and side scatter plot to eliminate debris. Within that gate live, CD45+, CD11b+ cells were sequentially identified and gated. Those cells were then analyzed as either CD11b+F4/80+, $CD11b^{int}GR1^{lo}$, or $CD11b^{hi}GR1^{hi}$ cells.

(Figure 2.6B), though LPS treatment resulted in a non-significant decrease compared to saline treated mice. Examination of the total number of infiltrating CD11b+F4/80+ cells demonstrated that LPS induced a significant increase in the macrophage infiltration compared to saline. Rather than inhibiting the number of infiltrating CD11b+F4/80+ cells as expected, SFN pre-treatment prior to LPS stimulation did not significantly change the number of infiltrating cells. In fact, the SFN pre-treatment, trended toward increasing this population of cells in the LPS treated animals (Figure 2.6C).

We next expanded our analysis to include an examination of myeloid cell populations using CD11b, GR1, and F4/80 surface markers. In vehicle treated mice, the myeloid compartment in the pouch infiltrate was composed predominantly of $CD11b^{int}GR1^{lo}$ cells, with a minor population of $CD11b^{hi}GR1^{hi}$ cells (representative flow plots are shown in Figure 2.7A with and quantification in Figure 2.7B and C). This quantification shows that LPS treatment resulted in a greater fraction of the $CD11b^{hi}GR1^{hi}$ population with a concomitant reduced fraction of $CD11b^{int}GR1^{lo}$ cells in the lavage fluid. SFN pre-treatment before LPS further shifted the fraction of cells found in the predominant $CD11b^{hi}GR1^{hi}$ population and reduced the contribution from $CD11b^{int}GR1^{lo}$ cells (Figure 2.7D and E). While the changes in relative populations of these cells could indicate a SFN-dependent decrease in infiltration of $CD11b^{int}GR1^{lo}$ cells, enumeration of the abundance of each cell type showed that LPS induced a significant increase in the absolute number of both infiltrating $CD11b^{int}GR1^{lo}$ and $CD11b^{hi}GR1^{hi}$ regardless of SFN pre-treatment (Figure 2.7D and E).

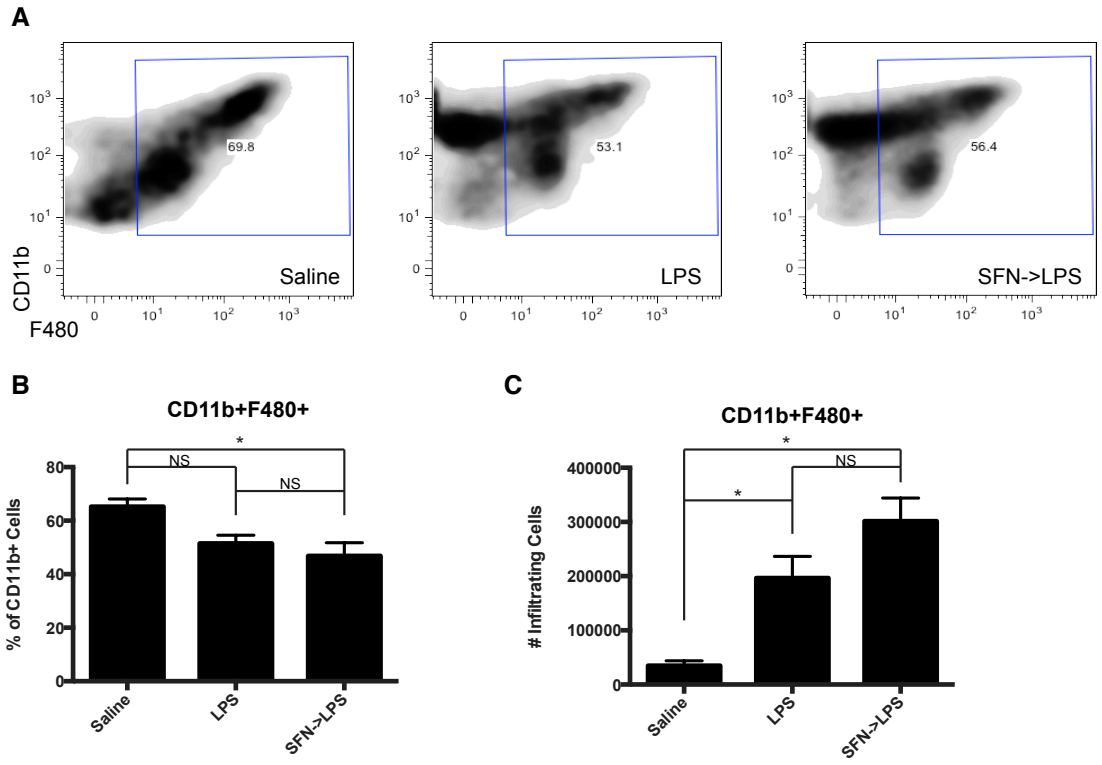


**Figure 2.6. SFN does not alter F4/80 macrophage infiltration in response to LPS stimulation *in vivo*.**

Mice were treated as depicted in figure 4B. Macrophages were identified as CD45+CD11b+F480+ cells by FACS; representative density plots are shown in (A). Quantitation of the percent F480+ macrophages (B) and the total number (C) demonstrated no significant differences between LPS and SFN pre-treated groups. N=5 for figure B and is representative of 3 independent experiments. N= 10-13 in figure C and data are pooled from 3 independent experiments per treatment. Results are displayed as the mean ± SEM. Significance is indicated by an asterisk and is defined as $p<0.05$.

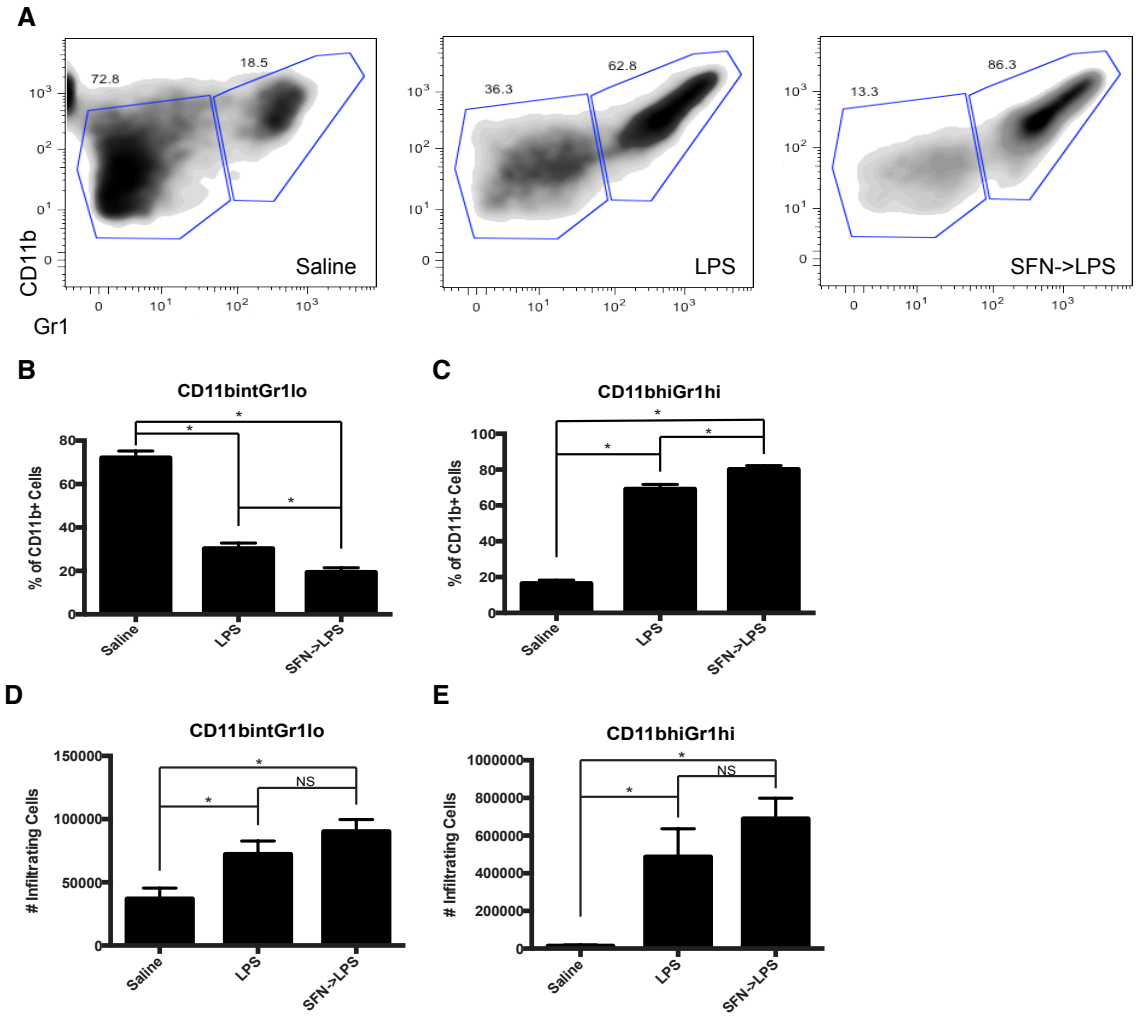


**Figure 2.7. SFN alters the percentages of CD11b$^{int}$GR1$^{lo}$ and CD11b$^{hi}$GR1$^{hi}$ cells in the air pouch following LPS treatment.**

Mice with air pouches were treated with of saline, 10µg LPS, or 100µg SFN 30 minutes before 10µg LPS, followed by incubation for 24 hours. Infiltrate was collected and stained for analysis by flow cytometry. Representative density plots of CD11b and Gr1 cell surface markers show the differences in CD11b$^{int}$GR1$^{lo}$ and CD11b$^{hi}$GR1$^{hi}$ cells in the air pouch (A). LPS alone decreased the percentage of CD11b$^{int}$GR1$^{lo}$ cells (B) and increased the percentage of CD11b$^{hi}$GR1$^{hi}$ cells (C) compared to saline control. SFN pre-treatment prior to LPS stimulation shifted

those populations even more than LPS alone. The absolute numbers of cells in these two populations (D and E) demonstrated significant increases in both between saline and LPS alone groups. SFN pre-treatment trended toward a further increase in both over LPS alone, but was not statistically significant. N=5 for figure B and is representative of 3 independent experiments. N= 10-13 in figure C and data are pooled from 3 independent experiments per treatment. Results are displayed as the mean ± SEM. Significance is indicated by an asterisk and is defined as $p<0.05$.

In fact, while not reaching statistical significance, SFN further increased the number of both cell types in the pouch lavage.

Finally, given that SFN inhibited macrophage migration *in vitro*, we examined the F4/80 status of the $CD11b^{int}GR1^{lo}$ and $CD11b^{hi}GR1^{hi}$ cell populations. As with the total F4/80 populations, these subpopulations did not differ in the LPS treated animals between the vehicle and SFN pre-treatment groups (Figure 2.8). Taken together, our data suggest that in this model, SFN does not exhibit the expected anti-inflammatory effect(s) on LPS induced cellular infiltrates. Instead, SFN trended towards enhancing the LPS-dependent effects on inflammatory cells numbers in the dorsal air pouch.

**A potential pro-inflammatory effect of SFN alone on immune cell infiltrates?**

Given these unexpected observations, we examined the effect of SFN alone on the pouch infiltrate. When compared to saline vehicle control, SFN induced a shift in the composition of the myeloid cell infiltrate, increasing the relative abundance of $CD11b^{hi}GR1^{hi}$ while decreasing the $CD11b^{int}GR1^{lo}$ cells (Figure 2.9A and C). Though the nature of this effect was similar to that seen with LPS treatment, the magnitude was far less. Moreover, while LPS induced a significant increase in the total cell number infiltrating the pouch, SFN alone did not (Figure 2.9G). However, when the total cell numbers were taken into account, SFN alone did induce a very modest, but statistically significant increase in $CD11b^{hi}GR1^{hi}$ and a trend towards an increased number of the other cell types examined as well (Figure 2.9B and D). SFN alone did not have any effect on infiltration of F4/80+ macrophages (Figure 2.9E and F).

Taken together, these observations argue against an anti-inflammatory role for SFN, and may in fact support a modest pro-inflammatory impact in this model.

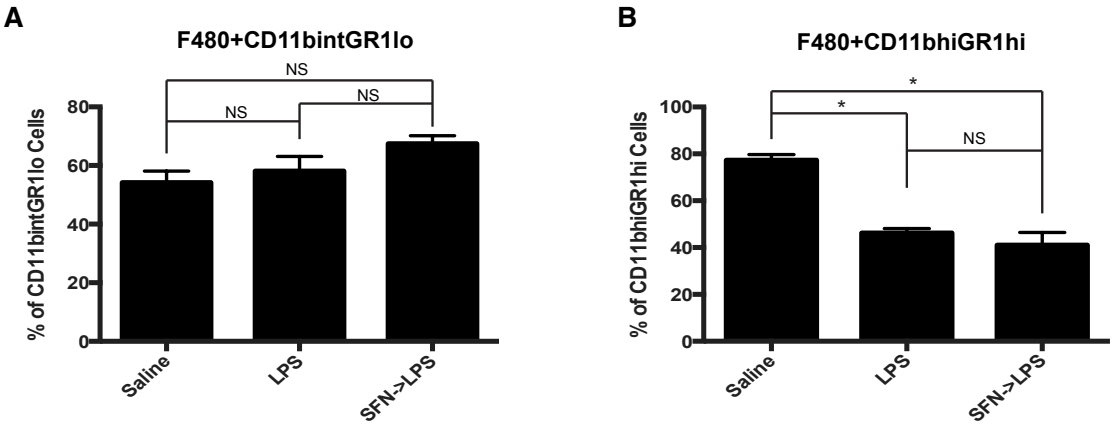


**Figure 2.8. SFN does not affect the F4/80 positivity within the CD11b$^{int}$GR1$^{lo}$ and CD11b$^{hi}$GR1$^{hi}$ cells that infiltrate the air pouch in response to LPS.**

FACS analysis was used to determine the F4/80 positivity of the CD11b$^{int}$GR1$^{lo}$ and CD11b$^{hi}$GR1$^{hi}$ cells that infiltrated into the air pouch after a 30 minute pre-treatment with SFN or saline prior to LPS stimulation for 24 hours. No significant differences were observed in the percentage of F4/80+ cells between the LPS alone and SFN pre-treated groups. The percent of F4/80+ cells within the CD11b$^{hi}$GR1$^{hi}$ population was significantly higher in the saline group compared to the LPS or SFN groups. N=5 per group. Results are displayed as the mean ± SEM. Significance is indicated by an asterisk and is defined as $p<0.05$.

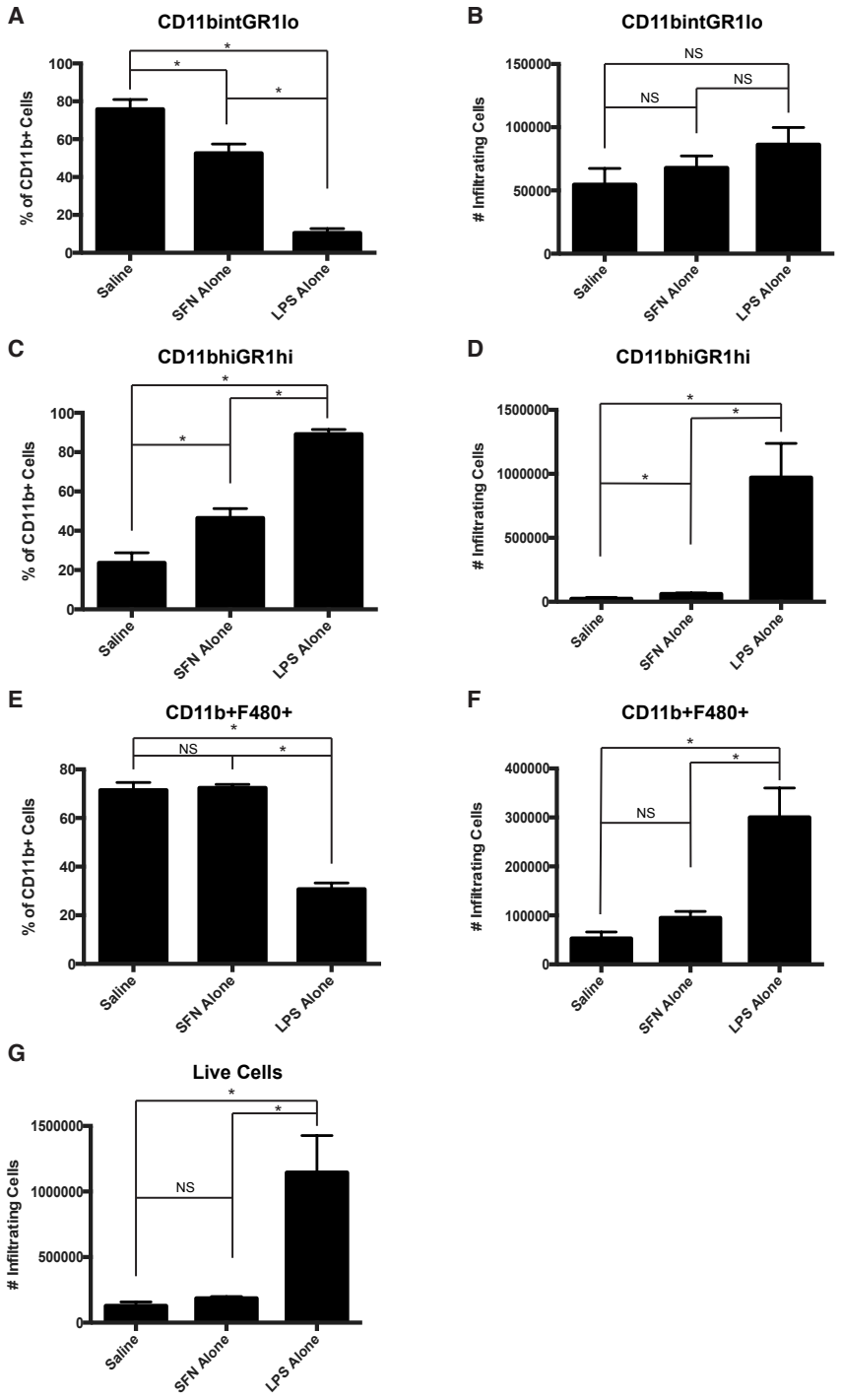


**Figure 2.9. SFN alone has a modest effect on immune cell infiltration in the air pouch.**

Mice with air pouches were treated with saline, SFN, or LPS alone for 24 hours prior to analysis of immune cell infiltrate. SFN alone had an intermediate effect on the

percent infiltration of $CD11b^{int}GR1^{lo}$ and $CD11b^{hi}GR1^{hi}$ cells compared to LPS and saline (A and C). There were no significant differences in the absolute number of $CD11b^{int}GR1^{lo}$ cells between any treatment groups (B). There was a small, but significant, increase in the number of infiltrating $CD11b^{hi}GR1^{hi}$ cells in the SFN group compared to the saline controls (D). No differences were seen between the saline and SFN groups in either percentage or number of infiltrating CD11b+F4/80+ macrophages (E and F). The total number of live cells was also not different between the saline and SFN treated groups and was much smaller than that of the LPS treated mice (G). N=5 for figure B and is representative of 3 independent experiments. N= 10-13 in figure C and data are pooled from 3 independent experiments per treatment. Results are displayed as the mean ± SEM. Significance is indicated by an asterisk and is defined as $p<0.05$.

## Discussion

While using a macrophage cell line and *ex-vivo* BMDMs we found that SFN blocked classic inflammatory signals (TNFα production, NF-κb activation, and NO production). In contrast, using a model of acute inflammation in mice, we found that SFN pre-treatment prior to LPS stimulation does not inhibit the development of the inflammatory cell infiltrate that would be suggested by these *in vitro* effects. In fact, SFN trended towards exacerbating the LPS induced inflammatory response, as well as exhibiting a modest but statistically significant pro-inflammatory effect when administered on its own. This was reflected in a relative increase in the $CD11b^{hi}GR1^{hi}$ neutrophil population with a corresponding decrease in the percentage of infiltrating $CD11b^{int}GR1^{lo}$ monocytes when compared to LPS alone. Owing to a high degree of variability in the total cell numbers infiltrating the air pouches the differences in the absolute number of neutrophils and monocytes between the LPS alone and SFN pre-treated groups failed to reach statistical significance. However, there was a strong trend toward SFN pre-treatment promoting more neutrophil and monocyte infiltration than LPS alone.

While our studies focused on a quantification of the cellular infiltrates, they did not consider the potential impacts of SFN on the function of the cells that respond to LPS stimulation. SFN has been demonstrated to enhance the phagocytic activity of peritoneal macrophages in Balb/c mice, while concomitantly reducing the TNFα secretion of those cells [21]. *In vitro* SFN pre-treatment of primary and HL60 neutrophils results in a reduced ROS response to PMA [20]. SFN is also able to inhibit ROS production of immune cells in HSV-infected brains of Balb/c mice [116].

Follow up studies could include functional analyses, such as quantification of ROS production or phagocytic ability, to determine whether the altered ratios of cells in the air pouch result in altered containment or resolution of the acute inflammatory response.

Macrophage infiltration was examined by F4/80 FACS analysis. Based on the ability of SFN to inhibit CSF-1-induced BMDM migration *in vitro* (Figure 2.3), we hypothesized that SFN pre-treatment prior to LPS *in vivo* would block macrophage infiltration into the pouch. Instead, we observed similar macrophage infiltration in response to LPS, at 24 hours post-stimulus, regardless of SFN-pretreatment. While not statistically significant, we did observe a trend toward an increase in F4/80 cells at the 24 hour time point. Given that macrophages are critical to the resolution of infection, this trend begs the question of whether there may be a more evident effect on the macrophage population later in the resolution stage.

