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THE ROLE OF BATF2 IN LPS/IFNγ POLARIZED MACROPHAGES

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine at the University of Kentucky

By

Marie Ann Gehman

Lexington, Kentucky

Co-Directors: Dr. Joseph P. McGillis, Associate Professor of Microbiology

and Dr. Donald Cohen, Professor of Microbiology

Lexington, Kentucky

2015

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ABSTRACT OF DISSERTATION

THE ROLE OF BATF2 IN LPS/IFNγ POLARIZED MACROPHAGES

Transcription factors regulate distinct macrophage functions by regulating gene expression in response to micro-environmental cues. This functional plasticity is critical for regulating innate and adaptive immune responses during infection and during chronic disease processes including inflammatory diseases and cancer. Microarray analysis of macrophages polarized to a pro-inflammatory (M1) phenotype with LPS and IFNγ revealed that basic leucine zipper transcription factor ATF-like 2 (Batf2), a member of the AP1 transcription factors, is selectively upregulated in M1 macrophages compared to anti-inflammatory IL-4-polarized (M2) macrophages. The initial hypothesis was that Batf2 is a master regulator of gene expression that orchestrates M1 polarization. To investigate a potential role of Batf2 during macrophage polarization, its expression in M1 polarized macrophages was examined. Batf2 mRNA appears within 60 minutes following LPS/ IFNγ treatment and is sustained for at least 48 hours. To address the hypothesis that Batf2 acts as a master transcriptional factor driving a functional M1 phenotype, we have established macrophage cell lines that constitutively express Batf2. Batf2 overexpression did not enhance key M1-associated genes, including iNOS and H2-Aa, but did enhance LPS/IFNγ-driven Cxcl10. Batf2 overexpression also failed to suppress key M2-associated genes including Fizz1 and Mrc1. Batf2 overexpression also failed to alter multiple non-immunity-related genes established or predicted to be downstream of Batf2 in macrophages or other cells. Overall, contrary to our initial hypothesis, constitutive Batf2 expression by itself does not appear to broadly induce M1 gene expression; rather, it appears to enhance only select genes. Since other Batf family members interact with members of the IRF family, I discuss the possibility that Batf2 works in conjunction with a limiting cofactor, possibly Irf family members and/or other regulatory proteins.

KEYWORDS: Transcription Factors, Batf2, AP-1,
Macrophage Polarization, LPS/IFNγ

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THE ROLE OF BATF2 IN LPS/IFNΓ POLARIZED MACROPHAGES

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To Mom and Dad

ACKNOWLEDGEMENTS

First and foremost I want to thank Dr. Joe McGillis, my doctoral advisor and a true mentor. His clever explanations of the material, forgiveness of mistakes, and encouragement of independent thinking have helped me grow in both science and teaching. I also want to thank Dr. Yasuhiro Suzuki, who taught me the value of precision, careful thought, and innovation. His insightful advice challenged me to carefully consider all angles, an invaluable skill.

I am also grateful to my doctoral committee and I wish to thank Dr. Don Cohen, Dr. Sarah D'Orazio, and Dr. Melinda Wilson. Their support, advice, and efforts on my behalf have gone above and beyond their required duties and have saved my sanity many times. Thank you to Dr. Penni Black for her knowledge and assistance searching the miRNA databases. I would also like to thank Dr. Beth Garvy, who somehow manages to take the time to lend a sympathetic ear and give excellent advice to students including myself.

I want to thank as well my friends and colleagues in the department for their support. In particular, I would never have survived without the wonderful Jane and Jeremi Mullins, Julia Walton and Sara Perkins, who have provided support and friendship both inside and outside the lab. Melissa Hollifield, the lab fairy godmother, and Robert Hayman IV have both saved many experiments from ending in tears. Last but not least, thank you to my mother and father, who have not only put up with me but supported me through the worst times in every way they could.

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LIST OF ABBREVIATIONS

AICE: AP-1-Irf element

Aid: Activation-induced cytidine deaminase

AP-1: Activator Protein-1

Arg1: Arginase 1

ATF: Activating transcription factor

Bach1: BTB and CNC homology 1

Batf: Basic leucine zipper transcription factor, ATF-like 2

Bcl: B cell CLL/lymphoma

BMDM: Bone marrow derived macrophages

bZIP: basic leucine zipper

Ccn1: CCN family member 1

Ccne1: Cyclin E

C/EBP: CCAAT/enhancer binding protein

Chi3l3: Chitinase 3-like-3/YM1

Clec7a: C-type lectin domain family 9

CRE: cAMP responsive element

Creg-1: Cellular repressor of E1A-stimulated genes 1

Csf1r: M-CSF receptor

CSR: Class switch recombination

Ctla4: Cytotoxic T-lymphocyte associated protein 4

CVID: Common variable immune deficiency

Cxcl: Chemokine (C-X-C motif) ligand

Cxcr: Chemokine (C-X-C motif) R

Ddit3: DNA-damage inducible transcript

EMT: Epithelial-mesenchymal transition

Erg: Ets-related gene

Fcer2: Fc fragment of IgE, low affinity II

FcR: Fc fragment of IgE, low affinity II

Ets: E26 transformation-specific

Figf: Fos-inducible growth factor; vascular endothelial growth factor D (Vegfd)

Fizz1: Found in inflammatory zone 1

Foxp3: Forkhead box P3

FRET: Fluorescence resonance energy transfer

GATA: GATA binding protein

GM-CSF: Granulocyte-macrophage colony stimulating factor

GSK3β: Glycogen synthase kinase 3 beta

HCC: Heaptocellular carcinoma

Hdac: Histone deacetylase

HIF-1α: Hypoxia Inducible Factor 1α

HLH: Helix-loop-helix

Hlx: H2.0-like homeobox

HSC: Hematopoietic stem cell

IC: Immune complex

Id2: Inhibitor of DNA binding 2

Ifi: IFNγ inducible protein

IFN: Interferon

IFNGR: IFNγ receptor

IL-1RA: IL-1 receptor antagonist

IL-12RB2: IL-12 receptor beta 2

IKKB: inhibitor of kappa light polypeptide gene enhancer in B cells

IL: Interleukin

iNOS: Inducible nitric oxide synthase

Irf: Interferon regulatory factor

JAK: Janus Activated Kinase

Klf2: Kruppel-like factor 4

LIF: Leukocyte inhibitory factor

LPS: Lipopolysaccharide

LTR: Long terminal repeats

MØ: Macrophage

M1: LPS/IFNγ-activated macrophages

M2: IL-2-activated macrophage

Maf: V-Maf avian musculoaponeurotic fibrosarcoma oncogene homolog

MAPK: Mitogen activated protein kinase

M-CSF: Monocyte-colony stimulating factor

MHCII: Major histocompatibility complex II

Mmp: Matrix Metalloproteinase

Monocyte-colony stimulating factor

Mrc: Mannose receptor C

MSCV: Murine stem cell virus

NCoR1: Nuclear receptor corepressor

NF-E2: Nuclear factor erythroid 2

NFAT: Nuclear factor of activated T cells

NF-κB: Nuclear factor kappa B

NK: Natural killer

NO: Nitric oxide

NRF: Nuclear respiratory factor

NSCLC: Non-small cell lung carcinoma

OTSCC: Oral tongue squamous cell carcinoma:

Parp: Poly (ADP-ribose) polymerase

PBMC: Peripheral bone marrow cells

Pp2a: Protein phosphatase 2

Raco: Ring-domain containing protein

Prp: Prion protein

Ror: RAR-related orphan receptor

Runx: Runt-related transcription factor

Stat: Signal transducer and activator of transcription

TAM: Tumor associated macrophage

Tcfec: Transcription factor EC

Tfh: Follicular helper T cell

TGF-β: Transforming growth factor beta

Tgm: Transglutaminase

Tgtp: T cell specific GTPase 1

Th1: Helper T cell type 1

Th2: Helper T cell type 2

Th17: Helper T cell type 17

TLR: Toll-like receptor

TNFα: Tumor necrosis factor alpha

TRE: Tetradecanoyl phorbol acetate-responsive element

Treg: Regulatory T cell

USF: Upstream stimulatory factor

Xcr1: Chemokine (X-C motif) receptor 1

Ym1: Chitinase 3-like-3

Chapter I

INTRODUCTION

1.1. Purpose

This study aims to examine the function of the transcription factor basic leucine zipper transcription factor, ATF-like 2 (Batf2) in the context of macrophage polarization. Master transcription factors commonly control transcriptional repertoires during development and polarization, and such a factor or even the major factors for macrophage polarization to the LPS/IFNγ activated macrophages (herein termed M1 macrophages) remains unclear. Batf2, a member of the Batf subset of AP-1 transcription factors involved in polarizing immune cells, was proposed as a master transcription factor for macrophage polarization. A series of studies were designed and carried out to demonstrate a role for Batf2 as master regulator. However, we found that Batf2 by itself did not induce M1 polarization.

1.2. Importance

Immune function plays a major role in the health of an individual. Appropriate inflammatory responses kill pathogens and tumor cells. Inappropriate inflammation, or lack of inflammation, contributes to many diseases including sepsis, atherosclerosis, meningitis, cancer, AIDS, *etc*. For this reason, we seek to understand and control the inflammatory processes: first, to understand which responses are appropriate in which situation; second, to initiate these responses at the correct time or dampen them when they are detrimental. This project focuses on the first goal by identifying transcription factors regulating multiple genes during M1 polarization of macrophages.

Although such transcription factors have been discovered for other cells, the study of macrophages has been complicated by their plasticity and sensitivity. Macrophages express polarization-associated genes in response to a large variety of common endogenous stimuli, mechanical stresses, and media components such as cholesterol, etc. Moreover, primary macrophage responses may be skewed by epigenetic programming *in vivo*. Prior studies have uncovered several transcription factors, but until recently no factor that acts similarly to master transcription factors in other cell types. This study makes use of a reductionist, highly controllable model to study gene expression control. We use a macrophage cell line isolated in an early myeloid precursor stage, genetically engineered to differentiate *in vitro* in response to a non-polarizing stimulus. Thus, we can detect gene changes upregulated or downregulated in the inflammatory state more clearly than other systems due to the minimal M1/M2 polarization in the resting state.

1.3. Results and analysis

In the context of the PUER macrophage cell system, we describe a recently discovered M1-associated transcription factor, Batf2. Batf2 belongs to the AP-1 group of bZIP transcription factors, and it was recently discovered that knockdown of Batf2 alters a variety of M1-assicated genes[1]. Contrary to expectations, we demonstrate that constitutive high expression of Batf2 alone in PUER cells did not induce most of the hallmark M1-associated genes or suppress most of the hallmark M2-associated genes, nor did it induce or suppress non-immune genes downstream of Batf2 or closely related Batf and Batf3 in other cells. However, it did appear to enhance LPS/IFNγ-induced expression of the M1-associated chemokine Cxcl10 and suppress expression the IL-4-

induced M2-associated enzyme Arg1. We further begin to explore the mechanisms of Batf2 activity by analyzing potentially limiting binding partners and cofactors and the genes they control. Binding partners required for Batf2 activity are present in PUER cells but not associated with polarization. On the other hand, potential cofactor Irfs, are expressed in M1-polarized cells and may be required for Batf2 activity.

Chapter II

BACKGROUND

2.1. Macrophages: Non-specific cells of the immune system with diverse function

2.1.1. Macrophages

Transcription factors serve a critical role in dictating macrophage differentiation from a myeloid precursor, as well as functional polarization and activation of monocytes and macrophages[2]. Macrophages arise from a precursor for all immune cells of myeloid lineage[3]. This highly heterogeneous group plays critical roles in non-specific defense and assists the adaptive defense against a variety of pathogens. It fulfills many homeostatic functions such as tissue repair, muscle regeneration, and iron transport[3]. These cells arise from a common myeloid progenitor, as opposed to the other lymphocytes that arise from a common lymphoid progenitor. This common myeloid progenitor differentiates, under the guidance of the key transcription factors that shape the chromatin landscape and initiate transcription of lineage-specific proteins, into macrophages and their cerebral counterpart microglia, dendritic cells, neutrophils, eosinophils, and basophils, and the more distantly related mast cells, megakaryocytes, and erythrocytes[2].

Macrophages play critical roles initiating and resolving infection-induced or sterile inflammation as well as maintaining tissue homeostasis. Here transcription factors also guide the translation of environmental cues into cellular activity[2]. Appropriate activation of transcription factors by signaling pathways orchestrates an appropriate response, such as generation of reactive oxygen species to damage an invading pathogen. Inappropriate activation of these pathways, however, can lead to the generation of detrimental responses, such as the generation of tissue-damaging inflammation in the

absence of infection in chronic inflammatory diseases[4,5]. Preventing and manipulating such inappropriate responses requires an understanding of the signaling pathways and transcription factors involved in each response. Therefore, we are investigating key transcription factors for specific macrophage functions.

2.1.2. Transcription factors integrate environmental cues during macrophage development

Each step in commitment to a lineage and thus to a function is under the control of a specific combination of chromatin remodeling and transcription factors[6]. The first stages of differentiation from a common stem cell occur during embryogenesis. Microglial and some resident tissue macrophages, notable for their self-renewal capacity, arise from the yolk sac hematopoietic stem cell (HSC) population[78]. Some HSCs, destined to become microglia, migrate to the brain region. Transcription factors direct microglia-specific marker expression around embryonic day 9 in mice[78]. Others migrate to various tissues of the embryo and become tissue macrophages residing long-term in the liver (Kupffer cells), kidney, spleen, lung, and skin (Langerhans cells). A second wave of myeloid cells develops later in the fetal liver from HSCs. This HSC pool differentiates into a common myeloid precursor, then macrophage and dendritic cell progenitor, then monocytic cell, then fully differentiated macrophage cells[3,8]. These fetal liver derived macrophages have two distinct types: those bound to be resident homeostatic tissue macrophages similar to the yolk-sac derived microglia and tissue macrophages, and those bound for immune-related activities such as fighting infection or healing wounds ("inflammatory" macrophages)[3,8].

In spite of the differences in environment and origin, these macrophage populations share some similar transcription factor requirements guiding their development. Some of the unique factors required for macrophage and microglial development have been defined. Early stages of differentiation require Runt-related transcription factor 1 (Runx1) and high levels of PU.1[8,9]. PU.1 and Runx1 recruit factors that modify histones and DNA, beginning to shape the epigenetic landscape so that available genes are hematopoietic cell specific[10,11]. Interferon regulatory factor 8 (Irf8) activity is required as well as PU.1 and Runx1 for the development of macrophages and microglia, likely in part as a cofactor with PU.1[7]. More recently, transcription factor EC 3 (Tcef3), CCAAT/enhancer binding protein-alpha (C/EBP-alpha), BTB and CNC homology 1 (Bach1), and cellular repressor of E1A-stimulated genes 1 (Creg-1) have been associated with macrophage core gene expression, although their importance for polarization requires further investigation[12]. However, there may also be differences between the development of yolk sac and fetal liver populations and differences in transcription factors required for macrophage types within these two lineages. Only peritoneal macrophages, for example, expressed high levels of transcription factor GATA binding protein 6 (GATA6) compared to splenic or lung macrophages or microglia in a murine transcriptome analysis[12,13].

Each macrophage subset likely responds in a unique manner to the environment in which it develops and expresses a unique signature of transcription factors, resulting in the wide variety of phenotypes in resting macrophages. Differentiation to these various tissue associated macrophages has been extensively studied and has been reviewed by Gautier *et al.* and Okabe and Medzhitov[12,13]. In summary, microglia and macrophages

share similar developmental pathways guided by transcription factors including PU.1, Runx1, and Irf8.

2.1.3. Polarization of macrophages

After development, transcription factor-guided polarization allows resting macrophage cells to respond uniquely to environmental cytokines. Often, this is accomplished through a few "master" transcription factors[2,14]. These transcription factors were first described as factors sufficient for determining cell type. T cells provide well described examples of immune cells controlled by master transcription factors. Tbet transduction drives helper T cell type 1 (Th1) cell differentiation, forkhead Box P3 (Foxp3) drives regulatory T cell (Treg) development, B cell CLL/lymphoma 6 (Bcl6) drives follicular helper T cell (Tfh) differentiation, GATA3 drives helper T cell type 2 (Th2) differentiation, and RORγt (encoded by RAR-related orphan receptor C gene [Rorc]) drives Helper T cell type 17 (Th17) differentiation[14–20]. These master transcription factors accomplish this by driving expression of key lineage- and function-specific genes and other transcription factors, thus controlling a specific transcriptional program of multiple effector proteins. For example, as reviewed in Lazarevic *et al.*, Tbet induces interleukin 12 (IL-12) receptor beta 2 (IL-12RB2), Runx3, chemokine (C-X-C motif) receptor 3 (Cxcr3), H2.0-like homeobox (Hlx), CC chemokine ligand 3 Ccl3, Ccl4, and IFNγ, and suppresses Rorc, IL-17A, IL-4, IL5, and IL-13[21]. Master transcription factors for macrophage polarization remain unclear, however, and this study contributes to current work on this topic.

Initially, macrophage function consisted of two categories: "classically activated"

(M1) or "alternatively activated" (M2)[22]. Bacterial cell wall component lipopolysaccharide (LPS) and cytokine interferon gamma (IFNγ) induce polarization to the "classically activated/inflammatory/M1" phenotype, promoting inflammation and infection clearance through production of Th1-associated responses, such as production of chemokine (C-X-C motif) ligand 10 (Cxcl10)[23,24]. In contrast, IL-4 induced an "alternative/M2" phenotype associated with Th2 responses and suppression of inflammatory responses, such as shunting L-arginine from nitric oxide (NO) production to ornithine and urea production by increased Arginase 1 (Arg1). These two classifications and the correlating gene expression, discussed below, are shown in Figure 2.1, page 46. Over time, however, more sensitive studies have continued to push the model of macrophage polarization away from this binary model and towards a spectrum of responses, even switching polarization states[8,22,25]. Still, four broad classifications have emerged that prove useful for simplification of discussion of macrophage polarization: M1 (classically activated or LPS/IFNγ activated), three alternative subsets M2a, M2b, and M2c, and the M2d/tumor associated macrophages (TAMs) [26].

Predominantly, LPS and/or IFNγ remain representative stimuli for inducing the M1, classically activated, phenotype, so named to correspond to the IFNγ-centered Th1 T cell response[23]. IL-1β, tumor necrosis factor alpha (TNFα), and IFNs also influence M1 macrophage polarization. LPS (through its receptor toll-like receptor 4 [TLR4]), IFNγ, IL-1β, and TNFα activate major adaptor protein MyD88 in the main mechanism of signal transduction. This adaptor in turn signals through inhibitor of kappa light polypeptide gene enhancer in B cells (IKKB) and mitogen activated protein kinase (MAPK) pathways to activate transcription factors nuclear factor kappa B (NF-κB) and Activator Protein 1

(AP-1), leading to expression of specific effector genes[27]. IFNs influence gene expression through Janus activated kinase/signal transducer and activator of transcription (JAK/Stat) pathways. Complete polarization is much more complex than simply activating Stat, however. Transcription factors induced by M1-polarizing stimuli important for inflammatory gene expression include Stat1, Stat2, NF-κB, AP-1, Irf3, Irf5 and HIF-1α (Figure 2.1)[2,8,12,28]. These each control multiple genes, but a unique 'master transcription factor' that governs polarization such as exists in other immune cells remains unclear[2,8]. Some studies have suggested Irf5 for this role, but recent evidence suggests that Batf2 and Irf1 may also broadly induce gene M1-associated gene expression in macrophages[1]. Mediated by genes induced by these transcription factors, M1-polarized macrophages participate in pathogen phagocytosis and pathogen destruction via iron restriction, phagosome acidification, nitric oxide production, and reactive oxygen species release, as well as releasing inflammatory cytokines such as IFNs, IL-12, IL-1β, and Cxcl10 to recruit and activate other inflammatory cells. Most macrophage studies agree on the characteristic M1-polarized macrophage markers and functions, which are described in several recent reviews[2,8,22]. In addition to the functions driven by the M1 transcriptional program, it also suppresses gene expression of alternative functions such as regulatory T cell recruitment and IL-10 secretion. All of these genes contains multiple transcription factor binding sites. Therefore, the various stimuli can have additive or synergistic effects, and opposing stimuli can attenuate expression. This allows a fine-tuning of the macrophage role in an inflammatory response, critical for avoiding an insufficiently weak or overly destructive response or for different environments.

In contrast to the M1 macrophages, the phenotypes of M2 macrophages range

widely enough to require multiple subsets to simplify discussion. To complicate the discussion, reviews from different authors rarely completely agree on M2 markers and phenotype. All M2 subsets tend to overlap in transcriptional repertoires, however, and all exert immunosuppressive activity when they affect the immune response. Mantovani *et al.* described the most commonly used classification schemes, defining M2a, M2b, and M2c subsets which reflect phenotypes alternatively activated macrophages, defined by polarizing cytokines[26]. The "M2a" subset, IL-4/IL13 activated, associates with Th2 cytokines and provides the best contrast to the M1 macrophages. IL-4/IL13 activation is most often used when comparing classical and alternative activation, with IL-10 providing another common alternative stimulus[29]. IL-4 and IL-10 elicit many of the same responses, but also have some differences; IL-4 stimulates the IL-4 receptor and activates both Stat3 and Stat6, while IL-10 stimulates the IL-10 receptor and activates Stat3[26,30–33]. IL-4 induces IL-10, while IL-10 induces Fc fragment of IgE, low affinity II (FcR/Fcer2*)*, and both induce mannose receptor C (Mrc1) and a key M2 enzyme Arg1. Importantly for our study, IL-10 also induces Batf2 (discussed in section 4.2 page 76). Genes strongly correlating with IL-4/IL13-activated M2a phenotype include Arg1*,* Ym1*,* Mrc1*,* transglutaminase (Tgm2) and Fizz1[26]. They counter the effects of M1 macrophages and play a role in protection against parasites, Th2 cell recruitment, tissue repair, and growth stimulation. Cytokines secreted by these cells include IL-10, TGF-β, and IL-1 receptor antagonist (IL-1RA)[22]. The Arg1 enzyme, a key M2 marker, provides important anti-parasitic, anti-inflammatory, and wound-repair function (described below). It is not unique to IL-4-polarized macrophages, since it is also induced by IL-10 and active in the M2b subset.

Other subset classifications may be used depending on the purpose of a given study, and designations differ between research groups. The subset designated M2b, activated by immune complexes (ICs), LPS, and viral long terminal repeats (LTR)/IL1R, parallels a memory immune response with B cell class switching and regulatory T cell recruitment. This subset produces high amounts of IL-10 and expresses major histocompatibility complex II (MHCII) and CD86[34,35]. The subset designated M2c, activated by IL-10, TGF-β, and glucocorticoids, serves as scavengers for cell debris and serves roles in the healing process. These express Arg1 like the M2a subset, but also CD163 and CD206[26]. Finally, another subset designated M2d but more commonly called tumor associated macrophages (TAMs) has been found in the context of tumors. This phenotype can be induced with IL-6, leukocyte inhibitory factor (LIF), and monocyte-colony stimulating factor (M-CSF). These produce some inflammatory and anti-inflammatory cytokines and chemokines, as well as vascular endothelial growth factor, and aid in tumor growth.

In summary, macrophages polarize to multiple functional states in response to various stimuli. These states include a Th1-cytokine-associated M1 state, a Th2-associted "M2a" state, and a variety of alternative M2 states functioning in wound repair, tissue homeostasis, tumor growth, and more. Based on control of functional states in other cells, it is reasonable to expect macrophages to be guided by master transcription factors governing broad gene expression. The master transcription factors for M1 polarization are unclear. The purpose of this study is to determine the function of one candidate for a master transcription factor, Batf2, and to identify Batf2-controlled M1-associated genes.

2.1.4. M1-associated gene Cxcl10

One of the genes influenced by the M1-associated transcription factor Batf2 is Cxcl10, previously known as interferon-inducible protein 10 (IP-10) or Crg-2 (cytokine responsive gene 2). Cxcl10 belongs to the small, inducible, secreted protein family classified as chemokines[36]. These proteins share structural and functional similarities: similar secondary structure with four similarly spaced cysteines, and the ability to attract other cells to the origin of chemokine production. "CXC" chemokines belong to the subfamily of chemokines characterized by having cys-x-cys as the first two cysteines[22]. Other subfamilies include CC, C, and CX3C[36]. Chemokines may promote inflammation, homeostasis, or both[22]. Cxcl10 is typically induced by pro-inflammatory stimuli[37–40]. In our study, Batf2 enhances Cxcl10 message induction, as demonstrated in Chapter 4 (see Figure 4.14) and discussed in Chapter 5 (see section 5.3).

Various stimuli generated during a pro-inflammatory state including IFNs, LPS, and TNFα with IFNγ rapidly stimulate Cxcl10 production from a variety of cells including macrophages, keratinocytes, fibroblasts, endothelial cells , mesangial cells, astrocytes, monocytes and neutrophils[41–44]. Indeed, the Cxcl10 promoter region contains sites for Irf1 (activated by interferon, IL6, leukemia inhibitory factor (LIF) or TLR signaling), Stat1 (activated by IFNβ or IFNγ), NF-κB (activated by TNFα, TLR ligands, or IL1), and AP-1 sites[27,37,42,45–47]. The importance of AP-1 in Cxcl10 induction remains unclear. Mutating the AP-1 site did not impact TNF-induced Cxcl10 in human airway smooth muscle cells, but decreased HCV infection-induced Cxcl10 in a separate study in human hepatoma cells[38,39]. In mice, deficiency of AP-1 inhibitory subunit JunB

decreased Cxcl10[48]. In contrast, expression is inhibited by Stat3 (activated by IL-10[49]. As a general rule, pro-inflammatory stimuli upregulate Cxcl10 expression in macrophages.

Cells responsive to Cxcl10 through the receptor CXCR3 are mainly polarized Th1 cells and natural killer (NK) cells but also macrophages, microglia, and dendritic cells (reviewed in Mantovani *et al.* and Liu *et al.*)[22,50]. These cells have cytotoxic activity to kill infected cells, or can produce inflammatory cytokines and chemokines to propagate an immune response. Unlike other CXC chemokines, Cxcl10 does not activate neutrophils[22,50]. Activation by Cxcl10 stimulates cells to migrate up the concentration gradient to the area of its origin. Once arrived, the recruited cells exert their protective functions such as resistance to intracellular pathogens and tumor cells[51]. In a typical scenario, IFNs from an infected cell may stimulate macrophages in the surrounding area to produce Cxcl10 that attracts Th1 and NK cells. These have the ability to destroy the infected cell and halt the viral production in that cell.

Besides chemotaxis, Cxcl10 has less well-characterized functions in immune and non-immune cells. Thymic or splenic Cxcl10 may play a role in T cell development and function, can stimulate adhesion of mature T cells to endothelial cells, and enhance anti-tumor activity[36,51,52]. Cxcl10 enhances T cell and NK cell development and function independently of chemotaxis, and may contribute to accessibility at sites of production. Cxcl10 may also enhance NK cell migration and cytolysis. Other cells that do not participate in the immune response may also be influenced by Cxcl10. Vascular smooth muscle cells' migration and proliferation may be enhanced, and angiogenesis is inhibited[51]. Cxcl10 may also contribute to apoptosis and regulate cell growth in some

cells[50]. The extent of these non-chemotactic functions *in vivo* are currently not well understood.

Cxcl10 production is important for protective responses to a variety of pathogens. Decreased Cxcl10 leads to increased susceptibility to *Legionella pneumophilia* and increased pathogenesis of cutaneous candidiasis[50]. Further, increased Cxcl10 is protective for in *Leishmania amazonesis* in mice, and Cxcl10 is protective during *Pneumocystis carinii* and *Cryptococcus neoformans* in brains of immunized mice[50 53]. In general, Cxcl10 is a chemokine induced by multiple M1 stimuli and promotes M1-associated responses. Despite its role in T cell and NK cell development and effector activity, the major role of Cxcl10 remains as a chemotactic signal for T and NK cells.

2.1.5. M2-associated gene Arginase 1 (Arg1)

Arginase 1 contrasts with Cxcl10 by association with M2 responses. Arginase 1 is a manganese metalloenzyme that catalyzes the conversion of L-arginine into ornithine and urea. In the liver, this is the final step in the urea cycle. In IL-4 or IL-13 polarized macrophages, it is considered a defining marker for alternative versus M1 polarization[54]. In our study, the M1-associated transcription factor Batf2 mildly suppresses IL-4-induced Arg1 expression.

Arginase 1 plays a critical role in immune function by promoting M2 responses and suppressing M1 responses that may damage tissue. One major consequence of arginase activity is decreased inflammation. By converting L-arginine to urea and ornithine, it sequesters the pool of L-arginine available for nitric oxide synthase, an M1-associated enzyme that produces the inflammatory and reactive nitric oxide to combat

pathogens[55–57]. Further, decreased L-arginine availability decreases Th2 cell responses[56]. Arginase activity also plays multiple roles in homeostasis and tissue repair. During homeostasis, the production of polyamines from L-ornithine by L-ornithine decarboxylase promotes cell growth[58]. Alternatively, L-ornithine aminotransferase converts ornithine to collagen in tissue repair.

Multiple studies confirm *in vitro* effects with roles for arginase activity during certain infections. Deficiency of Arg1 in myeloid cells worsens inflammation during *Schistosoma mansonii* infection due to hyperactive inflammation beyond the point of protection, thus hastening mortality[56]. Additionally, the decreased arginine availability may affect growth of pathogens. For example, *Toxoplasma gondii* lacks arginine synthesis ability, and arginine deficiency triggers conversion to the slow-growing bradyzoite stage[58,59]**.** Through these mechanisms, Arg1 impacts some infections.

Notably, some evidence suggests arginase undergoes multiple layers of regulation. Concentrations of polyamines may influence activity, as well as L-arginine (regulated in part by activity of the cationic amino acid transporter 2)[60]. Thus, arginase activity may be controlled independently of transcription.

In summary, arginase is a highly controlled enzyme that serves anti-inflammatory, anti-pathogen, and homeostatic functions. The main importance of arginase to our study is as a marker of IL-4-induced M2 macrophage polarization antagonizing M1 function.

2.2. Transcription factors: Dictating the functions of myeloid cells

2.2.1. Transcription factors bind DNA and dictate gene expression patterns

Differentiation, polarization, and function rely on specific transcription factors

that undergo regulation by a combination of multiple extracellular and intracellular signals and modifications. Transcription factors enhance or suppress transcription by altering the stability of the transcriptional apparatus[61]. They recognize and bind consensus elements: short, unique nucleotide sequences. These sequences are located within a gene's regulatory regions, reaching to a couple thousand base pairs upstream of the transcription start site, or at distant sites that loop around to contact the transcription apparatus. Binding often requires dimerization and/or ternary complex formation with other transcription factors and non DNA-binding cofactors[61,62]. The structure of the transcription factors promotes binding to specific DNA sequences, and transcription factors cluster into families by structure of the DNA-binding domain. The four most abundant are C2H2 zinc finger, homeodomain, helix-loop-helix (HLH), and basic leucine zipper (bZIP)[61]. Family groupings can be useful since transcription factor families tend to control genes with similar function. However, even transcription factors in the same family differ in function. Moreover, activity of any transcription factor may differ between cells.

Cell specificity and environmental specificity of transcription factors arises from both regulation at the transcription factor level and consensus element level[61]. At the transcription factor level, regulatory mechanisms include altering mRNA expression levels of any given transcription factor, altering mRNA stability, alternative splicing of that mRNA, post-translational modifications, and relative levels of opposing or consensus-modifying transcription factors[61]. Thus, any given transcription factor's mRNA message may be present only in some cells (at a low level for high specificity or a high level for increased/broader activity), spliced to an isoform with lesser or greater

activity, rapidly marked for degradation by ubiquitination, glycosylated for altered function, phosphorylated for enhanced/inhibited activity, in competition with an opposing transcription factor, and much more. PU.1, an important transcription factor for macrophage differentiation, provides an example of a cell-specific transcription factor with multiple levels of regulation. PU.1 mRNA levels are specifically induced in cells of hematopoietic lineage[9]. High levels of PU.1 differentiate CMPs to macrophages, overwhelming the effects of the antagonistic GATA3 that promotes mast cell differentiation. AP-1 homo- and heterodimers demonstrate multiple levels of regulation as well and also rely on a greater concentration of transcription-promoting subunits versus inhibiting subunits[62,63]. Additionally, AP-1 exemplifies transcription factor regulation by post-translational modifications, with cJun and cFos activity well characterized as dependent on appropriate phosphorylation[63–66]. Thus, cells generate and activate a specific set of active transcription factors for a given type and environment by regulating expression, alternative splicing, post-translational modification, and concentration of transcription factors.

At the consensus element levels, regulatory mechanisms involve altering accessibility via epigenetic modifications. The current model for cell development postulates early "pioneering" transcription factors that create a specific epigenetic landscape for each cell type, allowing any given set of transcription factors access to a unique set of genes[67]. PU.1, a pioneering transcription factor, again provides an important example in macrophages. PU.1 facilitates H2K4me1 deposition via remodeling enzymes[11,68,69], allowing access of and activation by transcription factors including NF-κB (activated by LPS, for example), Irf4 (role in M2 polarization), Irf5

(M1-associated cytokines including IL-12p40, IL-12p35, and IL23p19)[70], and Irf8 (M1-associated cytokines including IFNβ, IL-12p40, and iNOS, reviewed in Günthner *et al.*[4]). With these mechanisms, cells can achieve unique responses with a select set of active transcription factors.