Finally, we acknowledge the technical limitations of the air pouch model itself, which could be a factor in the unexpected effects that we observe. While the murine air pouch model has been used for decades as a model for diseases such as RA, it should be noted that it creates an artificial space that is then experimentally manipulated to mimic an acute inflammatory response. This distended subcutaneous space may respond differently to inflammatory stimuli than does a natural space such as the joint. In addition, we administered SFN directly into the air pouch to maximize bioavailability and to most closely mimic the *in vitro* treatment of cells with the agent. Therefore, our study models a local treatment with the intent of manipulating a local immune response. It is possible that a systemic delivery of

SFN may have yielded a different outcome. However, it is important to note that in human applications, more systemic modes of administration could lead to more unintended systemic effects.

Our *in vivo* data were surprising given that we confirmed several inhibitory effects of SFN on inflammatory signaling pathways *in vitro*. Specifically, we demonstrated that SFN inhibits LPS-induced NF-κB activation, TNFα and NO production, as well as blocking CSF-1-induced macrophage migration (Figures 2.1-2.3). These *in vitro* data are consistent with a large body of literature that uses similar *in vitro* effects to suggest potential therapeutic utility of SFN as an anti-inflammatory agent. However, the *in vivo* effects of SFN are supported by only a few studies. Youn *et al*. demonstrated that SFN pre-treatment reduced inflammation induced by intradermal injection of LPS, as measured by ear weight and H&E histology [25]. While the inflammatory cell response was not specifically examined in this study, these results are consistent with an anti-inflammatory effect of SFN. Kong *et al*. examined potential anti-inflammatory effects of SFN in a murine model of rheumatoid arthritis (RA). They demonstrated that *in vitro* treatment with SFN reduces synovial hyperplasia by inducing apoptosis in synoviocytes [15]. Similarly, Fragoulis *et al*. demonstrated that SFN induces apoptosis in TNFα-stimulated synoviocytes, but not in unstimulated synoviocytes [128].

In contrast, a handful of studies have reported potentially pro-inflammatory effects of SFN *in vivo*. Healthy Balb/c mice given intraperitoneal (IP) treatments of SFN had an overall increase in white blood cell count and an increase in bone marrow cellularity. These mice were also shown to have enhanced phagocytic

activity of peritoneal macrophages and increased levels of antigen-specific circulating antibodies upon antigen challenge [21]. A study in a model of B16 melanoma showed that IP administration of 500μg SFN enhanced NK cell lytic activity and increased serum levels of IL2 and IFNγ [129]. Shin *et al*. showed that SFN is able to increase class II-restricted antigen presentation by dendritic cells and peritoneal macrophages both *in vitro* and *in vivo* (20μM SFN subcutaneous administration) [130]. Oral administration of SFN (0.9μmol/day in 0.2mL) resulted in an increase of the Th1 response to contact hypersensitivity in older mice [131]. Taking these studies into account, SFN appears to be able to enhance the immune response in certain models regardless of the route of administration.

Together with our observations that SFN does not prevent, and may actually exacerbate LPS-induced inflammatory cell infiltrations in a simple model acute of inflammation, as well as having subtle pro-inflammatory effects on its own, these studies may encourage caution in application of SFN as an *in vivo* anti-inflammatory agent. As a naturally occurring compound that is also a chemopreventive agent, SFN is an attractive potential inflammatory drug. However, further studies are clearly needed to better elucidate the mechanisms by which SFN manipulates the immune system.

**Acknowledgements**

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# Chapter 3: MIF in the Murine Air Pouch Response to Acute Inflammation

## Introduction

MIF has been shown to be a critical factor in the acute inflammatory response. Bozza *et al*. demonstrated that $MIF^{-/-}$ mice are protected from the massive inflammatory infiltrate that is associated with sepsis [61]. This was corroborated by studies that demonstrated a similar effect using MIF blocking antibodies [60, 62]. SFN is a very effective inhibitor of the N-terminal tautomerase domain of MIF. We hypothesized that the tautomerase domain of MIF is critical for the acute response and that SFN could effectively inhibit the cellular response to an acute inflammatory stimulus such as LPS.

## Methods

### Recombinant MIF Preparation

Recombinant MIF was prepared using a modified version of the method published by Berhagen *et al.* [41]. In brief, BL-21 E. coli cells carrying pET MIF expression plasmid were cultured until the OD at 600nm reached 0.9. The culture was induced with 45mM IPTG and was incubated at 25°C overnight. The culture was pelleted by centrifugation and was frozen at -20°C. The bacteria were lysed in tris buffer with protease inhibitors (50mM Tris, 150mM NaCl, pH 7.5, 5µg/mL aprotinin, 5µg/mL leupeptin) by cycles of sonication on ice. The suspension was filtered through 0.45µm followed by 0.22µm filters. The filtrate was passed over a Q-sepharose column and was chased with tris buffer. The flow-through was diluted 1:8 with deionized water and was adsorbed onto a reverse phase columns

(Phenomenex Strata-X, 33µm, 200mg/3mL, catalog # 8B-S100-FBJ). The columns were washed with 20% acetonitrile. The column was eluted with 60% acetonitrile.

The eluted protein was vacuum dried and denatured in 8M urea in 20mM sodium phosphate buffer pH 7.2 with 5mM DTT. The denatured protein was placed into dialysis tubing (Thermo SnakeSkin, 3,500 MWCO, catalog # 68035) and were dialyzed multiple times against 20mM sodium phosphate buffer pH 7.2 with 5mM DTT at 4°C followed by 20mM sodium phosphate buffer pH 7.2.

1mM DTT was added, and the protein was split into two fractions. The rMIF was treated with 750µM sulforaphane or buffer for 3hr at RT. The proteins were dialyzed multiple times against 20mM sodium phosphate buffer pH 7.2 with 1mM DTT. The dialyzed protein was concentrated centrifugally using 3,000 KDa MWCO filters (Millipore Amicon Ultra Ultracel, cat # UFC800308).

Protein concentration was determined using Bradford protein assays. The purity and concentration was confirmed by coomasie gel. Tautomerase assays were performed to confirm activity in the purified protein and confirm the inhibition of the sulforaphane treatment. The protein was stored at 4°C until use.

**Tautomerase Assay**

Tautomerase activity was measured *in vitro* by detecting the tautomerization of L-dopachrome methyl ester (DCME) to 5,6-dihydroxyindole-2-corboxylic acid methyl ester, as previously described [26]. A 1:1 mixture of 8mM $NaIO_4$ (sodium periodate) (Sigma) and 4mM L-3.4-dihydroxyphenylalanine methyl ester hydrochloride (DOPA) (Sigma) was prepared and diluted 1:9 in tautomerase buffer

(25mM phosphate buffer with 1mM EDTA, pH6). Tautomerization is measured by a decolorization reaction quantified on a plate reader at 475nm every 10 second for 5 minutes. Data are plotted as negative velocity compared to a protein blank control to account for spontaneous decolorization. RAW cell and pouch lining protein extracts were prepared by lysis in MLB (MOPS lysis buffer: 50mM Mops pH7, 250mM NaCl, 5mM EDTA, 0.1% NP/40) plus protease inhibitors (2.5μg/mL aprotinin and 2.5μg/mL leupeptin) for 30 or 60 minutes, respectively, and normalized to 50μg using concentrations determined by Bradford assay.

**Bone Marrow-Derived Macrophage Preparation**

Femurs were harvested from mice and sterilized in 70% ethanol for 2 minutes. Bone marrow was isolated by lavage of the bone cavity with PBS. Cells were red cell lysed and plated at two million cells per 10cm plate. Media was replaced on day 3 and cells were collected for use on day 7.

**Migration Assay**

Cell culture transwell inserts (Falcon, 8μm pore size, 12 well format) were coated with 90μL of 100 μg/mL Type I collagen and allowed to dry completely. 100,000 bone marrow macrophages in 660μL of serum free αMEM were placed in the cell culture insert. For SFN treated samples, cells were pre-incubated with 5μM SFN for 10 minutes prior to plating. 1mL of media with the indicated chemoattractants was placed in the bottom chamber. Plates were incubated at 37°C at 5% $CO_2$ for 24 hours, after which media was aspirated from the inserts. The

inserts were stained with Crystal Violet and washed with $dH_2O$. Non-migrating cells were removed from the interior of the insert with a KimWipe (Kimberly Clark Professional). Migrated cells were viewed at 10x magnification and cells in 5 randomly selected fields were counted for each insert.

**Air pouch model of acute inflammation**

Wildtype C57Bl/6J mice bred in our colony at the University of Virginia or ordered from Charles River were used for air pouch experiments. Mice were sedated with isoflurane (Butler Schein) and 5mL of sterile filtered air was injected subcutaneously to form a dorsal pouch [124]. Three days following initial installation, pouches were re-inflated with 3mL sterile air. On day 7, the mice were pretreated with 100µg SFN (LKT Labs) in 500µl saline (or saline vehicle) by injection directly into the pouch. Following the 30 minute pre-treatment, acute inflammation was induced by injection of 10µg LPS in sterile saline, or saline vehicle into the pouch. Twenty-four hours later, cell infiltrates were harvested from the pouches by lavage using PBS (2x1mL), filtered through a 70uM pore filter to remove tissue debris, and immunophenotyped using flow cytometry. All animal experiments were done in compliance with guidelines instated by the University of Virginia Animal Care and Use Committee.

**Flow cytometry**

Flow cytometry was used to characterize the cellular infiltrates in the air pouch model. Cells were stained with a panel of markers including: LIVE/DEAD

Fixable Red Dead Stain (Invitrogen), CD45 PerCP (clone 30-F11, BD Biosciences), CD11b Pacific Blue (M1/70.15, Invitrogen), F4/80 APC-eFluor780 (BM8, eBioscience), and Gr1 Alexa Fluor-488 (RB6-8C5, Biolegend). CountBright counting beads (Invitrogen) were added to each sample to quantify total cell numbers for comparison. Populations were initially gated as live, CD45+, CD11b+ and were then defined as macrophages (F4/80+), monocytes ($CD11b^{int}GR1^{lo}$), or neutrophils ($CD11b^{hi}GR1^{hi}$ with gates set using fluorescence minus-one (FMO) controls (For example gating strategy, please see Supplementary Figure 1). Data was collected on a CyAN ADP LX 9 Color Flow Cytometer (Beckman Coulter) and analyzed using FlowJo software (Treestar, Ashland, OR).

### Statistics

Data were analyzed using one-way ANOVA. Significance was defined as $P<0.05$.

## Results

### SFN inhibits MIF tautomerase activity *in vitro*

As we have previously reported, SFN covalently modifies MIF at its N-terminal proline, inhibiting its enzymatic keto-enol tautomerase activity [26]. We confirmed that treatment of RAW264.7 cells with SFN dose-dependently inhibits MIF tautomerase activity (Figure 3.1A). In parallel, recombinant MIF protein was treated with SFN, the excess SFN removed, and the tautomerase activity assessed

compared to untreated MIF. Our data demonstrate that SFN completely blocks the tautomerase activity of MIF (Figure 3.1B).

**SFN Inhibits MIF-induced BMDM migration *in vitro***

To determine the effect of SFN on the ability of MIF to regulate macrophage migration, we examined the migration of primary murine bone marrow-derived macrophages (BMDM) in a transwell chamber assay. First, we showed that addition of SFN to MIF-containing media in the bottom chemotactic chamber blocks macrophage migration to the same extent as adding a MIF-neutralizing antibody, and that both treatments reduce migration to a level seen with serum-free media alone (Figure 3.2A). To confirm that the effects of SFN on BMDM migration were due to modification of MIF, rather than some other effect, we prepared rMIF protein inhibited by pre-incubation with SFN followed by removal of excess agent. This SFN-inhibited, tautomerase inactive MIF is significantly impaired in induction of BMDM migration. These data establish that inhibition of BMDM migration by SFN is due to its modification and inhibition of MIF. Moreover, they suggest that the tautomerase activity of MIF is important for its ability to induce macrophage migration and that SFN may be a good potential therapeutic for reducing macrophage migration during an acute immune response.

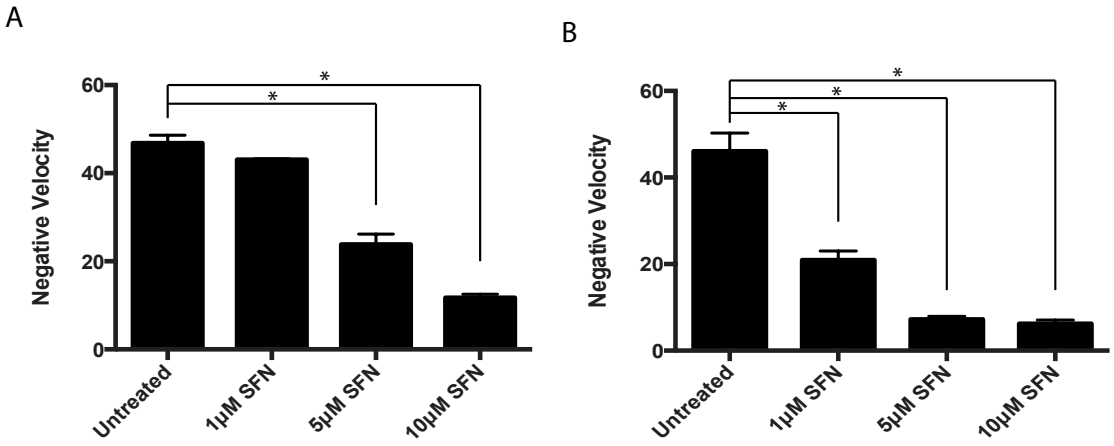


**Figure 3.1. SFN inhibits MIF tautomerase activity *in vitro*.**

A) RAW cells were stimulated with vehicle control or varying doses of SFN for 30 minutes. Protein lysates were normalized and assayed for tautomerase activity. SFN dose-dependently inhibited MIF tautomerase activity. B) Recombinant mouse MIF was treated with SFN and compared to untreated rmMIF or no protein control in a tautomerase assay. SFN treatment of rmMIF completely abolished tautomerase activity. N= 3-5 per group. Results are displayed as the mean ± SEM. Significance is indicated by an asterisk and is defined as $p<0.05$.

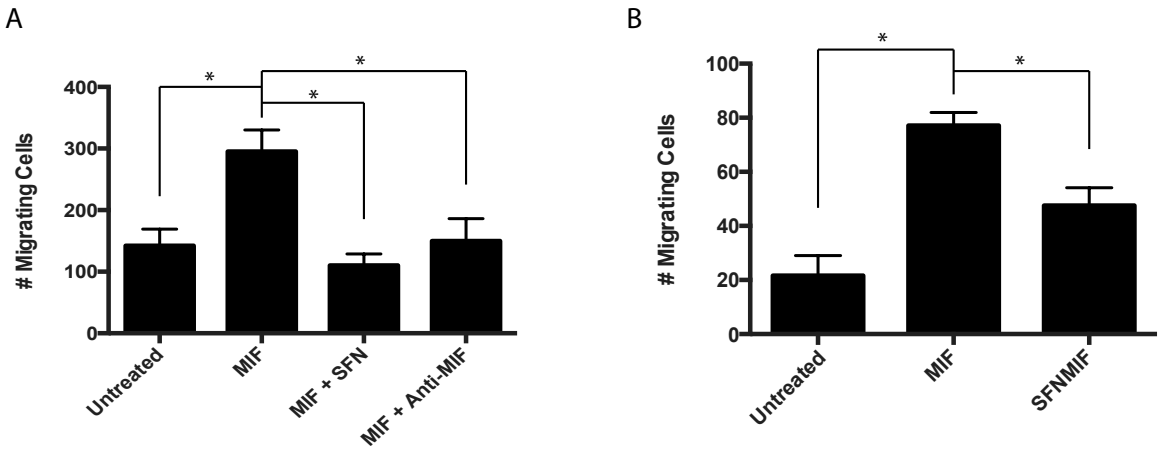


**Figure 3.2. SFN diminishes MIF-induced BMDM migration *in vitro*.**

A) Migration assays were set up in transwell dishes. BMDM were seeded into the top chamber to migrate toward media alone, 500ng/mL MIF, 500ng/mL MIF + 5μM SFN, or 500ng MIF + a neutralizing MIF antibody in the bottom well. MIF induced significant migration of BMDM compared to the control. The addition of either SFN or anti-MIF antibody abolished BMDM migration toward MIF. B) BMDM were seeded in transwell dishes to migrate toward media alone, 500ng/mL MIF, or 500ng/mL SFN-treated MIF. MIF induced BMDM migration that was blocked when the MIF was treated with SFN to abolish tautomerase activity. N= 4-6 per group. Results are displayed as the mean ± SEM. Significance is indicated by an asterisk and is defined as $p<0.05$.

**SFN inhibits MIF tautomerase activity *in vivo***

We next examined the effect of SFN on MIF tautomerase activity in an *in vivo* model, using the murine air pouch model of acute inflammation induced by LPS. Mouse air pouches were pre-treated with either saline control or 100ug SFN for 30 minutes prior to LPS stimulation. After 24 hours, we measured tautomerase activity in the pouch lining. LPS treated pouches exhibited similar tautomerase activity as saline controls. SFN inhibited tautomerase activity in both saline and LPS stimulated pouches, even 24 hours after SFN administration (Figure 3.3A). The inhibition of tautomerase activity by SFN was dose responsive (Figure 3.3B). Importantly, although other tautomerases exist in murine cells, the activity measured in this assay was entirely due to MIF, as no tautomerase activity was detected in the pouch lining of MIF knockout animals using this assay conditions (data not shown). Taken together, these data show that SFN is able to inhibit MIF tautomerase activity *in vivo*.

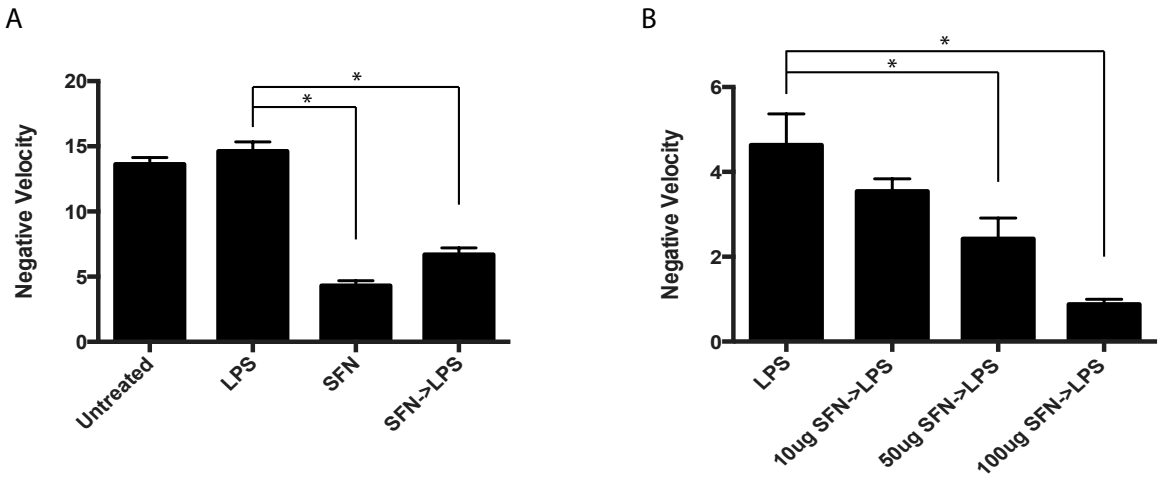


**Figure 3.3. SFN inhibits MIF tautomerase activity *in vivo*.**

Protein was isolated from the air pouch lining of mice and analyzed by *in vitro* tautomerase activity assay. A) Pouches were treated with saline, 10μg LPS alone, 100μg SFN alone, or 100μg SFN prior to 10μg LPS and cellular infiltrates harvested from the pouch after 24 hours. SFN alone and SFN pre-treatment to LPS stimulation both significantly decreased tautomerase activity compared to vehicle or LPS alone. B) A dose response of SFN pre-treatment prior to LPS stimulation for 24 hours was performed in air pouches with treatments of 10μg SFN, 50μg SFN, or 100μg SFN compared to LPS alone. The tautomerase activity within the pouch lining was dose-dependently inhibited by SFN. N= 2-12 per group. Results are displayed as the mean ± SEM. Significance is indicated by an asterisk and is defined as $p<0.05$ and were determined using one-way ANOVA.

**The absence of MIF does not impact immune cell infiltration in response to LPS *in vivo***

In order to demonstrate that inhibition of MIF by SFN could be a putative treatment strategy, we started by examining whether MIF is required for the cellular response to acute inflammation. We installed air pouches in both MIF WT and $MIF^{-/-}$ mice and challenged them with LPS alone, LPS plus rmMIF, or LPS plus SFN-treated rmMIF. The expectation based on the literature was that the $MIF^{-/-}$ mice would not have a robust cellular infiltrate into the pouch because of their MIF deficiency [61, 62]. In this initial experiment we observed that the response to LPS in $MIF^{-/-}$ mice trended toward being reduced compared to WT controls. Adding rmMIF trended toward rescuing that effect, whereas SFN-treated MIF did not (3.4A and B).

However, following this initial experiment we were unable to reproduce similar results to the original effect of a blunted immune response to LPS in $MIF^{-/-}$ mice in which those mice were protected from the normally lethal effects of a high dose of LPS [61]. To confirm that we were seeing the same lack of difference between $MIF^{-/-}$ and MIF WT mice that Honma *et al*. demonstrated, we compared larger numbers of WT and $MIF^{-/-}$ mice that were only stimulated with LPS [63]. This simplified version of the original experiments resulted in the same amount of cellular infiltration in response to LPS in the $MIF^{-/-}$ mice as in the WT (Figure 3.4C-E). This led us to conclude that the basis for our study was flawed because MIF was not actually critical for the acute immune response to LPS in this model. In light of

this development, we shifted our attention onto further examination of the effect of SFN on the acute response independent of MIF.