Previous studies on other immune cells have discovered "master transcription factors" that are sufficient to drive broad transcriptional programs and commit to a functional state. For T cells such master transcription factors include Tbet transduction driving Th1 cell differentiation, Foxp3 driving Treg development, Bcl6 driving Tfh differentiation, GATA3 driving Th2 differentiation, and RORγt driving Th17 differentiation[14–20]. Since the initial studies, the overall story has become somewhat more complicated with discoveries that cofactors act in concert with these master factors (Batf/Irf complexes being necessary for key Th2 genes along with GATA3, for example)[71]. For a discussion on the master regulators in T cells, refer to Oestreich and Weinmann, *et al* [72]. Still, broadly-acting master factors orchestrate the majority of T cell responses. Previous studies on master transcription factors in M1 macrophage polarization have been inconsistent due to variation in the highly plastic macrophages across different model systems. Recent studies have proposed Batf2 in combination with Irf1 as a complex governing M1 gene expression[1]. This project focuses on Batf2 and its role in regulating M1 gene expression.

In summary, specific cells produce specific responses via transcription factor activity regulated in multiple ways. Each contains a specific set of transcription factor mRNA and protein repertoire, with specific isoforms. Translation produces proteins which may be activated or suppressed by modification. Active transcription factors then

have access to specific gene regulatory regions, and master transcription factors can induce multiple genes in a transcriptional program.

2.2.2. AP-1 Transcription factors

The activator protein 1 (AP-1) group of transcription factors belong to the bZIP transcription factor family. One of the four most abundant transcription factor families, as reviewed in Vaquerizas *et al.*[61], AP-1 family members consist of a positively charged segment (basic region) mediating DNA interactions and leucine repeats (leucine zipper) mediating protein interactions. Two members must dimerize to bind DNA. Common functions of bZIP factors include mitogen signaling, stress responses, cytokine stimulation, brain and ocular development, development of dendritic cells, and differentiation of myeloid cells[65]. Importantly, bZIP factors are also involved in activation of macrophage cells.

AP-1 homo- or hetero-dimers recognize two similar consensus sequences: the AP-1 site, also known as a tetradecanoyl phorbol acetate-responsive element (TRE), and the cAMP responsive element (CRE). Several proteins of this group mix and match, forming activating and inhibitory complexes: Jun, cFos, cFos-related antigen 1 (Fra1), Fra2, FosB, JunB, and JunD[62,65,73]. Some sources divide these proteins into cFos and Jun families, with the cFos family consisting of cFos, Fra1, Fra2, and FosB and Jun family consisting of cJun, JunB, and JunD (refer to Foletta *et al.* for an excellent review of AP-1 factors)[74]. The Jun family members can form homo- or hetero-dimers with cFos family members, while cFos family members cannot homodimerize. More recently, the bZIP transcription factor ATF-like (Batf) family members Batf, Batf2, and Batf3, were found to have similar

structures, dimerization, and DNA-binding activities, and can be considered part of this group (Figure 2.2), reviewed in Murphy *et al*[65,75].

The cFos/Jun and Batf proteins, as basic leucine zipper (bZIP) transcription factors, contain leucine residues that form a 'zipper' connecting two dimerizing factors. Most members, such as cFos and cJun, contain a known transactivation domain at one end, a basic region for protein-protein interactions, and a DNA-binding region at the other end. Some members have two transactivation domains (cFos and FosB), while others lack the transactivation domain (Fra1 and Fra2), which can affect transcription rates[65,75]. The Batf family members Batf and Batf3 are truncated to the bZIP region and rely on dimerization to a partner with a transactivation domain, while Batf2 contains an N-terminus of unknown transactivation activity[76].

2.2.2.1. Regulation of AP-1 proteins

AP-1 proteins are regulated by complex and multifactorial mechanisms to provide differential gene expression in different cells and environments in light of the ubiquity of the dimers and their target sites[62]. Protein-protein interactions and phosphorylation of multiple residues provides the strongest regulatory mechanism. The consequences of dysregulation can be severe. The growth dysregulation and epithelial-mesenchymal transition of many cancers depends on dysregulation, for example. AP-1 activating stimuli include growth factors, mitogens, toxins, cytokines, stress signals, and pathogens; functional outcomes include cell development, proliferation, differentiation, and survival[74,77]. Regulation of activity takes place on multiple levels such as changes in transcription of AP-1 subunits (some subunits being inhibitory), mRNA stability, post-translational processing, turnover of pre-existing or newly synthesized subunits, and

specific interactions between the AP-1 dimers and other transcription factors or cofactors (reviewed in Foletta *et al.* 1996 and Hess *et al* 2004)[77,78].

Protein-protein interactions provide a critical point of regulation for both transactivation and DNA recognition (Table B2.1). These include 1) hetero- or homo-dimerization with related family members[63,73,75,79–83], 2) heterodimerization with other bZIP transcription factors, 3) ternary complex formation with other transcription factors, 4) ternary complex formation with other activators or coactivators. Closely related AP-1 members include cJun, JunB, JunD, cFos, FosB, Fra1, and Fra2; more distant bZIP transcription factors include V-Maf avian musculoaponeurotic fibrosarcoma oncogene (Maf), C/EBP, activating transcription factors (ATFs), upstream stimulatory factors (USFs), nuclear respiratory factor (NRF) and nuclear factor erythroid 2 (NF-E2); and known ternary complex-forming factors include SMADs, E26 transformations-specific (Ets) (of which the critical macrophage differentiation factor PU.1 is a part), glucocorticoid receptor, NFκB, CREB-binding protein, TATA-binding protein and nuclear factor of activated T cells (Nfat)[65]. Other cofactors include ring-domain containing protein (Raco)[84]. The members of the complex determine the gene to which the complex binds and its activity, allowing a high context specificity. For example, cJun homodimers can directly bind and negatively regulate the promoter region of matrix metalloproteinase 12 (Mmp12) and Mmp13, while cJun/cFos hereodimers positively regulate the same genes[63]. The complex formed also determines the preferred consensus sequence. While dimers of the closely related AP-1 family members usually prefer the TRE (5'-TGA(C/G)TCA-3') over the CRE (5'-TGACGTCA-3'), preference depends upon the members of the dimer[62,85]. Batf proteins complex with Irf4/Irf8 prefer the AP-1-Irf

element (AICE) 5'-TTTCnnnnTGACTAA-3' or 5'-GAAAATGAnTCA-3'[86]. Ternary complexes may also allow AP-1 proteins to activate gene expression without binding DNA at all; in the case of IL-1β, PU.1-C/EBP β complex binds Jun at its DNA-binding residues, preventing Jun from binding DNA directly, but still allowing its required transactivating activity[80]. To further add to the regulation through complex formation, at least some of the complexes formed change with changes in concentrations of AP-1 members present at any given time. For example, overexpression of cFos, which does not bind the AP-1 consensus sequence except as a heterodimer with Jun, blocks transactivation of the M-CSF receptor Csf1r presumably by saturating available AP-1 sites[87].

A second, critical level of AP-1 regulation involves post-translational modification. Hyperphosphorylation by MAPKs is a main regulatory mechanism of cJun and cFos activation, but can also suppress activity[64–66]. Typically, JNKs phosphorylate Jun proteins, and ERKs phosphorylate Fos proteins[65]. Mitogens or cell stress induce the MAPK, which travels to the nucleus and hyperphosphorylates pre-existing Jun or Fos proteins[64,80]. cJun then forms homodimers or heterodimers with cFos or other members and then binds to DNA to enhance transcription[88]. Mmp13 provides an example of the importance of phosphorylation. Unphosphorylated Jun/Jun homodimers act as repressors by recruiting nuclear receptor co-repressor 1 (NCoR1), a repressor that recruits DNA-modifying enzymes leading to transcriptional repression[63]. Phosphorylation allows dissolution of the complex and thus transcription. cFos is also regulated by phosphorylation[89]. In some cases, a member may have a phosphorylation site but no docking site for the kinase, and can only be phosphorylated when dimerized to a partner

with a docking site[65]. In contrast, ubiquitination and SUMOylation can repress transcriptional activity, specifically studied in the case of cJun and cFos, respectively[90,91]. Finally, redox state in the cell also controls the activity of AP-1 by reducing residues on the proteins, enabling the cell to respond appropriately[92,93].

In summary, the regulation of AP-1 activity is critical for correct cell function. Mechanisms include changes to levels of mRNA and AP-1 and non-AP-1 proteins and post-translational modifications. Relative levels of interacting proteins alter the transactivation activity and the DNA-binding location and ability of the AP-1-containing complexes.

2.2.2.2. Function of AP-1 proteins

AP-1 has a broad range of functions in all cell types. Activities include regulating stress response, tumor promotion, cell death, differentiation, immune cell function, and much more. Expression of many members is constitutive with activity induced rapidly after stimulus by post-translational modification such as hyperphosphorylation, as discussed above. Dimerization and subsequent phosphorylation allows DNA binding at TRE, CRE, or in complex formation with cofactors at variant sites. AP-1 can also regulate genes with alternative consensus elements and weak AP-1 sites by forming a ternary complex with other transcription factors. Occasionally, these complexes may regulate genes entirely independently of AP-1 DNA binding[62,80].

AP-1 is strongly implicated in tumorigenesis. Several of the AP-1 proteins, including cFos, FosB, and cJun, transform cells *in vitro*, and cJun and cFos are both viral oncoproteins[94]. AP-1 activity induces transcription of tumorigenic proteins including

Cyclin D, GM-CSF, and Kgf/Fgf7, and suppresses anti-tumor proteins such as p53[95,96]. On the other hand, AP-1 proteins in other contexts hinders tumor growth. JunB and JunD tend to have anti-tumor effects *in vivo*, possibly by inhibiting cJun. Moreover, suppression of JunB promotes spontaneous tumor formation independently of cJun[94]. Batf2 also exhibits tumor suppression activity in multiple cell types, largely by inhibiting cJun activity at various genes[95]. Specifically, this suppression results in decreased cell-adhesion molecule E-cadherin and decreased growth-promoting Cyclin E, among others. cJun/cFos heterodimers also regulate genes involved in tumor invasiveness, such as Mmp12, Mmp13, Mmp1, and Mmp3[63,94]. AP-1, therefore, has important roles for growth and differentiation in diverse cell types.

In addition to growth, AP-1 proteins promote the differentiation and function of many cell types including immune cells. In macrophages, cJun can interact with the major hematopoietic cell transcription factor PU.1, an important transcription factor for the differentiation of multiple cell lineages including macrophages and B cells. AP-1/PU.1 complexes are required for expression of a macrophage Fc receptor expressed during monocyte to macrophage differentiation [97]. Monocytic expression of Csf1r, receptor for the growth factor M-CSF and part of an essential pathway for growth, proliferation, and differentiation, also relies on AP-1/PU.1[87]. Indeed, cJun/cFos heterodimers promote expression of pro-inflammatory proteins including Mmp14[63,98]. Further, the cJun/Irf1/NF-κB complex serves as an 'enhanceosome' for inflammatory gene expression[4,99–101]. cFos and JunB play a non-essential but nevertheless influential role in monocytic differentiation. This effect was demonstrated in myeloblastic leukemic cells, in which expression of cFos or JunB increases their tendency to differentiate[74,102–

[104]. The Batf members have a more essential role in non-monocytic cells. Batf, for example, is required for differentiation of multiple immune cells including Th17 and Tfh CD4 T cells[76]. The Batf subfamily is rapidly gaining appreciation for its role in differentiation and function of multiple cell types, discussed more in depth below.

Clearly, AP-1 proteins play complex roles in a vast variety of cell types. Regulation of these proteins includes transcription, post-translational modification, dimerization, and ternary complex formation. Activity is highly dependent on cell type, epigenetic history, and environment. Because of this, Batf2's functions in macrophages cannot be extrapolated from studies of other AP-1 family members or even Batf2 functions in other cell types.

2.2.2.3. Batf subgroup: AP-1 proteins important for B, T, and cDC subset development and function

The Batf subgroup is composed of Batf (SFA2), Batf2 (SARI), and Batf3 (JDP/p21). Batf members, their function, and their interchangeability were recently reviewed in Murphy *et al*[76]. As mentioned above, this subgroup belongs to the bZIP family of transcription factors and falls into the category of AP-1 proteins based on homology to and dimerization with classical members such as cJun and cFos. Batf2 also recognizes the AP-1 TRE consensus and the similar AP-1 half of the AICE consensus (Figure 2.3). Notably, however, murine Batf and Batf3 proteins are significantly smaller than either cJun or cFos, with truncated C-terminal domains, and lack any likely transactivation domains[76]. Batf2 does contain a C-terminal domain, but does not contain known transactivation domains.

Batf and Batf3 are expressed in hematopoietic cells, and Batf2 is more broadly expressed in multiple cell types. Batf and Batf3 have roles in several cell types: Th1 and Th17 cells express both Batf2 and Batf3, Th2 cells express Batf and low amounts of Batf3, Tfh express Batf, and B cells, $CD103^{+}CD11b^{-}$ conventional dendritic cells (cDCs), and $CD8\alpha^{+}$ cDCs all express Batf3[76,83,105–107]. Recently, cap analysis gene expression (CAGE) also detected high expression of Batf in natural helper cells and macrophages, and Batf3 in macrophages[108]. Expression of Batf, Batf2, and Batf3 in macrophages have been confirmed in our studies and by others[1,109]. At this time, expression of Batf2 has two notable patterns: suppressed expression in cancer cells and induced expression in hematopoietic cells. Cancer cells downregulate or lose entirely expression of Batf2 compared to normal counterparts or adjacent tissues. M1 stimuli (IFNβ, IFNγ, and LPS) *in vitro* induce Batf2 expression in macrophages, and IL-12 treatment *in vivo* or *Toxoplasma gondii* infection *in vivo* (which induces IL-12 production) induces Batf2 expression in $CD103^{+}$ $CD11b^{-}$ cDCs and $CD8\alpha^{+}$cDCs[1,83,95].

In spite of the lack of transactivation domain, the Batf members clearly serve critical roles in transcription. Batf and Batf3 not only bind to TREs as heterodimers with another AP-1 protein, but also bind to AP-1-Irf consensus elements (AICEs) as a complex with Irf4 or Irf8. Batf3, induced Batf, or induced Batf2 forms complexes with Irf8 that induce genes for differentiation of $CD8\alpha^{+}$ cDCs and $CD103^{+}CD11b^{-}$ cDCs, inducing C-type lectin domain family 9, member A (Clec9a), chemokine (X-C motif) receptor (Xcr1), inhibitor of DNA binding 2 (Id2), and Spib[83,110]. Batf-induced transcription also plays important roles in cell development in B and some T cell subsets. In B cells, it is required for the expression of activation-induced cytidine deaminase (Aid) which is

required for affinity maturation and class-switch recombination (CSR)[107]. It is important for differentiation of Th17, Tfh cells, and Th9 by inducing essential genes including Rorc, IL17A, IL21, IL23R, Map, Bcl6, IL9, IL-10, Furin, Fes, Ets-related gene (Erg), and IL-1RA[71,76,106,111]. In Th2 cells Batf or Batf3 both induce GATA3, IL-4, IL-10, and cytotoxic T-lymphocyte-associated 4 (Ctla4) (Figure 2.2). Like Batf3, the importance of Batf in these cells is at least in part due to complex formations with Irf4 or Irf8 and subsequent binding to AICEs[86].

Interestingly, Batf3 and Batf can functionally compensate for each other, suggesting some redundancy in binding to both DNA elements and Irf proteins[83,86]. Batf2, on the other hand, can compensate for Batf3 in the cDC subsets, but not Batf or Batf3 in B cells or T cells, as discussed below. These prior studies clarify that the Batf family members are partially interchangeable and this interchangeability relies on ternary complex formation with Irf family members, but that Batf2 has unique activity.

2.3. Batf2: An AP-1 protein distinct from Batf and Batf3, with roles in cancer inhibition and immunity

Batf2 is a relatively little-known member of the AP-1 group of the bZIP transcription factors, but is gaining importance for its roles in tumor suppression and cell differentiation. Su *et al.* originally characterized it as an IFNβ -inducible early-response gene that inhibits tumor-promoting AP-1 activity. Currently, investigation into Batf2's value as an indicator of good prognosis as well as potential therapeutic target continues[95,108]. Additionally, Batf proteins are emerging as critical cofactors in the differentiation and function of many immune cells. Batf2 clearly behaves differently

from Batf and Batf3, and defining its specific contribution to immune cell functions is advancing our understanding of the transcriptional regulation of immune cells.

Mouse Batf2 shares 63% homology with human Batf2, which is 29.4 kDa. It is located on chromosome 19 in mice, and 11q13.1 in humans. Batf2 shares the functional domains characteristic of the AP-1 transcription factors: a basic region that binds DNA after dimerization and leucine zipper that binds other AP-1 and non-AP-1 proteins. Most Batfs have a truncated C-terminus compared to cFos, and lack known transactivation domains (Figure 2.2)[83]. Batf2, however, contains an extended C-terminus with unknown function that could encode some transactivation functions. Batf2 protein has 47% homology to Batf and 43% homology to Batf3. Conservation between Batf2 and the other Batf proteins is sufficient to allow some functional compensation. Of the other Batfs, Batf is required for $CD103^{+}CD11b^{+}$ and $CD8\alpha^{+}$ cDC development, and Batf3 for Th2, Th17, and Tfh cell development and function. While DNA-binding assays demonstrate Batf and Batf3 binding to AICE, Batf2's DNA-binding ability remains unknown[86]. Batf2's ability to compensate for Batf3's ability to guide Irf4/8 to DNA in dendritic cell development suggests DNA-binding activity, but no DNA-binding assays demonstrating Batf2 binding to the AICE have been published. Further, Roy *et al* state that Batf2 has no DNA-binding domain[1], Batf2-Jun heterodimers do not bind AP-1 consensus sequences as assessed by electromobility shift assays (EMSAs) and chromatin immunoprecipitation (ChIP) assays[95], and Batf2 inhibits cJun activity by inhibiting its binding ability[95,112]. At this time, Batf2's direct interaction with DNA remains unclear and further experiments are required to determine whether Batf2 binds a yet-unidentified site, or interacts with other DNA-binding proteins.

While Batf2-DNA interactions remain unclear, interactions with other proteins are better characterized. The leucine zipper motif of Batf2 mediates dimerization and ternary complex formation, and is structurally similar to Batf and Batf3[76]. Batf2 binds *in vivo* to cJun and inhibits its activity, and also *in vitro* to JunB with unexplored functional consequences[95,113]. Activity of the Batf subfamily depends largely on ternary complex formation. Batf and Batf3 guide non-DNA-binding transcription factors Irf4 and Irf8 to specific consensus sequences to initiate transcription. In some contexts (discussed below), Batf2 can compensate for a deficiency of Batf3 in a manner dependent upon the leucine zipper region, supporting the importance of protein-protein interactions for Batf2 activity.

In summary, Batf2 is a member of the bZIP family of transcription factors and the AP-1 and Batf subfamilies. It contains a DNA-binding domain with unclear function, and protein-binding domain similar to Batf and Batf3 able to dimerize with AP-1 members and form ternary complexes with other proteins including Irfs.

2.3.1. Batf2 Expression

2.3.1.1. Normal cells and tissues

Batf2 is expressed constitutively in multiple cell types and tissues, and loss tends to correlate with malignant growth and poor outcomes *in vivo*. Isolated cell types expressing Batf2 include common myeloid progenitor cells and macrophages, 'normal' immortal cell lines including primary human fetal astrocytes, mammary epithelial cells, prostate epithelial cells, pancreatic mesothelial cells, normal lung, and tongue epithelial cell lines[95,114–116]. It is expressed in many immune cells with the exception of T cells[76].

In vivo, Batf2 message is expressed in many normal tissues, although expression is widely variable between tissues and between samples[95,115,117–119]. Message levels are relatively high in colon, spleen, and pancreas; and moderate in heart, kidney, liver, lung, and prostate[95]. Low levels were detected in placenta, stomach, small intestine, and salivary gland. Brain, muscle, and testis do not express Batf2[95].

IFNs induce Batf2 expression many fold over baseline. In multiple cell lines including HeLa, IFNβ induces Batf2 within 2 hours, independent of protein synthesis[95,112,114,120]. The mRNA half-life under these conditions is 2 hours. IFNβ, IFNγ, and LPS also induce Batf2 in macrophages [1,109,121]. $CD8\alpha^+$ cDCs, and $CD11b^+CD103^+$ cDCs express Batf2 *in vivo* in response to intracellular pathogen *Toxoplasma gondii* or IL-12[83]. Thus, Batf2 is an IFN-inducible transcription factor with basal expression in multiple cells.

2.3.1.2. Batf2 in Cancers

Although Batf2 is expressed in many normal tissues, expression decreases in aggressive cancer cell lines and *in vivo* in tumors. Expression correlates with survival and inversely correlates with aggressiveness and likely plays a contributing role. Human leukemia cell lines and malignant counterparts of cell lines including glioma, prostate cancer, pancreatic cancer, breast cancer, melanoma, lung adenocarcinoma post-EMT, and oral tongue squamous cell carcinoma (OTSCC), decrease or eliminate Batf2 expression[95,114,115,117–119]. In general, Batf2 expression appears to generally correlate with normal cells with loss indicating progression of cancer cells. However, Batf2 may remain inducible even after basal expression is lost. In multiple cell lines in which Batf2

is decreased and in the HeLa cell line, IFNβ induces Batf2. Induction occurs within 2 hours, independent of protein synthesis[95,109,112,114]. The mRNA half-life under these conditions is 2 hours.

Similarly to cell line studies, Batf2 expression decreases in clinical tumor samples. Loss tends to correlate with aggressiveness while maintenance correlates with survival. For instance, the 52% positive expression in normal lung tissue samples decreases to 50% in paired non-small cell lung carcinoma (NSCLC) tissues[96]. It's also decreased in hepatocellular carcinoma (HCC), in OTSCC, and in colorectal cancers compared to healthy adjacent tissues, as well as in peripheral bone marrow cells (PBMCs) of leukemia patients compared to PBMCs of normal volunteers[117 115 112 122]. Batf2 expression inversely correlates with tumor size, differentiation, and metastasis[115–117,120]. Importantly, expression also correlates strongly with survival when compared to adjacent non-tumor tissues[115–117]. Batf2 may well have a causal role in survival, discussed below.

Mechanisms by which Batf2 decreases in cancers remains unclear. In normal cells, IFNβ directly induces expression, and Mda7/IL24 increases Batf2 expression by prolonging detectable mRNA from 1.5 hours to 3.5 hours via p38 MAPK pathway (half-life is 2 hours in HeLa)[114]. One study in chronic myeloid leukemia (CML) linked Batf2 expression inversely to BCR-ABL, JAK/Stat, and RAS/MAPK pathways[122]. Inhibition of BCR-ABL by imatinab mesylate increased Batf2 expression, as did inhibition of JAK/Stat and RAS/MAPK pathways. Therefore, Batf2 in non-hematopoietic cells is inducible by IFNs, enhanced by Mda7/IL24, and suppressible in at least some cell types by BCR-ABL, JAK/Stat, and RAS/MAPK pathways.

2.3.1.3. Hematopoietic cells

While loss of expression is notable in cancers, gain of expression is notable in hematopoietic cells of the immune system. Indeed, Batf2 expression increases during and at least 48 hours after M1 polarization of macrophages, serving important functional roles discussed below. IFNγ -induced Batf2 is important in cDC subsets, while IFNβ, IFNγ, and LPS all have roles in Batf2 induction in macrophage cells[1,83,109,121]. Some reports suggest LPS enhances Batf2 induction by IFNγ[1,83,121], and two reports found that LPS alone induces Batf2 in murine bone-marrow derived macrophages [1,83]. Bone marrow derived macrophages express minimal Batf2 mRNA in response to heat-killed *Listeria monocytogenes* and CpG[83]. In CD8α$^+$ cDCs and CD11b$^+$CD103$^+$ cDCs *in vivo*, Batf2 is inducible with IL-12 or infection with the intracellular pathogen *Toxoplasma gondii*[83]. *In vitro,* however, purified Flt3L-DC do not produce Batf2 in response to IL-12, nor in response to GM-CSF or pIC, but rather to LPS. In general, Batf2 is inducible in macrophages and CD8α$^+$ cDCs or CD11b$^+$CD103$^+$ cDCs by M1-associated stimuli, especially LPS and IFNγ.

2.3.1.4. Other correlations

Other clinical studies correlating Batf2 with disease outcomes have less clear connections. Microarray analysis of high-pain versus low-pain fibromyalgia found Batf2 to be among the top 10 most significantly differentially expressed genes in peripheral blood[123] . Increased Batf2 correlates with pain, but any hints at causality remain unclear. Batf2 is also highly upregulated in cases of common variable immune deficiency with

inflammation complications compared to cases without, in concert with other IFN-responsive genes. These studies may provide insight into the function of Batf2 or the mechanisms of these diseases. Although Batf2 may simply be responding to an inflammatory response involving IFNs, it is possible that Batf2 contributes as well; for example, by orchestrating macrophage polarization that in turn aggravates inflammation. Defining the functions of Batf2 paves the way for the development of therapies targeting Batf2 that may be useful in cancer and other diseases.

2.3.2. Batf2 Function

Initial studies identified Batf2 as an interferon-inducible negative regulator of AP-1 activity in transformed cancers. Based on this activity, these studies referred to the Batf2 protein as suppressor of AP-1, regulated by IFN (SARI). In vitro binding assays suggest that Batf2 cannot heterodimerize, but can form heterodimers *in vitro* with other AP-1 members JunB and cJun, as well as bZIP transcription factors C/EBPG, C/EBPA, and DNA-damage inducible transcript (Ddit3)[113]. Batf2 heterodimerizes with cJun *in vivo*[95]. Previous studies on Batf2 have demonstrated roles as a negative regulator of gene expression by AP-1 inhibition, a negative regulator of gene expression by participating in dephosphorylation complex, and a positive regulator of gene expression by interacting at AP-1-Irf consensus elements (AICEs)[83,86]. Negative gene regulation by AP-1 inhibition (specifically cJun sequestration) and by dephosphorylation (specifically of glycogen synthase kinase 3 beta [GSK3β], decreasing β -catenin) occurs in normal cells and is lost in cancer cells, while positive gene regulation at the AICE occurs in the development of dendritic cell subsets[83,95,124].

2.3.2.1. Cancer studies: Batf2 negatively regulates AP-1 and aids dephosphorylation of GSK3β

Studies on Batf2's role as a tumor suppressor lend insights into the mechanisms of Batf2's activity. As discussed above, decreased expression *in vitro* occurs post-EMT and in malignant cells compared to normal counterparts, and decreased Batf2 *in vivo* correlates with aggressiveness and survival. The tumor-suppressive activity occurs through two distinct mechanisms: AP-1 inhibition and β-catenin dephosphorylation. Batf2's affect as an AP-1 inhibitor appears to be the reason for its cancer-specific effects on growth and apoptosis and inhibition of proto-oncogenes. In various cancer cells, forced expression inhibits both anchorage-dependent and -independent cell growth and induces apoptosis[95,112]. This selective inhibition seems to arise from the reliance of these cancer cells on cJun, such that inhibition of cJun by Batf2 prevents gene expression for growth and survival. Batf2-sensitive cells show elevated cJun, perhaps reflecting increased reliance on cJun for growth and survival. Moreover, Batf2's growth-inhibiting activity in cancer cells is most pronounced during by Ras or Src oncogene transformation, which induces AP-1 activity[95].

A great deal of evidence indicates that Batf2 inhibits cJun activity by sequestration. Fluorescence Resonance Energy Transfer (FRET), immunofluorescence, and co-immunoprecipitation assays demonstrate Batf2 binding to cJun, and Batf2's protein-protein interaction motif (the leucine zipper) is required for its activity, demonstrating that Batf2 heterodimerizes with cJun and that heterodimerization is necessary for anti-tumor effects[95,112,113]. Moreover, forced Batf2 expression prevents

cJun from binding to the AP-1 consensus TRE as demonstrated by reporter assays, EMSAs, and AP-1-induced gene expression[95,112,124]. Therefore, little doubt remains that Batf2 acts as a negative regulator of cJun activity by binding to it and preventing its binding to AP-1 consensus. Yet, some evidence suggests that Batf2 can in fact drive transcription in hematopoietic cells, discussed below.

Batf2-inhibited AP-1 can suppress tumor growth by decreasing growth factors, altering cell cycle regulators, and interfering with communication with and modification to the extracellular environment. Batf2 inhibits the AP-1 dependent MET in colorectal cell lines; MET is both a proto-oncogene and a growth factor[112]. Additionally, Batf2 may alter cell cycle regulator expression to inhibit cell growth. Forced Batf2 expression inhibits cyclin D1 and cyclin E expression in HeLa cells[84]. These AP-1-regualted cyclins mediate transition into S phase, encouraging cell growth. Batf2 may thus block cell cycle in part by inhibiting cell cycle progression.

Batf2 also interferes with AP-1-dependent factors promoting invasiveness by communication with and modification of the extracellular environment, specifically CCN family member 1 (Ccn1)-induced integrin signaling, and MMP expression. Aggressive breast and prostate carcinoma cell lines displayed elevated AP-1 activity, elevated integrin-MAPK signaling, and enhanced invasiveness and anchorage-independent growth by both AP-1 and integrin-MAPK pathways. Batf2 transduction reduced the feed-forward loop of AP-1-induced cJun and Ccn1 expression[124]. Functionally, this resulted in reduced growth and invasiveness. Batf2 activity seems to vary between cells, however, since CCN1 was not affected by Batf2 in colorectal cancers[112]. Another way Batf2 influences the environment to prevent aggressive cancers is through Mmps. Mmps in

general contribute to EMT, and can increase growth factor availability by releasing molecules from the extracellular matrix, but Batf2 inhibits Mmp2 and Mmp7 by decreasing AP-1 expression[125 112]. Mmp2 in particular can increase tumor growth by promoting vascularization, and both Mmp2 and Mmp7 are associated with modulation of the immune response towards a tumor-promoting environment [125]. Therefore, Batf2 may modulate tumor growth through inhibition of integrin signaling and modification of the extracellular matrix, in addition to inhibition of cell cycle progression and growth factor signaling.

In addition to anti-cancer activity by AP-1 inhibition, Batf2 participates in a dephosphorylation complex that leads to B-catenin inhibition. Wang *et al* discovered this activity in lung adenocarcinoma[119]. Batf2 inhibits the epithelial-mesenchymal transition (EMT) through activation of GSK3β and subsequent inactivation of β -catenin. During EMT, cells change morphology, increase migratory ability, lose the adhesion factor and pre-EMT marker E-cadherin, and increase the intermediate filament vimentin. While lung adenocarcinoma cell lines pre-EMT express higher levels of Batf2, post-EMT cell lines lose Batf2 expression. Forced Batf2 in post-EMT cells, however, reverts cells back to pre-EMT morphology, migratory ability, E-cadherin expression, and vimentin expression. On the other hand, knockdown of Batf2 in pre-EMT cells increased post-EMT morphology, decreased E-cadherin, and increased vimentin. Batf2 therefore plays an important role in EMT in lung adenocarcinoma. These effects are not due to Batf2's transcriptional activity; rather, Batf2 inhibits the Wnt/ β-catenin signaling pathway by activating the β -catenin inhibitor GSK3β. Batf2's mechanistic role in this process remains unclear, but is required for GSK3β activation by protein phosphatase 2 (Pp2a).

The interaction involves physical contact between GSK3β, Pp2a, and Batf2. Possibly, Batf2 acts as a scaffold in this complex. Increased GSK3β activity results in decreased β -catenin and β –catenin-induced EMT-associated factors. Batf2 expression in post-EMT lung adenocarcinoma increases active GSK3β and inhibits β -catenin, including that induced through Wnt pathway activation.

Batf2 manipulation in xenografts demonstrates the importance of Batf2 *in vivo* as well as *in vitro*. Decreasing Batf2 in lung adenocarcinoma tumors with anti-Batf2 with small interfering RNA increased lymph-node metastases and metastatic sites with mesenchymal characteristics and reversed severity-associated changes to vimentin and E-cadherin[119]. In the reverse experiment using colorectal cancer cells, forced Batf2 expression led to decreased tumor size, proliferation, and proto-oncogene MET expression[112]. In a similar experiment, Batf2 abrogated Ccn1-induced growth and aggressiveness[114]. Therefore, the mechanisms by which Batf2 prevents tumor growth *in vitro* do indeed translate to *in vivo* models.

Clinical data also support Batf2's role as an anti-tumor transcription factor, and also suggest Batf2 may be a useful diagnostic marker or therapeutic target. Batf2 loss correlates with tumor aggressiveness in hepatocellular carcinoma, OTSCC, leukemic cell number in chronic myeloid leukemia, lung adenocarcinoma and lung squamous cell carcinoma, colorectal cancer, and esophageal squamous cell carcinoma[112,115–117,119,122,126].

Although the correlations with differentiation, metastasis, and invasiveness vary between cancer types, Batf2 may be a predictor of these factors in specific cancers. Moreover, Batf2 has a much more useful correlation in all cancers previously studied: survival. Batf2 served as an independent predictor of survival in hepatocellular

carcinoma, oral tongue squamous cell carcinoma, and colorectal cancer[112,115,117]. Therefore, Batf2 may prove useful in the future as a diagnostic marker predicting poor prognosis of various cancers, depending on the cell type.

Overall, Batf2's ability to inhibit AP-1 activity and β-catenin activity is revealed through studies on its role as a tumor-suppressor. *In vitro*, Batf2 inhibits growth factors, modulates communication and interaction the extracellular environment, suppresses B-catenin-mediated changes required for aggressiveness, and inhibits EMT. *In vivo*, Batf2 slows xenograft growth, and loss is associated with increased aggressiveness and decreased survival in multiple cancer types.