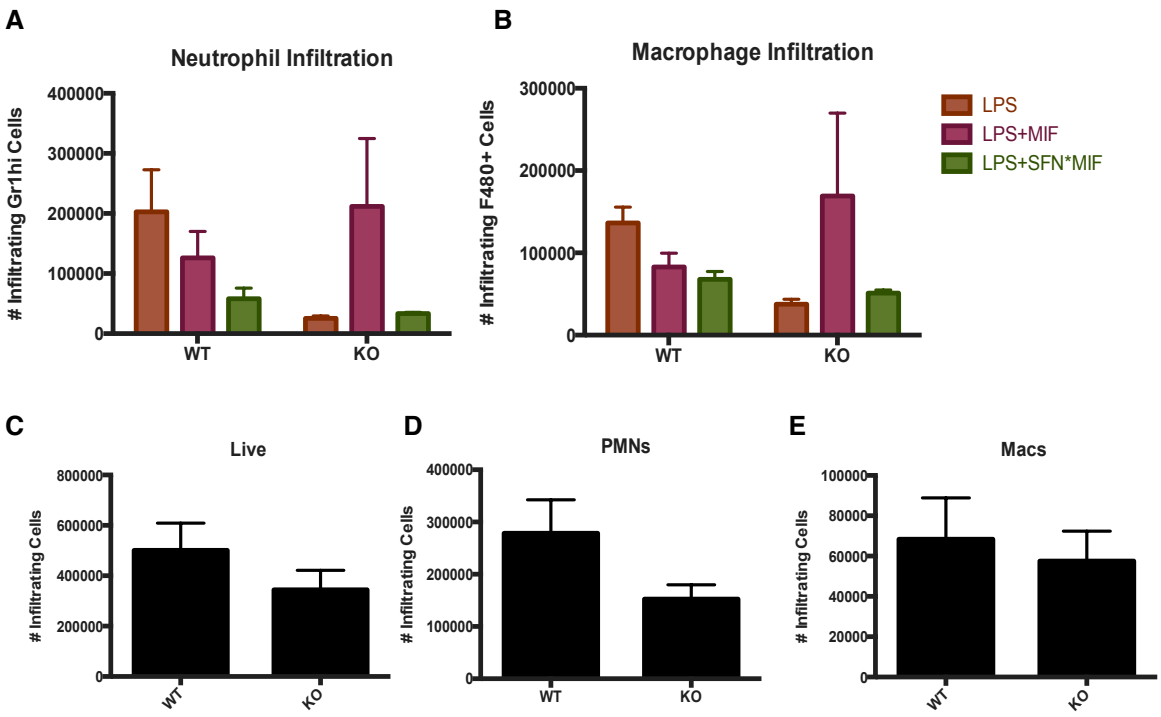


**Figure 3.4. LPS induces equivalent cellular infiltrates in MIF WT and MIF$^{-/-}$ mice.**

A and B) In a pilot experiment air pouches in MIF WT and MIF$^{-/-}$ mice were treated with LPS, rmMIF + LPS, or SFN-treated rmMIF (SFN*MIF) +LPS for 24 hours and infiltrating immune cells were quantified. High variation resulted in no significant differences, but there was a strong trend toward LPS inducing much smaller neutrophil (A) and macrophage (B) populations in the MIF$^{-/-}$ mice. N=4-5 per group. C-E) Repeat experiments in MIF WT and MIF$^{-/-}$ mice with air pouches treated solely with LPS were unable to produce any differences live (C), neutrophil (D), or macrophage populations. N= 5 per group and is representative of 3 independent experiments. Results are displayed as the mean ± SEM. Significance is indicated by an asterisk and is defined as $p<0.05$ and were determined using one-way ANOVA.

## Discussion

We approached this study based on our discovery of MIF as a target for SFN inhibition [26]. We hypothesized that SFN would exhibit anti-inflammatory properties similar to those previously observed with anti-MIF neutralizing antibodies or small molecule inhibitors [30, 62]. We demonstrate that *in vitro* modification of MIF by SFN inhibited MIF-induced migration of BMDM and that SFN administration inhibited MIF tautomerase activity *in vivo* in the pouch lining. Our intention to test *the in vivo* capacity of SFN to block MIF-induced macrophage infiltration into the pouch in response to LPS was thwarted by our inability to reproduce the published model that $MIF^{-/-}$ mice have a very reduced immune response to LPS stimulation compared to WT mice [61].

Others have shown that SFN treatment of peritoneal macrophages influences their phagocytic and cytokine secreting abilities [9, 21]; however, ours is the first report to show that SFN pre-treatment of BMDM inhibits MIF-induced BMDM migration. These *in vitro* data suggest that SFN could act as an anti-inflammatory compound by targeting MIF, specifically the tautomerase domain. Al-Abed *et al.* demonstrated that by inhibiting the tautomerase domain of MIF with the small molecule ISO-1 they not only inhibited TNFα secretion of macrophages, but were able to protect mice from sepsis using this drug [30]. We had hypothesized that we would see a similar effect using SFN in an LPS-induced model of acute inflammation in the murine air pouch.

Testing that hypothesis was complicated by our unexpected finding that $MIF^{-/-}$ mice treated with LPS had the same number of responding immune cells as MIF

WT mice. This was surprising due to the reports in the literature claiming that MIF is crucial for the septic immune response [60-62]. Interestingly, when we began to delve further into the literature in response to our data, we uncovered a small report from a Japanese group around the same time as the other papers implicating MIF in the septic response. This paper, by Honma *et al*., demonstrated that in the $MIF^{-/-}$ mice that they had generated they did not see any protection from septic shock [63]. Taken together with our own data and that of Honma *et al*., this brings into question whether the other reports were truly seeing a protective effect from the absence of MIF, or if there is some other factor confounding these studies.

# Chapter 4: MIF Deficiency Does Not Alter Glucose Homeostasis of Adipose Tissue Inflammatory Cell Infiltrates during Diet-Induced Obesity

Sarah J Conine and Janet V Cross

(Modified from Obesity Journal)

## Abstract

*Objective*: Circulating Macrophage Migration Inhibitory Factor (MIF) levels have been shown to positively correlate with body mass index (BMI) in humans. Our objective in this study was to determine the effects of MIF deficiency in a model of high fat diet-induced obesity.

*Design and Methods*: MIF wild type (MIF WT) and MIF deficient ($MIF^{-/-}$) C57Bl/6J mice were fed a high fat diet (HFD) for up to 15 weeks. Weight and metabolic responses were measured over the course of the disease. Immune cell infiltrates in visceral and subcutaneous adipose tissue were examined by flow cytometry. *Results*: There was no difference in weight gain or adipose tissue mass in $MIF^{-/-}$ mice compared to MIF WT mice. Both groups fed HFD developed glucose intolerance at the same rate and had similar elevations in fasted blood insulin. MDSC abundance was evaluated and showed no MIF-dependent differences. Macrophages were elevated in the visceral adipose tissue of obese mice, but there was no difference between the two groups.

*Conclusions*: While high fat diet feeding induced obesity with the expected perturbations in glucose homeostasis and adipose tissue inflammation, the presence or absence of MIF had no effect on any parameter examined.

## Introduction

Obesity is a multifactorial disease affecting numerous tissues and physiological processes [80]. Metabolic dysregulation during obesity leads to problems such as glucose intolerance, insulin resistance, and Type 2 diabetes [132, 133]. Chronic

inflammation also arises as a result of excessive weight gain [134, 135]. Adipocytes that are damaged or stressed by high lipid intake produce signals that reprogram the inflammatory response from a maintenance Th2/M2 response to a proinflammatory Th1/M1 response [136-138]. While not completely understood, these immunological changes are thought to contribute to the damage of adipocytes that aids in metabolic disruption resulting in problems such as insulin resistance [139].

Researchers and clinicians alike have become very interested in the potential roles of Macrophage Migration Inhibitory Factor (MIF) in the development of obesity. Human studies have demonstrated a strong positive correlation between body mass index (BMI) and circulating MIF levels [28, 84]. It has also been shown that MIF mRNA levels in the visceral white adipose tissue (vWAT) positively correlate with percentage of body fat [85]. Mice that are deficient in MIF are protected from the onset of glucose intolerance in the context of a pro-atherogenic model ($LDLR^{-/-}$) [86]. These data have led to speculation that MIF contributes to some of the complications of obesity and that inhibition of MIF may provide a therapeutic approach for some of the co-morbidities of the disease [140, 141].

MIF is a widely expressed protein that participates in pathways significant for both metabolism and regulation of the immune system. MIF promotes insulin secretion in the pancreas [87]. The absence of MIF protects beta cells from fatty acid-induced apoptosis and helps preserve beta cell function [87, 142]. MIF deficient mice have a more rapid onset of age-induced glucose intolerance, although they exhibit no differences in glucose metabolism compared to WT mice throughout

most of their lives [90]. MIF deficient mice are also less susceptible to streptozotocin (STZ)-induced diabetes, in that multiple low doses of STZ failed to elevate blood glucose in $MIF^{-/-}$ mice to the same degree as MIF WT [88, 89].

MIF has been shown to regulate several distinct inflammatory processes [143]. The protein was originally identified and named for its ability to inhibit the random migration of macrophages in vitro [31] and has been shown to recruit macrophages in a CCL2-dependent manner in vivo [33]. Using histologic staining for the MAC-3 surface marker, the study in $LDLR^{-/-}$ mice demonstrated that mice that also lacked MIF ($LDLR^{-/-}MIF^{-/-}$) exhibited fewer macrophages infiltrating into epididymal adipose tissue [86]. In the 4T1 breast cancer model, our group has shown that tumor-derived MIF positively regulates the abundance of monocytic MDSCs into the tumor [55]. MIF has also been shown to promote alternative activation of tumor associated macrophages (TAMs) and suppressive function of both TAMs and MDSCs [144].

MDSCs are known to suppress T cells, and to some extent NK cells, and are best characterized in the context of cancer [96, 102]. MDSCs are understudied in obesity; however, Xia *et al*. have demonstrated that MDSCs are increased during obesity and that they contribute to protection from both inflammation and development of insulin resistance that result from obesity [99]. The monocytic subtype (mMDSC) is known to be more suppressive of T cells than the granulocytic population (gMDSC), and can also be induced to differentiate into macrophages under certain conditions [96, 101, 103]. To our knowledge, the potential roles of the two recognized MDSC subsets, granulocytic MDSCs and monocytic MDSCs, have not

been explored in any model of obesity.

The clinical data from obesity studies and the potential importance of MIF in both metabolism and immune cell infiltration led us to explore the effects of MIF deletion in the context of diet-induced obesity. Based on our observations in the 4T1 tumor model, we hypothesized that MIF would promote the prevalence of mMDSCs in obese vWAT and that this would contribute to the proinflammatory environment that promotes diet-induced glucose intolerance. Thorough studies of high fat diet-fed MIF WT and $MIF^{-/-}$ mice demonstrate that the absence of MIF does not alter obesity, inflammatory cell accumulation in vWAT, or the development of high fat diet-induced glucose intolerance.

## Methods

### Animals

Heterozygous $MIF^{-/+}$ Balb/c mice (31) were obtained from cryopreserved stocks maintained at Jackson Labs (Bar Harbor, ME). Mice were backcrossed onto C57Bl/6 using speed congenic microsatellite marker screening to allow completion of the backcross in six generations. Male 6- to 8-week old homozygous MIF wild type (MIF WT) and MIF deficient ($MIF^{-/-}$) C57Bl/6 mice were fed either normal chow (Harlan 7012) or 60% fat/kcal diet (High Fat Diet (HFD), Research Diets, New Brunswick, NJ) for up to 15 weeks. Mass of the animals was monitored over the course of HFD feeding. All experiments were conducted in accordance with guidelines of the University of Virginia Animal Care and Use Committee.

**Isolation of Stromal-vascular Fraction (SVF)**

At time of harvest, mice were perfused immediately post-mortem with PBS and visceral and subcutaneous adipose depots collected. Weights of these adipose depots were recorded, and lymph nodes were excised from the sWAT prior to further processing. Adipose tissue was finely minced in a solution of Krebs-Ringer-HEPES (KRH) Buffer plus 2.5% BSA and then digested with 2 mg of collagenase I (Worthington Biochemical, Lakewood, NJ) per gram of fat at 37°C for 45-60 minutes.

Tissue fragments were removed from the digests by straining through a 400μm pore mesh (Sefar, Buffalo, NY). The stromal-vascular cell fraction (SVF) was pelleted by centrifugation at 400xg for 10 minutes at 4°C. Floating cells (adipocytes) were removed by aspirating the supernatant and the SVF pellet was washed again before filtration through a 70μm pore filter to obtain a final single cell suspension.

**Flow Cytometry**

Flow cytometry was used to immunophenotype the isolated SVF with the following stains/antibodies: LIVE/DEAD Fixable Red Dead Stain (Invitrogen), CD45 PerCP (clone 30-F11, BD Biosciences), CD11b Pacific Blue (M1/70.15, Invitrogen), F4/80 APC-eFluor780 (BM8, eBioscience), Ly6C APC (HK1.4, Biolegend) and Ly6G FITC (1A8, BioLegend). Single cells were gated by forward and side scatter followed by gating for live, CD45+, CD11b+ to identify the myeloid cells. Further gating of Ly6C and Ly6G was utilized to discern mMDSC and gMDSC subtypes, while F480+ cells were defined as macrophages. A representative gating strategy is provided in Figure 4.8. CountBright counting beads (Invitrogen) were included in all samples to

allow for determination of absolute cell numbers. Flow cytometry was performed using the CyAN ADP LX 9 Color Flow Cytometer (Beckman Coulter). Data analysis was performed using FlowJo software (Tree Star, Ashland, OR) and gates were set based on fluorescence minus one (FMO) controls.

**Liver Histology**

Livers from mice that had been fed a HFD for 15 weeks were harvested following perfusion. The tissue was fixed for 24 hours in formalin, embedded in paraffin, sectioned, and stained with H&E.

**Measurement of Blood Glucose and Blood Insulin Levels**

To obtain random-fed blood samples, mice that had been on HFD for 12 weeks were separated into individual cages the night before blood collection and provided food and water ad libitum. The following morning (15-16 hours later) 2-3 mm of the tail of each mouse was amputated, and blood was collected to measure blood glucose using a glucose meter (Accu-Chek Advantage, Roche), following which 25-40µl of blood was drawn into a heparinized capillary tube. Plasma was isolated by centrifugation at 1600xg for 10 minutes at 4°C. Samples were stored at -20°C for batched assay.

For blood collection from fasted mice, animals fed a HFD for 11 weeks were separated into individual Sani-Chip (Harlan) bedding cages with water, but no food and fasted for 15-16 hours (overnight). Blood samples and glucose measurements were taken as described above, except that 50-75µl of blood was required from

fasted mice. Plasma was isolated as described above and stored at -20°C.

Blood insulin levels in both fasted and random-fed mice were measured using an Ultrasensitive Insulin ELISA kit (ALPCO, Salem, NH) per manufacturer's instructions, except that detection was enhanced by substituting Amplex Red in phosphate buffer in place of the substrate provided [145].

**Intraperitoneal Glucose Tolerance Tests**

Mice were weighed and separated into individual Sani-Chip cages to fast for 15-16 hours with water, but no food. Baseline blood glucose measurements were taken using a glucose meter and blood from the tail of each mouse. Immediately after the baseline reading, each mouse was given an intraperitoneal (IP) injection of 1.4 g/kg of D-glucose. Subsequent blood glucose readings were taken at 15, 30, 45, 60, 90, and 120 minutes post-injection. Data were averaged and graphed as blood glucose as a function of time.

**Statistics**

Data are displayed as mean ± SEM and were analyzed using one-way ANOVA, two- way ANOVA, or unpaired two-tailed Student's t-test as indicated in the figure legends. Significance was defined as $P<0.05$ and is indicated by a single asterisk in the figures.

## Results

### MIF Deficiency Does Not Impact Weight Gain During Diet-Induced Obesity

The observed correlations between MIF levels and obesity in both humans and other animal models prompted us to determine if absence of MIF in a mouse model of diet-induced obesity would result in changes in weight gain over the course of the disease [28, 84, 142, 146]. MIF WT and MIF$^{-/-}$ C57Bl/6 mice were placed on HFD for 15 weeks. Total weight was tracked throughout the course of HFD feeding. We found that HFD-fed mice gained significantly more weight than their chow-fed controls; however, there was no difference in weight gain between the MIF WT and MIF$^{-/-}$ groups (Fig 4.1 A).

It is possible that while there was no difference in the overall weight gain between MIF WT and MIF$^{-/-}$ mice, these two groups might vary in the distribution of increased fat mass between the primary adipose tissue depots. Mice were sacrificed after 15 weeks on either HFD or normal chow diet for quantification of visceral adipose tissue (vWAT) and subcutaneous adipose tissue (sWAT) masses. While both MIF WT and MIF$^{-/-}$ mice fed HFD had significantly larger fat depots compared to their chow-fed counterparts, both obese WT and obese MIF$^{-/-}$ animals gained sWAT and vWAT masses equivalently (Fig 4.1 B and C).

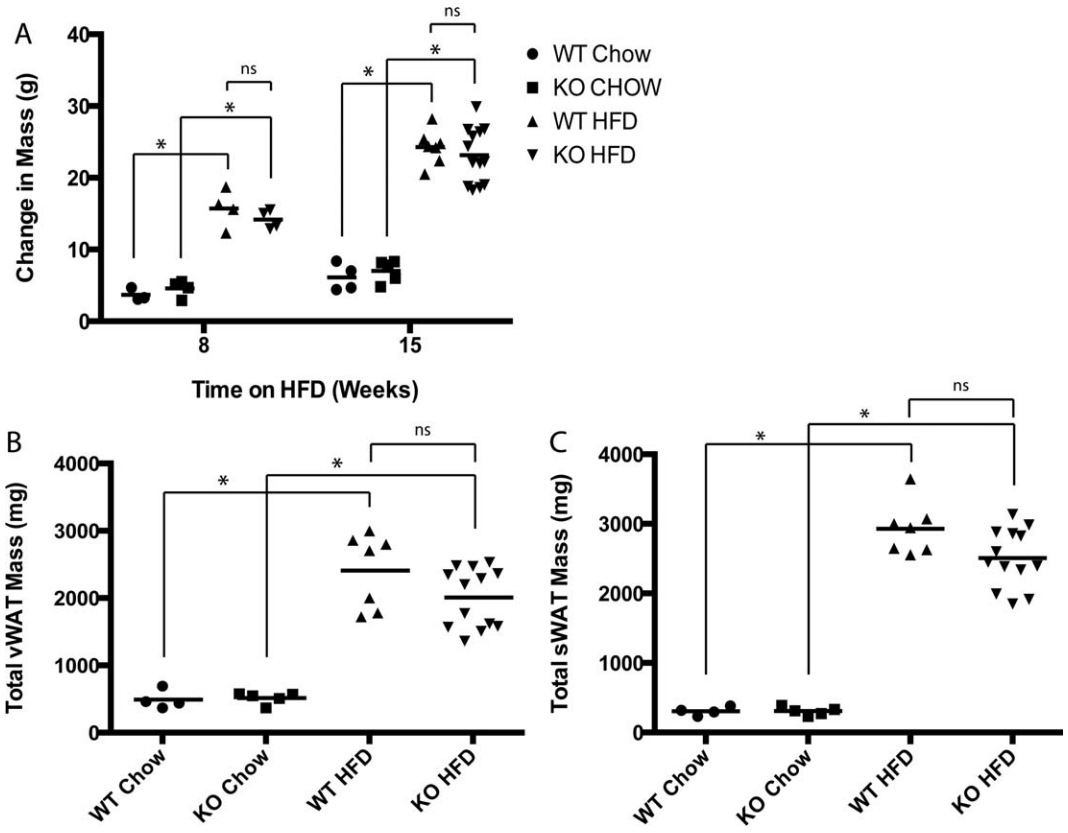


**Figure 4.1. MIF deficiency does not impact weight gain or WAT mass during high fat diet-induced obesity.** A) 6-8 week old WT and MIF$^{-/-}$ C57Bl/6 mice were fed a normal chow or HFD for 15 weeks. Change in total mass of chow-fed or HFD-fed WT and MIF$^{-/-}$ C57Bl/6 mice is shown after 8 and 15 weeks of diet. HFD-fed mice are significantly heavier than chow controls ($p<0.05$), however, there are no differences between WT and MIF$^{-/-}$ mice. B) Total vWAT and C) sWAT mass are not different between WT and MIF$^{-/-}$ mice after 15 weeks of HFD. N= 4-13 per group. Statistical significance was tested using one-way ANOVA.

To determine if MIF could be important in the early stages of obesity, we placed 4 week old mice on HFD for either 1 or 4 weeks. We found no difference between the weights of MIF WT and MIF$^{-/-}$ mice at these stages, nor was there any difference in vWAT mass (Figure 4.2 A and B). Taken together, these data suggest that MIF does not play a direct role in weight gain or distribution of expanding adipose tissue mass during the development of obesity in this model.

**MIF Does Not Influence MDSC or Macrophage Infiltration into vWAT During Weight Gain**

The inflammation that results from increased weight gain has pleiotropic effects on the organism, but is still poorly understood. MIF has been shown to be important in the recruitment of immune cells such as macrophages, neutrophils, and MDSCs in models of cancer and arthritis [33, 55, 56]. Knowing that MIF is involved in the regulation of many inflammatory processes in various disease settings, we set out to determine if MIF influences MDSC or macrophage prevalence in obese adipose tissue.

Visceral and subcutaneous adipose tissues were harvested from MIF WT and MIF$^{-/-}$ mice that had been fed a HFD for 15 weeks. The stromal-vascular fraction (SVF) from these depots was isolated and stained for monocytic MDSCs (Ly6Chi, Ly6Glo), granulocytic MDSCs (Ly6Ghi, Ly6Clo), and macrophages (F4/80+). Neither diet nor genotype induced differences in mMDSC or gMDSC prevalence in the vWAT (Fig 4.3 A, Fig 4.4 A), although the obese vWAT showed an increase in total MDSCs compared to chow controls (Fig 4.4 E). As expected, we

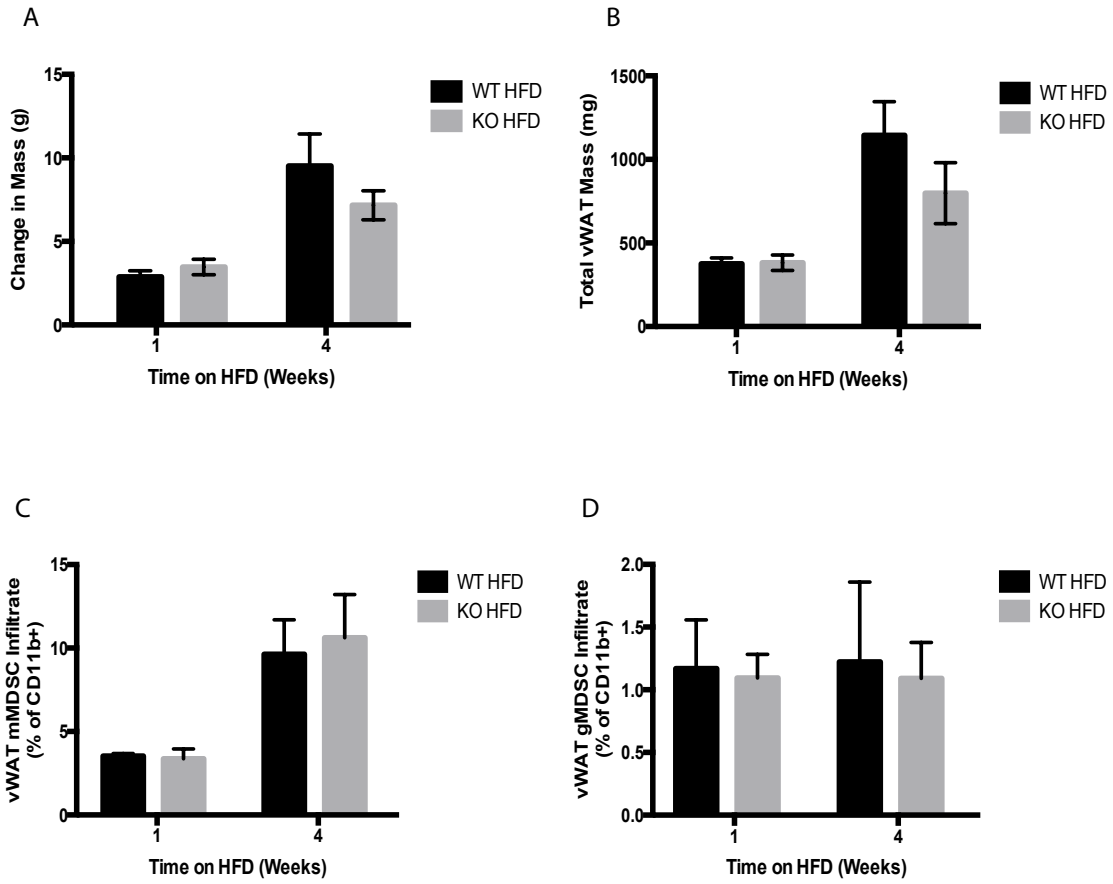


**Figure 4.2. No MIF-dependent differences are seen in weight gain, vWAT mass, or MDSC abundance after 1 or 4 weeks of HFD.** 4 week old C57Bl/6 WT and MIF$^{-/-}$ mice were fed a HFD for 1 or 4 weeks. No differences were seen in (A) total weight gain or (B) vWAT mass between the WT and MIF$^{-/-}$ mice at 1 and 4 weeks. SVF was isolated from the vWAT of these mice after either 1 or 4 weeks and stained for flow cytometry. No significant differences were seen in the infiltration of either (C) mMDSCs (Ly6C$^{hi}$, Ly6G$^{lo}$) or D) gMDSCs (Ly6G$^{hi}$, Ly6C$^{lo}$) populations in MIF WT versus MIF$^{-/-}$ adipose tissue depots. N= 3-5 per group. Statistics were calculated by Students t-test.

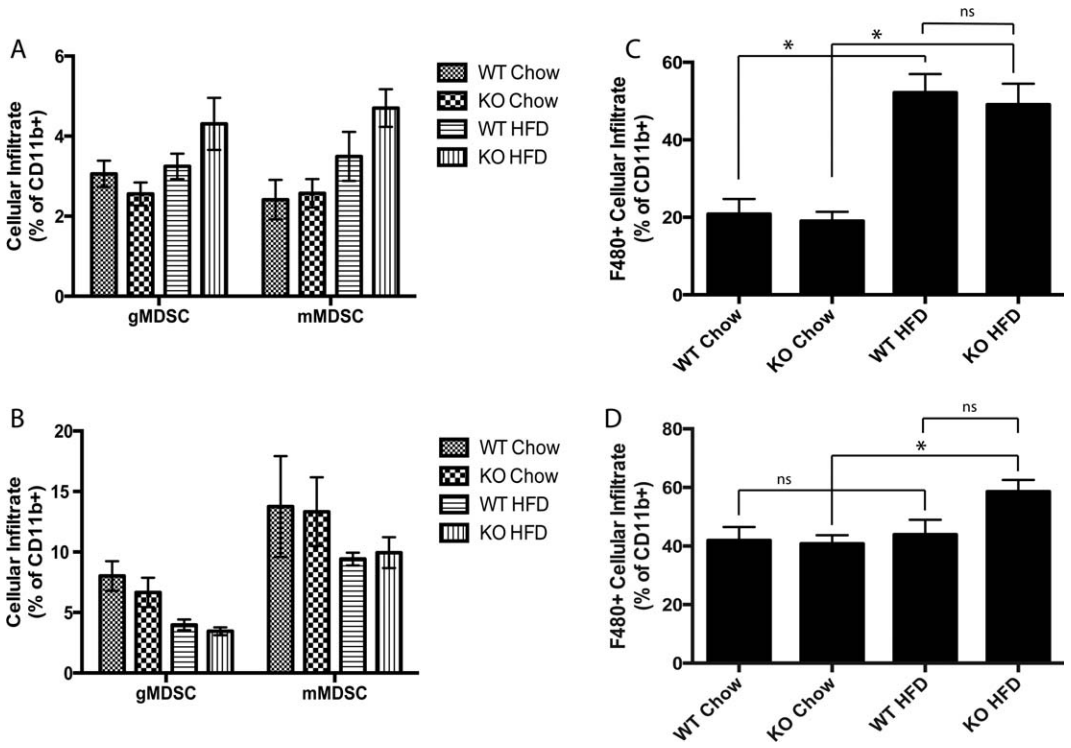


**Figure 4.3. MIF deficiency does not affect MDSC or macrophage abundance during diet-induced obesity.** A-D) The stromal-vascular fraction (SVF) was purified from the vWAT and sWAT of WT and MIF$^{-/-}$ mice that had been fed either chow or HFD for 15 weeks. Cells were stained with a live/dead marker along with fluorescent antibodies for the cell surface markers CD45, CD11b, Ly6C, Ly6G, and F480. Among the live, CD45+, CD11b+ cell populations, no significant differences were seen in the gMDSC (Ly6G$^{hi}$, Ly6C$^{lo}$) or mMDSC (Ly6C$^{hi}$, Ly6G$^{lo}$) populations in the vWAT (A) or sWAT (B) of MIF WT versus MIF$^{-/-}$ mice. Macrophages (F480+) were elevated in the vWAT (C) of obese mice ($p<0.05$), however, no significant differences were seen in the vWAT (C) or sWAT (D) between WT and MIF$^{-/-}$ mice on HFD. N= 4-13 per group. Statistical significance was tested using one- or two-way ANOVA. Asterisks indicate differences from chow controls.

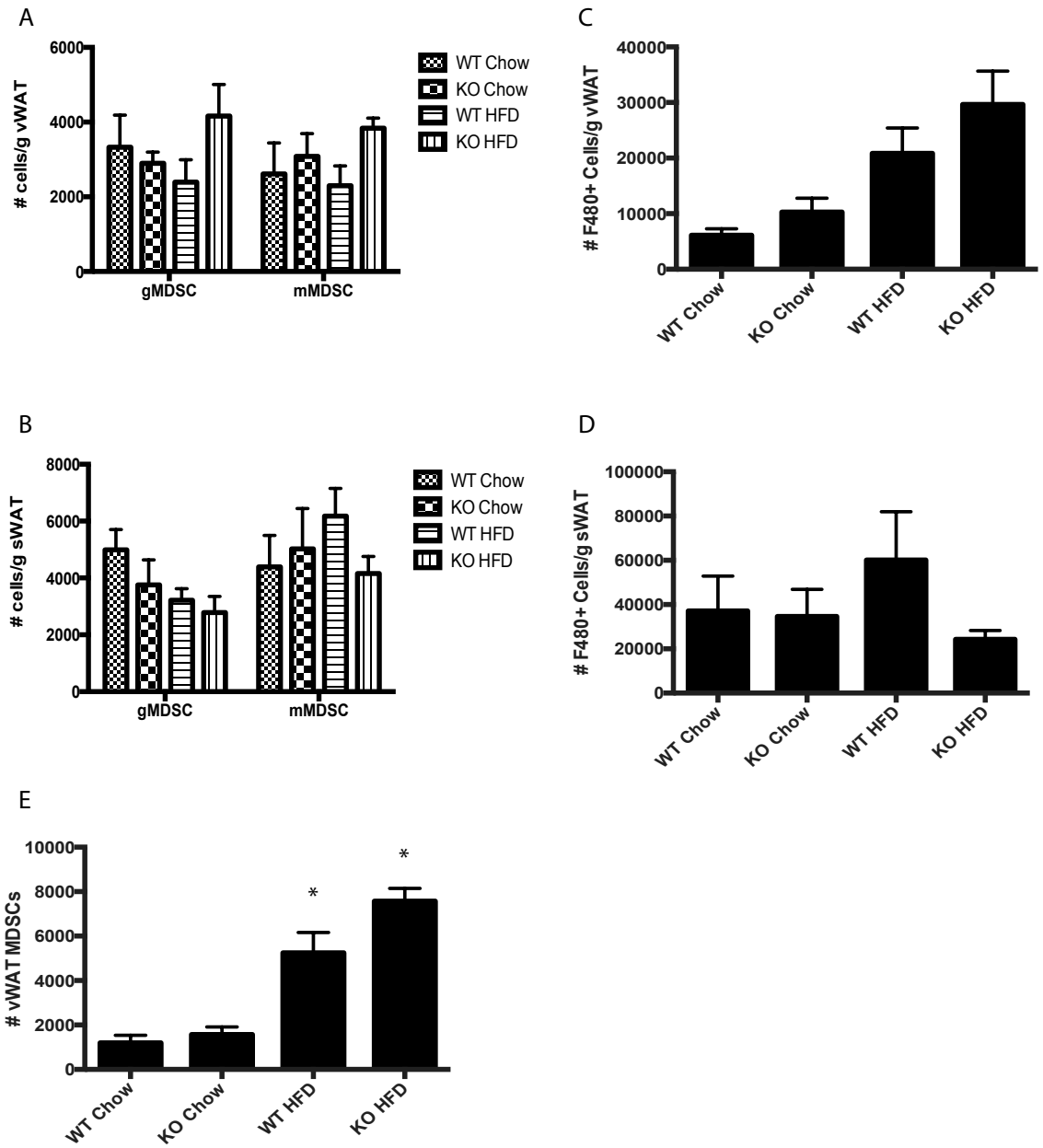


**Figure 4.4. The number of macrophages and MDSCs infiltrating in the adipose tissue does not change in the absence of MIF.** Total numbers of cells per gram of fat were calculated for the samples shown in Figure 2. Briefly, WT and MIF$^{-/-}$ mice were fed a HFD for 15 weeks. SVF was isolated and stained for flow cytometry analysis. Counting beads were used to quantify cells numbers and cells were gated on live, CD45+, CD11b+ populations before further gating into A and B) mMDSCs

(Ly6C-hi, Ly6G-lo), gMDSCs (Ly6G-hi, Ly6C-lo), and C and D) macrophages (F480+). No significant differences were seen between MIF WT and MIF$^{-/-}$ groups in gMDSCs, mMDSC, or F480+ macrophages in either the vWAT (A and C) or the sWAT (B and D). E) Total number of MDSCs (mMDSC + gMDSC) were quantified and showed an increase in obese vWAT over chow controls. N= 4-13 per group. Statistical significance was tested using one- or two-way ANOVA. Asterisks indicate differences from chow controls.

observed an increase in F4/80+ macrophages in the vWAT of HFD-fed mice; however, the absence of MIF did not lead to any change in the macrophage population as compared to the WT HFD group (Fig 4.3B, Fig 4.4C). No differences in any of the three immune cell populations were found between MIF WT and MIF$^{-/-}$ mice on HFD in the sWAT (Fig 4.3 B and D, Fig 4.4 B and D). Interestingly, MIF$^{-/-}$ mice on HFD demonstrated a statistically significant increase in the percentage of infiltrating F480+ cells compared to the chow controls. This difference was not seen between the MIF WT HFD and MIF WT chow mice (Fig 4.3 D). Immunophenotyping of the visceral fat depots of mice that had been fed HFD for only 1 or 4 weeks also demonstrated no significant differences in MDSC infiltration at earlier time points (Figure 4.2 C and D). These data demonstrate that the absence of MIF does not lead to differences in the abundance of MDSCs or macrophages in obese adipose tissue.

Knowing that excess lipids that are not successfully stored in the adipose depots are often deposited in the liver [76], we sought to determine if MIF deficiency might alter lipid deposition in the liver. We harvested livers from mice that had been on HFD for 15 weeks and stained sections with H&E. There was a marked increase in lipid accumulation in the livers of all obese animals; however, we saw no difference due to the presence or absence of MIF (Fig 4.5).

**MIF Deficiency Does Not Influence the Development of Glucose Intolerance**

Given the apparent absence of differences in weight gain and immune cell populations in the adipose tissue, we wanted to determine if there was a difference

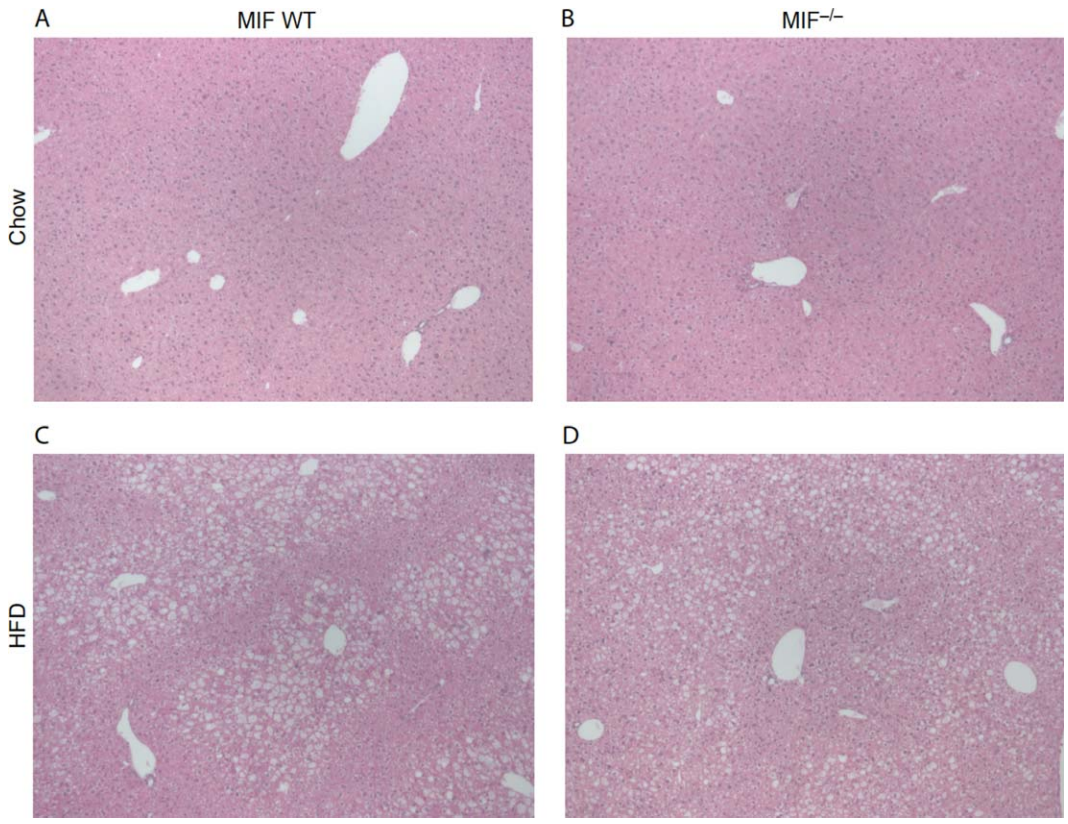


**Figure 4.5. Fat deposition is the same in the livers of obese WT and MIF$^{-/-}$ mice.** Livers from 15 week chow-fed and HFD-fed WT and MIF$^{-/-}$ mice were processed for histologic sections and stained with H&E. Chow-fed livers appear normal (A and B) compared to HFD-fed livers (C and D), which have marked lipid deposition. No MIF-dependent differences were observed in any condition tested. 8x magnification.

in the metabolic responses of MIF WT and MIF$^{-/-}$ mice after an extended period on HFD. As a functional readout of glucose metabolism, intraperitoneal glucose tolerance tests (IPGTTs) were performed on cohorts of mice that had been on a HFD for 5, 8, or 12 weeks. As expected, a delay in the response to glucose challenge was observed in obese mice at all three time points. However, there was no difference in glucose response between the MIF WT and MIF$^{-/-}$ mice, suggesting that the absence of MIF neither inhibits nor promotes the development of glucose intolerance (Fig 4.6 A-C).

Differences in insulin secretion could account for similar IPGTT curves that mask an underlying metabolic perturbation. Therefore, we measured both random-fed and fasted blood insulin levels in these mice. HFD groups showed slight elevations in their blood insulin levels in both random-fed and fasted conditions. However, there was no significant difference in insulin levels between the obese MIF WT and MIF$^{-/-}$ groups (Fig 4.7 B and D). Random-fed and fasted blood glucose concentrations were monitored concurrently with insulin levels and while there was a slight elevation of fasted HFD glucose levels in obese mice compared to chow fed controls, again, there was no indication that the absence of MIF had any effect (Fig 4.7 A and C). Taken together, these data suggest that the presence or absence of MIF alone is not sufficient to change the development of insulin resistance during diet-induced obesity.

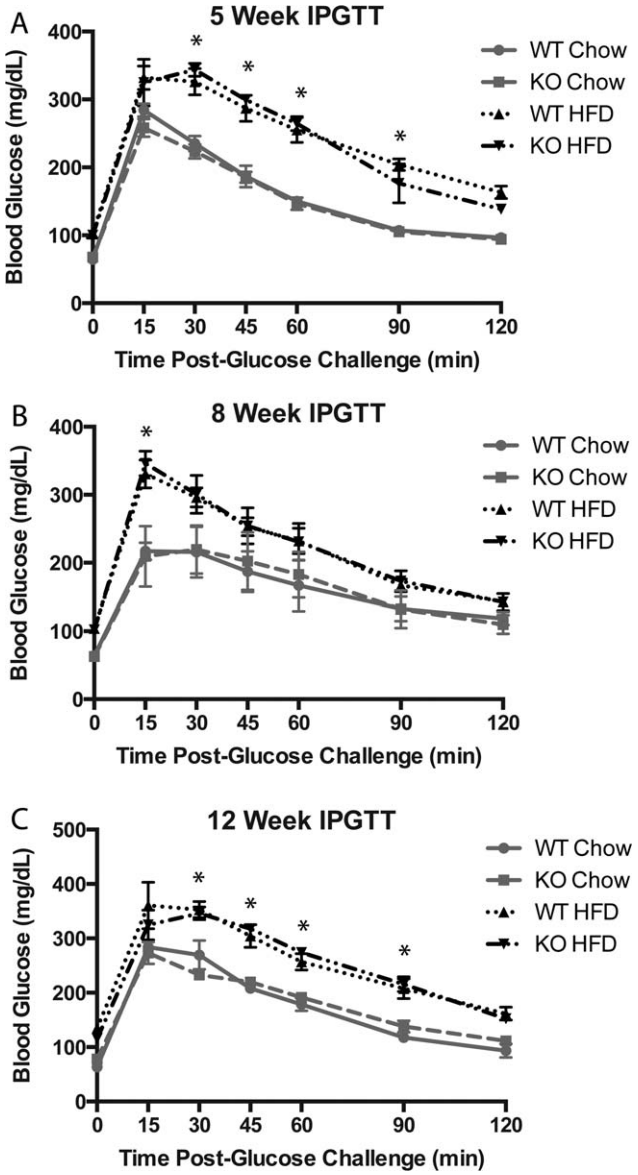


**Figure 4.6. MIF deficiency does not impact the development of glucose intolerance during diet-induced obesity.** Intraperitoneal glucose tolerance tests (IPGTTs) were performed on WT and MIF$^{-/-}$ mice after 5 (A), 8 (B), and 12 (C) weeks of HFD. At all time points, chow-fed controls respond normally to glucose challenge, whereas HFD-fed mice have a delayed response in normalizing blood glucose levels, indicating the development of glucose intolerance. No significant

differences were measured between the WT and MIF$^{-/-}$ groups on either diet. Asterisks indicate time points at which both HFD groups were significantly different from both chow groups ($p<0.05$). N= 3-4 per group. Statistical significance was tested using one-way ANOVA.