2.3.3. Hematopoietic cell development and function: Batf2 as an AP-1 transcription factor

Although Batf2 has a negative regulatory role in some conditions, studied in multiple non-hematopoietic cell types, it also has a positive regulatory role, studied in hematopoietic cell development and function alongside closely related Batf and Batf3. Batf and Batf3 have been known transcription factors important for multiple immune cells. Batf3 forms a ternary complex with Irf8 or Irf4 to drive expression of genes for CD8α^+ and related CD103+CD11b- cDCs including CD103 and Xcr[76]. Normally, these cells do not produce Batf2, and thus mice deficient in Batf3 lack this population; however, *Toxoplasma gondii* infection or IL-12 treatment *in vivo* induces Batf2, which can partially compensate for Batf3 deficiency in the development of these CD subsets[83]. Table B2 lists the situations in which Batf2 can and cannot compensate for other Batfs. This suggests that Batf2 does have some transcriptional activation activity.

Interestingly, Batf2 seems unique among the Batf factors, and its role in activating transcription remains unclear. Batf and Batf3 are largely interchangeable in the development of cells, complexing with Irf4 or Irf8 and subsequently binding to the AP-1/Irf Composite Element (AICE) (5'-TTTCnnnnTGACTAA-3' or 5'-GAAATGAnTCA-3') in regulatory regions of many genes necessary for immune cell development (Figure 2.3). Batf and Batf3 drive Th2 cell development, being necessary for genes including GATA3, IL-4, IL-10, and *Ctla4*. Batf or ectopic Batf3 is required for genes involved in B cell class switching (Aid), Th17 development (Rorc, IL17A, IL21, IL23R), and Tfh development (Maf, Bcl6) and Th9 development (IL21, IL-10, Furin, Fes, Erg, IL-1RA)[76,111]. Batf2, however, can only compensate for Batf3 in the cDC subsets, and not Batf3 in Th2 development, or Batf in B cells, Th17, or Tfh cells.

Two components critical for the function of the bZIP transcription factors are the leucine zipper, critical for protein-protein interaction, and the basic region, critical for DNA binding. Batf2 presumably binds to Irf4 or Irf8 to compensate for Batf3 development in the cDC subsets, and heterodimerizes with cJun as demonstrated in cancer cell studies. However, it has not been shown to bind DNA. Fusion of the Batf2 basic region with Batf3 leucine zipper produces a chimera that functions in cDCs but not Th17, similar to wild-type Batf2. Moreover, Roy *et al.* in an investigation of Batf2 in macrophage cells asserts that Batf2 does not contain a DNA-binding domain[109].

Recently, Batf2's importance in macrophage polarization was explored in BMDMs. As in other immune cells, this study suggests the requirement for a cofactor, specifically the M1-specific Irf1. Batf2 knockdown with small interfering RNA (siRNA) abolished the expression of several genes important to macrophage polarization. Batf2

complexed with Irf1, and knockdown of Irf1 mimicked knockdown of Batf2. However, recognition of a consensus region by Batf2-Irf1 complex was not explored, so it remains unknown whether this complex indeed recognizes a consensus element, perhaps the AICE or simply the Irf1 element, in macrophages. Batf2's function *in vivo* in regards to immune function remains only briefly explored. Batf2-/- mice, produced by Tussiwand *et al.,* appear to possess normal hematopoietic cell populations at homeostasis[83]. The only macrophage populations altered significantly in these mice were the lung-resident $CD103^{-}CD11b^{-}$, and this deficiency only appeared after *Toxoplasma gondii* infection. Whether the macrophages contribute to the increased mortality is unclear. Of note, this study focused on dendritic cell populations, so macrophage populations were not reported in detail.

In summary of previous studies, Batf2 has important roles as both a tumor-suppressor and inhibitor of AP-1 activity, as well as a differentiation and polarization factor in some immune cells as an activator of AP-1 activity. This study complements the previous and current work on Batf2 by exploring its function in a macrophage cell line with advantages over BMDMs. The conclusions of Batf2 having an activating role on its own are contradicted, but the importance of a cofactor or other environmental factor specific to M1-polarized macrophages is supported.

2.3.4. Studying Batf2's role as a transcription factor in PUER macrophages

The work in this dissertation adds to this body of research by constitutively expressing Batf2 in an *in vitro* macrophage system, the PUER cells. Initial microarray analysis from our lab found Batf2 highly upregulated in LPS/IFNγ (M1) polarized

macrophages relative to resting or IL-4 (M2) polarized PUER macrophages, in agreement with the recent studies in bone marrow macrophages. My hypothesis was that Batf2 is a master regulator of M1 polarized macrophages. To test this hypothesis, I investigated the function of Batf2 in the PUER macrophage model.

2.3.4.1. Model system: PUER macrophages

Previous studies have multiple discrepancies due in part to sensitivity and plasticity of macrophages to a wide variety of stimuli and to variation in animal colonies. PUER cells uniquely permit study of differentiation of macrophages without the previous epigenetic programming seen in primary macrophages, macrophage-differentiation cytokines M-CSF and granulocyte-macrophage colony stimulating factor (GM-CSF), stress, or polarizing stimuli during purification. All of these can alter gene expression independently of the experimental stimulus and may account for much of the variability in the literature that used cells isolated from animals[23,127,128]. PUER cells are common myeloid progenitors from fetal liver of PU.1$^{-/-}$ mice constructed in the lab of Harinder Singh. Homozygous knockout of PU.1 is embryonic or newborn lethal. In the liver, the development of the myeloid lineages is blocked at the CMP stage. IL-3 dependent fetal CMPs were cloned and myeloid development potential was restored by inserting a gene for a chimeric protein containing the PU.1 DNA binding domain with the hormone binding domain of a modified tamoxifen-sensitive estrogen receptor[9]. Thus, these progenitor cells can be differentiated *in vitro* to multiple cells of the hematopoietic lineage and provide an attractive tool for studying the factors required for cell differentiation.

Our collaborator Dr. Peter Laslo previously used PUER cells for studying factors for differentiation macrophages and neutrophils[129]. Differentiation to macrophages can be induced by treatment with tamoxifen, rather than commonly-used differentiating cytokines M-CSF and GM-CSF that can alter gene expression of polarization-associated genes[127]. Such *in vitro* polarization results in a highly controllable macrophage differentiation and polarization model subject to minimal *in vivo* polarizing stimuli or accumulation of relevant epigenetic changes, thereby, minimizing environment-induced factors. We took advantage of this system to build on the differentiation factor studies to examine transcriptional regulation in M1- and M2-polarized macrophages.

2.3.5. Summary

This project investigates the hypothesis that Batf2 controls M1-specific gene expression, thus contributing to the inflammatory functions of these cells. To achieve this end, we use an *in vitro* model in which CMPs are first differentiated to macrophages *in vitro* to avoid potential polarizing stimulation present in *in vivo* models. We then polarize them to the M1 phenotype with LPS and IFNγ, producing M1-polarized macrophages with minimal environment-induced variability. Gene expression analyses revealed Batf2 as a novel M1-associated transcription factor. The role of Batf2 as a putative master transcription factor was explored by creating CMPs constitutively expressing Batf2. Constitutive Batf2 expression alone does not induce M1-specific gene expression, but it enhances IFNγ/LPS-induced Cxcl10 expression and suppresses IL-4-induced Arg1 expression. Further, the study begins to address the possibility of a required M1-associated cofactor or condition for Batf2's activity, including binding

partners. In agreement with the recent studies on Batf2, Batf2 is an M1-associated transcription factor that likely controls gene expression in conjunction with an M1-associated cofactor.

Table 2.1: Select mechanisms of gene expression regulation

Level of Regulation	Mechanism	References	Tested in this Study?
Protein binding/ Cofactor	AP-1 Dimerization Partners	Jun/Jun represses Mmp13, Jun/Fos activates[52]	Yes (Figure 4.17)
	Non-AP-1 Dimerization Partners	Fos/Jun proteins prefer TRE[a] and ATF/Jun proteins prefer CRE[a][73]	No
	Ternary Complex Members	Batf3/Irf4 activation complexes	Yes (Figure 4.19)
Post-translational modification	Phosphorylation	Phosphorylation of cFos[76]	No
	SUMOylation	Regulation of AP-1 dimers[77]	
	Redox state	Regulation of cFos[78]	
Promoter/enhancer accessibility	Epigenetic Modification	PU.1 and Runx1 remodel chromatin to determine macrophage lineage[6,7,57,58]	No

[a]TRE: Phorbol Acetate-Responsive Element; CRE:cAMP-Responsive Element

Table 2.2: Compensatory ability of Batf2 for other Batf proteins.

Cell Type	Product/ Outcome Detected[a]	Original Inducer	Means of Batf2 Expression[a]	Compensates? [a]
T cells	IL17	BATF	Retroviral in BATF$^{-/-}$	No
B Cells	Class Switch Recombination	BATF	Retroviral in BATF$^{-/-}$	No
CD8α^{+}cDCs[a]	Development	BATF3	Retroviral in BATF$^{-/-}$mice and bone marrow cells	Yes
CD103^{+}CD11b^{-} cDCs[a]	Development	BATF3	BATF2 DBD[c] on BATF LZ[d]	Approx. 50%
Th17	Development	BATF	BATF2 DBD[c] on BATF LZ[d]	No

[a]Data from Tussiwand, *et al.*[83]

[b]CD8α^{+}cDCs and CD103^{+}CD11b^{-} cDCs are largely the same except for tissue location

[c]The DNA-Binding Domain (DBD) mediates interaction with DNA

[d]The Leucine Zipper (LZ) motif mediates interactions with other proteins

Figure 2.1: Genes associated with various macrophage states.

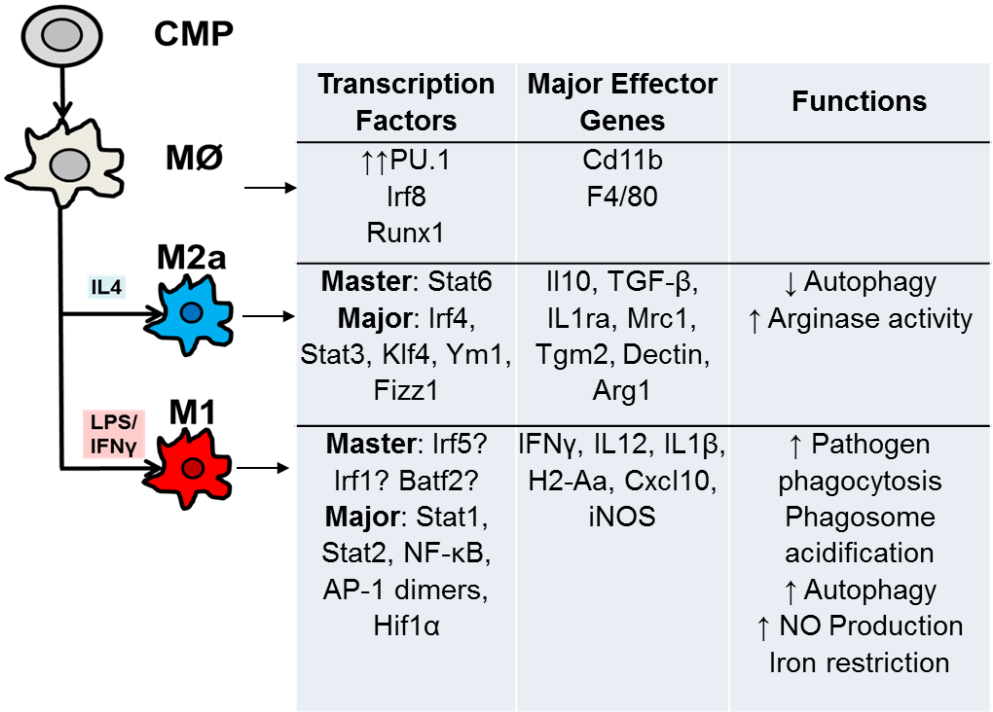


Figure 2.1: Genes associated with various macrophage states. Common myeloid progenitor cells (CMPs) are directed to the macrophage lineage and various functional states by a handful of master transcription factors that induce the largest number of lineage- or function-specific genes and some major transcription factors the also induce several critical genes. Many of the transcription factors and key functional genes defining macrophages and their functional states have been defined, although gaps remain. Resting macrophages develop from high expression of transcription factors including SpiB, interferon regulatory factor 8 (Irf8), and runt-related transcription factor 1 (Runx1) and express Cd11b and F4/80 (Emr1). Treatment of resting macrophages with Th2 cytokine IL-4 leads to induction of master transcription factor Stat6, and major transcription factors Irf4, Signal Transducer and Activator of Transcription 3 (Stat3), Kruppel-Like Factor 4 (Klf4). These in turn induce expression of genes including interleukin 10 (IL-10), transforming growth factor beta (TGF-β), mannose receptor C

(Mrc), transglutaminase 2 (Tgm2), dectin (C-type lectin domain family 7 member A [Clec7a]), arginase 1 (Arg1), Ym1 (chitinase 3-like-3 [Chi3l3]), found in inflammatory zone 1 (Fizz1*),* and IL1R. These "M2a" polarized macrophages demonstrate decreased autophagy and increased arginase activity. Treatment of resting macrophages with the Th1 cytokine IFNγ and bacterial cell wall component LPS, in contrast, polarizes macrophages to an M1 state. The transcription factors for M1 polarization are less well defined. Irf5, Irf1, and basic leucine zipper transcription factor, ATF-like 2 (Batf2), all have roles in broad M1-associated gene expression. Other major transcription factors include Stat1, nuclear factor kappa B (NF-κB), the cFos and cJun proteins that form activator protein-1 (AP-1) dimers, and hypoxia inducible factor 1α (Hif1a). M1 macrophages express IFNγ, IL-12, IL-1β, H2-Aa, Cxcl10, and inducible nitric oxide synthase (iNOS). Functionally, these cells increase pathogen phagocytosis, increased acidification of phagosomes, increased autophagy, increased nitric oxide (NO) production, and iron restriction.

Figure 2.2: Batf2 is a member of the AP-1 group of bZIP transcription factors.

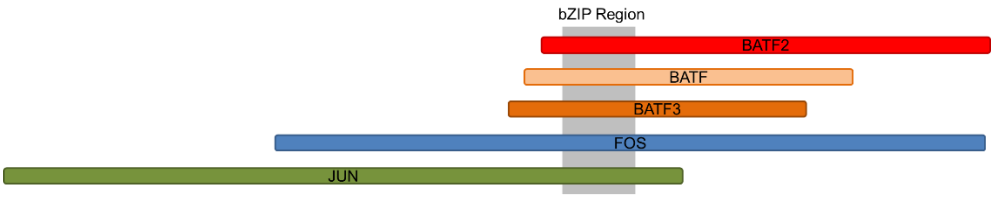


Figure 2.2: Batf2 is a member of the AP-1 group of bZIP transcription factors. The diagram above shows the alignment of the Batf proteins, centered on the bZIP regions that mediate protein-protein and protein-DNA interactions. cFos and cJun are included for reference. The homology between Batf2 and Batf is 47% and between Batf2 and Batf3 is 43%.

Figure 2.3: Functions of Batf and Batf3 in immune cell development and function.

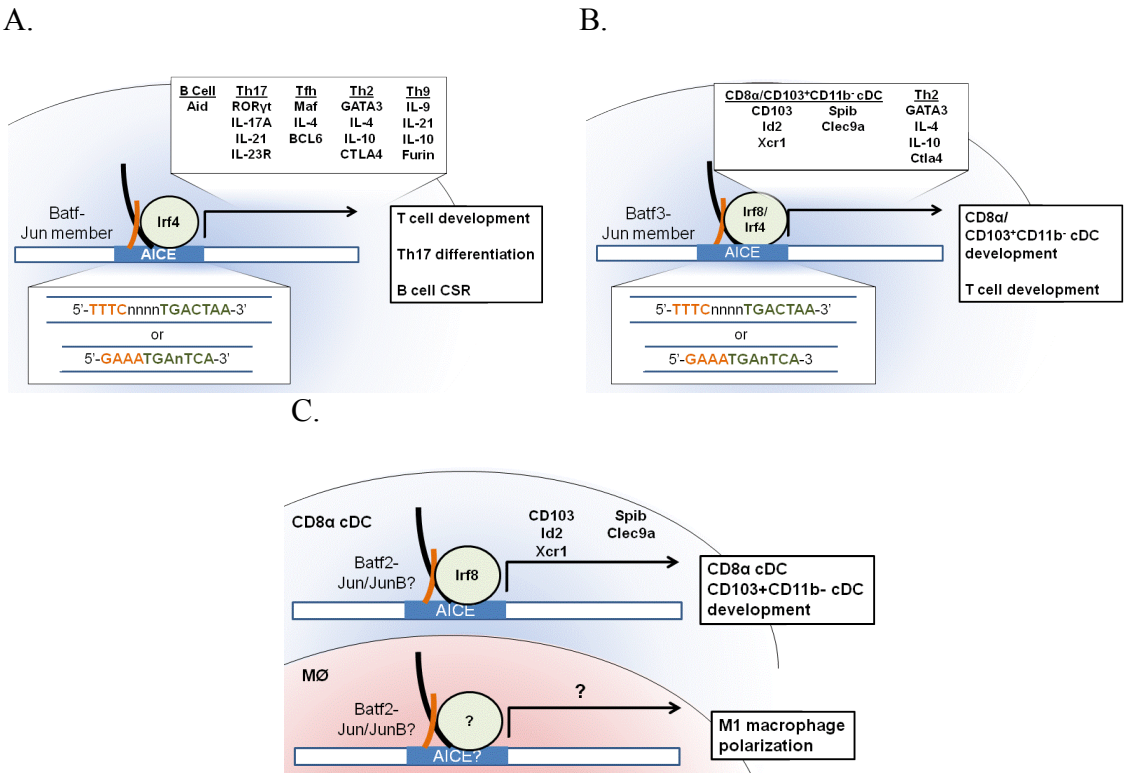


Figure 2.3: Functions of Batf and Batf3 in immune cell development and function. **A.** Batf, present in B cells, Th17, Tfh, Th2, and Th9 cells, dimerizes with another AP-1 protein, and further complexes with Irf1. This ternary complex recognizes the AICE (5'-TTTCnnnnTGACTAA-3' or 5'-GAAATGAnTCA-3') and drives gene expression including AID (B cells, for class-switching); Rorc (encodes RORγt), IL17A, IL21, IL23R (Th17, development and function); V-**Maf** Avian Musculoaponeurotic Fibrosarcoma Oncogene Homolog (Maf), B cell CLL/lymphoma (Bcl6) (Tfh, development and function); GATA3, IL-4, IL-10, and Cytotoxic T-lymphocyte associated 4 (Ctla4) (Th2, development and function); IL9, Furin, Fes, Erg, IL-1RA (Th9, development and function). **B.** Batf3, present in CD8α$^+$ cDCs, CD11b$^+$CD103$^-$ cDCs, and Th2 cells, dimerizes with another AP-1 protein and further complexes with Irf4 or Irf8. This ternary

complex recognizes the AICE and drives gene expression including CD103, Inhibitor of DNA binding 2 (Id2), chemokine (X-C motif) receptor 1 (Xcr1), Spib, Clec9a ($CD8\alpha^+$, $CD11b^+CD103^+$ cDC, development and function); GATA3, IL-4, IL-10, and Ctla4 (Th2, development and function). **C.** In $CD8\alpha^+$ cDCs and $CD11b^+CD103^+$cDCs, Batf2 can compensate for Batf3. Batf3 or Batf2 dimerizes with an AP-1 protein; in vitro, Batf2 binds cJun or JunB. The heterodimer forms a ternary complex with Irf4 or Irf8 that recognizes the AICE (5'-TTTCnnnnTGACTAA-3' or 5'-GAAATGAnTCA-3'), driving expression of critical genes for development and function including CD103, Id2, Xcr1, Spib, and Clec9a. Macrophages express high amounts of Batf2 after M1 polarization; macrophages simultaneously express cJun, JunB, and Irf8, but Batf2's cofactors remain to be defined.

Chapter III

MATERIALS AND METHODS

3.1. Reagents and antibodies

The following cytokines and reagents were used to differentiate or polarize cells: 4-Hydroxytamoxifen (tamoxifen/4-OHT) (Calbiochem, Billerica, MA), γ-irradiated lipopolysaccharide (LPS) from *E. coli* 011184 (Sigma-Aldrich, St. Louis, MO), murine rIFNγ, murine rIL-10, and murine rIL-4 (Peprotech, Rocky Hill, NJ). PUER cells were provided by Dr. Harinder Singh (University of Chicago) and maintained in IMDM without phenol red (GIBCO, Grand Island, NY), supplemented with 10% fetal bovine serum (Advantage Grade, Atlanta Biologicals) 100 U/ml penicillin, 100 µg/ml streptomycin, and 28.2 µg/ml L-glutamine (GIBCO). Growth was maintained with the addition of 12.5ng/ml IL-3 (Peprotech). Cultures were maintained at 37ºC, 5% CO_2. For transfections, Optimem-1 media was used (GIBCO) with TransIT2020 Reagent (Mirus, Madison, WI). Transductions were perfumed with polybrene solution (Millipore, Billerica, MA).

The following antibodies were used for Western blots: polyclonal rabbit anti-human Batf2 (Aviva Systems Biology, San Diego, CA), mouse anti-rabbit GAPDH (71.1) (Sigma-Aldrich), and polyclonal rabbit anti-mouse iNOS/NOS Type II (BD Transduction Laboratories) and the secondary antibodies used were goat anti-rabbit IRDye680 and goat anti-mouse IRDye800CW (LI-COR Lincoln, NE).

3.2 PUER macrophage differentiation and polarization

PUER cells are myeloid progenitor cells with PU.1 under the control of the

estrogen receptor. The cells were originally isolated from fetal liver of PU.1$^{-/-}$ mice, then transduced with the chimeric construct composed of a PU.1 derivative fused to the tamoxifen responsive ligand binding domain of the estrogen receptor by Walsh *et al* [5].

To differentiate the PUER cells to macrophages, PUER were seeded at $0.2x10^6$-$0.4x10^6$ per cm^2 in polystyrene tissue culture flasks. One hundred nM tamoxifen was added to the IMDM-based growth media described above[109] and cells were differentiated for 4 days. On day 4, one set of flasks was used for analysis. The remainder were treated with polarizing cytokines or were treated with fresh media as control. At the time of polarization, cultures consisted of both non-adherent and adherent cells. Non-adherent cells were pooled, pelleted, washed, and re-distributed evenly to the flasks that contained the adherent cells. Earlier analysis in our lab and in Dr. Singh's lab were unable to identify any differences between adherent and non-adherent cells (data not shown). To polarize cells to M1 phenotype, 20 ng/ml IFNγ and 100 ng/ml LPS were added. To polarize cells to M2, 4 ng/ml IL-4 was added. An additional group left unstimulated served as a negative congrol. For alternative polarizations, recombinant murine IL-10 was added at 1-100ng/ml. Cells were then incubated for 2 days for typical experiments, or as otherwise described.

3.3. Cloning and transduction of Batf2 expression vector

Batf2 in the pCMV-SPORT6 plasmid was obtained from Open Biosystems and cloned into the pMigR1 murine retroviral vector (pMigR1 courtesy of Harinder Singh). The pMigR1 vector has previously demonstrated high efficiency cloning into hematopoietic lineage cells[130]. This vector drives gene expression with a murine stem

cell virus (MCSV) long terminal repeat (LTR) and allows for GFP selection by following the MCS with an internal ribosomal entry site for simultaneous expression of GFP[130]. To subclone Batf2, *EcoRI* and *XhoI* restriction enzyme recognition sequences were introduced in the flanking regions of the Batf2 open reading frame (ORF) by site-directed mutagenesis. For site-directed mutagenesis, the QuikChange Lightning kit was used (Agilent Technologies, La Jolla, CA), following the manufacturer's protocol. The Batf2 ORF was isolated from pCMV-SPORT6 by *EcoRI* and *XhoI* digestion followed by gel purification. At the same time, the vector pMigR1 was prepared by digestion with *EoRI* and *XhoI* and similarly purified by gel purification. pMigR1-Batf2 was made by ligation of these gel-purified Batf2 ORF and pMigR1 vector. The resulting plasmid (shown in Figure 3.1) was verified by sequencing and transfected into *E. coli* for future experiments.

To create PUER-Batf2 cells, pMigR1-Batf2 viral particles were created with a packaging cell line and then transduced into PUER at the progenitor (CMP) stage. Empty vector pMigR1 was transduced in parallel as a control. Purified pMigR1-Batf2 was transfected into the retroviral packaging cell line PLAT-E (Harinder Singh), resulting in the viral particles used for transduction. At the time of transfection, PLAT-E were at 80-90% confluency in a 15cm^2 cell culture plate containing Optimem-1 media with 210 µg of and 63 µg of DNA per 15 ml media. PLAT-E were incubated overnight at 32°C, then the media was removed and replaced with 14 ml cIMDM as prepared above but without antibiotics. Transfected PLAT-E cells were then incubated an additional 3 days at 37 °C. Supernatant containing viral particles was harvested the next day and immediately used for transduction. PUER cells were transduced with virus by “spin

transduction." For the transduction, PUER cells were seeded at $3x10^6$ in 12 mls of viral supernatant total and 2 mL was placed into each well of a 6-well flat-bottom plates and spun at 2200 rpm for 2 hours at room temperature. After spin, cells were incubated at 37 °C for 30 minutes, washed, and then seeded in cIMDM supplemented with antibiotics and IL3 as described above. After two days incubation at 37 °C, transduced cells were selected by flow cytometry sorting for GFP^+ cells. Sorting was done in the Flow Cytometry Core Facility of the University of Kentucky by Jennifer Strange. After two rounds of sorting, the transduced cells were greater than 98% GFP^+ and used for experiments. Individual clones were created by sorting the cells a 3rd time for GFP into 98-well plates, 1 cell/well. These clones were used for some experiments, with similar results to the cells following 2 sorts.

Constitutive expression of Batf2 mRNA is shown in Chapter 4 (Figure 4.12). We were unable to confirm constitutive Batf2 protein, however, due to inconsistent antibody quality. Initial Western blots confirming Batf2 protein in M1-polarized PUER were performed with one lot of AVIVA polyclonal anti-Batf2 antibody. Subsequent batches of the anti-Batf2 from the same vender and from three other companies did not work with Batf2 Western blots. I performed extensive troubleshooting with alternative antibody concentration, protein extraction methods, secondary antibodies, *etc*. and was unable to produce usable blots. In these analyses, the M1-specific marker iNOS was clearly detectable, suggesting the problem lies with the antibodies and not with the methodology, polarization, or other reagents (Figure 4.13). Thus, I was unable to do further analysis of Batf2 protein. Recent commentaries suggest that this problem is increasingly common[131]. Therefore, we cannot confirm constitutive protein expression in PUER-

Batf2 cells.

3.4. RNA extraction and analysis with RT-PCR

After 4 days of differentiation (D4) or 2 additional days of polarization (MØ, M1, and M2), RNA was extracted for analysis using TriReagent (Molecular Research Center, Cincinnati, OH) following the manufacturer's instructions. Briefly, both suspended and adherent cells were collected for analysis: adherent cells were treated with TriReagent, while suspended cells were pelleted at 1000 xg, washed, and combined with the adherent cells. RNA was separated by addition of chloroform (Sigma) followed by centrifugation at 20,000 xg for 15 minutes. RNA was then precipitated by addition of isopropanol (Fisher), washed with 70% ethanol (maintained at -20 °C), and resuspended in RNAse/DNAse-free H_2O (Ambion, Grand Island, NY). RNA concentration was measured using a Nanodrop 2000 (Thermo).

cDNA was synthesized from 4ug of extracted RNA using a GE First Strand cDNA Synthesis Kit (GE Healthcare, Pittsburg, PA) following the manufacturer's instructions, using random primers. mRNA levels were analyzed using semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR). Specific primers shown in Table 3.1 were used for 28-35 amplification cycles, and products were visualized by ethidium bromide staining and digital photography. For each primer pair the number of cycles used was based on prior analysis using known template amounts to determine a specific number of cycles that was near the 50% level on the amplification curve. Band intensity was calculated using ImageJ and normalized to the average signal of the loading controls Gapdh, B-actin, and Hprt.

3.5. Protein extraction and Western blot analysis

For protein extraction, both suspended and adherent cells were collected. Adherent cells were removed with a cell scraper. Lysis of cells was performed by suspension in RIPA buffer (Pierce, Rockford, IL) with protease inhibitors (Sigma) and passage through a 28 ½ G needle, and 15 minute incubation on ice with occasional mixing. Protein concentration was determined using BCA assay as per manufacturer's instruction (Pierce). Forty µg of protein was used for Western blots under reducing conditions unless otherwise indicated. Protein was mixed with sample buffer (Bio-Rad) supplemented with 2-mercaptoethanol (Sigma) and incubated at 90 C for 5 minutes. Samples were then run with appropriate standards on a 4-20% tris-glycine gel (Bio-Rad, Hercules, CA) at 100V, with buffer containing 0.1% sodium dodecyl sulfate. Proteins were transferred to 0.2 µm nitrocellulose membrane. The Odyssey Infrared Imaging System was used to visualize target proteins. Following transfer, the membranes were blocked in Odyssey PBS blocking buffer (LI-COR) for 1 hour and incubated at 4ºC overnight with rabbit anti-human Batf2 at 1:200 dilution or iNOS at 1:2000 dilution and mouse anti-rabbit GAPDH clone 71.1, cross-reactive to mouse GAPDH, at 1:40,000. Membranes were washed for 3 times with PBS-0.1% Tween for 5 minutes each, then incubated in blocking buffer with goat anti-rabbit IRDye690 and goat anti-mouse IRDye800 antibodies at 1:15000. After washing, the membranes were imaged with Odyssey Infrared Imager.

3.6. Cxcl10 and Total Protein Assays

Total protein was used as a correlate of cell number to normalize Cxcl10 that was measured in the culture supernatants. To verify that total protein correlates with cell number, a 24-well flat-bottomed cell culture plate was seeded with various concentrations of PUER-pMigR1 and PUER-Batf2 cells and differentiated for 4 days with tamoxifen. After differentiation, the plates were spun at 1000 xg, 10 min, then analyzed for total protein or cell number. For total protein, the cells from two series of aliquots were harvested, washed with PBS, and lysed in RIPA buffer (Pierce). Total protein was quantified from lysate with BCA Protein Assay Kit (Pierce) as per manufacturer's protocol. For cell number, cells were harvested from two parallel series of aliquots, washed in PBS, and counted using a hemocytometer. As clearly shown in Figure 3.2, cell number correlates linearly with total protein, validating our use of total protein concentration to normalize Cxcl10 measurements.

Secreted Cxcl10 was analyzed using the murine IP-10 Mini ELISA Development Kit (Peprotech), following manufacturer's protocol. Cells in 24-well tissue-culture plates were differentiated for 4 days and polarized an additional day as described above. Plates were spun at 1000xg, 10 min. Supernatant was used for Cxcl10 analysis, and cells used for total protein analysis. For Cxcl10, supernatant was added to 96-well plates coated with rabbit anti-mIP-10. Biotinylated rabbit anti-mIP-10 were then added, followed by avidin-HRP conjugate and then ABTS liquid substrate. Absorbance values correlating to substrate conversion for each kit were detected using a Spectramax M5 plate reader with SoftMaxPro software. Comparisons between PUER-MigR1 and PUER-Batf2 were done by 2-tailed Student's T test, 2-tailed (n =3), with equal variance. Statistical significance

was accepted at $P < 0.05$.

3.7. shRNA subcloning and transduction

To knock down Batf2 expression, three small-hairpin RNAs (shRNAs) were designed using a program created by the Hammond lab at RNAi Central (http://cancan.cshl.edu/RNAi_central/RNAi.cgi?type=shRNA) and subcloned into a retroviral vector (murine stem cell virus [pMSCV]), resulting in three different knockdown vectors (shBF2.1, shBF2.2, and shBF2.3). pMSCV containing shRNA targeting firefly luciferase served as a negative control.

The 22mer shRNAs produced by the Hammond lab's algorithm for shBF2.1, shBF2.2, and shBF2.3 contain two regions complementary to each other such that the resulting transcript forms a short hairpin. These regions are also complementary to the Batf2 CDS such that the short hairpin, after process by the cell, targets Batf2 message for degradation. The sequences are as follows:

shRNA1

TGCTGTTGACAGTGAGCGAACTCTCCAGCTGCTCTGGGACTAGTGAAGC

CACAGATGTAGTCCCAGAGCAGCTGGAGAGTCTGCCTACTGCCTCGGA

shRNA2

TGCTGTTGACAGTGAGCGAGTCCATGCTCTCATGAGTCTCTAGTGAAGC

CACAGATGTAGAGACTCATGAGAGCATGGACCTGCCTACTGCCTCGGA

shRNA3

TGCTGTTGACAGTGAGCGCCCACTCATTGGCAGAAGTCATTAGTGAAGC

CACAGATGTAATGACTTCTGCCAATGAGTGGATGCCTACTGCCTCGGA

Primers complementary to 5' and 3' ends containing restriction enzyme sites *EcoR1* and *Xho1* were used for amplification to produce oligos for subcloning. Resulting oligos were purified by electrophoresis on an agarose gel, digested with *Ecor1* and *Xho1* and subcloned into the pMSCV vector similarly purified and enzyme-digested. Correct insertion of the oligos was confirmed by sequencing. The pMSCV vector is useful for transduction of hematopoietic cells, and contains the Lac operon for selection in bacteria and hygromycin resistance for selection in eukaryotic cells. pMSCV-shBF2.1, -shBF2.2, and –shBF2.3 were transfected into *E. coli* for amplification, with successfully transfected cells selected by blue-white color selection. Plasmids were later purified using plasmid purification kits (Qiagen), analyzed for the presence of the shRNA oligo, and purified using agarose gel purification. Purified plasmids were packaged into retroviral virions using packaging cell line PLAT-E, and virions were used to transduced PUER cells by spin transduction as described above for creation of PUER-Batf2 cells. Unlike PUER-Batf2, successfully transduced cells were selected by growth in hygromycin-containing cIMDM. Cultures grew from shBF2.2 and shBF2.3 –transduced cells, but these demonstrated levels of Batf2 equivalent with untransduced PUER after polarization with LPS/IFNγ (data not shown). shBF2.1-transduced cultures failed to grow after multiple attempts using the pMSCV retroviral system or a lentiviral vector system in collaboration with the University of Kentucky Genetic Technology Core Facility.