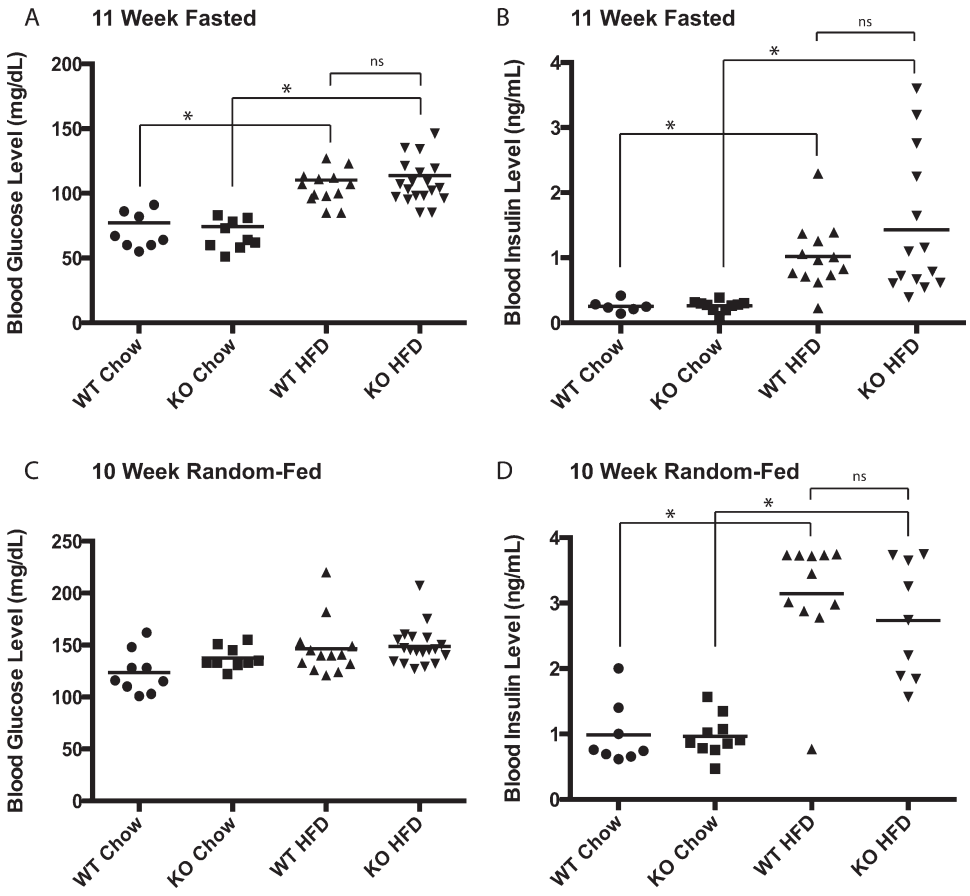


**Figure 4.7. WT and MIF$^{-/-}$ mice have comparable fasted and random-fed blood glucose and blood insulin levels in response to HFD.** A and B) After 11 weeks of HFD, WT and MIF$^{-/-}$ mice were fasted overnight (approximately 16 hours). Blood glucose levels (A) and blood insulin (B) were slightly elevated in obese compared to normal mice, however, no significant MIF-dependent differences were observed. C and D) During week 10 of HFD feeding, random-fed blood glucose (C) and blood insulin levels (D) were quantified. Random-fed blood insulin was increased in the chow compared to HFD groups ($p<0.05$); however, there were no significant differences between the WT and MIF$^{-/-}$ groups. N= 8-19 per group. Statistical significance was tested using one-way ANOVA.

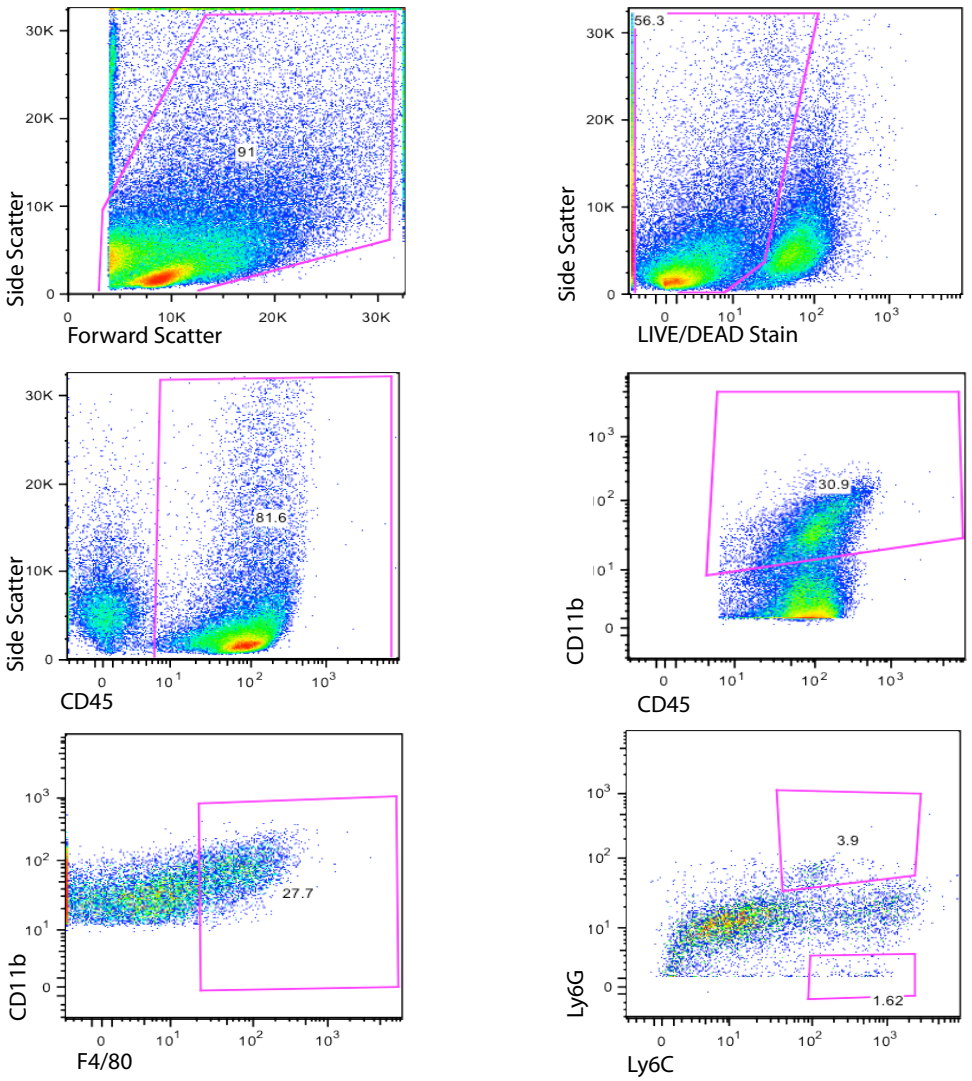


**Figure 4.8. Gating strategy for immunophenotyping of MDSCs and macrophages in WAT.** Cells were gated on forward and side scatter for intact cells. This was followed by sequential gating for live cells, CD45+, and CD11b+ cells to identify the myeloid lineage. mMDSCs were further identified by $Ly6C^{hi}$, $Ly6G^{lo}$. gMDSCs were characterized by $Ly6G^{hi}$, $Ly6C^{lo}$. Finally, macrophages were gated on using the F480+ parameter. Gates were set on the basis of fluorescence minus one (FMO) controls. Counting beads were included and used to determine absolute cell number.

## Discussion

We have shown that in a mouse model of diet-induced obesity, the absence of MIF does not impact weight gain or adipose tissue mass. The human data showing correlations between BMI and MIF [28, 84] might lead one to expect that lack of MIF would result in decreased weight gain compared to WT. Given our data, we believe that the increase in MIF seen during obesity may be an effect, rather than a cause of weight gain and increased adiposity. For example, it is known that preadipocytes increase MIF secretion during the differentiation process in which mature adipocytes are formed [147]. Obesity results in an increased rate of preadipocyte differentiation to accommodate increased nutrient intake, which might be predicted to lead to more MIF secretion from the adipose tissue [148].

In this study, we have demonstrated that MIF does not impact the abundance of mMDSC, gMDSC, or F4/80+ macrophage populations in the vWAT. These data do not support our initial hypothesis that MIF expression would positively regulate the prevalence of mMDSCs in adipose tissue during obesity, as we have shown in a breast cancer model [55]. We did see the expected increase in macrophages and total MDSCs due to the onset of obesity (Fig. 4.3, Fig 4.4); however, we did not observe any MIF-dependent changes in myeloid cell infiltration into vWAT during obesity. While our study carefully examined the number of cells infiltrating into the fat, we did not address the function of these cells. Therefore, it remains possible that MIF could exert some effect on the role that MDSCs or macrophages play once they enter into the vWAT. In particular, it is possible that MIF may influence the relative abundance of M1 vs M2 macrophages within the adipose tissue. The M1 vs M2

phenotype has been shown to play a role in the pathogenesis of obesity [149] however, even if there is a MIF dependent alteration in MDSC and/or macrophage function, that difference does not manifest in any changes in weight gain or in the metabolic effects of glucose intolerance as obesity develops.

Others have shown that MIF is a positive regulator of insulin secretion from pancreatic islets [87]. It has also been demonstrated that MIF deficiency protects pancreatic islets from fatty acid induced apoptosis *in vitro* [142]. These studies are compatible with our hypothesis that $MIF^{-/-}$ mice fed a HFD would develop glucose intolerance at a slower rate than obese WT mice. Instead, we observed that both groups develop similar glucose intolerance and increased insulin levels on HFD regardless of MIF expression. This suggests that MIF is not a key regulator of the damage that leads to obesity-related disruption of glucose homeostasis and therefore is not a promising therapeutic target for modulation of conditions such as Type 2 diabetes. It is possible that we did not see the predicted metabolic changes in $MIF^{-/-}$ mice because of compensatory pathways that could make up for MIF deficiency or MIF overexpression.

The role of MIF in the development of glucose intolerance has been studied in the context of an atherogenic mouse strain ($LDLR^{-/-}$). In this model, MIF deficient $LDLR^{-/-}$ mice fed a chow diet developed glucose intolerance at a slower rate than $LDLR^{-/-}$ mice that express MIF. The two groups of mice became obese at the same rate and had no differences in fat mass [86]. This study differs from ours in that the effect of MIF deficiency was examined on an atherosclerosis prone background ($LDLR^{-/-}$) and the composition of the diets was not the same. Either of these factors

could result in alterations in both the metabolic and immune responses of the mice. Based on our results, we would contend that while MIF deficiency is protective in the context of genetic predisposition for atherosclerosis, it does not exert an effect in a high fat diet model of obesity.

Serre-Beinier *et al.* demonstrated that MIF$^{-/-}$ mice develop age-induced glucose intolerance. Their data showed that MIF WT and MIF$^{-/-}$ mice had no significant differences in glucose tolerance at 2 or 4 months of age. The latest timepoint tested, 12 months, was the only one to show that MIF$^{-/-}$ mice were more glucose intolerant than MIF WT mice [90]. It is possible that our mice would have shown similar changes over time had our study extended as long. In a different model, Harper *et al.* demonstrated that MIF$^{-/-}$ mice on a C57BL/6J x 129/SvJae background exhibit increased longevity and are significantly smaller when compared to MIF wild type controls [150]. This may be a strain-specific difference, as MIF$^{-/-}$ mice on a pure C57BL/6J background are no different in size than their MIF wild type litter mate controls [151], though this study did not address longevity. It is important to note that while there are several studies that have looked at the role of MIF in control of various metabolic parameters, none have compared obese MIF WT and MIF$^{-/-}$ animals to assess diet-induced glucose intolerance, and our data clearly point toward MIF expression not being a key factor in the dysregulated glucose homeostasis resulting from diet-induced obesity.

Our study was designed to determine if MIF would be a good therapeutic target for treating obesity-related metabolic and immunologic co-morbidities. There has been much speculation in the literature that controlled regulation of MIF during

obesity may benefit patients; however, up until this point no one had investigated the impact of MIF deletion in an animal model of diet-induced obesity. We provide the first report that the absence of MIF alone is not sufficient to alter either glucose homeostasis or the inflammatory changes that accompany HFD-induced obesity. Taken together, our results suggest that MIF inhibition may not be a useful approach for correction of these aspects of obesity-related disease development.

## Disclosure

The authors declared no conflict of interest.


## Acknowledgements

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# Chapter 5: Discussion

**MIF as a Therapeutic Target in Acute Inflammation**

In our second results chapter, we demonstrated that in our hands MIF is not a critical regulator of the acute inflammatory response induced by LPS. We used $MIF^{-/-}$ mice in the dorsal air pouch model to show that LPS stimulation resulted in the same degree of immune cell infiltration into the air pouch as compared to MIF WT mice. This result was not what we had anticipated based on the preponderance of the MIF literature.

Others had reported seeing a protection from lethality in septic murine models of MIF deficient mice [61]. Administration of an anti-MIF antibody to septic mice had also been shown to be protective against further complications [60, 62]. Thus, it became commonplace to see review articles report the importance of MIF in the acute response and how inhibition or genetic deletion of MIF protected from the lethality induced by septic conditions [27, 43, 152]. It is of note that we also attempted to reproduce the published effects of protection from lethal septic shock in MIF deficient mice in this LPS lethality model, and were unable to see an effect (data not shown).

After multiple experiments comparing MIF WT and $MIF^{-/-}$ mice failed to produce any difference in the immune response to LPS in the air pouch model, we revisited the literature. There is a single report that emerged not long after the initial reports of the importance of MIF in sepsis that showed that the Japanese group led by Honma *et al*. could not produce any difference in the response to LPS in their $MIF^{-/-}$ mice compared to WT controls [63]. This led us to question the validity of MIF as the sole factor in the results published by Bozza *et al.* and Calandra *et al.*

There are many factors that can confound animal experiments that might not be within the control of the investigator. An example of this is the bacterial gut flora in mice. It has been demonstrated that differences in gut flora can cause very different results to be observed in mice that are genetically identical [153]. Given that the animals in the discrepant studies were housed in different facilities, we cannot rule out a confounding factor such as this. Given our own results, we must conclude that the role of MIF in acute inflammation is not as clear as the literature would lead one to believe. Due to the ubiquitous expression and many roles of MIF within the organism, it is a very hard protein to target in any disease without causing off-target effects. The controversy within the field as to the role of MIF in the response to acute inflammation suggests that investigators and clinicians should use caution when considering approaches to target MIF in this setting.

**Is the Tautomerase Activity of MIF Important?**

It is still unknown whether or not the tautomerase domain is a physiologically active and important part of the MIF protein. One of the primary goals of the air pouch study was to utilize tools such as SFN and genetic manipulation of recombinant MIF to determine whether the tautomerase domain is critical for MIF-dependent responses to acute stimuli. Our study was inconclusive in this regard due to the failure of what we expected to be a MIF-dependent model. However, we did show that SFN treatment of cells and recombinant MIF protein reduces MIF-induced BMDM migration *in vitro* (Figure 3.2). We have also

demonstrated the importance of the tautomerase domain in the 4T1 model of breast cancer [55].

A point of contention in the literature is whether or not the tautomerase activity of MIF, and therefore the inhibition of MIF by SFN (and other characterized inhibitors), is biologically relevant. Currently, there are groups on each side of the fence within the MIF field. Some would say that the tautomerase activity is irrelevant, perhaps a leftover relic that was never completely phased out by evolution. This is based on the fact that no one has been able to identify an *in vivo* target. These groups would suggest that any biological effects seen as a result of SFN binding to MIF could be due to steric hindrance or changes in protein conformation that inhibit binding to the MIF receptors rather than loss of enzymatic function [154]. It is not clear why researchers have been unable to identify a substrate for MIF tautomerase. We speculate that it could be that the reaction happens so rapidly that it is difficult to isolate *in vitro* or that perhaps there is some sort of additional tertiary requirement for enzymatic function (eg. Monomer vs. trimer with itself or co-factors).

Others, our group included, believe that the tautomerase activity of MIF is important and biologically relevant. MIF is active in many different systems and can have varying effects depending on the scenario, suggesting that enzymatic activity may complement receptor binding in order to provide MIF with such versatility. Adamali *et al.* conducted perhaps the most compelling study that supports the hypothesis that MIF tautomerase activity is relevant, especially in disease settings [47]. They used a MIF tautomerase null genetic mouse model in which the essential

proline has been mutated to a glycine (P1G), rendering the enzymatic activity ineffective without the binding of an inhibitor that could potentially inhibit receptor binding. These mice were infected with *Pseudomonis aeruginosa* to model cystic fibrosis, and showed significantly less pathology in comparison to infected mice that retained the tautomerase activity [47]. However, dissention within the field is evident in another report by some of the same authors, using the same P1G mice, that concludes that the intermediate phenotype that these mice produce in their study of MIF signaling and tumor growth indicates the lack of a functional role of the tautomerase in their model [154]. Our studies in the 4T1 model demonstrating that tumors expressing MIF with the P2G mutation exhibit decreased growth and mMDSC infiltration also support a physiological role for the tautomerase [55].

**SFN as a MIF Inhibitor and Potential Therapeutic Agent**

The initial goal of our air pouch project was to evaluate the efficacy of SFN as a MIF-dependent inhibitor of the acute immune response (Chapter 3). We were forced to change course to focus on MIF-independent effects of SFN on the acute immune response (Chapter 2); however, prior to that shift we were able to perform some studies on the effect of SFN on MIF in the pouch lining. We demonstrated that local administration of SFN is able to inhibit MIF tautomerase activity in the pouch lining (Figure 3.3). We also showed that SFN significantly reduces MIF-induced BMDM migration *in vitro* (Figure 3.2). These data indicate that SFN is able to target MIF both *in vitro* and *in vivo* in our model systems.

However, further analysis of the effect of SFN on the cellular response to LPS *in vivo* suggests that SFN may have MIF-independent effects that are more physiologically significant than any MIF-dependent effects in this model. Our hypothesis based on the literature and the *in vitro* migration data was that SFN would inhibit MIF-induced cellular infiltration into the air pouch, primarily of macrophages. What we observed instead was that SFN did not inhibit the number of macrophages that infiltrated into the pouch 24 hours after LPS stimulation, and that SFN actually seemed to increase the number of infiltrating cells (Figures 2.4, 2.6). This could mean one of two things: the tautomerase activity of MIF is irrelevant for the acute response to LPS and therefore SFN has no effect on cellular infiltration, or SFN acts on multiple targets *in vivo* and is able to exert MIF-independent effects on immune cell trafficking.

SFN has been shown to have effects on endothelial cells that result in regulation of the immune system. Adhesion molecules are down-regulated in SFN treated endothelial cells, resulting in reduced monocyte adhesion [22]. SFN also inhibits IL-8 and MCP-1 secretion by endothelial cells [23]. It is possible that SFN is also affecting endothelial cell functions such as these in the air pouch model. Given the unexpected results of SFN pre-treatment in our pouch experiments, we recognize the importance of examining the impact of SFN on endothelial cells and chemotactic gradients as a potential mechanism for the lack of anti-inflammatory of SFN in this model.

**Limitations of the Murine Air Pouch Model**

The air pouch model is often utilized for imitation of the joint or as an easily accessible space for testing drugs and evaluating the immune response [64, 124]; however, there are limitations to every model. The most obvious drawback to the air pouch model is that it creates a superficial space that has been modified from what occurs in the body naturally. The subcutaneous space does not normally have a very large volume, and the air pouch intentionally creates a large volume of space for experimental manipulation. This manipulation of the layers of tissue surrounding the pouch may result in unknown changes to the cells in the pouch area and may not result in a perfect mimic of the desired disease state response.

The installation of the pouch itself causes stress on the mouse. It is not unlikely that the disruption of membrane adhesion and stretching of tissue as a result of the air used to inflate the pouch would activate stress responses in the cells that compose the pouch [155, 156]. The insult of the pouch installation also causes a certain degree of inflammation. Our data of saline treated pouches show that there is a low level (compared to LPS stimulated pouches) of immune cells that infiltrate the pouches (Figure 2.4). Macrophages also compose a significant portion of the pouch lining, perhaps in response to the stretching of the layers of the skin and the damage that is caused as a result [64]. This low level of inflammation can be partially controlled for using vehicle control mice; however, it is unknown how the signaling events that occur upon installation of the pouch affect the responses to further experimental manipulation.

Lastly, the air pouch model of acute inflammation is a murine model and therefore is an imperfect model for the human acute inflammatory response. Discrepancies between the human and mouse immune systems make translation from one species to another difficult in many models. There are known temporal differences in certain types of immune responses between mice and humans; for example, it can take years for the symptoms of graft versus host disease to manifest in humans, whereas mouse models begin to show effects mere weeks after transplantation. The speed at which changes occur in the mouse can result in differences in how the disease progresses at various stages and cause difficulties in drawing parallels between species at certain points [157]. There are also many overall differences in the immune systems between mice and humans. A few examples include: humans have a high percentage of neutrophils in circulation whereas mice have a higher percentage of lymphocytes, some TLR expression and activation varies between species, and cytokine production from certain cell types can be different in humans versus mice [158]. Hence, while mouse models are certainly necessary for the course of research, it is important to keep in mind that the reason why so many clinical trials end prematurely may be due in part to differences between these species.

**MIF as a Therapeutic Target in Obesity**

We demonstrated using a mouse model of diet-induced obesity that global deletion of MIF does not impact weight gain, MDSC or macrophage infiltration into WAT, or development of insulin resistance. This was a surprising result given the

links that had been demonstrated between increase in weight gain and MIF levels in humans. More than one group established that obese patients have elevated MIF levels in both their circulation and adipose tissue, and that there is a strong positive correlation between circulating MIF and BMI [28, 84, 85]. Consequently, it had been suggested in the literature that MIF should be evaluated as a potential therapeutic target for obesity-related conditions [140, 141]. We designed our study to test whether the absence of MIF affected the course of obesity and showed that in this model, it does not.