3.8. Nitrate detection and arginase activity assays

To determine iNOS activity, supernatants from control and polarized PUER macrophages were analyzed using a Griess Reaction in a colorimetric assay kit

(EnzyChrom Nitric Oxide Synthase Assay) as per manufacturer's instructions (BioAssay Systems, Hayward, CA.) Briefly, cells were seeded and differentiated to macrophages as described above in cIMDM with Tamoxifen and IL3. Cells were then polarized with LPS/IFNγ (M1) or IL-4 (M2), or left unstimulated. After 24 hours, supernatants were removed and centrifuged at 10,000 x g to remove cells. Nitrate in these supernatants was analyzed using the kit reagents that employ the Griess reaction, in which nitrate is reduced to nitrite for 5 min at 60 °C. The reaction generates proportional indicator product that can be quantified at 540 nm.

To determine arginase activity, conversion of L-arginine to urea in total cell lysates was analyzed using a kit as per manufacturer's instructions (Sigma). Briefly, cells were seeded, differentiated to macrophages, and polarized as described above. After 24 hours, cells were harvested by scraping and washed 1-2 times by pelleting cells at 1000 x g, 4 C, 10 minutes and resuspension in PBS. Total cell lysates were made using the lysis buffer suggested in the kit containing 0.4% Triton X-100, pepstatin, and leupeptin at the recommended concentration of 100 ul per 10^6 cells. Cells were incubated in the lysis buffer on ice for 10 minutes. Insoluble material was then removed by centrifugation at 14,000xg at 4 C, 10 minutes, and supernatants were aliquoted onto a 96 well plate for analysis. The reaction of supernatants with reaction buffer and Mn solution and controls continued for 2 hours at 37 C, after which the urea detection reagent was added and the amount of urea was analyzed using a plate reader.

3.9. BMDM isolation

Bone marrow harvested from BALB/c mouse femurs was suspended in PBS, then pelleted at 400 x g and resuspended in complete DMEM using a syringe with an 18 G needle. Cells were then counted on a hemocytometer and 4 x 106 cells were seeded in a 10 cm tissue culture dishes with 50 ng/mL M-CSF (Peprotech). Three days after seeding, additional complete RMPI media with M-CSF was added. On day 7, cells were washed with PBS, washed with Ca/Mg free PBS with 0.5 mM EDTA, lifted with Cellstripper non-enzymateic cell dissociation solution ((Mediatech) pelleted at 450 x g for 5 min, and resuspended in complete DMEM at 0.5x106 cells/mL. These cultures were polarized and used for mRNA analysis as described for PUER above.

Table 3.1: Primers used in RT-PCR analysis.

A.

Arg1	5'- CCCTGGGGAACACTATATAATAAAAA-3' 5'- GTGTTCACAGTACTCTTCACCTCCT-3'
Ccl5	5'- ACCATATGGCTCGGACACCACTCC-3' 5'- AGATGCCCATTTTCCCAGGACCGA-3'
Ccne1 (Cyclin E)	5'- ATTGCCAAGATTGACAAGACTGTG-3' 5'- CGTCTCTCTGTGGAGCTTATAGAC-3'
cFos	5'- CCAAACTTCGACCATGATGTTCTC-3' 5'- GATAAAGTTGGCACTAGAGACGGA-3'
cJun	5'- CATTTTCTCACCAACTGCTTGGAT-3' 5'- TCTCCCTTTTCTTTACAGTCTCGG-3'
Csf1r	5'- AGGCTAAAGTCCTTGACAGCAATA-3' 5'- TTGAGGATAACGTTGAATCCCACT-3'
Cxcl10	5'- CATGAACCCAAGTGCTGCCGTCAT-3' 5'- AAAGGGGAGTGATGGAGAGAGGCT-3'
Figf/Vegfd	5'- GAAACAACTGCTTAGTCATCGGTA-3' 5'- CTTGAAGAATGTGTTGGTTGTCTT-3'
Fizz1	5'- TGAGACCATAGAGATTATCGTGGA-3' 5'- ACATTTAAAGGCACATGAGTCAGA-3'
Fra1	5'- ACAGAGGTTCATCTGGAGAGGT-3'-3' 5'- CAGTGGGTCCCAGGAAATGAG-3'-3'
Fra2	5'- GAAGAAAACCACCCTGTTTCCTCT-3' 5'- ATAGGGATTGGACATGGAGGTGAT-3'
H2-Aa	5'-CAGCCTCTGTGGAGGTGAAGACGA-3' 5'-TTGGGGAACACAGTCGCTTGAGGA-3'
Hdac9	5'-GAGGCAAGAACAGGAAGTAGAGAG-3' 5'-TGAATGAAGTGACAAGATTTCCAT-3'
Id2	5'-GAGCTTATGTCGAATGATAGCAAA-3' 5'-ATACTGGGTCCTTCTGGTATTCAC-3'
Bcl6	5'-ACTTCATGTACACATCTAGGCTCAAC-3' 5'-GTATTTCTCAGTGGCATATTGTTCTC-3'
Ifi204	5'-ACCAAAGTTAGTGTGTGGAGAACA-3' 5'-AGGAGTTGCATTAGCTCTTTGTCT-3'
Ifi205	5'-ACTTCCACAGCCCAGAAAAGGAAAG-3' 5'-TCAAACGGGTCTGTTGCAGTGAG-3'
IL-1β	5'-AAGTGATATTCTCCATGAGCTTTGTA-3' 5'-CTTTGCTCTTGACTTCTATCTTGTTG-3'
iNOS	5'-GATGCTGCCACCTTGGAGTTCACC-3' 5'-GTACCAACCATTGAAGGGGCAGGC-3'

Table 3.1 (cont.)

Irf1	5'-GGAAGTGAAGGATCAGAGTAGGAA-3' 5'-TTCATAAGGTCTTCGGCTATCTTC-3'
Irf2	5'-AAGAAGATTTTCCAGATCCCCTGG-3’ 5'- GGTTCTTGCTTGATGTGCTTAAC-3’
Irf3	5'- AGTGTATGAGTTTGTGACTCCAGG-3’ 5'- GAACTCCCATTGTTCCTCAGCTA-3’
Irf4	5'-TTCCCTACCCGGACGACAATGGAC-3' 5'-GGAGCGGTGGTAATCTGGAGTGGT-3'
Irf5	5'-AGCTGCTAGATGTCCTGGACCGTG-3' 5'-ATGCTGTCTGCCGACCAAGAAAGC-3'
Irf7	5'-AGCGAAGAGGCTGGAAGACCAACT-3' 5'-CTTGGGGTTTGGAGCCCAGCATTT-3'
Irf9	5'- GTAGAGATTTGGCCCAGTACTTC-3' 5'- ACGGTGATAAGAACCATCACAGAA-3'
JunB	5'- GCCTTTCTATCACGACGACTCTTA-3' 5'- CTGATCCCTGACCCGAAAAGTAG-3'
JunD	5'- GGGGTACAGAGTGAGATTCTGTTT-3' 5'- TCATATTCCCAAGTGTGGCAGTAA-3'
Mmp13	5'-AAGCAGTTCCAAAGGCTACAACT-3' 5'-AGATAAACATAAGGTCACGGGATG-3'
Mrc1	5'-TCAGAAATGGGAGTGTAAGAATGA-3' 5'-ACAGCTTGTCTTTGTCGTAGTCAG-3'
Parp12	5'-GGATGTGCCGTGCAGACCACA-3' 5'-GGCCCAAAGAGCATCCCAGACTACT-3'
Parp14	5'-GCCTCCTGGGGTGGACGAGT-3' 5'-ATGCCAGAGAGCACTGCGGA-3'
Prp	5'-CTCTTTGTGACTATGTGGACTGATGT-3' 5'-GCCTGTAGTACACTTGGTTAGGGTAG-3'
Stat1	5'-TGGACGACCAGTACAGCCGCT-3' 5'-CCTTCTCTGTTCTGAGAGGTTTTGCA-3'
Stat2	5'-TGGCCCTACCCAGTTGGCTGA-3' 5'-TGGCTCTGATGGGGGTCTGAAGAC-3'
Tcfec	5'-TGCGCTGGAACAAAGGGACCATTC-3' 5'-TTCCTCTCAGGATGGGTCTGCTGC-3'
Tgfb1	5'-AAGCGGACTACTATGCTAAAGAGG-3' 5'-ACGCCAGGAATTGTTGCTATATT-3'
Tgtp	5'-TGCCTGGCATTGGGACCACTAACT-3' 5'-GCTTGTGGGCTGGGAGATCCTGTA-3'
Ym1	5'-AGAATCTGTGGAGAAAGACATTCC-3' 5'-TAGAAGGGTCACTCAGGATAAAGG-3'

Table 3.1 (continued)

B.

1 F	5'- GGGCCTCCATAGGTCACTGG -3'
1 R	5'- AAGGATTCGTGCTGGTGCAG -3'
2 F	5'- GTCTTGTAGATCTCTTCTGTGCCA -3’
All F	5'- AAGAAGAAGCAGAAGAACCGAGT -3'
All R	5'- CTAGAGGTGTCCTCACCATGAGA -3'
3 F	5'- TGACTGAGACATTCTAGGCAGC -3’
3 R	5' -CGTGGTTCTGTTTCTCCAAGGATT -3’

A. Sequences of primers used for gene detection.

B. Sequences of primers used for Batf2 isoform analysis.

Figure 3.1: Vector for constitutive Batf2 expression.

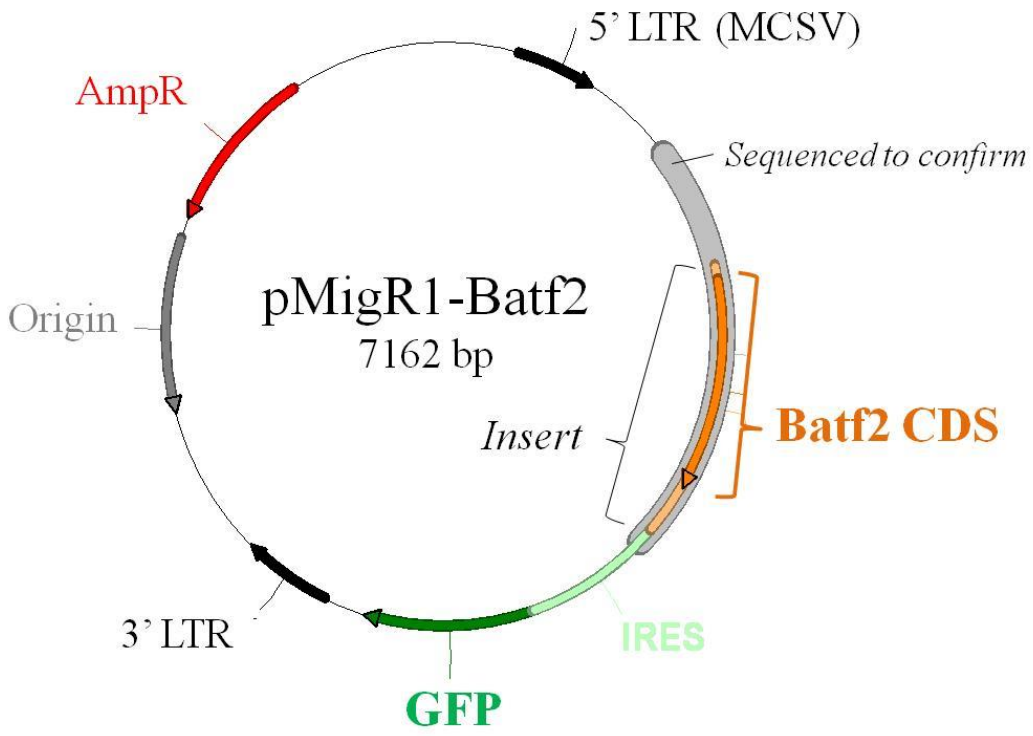


Figure 3.1: Retroviral vector for constitutive Batf2 expression. Batf2 in the pCMV-SPORT6 (Open Biosystems) was modified by introducing *EcoRI* and *XhoI* restriction enzyme recognition sequences were introduced in the flanking regions of the Batf2 open reading frame (ORF) with site-directed mutagenesis. The long terminal repeats (LTRs) are from the murine stem cell virus (MSCV).

Figure 3.2: Cell number correlates with total protein.

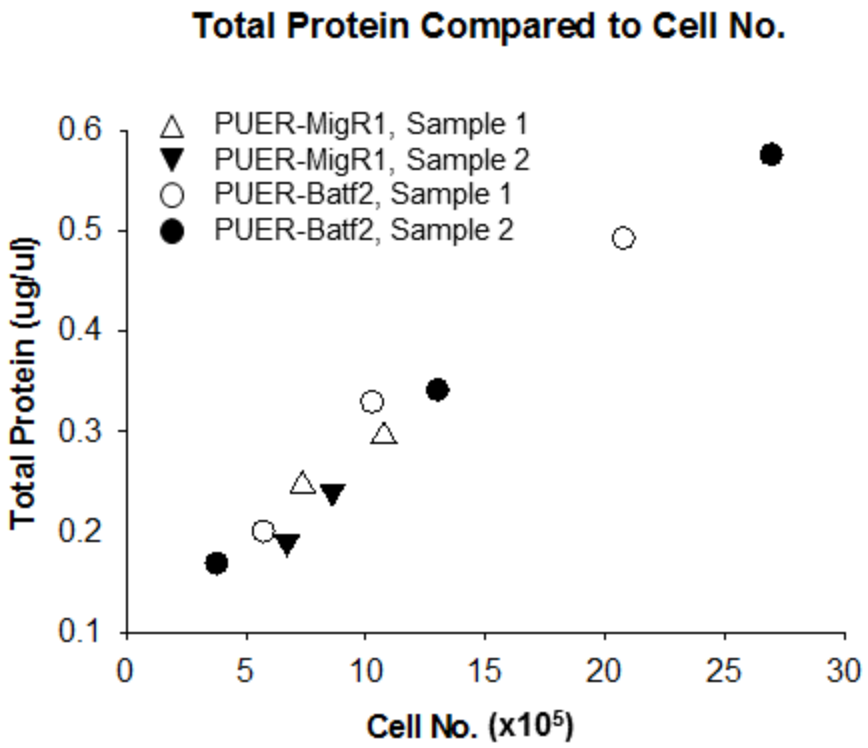


Figure 3.2: Cell number correlates with total protein. As cell number increases, total protein extracted also increases linearly. PUER-MigR1 and PUER-Batf2 cells were seeded at various concentrations in a 24-well plate and polarized with tamoxifen as described elsewhere in this section. After differentiation, cells were harvested washed with PBS, then either counted with a hemocytometer or lysed. Lysed cells were analyzed by BCA analysis for total protein.

Chapter IV

RESULTS

4.1. Transcriptional programs of resting, LPS/IFNγ-activated (M1), and IL-4-activated (M2) PUER macrophages

The overall goal of this project is to understand the mechanisms governing macrophage polarization to an inflammatory phenotype by describing putative novel master transcription factors required for key M1 gene expression. In this study, I investigate and rule out the ability for the highly expressed, M1-associated, and novel transcription factor Batf2 expression by itself to induce expression of key genes for M1 polarization. I demonstrate a role for Batf2 in enhancing LPS/IFNγ induction of key inflammatory chemokine Cxcl10 mRNA and suppressing the IL-4 induction of the anti-inflammatory effector protein Arginase 1. I further postulate reasons for the failure of Batf2 alone to induce major M1 gene expression or alter phenotype, the most likely possibility being a requirement for a cofactor such as an Irf family member.

4.1.1. Differentiation and polarization of the PUER cell line provides an in vitro system for determining gene changes due to polarization

Macrophage cells have a wide repertoire of functions, from phagocytosis of pathogens to secretion of growth factors. Macrophages have different functional states tailored to specific environments, named 'polarization states.' During infection, macrophages can be divided into two polarization states based on the T cell response type that produces their polarizing cytokines[2]. M1 polarized macrophages support inflammatory Th1 type immune responses that function in elimination of microbial

pathogens. This state is associated with cytokines such as Ccl5 and IL-1β, enzymes such as iNOS and chemokines such as Cxcl10[22]. M2 polarized macrophages more often participate in repair and can counteract M1 responses. These cells secrete cytokines such as TGF-β1, enzymes such as Arg1 and MMPs, and downregulation of Th1 type responses. Typically, the bacterial cell wall component LPS and the inflammatory Th1-associated cytokine IFNγ are used to imitate an infection and polarize cells to M1, while Th2-associated cytokine IL-4 or closely-related IL13 are used to imitate an anti-inflammatory M2 response in experimental systems[2].

Gene expression and thus function in response to polarizing stimuli occurs under the guidance of transcription factors. Given that a few master transcription factors govern each stage of development and differentiation of most cells, such as PU.1, Runx and Irf8 during macrophage development, it follows that macrophages likely require a master transcription factor for polarization. Our overall goal is to identify these master transcription factors for M1 polarization. Although master transcription factors have been determined for development, identification of factors guiding polarization states has been complicated by the unique plasticity of mature macrophages in that many non-polarizing stimuli present in the environment alter gene expression. Therefore, my studies use the PUER cell line, a system which provides a highly controllable environment.

PUER cells are IL3-dependent common myeloid progenitor (CMP) cells with PU.1 under the control of the estrogen receptor[9]. PUER cells uniquely permit study of macrophages differentiation without the previous epigenetic programming, polarizing stimulation during purification, or commonly-used differentiating cytokines M-CSF and

GM-CSF that can alter gene expression, and with controls for time in culture. These are described in Chapter 3, page 52[127,128]. Briefly, Walsh *et al.* created these cells by isolating fetal liver cells from PU.1$^{-/-}$ mice, then transducing them with a chimeric PU.1 transcription factor attached to a modified estrogen receptor regulatory domain[9]. These cells have the ability to differentiate into multiple hematopoietic cell lineages after treatment with appropriate cytokines. PUER are differentiated to resting macrophages by tamoxifen with IL-3, then polarized to inflammatory phenotype (M1) with IFNγ/LPS, or to alternative phenotype (M2, sometimes referred to as M2a) with IL-4[9,132]. This use of PUER produces polarized macrophages in a highly controlled environment.

Our collaborator, Dr. Peter Laslo (University of Leeds) used the PUER cell system to study macrophage and neutrophil differentiation and identify factors that regulate their differentiation[129]. We extend use of this system to analysis of macrophage polarization. We first validated the PUER system for studying macrophage polarization by confirming expression of known key M1- and M2-associated genes and activity of key M1- and M2-associated enzymes. To do this, PUER cells were differentiated to macrophages and polarized, and expression of M1 and M2 specific genes was analyzed. PUER CMPs were first differentiated for 4 days with tamoxifen and IL3, then treated to produce three groups. Treatments included: i) additional 2 day culture in new media in parallel with polarized cells (MØ), ii) 2 days in fresh media supplemented with 20ng/ml IFNγ and 100 ng/ml LPS (M1), and iii) 2 days in fresh media supplemented with 4 ng/ml IL-4 (M2) (Figure 4.1). An additional control, macrophage differentiated for 4 days and immediately processed, ruled out any shifts in gene expression due to time in culture. RNA and protein analyses were performed at the MØ, M1, and M2 state for known

markers. To analyze RNA, we use semi-quantitative reverse-transcription PCR. This has the advantage of being a rapid and inexpensive method to roughly quantify gene expression when the sensitivity of real-time reverse-transcriptase PCR is unnecessary. This method was used for several known M1- and M2-associated genes.

As shown in Figure 4.2, M1-polarized macrophages expressed high levels of established M1-associated genes iNOS and Ccl2, and M2-polarized macrophages expressed high levels of M2-associated genes Arg1 and Fizz1. Our lab extended this analysis to a wider panel of M1 and M2 specific genes and found that most are appropriately upregulated in the PUER model systems, including H2-Aa, Irg1, Stat1, and Mrc1 (data not shown). To confirm that PUER macrophages polarized to M1 or M2 states are functional macrophages, activity of the key enzymes iNOS (M1) and Arg1 (M2) were measured. Nitric oxide production, indicative of iNOS activity, was minimal in resting or M2-polarized macrophages, but increased in M1-polarized macrophages (Figure 4.2). Similarly, Arginase activity was undetectable or low in lysates from resting or M1-polarized macrophages, but increased in M2-polarized macrophages. Taken together, these results indicate that PUER-derived macrophages polarize to M1 or M2 states after stimulation with LPS/ IFNγ or IL-4, respectively.

To comprehensively survey gene expression changes in polarized PUER macrophages, a microarray analysis was performed at the UK Microarray Core Facility using the GeneChip ® Mouse Exon 1.0 ST Array to analyze gene expression in PUER macrophages in the MØ, M1, and M2 state. Microarray analysis confirmed that PUER cells express macrophage-specific but distinct transcriptional programs for differentiated resting, M1-polarized, or M2-polarized macrophages. All states expressed macrophage

markers Emr1 (F4/80) and Itgam (CD11b) (data not shown) confirming previous studies using PUER to study macrophages[9,129,132]. M1-polarized PUER macrophages compared to unpolarized macrophages revealed an expected increase in M1-associated genes, including Irf1, Stat1, Cxcl10, and key M1 enzyme iNOS and lack of upregulation of M2-associated genes including Mmp13, Fizz1, Mrc1, and the key M2 enzyme Arg1 (Table 4.1). Many were confirmed by semi-quantitative reverse-transcriptase PCR (RT-PCR) (data not shown). Similarly, M2 -polarized PUER macrophages upregulated M2-associated genes Mmp13, Fizz1, Mrc1, and key M2 enzyme Arg1. Likewise, many were confirmed with RT-PCR including Fizz1 and Arg1. M2-polarized PUER macrophages did not increase expression of M1-associated genes Irf1, Stat1, Cxcl10, and key M1 enzyme iNOS. Importantly, there were no significant gene expression changes between the day 4 and the MØ unpolarized macrophages. Therefore, PUER cells differentiate to macrophages and polarized to M1 and M2 states not only express the appropriate markers, but also display established M1 and M2 function.

Originally, many investigators thought that macrophage polarization to M1or M2 states was more stable or permanent. However, macrophages are now appreciated to be more plastic and exist along a spectrum of idealized polarization states. For example, adequate stimuli can drive a macrophage cell from an inflammatory state to an anti-inflammatory state[133]. To determine whether PUER macrophages also exhibit this ability, PUER cells were polarized with M1 or M2 stimuli followed by the opposing stimuli. PUER cells were differentiated to macrophages with tamoxifen and then polarized for 2 days with LPS/ IFNγ or IL-4 as before. These resulting polarized macrophages were then treated with the same stimuli or with opposite stimuli for an

additional 48 hours (M1 with IL-4, M2 with LPS/IFNγ). Lanes 1 to 3 in Figure 4.3[1] show that tamoxifen-differentiated PUER macrophages strongly express mRNA for the M1 marker iNOS after 48 hours of polarization with LPS/IFNγ, or strongly expressed mRNA of M2 marker Arg1 after 48 hours of polarization with IL-4, as expected. To determine whether polarization was reversible, M1-polarized macrophages were then treated with IL-4 and M2-polarized macrophages were treated with LPS/IFNγ for an additional 48 hours. M1 polarized cells treated with IL-4 slightly upregulated Arg1 and downregulated iNOS, while M2 polarized cells treated with LPS/IFNγ strongly upregulated iNOS and strongly downregulated Arg1 (Figure 4.3). These results suggest polarized PUER macrophages exhibit plasticity, with M2-polarized macrophages more readily reversible than M1- polarized macrophages under the conditions used in this study.

In summary, our studies provide proof of concept that PUER cells can be used as a model system to study macrophage polarization. Therefore, PUER-derived macrophages present a good model for my studies on transcription factors involved in macrophage polarization. Moreover, PUER macrophage repolarization to M1 and somewhat to M2, demonstrate a plasticity that is a feature of macrophages in the animal.

4.1.2. Identification of novel transcription factors during sustained M1 or M2 polarization

Many studies on macrophage transcription factors analyze gene expression at early time points after polarization. We focused on transcription factors altered at 48

[1] The data shown in Figure 4.3 are from an experiment done with the assistance of Mr. Ben Taylor and Dr. Joe McGillis

hours after stimulation to identify transcription factors involved in long-term maintenance of polarization. Moreover, transcription factors may be altered briefly due to fresh serum (such as serum response factors) or serve only a temporary role during initial polarization while not playing a long-term role maintaining polarization. It is important to distinguish between these two phases because *in vivo* inflammatory responses are often beneficial during initial clearance of infection but damaging to the host in the long term. Having established the PUER macrophage model as useful for analyzing polarization-associated gene expression, we used this system to identify transcription factors highly increased or decreased at 48 hours after polarization. Twenty-three total transcription factors were increased in M1-polarized macrophages, and 5 were increased in M2-polarized macrophages (Figure 4.4). M1-associated macrophage transcription factors included the previously known Irf1 and Stat1, but also novel Ifi204, Id2, and Batf2. M2-associated transcription factors included known Tcfec and novel Hdac9 and Zpf609. Batf2 was one of the most highly upregulated transcription factors upon M1 polarization, increasing nearly 10-fold relative to unpolarized macrophages. Moreover, it belongs to the AP-1 group of transcription factors, interacts with AP-1 signaling in non-immune cells, and bears close resemblance to key transcription factors important for development of other hematopoietic cells, Batf and Batf3. These last observations also support the case for Batf2/s potential as a key M1 transcription factor. We therefore selected Batf2 for further investigation as a putative master transcription factor during M1 polarization.

4.2. Batf2 expression in PUER common myeloid progenitors and macrophages

4.2.1. Batf2 is rapidly upregulated in IFNγ-stimulated macrophages

Our initial hypothesis was that Batf2 is a master transcription factor for LPS/IFNγ (M1) polarization. The first goal was to confirm the microarray data that Batf2 selectively increases in M1-polarized PUER macrophages. To do this, PUER macrophages were differentiated and polarized as in the microarray analysis (Figure 4.1). PUER macrophage progenitors were differentiated to macrophages for 4 days with tamoxifen, then polarized to M1 with LPS/IFNγ or to M2 with IL-4 for an additional 2 days. Total RNA was extracted and analyzed by semi-quantitative RT-PCR. Undifferentiated monocytes and unpolarized or IL-4 polarized macrophages express little or no Batf2, while macrophages stimulated with classical activation stimuli IFNγ and LPS highly upregulate Batf2 mRNA and protein, shown in Figure 4.5 A and B, respectively. These results confirm that Batf2 is selectively induced in M1 PUER macrophages at 48 hours. In our model system we were interested in this later time point for sustained polarization as explained in the previous section, but it is also important to know whether Batf2 plays a role in the initial polarization. We therefore stimulated PUER macrophages with LPS/IFNγ and analyzed Batf2 mRNA expression at various time points from 0.5 hours to 24 hours. As shown in the representative experiment in Figure 4.6, Batf2 message appears 1 hour after polarization with LPS/IFNγ, peaks between 2 and 8 hours, but remains sustained at 48 hours. These results support a role for Batf2 in both the transition from resting to the M1 polarized state as well as sustained M1 polarization.

To confirm that Batf2 expression in the PUER M1-polarized macrophages is relevant to the induced M1 state in primary macrophages, a study was done with murine bone-marrow-derived macrophages (BMDMs). BMDMs were purified from mice and polarized to M1 with LPS/IFNγ, polarized to M2 with IL-4, or left unstimulated for 24 hours. Confirming the results in PUER, M1-polarized cells strongly upregulated Batf2 mRNA as seen in Figure 4.7[2]. In contrast to PUER, the BMDMs also expressed Batf2 after IL-4 stimulation. Batf2 expression by IL-4 polarization was moderate compared to the strong upregulation by LPS/IFNγ polarization. These cells may have been epigenetically programmed or primed to M1-associated gene expression *in vivo*, altering gene expression, or the stress of purification and stresses in cell culture may have driven all treatment groups towards M1. The data demonstrates, however, that Batf2 is induced in macrophages in response to LPS/IFNγ, agreeing with previous reports of Batf2 as part of an IFN response[1,83,95].

For many genes, multiple isoforms exist and may have different functions. The National Center for Biotechnology (NCBI) database contains sequences for four different Batf2 mRNA isoforms and proteins, but no studies have reported on the representation of isoforms in any cell type or the functionality of the different isoforms. To determine which isoforms are present in macrophages during peak Batf2 mRNA expression and sustained polarization, cells were polarized for 8 hours (peak Batf2 mRNA expression) and 48 hours (sustained Batf2 mRNA expression). Total RNA extracts from polarized cells were analyzed by RT-PCR using isoform-specific primers. Note that no primers can be made specific to the reference sequence since X1 isoforms include the entire reference

[2] The data shown in Figure 4.7 are from an experiment done with the assistance of Mr. Robert Hayman IV and Dr. Joe McGillis

sequence (Figure 4.8). Two isoforms were detected: X1 and X3. Since universal Batf2 primers produced a robust product while X1 and X3 are only weakly detectable, it is likely the reference sequence makes up part of the total isoforms, but this remains to be conclusively determined. Most differences in message lie outside the reading frame, and protein products differ only in a brief segment at the C-terminal region. A notable exception is the truncated isoform "CRA_a" (NCBI accession EDL33225.1). X1 differs from the reference sequence in a missing Q residue in the bZIP region. No functional correlations are known. This data provides the first report of the existence of multiple Batf2 isoforms in any cell type. For protein isoforms, the antibody to Batf2 has not been tested. The antibody was raised to a region of the human Batf2 overlapping a C-terminal segment of bZIP region common to all isoforms except the truncated "CRA_a." The isoforms share 94% pairwise identity in this region. Therefore, the antibody should recognize products of the reference, X1, and X3 message present in macrophages. However, this remains unverified. This data suggests that LPS/IFNγ activation upregulates multiple isoforms, including X1 and X1, and possibly the reference sequence. The functional consequences remain unknown.

4.2.2. Batf2 is upregulated in response to IFNγ or IL-10, but not LPS

Typically, LPS and IFNγ are used as *in vitro* inflammatory stimuli to model M1 polarization, and both are required for full M1 polarization. *In vivo*, however, macrophages see a wide variety of stimuli that polarize them to variations of the inflammatory and anti-inflammatory phenotypes, depending on the environment. In some contexts, for example, IFNγ may be present without TLR4 agonists like LPS,

activating different macrophage functions than LPS/IFNγ in combination. IFNγ signals through JAK/Stat pathways, and LPS signals through MyD88/AP-1 and MyD88/NF-κB. IFNγ activates a receptor composed of IFNγ-receptor 1 (IFNGR1) and IFNGR2, which activates JAK2, which then activates Stat1 transcription factor homodimers. TLR4 and MyD88 activate IKK, PI3K, and MAPKKs, which activate NF-κB and AP-1 transcription factors. To determine which pathway(s) control Batf2 in PUER macrophages, we tested for Batf2 mRNA in response to LPS alone, IFNγ alone, or LPS/ IFNγ. Expression was analyzed at 8, 24, and 48 hours using semi-quantitative RT-PCR with specific primers. The 8 and 24 hour timepoints were included to account for possible differences in peak gene expression in response to LPS *vs* IFNγ. Figure 4.9 shows that IFNγ alone induced Batf2 at 8, 24, and 48 hours, while LPS did not. Additionally, IFNγ-induced Batf2 message roughly compares to IFNγ /LPS-induced Batf2 message quantitatively. Taken together, the results suggest that IFNγ induces Batf2 in PUER macrophages, while LPS does not contribute to Batf2 expression.

Like IL-4, IL-10 also opposes inflammatory stimuli[29,33,57]. However, the transcriptional repertoire of IL-10- activated macrophages differs somewhat from IL-4-actived macrophages, and overlaps partly with the IFNγ-induced repertoire[26,31]. IL-4 activated macrophages express IL-10, for example, while IL-10 activated macrophages express FceRII[26]. Both induce the mannose receptor (Mrc1) and Arg1. IL-4 stimulates the IL-4 receptor, resulting in activation of Stat3 and Stat6. IL-10 stimulates the IL-10 receptor, resulting in activation of Stat3 but not Stat6[29,30,32,33]. To determine whether the IL-10-activated pathway differed from the IL-4 pathway in Batf2 induction, we polarized PUER macrophages as before with LPS/ IFNγ or IL-4, and in parallel stimulated

macrophages with 1, 10, or 100ng/ml IL-10. In contrast to the minimal expression of Batf2 in unstimulated or IL-4-polarized macrophages, all three IL-10-treated macrophages expressed Batf2 at 48 hours (Figure 4.10). Expression was not dose-dependent and not as robust as LPS/IFNγ treatment. It is possible that the minimum dose used, 1 ng/ml, elicits the maximal response for IL-10. In this case, this suggests that the maximal LPS/IFNγ response surpasses the maximal IL-10 response.

In summary, LPS/IFNγ induces three isoforms of Batf2 mRNA in PUER macrophages, although protein products remain unknown. IFNγ and, unexpectedly, IL-10 induce Batf2, while LPS does not. After LPS/ IFNγ stimulation, Batf2 mRNA appears within one hour, peaks before 16 hours, and is maintained for over 48 hours. This data demonstrates that Batf2 expression in macrophages is controlled by signaling pathways activated by both IFNγ and IL-10, but not by IL-4 or LPS.

4.2.3. Batf2 expression precedes select M1 genes

If Batf2 is important for generating the M1 phenotype in macrophages, we would expect that Batf2 expression would proceed expression of genes selectively upregulated in M1 macrophages. To determine whether Batf2 is induced prior to induction of major M1-related genes, we compared the timecourse of the expression of various M1-associated genes with expression of Batf2. In these studies PUER macrophages were differentiated with tamoxifen and IL-3 and polarized with LPS/IFNγ or left unstimulated. Total RNA was extracted at time points from 0 to 24 hours and analyzed by semi-quantitative RT-PCR with specific primers.

Shown in Figure 4.11 are two genes consistently and highly upregulated in our M1-polarized PUER macrophages, Cxcl10 and interferon-activated protein 204 (Ifi204). Cxcl10, a chemokine attracting inflammatory cells, is a major marker for M1-polarized macrophages[28,44,51,134] . Ifi204/human myeloid nuclear differentiation antigen (MNDA) belongs to the p200 family of transcription factors, expressed in response to IFNα, IFNγ, or LPS in mature granulocytes, in monocytes, and in activated macrophages (reviewed in Gariglio *et al.*)[135]. The p200 family of transcription factors participates in cell cycle inhibition, apoptosis, and differentiation in some cells including monocytes. While its specific role in M1 polarization remains unclear, it is consistently and robustly induced and therefore serves as a good indicator of M1 polarization. Expression of both Cxcl10 and Ifi204 is low or absent in unpolarized PUER macrophages (Figure 4.11). For Cxcl10, a slight increase was seen at 30 minutes after polarization and peaked from 1 to 8 hours. These kinetics are similar to Batf2, which is expressed at 30 minutes but strongly expressed at 1 hour. This does not support an argument for Batf2-induced Cxcl10. However, there is a marked enhancement of Cxcl10 at 1 hour, subsequent to induction of Batf2, allowing for a role of Batf2 in enhancement of Cxcl10. In contrast, Ifi204 induction occurs much later after polarization, about 2 hours after treatment, after Batf2 induction. Therefore, Batf2 mRNA induction in polarized cells occurs prior to induction or enhancement of select M1 genes, allowing the possibility that Batf2 lies upstream of these genes during M1 polarization.

4.2.4. Summary of Batf2 expression data

To summarize this data on Batf2 expression, Batf2 is highly induced in LPS/IFNγ (M1) -polarized macrophages within 1 hour of polarization, and expression is dependent on IFNγ but not LPS. Batf2 induction correlates with expression of Th1-supportive gene expression, but not IL-4-induced Th2-associated gene expression. Surprisingly, the anti-inflammatory cytokine IL-10 also moderately induced Batf2 expression, suggesting Batf2 induction occurs by multiple signals. After LPS/IFNγ, Batf2 induction precedes select genes important for the M1 phenotype. Overall, mRNA analysis of Batf2 in response to multiple stimuli and in relation to other M1-associated genes supports the possibility that Batf2 contributes to M1-associated gene expression.

4.3. Constitutive Batf2 expression alone does not drive gene expression, but enhances select M1 genes or suppresses select M2 genes

4.3.1. Constitutive Batf2 expression may enhance select LPS/IFNγ-induced M1 genes in conjunction with other factors

Having established that Batf2 is strongly induced in M1-polarized macrophages, but not M2-polarized macrophages, and induced prior to at least some M1-associated genes, we set out to identify genes regulated by Batf2. I therefore created a PUER cell line that constitutively expresses Batf2, herein called PUER-Batf2. I hypothesized that genes induced by Batf2 in M1-polarized macrophages would be induced in PUER-Batf2 cells in the absence of cytokine and LPS stimulation. To test this, expression of several M1-associated genes was analyzed by semi-quantitative RT-PCR. Forced expression of a single transcription factor in this way has successfully been used to identify genes downstream of other transcription factors, such as Klf4 overexpression enhancing

expression of Arg1, Mrc1, Pdcd1lg2, Pparg, and others (reviewed in Liao *et al.*)[136]. Based on the mRNA analyses described in the previous section placing Batf2 within M1-associated response, I hypothesized that many key M1-associated genes such as Cxcl10, iNos, Ifi204, and H2-Aa would be induced PUER cells constitutively expressing Batf2.

To construct cells that constitutively express Batf2, PUER cells were transduced with a retroviral vector containing a Batf2 cDNA (pMigR1-Batf2). A murine Batf2 cDNA was subcloned in the multiple cloning site of the pMigR1 vector[130]. The pMigR1 vector drives gene expression with a MCSV LTR, and allows for GFP selection by following the multiple cloning site with an internal ribosomal entry site for simultaneous expression of GFP. This vector was transfected into the packaging cell line PLAT-E, and PUER (at progenitor stage) were treated with the resulting supernatant containing retroviral particles as described in Chapter 3. Cells then underwent two rounds of fluorescence-activated cell sorting to select for GFP^+ cells. First, expression of Batf2 was confirmed in these cultures at the common myeloid progenitor stage (undifferentiated) and unpolarized macrophage stage (tamoxifen and IL-3 treated) by RT-PCR. While undifferentiated and tamoxifen-differentiated PUER or vector-transduced (PUER-pMigR1) cells expressed undetectable to minimal Batf2 message, Batf2-transducaed (PUER-Batf2 cells) had significant expression of Batf2 message, as shown in Figure 4.I2 B. This suggests that PUER-Batf2 cells constitutively express Batf2 mRNA at levels surpassing LPS/IFNγ-induced Batf2.

In order to identify genes potentially induced by Batf2, we analyzed expression of multiple M1-associated genes in PUER-Batf2 compared to the vector-transduced PUER-pMigR1 using RT-PCR. Unexpectedly, undifferentiated and tamoxifen-differentiated

PUER-Batf2 cells did not demonstrate expression of any of the multiple M1-associated genes, including the key M1 gene Cxcl10 (lane 1 of Figure 4.14 and data not shown). Table 4.2 contains a list of all genes tested. This suggests that Batf2 alone does not induce M1 gene expression, as predicted for an M1 master transcription factor.

Although high constitutive Batf2 mRNA expression alone did not drive gene expression, this does not entirely rule out a role for Batf2 as a part of a transactivating transcription complex in M1 polarized macrophages. The activity of AP-1 family members is highly sensitive to context, so activity often requires not only expression of the factor in question but also a permissive intracellular environment composed of dimerization partners, cofactors, post-translational modification enzymes, and chromatin modifications. For example, cFos requires cJun for both activity as activator and as inhibitor, depending on the cell type and gene examined, and phosphorylation of specific residues in both cFos and cJun[64,87]. Moreover, many functions of this family require additional cofactors for their DNA binding activity. The closely related Batf and Batf3, for example, requires Irf4 or Irf8 for transactivation of genes necessary for several T cell subsets including Th17, Tfh, Th2, and Th9[76,111]. Therefore, Batf2 activity may require necessary cofactors or conditions absent in the CMP stage or resting macrophage stage.

To examine this possibility, we tested gene expression in conditions permissive for Batf2 activity. Since Batf2 is expressed in M1-polarized macrophages, M1-polarized macrophages must contain the necessary cofactors and conditions for Batf2 function if Batf2 is important for M1 polarization. Enhanced Batf2 expression in the M1 state such as in M1-polarized PUER-Batf2 cells might act with other regulatory factors to increase downstream gene expression in M1-polarized cells. To determine whether this occurs,

we next analyzed select M1-associated gene expression in M1-polarized PUER-Batf2 cells compared to controls. PUER, PUER-pMigR1, and PUER-Batf2 were polarized as in previous gene expression assays. Cells were differentiated with tamoxifen for 4 days then polarized with LPS/IFNγ to M1 or IL-4 to M2 or left unstimulated. We first analyzed the protein production of key M1 enzyme iNOS to ensure that cells constitutively expressing Batf2 polarized normally. iNOS protein was detected in PUER-Batf2, demonstrating that these cells functionally polarize to M1 (Figure 4.13). Next, we asked whether PUER-Batf2 upregulated M1-associated gene expression (see Figure 4.14 for Cxcl10, Figure 4.16 for Csf1r, and Figure 4.19). After polarization, high constitutive Batf2 mRNA expression enhanced LPS/IFNγ induced Cxcl10 expression, suggesting there is an additional condition or factor required for Batf2 activity, as clearly shown in Figure 4.14. However, as also shown in this figure, this increase in message failed to translate into increased protein. No other M1-associated genes tested demonstrated consistent enhancement in M1-polarized PUER-Batf2 compared to PUER and vector controls, including key M1-associated genes Irf1 and iNOS and (refer to Table 4.2 for the list of all genes tested). Interestingly, Batf2 does not seem to enhance Cxcl10 mRNA except in the context of M1 polarization, when the cellular environment expresses multiple different factors, shifts in metabolism, different enzymes, etc. This suggests that Batf2 requires an additional condition such as a dimerization partner, cofactor, post-translational modification, or chromatin modification for its activity. These data show that constitutive high levels of Batf2 expression alone does not induce expression of key M1-associated genes, but in M1-polarized cells enhances select LPS/IFNγ-induced

expression of select M1-associated genes, suggesting that Batf2 requires a cofactor or condition.

4.3.2. Constitutive Batf2 expression may downregulate select IL-4-induced genes in conjunction with other factors

Based on the data in the previous sections, the modified hypothesis is that Batf2 induces M1-associated gene expression in concert with a necessary cofactor or condition present only in M1-polarized cells. However, constitutive Batf2 expression only enhanced Cxcl10 in M1 state out of the genes tested. This could be due to Cxcl10 being very sensitive to Batf2 induction. Another explanation, however, could be that Batf2's main activity is as a repressor of M2 functions rather than an activator of M1 functions. In cancer studies, for example, Batf2 suppresses activity at the AP-1 element by sequestering cJun into an inactive dimer, suppressing transcription of genes including Cyclin E in HeLa cells[95] (see Chapter 2 page 34 for more detail).

To determine if Batf2 suppresses M2-associated genes, we analyzed gene expression in IL-4-polarized M2 PUER-Batf2 macrophages compared with PUER and PUER-pMigR1 cells. Cells were differentiated for 4 days with tamoxifen and IL3, then polarized with IL-4 or left unstimulated. M2-associated genes as defined in this study refers to either absent/minimal in PUER macrophages and upregulated upon IL-4 treatment, or expressed in resting state and downregulated in M1 stimuli but upregulated or maintained in M2 state. This is shown in Figures R2 for Arg1 and Fizz1 and Irf4 in Figure 4.19 A. It is worth noting that resting macrophages are more similar in many respects to M2-polarized macrophages, and M2-associated genes are sometimes

expressed in the resting stage to a lesser extent than M2-polarized macrophages. As shown in Figure 4.15, PUER-Batf2 cells failed to upregulate Arg1 mRNA after IL-4 stimulation compared to PUER and PUER-pMigR1. This effect was consistent across three experiments. Thus, Batf2 expression reduces IL-4-induced Arg1 mRNA expression.

Of note, an experiment suggested that unpolarized macrophage PUER cells with forced Batf2 expression also express higher levels of the M2-associated gene Mmp13 (discussed further in the context of AP-1 activity below, see Figure 4.16). This may give hints to pathways Batf2 interacts with in macrophages, but is not useful to our goal of identifying factors involved in the M1 polarization since Mmp13 is absent in M1 state. Overall, constitutive high levels of Batf2 message as seen in PUER-Batf2 suppresses IL-4-induced Arg1 message but does not significantly suppress multiple other IL-4-induced gene expression. This data does not support the hypothesis that Batf2 alone promotes M1 polarization by suppressing M2-associated gene expression, but Batf2 might suppress M2-associated gene expression with a necessary cofactor or condition.

4.3.3. Constitutive Batf2 expression does not alter expression of genes that are 1) Batf2-regulated in non-immune cells, 2) cJun-regulated in macrophages, 3) compensatorally regulated by Batf2 in DC subsets

We initially hypothesized that Batf2 by itself promotes M1 polarization as a master transcription factor inducing M1-associated gene expression. Alternatively, since this does not seem to be the case, Batf2 could promote M1 polarization as a factor downregulating M2-associated genes such as Arg1, Tcfec, and Mrc1. However, while

Batf2 alone enhances Cxcl10 and suppresses Arg1 mRNA, it fails to cause the broad changes we expected. Most of the M1 or M2-associated genes I examined support other immune cells or are involved in microbicidal activity. Another possibility is that Batf2 controls genes that encode more supportive functions such as stress responses to the microbicidal reactive species released during inflammation, or structural changes supporting chemotaxis, *etc*.[92,93,137,138]. AP-1 has well described roles in cellular stress responses in other cells, responding to stimuli such as intracellular oxidative stress[92,93]. Moreover, Batf2 exhibits anti-tumorigenic activity through integrin signaling and anchorage-independent cell growth[124]. Based on this information, Batf2 might reasonably play a critical role in M1 polarization by regulating antioxidants, extracellular matrix, cell cycle, *etc*. We therefore explored whether Batf2 impacted three categories of non-immune genes: 1) genes regulated by Batf2 in non-immune cells, 2) genes regulated by cJun (which Batf2 represses) in macrophages, 3) genes regulated by Batf and Batf3, with which Batf2 shares function in some situations. These three categories of genes were investigated in tamoxifen-differentiated PUER, PUER-pMigR1, and PUER-Batf2 in unpolarized, LPS/IFNγ (M1)-polarized, and IL-4 (M2)-polarized cells at 48 hours after stimulation.

4.3.3.1. Genes directly regulated by Batf2

Batf2 suppresses Ccne1 (encoding Cyclin E protein) in non-hematopoietic cells by sequestering cJun[95]. Cyclin E is needed for cell cycle progression from the G1 to S phase[139,140]. Macrophages undergo cell cycle arrest upon activation with LPS or IFNγ[141]. Thus, Batf2 might support polarization in part through inhibiting Cyclin E and

subsequent cell cycle progression. Cyclin E expression was therefore compared in PUER, PUER-pMigR1, and PUER-Batf2. M1-polarized and M2-polarized cells were also compared to resting macrophages, to determine whether Batf2-mediated cJun enhancement or suppression requires an M1-associated cofactor or condition. In this case, suppression would occur in M1-polarized PUER-Batf2 but not resting macrophages and possibly not M2-polarized macrophages. As seen in Figure 4.16 A, high constitutive Batf2 mRNA expression did not markedly suppress levels in unpolarized, M1-polarized, or M2-polarized cells. This data demonstrates that Batf2 expression alone does not regulate Cyclin E in macrophages. Moreover, the failure of Batf2 to suppress Cyclin E here, in contrast to previous studies on non-hematopoietic cells, suggests that Batf2 has differential activity in different cell types.

4.3.3.2. Genes regulated by Batf2's dimerization partner cJun

Because Batf2 suppresses cJun activity in some cells and transactivates genes as a heterodimer with another AP-1 member (possibly cJun or JunB) in CD8α^+ and CD103$^+$CD11b$^-$ cDCs, Batf2 could be acting as an inhibitor or an activator in macrophages. That constitutive Batf2 expression alone failed to activate expression of major M1-associated genes was not necessarily surprising given the literature suggesting that Batfs requires cofactors. Batf2's suppressive activity, however, has not been reported to require a cofactor, and forced expression of Batf2 does suppress AP-1 activity in prior studies [95,124]. Therefore, I expected constitutive Batf2 expression to suppress genes downstream of cJun in macrophages. Three genes regulated by cJun in macrophages were specifically tested for induction or suppression by Batf2: Csf1r, IL-1β, and Mmp13.

Behre *et al*. demonstrated that cJun/PU.1 complexes induce M1-associated Csf1r in M1-polarized macrophages[87]. PUER macrophages contain PU.1, so it is reasonable to expect that PU.1/cJun complexes drive Csf1r expression in our cells and that Batf2 might suppress cJun and thus Csf1r expression. However, Batf2 expression did not markedly alter Csf1r expression in M1 PUER macrophages (Figure 4.16 B). Grondin et al. demonstrated that cJun/PU.1 also regulates IL-1β, but in complex with the additional factor C/EBP that is also present in PUER macrophages [80]. Therefore, Batf2 might also suppress IL-1β message expression by suppressing cJun. PUER-Batf2 did not, however, express markedly different levels of IL-1β in the M1 state compared to control cells in the resting MØ state (Figure 4.16 A). Finally, cJun also regulates Mmp13 in macrophages by a mechanism more complex than simple transactivation, described in Ogawa, *et a*l. and Glass, *et al.* For Mmp13 regulation, unphosphorylated cJun (inactive) normally negatively controls Mmp13 through recruitment of the corepressor NCoR1 and subsequent silencing histone modifications. TLR signaling leads to phosphorylated (active) cJun in macrophages[63,98]. Like Csf1r and IL-1β, M1-polarized PUER-Batf2 did not display markedly suppressed Mmp13 expression compared to M1-polarized controls (Figure 4.16 B). Interestingly, PUER-Batf2 cells display markedly increased basal IL-1β and Mmp13. This may provide insight into pathways with which Batf2 interacts, but requires further studies to identify binding partners. With relevance to our study, failure of high constitutive Batf2 mRNA expression to alter these three representative cJun-regulated genes suggests that Batf2 does not alter cJun's activity in M1-polarized macrophages.

4.3.3.3. Genes regulated by Batf2 in compensation of Batf2 deficiency

Batf2 or Batf compensate for Batf3 in CD8α cDCs and the related CD103^{+}CD11b^{-} cDCs by forming complexes with Irf4/Irf8 that transactivate transcription at AP-1-Irf Composite Elements (AICE)[110] (see Figure 2.2, page 48). Irf8 is present in macrophages under all conditions, and Irf4 is present in M2-polarized macrophages (see Irf discussion on page 148 for further analysis, as well as Figure 19 A). Constitutive expression of Batf2 might therefore support M1 polarization by enhancing expression of genes controlled by these Batf-Irf complexes, including *Id2* [142]. Id2 plays a role in fetal liver macrophage differentiation by countering differentiation-promoting Retinoblastoma protein (Rb)[143] and is expressed in PUER macrophages as shown in Figure 4.16 C although its function in polarization has not been described. This figure shows that PUER-Batf2 cells do not show enhanced expression of *Id2* in unpolarized, M1-polarized, or M2-polarized states, suggesting that Batf2 expression alone does not enhance the same genes as Batf-Irf complexes in the cDC subsets. Additionally, although Batf2 cannot compensate for loss of Batf in Tfh cells, macrophages may contain factors necessary for Batf2 and absent from Tfh cells. We therefore tested the Batf-induced T cell gene *Bcl6* [83]. *Bcl6* induction might assist macrophage polarization by promoting the necessary structural changes needed for cytokinesis, cell motility, and other functions[138]. However, high constitutive Batf2 mRNA expression Batf2 expression fails to enhance Bcl6 expression in unpolarized, M1-polarized, or M2-polarized macrophages (Figure 4.16 C). Therefore, Batf2 expression alone in macrophage cells does not regulate genes downstream of Batf2 in CD8α and CD103^{+}CD11b^{-} cDCs, nor does it induce expression of genes controlled by Batf or Batf3 in T cells.

In this survey of select genes, Batf2 did not induce genes regulated by Batf2 in non-hematopoietic cells (Cyclin E), by cJun in non-hematopoietic cells or macrophage cells (Csf1r, IL-1β, and Mmp13), by Batf2 compensationally in CD8α and $CD103^{+}CD11b^{-}$ cDCs (Id2) or Tfh cells (Bcl6). These results suggest that Batf2 regulation differs between cell types and that BAT2 function differs in macrophages from other cell types. However, whether Batf2 functions overlap in macrophages and other cells requires a much more extensive analysis.

4.3.4. Possibility that Batf2 plays a role in growth or survival

Batf2 appears to require a cofactor or condition present in M1-polarized cells to enhance gene expression, but in these cells Batf2 activity from naturally-induced protein may be at maximum. This could account for the lack of changes to the array of M1 genes tested here. Therefore, we attempted to examine genes induced by Batf2 by knocking down Batf2 using small-hairpin RNA (shRNA). Three different shRNA constructs were transduced into PUER cells using the retroviral vector pMSCV. Although all three shRNA constructs were successfully transduced into PUER as well as the control plasmid encoding shRNA against firefly luciferase, two of the resulting shBatf2-transduced cultures displayed normal Batf2 expression and one failed to grow (data not shown). Batf2 mRNA is normally only minimally detectable in healthy PUER cells (see Figure 4.5). However, Batf2 amounts in newly-transduced cultures remain unknown. Batf2 expression may be important for growth and survival shortly after transduction.

4.4. Regulation of Batf2 activity: Requirement for additional M1-associated factors

Based on the survey of gene expression in the previous sections, high constitutive Batf2 mRNA expression alone does not appear to suppress cJun activity (Csf1r, IL-1β, and Mmp13) as in other cells, or enhance transcription at the AICE (Id2) as in subsets of cDCs[143]. This does not rule out a role for Batf2 protein in macrophage polarization, however, given the many layers regulation of gene expression in general and AP-1 activity in particular (Table 4.2). The complex requirements for activity of the AP-1 related transcription factors provide a means of fine control over transcription for any given downstream gene. Some of the mechanisms for regulation include: presence of a positively-regulating dimerization partner (although appropriate cofactors may compensate), absence of any negatively regulating dimerization partners, non-AP-1 cofactors (if necessary), post-translational modifications, mainly phosphorylation but also avoidance of SUMOylation, and redox potential[64–66,77,80,92,93,144,145]. Moreover, gene expression in general requires correct epigenetic landscape through modification of the DNA or chromatin, exemplified by PU.1[10]. If our PUER cells or PUER-Batf2 cells lack activating factors or have an overabundance of repressive factors, then this could prevent us from detecting any changes in genes that are usually regulated by Batf2 *in vivo*.

Using gene expression studies, we began to explore possible restrictions of Batf2 activity by analyzing the presence or absence of factors that interact with AP-1. AP-1 proteins interact with a complex network of factors. These interactions include multiple categories, including dimerization with AP-1 proteins, dimerization with more distantly related bZIP proteins, ternary complex formation with DNA-binding transcription factors, and complex formation with non-DNA binding transcription factors. Our preliminary

study examined select representative genes from two categories: 1) other AP-1 dimerization partners, and 2) ternary complex members.

4.4.3. Other AP-1 members are present and may dimerize with Batf2 or a Batf2 partner

AP-1 family members require dimerization with another AP-1 member which provides an opportunity to regulate DNA binding specificity and activity[63,73,75,80–83]. Some members, such as cJun, recognize the AP-1 consensus elements when in a dimer and contain transactivation domains, and thus more often activate than repress gene expression[79,81,82] . Other members, such as cFos, activate transcription in a heterodimer with a DNA-binding member but cannot bind DNA as a homodimer. Other members, such as JunB, lack a transactivation domain and may decrease activity. Still other members may prevent DNA recognition entirely at some consensus elements. Batf2 itself, for example, prevents cJun from binding to the AP-1 consensus[95,112,124]. Which homo- or hetero-dimers form is in part determined by relative levels of possible partners. Therefore, Batf2 activity in macrophages could be limited by available activating partners or overabundance of repressive partners.

We first looked for the presence or absence of known *in vitro* Batf2 partners cJun and JunB. Multiple studies support Batf2-cJun dimerization[95,112,113,124]. JunB binds Batf2 FRET assays but lacks direct evidence in the cell. Expression of AP-1 family members was analyzed as above by RT-PCR in unpolarized, LPS/IFNγ (M1)-polarized, or IL-4 (M2)-polarized PUER, PUER-pMigR1, and PUER-Batf2. Unlike previous analyses, the important data here is the presence or absence of the binding partner rather than the effect

of Batf2 expression on the relative levels: either the factor is absent and unavailable for Batf2 interaction, or the factor is present and Batf2 could interact. Additionally, gene expression was assessed for M1 or M2 association: an M1-associated gene may be more likely to be the dimerization partner for Batf2. Both cJun and JunB message are detectable in macrophages, but are not associated with M1 polarization at 48 hours (Figure 4.17). Therefore, Batf2 activity in the M1 state does not appear to result from increased Batf2/cJun or Batf2/JunB dimers. However, the activity of these proteins may be regulated in ways not detectable by message analysis. First, they may be bound up by another dimerization partner and thus be unavailable to Batf2. Second, they may be regulated post-transcriptionally. To explore whether cJun or JunB might be dimerizing with another AP-1 member in the M1 state, shifting dimer balance to Batf2/cJun or Batf2/JunB, we tested many other AP-1 members. JunD, Batf, cFos, and Fra2 were all present in macrophages under all conditions at 48 hours, and were not altered by polarization (Figure 4.17). This does not support a role for these factors in macrophage polarization, but they also may be regulated post-transcriptionally or through dimerization with other bZIP proteins. Batf3, on the other hand, is M2-associated. Figure 4.17 shows that Batf3 message is downregulated in M1-polarized cells and upregulated in M2-polarized cells. Fra1, in contrast, was not detected at 48 hours, but is upregulated in M1 polarized macrophages at 8 hours. Batf3 and Fra1, at early time points, may have a role in macrophage polarization. None of the patterns of expression change in PUER-Batf2 cells.

Fra1, which transiently increased in M1-polarized macrophages, binds both cJun and JunB and so could potentially sequester a dimerization partner of Batf2 in PUER-

Batf2, thus limiting activity at early time points. Other interactions may also exist *in vivo* that are not predicted by *in vitro* screening, and a homo- or hetero-dimer with activity in one context may have different activity in another. Therefore, downstream genes were examined as a better correlate of activity. High constitutive Batf2 mRNA expression does not alter Figf/Vegfd, a gene reportedly regulated by Fra1, suggesting no change in Fr-1 activity (Figure 4.18)[146]. Although constitutive Batf2 expression might be predicted to inhibit cJun activity as in other cells, Csf1r , reportedly regulated by PU.1/cJun [87], also did not change, suggesting no significant negative interaction of Batf2 with PU.1-cJun complexes. Interestingly, a cJun-regulated genes IL-1β and Mmp13 appear to be upregulated in M2-polarized PUER-Batf2 (Figure 4.16 B). IL-1β and Mmp13 are not normally expressed simultaneously with Batf2, being M2-associated genes. Future investigation into the pathways by which Batf2 enhances Mmp13 expression under these conditions might be helpful in defining the activity of Batf2.

Overall, analysis of AP-1 factors reveals that only Batf3 and Fra1 message change with polarization. *In vitro* Batf2 binding partners cJun and JunB remain stable, but could interact with Fra1. Figf/Vegfd and Csf1r, reportedly controlled by Fra1 and PU.1-cJun, respectively, remain unaltered by high constitutive Batf2 mRNA expression. This suggests that Batf2 may not interact significantly with these proteins in macrophages. However, Batf2 might interact with cJun/cFos pathways in non-physiologically relevant context, as suggested by derepression of cJun/cJun-repressed Mmp13 in unpolarized macrophages.

4.4.2. Presence of possible ternary cofactors

In addition to dimerization, transcriptional activation by AP-1 at some sites requires ternary complex formation. AP-1 consensus TREs were the first AP-1 target sites to be defined and AP-1 heterodimers alone possess high affinity for these sites; however, this transcriptional repertoire expands when AP-1 dimers complex further with cofactors important for immune cell development and function such as Irf4/8, Ets, nuclear factor of activated T cells (NFAT) family, SMAD family, C/EBPB and PU.1 [62,87]. These complexes expand the transcriptional repertoire to genes with AP-1-Irf composite elements (AICE) Ets-Irf composite elements (EICE), C/EBPB promoter sequences, and PU.1 promoter sequences, respectively. Defining the Batf2 cofactors present in macrophages allows predictions of which ternary complexes form and which genes can be activated by those complexes. Moreover, lack of a Batf2 cofactor might explain the failure of constitutive high Batf2 expression to activate genes.

To begin to explore which cofactors are present in M1-polarized PUER macrophages, we began with expression analysis of the Irf family members. Irf1, 4, and 8 bind to Batf2. Batf2/Irf1 complexes have been tested directly by immunoprecipitation assays in mouse-derived macrophages, while Batf2-Irf4 and Batf2-Irf8 interactions can be implied by its ability to compensate for Batf3 deficiency in CD8α/CD103^{+}CD11b^{-} cDC development *in vivo*, described in Chapter 2, Table B2[1,83]. Moreover, Irf1 is important for macrophage polarization and the induction of inflammatory genes including iNOS, in part by complexing with cJun and NF-κB (see Gunthner *et al.* for a review of Irf functions in macrophages)[1,4,99,147–149]. Irf5 has previously been proposed as a master regulator of M1 polarization, but not reported to interact with AP-1 factors [70]. Irf7

contributes to M1 polarization in microglia[1,150]. Irf2, 3, and 9 were also tested, although there are no reports of these interacting with AP-1 or playing a large role in M1 macrophage polarization. As before, PUER macrophages were differentiated for four days with tamoxifen, then polarized an additional 8 or 48 hours with LPS/ IFNγ to inflammatory/M1 phenotype or with IL-4 to alternative/M2 phenotype or left unstimulated (Figure 4.1). Following polarization, mRNA was extracted and analyzed using semi-quantitative RT-PCR for the presence or absence of the binding partners using specific primers.

The results shown in Figure 20 indicate that Irf4 and Irf8 are not associated with long-term M1 polarization. Irf4 message is strongly associated with M2 polarization in both PUER-Batf2 and control cells at 8 hours and 48 hours (Figure 4.19 A). Irf8, on the other hand, is only minimally expressed in polarized cells. Irf1, in contrast, is highly associated with M1 polarization, confirming previous reports. Irf7 appears to be M1-associated, but in 2 out of 4 experiments PUER cells expressed similar levels of Irf7 mRNA in M1-polarized state as in resting state (data not shown). Unexpectedly, Irf5 was not expressed. Irf2, 3, and 9 expression does not vary markedly between conditions.

This data supports the recent suggestion that Irf1 plays a role in macrophage polarization, suggests Irf7 might also support polarization, and contradicts reports of Irf5's importance for macrophage polarization. Irf1 and Irf7 may both function in part by binding to Batf2, although more study is needed. Moreover, other non-Irf proteins may well serve as cofactors for Batf2. Future protein-protein and protein-DNA binding studies are needed to further define interactions.

4.5. Summary: Batf2 is an M1-associated transcription factor not regulating select genes alone, but requiring additional factors

In this study, we describe Batf2 as an LPS/IFNγ-induced, M1-specific transcription factor with activity likely dependent on additional M1-associated factors or processes. Constitutive high Batf2 expression alone enhanced LPS/IFNγ-induced Cxcl10 message but not protein, and suppressed IL-4-induced Arg1 message. Since AP-1 proteins in general require additional factors for activity, including proper dimerization partners, non-AP-1 cofactors, adequate chromatin modifications, and post-translational modifications, Batf2 also likely requires a necessary cofactor that is limiting Batf2 activity in our cells. An Irf, possibly Irf1, may play this role, especially considering recent reports showing Batf2 interaction with Irf1 in macrophages.

Table 4.1: Microarray analysis of select novel and established M1 or M1 associated genes in polarized PUER macrophages.

A.

Type	Gene	Established or Novel M1 or M2-Associated	Fold change M1/MØ	P[a]
Transcriptional Regulator	Irf1	Established M1[1,109,151,152]	21.8	6.76e-9
	Stat1	Established M1[151,153]	15.4	6.44e-7
	Batf2/SARI	Novel	10.0	8.28e-7
	Ifi204/p204	Novel	8.3	2.67e-7
	Id2	Novel	3.5	6.54e-5
Chemokine	Ccl5/Rantes	Established M1[154,155]	68.8	4.4e-10
	Cxcl10/IP-10	Established M1[44,109,134,151]	101	4.3e-9
	CXCL9	Established M1 [109,151,156]	28.0	6.1e-8
Cytokine	IL-1β	Established M1[151,157–159]	4.5	6.4e-7
Enzyme	iNOS/NOS2	Established M1 [55,57,109,160]	55.4	1.7e-10
T-Cell Ligand	H2-Aa	Conflicting[109,161]	36.6	1.2e-7
Transcriptional Regulators	Hdac9	Novel	0.94	5.9e-3
	ZFP609	Novel	0.96	2.9e-3
	Tcfec/Tfec	Established M2[162]	0.34	2.7e-4
Enzymes	Arg1	Established M2[57,160]	0.99	1.9e-3
	Mmp13/Collagenase 3	Conflicting	1.0	3.0e-4
Mitogen	Figf /Vegfd	Novel	1.1	2.3e-8
Regulator Protein	Myc	EstablishedM2[163]	0.27	9.6e-5
Receptor	Mrc1/Mmr	Established M2[164]	0.11	1.7e-7
Secreted Proteins	Ym1/Chil3	Established M2[160,165,166]	0.71	4.5e-6
	Fizz1/Retnlb	Established M2[160,165]	0.89	2.6e-4

Table 4.1 (continued)
B.