The initial focus of our study was on the vWAT immune cell populations. It had been shown by Xia *et al*. that MDSCs are increased in obese murine adipose tissue and that their presence confers protection against insulin resistance [99]. We did observe an overall increase in MDSCs, as Xia *et al*. did in their report. However, this increase was MIF-independent. Our studies in the 4T1 model of breast cancer support a role for MIF in controlling the abundance of mMDSCs within the tumor; therefore we examined the infiltration of MDSCs into vWAT in a MIF-dependent manner [55]. We demonstrated that the global absence of MIF did not change the number or percent of either MDSC subtype or total F4/80+ macrophages within the vWAT. This could indicate that MIF does not regulate myeloid cell prevalence within obese adipose tissue. However, it could also be a result of using a global deletion approach. In our tumor studies, we utilized a MIF depletion model in the tumor as opposed to the mouse or mouse and tumor. That allows for some discrimination of tumor- versus host-derived MIF. Our model does not allow for the ability to distinguish between adipose-derived MIF and MIF from the rest of the

mouse. The ubiquitous expression of MIF and its involvement in the regulation of so many other systems may mask the effect of adipose-derived MIF on the immune system as a result of obesity. Future studies of conditional knockout mice focusing on MIF in the adipose tissue may be more informative than this initial global MIF knockout model. However, it is of note that our global depletion strategy is indicative of how a systemic MIF inhibitor would work in the context of MIF as a therapeutic target in obesity.

Upon observing a lack of difference in weight gain or MDSC infiltration into obese vWAT, we followed up with disease endpoint studies to determine whether global ablation of MIF resulted in metabolic alterations compared to obese WT mice. We observed no MIF-dependent differences in blood glucose, blood insulin, or glucose tolerance in these experiments (Figure 4.6, 4.7). These results were surprising because it has been shown that MIF can stimulate islet cells to secrete insulin [87] and that the lack of MIF protects pancreatic islets from fatty acid-induced apoptosis [142], which would be consistent our hypothesis that obese $MIF^{-/-}$ mice would not develop insulin resistance to the same degree as WT mice. Another compelling study demonstrated that MIF deficiency in $LDLR^{-/-}$ mice protected them from developing glucose intolerance [86]. This suggests that perhaps the role of MIF in glucose metabolism is more important in the context of atherosclerosis. The mice in the Verschuren *et al*. study were fed a regular chow diet and did not gain weight any differently in the presence or absence of MIF [86], which could also imply that the fat content of the diet may have an impact on the role of MIF in glucose metabolism.

Even if we had seen different results in this study, MIF would be a difficult protein to target as an effective anti-obesity therapy. MIF is involved in so many capacities throughout the organism that avoiding off-target effects could be extremely problematic. If a specific role or mechanism for MIF is identified in obesity, it may be easier to elucidate the pathway leading to that particular effect and attempt to target a more specific factor in the pathway and avoid some of the side effects that would come with systemic, or even local, MIF inhibition. On the other hand, the sheer number of roles that MIF plays in the organism implies that if MIF were to be inhibited many of these processes can probably be maintained by redundant pathways. This leads us to speculate that MIF inhibition would primarily affect MIF-dependent processes that are enhanced by disease progression, while normally functioning processes would remain intact through management by other mediators.

**Limitations of the Murine Diet-Induced Obesity Model**

In general, the rodent model of diet-induced obesity (DIO) closely mimics the obese state of increased adipose tissue mass and chronic inflammation developed by humans. Most humans become obese from eating foods that are high in fat over time, making the DIO model representative of more of the population than some of the genetic models of obesity (eg. *ob/ob* and *db/db* mice). Mice on HFD typically rapidly gain weight as humans would on a similar diet, and gene expression modifications as a result of HFD are fairly consistent between rats and humans [108].

There are, however, some key differences between species that could make it difficult to extrapolate from one species to another. For humans, there are important psychological cues associated with eating and food choice. Rodent models of HFD typically do not take those factors into account in that there is a single food source available freely at all times [159]. The choice of food and when to eat has been removed from the picture. Another difference is that there are certain strains of mice that are susceptible to the development of obesity (eg. C57Bl/6) and others that are not (eg. Balb/c) [160, 161]. Researchers almost always utilize the models that promote weight gain, which may only accurately model part of the human population. It may be useful to also study the strains that do not gain weight as easily because there is certainly a human population that may eat a poor diet for a longer period of time before gaining enough weight to be classified as obese and it is worth understanding that physiological model as well. Most obesity studies are carried out in male mice to avoid the complications that arise because of the estrous cycle [108, 162]. Therefore, it is still important to perform the same experiments in a female model to ensure that should a study be translated into humans it does not have unexpected effects in females as a result of the differences in hormones.

# Chapter 6: Future Directions

**Alternative Dosing Strategies and Activity of SFN in the Air Pouch**

Having shown that SFN has an effect on the immune cell infiltration into the air pouch in response to LPS, one of the next logical steps is to perform alternative dosing strategies to further examine the dynamics of this response. We have looked at the impact of a single dose of SFN on immune cell infiltration at 24 hours post-LPS stimulation. It is possible that multiple doses of SFN after the LPS stimulus and in combination with SFN pre-treatment exert a different effect than a single pre-treatment dose. In order to start to uncover the mechanism of action of SFN in this model it is important to elucidate the spatial and temporal activity of SFN, i.e. where in the tissue is the SFN is active, at what times, and for how long.

*Alternative Dosing Strategies*

We propose to determine whether the addition of further doses of SFN throughout the course of the 24 hour experiment will have an effect. Pharmacokinetic studies of SFN in rats suggest that orally administered SFN results in peak circulating concentrations after 1-2 hours and that the half-life is roughly 2.2 hours [16]. Taking the half-life of SFN into account, it would be interesting to test various combinations of SFN administration at 2, 4, 12, and 22 hours post-LPS stimulation. Given the fact that our current study utilized only a single treatment of SFN with a harvest 24 hours after administration, we hypothesize that further doses of SFN will increase the time that SFN is bioavailable, increase the difference in cellular infiltrate at 24 hours, and result in functional differences in the infiltrating immune cells as a result of increased SFN exposure. We speculate that multiple

doses of SFN across the course of 24 hours will lead to an effect on the infiltrating immune cells that will likely result in alteration of cellular functions through pathways such as NF-κB.

*Activity of SFN in the Pouch*

We also propose to determine the activity of SFN during the course of the original 24 hour experiment. This will require various time points in order to measure activity and concentration of SFN in the tissues at times such as 30 minutes post-LPS stimulation, 3, 6, 12, and 18 hours after stimulus. These time points will give a good overview of the reaction to SFN and its bioavailability throughout the course of the experiment. Our primary method of measuring SFN activity is through protein lysis of tissue or cellular samples followed by tautomerase assay (see methods sections). We can monitor SFN concentration in the circulation using either LC/MS [16] or by measuring dithiocarbamate (DTC) metabolites of SFN in a cyclocondensation reaction [163, 164]. Based on previous studies of the pharmacokinetics of SFN in rats and humans [16, 163], we expect SFN concentrations to peak at about 20μM in the circulation and tissue roughly 1-2 hours after administration. There may be some variability in this with our model because previous studies have analyzed oral administration whereas we have a local administrative approach directly into the pouch. It is possible that the SFN will be taken up more quickly because it does not pass through the GI tract. Functional activity of SFN will be monitored at concurrent time points by MIF tautomerase activity assay.

While monitoring SFN activity and concentrations in the blood, we will concurrently measure SFN concentrations and activity in the infiltrating immune cells, pouch lining, and liver. We do not know yet what cells SFN is acting on to exert its effect on the relative percent of infiltrating monocytes and neutrophils. We speculate that SFN is altering signaling of pathways such as chemokine gradients or adhesion molecule expression in endothelial cells in the pouch wall. However, we do not know the kinetics of that proposed response, nor do we know if SFN acts on the immune cells themselves or peripheral organs, such as the liver. To monitor SFN activity of immune cells and peripheral tissues we could utilize the same methods described above, or indirectly measure SFN activity by quantifying gene and protein expression of known targets such as NF-κB, nrf2, or NAD(P)H:quinone oxidoreductase (NQO1) [163]. We expect that SFN will have a measurable concentration up to roughly 4 hours [16] in the pouch lining and liver, with functional effects lasting at least 24 hours; however, because of the delay in immune cell infiltrate compared to the bioavailability of SFN, we do not expect that the single dose of SFN will have acted on the majority immune cells themselves as they will likely have primarily infiltrated at a later time point.

**Temporal Effects of SFN in the Air Pouch Inflammatory Response**

We have shown in this work that SFN pre-treatment prior to LPS stimulation has an effect on the relative proportions of monocytes and neutrophils at 24 hours post-LPS. This was the point of analysis chosen for this study because it had an immune response that was composed of substantial populations of both neutrophils

and macrophages. At this time point neutrophils are still the predominant cell type, suggesting that the immune response is still in the acute phase and that it has not yet started the clearing process of resolution of the infection. During the resolution stage, there is a switch from a preponderance of neutrophils set to kill the pathogen to a predominance of macrophages that infiltrate to clear up the debris.

*Effects of SFN on Resolution of the Infection*

We propose to analyze a time point after 24 hours (eg. 36 or 48 hours) to determine if SFN pre-treatment prior to LPS stimulation has an impact on the number of cells that infiltrate during the resolution of the infection. The hypothesis for this experiment is that SFN pre-treatment would dampen the macrophage response at this later time point. This is based on the *in vitro* observation (Figures 2.3 and 3.2) that SFN inhibits BMDM migration toward both CSF-1 and MIF. This may be variable based on the bioavailability of SFN in the mouse. Incorporation of multiple doses of SFN, based on the pharmacokinetic studies described above, may be a preferred method for testing this hypothesis at such a late time point (see discussion of SFN activity and alternative dosing strategies above).

*Effects of SFN on Gene Expression in the Pouch Lining*

Since the effects of SFN in the air pouch seem to be MIF-independent, gene expression analysis of the pouch lining is one approach that could be applied to identify a mechanism to explain the effects that SFN pre-treatment has on the cellular infiltrate in response to LPS. Pessler *et al.* performed a study of gout using

the murine air pouch model and determined that the optimal time to measure gene expression changes in the pouch lining is one hour post-stimulation [165]. We propose examining transcript levels of candidate genes in the pouch linings of mice treated with LPS alone versus SFN pre-treatment to LPS (and vehicle control) at one hour post-treatment as an initial screen for immunological targets of SFN. Given the change in populations that we observed at 24 hours (Figure 2.7), we expect to see changes in either transcriptional regulators of the immune system or chemokines. SFN has been shown to inhibit NF-κB activation *in vitro*; therefore, it would not be unexpected for SFN to act through NF-κB to affect changes in proinflammatory signaling molecules such as TNFα [9].

Very little is known about any potential effect that SFN may have on chemokine expression. However, SFN has been shown to inhibit IL-8 and MCP-1 synthesis in a human epithelial cell line; therefore it is worth examining whether any myeloid cell chemokine transcripts are changed in the pouch lining in response to SFN-pretreatment [23]. A decrease in MCP-1 transcript levels would indicate a potential for differential macrophage migration, as we observed in transwell assays using CSF-1 as a chemoattractant (Figure 2.3), and could support the hypothesis that while macrophages infiltration is not significantly different at 24 hours, it may be affected during the resolution stage. IL-8 (CXCL8) is a neutrophil chemoattractant; therefore, we might expect it to be slightly elevated in the SFN pre-treated group compared to LPS alone because there is a trend toward increased neutrophil chemotaxis in that group, which could be induced by IL-8. If IL-8 is not inhibited in the air pouch as it is in the *in vitro* study by Starrett *et al*. using cigarette

smoke, we might then hypothesize that SFN has different effects on IL-8 expression based on the stimulus [23].

**Functional Studies of Air Pouch Immune Infiltrate**

In the present study, we have simply shown that there is a SFN-dependent difference in the percentages of infiltrating cells in response to LPS. We have not examined whether there is a functional difference in these cells due to the introduction of SFN prior to LPS stimulation. In future studies, we propose examining the cellular infiltrate in the pouch for functional differences in immune cells in SFN pre-treated versus LPS alone groups. There is precedent in the literature that supports the hypothesis that SFN may affect the function of macrophages and neutrophils. SFN can inhibit ROS production by neutrophils in both *in vitro* (in primary human neutrophils and in the HL60 cell line) and *in vivo* models (murine neutrophils in HSV-infected brains) [20, 116]. Peritoneal macrophages from healthy Balb/c mice show increased phagocytic ability as well as decreased TNFα secretion when treated with SFN *in vitro* [21].

Based on these studies, we predict that harvesting the immune cells from pouches after the 24 hour experiment and measuring functional parameters such as phagocytosis and ROS production will reveal differences between the SFN pre-treated and LPS alone groups. We do not know the bioavailability of SFN within the pouch after a single pre-treatment; therefore, it would be prudent to follow up the alternative dosing strategy studies with these functional studies to compare whether multiple doses of SFN has a more significant effect on the function of

infiltrating immune cells than a single pre-treatment dose. We would expect a more significant impact on functional parameters with increasing treatments of SFN.

**Functional Studies of MDSCs in Obese MIF$^{-/-}$ Mice**

Our obesity study clearly demonstrated that there is no MIF-dependent change in the MDSC or macrophage populations in the WAT during HFD-induced obesity (Figure 4.3). However, we did not evaluate whether the lack of MIF in this model has an effect on the function of WAT infiltrating immune cells. MIF has been shown to be an important regulator of T cell and NK activity [166, 167]. Another study showed that MIF is important for regulation of the trafficking of MDSCs, but we have not yet analyzed the functional importance of MIF in these cells in the context of obesity [55].

We propose to use FACS to isolate MDSC populations from obese MIF WT and MIF$^{-/-}$ mice. We would purify MDSCs by CD11b+ and GR1+ cell surface markers for isolation of the entire MDSC population, or with Ly6C versus Ly6G for the monocytic and granulocytic MDSC populations, respectively. T cell suppression assays would be performed *in vitro* with MDSCs to determine whether the absence of MIF affects their suppressive phenotype. We would also perform qPCR on these isolated cells to measure transcript levels of iNOS and arginase, both of which are hallmarks of MDSC suppressive function [98, 103, 168]. We do not expect to see a MIF-dependent difference in MDSC function in obese mice. We analyzed disease endpoints such as weight gain and blood glucose and insulin levels as indicators of metabolic disruption and did not see any MIF-dependent differences. Immune cells

play an important role in the development of metabolic disorders in obesity; therefore, if the function of MDSCs was compromised, we would have expected to see that reflected in those systemic parameters. Despite the hypothesis that we will not see a MIF-dependent difference in these experiments, they are still critical in furthering our understanding of the effects of MIF both in general and in the context of obesity.

For completeness of the study, it may be pertinent to examine similar parameters in other cell types. As a pro-inflammatory mediator of many different cell types and pathways, MIF could be acting on macrophages, T cells, or other immune cells in the vWAT. FACS sorting could be utilized to isolate M1 versus M2 macrophages and various T cell populations to assay for MIF-dependent effects in these populations. We could also apply a screening-type approach using gene analysis of obese tissue to suggest inflammatory genes that are differentially regulated in obese MIF WT versus MIF$^{-/-}$ mice and hone in on immune cells that are likely impacted by those changes in gene expression within the tissue.

**Conditional MIF$^{-/-}$ Studies in the DIO Model**

It was mentioned in the discussion that MIF expression from the many MIF-secreting cell types throughout the mouse could mask any effect that adipose-derived MIF has in the context of obesity. We propose to tackle this problem using a conditional MIF$^{-/-}$ mouse that has been engineered to delete MIF from the WAT at the desired time point (Tet-inducible model in the cre-loxP system with a WAT-specific gene such as *fab4/aP2*) [169, 170]. We would induce WAT deletion of MIF

one week prior to starting HFD feeding and monitor weight, blood glucose, and blood insulin over the course of an eight-week diet. Other time points may be relevant additions to this study, but we saw significant changes in our obese mice compared to chow at 8 weeks of HFD; therefore, it is a good starting point to look for phenotypic changes. If time is not an issue, it may be prudent to conduct a longer study to allow more time to MIF-dependent changes to occur in the adipose tissue as the disease progresses. At the end of the experiment we would harvest WAT and examine the infiltrating immune cell populations by flow cytometry. We speculate that the absence of MIF in the WAT will result in fewer immune cells, particularly MDSCs and M1 macrophages, infiltrating the WAT. We hypothesize that this difference will occur because we have seen that MIF is a chemotactic signal for BMDM *in vitro* (Figure 3.2) and that mMDSCs are more prevalent in MIF expressing tumors in mice [55].

**The Effect of SFN on the Development of Obesity**

One of the overarching goals upon embarking on our study of MIF in the immune response to obesity was the eventual aim of testing the effects of the MIF inhibitor SFN on the obese mouse. It has been shown that 5-times weekly IP treatments of SFN are able to reduce blood pressure, cardiac hypertrophy, and cardiac fibrosis in a murine model of STZ-induced Type 1 diabetes [171]. Another group studying a similar STZ-induced model of T1D demonstrated that daily SFN treatment prevented mice from developing the same degree of diabetic

nephropathy as untreated mice [172]. This leads us to wonder if SFN could ameliorate some of the symptoms of T2D in our model of diet-induced obesity.

We propose two different approaches to examining the effects of SFN on T2D in a DIO model. The first is a question of whether SFN can be used as a preventative agent prior to development of the disease. For these experiments we would administer 5-times weekly IP treatments [171] of SFN to mice from the age of 4 weeks to 8 weeks and place the mice on HFD from 6 weeks of age to 14 weeks of age. Blood glucose would be measured bi-weekly and blood insulin once a month over the course of the experiment. The second experiment is an examination of the corrective therapeutic effect of SFN. We would place mice on HFD at 6 weeks of age for 8 weeks and give SFN treatments 5 times a week from 10 to 14 weeks of age. Blood glucose and insulin would be measured as in the first scenario. We expect SFN to have a small, but significant, effect in lowering blood glucose and insulin levels, based on the success of the T1D studies [171, 172]. We suspect that the two sets of experiments would convey similar benefits to the mice and that to have a more significant effect one would have to administer SFN starting before onset of HFD and running the entire course of the experiment. If there was to be a protective effect of SFN in either scenario, it would support the idea of encouraging obese individuals to add larger quantities of SFN-containing vegetables, such as broccoli, to their diets. This could be followed by similar studies that replace the IP treatment with a feeding regimen where broccoli sprouts (high in the precursor to SFN) are given to mice as part of a powered diet to assess the effects of oral SFN from a relevant food source.

**The Effect of MIF in the Contribution of Obesity to the Development of Cancer**

It has been shown that obesity predisposes people to developing cancer [72]. The reason for that is as yet unknown. Our lab has demonstrated that MIF is critical for the progression of cancer in the murine 4T1 breast tumor model. The absence of intratumoral MIF in this model results in reduced tumor burden as well as a reduction in the number of intratumoral monocytic MDSCs. Similar observations were made in the CT26 model [55]. It would be interesting to examine whether there is a similar effect on tumor growth and MDSC tumor infiltration in obese mice bearing MIF-deficient tumors.

We hypothesize that obese mice given cancer that does not express MIF would have a slower tumor growth rate and lower intratumoral mMDSC infiltration than obese mice with MIF WT tumors. We would test this by feeding C57Bl/6 mice high fat diet until significant weight had been gained (about 4-8 weeks). Tumors, such as LLC (MIF WT or MIF-depleted), would then be implanted subcutaneously into the flanks of the obese mice. Tumor growth rates in obese mice would be compared to tumor growth rates in normal chow control mice (MIF WT or $MIF^{-/-}$). Tumor size would be measured using electronic calipers and FACS analysis would be performed on digested tumors to determine the extent of intratumoral immune cell infiltration. We expect that the absence of MIF in the tumors of these obese mice would reduce the intratumoral mMDSC population and result in a delay in tumor progression. This would indicate that local inhibition of MIF within the tumor itself could be considered as a potential adjuvant therapy in obese cancer patients.

**Final Summary Statement**

Our goal in this work was to examine the effects of SFN as a MIF inhibitor in both acute and chronic inflammation. Using the air pouch model of LPS-induced inflammation, we have shown that SFN modulates the acute immune response and may exhibit pro-inflammatory, rather than the anticipated anti-inflammatory, effects. SFN inhibits MIF tautomerase in the pouch lining, but we suspect that SFN is acting through MIF-independent pathways to alter monocyte and neutrophil populations in the pouch in response to LPS. Our results from air pouch studies in $MIF^{-/-}$ mice have led us to conclude that MIF plays a complex role in the acute immune response that requires further investigation to ascertain the true effects of MIF in the acute inflammatory response.

We employed a HFD-induced model of obesity to examine the effects of MIF on the chronic immune response to obesity. Our studies showed that MIF WT and $MIF^{-/-}$ mice gained weight and adipose tissue mass equivalently. Accumulation of MDSCs and macrophages in the vWAT is not a MIF-dependent process, nor is the development of insulin resistance in this model. Despite human studies suggesting the utility of targeting MIF in obese patients [28, 84, 140], the lack of difference in a global MIF knockout model suggests that systemic treatment using SFN may not be an effective anti-inflammatory therapy for obese patients.

Overall, we submit that SFN has previously unrecognized effects on the acute immune response *in vivo*, that MIF is a highly complex immune mediator in both acute and chronic inflammation, and that the application of SFN as a MIF inhibitor in immunological disease processes should be approached with caution.