Type	Gene	Established or Novel M1 or M2-Associated	Fold change M2/MØ	P
Transcriptional Regulator	Irf1	Established M1 [1,109,151,152]		6.76e-9
	Stat1	Established M1[151,153]	0.94	6.44e-7
	Batf2/SARI	Novel	0.82	8.28e-7
	Ifi204/p204	Novel	0.84	2.67e-7
	Id2	Novel	1.1	6.54e-5
Chemokine	Ccl5/Rantes	Established M1[154,155]	1.1	4.4e-10
	Cxcl10/IP-10	Established M1[44,109,134,151]	1.1	4.3e-9
	CXCL9	Established M1 [109,151,156]	1.3	6.1e-8
Cytokine	IL-1β	Established M1[151,157–159]	0.74	6.4e-7
Enzyme	iNOS/NOS2	Established M1 [55,57,109,160]	1.2	1.7e-10
T-Cell Ligand	H2-Aa	Mixed[109,161]	1.1	1.2e-7
Transcriptional Regulator	Hdac9	Novel	3.9	5.9e-3
	ZFP609	Novel	2.4	2.9e-3
	Tcfec/Tfec	Established M2[162]	2.0	2.7e-4
Enzymes	Arg1	Established M2[57,160]	1.4	1.9e-3
	Mmp13/Collagenase 3	Mixed	0.79	3.0e-4
Mitogen	Figf /Vegfd	Novel	29.8	2.3e-8
Regulator Protein	Myc	Established M2[163]	1.13	9.6e-5
Receptor	Mrc1Mmr	Established M2[164]	0.42	1.7e-7
Secreted Proteins	Ym1/Chil3	Established M2[160,165,166]	1.65	4.5e-6
	Fizz1/Retnlb	Established M2[160,165]	3.97	2.6e-4

[a]P-value determined by ANOVA for comparison of day 4, MØ, M1, and M2 groups (n=3)

A. M1-polarized gene changes compared to MØ.

B. M2-polarized gene changes compared to MØ.

Table 4.2: Genes tested by microarray and RT-PCR analyses

Gene	Relevance	Microarray M1/MØ[a]	Microarray M2/MØ[b]	RT-PCR M1/ MØ[c]	RT-PCR M2/MØ[d]
Ccne1 (Cyclin E)	Decreased by forced Batf2 in HeLa cells	Var1[e] NC[f] Var2 ND/NC	Var1 NC Var2 ND/NC	NC[e] 8hr ↓[e] 48hr	NC 8hr NC 48hr
Csf1r	M1-associated cytokine receptor Induced by PU.1-AP-1 in macrophages	3.2	0.2	NT 8hr ↑[e] 48hr	NT 8hr NC 48hr
IL-1β	Induced by cJun/PU.1-CEBPB in macrophages	4.5	0.7	NT 8hr ↓/↑[e] 48hr	NT 8hr ↓/ND 48hr
Mmp13	Induced by cJun in macrophages	1.2	5.9	NT 8hr ↓/NC 48hr	NT 8hr ↑ 48hr
Figf	Induced by Fra1	1.1	29.8	NC 8hr ↓/↑ 48hr	↑ 8hr ↑ 48hr
cJun	AP-1 family member, binds Batf2 *in vitro* Batf2 suppresses activity	0.9	0.7	NC 8hr NC 48hr	NC 8hr NC 48hr
JunD	AP-1 family member	NC	0.6	NC 8hr NC 48hr	NC 8hr NC 48hr
JunB	AP-1 family member, binds Batf2 *in vitro*	NC	NC	↑ 8hr NC 48hr	NC 8hr NC 48hr
Fra1	AP-1 family member Induces Figf expression	NC	NC	↑ 8hr ↓/↑ 48hr	↓ 8hr ↓/↑ 48hr
Fra2	AP-1 family member	0.8	0.4	NC 8hr NC 48hr	NC 8hr NC 48hr
cFos	AP-1 family member	0.3	0.2	NC 8hr ↓/↑ 48hr	↓ 8hr ↓/↑ 48hr

Table 4.2 (Continued)

Gene	Relevance	Microarray M1/MØ[a]	Microarray M2/MØ[b]	RT-PCR M1/ MØ[c]	RT-PCR M2/MØ[d]
Arg1	M2-associated enzyme[g] IL-4-induced message suppressed in pMigR1-Batf2	NC/ND	NC/ND	NC 8hr ↓ 48hr	NA 8hr ↑ 48hr
Cxcl10	M1-associated cytokine[g] LPS/IFNγ-induced message enhanced in pMigR1-Batf2	101	NC	NT 8hr ↑ 48hr	NT 8hr ND4 8hr
Batf	Batf family members[76]	NC	NC	NC 8hr NC 48hr	NC 8hr NC 48hr
Batf3		NC/ND	1.4	NT 8hr ND/NC 48hr	NT 8hr ↑ 48hr
Id2	Potential Batf2-inducible genes	3.4	NC	NC 8hr NC 48hr	↑ 8hr NC 48hr
Bcl6		2.8	NC	NT 8hr NC 48hr	NT 8hr NC 48hr
Irf1	M1-associated Irf[g] transcription factors	21.8	NC	↑ 8hr ↑ 48hr	NC 8hr NC 48hr
Irf5	Potential Batf2 cofactors[76,83,86]	ND	ND	ND 8hr ND 48hr	ND 8hr ND 48hr
Irf7		7.7	1.2	↑ 8hr ↑ 48hr	↑ 8hr NC 48hr
Irf9		3.2	0.7	NC 8hr NC 48hr	NC 8hr NC 48hr
Irf4	Known Batf2 cofactors[83,86]	NC/ND	NC/ND	↑ 8hr (minor) ND 48hr	↑ 8hr ↑ 48hr
Irf4		NC/ND	NC/ND	↑ 8hr NC/ND 48 hr	NC/ND 8 hr NC/ND 48 hr
Irf2	Potential Batf2 cofactors[76,83,86]	1.8	0.9	NC 8hr NC 48hr	NC 8hr NC 48hr
Irf3		NC	NC	NC 8hr ↓/↑ 48hr	NC 8hr NC 48hr

Table 4.2 (continued)

Gene	Relevance	Microarray M1/MØ[a]	Microarray M2/MØ[b]	RT-PCR M1/ MØ[c]	RT-PCR M2/MØ[d]
Ccl5	M1 or inflammatory-stimulus associated cytokine, established or tentative[g]	68.1	1.1	NT 8hr ↑ 48hr	NT8hr NC 48hr
H2-Aa		36.6	1.1	NT 8hr ↑ 48hr	NT 8hr NC 48hr
Id2		3.4	NC	NT 8hr ↑ 48hr	NT 8hr NC 48hr
Ifi204		8.3	0.8	NT 8hr ↑ 48hr	NT 8hr NC 48hr
Ifi205		8.8	NC	NT 8hr ↑ 48hr	NT 8hr NC 48hr
iNOS		5.4	1.2	NT 8hr ↑ 48hr	NT 8hr ND 48hr
Tgtp		97.8	1.2	NT 8hr ↑ 48hr	NT 8hr NC 48hr
Parp12		7.8	0.9	NT 8hr NC 48hr	NT 8hr NC 48hr
Parp14		12.8	NC	NT 8hr NC 48hr	NT 8hr NC 48hr
Stat1		12.7	9.8	NT 8hr NC 48hr	NT 8hr NC 48hr
Stat2		6.6	0.9	NT 8hr NC 48hr	NT8hr NC 48hr

Table 4.2 (continued)

Gene	Relevance	Microarray M1/MØ[a]	Microarray M2/MØ[b]	PUER M1/ MØ[c]	PUER M2/MØ[d]
Fizz1	M2-associated proteins, established or tentative[g]	0.9	4.0	NC 8hr ↓ or ND 48hr	↑ 8hr ↑ 48hr
Hdac9		0.9	3.2	NT 8hr ↓ 48hr	NT 8hr ↑ or NC 48hr
Mmp13		1.2	5.9	NT 8hr ↓ or NC 48hr	NT 8hr ↑ 48hr
Mrc1		0.1	0.4	NT 8hr ↓ 48hr	NT 8hr NC 48hr
Prp		1.1	1.6	NT 8hr ↓/↑ 48hr	NT 8hr ↓/↑ 48hr
Tcfec		0.3	2.0	NT 8hr ↓ 48hr	NT 8hr NC 48hr
Tgfb1		NC	NC	NT 8hr NC 48hr	NT 8hr NC 48hr
Ym1		0.7	1.6	NT 8hr ↓ or NC 48hr	NT 8hr ↓ or NC 48hr

[a]M1/MØ for microarray data: Fold change in signal of M1 over MØ 48 hours after polarization

[b]M2/MØ for microarray data: Fold change in signal of M2 over MØ 48 hours after polarization

[c]M1/MØ for RT-PCR data: Direction of change in signal from MØ to M1 8 or 48 hours after polarization

[d]M2/MØ for RT-PCR data: Direction of change in signal from MØ to M2 8 or 48 hours after polarization

[e]Cyclin E has two variants

[f]NC=No change (includes slight changes that not clearly significant); ND=Not detected; NT=Not Tested; ↑=Increased; ↓=Decreased; ↓/↑ = Variable across multiple experiments and cell types

[g] References for established M1/M2 markers or novel M1/M2-associated genes are included in Table 4.1

Figure 4.1: Schematic of macrophage differentiation and polarization.

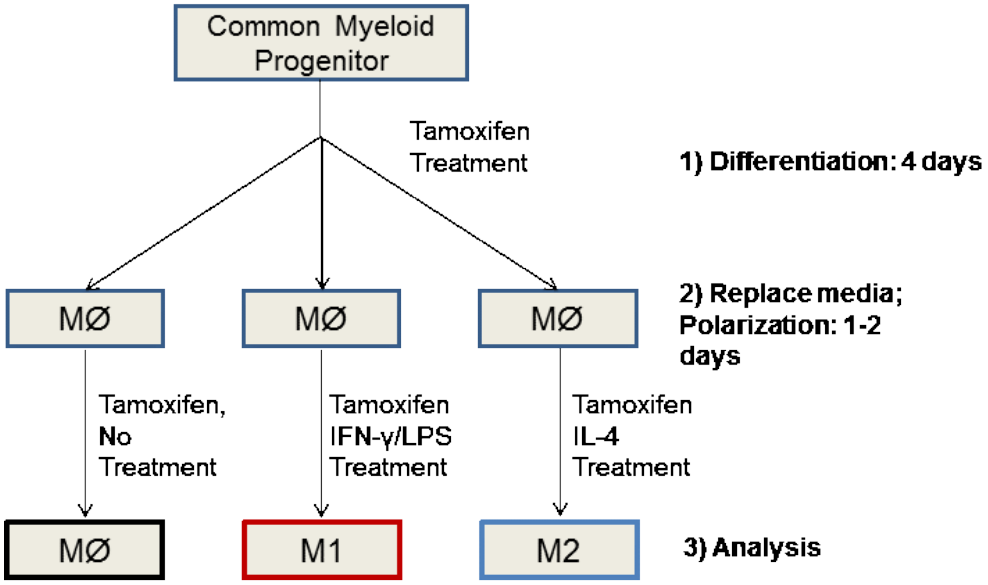
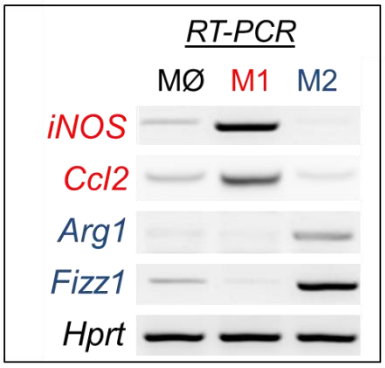


Figure 4.1: Schematic of macrophage differentiation and polarization. To differentiate the PUER cells to macrophages, PUER were seeded at $2x10^5$-$4x10^5$ cells per cm^2 in polystyrene tissue culture flasks and tamoxifen was added to media supplemented with 5ng/ml IL-3 for 4 days. On day 4 cells were treated with fresh control media (M0 group) or fresh media with cytokines for an additional 48 hours, or at times indicated in individual experiments. In some cases, experiments lasting 24 hours or less did not receive fresh media in order to minimize the short-term effects of adding fresh media. To polarize cells to M1 phenotype, 20 ng/ml IFNγ and 100 ng/ml LPS were added. To polarize cells to M2, 4 ng/ml IL-4 was added. Cells were analyzed after 2 days, or as otherwise described.

Figure 4.2: PUER macrophages polarize to M1 or M2.

A.



B.

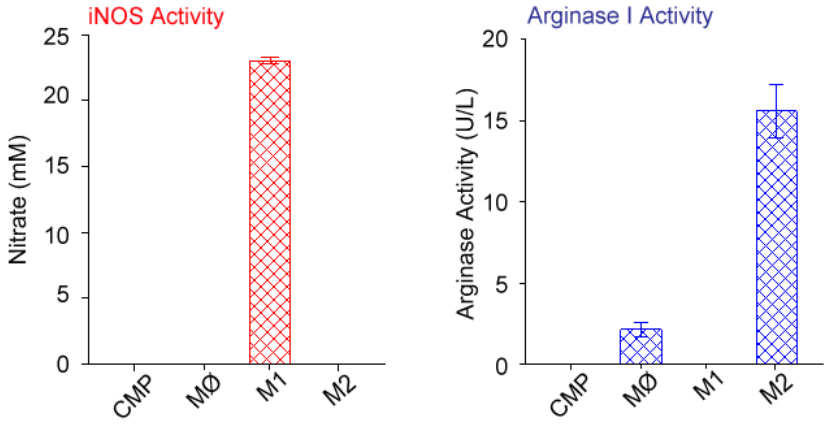


Figure 4.2: PUER macrophages polarize to M1 or M2. M1/M2 polarization of PUER macrophages was confirmed by expression of M1 and M2 specific genes and enzyme activities. Briefly, PUER cells at the common myeloid progenitor stage (CMP) or differentiated to macrophages and polarized by treatment with 100 ng/ml LPS and 20 ng/ml IFNγ (M1) or 4 ng/ml IL-4 (M2) for 48 hours, or left unstimulated (MØ), were analyzed as described in Chapter 3. **A.** Expression of M1 and M2 specific genes was measured by RT-PCR. Total cellular RNA was isolated 48 hours after treatment, reverse transcribed and specific genes for M1 (iNOS and Ccl2) or M2 (Arg1 and Fizz) were amplified by PCR using the primers listed in the materials and methods. The PCR

products were separated on agarose gels, stained with ethidium bromide and photographed. The images were inverted for clarity. LPS/IFNγ or IL-4 treatment resulted in the expected upregulation of M1 specific genes iNOS and Ccl2 and M2 specific genes Fizz1 and Arg1. **B.** Enzyme activities of the M1 specific enzyme iNOS or M2 specific enzyme Arginase 1 were measured in PUER cells 24 hour after treatment with LPS/IFNγ or IL-4. For iNOS activity, nitrate concentration in the culture supernatants was measured using the Griess reagent, which measures nitrate in the supernatant. For Arginase 1 activity cell extracts were prepared and arginase activity was measured by urea production, a product of arginase activity. LPS/IFNγ treatment caused an approximately 23 fold selective upregulation of iNOS activity, whereas IL-4 treatment selectively upregulated arginase activity by about 6 fold.

Figure 4.3: PUER macrophage polarization is reversible.

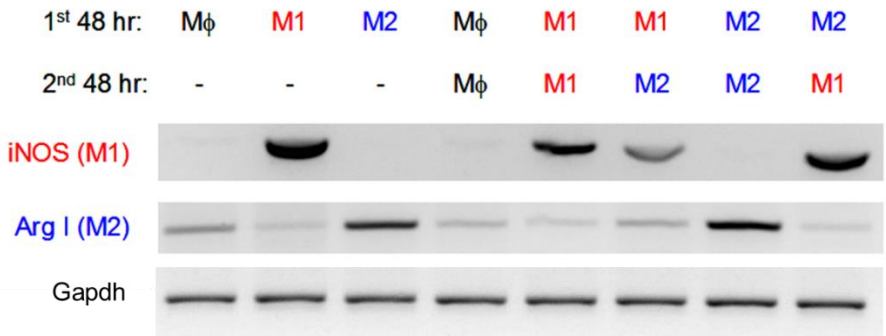


Figure 4.3: PUER macrophage polarization is reversible. PUER cell re-polarization after initial polarization and repolarization was measured by gene expression of iNOS or Arg1. 100 ng/ml LPS and 20 ng/ml IFNγ (M1) or 4 ng/ml IL-4 (M2) for 48 hours, or left unstimulated (MØ), as described in Chapter 3. For baseline comparison, cells in the first three lanes were polarized for 48 hrs as in Figure 4.2, RNA was extracted and iNOS and Arg1 expression were detected by RT-PCR. The products were separated on agarose gels, stained with ethidium bromide and photographed. The images have been inverted for clarity. The cells in lanes 4 to 8 were polarized for an additional 48 hours with the same stimuli (lanes 4, 5 &7) or the opposing stimuli (lanes 6 & 8). iNOS and Arg1 expression in PUER cells repolarized from M1 to M2 or M2 to M1 shows an almost complete reversal for M2 to M1 and a partial reversal of M1 to M2. *(credit: B. Taylor and J. McGillis)*

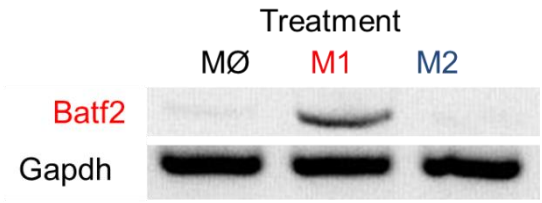
Figure 4.4: Transcription factors upregulated in M1 or M2 polarized PUER macrophages.

Microarray *Up regulated Transcriptional Regulators*			
M1		**M2**	
(23 total)	*vs. C*	*(5 total)*	*vs. C*
Irf1	21.8	Hdac9	3.9
Stat1	15.4	ZFP609	2.4
Batf2	10.0	Tcfec	2.0
Ifi204	8.3		
Id2	3.5		

Figure 4.4: Transcription factors upregulated in M1 or M2 polarized PUER macrophages. Transcription factors upregulated in M1 or M2 polarized macrophages were identified by microarray analysis. Cells were treated with100 ng/ml LPS and 20 ng/ml IFNγ (M1) or 4 ng/ml IL-4 (M2) for 48 hours, or left unstimulated (MØ), as described in Chapter 3. At 48 hours, mRNA was extracted and a microarray analysis performed in the UK Microarray Core Facility using the GeneChip® Mouse Exon 1.0 ST Array. Transcription factors upregulated or downregulated more than two fold were selected. Numbers indicate fold increase compared to unstimulated. Irf1 and Stat1 were previously reported to be upregulated in M1 and Tcfec found to be upregulated in M2. Batf2, Ifi204, and Id2 are novel transcription factors in M1 polarized macrophages. Hdac9 and ZFP609 are novel transcription factors in M2 polarized macrophages. A full list of genes in the microarray is found in Table 4.1.

Figure 4.5: Classical activation upregulates Batf2 mRNA and protein.

A.



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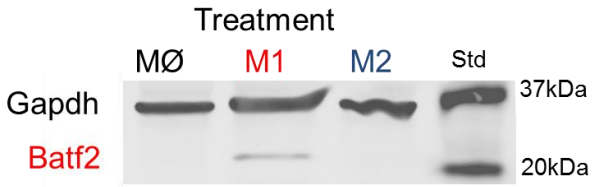


Figure 4.5: Classical activation upregulates Batf2 mRNA and protein. Batf2 mRNA (A) and protein (B) were confirmed in PUER macrophages by RT-PCR (A) and Western blot (B). PUER cells were polarized with 100 ng/ml LPS and 20 ng/ml IFNγ (M1) or 4 ng/ml IL-4 (M2) for 48 hours, or left unstimulated (MØ), then analyzed as described in Chapter 3. **A.** Total RNA was extracted and analyzed using RT-PCR with specific primers. The products were separated on agarose gels, stained with ethidium bromide and photographed. The images have been inverted for clarity. **B.** Total protein extracts were analyzed by Western blot with fluorescence detection methods. Standards denoting 37 kDa and 20 kDa are marked on the right, and Batf2 and Gapdh bands are marked on the left. Batf2 expression is unique to M1-polarized macrophages.

Figure 4.6: Kinetics of Batf2 induction by LPS/IFNγ.

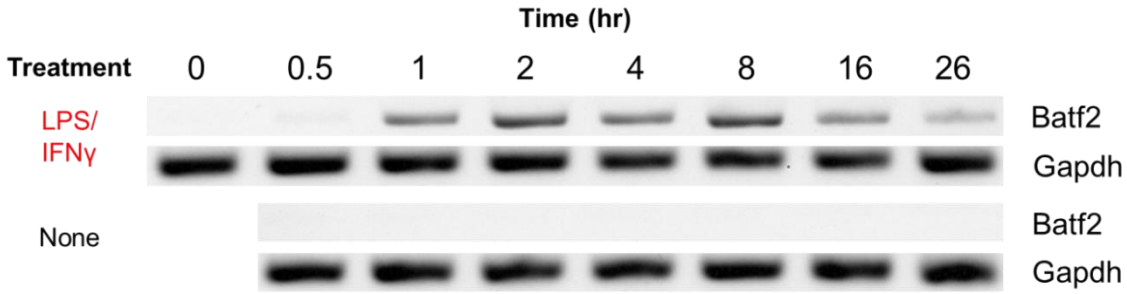


Figure 4.6: Kinetics of Batf2 induction by LPS/IFNγ. Batf2 mRNA at various time points was confirmed in PUER macrophages by RT-PCR. PUER cells were polarized with 100 ng/ml LPS and 20 ng/ml IFNγ (M1) or 4 ng/ml IL-4 (M2) for 48 hours, or left unstimulated (MØ), then analyzed as described in Chapter 3. Total RNA was extracted and analyzed using RT-PCR with specific primers. The products were separated on agarose gels, stained with ethidium bromide and photographed. The images have been inverted for clarity. Batf2 message appears 1 hour after polarization and seems to peak around 8 hours. Marked expression above baseline is shown here up to 26 hours.

Figure 4.7: Classical activation upregulates Batf2 mRNA in BMDM.

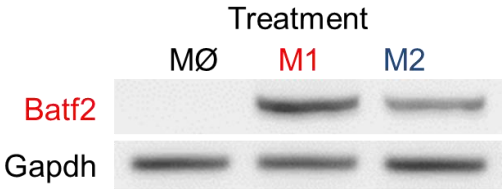
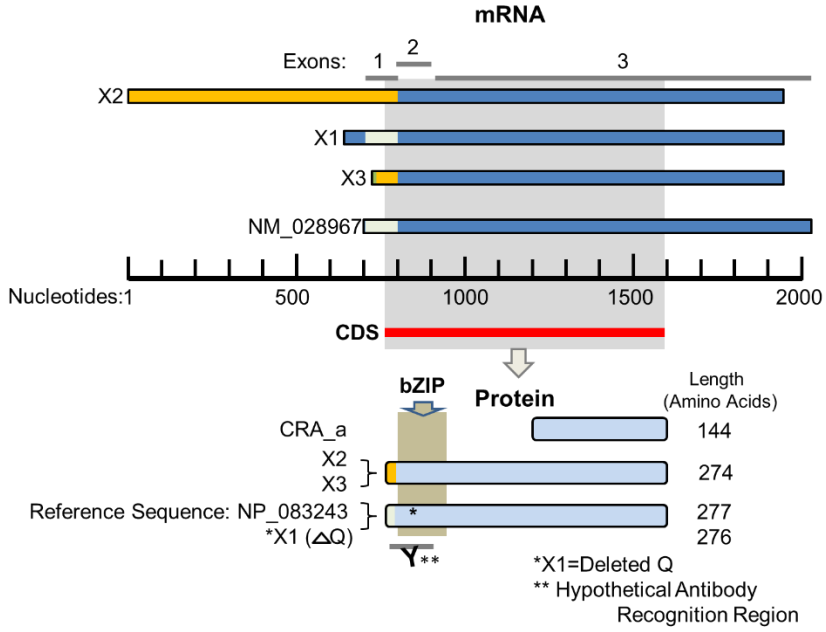


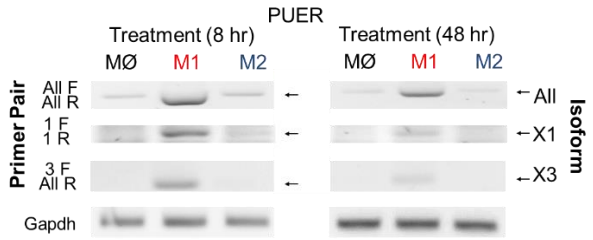
Figure 4.7: Classical activation upregulates Batf2 mRNA in BMDM. Batf2 expression in bone marrow derived macrophages (BMDM) was determined by RT-PCR. BMDM were polarized with 100 ng/ml LPS and 20 ng/ml IFNγ (M1) or 4 ng/ml IL-4 (M2) for 48 hours, or left unstimulated (MØ), then analyzed as described in Chapter 3. Total RNA was extracted and analyzed using RT-PCR with specific primers. The products were separated on agarose gels, stained with ethidium bromide and photographed. The images have been inverted for clarity. Both M1 and M2 polarization induced Batf2 expression; however, Batf2 expression in M2 polarized cells did not reach the amounts in M1 polarized cells. Moreover, additional gene analysis suggested that BMDMs were partially M1 polarized, even after M2 stimulus (data not shown). *(Credit: R. Hayman IV and J. McGillis)*.

Figure 4.8: Classical activation upregulates multiple Batf2 mRNA isoforms.

A.



B.



C.

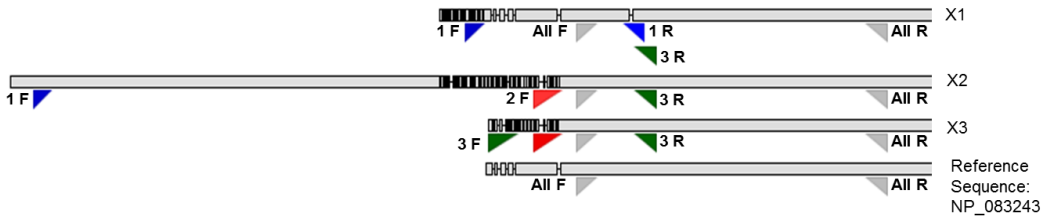


Figure 4.8: Classical macrophage activation upregulates multiple Batf2 mRNA isoforms.

A. Known and predicted mRNA isoforms and protein variants in the National Center for Biotechnology (NCBI) database. Four mRNA isoforms are predicted to exist for Batf2: the reference sequence NM_028967, isoform X1, isoform X2, and isoform X3. Colors of the RNA correspond to sequence similarities (for example, the dark blue is shared by all

isoforms). These translate into four protein variants: the reference sequence, variant X1 (which differs from the reference sequence by a glutamine residue in the bZIP region), variant X2, and variant X3. The isoform specificity of the antibody used in Figure 4.5 is unknown, but was raised to the indicated region (**) that is conserved in all full-length products with 94% identity. **B**. Detection of Batf2 isoforms X1 and X3. PUER cells were polarized with 100 ng/ml LPS and 20 ng/ml IFNγ (M1) or 4 ng/ml IL-4 (M2) for 8 or 48 hours, or left unstimulated (MØ), then analyzed as described in Chapter 3. Total RNA was extracted and analyzed using RT-PCR with isoform-specific primers. The products were separated on agarose gels, stained with ethidium bromide and photographed. The images have been inverted for clarity. Isoforms were confirmed by sequencing (data not shown). The product indicated as "X3" could be produced from both X2 and X3; however, reactions using primers "1 F" and "All R" yielded no product the length of isoform X2. **C**. Alignment of primers to the three mRNA isoforms detected in M1-polarized PUER cells: X1, X2, X3, and the reference sequence. Dark areas indicate regions of variation between isoforms. Triangles indicate the primers used to detect the isoforms and correspond to the primers on the left in (B).

Figure 4.9: IFNγ, but not LPS, induces Batf2 expression

A. B.

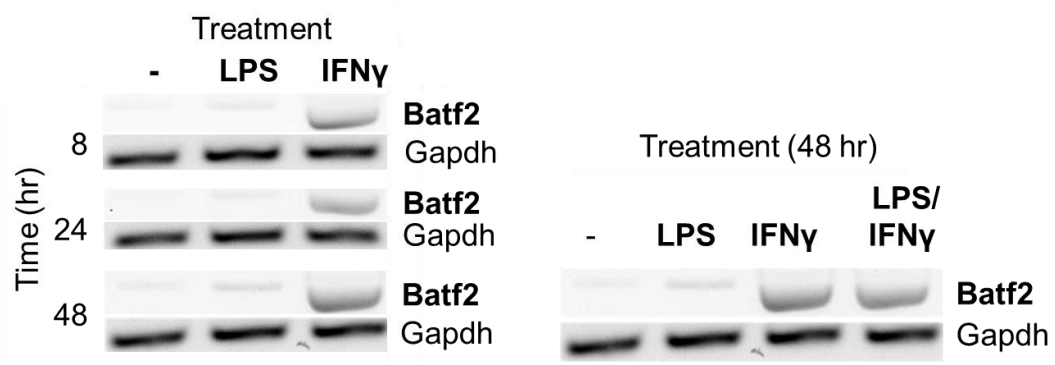


Figure 4.9: IFNγ, but not LPS, induces Batf2 expression. Batf2 expression in response to LPS or IFNγ alone (A) or compared to LPS/IFNγ combination (B). PUER cells were polarized with 100 ng/ml LPS and/or 20 ng/ml IFNγ for 8, 24, or 48 hours, or left unstimulated (MØ), then analyzed as described in Chapter 3. Total mRNA was extracted and analyzed using RT-PCR with specific primers. The products were separated on agarose gels, stained with ethidium bromide and photographed. The images have been inverted for clarity. Unstimulated and LPS-stimulated cells had minimal Batf2 mRNA, while cells treated with IFNγ by itself expressed Batf2 at roughly comparable levels to cells treated with LPS and/IFNγ.

Figure 4.10: Both LPS/IFNγ and IL-10 induce Batf2 expression.

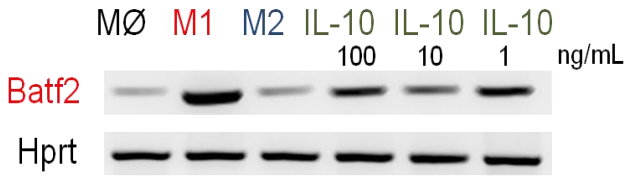


Figure 4.10: Both LPS/IFNγ and IL-10 induce Batf2 expression. IL-10-induced Batf2 expression *vs* LPS/IFNγ-induced Batf2 expression. PUER cells were polarized with 100 ng/ml LPS and 20 ng/ml IFNγ (M1), 4 ng/ml IL-4 (M2), or IL-10 at the listed concentrations for 48 hours, or left unstimulated (MØ), then analyzed as described in Chapter 3. Total RNA was extracted and analyzed using RT-PCR with specific primers. The products were separated on agarose gels, stained with ethidium bromide and photographed. The images have been inverted for clarity. Concentrations of IL-10 from 1-100 ng/ml induce Batf2 expression.

Figure 4.11: Induction of select M1-associated genes occurs subsequent to Batf2 induction.

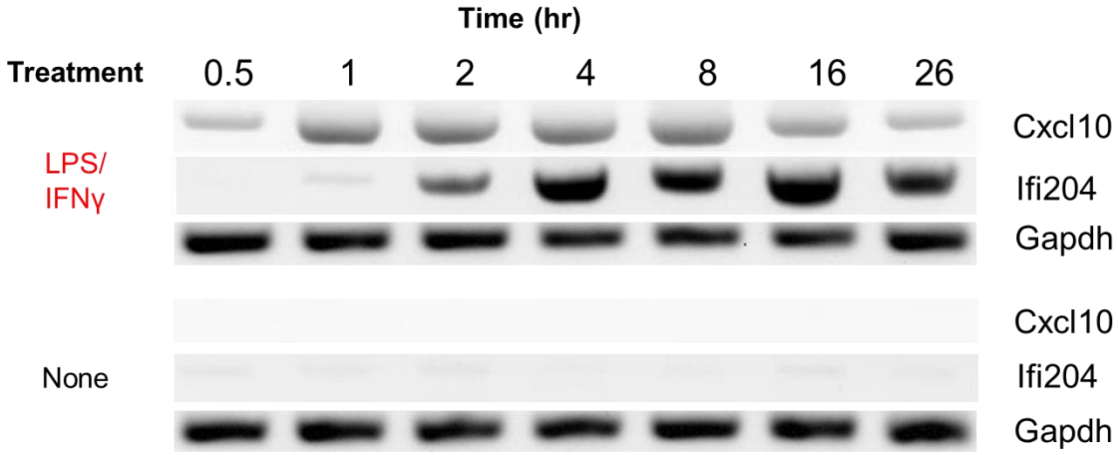
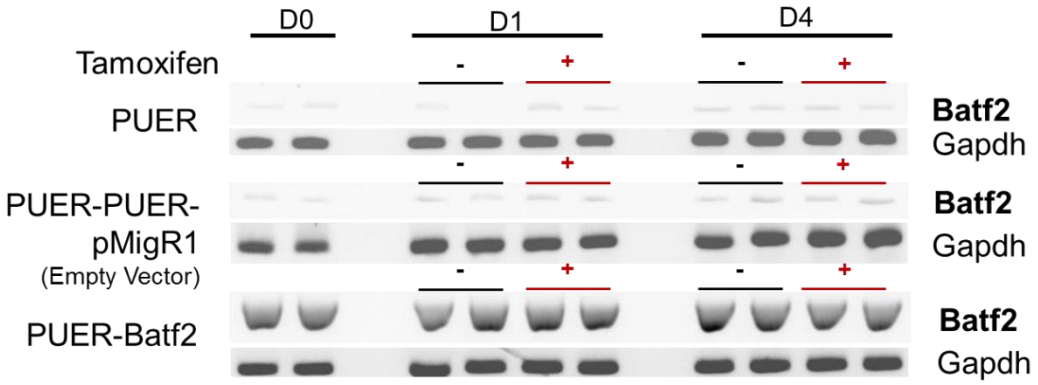


Figure 4.11: Induction of select M1-associated genes occurs subsequent to Batf2 induction. Two M1-associated genes, Cxcl10 and Ifi204, increase markedly subsequent to Batf2 induction. PUER cells were polarized with 100 ng/ml LPS and 20 ng/ml IFNγ (M1) or 4 ng/ml IL-4 (M2) for 48 hours, or left unstimulated (MØ), then analyzed as described in Chapter 3. Total RNA was extracted and analyzed using RT-PCR with specific primers. The products were separated on agarose gels, stained with ethidium bromide and photographed. The images have been inverted for clarity. Chemokine Cxcl10 and transcription factor Ifi204 are induced by IFNγ and LPS in macrophages with slightly different kinetics.