**List of Figures:**

# Chapter 7: References

1. Abbas, A. K., Lichtman, A. H., Pillai, S. (2010) Cellular and Molecular Immunology. Saunders Elsevier, Philadelphia, PA, USA.
2. Weiss, U. (2008) Inflammation. Nature 454, 427.
3. Medzhitov, R. (2008) Origin and physiological roles of inflammation. Nature 454, 428-35.
4. Cheung, K. L. and Kong, A. N. (2010) Molecular targets of dietary phenethyl isothiocyanate and sulforaphane for cancer chemoprevention. AAPS J 12, 87-97.
5. Clarke, J. D., Dashwood, R. H., Ho, E. (2008) Multi-targeted prevention of cancer by sulforaphane. Cancer Lett 269, 291-304.
6. Juge, N., Mithen, R. F., Traka, M. (2007) Molecular basis for chemoprevention by sulforaphane: a comprehensive review. Cell Mol Life Sci 64, 1105-27.
7. Sharma, R., Sharma, A., Chaudhary, P., Sahu, M., Jaiswal, S., Awasthi, S., Awasthi, Y. C. (2012) Role of 4-hydroxynonenal in chemopreventive activities of sulforaphane. Free Radic Biol Med 52, 2177-85.
8. Guo, S., Qiu, P., Xu, G., Wu, X., Dong, P., Yang, G., Zheng, J., McClements, D. J., Xiao, H. (2012) Synergistic anti-inflammatory effects of nobiletin and sulforaphane in lipopolysaccharide-stimulated RAW 264.7 cells. J Agric Food Chem 60, 2157-64.
9. Heiss, E., Herhaus, C., Klimo, K., Bartsch, H., Gerhäuser, C. (2001) Nuclear factor kappa B is a molecular target for sulforaphane-mediated anti-inflammatory mechanisms. J Biol Chem 276, 32008-15.
10. James, D., Devaraj, S., Bellur, P., Lakkanna, S., Vicini, J., Boddupalli, S. (2012) Novel concepts of broccoli sulforaphanes and disease: induction of phase II antioxidant and detoxification enzymes by enhanced-glucoraphanin broccoli. Nutr Rev 70, 654-65.
11. Gao, X. and Talalay, P. (2004) Induction of phase 2 genes by sulforaphane protects retinal pigment epithelial cells against photooxidative damage. Proc Natl Acad Sci U S A 101, 10446-51.
12. Zhang, Y., Talalay, P., Cho, C. G., Posner, G. H. (1992) A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure. Proc Natl Acad Sci U S A 89, 2399-403.
13. Conaway, C. C., Getahun, S. M., Liebes, L. L., Pusateri, D. J., Topham, D. K., Botero-Omary, M., Chung, F. L. (2000) Disposition of glucosinolates and sulforaphane in humans after ingestion of steamed and fresh broccoli. Nutr Cancer 38, 168-78.
14. Clarke, J. D., Hsu, A., Riedl, K., Bella, D., Schwartz, S. J., Stevens, J. F., Ho, E. (2011) Bioavailability and inter-conversion of sulforaphane and erucin in human subjects consuming broccoli sprouts or broccoli supplement in a cross-over study design. Pharmacol Res 64, 456-63.
15. Kong, J. S., Yoo, S. A., Kim, H. S., Kim, H. A., Yea, K., Ryu, S. H., Chung, Y. J., Cho, C. S., Kim, W. U. (2010) Inhibition of synovial hyperplasia, rheumatoid T cell activation, and experimental arthritis in mice by sulforaphane, a naturally occurring isothiocyanate. Arthritis Rheum 62, 159-70.
16. Hu, R., Hebbar, V., Kim, B. R., Chen, C., Winnik, B., Buckley, B., Soteropoulos, P., Tolias, P., Hart, R. P., Kong, A. N. (2004) In vivo pharmacokinetics and

regulation of gene expression profiles by isothiocyanate sulforaphane in the rat. J Pharmacol Exp Ther 310, 263-71.
17. Lin, W., Wu, R. T., Wu, T., Khor, T. O., Wang, H., Kong, A. N. (2008) Sulforaphane suppressed LPS-induced inflammation in mouse peritoneal macrophages through Nrf2 dependent pathway. Biochem Pharmacol 76, 967-73.
18. Zhu, M., Zhang, Y., Cooper, S., Sikorski, E., Rohwer, J., Bowden, G. T. (2004) Phase II enzyme inducer, sulforaphane, inhibits UVB-induced AP-1 activation in human keratinocytes by a novel mechanism. Mol Carcinog 41, 179-86.
19. Koo, J. E., Park, Z. Y., Kim, N. D., Lee, J. Y. (2013) Sulforaphane inhibits the engagement of LPS with TLR4/MD2 complex by preferential binding to Cys133 in MD2. Biochem Biophys Res Commun 434, 600-5.
20. Dias, I. H., Chapple, I. L., Milward, M., Grant, M. M., Hill, E., Brown, J., Griffiths, H. R. (2013) Sulforaphane restores cellular glutathione levels and reduces chronic periodontitis neutrophil hyperactivity in vitro. PLoS One 8, e66407.
21. Thejass, P. and Kuttan, G. (2007) Immunomodulatory activity of Sulforaphane, a naturally occurring isothiocyanate from broccoli (Brassica oleracea). Phytomedicine 14, 538-45.
22. Liu, Y. C., Hsieh, C. W., Weng, Y. C., Chuang, S. H., Hsieh, C. Y., Wung, B. S. (2008) Sulforaphane inhibition of monocyte adhesion via the suppression of ICAM-1 and NF-kappaB is dependent upon glutathione depletion in endothelial cells. Vascul Pharmacol 48, 54-61.
23. Starrett, W. and Blake, D. J. (2011) Sulforaphane inhibits de novo synthesis of IL-8 and MCP-1 in human epithelial cells generated by cigarette smoke extract. J Immunotoxicol 8, 150-8.
24. Wu, L., Noyan Ashraf, M. H., Facci, M., Wang, R., Paterson, P. G., Ferrie, A., Juurlink, B. H. (2004) Dietary approach to attenuate oxidative stress, hypertension, and inflammation in the cardiovascular system. Proc Natl Acad Sci U S A 101, 7094-9.
25. Youn, H. S., Kim, Y. S., Park, Z. Y., Kim, S. Y., Choi, N. Y., Joung, S. M., Seo, J. A., Lim, K. M., Kwak, M. K., Hwang, D. H., Lee, J. Y. (2010) Sulforaphane suppresses oligomerization of TLR4 in a thiol-dependent manner. J Immunol 184, 411-9.
26. Cross, J. V., Rady, J. M., Foss, F. W., Lyons, C. E., Macdonald, T. L., Templeton, D. J. (2009) Nutrient isothiocyanates covalently modify and inhibit the inflammatory cytokine macrophage migration inhibitory factor (MIF). Biochem J 423, 315-21.
27. Calandra, T. and Roger, T. (2003) Macrophage migration inhibitory factor: a regulator of innate immunity. Nat Rev Immunol 3, 791-800.
28. Dandona, P., Aljada, A., Ghanim, H., Mohanty, P., Tripathy, C., Hofmeyer, D., Chaudhuri, A. (2004) Increased plasma concentration of macrophage migration inhibitory factor (MIF) and MIF mRNA in mononuclear cells in the obese and the suppressive action of metformin. J Clin Endocrinol Metab 89, 5043-7.

29. Zhou, J., Joplin, D. G., Cross, J. V., Templeton, D. J. (2012) Sulforaphane inhibits prostaglandin E2 synthesis by suppressing microsomal prostaglandin E synthase 1. PLoS One 7, e49744.
30. Al-Abed, Y., Dabideen, D., Aljabari, B., Valster, A., Messmer, D., Ochani, M., Tanovic, M., Ochani, K., Bacher, M., Nicoletti, F., Metz, C., Pavlov, V. A., Miller, E. J., Tracey, K. J. (2005) ISO-1 binding to the tautomerase active site of MIF inhibits its pro-inflammatory activity and increases survival in severe sepsis. J Biol Chem 280, 36541-4.
31. David, J. R. (1966) Delayed hypersensitivity in vitro: its mediation by cell-free substances formed by lymphoid cell-antigen interaction. Proc Natl Acad Sci U S A 56, 72-7.
32. Bloom, B. R. and Bennett, B. (1966) Mechanism of a reaction in vitro associated with delayed-type hypersensitivity. Science 153, 80-2.
33. Gregory, J. L., Morand, E. F., McKeown, S. J., Ralph, J. A., Hall, P., Yang, Y. H., McColl, S. R., Hickey, M. J. (2006) Macrophage migration inhibitory factor induces macrophage recruitment via CC chemokine ligand 2. J Immunol 177, 8072-9.
34. Conroy, H., Mawhinney, L., Donnelly, S. C. (2010) Inflammation and cancer: macrophage migration inhibitory factor (MIF)--the potential missing link. QJM 103, 831-6.
35. Esumi, N., Budarf, M., Ciccarelli, L., Sellinger, B., Kozak, C. A., Wistow, G. (1998) Conserved gene structure and genomic linkage for D-dopachrome tautomerase (DDT) and MIF. Mamm Genome 9, 753-7.
36. Calandra, T., Bernhagen, J., Metz, C. N., Spiegel, L. A., Bacher, M., Donnelly, T., Cerami, A., Bucala, R. (1995) MIF as a glucocorticoid-induced modulator of cytokine production. Nature 377, 68-71.
37. Kleemann, R., Hausser, A., Geiger, G., Mischke, R., Burger-Kentischer, A., Flieger, O., Johannes, F. J., Roger, T., Calandra, T., Kapurniotu, A., Grell, M., Finkelmeier, D., Brunner, H., Bernhagen, J. (2000) Intracellular action of the cytokine MIF to modulate AP-1 activity and the cell cycle through Jab1. Nature 408, 211-6.
38. Mitchell, R. A., Metz, C. N., Peng, T., Bucala, R. (1999) Sustained mitogen-activated protein kinase (MAPK) and cytoplasmic phospholipase A2 activation by macrophage migration inhibitory factor (MIF). Regulatory role in cell proliferation and glucocorticoid action. J Biol Chem 274, 18100-6.
39. Noels, H., Bernhagen, J., Weber, C. (2009) Macrophage migration inhibitory factor: a noncanonical chemokine important in atherosclerosis. Trends Cardiovasc Med 19, 76-86.
40. Waeber, G., Calandra, T., Bonny, C., Bucala, R. (1999) A role for the endocrine and pro-inflammatory mediator MIF in the control of insulin secretion during stress. Diabetes Metab Res Rev 15, 47-54.
41. Bernhagen, J., Mitchell, R. A., Calandra, T., Voelter, W., Cerami, A., Bucala, R. (1994) Purification, bioactivity, and secondary structure analysis of mouse and human macrophage migration inhibitory factor (MIF). Biochemistry 33, 14144-55.

42. Verjans, E., Noetzel, E., Bektas, N., Schütz, A. K., Lue, H., Lennartz, B., Hartmann, A., Dahl, E., Bernhagen, J. (2009) Dual role of macrophage migration inhibitory factor (MIF) in human breast cancer. BMC Cancer 9, 230.
43. Santos, L. L. and Morand, E. F. (2009) Macrophage migration inhibitory factor: a key cytokine in RA, SLE and atherosclerosis. Clin Chim Acta 399, 1-7.
44. Rosengren, E., Bucala, R., Aman, P., Jacobsson, L., Odh, G., Metz, C. N., Rorsman, H. (1996) The immunoregulatory mediator macrophage migration inhibitory factor (MIF) catalyzes a tautomerization reaction. Mol Med 2, 143-9.
45. Lubetsky, J. B., Swope, M., Dealwis, C., Blake, P., Lolis, E. (1999) Pro-1 of macrophage migration inhibitory factor functions as a catalytic base in the phenylpyruvate tautomerase activity. Biochemistry 38, 7346-54.
46. Rosengren, E., Aman, P., Thelin, S., Hansson, C., Ahlfors, S., Björk, P., Jacobsson, L., Rorsman, H. (1997) The macrophage migration inhibitory factor MIF is a phenylpyruvate tautomerase. FEBS Lett 417, 85-8.
47. Adamali, H., Armstrong, M. E., McLaughlin, A. M., Cooke, G., McKone, E., Costello, C. M., Gallagher, C. G., Leng, L., Baugh, J. A., Fingerle-Rowson, G., Bucala, R. J., McLoughlin, P., Donnelly, S. C. (2012) Macrophage migration inhibitory factor enzymatic activity, lung inflammation, and cystic fibrosis. Am J Respir Crit Care Med 186, 162-9.
48. Kleemann, R., Kapurniotu, A., Frank, R. W., Gessner, A., Mischke, R., Flieger, O., Jüttner, S., Brunner, H., Bernhagen, J. (1998) Disulfide analysis reveals a role for macrophage migration inhibitory factor (MIF) as thiol-protein oxidoreductase. J Mol Biol 280, 85-102.
49. Hudson, J. D., Shoaibi, M. A., Maestro, R., Carnero, A., Hannon, G. J., Beach, D. H. (1999) A proinflammatory cytokine inhibits p53 tumor suppressor activity. J Exp Med 190, 1375-82.
50. Mitchell, R. A., Liao, H., Chesney, J., Fingerle-Rowson, G., Baugh, J., David, J., Bucala, R. (2002) Macrophage migration inhibitory factor (MIF) sustains macrophage proinflammatory function by inhibiting p53: regulatory role in the innate immune response. Proc Natl Acad Sci U S A 99, 345-50.
51. Leng, L., Metz, C. N., Fang, Y., Xu, J., Donnelly, S., Baugh, J., Delohery, T., Chen, Y., Mitchell, R. A., Bucala, R. (2003) MIF signal transduction initiated by binding to CD74. J Exp Med 197, 1467-76.
52. Shi, X., Leng, L., Wang, T., Wang, W., Du, X., Li, J., McDonald, C., Chen, Z., Murphy, J. W., Lolis, E., Noble, P., Knudson, W., Bucala, R. (2006) CD44 is the signaling component of the macrophage migration inhibitory factor-CD74 receptor complex. Immunity 25, 595-606.
53. Bernhagen, J., Krohn, R., Lue, H., Gregory, J. L., Zernecke, A., Koenen, R. R., Dewor, M., Georgiev, I., Schober, A., Leng, L., Kooistra, T., Fingerle-Rowson, G., Ghezzi, P., Kleemann, R., McColl, S. R., Bucala, R., Hickey, M. J., Weber, C. (2007) MIF is a noncognate ligand of CXC chemokine receptors in inflammatory and atherogenic cell recruitment. Nat Med 13, 587-96.
54. Tarnowski, M., Grymula, K., Liu, R., Tarnowska, J., Drukala, J., Ratajczak, J., Mitchell, R. A., Ratajczak, M. Z., Kucia, M. (2010) Macrophage migration inhibitory factor is secreted by rhabdomyosarcoma cells, modulates tumor

metastasis by binding to CXCR4 and CXCR7 receptors and inhibits recruitment of cancer-associated fibroblasts. Mol Cancer Res 8, 1328-43.
55. Simpson, K. D., Templeton, D. J., Cross, J. V. (2012) Macrophage migration inhibitory factor promotes tumor growth and metastasis by inducing myeloid-derived suppressor cells in the tumor microenvironment. J Immunol 189, 5533-40.
56. Santos, L. L., Fan, H., Hall, P., Ngo, D., Mackay, C. R., Fingerle-Rowson, G., Bucala, R., Hickey, M. J., Morand, E. F. (2011) Macrophage migration inhibitory factor regulates neutrophil chemotactic responses in inflammatory arthritis in mice. Arthritis Rheum 63, 960-70.
57. Makita, H., Nishimura, M., Miyamoto, K., Nakano, T., Tanino, Y., Hirokawa, J., Nishihira, J., Kawakami, Y. (1998) Effect of anti-macrophage migration inhibitory factor antibody on lipopolysaccharide-induced pulmonary neutrophil accumulation. Am J Respir Crit Care Med 158, 573-9.
58. Gregory, J. L., Leech, M. T., David, J. R., Yang, Y. H., Dacumos, A., Hickey, M. J. (2004) Reduced leukocyte-endothelial cell interactions in the inflamed microcirculation of macrophage migration inhibitory factor-deficient mice. Arthritis Rheum 50, 3023-34.
59. Cheng, Q., McKeown, S. J., Santos, L., Santiago, F. S., Khachigian, L. M., Morand, E. F., Hickey, M. J. (2010) Macrophage migration inhibitory factor increases leukocyte-endothelial interactions in human endothelial cells via promotion of expression of adhesion molecules. J Immunol 185, 1238-47.
60. Bernhagen, J., Calandra, T., Mitchell, R. A., Martin, S. B., Tracey, K. J., Voelter, W., Manogue, K. R., Cerami, A., Bucala, R. (1993) MIF is a pituitary-derived cytokine that potentiates lethal endotoxaemia. Nature 365, 756-9.
61. Bozza, M., Satoskar, A. R., Lin, G., Lu, B., Humbles, A. A., Gerard, C., David, J. R. (1999) Targeted disruption of migration inhibitory factor gene reveals its critical role in sepsis. J Exp Med 189, 341-6.
62. Calandra, T., Echtenacher, B., Roy, D. L., Pugin, J., Metz, C. N., Hültner, L., Heumann, D., Männel, D., Bucala, R., Glauser, M. P. (2000) Protection from septic shock by neutralization of macrophage migration inhibitory factor. Nat Med 6, 164-70.
63. Honma, N., Koseki, H., Akasaka, T., Nakayama, T., Taniguchi, M., Serizawa, I., Akahori, H., Osawa, M., Mikayama, T. (2000) Deficiency of the macrophage migration inhibitory factor gene has no significant effect on endotoxaemia. Immunology 100, 84-90.
64. Edwards, J. C., Sedgwick, A. D., Willoughby, D. A. (1981) The formation of a structure with the features of synovial lining by subcutaneous injection of air: an in vivo tissue culture system. J Pathol 134, 147-56.
65. Bottomley, K. M., Griffiths, R. J., Rising, T. J., Steward, A. (1988) A modified mouse air pouch model for evaluating the effects of compounds on granuloma induced cartilage degradation. Br J Pharmacol 93, 627-35.
66. Romano, M., Faggioni, R., Sironi, M., Sacco, S., Echtenacher, B., Di Santo, E., Salmona, M., Ghezzi, P. (1997) Carrageenan-induced acute inflammation in the mouse air pouch synovial model. Role of tumour necrosis factor. Mediators Inflamm 6, 32-8.

67. Wilkinson, L. S., Moore, A. R., Pitsillides, A. A., Willoughby, D. A., Edwards, J. C. (1993) Comparison of surface fibroblastic cells in subcutaneous air pouch and synovial lining: differences in uridine diphosphoglucose dehydrogenase activity. Int J Exp Pathol 74, 113-5.
68. CDC (2010) Vital Signs: State-Specific Obesity Prevalence Among Adults. Volume 59CDC, Morbidity and Mortality Weekly Report 1-5.
69. WHO (2011) WHO Media Centre Fact Sheet: Obesity and Overweight.
70. Cancello, R. and Clément, K. (2006) Is obesity an inflammatory illness? Role of low-grade inflammation and macrophage infiltration in human white adipose tissue. BJOG 113, 1141-7.
71. Tran, T. T. and Kahn, C. R. (2010) Transplantation of adipose tissue and stem cells: role in metabolism and disease. Nat Rev Endocrinol 6, 195-213.
72. Harvey, A. E., Lashinger, L. M., Hursting, S. D. (2011) The growing challenge of obesity and cancer: an inflammatory issue. Ann N Y Acad Sci 1229, 45-52.
73. CDC (2011) Obesity and Overweight for Professionals: Data and Statistics: Adult Obesity. CDC.
74. Flegal, K. M., Carroll, M. D., Ogden, C. L., Curtin, L. R. (2010) Prevalence and trends in obesity among US adults, 1999-2008. JAMA 303, 235-41.
75. Ibrahim, M. M. (2010) Subcutaneous and visceral adipose tissue: structural and functional differences. Obes Rev 11, 11-8.
76. O'Rourke, R. W. (2009) Inflammation in obesity-related diseases. Surgery 145, 255-9.
77. Ferris, W. F. and Crowther, N. J. (2011) Once fat was fat and that was that: our changing perspectives on adipose tissue. Cardiovasc J Afr 22, 147-54.
78. Duffaut, C., Galitzky, J., Lafontan, M., Bouloumié, A. (2009) Unexpected trafficking of immune cells within the adipose tissue during the onset of obesity. Biochem Biophys Res Commun 384, 482-5.
79. Murano, I., Barbatelli, G., Parisani, V., Latini, C., Muzzonigro, G., Castellucci, M., Cinti, S. (2008) Dead adipocytes, detected as crown-like structures, are prevalent in visceral fat depots of genetically obese mice. J Lipid Res 49, 1562-8.
80. Lumeng, C. N. and Saltiel, A. R. (2011) Inflammatory links between obesity and metabolic disease. J Clin Invest 121, 2111-7.
81. Neels, J. G. and Olefsky, J. M. (2006) Inflamed fat: what starts the fire? J Clin Invest 116, 33-5.
82. Zeyda, M. and Stulnig, T. M. (2007) Adipose tissue macrophages. Immunol Lett 112, 61-7.
83. Xu, H., Barnes, G. T., Yang, Q., Tan, G., Yang, D., Chou, C. J., Sole, J., Nichols, A., Ross, J. S., Tartaglia, L. A., Chen, H. (2003) Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. J Clin Invest 112, 1821-30.
84. Ghanim, H., Aljada, A., Hofmeyer, D., Syed, T., Mohanty, P., Dandona, P. (2004) Circulating mononuclear cells in the obese are in a proinflammatory state. Circulation 110, 1564-71.

85. Alvehus, M., Burén, J., Sjöström, M., Goedecke, J., Olsson, T. (2010) The human visceral fat depot has a unique inflammatory profile. Obesity (Silver Spring) 18, 879-83.
86. Verschuren, L., Kooistra, T., Bernhagen, J., Voshol, P. J., Ouwens, D. M., van Erk, M., de Vries-van der Weij, J., Leng, L., van Bockel, J. H., van Dijk, K. W., Fingerle-Rowson, G., Bucala, R., Kleemann, R. (2009) MIF deficiency reduces chronic inflammation in white adipose tissue and impairs the development of insulin resistance, glucose intolerance, and associated atherosclerotic disease. Circ Res 105, 99-107.
87. Waeber, G., Calandra, T., Roduit, R., Haefliger, J. A., Bonny, C., Thompson, N., Thorens, B., Temler, E., Meinhardt, A., Bacher, M., Metz, C. N., Nicod, P., Bucala, R. (1997) Insulin secretion is regulated by the glucose-dependent production of islet beta cell macrophage migration inhibitory factor. Proc Natl Acad Sci U S A 94, 4782-7.
88. Stosic-Grujicic, S., Stojanovic, I., Maksimovic-Ivanic, D., Momcilovic, M., Popadic, D., Harhaji, L., Miljkovic, D., Metz, C., Mangano, K., Papaccio, G., Al-Abed, Y., Nicoletti, F. (2008) Macrophage migration inhibitory factor (MIF) is necessary for progression of autoimmune diabetes mellitus. J Cell Physiol 215, 665-75.
89. Sanchez-Zamora, Y., Terrazas, L. I., Vilches-Flores, A., Leal, E., Juárez, I., Whitacre, C., Kithcart, A., Pruitt, J., Sielecki, T., Satoskar, A. R., Rodriguez-Sosa, M. (2010) Macrophage migration inhibitory factor is a therapeutic target in treatment of non-insulin-dependent diabetes mellitus. FASEB J 24, 2583-90.
90. Serre-Beinier, V., Toso, C., Morel, P., Gonelle-Gispert, C., Veyrat-Durebex, C., Rohner-Jeanrenaud, F., Calandra, T., Roger, T., James, R. W., Montet, X., Bühler, L., Bosco, D., Berney, T. (2010) Macrophage migration inhibitory factor deficiency leads to age-dependent impairment of glucose homeostasis in mice. J Endocrinol 206, 297-306.
91. Cripps, J. G. and Gorham, J. D. (2011) MDSC in autoimmunity. Int Immunopharmacol 11, 789-93.
92. Gabrilovich, D. I. and Nagaraj, S. (2009) Myeloid-derived suppressor cells as regulators of the immune system. Nat Rev Immunol 9, 162-74.
93. Sica, A. and Bronte, V. (2007) Altered macrophage differentiation and immune dysfunction in tumor development. J Clin Invest 117, 1155-66.
94. Condamine, T. and Gabrilovich, D. I. (2011) Molecular mechanisms regulating myeloid-derived suppressor cell differentiation and function. Trends Immunol 32, 19-25.
95. Kusmartsev, S. and Gabrilovich, D. I. (2006) Role of immature myeloid cells in mechanisms of immune evasion in cancer. Cancer Immunol Immunother 55, 237-45.
96. Gabrilovich, D. I., Ostrand-Rosenberg, S., Bronte, V. (2012) Coordinated regulation of myeloid cells by tumours. Nat Rev Immunol 12, 253-68.
97. Movahedi, K., Guilliams, M., Van den Bossche, J., Van den Bergh, R., Gysemans, C., Beschin, A., De Baetselier, P., Van Ginderachter, J. A. (2008) Identification of discrete tumor-induced myeloid-derived suppressor cell subpopulations with distinct T cell-suppressive activity. Blood 111, 4233-44.

98. Peranzoni, E., Zilio, S., Marigo, I., Dolcetti, L., Zanovello, P., Mandruzzato, S., Bronte, V. (2010) Myeloid-derived suppressor cell heterogeneity and subset definition. Curr Opin Immunol 22, 238-44.
99. Xia, S., Sha, H., Yang, L., Ji, Y., Ostrand-Rosenberg, S., Qi, L. (2011) Gr-1+ CD11b+ myeloid-derived suppressor cells suppress inflammation and promote insulin sensitivity in obesity. J Biol Chem 286, 23591-9.
100. Umemura, N., Saio, M., Suwa, T., Kitoh, Y., Bai, J., Nonaka, K., Ouyang, G. F., Okada, M., Balazs, M., Adany, R., Shibata, T., Takami, T. (2008) Tumor-infiltrating myeloid-derived suppressor cells are pleiotropic-inflamed monocytes/macrophages that bear M1- and M2-type characteristics. J Leukoc Biol 83, 1136-44.
101. Tu, S. P., Jin, H., Shi, J. D., Zhu, L. M., Suo, Y., Lu, G., Liu, B., Wang, T., Yang, C. S. (2011) Curcumin induces the differentiation of myeloid-derived suppressor cells and inhibits their interaction with cancer cells and related tumor growth. Cancer Prev Res (Phila).
102. Youn, J. I., Nagaraj, S., Collazo, M., Gabrilovich, D. I. (2008) Subsets of myeloid-derived suppressor cells in tumor-bearing mice. J Immunol 181, 5791-802.
103. Dietlin, T. A., Hofman, F. M., Lund, B. T., Gilmore, W., Stohlman, S. A., van der Veen, R. C. (2007) Mycobacteria-induced Gr-1+ subsets from distinct myeloid lineages have opposite effects on T cell expansion. J Leukoc Biol 81, 1205-12.
104. Nagaraj, S., Gupta, K., Pisarev, V., Kinarsky, L., Sherman, S., Kang, L., Herber, D. L., Schneck, J., Gabrilovich, D. I. (2007) Altered recognition of antigen is a mechanism of CD8+ T cell tolerance in cancer. Nat Med 13, 828-35.
105. Toh, B., Wang, X., Keeble, J., Sim, W. J., Khoo, K., Wong, W. C., Kato, M., Prevost-Blondel, A., Thiery, J. P., Abastado, J. P. (2011) Mesenchymal transition and dissemination of cancer cells is driven by myeloid-derived suppressor cells infiltrating the primary tumor. PLoS Biol 9, e1001162.
106. Yang, L., DeBusk, L. M., Fukuda, K., Fingleton, B., Green-Jarvis, B., Shyr, Y., Matrisian, L. M., Carbone, D. P., Lin, P. C. (2004) Expansion of myeloid immune suppressor Gr+CD11b+ cells in tumor-bearing host directly promotes tumor angiogenesis. Cancer Cell 6, 409-21.
107. Deng, Z. B., Liu, Y., Liu, C., Xiang, X., Wang, J., Cheng, Z., Shah, S. V., Zhang, S., Zhang, L., Zhuang, X., Michalek, S., Grizzle, W. E., Zhang, H. G. (2009) Immature myeloid cells induced by a high-fat diet contribute to liver inflammation. Hepatology 50, 1412-20.
108. Vickers, S. P., Jackson, H. C., Cheetham, S. C. (2011) The utility of animal models to evaluate novel anti-obesity agents. Br J Pharmacol 164, 1248-62.
109. Reuter, T. (2007) Diet-induced models for obesity and type 2 diabetes. In *Drug Discovery Today: Disease Models*, Volume 4 3-8.
110. Winzell, M. S. and Ahrén, B. (2004) The high-fat diet-fed mouse: a model for studying mechanisms and treatment of impaired glucose tolerance and type 2 diabetes. Diabetes 53 Suppl 3, S215-9.
111. Dinkova-Kostova, A. T. and Kostov, R. V. (2012) Glucosinolates and isothiocyanates in health and disease. Trends Mol Med 18, 337-47.
112. Healy, Z. R., Liu, H., Holtzclaw, W. D., Talalay, P. (2011) Inactivation of tautomerase activity of macrophage migration inhibitory factor by

sulforaphane: a potential biomarker for anti-inflammatory intervention. Cancer Epidemiol Biomarkers Prev 20, 1516-23.
113. Brown, K. K., Blaikie, F. H., Smith, R. A., Tyndall, J. D., Lue, H., Bernhagen, J., Winterbourn, C. C., Hampton, M. B. (2009) Direct modification of the proinflammatory cytokine macrophage migration inhibitory factor by dietary isothiocyanates. J Biol Chem 284, 32425-33.
114. Kallifatidis, G., Rausch, V., Baumann, B., Apel, A., Beckermann, B. M., Groth, A., Mattern, J., Li, Z., Kolb, A., Moldenhauer, G., Altevogt, P., Wirth, T., Werner, J., Schemmer, P., Büchler, M. W., Salnikov, A. V., Herr, I. (2009) Sulforaphane targets pancreatic tumour-initiating cells by NF-kappaB-induced antiapoptotic signalling. Gut 58, 949-63.
115. Kwon, J. S., Joung, H., Kim, Y. S., Shim, Y. S., Ahn, Y., Jeong, M. H., Kee, H. J. (2012) Sulforaphane inhibits restenosis by suppressing inflammation and the proliferation of vascular smooth muscle cells. Atherosclerosis 225, 41-9.
116. Schachtele, S. J., Hu, S., Lokensgard, J. R. (2012) Modulation of experimental herpes encephalitis-associated neurotoxicity through sulforaphane treatment. PLoS One 7, e36216.
117. Noyan-Ashraf, M. H., Sadeghinejad, Z., Juurlink, B. H. (2005) Dietary approach to decrease aging-related CNS inflammation. Nutr Neurosci 8, 101-10.
118. Heiss, E. and Gerhäuser, C. (2005) Time-dependent modulation of thioredoxin reductase activity might contribute to sulforaphane-mediated inhibition of NF-kappaB binding to DNA. Antioxid Redox Signal 7, 1601-11.
119. Cross, J. V., Foss, F. W., Rady, J. M., Macdonald, T. L., Templeton, D. J. (2007) The isothiocyanate class of bioactive nutrients covalently inhibit the MEKK1 protein kinase. BMC Cancer 7, 183.
120. Ulett, G. C., Ketheesan, N., Hirst, R. G. (2000) Cytokine gene expression in innately susceptible BALB/c mice and relatively resistant C57BL/6 mice during infection with virulent Burkholderia pseudomallei. Infect Immun 68, 2034-42.
121. Pattyn, F., Robbrecht, P., De Paepe, A., Speleman, F., Vandesompele, J. (2006) RTPrimerDB: the real-time PCR primer and probe database, major update 2006. Nucleic Acids Res 34, D684-8.
122. Kim, S., Elkon, K. B., Ma, X. (2004) Transcriptional suppression of interleukin-12 gene expression following phagocytosis of apoptotic cells. Immunity 21, 643-53.
123. Lee, J. M., Chan, K., Kan, Y. W., Johnson, J. A. (2004) Targeted disruption of Nrf2 causes regenerative immune-mediated hemolytic anemia. Proc Natl Acad Sci U S A 101, 9751-6.
124. Kadl, A., Galkina, E., Leitinger, N. (2009) Induction of CCR2-dependent macrophage accumulation by oxidized phospholipids in the air-pouch model of inflammation. Arthritis Rheum 60, 1362-71.
125. Singh, S. V., Warin, R., Xiao, D., Powolny, A. A., Stan, S. D., Arlotti, J. A., Zeng, Y., Hahm, E. R., Marynowski, S. W., Bommareddy, A., Desai, D., Amin, S., Parise, R. A., Beumer, J. H., Chambers, W. H. (2009) Sulforaphane inhibits prostate carcinogenesis and pulmonary metastasis in TRAMP mice in association with increased cytotoxicity of natural killer cells. Cancer Res 69, 2117-25.

126. Hamsa, T. P., Thejass, P., Kuttan, G. (2011) Induction of apoptosis by sulforaphane in highly metastatic B16F-10 melanoma cells. Drug Chem Toxicol 34, 332-40.
127. Davies, D. E., Stevens, A. J., Houston, J. B. (1992) Use of the rat air pouch model of inflammation to evaluate regional drug delivery. Agents Actions Spec No, C109-11.
128. Fragoulis, A., Laufs, J., Müller, S., Soppa, U., Siegl, S., Reiss, L. K., Tohidnezhad, M., Rosen, C., Tenbrock, K., Varoga, D., Lippross, S., Pufe, T., Wruck, C. J. (2012) Sulforaphane has opposing effects on TNF-alpha stimulated and unstimulated synoviocytes. Arthritis Res Ther 14, R220.
129. Thejass, P. and Kuttan, G. (2007) Modulation of cell-mediated immune response in B16F-10 melanoma-induced metastatic tumor-bearing C57BL/6 mice by sulforaphane. Immunopharmacol Immunotoxicol 29, 173-86.
130. Shin, S. and Kim, K. (2011) Sulforaphane Enhances MHC Class II-Restricted Presentation of Exogenous Antigen. Volume 19The Korean Society of Applied Pharmacology, Biomolecules & Therapeutics 77-83.
131. Kim, H. J., Barajas, B., Wang, M., Nel, A. E. (2008) Nrf2 activation by sulforaphane restores the age-related decrease of T(H)1 immunity: role of dendritic cells. J Allergy Clin Immunol 121, 1255-1261.e7.
132. Shoelson, S. E., Herrero, L., Naaz, A. (2007) Obesity, inflammation, and insulin resistance. Gastroenterology 132, 2169-80.
133. Kahn, B. B. and Flier, J. S. (2000) Obesity and insulin resistance. J Clin Invest 106, 473-81.
134. Hotamisligil, G. S., Shargill, N. S., Spiegelman, B. M. (1993) Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science 259, 87-91.
135. Gregor, M. F. and Hotamisligil, G. S. (2011) Inflammatory mechanisms in obesity. Annu Rev Immunol 29, 415-45.
136. Kanneganti, T. D. and Dixit, V. D. (2012) Immunological complications of obesity. Nat Immunol 13, 707-12.
137. Harford, K. A., Reynolds, C. M., McGillicuddy, F. C., Roche, H. M. (2011) Fats, inflammation and insulin resistance: insights to the role of macrophage and T-cell accumulation in adipose tissue. Proc Nutr Soc 70, 408-17.
138. Chatzigeorgiou, A., Karalis, K. P., Bornstein, S. R., Chavakis, T. (2012) Lymphocytes in obesity-related adipose tissue inflammation. Diabetologia 55, 2583-92.
139. Donath, M. Y. and Shoelson, S. E. (2011) Type 2 diabetes as an inflammatory disease. Nat Rev Immunol 11, 98-107.
140. Finucane, O. M., Reynolds, C. M., McGillicuddy, F. C., Roche, H. M. (2012) Insights into the role of macrophage migration inhibitory factor in obesity and insulin resistance. Proc Nutr Soc 71, 622-33.
141. Stojanovic, I., Saksida, T., Stosic-Grujicic, S. (2012) Beta cell function: the role of macrophage migration inhibitory factor. Immunol Res 52, 81-8.
142. Saksida, T., Stosic-Grujicic, S., Timotijevic, G., Sandler, S., Stojanovic, I. (2012) Macrophage migration inhibitory factor deficiency protects pancreatic islets from palmitic acid-induced apoptosis. Immunol Cell Biol 90, 688-98.

143. Hoi, A. Y., Iskander, M. N., Morand, E. F. (2007) Macrophage migration inhibitory factor: a therapeutic target across inflammatory diseases. Inflamm Allergy Drug Targets 6, 183-90.
144. Yaddanapudi, K., Putty, K., Rendon, B. E., Lamont, G. J., Faughn, J. D., Satoskar, A., Lasnik, A., Eaton, J. W., Mitchell, R. A. (2013) Control of tumor-associated macrophage alternative activation by macrophage migration inhibitory factor. J Immunol 190, 2984-93.
145. Lansey, M. N., Walker, N. N., Hargett, S. R., Stevens, J. R., Keller, S. R. (2012) Deletion of Rab GAP AS160 modifies glucose uptake and GLUT4 translocation in primary skeletal muscles and adipocytes and impairs glucose homeostasis. Am J Physiol Endocrinol Metab 303, E1273-86.
146. Velicˇkovic , N. s., Djordjevic, A., Vasiljevic´, A., Bursac´, B., Vojnovic´ Milutinovic, D., Matic, G. (2012) Tissue-specific regulation of inflammation by macrophage migration inhibitory factor and glucocorticoids in fructose-fed Wistar rats. In *Tissue-specific regulation of inflammation by macrophage migration inhibitory factor and glucocorticoids in fructose-fed Wistar rats*, British Journal of Nutrition.
147. Skurk, T., Herder, C., Kräft, I., Müller-Scholze, S., Hauner, H., Kolb, H. (2005) Production and release of macrophage migration inhibitory factor from human adipocytes. Endocrinology 146, 1006-11.
148. Hausman, D. B., DiGirolamo, M., Bartness, T. J., Hausman, G. J., Martin, R. J. (2001) The biology of white adipocyte proliferation. Obes Rev 2, 239-54.
149. Morris, D. L., Singer, K., Lumeng, C. N. (2011) Adipose tissue macrophages: phenotypic plasticity and diversity in lean and obese states. Curr Opin Clin Nutr Metab Care 14, 341-6.
150. Harper, J. M., Wilkinson, J. E., Miller, R. A. (2010) Macrophage migration inhibitory factor-knockout mice are long lived and respond to caloric restriction. FASEB J 24, 2436-42.
151. Fingerle-Rowson, G., Petrenko, O., Metz, C. N., Forsthuber, T. G., Mitchell, R., Huss, R., Moll, U., Müller, W., Bucala, R. (2003) The p53-dependent effects of macrophage migration inhibitory factor revealed by gene targeting. Proc Natl Acad Sci U S A 100, 9354-9.
152. Santos, L. L. and Morand, E. F. (2006) The role of macrophage migration inhibitory factor in the inflammatory immune response and rheumatoid arthritis. Wien Med Wochenschr 156, 11-8.
153. Björkstén, B. (2008) Environmental influences on the development of the immune system: consequences for disease outcome. Nestle Nutr Workshop Ser Pediatr Program 61, 243-54.
154. Fingerle-Rowson, G., Kaleswarapu, D. R., Schlander, C., Kabgani, N., Brocks, T., Reinart, N., Busch, R., Schütz, A., Lue, H., Du, X., Liu, A., Xiong, H., Chen, Y., Nemajerova, A., Hallek, M., Bernhagen, J., Leng, L., Bucala, R. (2009) A tautomerase-null macrophage migration-inhibitory factor (MIF) gene knock-in mouse model reveals that protein interactions and not enzymatic activity mediate MIF-dependent growth regulation. Mol Cell Biol 29, 1922-32.

155. Hinz, B., Mastrangelo, D., Iselin, C. E., Chaponnier, C., Gabbiani, G. (2001) Mechanical tension controls granulation tissue contractile activity and myofibroblast differentiation. Am J Pathol 159, 1009-20.
156. Aplin, A. E., Howe, A., Alahari, S. K., Juliano, R. L. (1998) Signal transduction and signal modulation by cell adhesion receptors: the role of integrins, cadherins, immunoglobulin-cell adhesion molecules, and selectins. Pharmacol Rev 50, 197-263.
157. Schroeder, M. A. and DiPersio, J. F. (2011) Mouse models of graft-versus-host disease: advances and limitations. Dis Model Mech 4, 318-33.
158. Mestas, J. and Hughes, C. C. (2004) Of mice and not men: differences between mouse and human immunology. J Immunol 172, 2731-8.
159. Halford, J. C., Boyland, E. J., Blundell, J. E., Kirkham, T. C., Harrold, J. A. (2010) Pharmacological management of appetite expression in obesity. Nat Rev Endocrinol 6, 255-69.
160. Kim, E. J., Choi, M. R., Park, H., Kim, M., Hong, J. E., Lee, J. Y., Chun, H. S., Lee, K. W., Yoon Park, J. H. (2011) Dietary fat increases solid tumor growth and metastasis of 4T1 murine mammary carcinoma cells and mortality in obesity-resistant BALB/c mice. Breast Cancer Res 13, R78.
161. Nishikawa, S., Yasoshima, A., Doi, K., Nakayama, H., Uetsuka, K. (2007) Involvement of sex, strain and age factors in high fat diet-induced obesity in C57BL/6J and BALB/cA mice. Exp Anim 56, 263-72.
162. Asarian, L. and Geary, N. (2006) Modulation of appetite by gonadal steroid hormones. Philos Trans R Soc Lond B Biol Sci 361, 1251-63.
163. Cornblatt, B. S., Ye, L., Dinkova-Kostova, A. T., Erb, M., Fahey, J. W., Singh, N. K., Chen, M. S., Stierer, T., Garrett-Mayer, E., Argani, P., Davidson, N. E., Talalay, P., Kensler, T. W., Visvanathan, K. (2007) Preclinical and clinical evaluation of sulforaphane for chemoprevention in the breast. Carcinogenesis 28, 1485-90.
164. Ye, L., Dinkova-Kostova, A. T., Wade, K. L., Zhang, Y., Shapiro, T. A., Talalay, P. (2002) Quantitative determination of dithiocarbamates in human plasma, serum, erythrocytes and urine: pharmacokinetics of broccoli sprout isothiocyanates in humans. Clin Chim Acta 316, 43-53.
165. Pessler, F., Mayer, C. T., Jung, S. M., Behrens, E. M., Dai, L., Menetski, J. P., Schumacher, H. R. (2008) Identification of novel monosodium urate crystal regulated mRNAs by transcript profiling of dissected murine air pouch membranes. Arthritis Res Ther 10, R64.
166. Apte, R. S., Sinha, D., Mayhew, E., Wistow, G. J., Niederkorn, J. Y. (1998) Cutting edge: role of macrophage migration inhibitory factor in inhibiting NK cell activity and preserving immune privilege. J Immunol 160, 5693-6.
167. Bacher, M., Metz, C. N., Calandra, T., Mayer, K., Chesney, J., Lohoff, M., Gemsa, D., Donnelly, T., Bucala, R. (1996) An essential regulatory role for macrophage migration inhibitory factor in T-cell activation. Proc Natl Acad Sci U S A 93, 7849-54.
168. Rodríguez, P. C. and Ochoa, A. C. (2008) Arginine regulation by myeloid derived suppressor cells and tolerance in cancer: mechanisms and therapeutic perspectives. Immunol Rev 222, 180-91.

169. Polak, P., Cybulski, N., Feige, J. N., Auwerx, J., Rüegg, M. A., Hall, M. N. (2008) Adipose-specific knockout of raptor results in lean mice with enhanced mitochondrial respiration. Cell Metab 8, 399-410.
170. Sun, Y., Chen, X., Xiao, D. (2007) Tetracycline-inducible expression systems: new strategies and practices in the transgenic mouse modeling. Acta Biochim Biophys Sin (Shanghai) 39, 235-46.
171. Bai, Y., Cui, W., Xin, Y., Miao, X., Barati, M. T., Zhang, C., Chen, Q., Tan, Y., Cui, T., Zheng, Y., Cai, L. (2013) Prevention by sulforaphane of diabetic cardiomyopathy is associated with up-regulation of Nrf2 expression and transcription activation. J Mol Cell Cardiol 57, 82-95.
172. Cui, W., Bai, Y., Miao, X., Luo, P., Chen, Q., Tan, Y., Rane, M. J., Miao, L., Cai, L. (2012) Prevention of diabetic nephropathy by sulforaphane: possible role of Nrf2 upregulation and activation. Oxid Med Cell Longev 2012, 821936.