Figure 4.12: PUER-Batf2 constitutively express Batf2 mRNA.

A.



B.

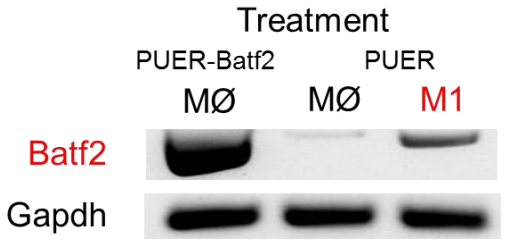


Figure 4.12: PUER-Batf2 cells constitutively express Batf2 mRNA. PUER cells were transduced with retroviral vector pMigr1-Batf2 to create cells that constitutively express Batf2 (PUER-Batf2).To control for off-target effects due to the retroviral vector, PUER cells were also transduced with the empty vector pMigr1 (PUER-pMigR1). Batf2 and Gapdh expression were also analyzed as described in Chapter 3. Total RNA was extracted and analyzed using RT-PCR with specific primers. The products were separated on agarose gels, stained with ethidium bromide and photographed. The images have been inverted for clarity. **A.** PUER, PUER-pMigR1, and PUER- Batf2 were analyzed at common myeloid progenitor stage (D0) or differentiated to resting macrophages with tamoxifen for the 1 or 4 days. In contrast to unpolarized PUER cells

or macrophages that express little Batf2 message in resting state, PUER-Batf2 constitutively express abundant Batf2 message at all stages. **B.** PUER-Batf2 comparison to resting or M1-polarized PUER. Batf2 message is highly expressed even compared to M1-polarized PUER levels.

Figure 4.13: PUER-Batf2 cells polarize to M1 phenotype after LPS/IFNγ.

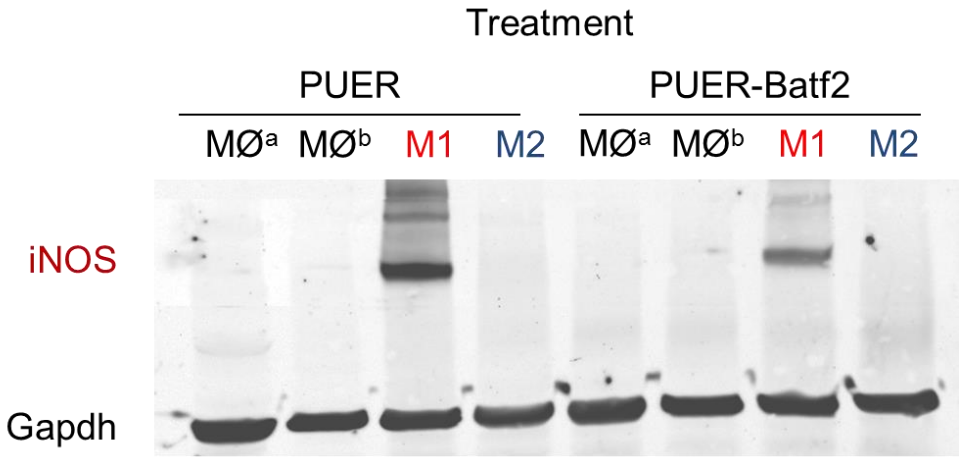
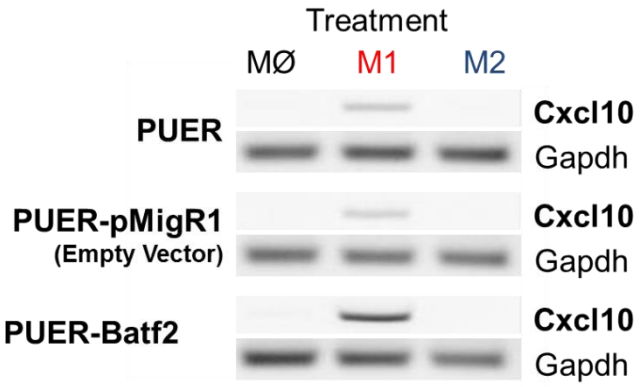


Figure 4.13: PUER-Batf2 cells polarize to M1 phenotype after LPS/IFNγ. M1 polarization was verified by detection of the M1-associated iNOS protein. PUER cells and Batf2-transduced cells (PUER-Batf2) were analyzed in cells differentiated for 4 days and analyzed immediately (MØ[a]) or differentiated to macrophages with tamoxifen treatment and then were polarized with 100 ng/ml LPS and 20 ng/ml IFNγ (M1) or 4 ng/ml IL-4 (M2) for 48 hours, or left unstimulated (MØ[b]). iNOS protein in cell extracts was analyzed by Western blot with fluorescence detection as described in Chapter 3. Gapdh was used as a loading control. M1-polarized PUER and PUER-Batf2 produce iNOS, indicating proper M1 polarization.

Figure 4.14: Constitutive expression of Batf2 enhances LPS/IFNγ-induced Cxcl10 gene mRNA expression.

A.



B.

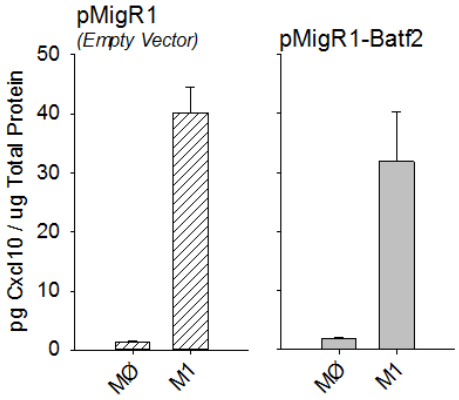


Figure 4.14: Constitutive expression of Batf2 enhances LPS/IFNγ-induced Cxcl10 mRNA expression. M1-associated chemokine Cxcl10 mRNA and protein in macrophages constitutively expressing Batf2 compared to controls was detected by RT-PCR and Western blot, respectively. **A.** Cxcl10 mRNA. PUER cells, vector-transduced cells (PUER-pMigR1) and Batf2-transduced cells (PUER-Batf2) were polarized with 100 ng/ml LPS and 20 ng/ml IFNγ (M1) or 4 ng/ml IL-4 (M2) for 48 hours, or left

unstimulated (MØ), then analyzed as described in Chapter 3. Total RNA was extracted and analyzed using RT-PCR with specific primers. Gapdh was included as a loading control. The products were separated on agarose gels, stained with ethidium bromide and photographed. The images have been inverted for clarity. Similar results were observed in 3 out of 4 independent experiments. While there was a clear trend, the enhancement of Cxcl10 message in PUER-Batf2 M1-polarized macrophages from these three experiments pooled was not significant after quantitation with Image J due to high standard deviation. **B.** Cxcl10 protein. Vector-transduced cells (PUER-pMigR1) and Batf2-transduced cells (PUER-Batf2) were polarized for 24 hours with 100 ng/ml LPS and 20 ng/ml IFNγ or left unstimulated (MØ), then Cxcl10 levels in the medium were analyzed as described in Chapter 3. Supernatant was removed for Cxcl10 analysis and the cells were harvested for total protein quantification for normalization. Cxcl10 protein in supernatant was assayed by ELISA, and total protein was extracted from cell fraction and used as a correlate of cell number (see Chapter 3) for the correlation of protein and cell number). The difference between PUER-MigR1 and PUER-Batf2 is not statistically significant ($p=0.204$) demonstrating that Batf2-enhanced LPS/IFNγ-induced Cxcl10 mRNA does not translate into enhancement of Cxcl10 protein secretion.

Figure 4.15: Constitutive expression of Batf2 downregulates IL-4 induced Arg1 mRNA.

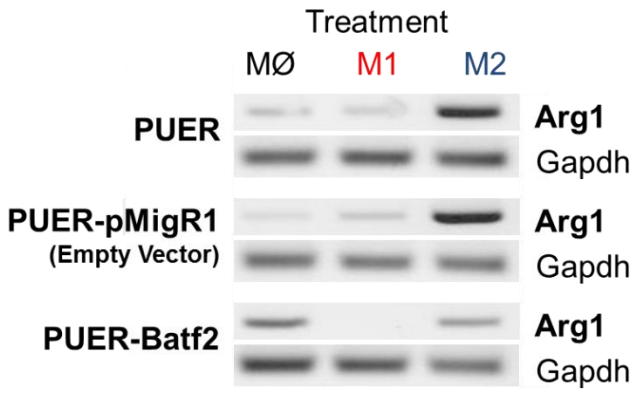


Figure 4.15: Constitutive expression of Batf2 downregulates IL-4 induced Arg1 mRNA. Arginase 1 (Arg1) in macrophages constitutively expressing Batf2 compared to controls was determined by RT-PCR. PUER cells, vector-transduced cells (PUER-pMigR1) and Batf2-transduced cells (PUER-Batf2) were polarized with 100 ng/ml LPS and 20 ng/ml IFNγ (M1) or 4 ng/ml IL-4 (M2) for 48 hours, or left unstimulated (MØ), then analyzed as described in Chapter 3. Total RNA was extracted and analyzed using RT-PCR with specific primers. Gapdh was analyzed as a loading control. The products were separated on agarose gels, stained with ethidium bromide and photographed. The images have been inverted for clarity. Similar suppression of Arg1 expression compared to unpolarized macrophages was observed in 3 out of 4 independent experiments. PUER-Batf2 cells suppress Batf2-induced Arg1 expression relative to PUER and PUER-pMigR1. While there was a clear trend, the suppression by Batf2 from these three experiments combined was not significant after quantitation with ImageJ due to high standard deviation.

Figure 4.16: High constitutive Batf2 mRNA expression does not alter known or potential genes downstream of Batf2 in macrophages.

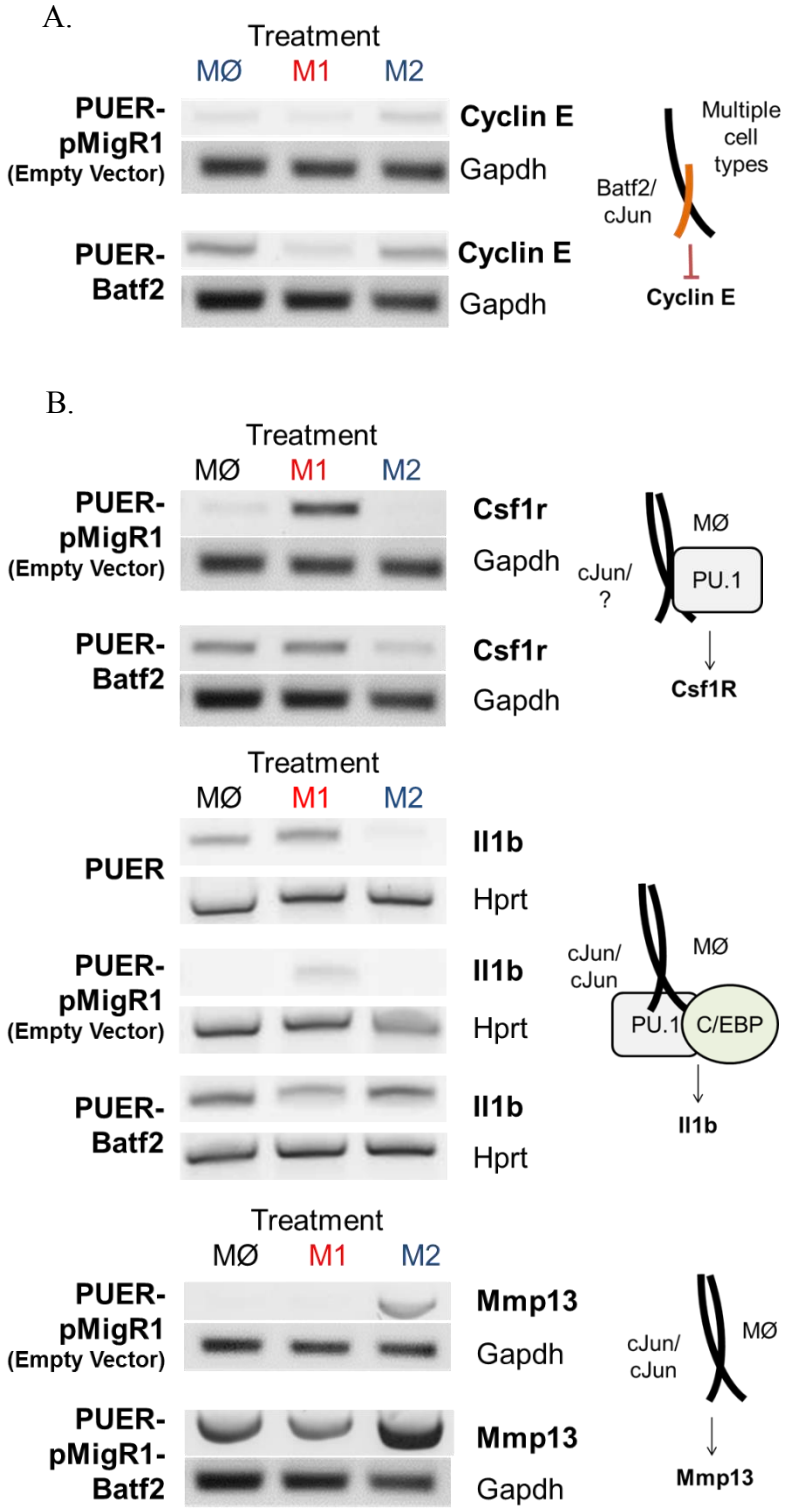


Figure 4.16 (continued)

C.

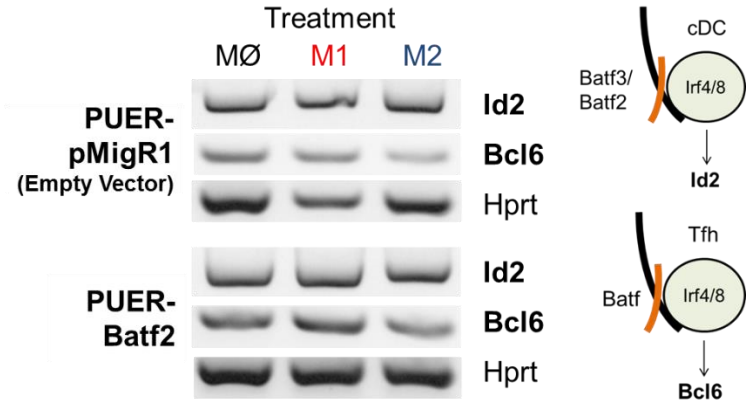
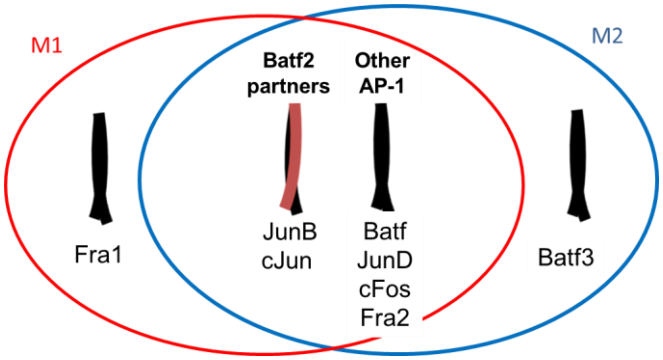


Figure 4.16: High constitutive Batf2 mRNA expression does not alter known or potential genes downstream of Batf2 in macrophages. PUER cells, vector-transduced cells (PUER-pMigR1) and Batf2-transduced cells (PUER-Batf2) were polarized with 100 ng/ml LPS and 20 ng/ml IFNγ (M1) or 4 ng/ml IL-4 (M2) for 48 hours, or left unstimulated (MØ), then analyzed as described in Chapter 3. Total RNA was extracted and analyzed using RT-PCR with specific primers. Either Gapdh or Hprt were included as a loading control. The products were separated on agarose gels, stained with ethidium bromide and photographed. The images have been inverted for clarity. **A**. Expression of cJun-suppressible Ccne1 (Cyclin E). Batf2 suppression of cJun suppresses expression of expression of Ccne1 (encoding the Cyclin E cell cycle protein) in multiple cell types. Expression in PUER-Batf2 cells compares to control cells, suggesting Batf2 does not interfere with cJun-driven gene expression. Lack of induction in PUER-Batf2 was observed in 2 out of 2 independent experiments. **B.** Expression of cJun-inducible Csf1r, Il1b, and Mmp1.3 Csf1r, Il1b, and Mmp13 are induced in macrophages by cJun and the cofactors pictured to the right of the gel. cJun homodimer induces Il1b in conjunction

with macrophage transcription factors PU.1 and C/EBP, cJun and an undefined AP-1 dimerization partner to induce Csf1r in conjunction with PU.1. Phosphorylated cJun/cFos activates Mmp13 transcription while unphosphorylated cJun/cJun represses Mmp13. Basal expression of Mmp13 is increased in PUER-Batf2 cells but not enhanced by LPS/IFNγ treatment, suggesting Batf2 does not enhance these genes in M1-polarized macrophages. Lack of suppression in PUER-Batf2 was observed in 3 out of 3 experiments for Csf1r and Mmp13, and 2 out of 2 experiments for Il1b. **C.** Expression of Batf2-inducible Id2**.** Id2 is induced by forced Batf2 in compensation for a deficiency of Batf3 in cDC subsets. Bcl6 is induced by Batf in Tfh cells, but Batf2 cannot compensate in these cells. PUER-Batf2 express Id2 and Bcl6 comparably in PUER-Batf2 and controls, suggesting Batf2 activity differs in macrophages and the cDC subsets and that Batf2 does not have similar activity in macrophages as Batf3 or Batf in other cells. Lack of enhancement in PUER-Batf2 relative to controls was observed in 3 out of 4 experiments for Id2 and 2 out of 2 experiments for Bcl6.

Figure 4.17: Dimerization partners are present in PUER cells.

A.



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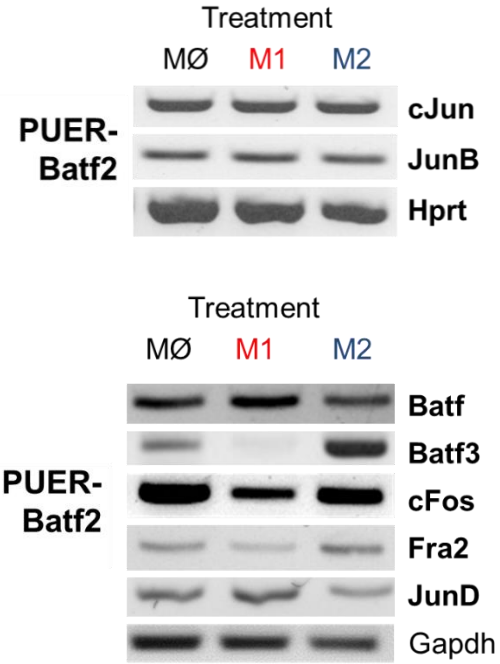


C.

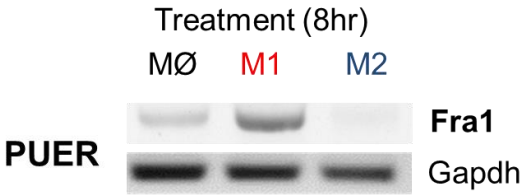


Figure 4.17: Dimerization partners are present in PUER cells. Presence of mRNA for other AP-1 proteins was determined by RT-PCR. PUER cells, vector-transduced cells and Batf2-transduced cells (PUER-Batf2) were polarized with 100 ng/ml LPS and 20 ng/ml IFNγ (M1) or 4 ng/ml IL-4 (M2) for 48 hours, or left unstimulated (MØ), then analyzed as described in Chapter 3. Total RNA was extracted and analyzed using RT-PCR with specific primers. Either Gapdh or Hprt was included as a loading control. The products were separated on agarose gels, stained with ethidium bromide and photographed. The images have been inverted for clarity. One representative experiment out of three to four (for the 48-hour polarizations) or two (for 8 hour polarization) is shown for clarity. **A.** Summary of the representation of AP-1 proteins in PUER macrophages, determined by RT-PCR at 8 and 48 hours. cJun dimerizes with Batf2 functionally in vivo, and JunB dimerizes with Batf2 in fluorescence resonance energy transfer (FRET) assays by Reinke *et al.*[113]. The remaining AP-1 proteins did not bind to Batf2 in FRET assays. **B.** Batf**,** cFos**,** Fra2**,** and JunD are not associated with any polarization state. Batf3 is associated with M2 polarized cells during sustained M2 polarization. The presence of the AP-1 factors leave open the possibility that Batf2 interacts with their activity either directly by dimerization with them or indirectly by sequestering a binding partner. **C.** Fra1 is associated with M1-polarized cells at 8 hours but not during long-term maintenance of polarization (data not shown). This suggests Fra1 may be involved with early M1 polarization, either in conjunction with Batf2 or alone, but does not necessarily suggest involvement during sustained polarization. It is worth noting that AP-1 proteins are highly regulated by post-translational modifications and therefore activity may not correlate to mRNA expression.

Figure 4.18: Constitutive Batf2 expression does not appear to interfere with Fra1 signaling.

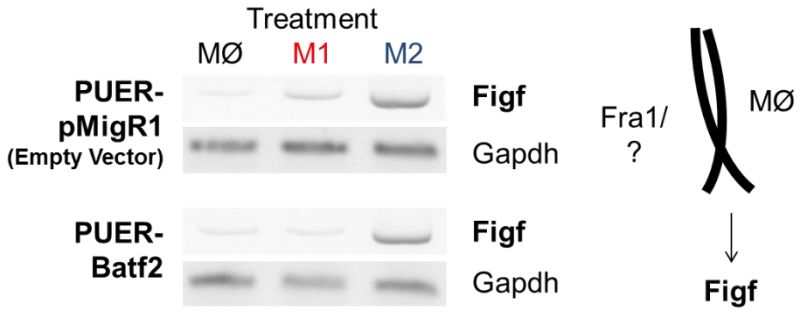
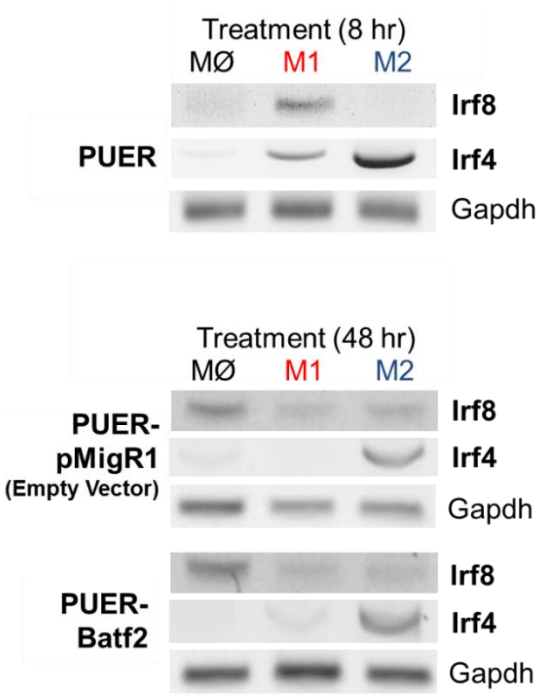


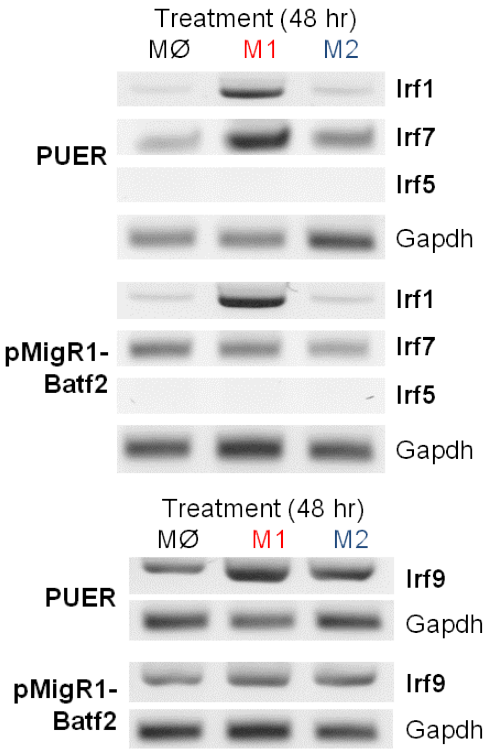
Figure 4.18: High constitutive Batf2 mRNA expression does not appear to interfere with Fra1 signaling. The Fra1-inducible gene Figf was detected in PUER cells constitutively expressing Batf2 compared to controls by RT-PCR. PUER cells, vector-transduced cells (PUER-pMigR1) and Batf2-transduced cells (PUER-Batf2) were polarized with 100 ng/ml LPS and 20 ng/ml IFNγ (M1) or 4 ng/ml IL-4 (M2) for 48 hours, or left unstimulated (MØ), then analyzed as described in Chapter 3. Total RNA was extracted and analyzed using RT-PCR with specific primers. Gapdh was included as a loading control. The products were separated on agarose gels, stained with ethidium bromide and photographed. The images have been inverted for clarity. Similar results were observed in 3 independent experiments. Figf is induced by Fra1 in macrophage cells, and Fra1 dimerizes with Batf2 *in vitro*. Figf expression was not suppressed in PUER-Batf2 cells that highly express Batf2 constitutively compared to controls in 3 out of 3 experiments, suggesting Batf2 does not interfere with Fra1 activity.

Figure 4.19: mRNA expression of known and potential Batf subfamily cofactors, the Irf transcription factors.

A.



B.



C.

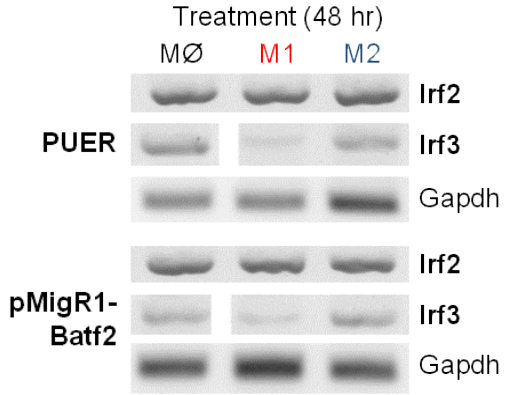


Figure 4.19: mRNA expression of known and potential Batf subfamily cofactors, the Irf transcription factors. Expression of Irf family members in PUER, PUER-pMigR1, and PUER-Batf2 cells. PUER cells, vector-transduced cells (PUER-pMigR1) and Batf2-

transduced cells (PUER-Batf2) were polarized with 100 ng/ml LPS and 20 ng/ml IFNγ (M1) or 4 ng/ml IL-4 (M2) for 48 hours, or left unstimulated (MØ), then analyzed as described in Chapter 3. Total RNA was extracted and analyzed using RT-PCR with specific primers. Gapdh was included as a loading control. The products were separated on agarose gels, stained with ethidium bromide and photographed. The images have been inverted for clarity. Similar results were observed in 3 independent experiments for the 48 hour timepoints. **A.** Expression of known Batf family cofactors Irf4 and Irf8. Irf4 and Irf8 form a ternary complex with AP-1 dimers containing Batf family members and subsequently bind to the AP-1/Irf Composite Element (AICE) (5'-TTTCnnnnTGACTAA-3' or 5'-GAAATGAnTCA-3'). At 8 hours, Irf8 and Irf4 are both expressed in M1-polarized macrophages, although Irf4 is more strongly expressed in M2-polarized macrophages. At 48 hours, Irf8 is minimally expressed and Irf4 is not detectable in M1-polarized macrophages. Therefore, Irf4 and Irf8 may interact with Batf2 at 8 hours but not 48 hours in M1-polarized PUER macrophages. **B.** Expression of M1-associated Irf1, Irf7, Irf9, and Irf5. Irf1 binds to Batf2 in macrophages and plays a role in expression of M1associated genes. Irf7 and Irf 9 have no demonstrated interactions with Batf2. Irf1 and Irf7 are expressed in M1-polarized macrophages at 48 hours, while Irf5 is not detectable. Therefore, Irf1, Irf7, and Irf9 could potentially interact with Batf2 at 48 hours in M1-polarized PUER macrophages. **C.** Irf2 and Irf3 are not M1-associated. Irf2 and Irf9 are expressed in M1-polarized macrophages at 48 hours, while Irf3 is not detectable. Therefore, Irf2 could potentially interact with Batf2 at 48 hours in M1-polarized PUER macrophages, but not Irf3.

Chapter V

DISCUSSION

5.1. Summary

The major question addressed in this project is how specific genes affect macrophage polarization; more specifically, the role of the transcription factor Batf2 in sustained inflammatory (M1) polarization. My initial hypothesis was that Batf2 alone is a master regulator of M1 polarization. However, this was not the case. Constitutive high Batf2 expression alone did not drive expression of M1-associated genes, but did moderately enhance LPS/IFNγ-induced Cxcl10 mRNA and suppress IL4-induced Arg1 mRNA. Additionally, Batf2 activity in macrophages does not appear to be similar to its roles in other cells. These results considered alongside the previously established importance of cofactors to the activity of Batf subfamily members activity suggests that Batf2 activity requires a necessary cofactor, and lead the way for future studies investigating the cofactors that interact with Batf2 and the genes regulated by Batf2-cofactor complexes in sustained M1 polarization state.

In this discussion I address first the evidence suggesting Batf2 drives M1-specific gene expression. Next, I address the moderate polarization-dependent effects of Batf2 and extrapolate those results to hypothesize functions of Batf2 during immune responses. I then present possible implications of the inability of Batf2 alone to drive M1-associated gene expression, as well as differences in Batf2 activity between other cells and macrophages. Finally, I consider several potential factors that might contribute to the lack of Batf2 activity from constitutive high expression alone, and focus in on the most likely solution: that Batf2 requires a necessary cofactor. Based on previous studies, I

focus on the Irf family members, discussing the potential of each for being the necessary cofactor of Batf2.

5.2. Batf2 is rapidly induced in M1-polarized macrophages

Several pieces of evidence strongly support Batf2 as a master transcription factor in sustained M1 polarization. First, Batf2 expression is specific to the M1 state. Second, Batf2 is highly expressed relative to other M1-specific transcription factors. Third, Batf2 mRNA appears early after LPS/IFNγ stimulation. Finally, Batf2 subfamily members have established roles as master transcription factors in development and function of other immune cells.

Batf2's specificity to the M1 polarization state in the PUER model system supports the hypothesis that it regulates genes important for M1 functions. In general, LPS/IFNγ-activated M1 functions support a type 1 inflammatory response involving the production of effector proteins such as nitric oxide, activation of effector functions such as phagocytosis, and recruitment and support of Th1-type T cells. In our PUER cells, Batf2 correlated with upregulation of the anti-microbial and phagocytosis-supporting enzyme iNOS, inflammatory cell-recruiting chemokine Cxcl10, and multiple genes consistently associated with multiple models of M1 macrophages including Irf1, Irf7 and Ccl5. In contrast, Batf2 was not expressed in IL-4-activated M2 polarization (denoted specifically by some authors as M2a). M2 polarization supports type 2 inflammatory responses with effector proteins such as NO-antagonizing Arg1 and with proteins supporting Th2-type T cells. Batf2 expression inversely correlated with multiple M2a-associated genes including Arg1, Fizz1, and Irf4. In PUER cells, resting macrophages

displayed only minimal amounts of Batf2, if any. On the other hand, bone marrow derived macrophages (BMDMs) did express Batf2 mRNA in the M2-polarized state. This could be due to previous *in vivo* influences, the isolation procedures, or stresses from cell culture partially polarizing the highly plastic macrophage partially to M1. Although we use a reductionist model with discrete M1/M2 subsets for study, in reality macrophages fall on a spectrum. Some M2 subsets, such as BMDM in our study, may be closer to M1 on the spectrum. *In vivo*, macrophages likely exist in states with some M1 functions and some M2 functions. Roy *et al.* and Tussiwand *et al.* examined macrophages and dendritic cells *ex vivo* and found low basal Batf2 expression compared to infected mice or *in vitro* stimulated cells[1,83]. Whether this compares to the level in our BMDMs is not clear. Batf2 expression in basal levels or M2-associated macrophages *in vivo* requires further exploration. The analyses in human cancer studies and IFN-associated diseases (see Background section) suggest that Batf2 is indeed somewhat variable *in vivo*. As future studies define the genes induced or repressed by Batf2, the consequences of Batf2 expression in macrophages exposed to M2 stimuli such as IL-4 will become clearer. For the purposes of this study, we are focusing on the fact that Batf2 associates highly with IFNγ but not IL-4 activation in our reductionist and highly controllable model, and this agrees with previous reports on Batf2's correlation with inflammatory responses[1,83,95,108,109,119].

Typically, LPS and IFNγ are the model *in vitro* inflammatory stimuli for the M1 state. *In vivo*, macrophages see a wide variety of stimuli that polarize them to variations of the inflammatory phenotype depending on the environment. In some contexts, for example, IFNγ may be present without TLR4 agonists such as LPS, activating different

macrophage functions than LPS/IFNγ in combination. IFNγ signals through IFNGR1/2 and activates the JAK1/2 and Stat1/1 pathway, while TLR4 signals through MyD88, which then activates the MAPK pathway and transcription factor AP-1, or activates the transcription factors NF-κB or Irf3. Our study began to address the potential for Batf2 activity in multiple environments by assessing differential induction by LPS or IFNγ. TLR4 stimulation by LPS was not necessary for Batf2 expression in PUER macrophages, so Batf2 may have activity in contexts where TLR4 stimulation is minimal but IFNγ is high. This is consistent with the developing understanding on Batf2 expression. Batf2 was initially discovered as an IFNβ -inducible gene in multiple cell types, and IFNβ has continued to be the main stimulus in studies on Batf2's tumor-suppressing effect[95,112,114]. These studies demonstrate that Batf2 has a role in functions outside of bacterial infections and TLR4-stimulating damage, and open the possibility that IFN-induced Batf2 might drive damaging inflammatory genes in situations when inflammation is inappropriate. For example, Batf2 is highly upregulated in common variable immune deficiency patients with autoimmune complications versus those without, and in fibromyalgia with high pain versus low pain[123,167]. In these cases, Batf2 might be a good target for anti-inflammatory therapeutics.

Two previous studies have examined Batf2 expression in macrophages in response to LPS and/or IFNγ, in part agreeing and in part disagreeing with our results. Tussiwand *et al.* found that IFNγ alone can induce Batf2 in BMDM, similar to our PUER cells[83]. Contrary to our results, however, Batf2 was also induced by LPS, although this experiment was in peritoneal macrophages. Roy *et al.* has recently done further studies on the effects of Batf2 in macrophages. As in our study and in Tussiwand *et al*., Batf2

was induced by IFNγ alone. Contrary to our results but agreeing with Tussiwand *et al.*'s experiments with BMDM cells, Batf2 was also inducible by LPS[1,109]. Further, a genome-wide association study by Chmielewski *et al.* found synergism between IFNγ and TLR4 for Batf2 induction in vascular smooth muscle cells[121]. It is possible that the macrophages in the Roy *et al.* and Tussiwand *et al.* studies, being isolated from mice or human patients, experienced some mildly polarizing stimuli *in vivo* or during the isolation procedure that primed them for Batf2 expression in response to LPS, or were epigenetically programmed. One of the advantages of PUER cells is the ability to differentiate and polarize the cells with minimal polarizing stimulation. Future studies could examine these pathways with a wider variety of macrophage cells to determine the consistency of LPS-induced Batf2 expression. Regardless, Batf2 clearly belongs to in an LPS/IFNγ-induced M1 and not an IL-4-induced M2 transcriptional program in PUER cells.

Interestingly, Batf2 also increased in response to IL-10 stimulation, demonstrating that Batf2 may also be induced by factors more often associated with M2 phenotypes. IL-10 in combination with TGF-β and glucocorticoids activates macrophages to an alternative phenotype. IL-10/TGF-β/glucocorticoids-induced macrophages produce IL-10, TGF-β, and Ccr2. This polarization state functions in wound healing and cell debris scavenging, and has more in common with the IL-4-induced M2 state than the LPS/IFNγ-induced M1 state[31,33,34,128]. LPS/IFNγ activates Stat1, while both IL-4 and IL-10 activate Stat3, so Batf2 induction by IL-10 was unexpected. Nevertheless, IL-10-activated macrophages do express some overlapping genes with IFNγ and LPS[31,33]. Therefore, Batf2 may be induced by an IL-10-activated pathway distinct from Stat3. Additionally,

IL-10-induced Batf2 was not dose-dependent in the experiment shown in Figure 4.10, suggesting that all three concentrations of IL-10 (1, 10, and 100ng/ml) elicited maximal Batf2 expression on the plateau of the dose-response curve. Therefore, the pathway inducing Batf2 in response to IL-10 may have a lesser maximal response than LPS/IFNγ, or the maximal response in response to LPS/IFNγ may be from a combination of the IL-10-induced pathway and an LPS/IFNγ-exclusive pathway. Our study and those of other support an important role for Batf2 in polarization to an M1 phenotype, but Batf2-induced gene expression may also have functions in other contexts (as discussed in the Background section on Batf2's role in cancers). IL-10-induced Batf2 may induce some functions needed in specific environments containing IL-10 as well as environments containing LPS and IFNγ. Future studies may seek to confirm IL-10 induction of Batf2 and examine the pathways inducing Batf2, as well as the role of IL-10-induced Batf2 in the context of wound healing and debris scavenging. Batf2 may serve in this context to activate phagocytic abilities during scavenging, or receptors detecting foreign molecules such as bacterial mannose by the mannose receptor (Mrc1).

In addition to its association with the LPS/ IFNγ-induced M1 state, the rapid induction of Batf2 message and high expression levels at later time points support a role for Batf2 in the MØ to M1 transition and sustained M1 polarization. Batf2's appearance as early as 1 hour after stimulation suggests it may be an immediate-early gene as well for M1 polarization. This would mean that the factor inducing Batf2 is constitutively present and rapidly activated upon stimulation, allowing Batf2 among other immediate-early genes to activate downstream gene expression early after stimulation when macrophages are shifting out of a resting state and into an inflammatory state. The

approximately10-fold increase in Batf2 after polarization approaches the major inflammatory transcription factor Stat1, which was increased 12.7-fold. This robust expression of Batf2 may support our hypothesis that Batf2 is important for sustained M1 polarization. It is worth considering, however, that Batf2 does not necessarily need to be an immediate-early gene to be critical for sustained M1 polarization. Several transcription factors are expressed early but transiently such as Fra1[168]. Another transcription factor could have conceivably fulfilled Batf2's role initially, with Batf2 being a critical factor only later. Instead, the data suggest that Batf2 seems to be important for initial polarization as well as long-term polarization. Once genes downstream of Batf2 are identified, studies knocking down Batf2 and examining early expression of these genes could clarify the timeframe of Batf2 importance and confirm whether it indeed is important for initial polarization, as this data suggests.

The hypothesis that Batf2 is important for M1 polarization, sustained in particular but likely also immediate, is backed by a large body of research demonstrating the importance of Batf subfamily members in immune cell differentiation and function. Batf3 drives genes for the differentiation of $CD8\alpha^+$ cDCs and $CD103^+CD11b^-$ cDCs, inducing Clec9a, Xcr1, Id2, and Spib. Batf induces genes for CSR in B cells (Aid), and genes for differentiation of Th17, Tfh cells, and Th9 including Rorc, Il17A, Il21, Il23R, Maf, Bcl6, ILl9, Il21, Il10, Furin, Fes, Erg, and IL-1RA. In Th2 cells Batf or Batf3 both induce GATA3, Il4, Il10, and Ctla4. Batf, Batf2, and Batf3 all share homology and have partially overlapping functions in some cells [76,111]. Batf2's ability to compensate for Batf3 is discussed more in depth below. Based on Batf2's association to M1 polarized macrophages, rapid and robust expression, and prior knowledge of the Batf subfamily

members, it is reasonable to predict that Batf2 could function as an inducer of an array of M1-associated genes in macrophages.

5.3. Constitutive Batf2 expression alone enhances select M1 genes or suppresses select M2 genes

If Batf2 was a master transcription factor for M1 macrophage polarization as initially predicted, similar to the Th1 master transcription factor Tbet, then constitutive Batf2 expression in resting macrophages should have induced expression of M1-specific genes. This turned out not to be the case. On the other hand, Roy *et al.* recently uncovered a role for Batf2 in a broad array of M1-associated genes using knockdown studies [1]. This apparent contradiction, as well as our mild polarization-exclusive changes, can be most simply explained as a requirement for a necessary cofactor (discussed in more detail below). In spite of the lack of broad changes, constitutive Batf2 expression enhanced LPS/IFNγ-induced Cxl10 and suppressed IL-4-induced Arg1. Neither Cxcl10 nor Arg1 was mentioned in the knockdown studies by Roy *et al.* These results, along with prior studies on Cxcl10 and Arg1 regulation, provide a starting point for future studies on Batf2 control of these genes. Batf2 is known to inhibit cJun activity at the AP-1 consensus by preventing DNA binding[95]. The murine Cxcl10 promoter contains an AP-1 consensus site although it is not required for Cxcl10 expression in some contexts. For example, Cxcl10 was not inducible by the AP-1 activator PMA[40,51]. Moreover, AP-1-mediated enhancement contradicts Batf2's known role as an *inhibitor* of cJun activity by preventing cJun binding to these sites[95]. This would not contradict our results if Batf2 or a Batf2-containing complex recognizes and activates from a consensus

other than the AP-1 site. In contrast to Cxcl10, Arg1 contains an AP-1 consensus on the promoter that significantly drives Arg1 expression in macrophages via cFos in response to macrophage stimulating protein (MSP), and another that drives expression in rat aortic endothelial cells via cJun/ATF2 in response to thrombin[47,169]. Thus, Batf2 could inhibit Arginase 1 expression via the AP-1 consensus site in M2 associated genes.

Another explanation for our results that Batf2 message itself may be preventing Cxcl10 message degradation through RNA interference. Currently, there is substantial interest in the importance of small interfering RNAs (siRNAs) in macrophage polarization[170] (siRNA)-mediated gene knockdown is now standard in mice and has potential for human therapies as well. In consultation with Dr. Penni Black (College of Pharmacy, University of Kentucky) we asked whether it might be possible that the presence of Batf2 mRNA itself could regulate Cxcl10. Interestingly, analysis using miRDB (http://www.mirdb.org) found that miR-296 is both predicted to bind both Cxcl10 message and Batf2 message[3]. Batf2 may, therefore, sequester and act as a sink for Cxcl0-degrading miR-296. This could contribute to the enhanced Cxcl10 mRNA induction in our cells, especially given the lack of enhancement of any other genes tested. This type of regulation, by sequestering a regulatory miRNA, presents a novel mechanism of gene regulation and requires experimental verification. An interesting further experiment would be to transduce a translation-defective Batf2 mRNA to analyze whether this can enhance Cxcl10. Future studies could evaluate further the interaction of miR-293 with Cxcl10 and Batf2, and determine whether this interaction occurs *in vivo*. This avenue of research, although departing from studies on Batf2, could lead to a deeper understanding

[3] *Personal communication, Dr. Penni Black, College of Pharmacy, University of Kentucky*

of the ramifications of overexpression vectors in research, and to helpful therapeutics using miRNAs to decrease Cxcl10 and therefore decrease T and NK cell influx in inflammatory diseases.

One approach to confirm a role for Batf2 in enhancing LPS/IFNγ-induced Cxcl10 and suppressing IL-4-induced Arg1 is to knock down Batf2 and analyze the changes in these genes. Roy *et al*. successfully knocked down Batf2 and found decreased expression of an array of M1-associated genes[1]. This study did not mention Cxcl10 or M2-associated genes. We did attempt to create PUER cells constitutively expressing small-hairpin RNAs (shRNAs) to downregulate Batf2 message (see Section 4.3.3 on page 85). However, transduced cells from two shRNA sequences did not demonstrate decreased Batf2, and a third did not grow (data not shown). Batf2 is minimally expressed in healthy PUER cells prior to polarization, but Batf2 levels have not been tested in newly transduced cells. It is possible that Batf2 suppression by our specific shRNA strategy or vector system is toxic or growth-prohibitive in PUER cells. This contrasts with the study by Roy *et al.* that successfully stably transduced BMDMs with shRNA knocking down Batf2, and with Batf2's known role as a growth suppressant in cancer cells[1,95]. Further studies could determine the lethality or growth inhibition of Batf2 in PUER cells by treatments with small interfering RNA treatments, which permit transient knockdowns.

5.4. Possible cofactor proteins or processes required for Batf2 activity

Surprisingly, constitutively high Batf2 expression in the common myeloid progenitor stage did not drive multiple M1-associated genes. This approach has successfully uncovered other master regulators in particular in T cells such as Tbet (Th1

cells) Foxp3 (Treg), Bcl6 (Tfh), GATA2 (Th2) and RORγt (Th17)[14–20]. Recently, Roy *et al.* confirmed the importance of Batf2 for the expression of multiple M1-associated genes and repression of multiple M2-associated genes in murine macrophages using knockdown studies[1]. While constitutively high Batf2 expression in our study did enhance Cxcl10 mRNA expression and suppress Arg1 expression, this effect was moderate and specific to polarized cells. Therefore, Batf2 must be undergoing some level of post-transcriptional regulation. Transcription factors undergo multiple post-transcriptional regulatory mechanisms (see Table 2.2 in Chapter 1). In particular, the ubiquitous AP-1 proteins undergo many layers of regulation that provide fine control of transcription of any given downstream gene. These layers include: presence of a positively-regulating dimerization partner (although appropriate cofactors may compensate[80]); absence of any negatively regulating dimerization partners; non-AP-1 cofactors (if necessary); post-translational modifications, including phosphorylation, avoidance of SUMOylation, ubiquitination, and redox potential.

Based on our data and previous studies on Batf members, the simplest and most likely explanation is that a necessary cofactor, also upregulated upon polarization, is required for Batf2 activity. First, Batf2 enhances Cxcl10 message only in the polarized state, when the cofactor would also be elevated. Second, Batf proteins exert their known activities in other immune cells via protein-protein binding, presumably to a coregulatory factor. Third, knockdown of Batf2 leads to changes in gene expression not seen in constitutive Batf2 mRNA expression[1,76]. Cofactor binding also provides a mechanism for Batf2's cell specific activity and might explain why Batf2 suppresses Cyclin E in cancer cells and Id2 in $CD103^{+}CD11b^{-}$ cDCs but suppresses neither in our macrophages (Figure

4.16A). This evidence suggests not only that Batf2 functions in conjunction with a necessary cofactor, but that Batf2 target genes differ between different cell types. This could be due to unique cofactors that modify Batf2 activity, or differences in gene accessibility through chromatin modification. Finally, Batf2 must associate with Irf4/Irf8 in order to compensate for Batf3 deficiency in $CD103^{+}CD11b^{-}$ cDCs development and function[83]. This evidence suggests that a cofactor is probably required for Batf2 activity in macrophages. However, other mechanisms could also be at play. Although Batf2 sequesters cJun away from DNA binding, constitutive expression of excess Batf2 did not seem to seem to affect cJun activity, since the PUER-Batf2 cells expressed equal amounts of three genes downstream of cJun in macrophages (Csf1r, IL-1β, Mmp13). This might be due to post-translational regulation of the Batf2 protein, cJun, or both. Thus, it is worth considering other mechanisms that might alter the activity of Batf2.

In this study, we began to explore two of the likely mechanisms regulating Batf2 activity by examining the presence, absence, and polarization state association of known or likely proteins with which Batf2 interacts and that lie downstream of pathways with which Batf2 interacts. Two categories of interactants tested include a) dimerization partners and b) ternary complex cofactors.

5.4.1. Dimerization partners

The balance of AP-1 proteins present in a cell may be a key factor regulating Batf2 activity. Dimerization is necessary for activity of bZIP proteins ontranscription[81,82]. Thus, the balance of binding partners present could regulate gene expression by shifting the predominant heterodimers, which have different transactivation

activity. Activity of AP1 activity depends on relative ratios of binding partners, demonstrated in several instances by altering cJun, cFos, Fra2, and Batf proteins[63,73,75,79,83,171,172]. cJun/cJun versus cJun/cFos dimers at the Mmp12 and Mmp13 locus provide an example of the importance of correct partners. cJun/cJun heterodimers repress transcription by recruiting repressors, while cJun/cFos heterodimers activate transcription through their transactivation domains[63]. Batf2 itself provides another example of the influence of relative levels of dimerization partners; low levels of Batf2 in multiple cancer cell lines allow cJun to bind DNA and activate transcription, while high expression of Batf2 in those cells sequesters cJun from DNA binding and thereby inhibits transcription [95,124]. Based on these observations, I expect that Batf2 dimerization could alter activity of a dimerization partner in two ways, depending on interacting proteins or complexes present. This could occur by 1) assisting a dimerization partner in DNA binding, directly activating or repressing of the downstream gene, or 2) preventing a dimerization partner binding to DNA, indirectly preventing activation or derepressing the downstream gene. The former occurs when Batf2 replaces Batf3 (which heterodimerizes with cJun, JunB, or JunD) in Batf3-Irf4 ternary complexes driving $CD103^{+}CD11b^{-}$ cDC development[83]. The latter occurs when Batf2 sequesters cJun in multiple non-immune cells, preventing activation of tumor promoting genes [95]. Predicting the effect of Batf2 on other AP-1 proteins in macrophages is complicated by the lack of *in vivo* information on Batf2's possible dimerization partners. Batf2 binds to cJun *in vivo* and JunB in fluorescence resonance energy transfer (FRET) protein-protein binding assays, while none of the other AP-1 proteins bound Batf2 strongly in FRET assays[95,113]. cJun and JunB expression is not associated with M1 polarization, but either could be Batf2 binding

partners in PUER macrophages since they were both detectable at 48 hours, Batf2 interacts with cJun, and JunB has been implicated previously in macrophage activation [159]. However, the mRNA kinetics does not support a role for either in maintaining long-term M1 polarization with Batf2, since neither cJun nor JunB increased in M1-polarized cells at 8 or 48 hours. A previous study on BMDMs observed an increase in cJun at 24 hours after IFNγ polarization[109]. It is possible that these proteins play a transient role at early time points in PUER macrophage polarization. Further, cJun message does not necessarily indicate activity, since post-translational modifications, largely phosphorylation, commonly regulate cJun activity. Constitutive Batf2 expression even at the high levels in our PUER-Batf2 cells does not appear to be functionally sequestering cJun protein, however, as three genes downstream of cJun (Ccne1 [Cyclin E], Csf1r, and IL-1β) were comparable in control cells and in cells highly and constitutively expressing Batf2 mRNA.

In contrast to cJun and JunB, cFos, Fra1, Fra2, JunD, Batf, and Batf3 do not dimerize significantly to Batf2 in large-scale binding assays[113]. Batf2 is more likely to interact with these by sequestering another binding partner. Like cJun and JunB, expression of these factors is not M1-associated at later time points but mRNA levels do not necessarily correlate with activity. cFos message was elevated transiently hours after IFNγ polarization in a separate study[109], which might suggest a transient role at early time points, but not long-term maintenance of M1 polarization state. Fra1 was the only factor increased markedly after M1 polarization, but at 48 hours was detected sporadically in unpolarized as well as polarized cells, with no correlation to any specific treatment. Constitutive, high Batf2 expression does not appear to interact with Fra1 activity since

PUER-Batf2 do not exhibit enhanced expression of Figf, a gene induced by Fra1 transfection in mouse mammary epithelial carcinoma cells[146]. Therefore, the AP-1 proteins that do not likely dimerize with Batf2 but may be indirectly regulated by Batf2 are not associated with sustained M1 polarization by mRNA analysis, and unlikely to play a significant role in M1-promoting gene expression changes mediated by Batf2.

Overall, mRNA analysis alone does not suggest either of the putative (JunB) or verified (cJun) Batf2 dimerization partners as a partner in long-term Batf2 activity. However, mRNA analysis at 8 and 48 hours alone may not be a good indication of activity. In spite of a lack of significant message increase, the recent study mentioned above by Roy *et al.* reports that the kinetics of JunB most closely match the kinetics of enriched cJun/cFos consensus-driven message in IFNγ-polarized BMDMs at 2-24 hours, and the authors suggest that JunB plays a role in polarization [1]. This agrees with previous studies implicating JunB in macrophage polarization[159]. JunB may well be the most likely candidate for Batf2 heterodimers, although this raises the question of how it continues to drive M1-associated gene expression after expression returns to baseline at later time points. Future studies on the Batf2 activity must analyze the heterodimers that form *in vivo* at various time points after polarization and the activity of these heterodimers.

5.4.2. Ternary complex cofactors

Alongside dimerization, ternary complex formation may also regulate Batf2 activity during M1 polarization. As mentioned above, the simplest interpretation of the evidence supports the existence of a necessary cofactor for Batf2. Further, precedents in

other systems suggest that the cofactor is a ternary complex cofactor. Ternary complex cofactors guide Batf proteins to specific consensus sites in other immune cells including T cells, B cells, and some subsets of DCs (reviewed in Murphy *et al.*[76]). Cofactor binding also provides a mechanism for Batf2's cell specific activity and might explain why Batf2 suppresses Cyclin E in cancer cells and Id2 in $CD103^{+}CD11b^{-}$ cDCs but does neither in macrophages.

Batf provides an example of the manner by which cofactors dictate different roles for Batf subfamily members in T cells and B cells (reviewed in Li *et al.*[173]). In B cells, Batf is important for class-switch recombination[107]; while in T cells it is important for differentiation of various T cell subsets and for expression of various cytokines and transcription factors such as Rorc, Rora, and Ahr. The major difference between these two cell lineages is the presence of the Irf4-binding proteins PU.1 and Spib in B cells. Irf4 monomers have autoinhibitory activity preventing DNA binding at the concentrations found in T and B cells, but PU.1, Spib, and Batf bind and disinhibit Irf4. In B cells, Irf4 binds PU.1 or Spib, and guides the complex to ETS-Irf consensus elements (EICEs) (5'-GGAAnnGAAA-3'), leading to gene expression. T cells, however, lack these factors so Irf4 binds instead to Batf-cJun heterodimers, and the complex subsequently binds AP-1-Irf composite elements (AICEs) (TGAnTCAnnnnGAAA or GAAATGAnTCA). AICE binding by Batf-cJun-Irf4 complexes also occurs in B cells, but to a much lesser extent, possibly due to relative abundance of each complex, or possibly due to another factor yet to be identified. Thus, Batf2 activity in M1-polarized macrophages likely depends on the unique cofactor repertoire in these cells relative to other cells that use Batf2 and other Batfs.

Irf family members are candidates for being Batf2 cofactors in PUER cells. Irf1 and Irf7 are most likely to have a role in long-term polarization based on mRNA expression analysis, since both are elevated during sustained M1 polarization. Irf8 is associated with early M1 polarization, so potentially plays a role during early shift from resting to M1. Previous studies suggest that Irf9 and Irf5 induce M1-associated gene expression, but Irf9 is not M1-associated in our study and Irf5 is not expressed. Irf2, Irf3, and Irf4 are not likely to support M1 polarization. Irf2 was constitutively expressed, Irf3 was somewhat decreased upon M1 polarization, and. Irf4 was associated with sustained M2 polarization. Prior studies do not report roles for either of them in M1 polarization

5.4.2.1. Irf4 and Irf8: Hematopoietic-specific known Batf subfamily cofactors

Irf4 and Irf8 are hematopoietic-specific Irf family members that bind with Batf-subfamily proteins, including Batf2, and are therefore candidates for Batf2 cofactors in macrophages. Batf-Irf4/Irf8 complexes are critical for development of other immune cells by driving transcription at AICEs. Although no DNA-binding assays have been published demonstrating Batf2-Irf4/Irf8 complexes binding to AICE elements, previous data suggests Batf2 does have transactivation activity through DNA binding. For example, the protein-protein interaction domain (the leucine zipper) allows Batf2 to functionally compensate for Batf3 deficiency in $CD103^{+}CD11b^{-}$cDC/$CD8\alpha^{+}$ cDC development. This suggests that it either activates gene expression from AICEs or an alternative site located in most of the same promoter or enhancer regions[83]. Therefore, Irf4 has the potential to drive gene expression in PUER cells in concert with Batf2. However, Irf4 is M2-associated in our study and in previous reports, acting as an

antagonist in TLR signaling[4,8]. Irf4 has known activity in macrophages both at the interferon stimulated response element (ISRE) by interacting with other Irf family members as well as at the Ets/Irf composite elements (EICE) by interacting with PU.1, but has no activity alone due to an autoinhibitory domain[174–177]. In contrast, Irf8 is associated with M1 polarization in our study at earlier time points but expressed equally in unpolarized and polarized PUER macrophages at 48 hours. Irf8 is induced by LPS and/or IFNγ and in turn induces M1-specific cytokines including IL-1β, TNFα, IL-12, IL-6, and iNOS [178]. Irf8 contains a similar protein-binding domain to Irf4, and also interacts with other Irf family members to transactivate at ISRE or with PU.1 at EICE[175–177,177]. Notably, transactivation tends to be weaker than Irf4, and it seems to lack transactivation activity alone[179,180]. If that is the case, then how Batf-Irf8 complexes activate transcription requires clarification, since Batf also lacks transactivation activity on its own[83,180]. Unlike Irf4, Irf8 is IFNγ -inducible and associated with M1-polarized macrophages[181]. It is important for FcgR1, LPS/IFNγ-induced IL-12p40, and iNOS, as well as directing myeloid progenitors to the macrophage instead of granulocyte lineage[182]. *In vivo* Irf8 is important for proper development and response of macrophage populations in bone marrow[183]. In PUER, we find Irf8 expressed under all conditions at 48 hours, but it is M1-associated at early timepoints. Irf8 may be important for initial polarization, and then sustained expression continues to contribute to maintenance of M1-associated gene expression. Therefore, although Irf4 is not M1-associated, Irf8 is an early M1-associated Batf-subfamily cofactor that may be a cofactor for Batf2.

Overall, although initially promising, the evidence does not suggest a role for the hematopoietic Irf family members Irf4 and Irf8 as Batf2 cofactors with roles in sustaining

M1 macrophage polarization. Batf2 and Irf4 or Irf8 expression do not coincide, since CMPs, mature macrophages, and M2-polarized macrophages lack Batf2, while M1-polarized macrophages cells lack Irf4 and do not upregulate Irf8 throughout sustained polarization. Moreover, previous studies have detected Irf8 significantly upregulated at 2 hours after IFNγ polarization, so it may be a factor at earlier time points, but Batf2 must interact with another cofactor for activity during sustained M1 polarization[109].

5.4.2.2. M1-associated Irf1 and Irf7 may interact with Batf2 and play a role in M1 polarization

Although not supported as a Batf/Irf cofactor by the initial studies described above, Irf1 has recently become a likely candidate for driving gene expression in concert with Batf2 in macrophages. Recent studies demonstrate that Irf1 interacts with Batf2 in M1-polarized macrophages. Moreover, Irf1 is induced by IFNγ and TLR stimulation, strongly and consistently associates with M1 polarization, and is known to act in concert with cofactors including NF-κB and cJun[1,4,109,184]. Roy *et al* made the discovery that Irf1 binds to Batf2 in M1-polarized macrophages, and further demonstrated that loss of Irf1 recapitulates many of the same changes as loss of Batf2[1]. Irf1 still lacks confirmation as a necessary cofactor of Batf2 in macrophages, however, and has some important differences with known Batf subfamily cofactors Irf4 and Irf8. Irf1 binds to ISREs and drives gene expression without the necessity of a Batf member, while Batf-Irf complexes were originally found because Irf4 and Irf8 are necessary for cell development or function but do not drive gene expression alone. In dendritic cells, Irf4/8 guides the Batfs preferentially from the conventional AP-1 consensus sequence to an AP-1/Irf consensus

element, termed the AICE[83,86]. Glasmacher *et al*'s study defining the AICE did explore a role for Irf1 along with Irf3, since they are structurally similar to Irf4 and Irf8. They found that these factors did not assemble on AICE sites with Batf-JunB complexes as did Irf4 and Irf8. Moreover, Irf1 is also found in B cells but Batf2 cannot compensate for Batf in these cells, suggesting Batf2-Irf1 complexes do not function like Batf-Irf4/8 complexes[83]. This evidence taken together suggests that Batf-Irf1 complexes do not have the same activity as Batf-Irf4/8 complexes.

However, if Irf1 can bind ISREs on its own and does not bind to AICEs in conjunction with Batfs, why does knockdown of Irf1 and Batf2 result in similar transcriptional profiles? One possibility is that Batf2-Irf1 complexes may recognize an alternative consensus. In theory, this alternative consensus might be present in the critical promoters for DC development and macrophage polarization (where Batf2 has activity and interacts with Irfs), but not critical genes in T cell development and function and B cell activation. There may also be another cofactor required to stabilize Batf2-Irf1 complexes that is not present in T and B cells.

Our finding that Irf1 increases selectively in M1-polarized macrophages contrasts with other reports of Irf1 increased in M2-polarized cells, but agrees with Irf1's consistent association with M1 polarization[4,109]. Irf1 could drive M1 associated genes in M1-polarized but not M2-polarized cells if Batf2 mediates Irf1's effects, or if M1-specific post-translational modification activates Irf1[184]. Future studies must examine the promoters of genes for Batf-Irf1 consensus binding, as well as exploring other potential cofactors that may be part of a larger transcriptional activation complex.

Irf7 is also M1-associated at 48 hours in our PUER macrophages, and may have relevance to M1 polarization. Unlike Irf1, Irf4, and Irf8, Irf7 is not specific to hematopoietic cells, but nonetheless is involved in macrophage differentiation and type I interferon induction, as well as M1-like polarization in microarray [4,150]. Interestingly, Irf7 basal expression is increased in PUER cell constitutively expressing Batf2. Irf7-interacting pathways would provide a reasonable start for future studies examining Batf2-interacting pathways.

5.4.2.3. M1-associated Irf5 and Irf9, and M1-unassociated Irf2 and Irf3

Irf5 and Irf9 both are notable as previously proposed master regulators of M1 polarization and mediators of LPS-activated transcription[4,70]. However, Irf5 was not detected in our PUER macrophages in resting or polarized states, suggesting this factor is dispensable for macrophage polarization. In our study, Irf9 was not exclusive to M1 state. Irf9 might still have M1-promoting roles under some conditions by propagating type 1 IFN signaling and inducing Cxcl10 in concert with Stat1 homodimers[185]. It might also be active selectively in M1-polarized macrophages without a change in mRNA expression by post-translational regulation or cofactor interaction.

Based on the literature, other ubiquitously-expressed Irf family members have activity in macrophages, but are unlikely to serve a broad role. Irf2 and Irf3 were constitutively expressed under all conditions. Irf2 is not a likely contributor to M1 polarization because expression is not M1-associated and because it can inhibit Irf1[101,186]. Irf3 may promote either M1- or M2- associated cytokines. On the one hand, Irf3 induces Ccl5; on the other, Irf3 suppresses IL-1A and IL-23[187,188]. These Irf family members

provide examples of a minor importance of Irf family members and Irf-containing complexes inducing macrophage genes, but other Irf family members are more likely to play a role

In summary, studies on the Batf subfamily suggest that Irf family members present the most likely candidates for being cofactors for Batf2. Irf1, Irf4, and Irf8 are established Batf2-interacting factors, but only Irf1 is strongly M1-associated. Batf2manipulation by itself may fail to alter genes in our cells simply due to limited availability of Irf1 or another Irf. Still, Batf2's interaction with a cofactor or multiple cofactors has not been definitively determined. Batf2-Irf1 has not yet been evaluated for DNA-binding. Batf2's putative cofactor remains to be definitively determined. Future studies should examine complexes formed with Batf2 in macrophages. Knock-out and restoration methods could examine the ability of each of these complexes to drive macrophage polarization.

5.4.3. Other regulatory mechanisms: Chromatin state and post-translational modification

Two more major regulatory mechanisms that likely regulate Batf2 activity merit some discussion: epigenetic modifications and post-translational modification. Chromatin modification regulates gene accessibility to multiple transcription factors, while post-translational modification regulate individual transcription factors. Phosphorylation is of particular importance to AP-1 proteins[64–66,66,145]. Other regulatory modifications for AP (reviewed by Foletta *et al*.[74,77]) include SUMOylation of cFos, ubiquitination of cJun in Xia *et al*., and oxidation or reduction of cJun[77,91–93]. Both

chromatin state and post-translational mechanisms likely mediate to a major extent some of the differences seen in Batf2 activity in various cell types.

Chromatin structure is an important determinant of cell specificity that presents an avenue of research for future studies on the differences in Batf2's activity in macrophages relative to other cells. Chromatin structure refers to the modifications and tightness of the collective bundle of DNA-wrapped protein histones called nucleosomes. Remodeling enzymes modify DNA or histones, tightening or loosening interactions of DNA with the proteins. Tightening blocks access to transcription factors, and loosing permits access and transcription. The pattern of closed and open chromatin is to some extent determined by lineage-specific 'pioneer factors' that can access regions inaccessible to other transcription factors and recruit remodeling enzymes that open specific regions. This results in linage-specific gene, reviewed in Smale *et al.*[6]. Additionally, external stimuli can to some extent cause remodeling, providing a means of environment-specific gene expression. In macrophages, Runx1 transiently permits PU.1 transcription. Macrophage lineage-determining pioneer factor PU.1 along with Runx1 mediate gene accessibility by facilitating H2K4me1 deposition via remodeling enzymes expression[10,11,68,69]. Thus, chromatin structuring during development by factors like PU.1 may open up different sets of enhancers and promoters to Batf2 in macrophages, $CD103^{+}CD11b^{-}$cDCs, and cancer cell lines. It is also interesting to note that Hdac proteins also seem to be playing roles cell differentiation and our lab has strong evidence that Hdac9 is important for the M2 state (McGillis, unpublished data). Thus interaction with chromatin modifying proteins and complexes may be another potential binding partner for Batf2 mediated regulatory activities.

Not only may lineage-specific epigenetic modifications mediate differences in Batf2-induced or suppressed genes between different cells, it may also contribute to the differences in Batf2 expression in response to LPS stimulation seen in PUER cells compared to bone-marrow derived macrophages in the Roy *et al* study[1]. LPS stimulation of PUER does not induce Batf2 expression, while Roy *et al* reported that LPS synergistically with IFNγ as well as independently induces Batf2 expression in murine BMDMs. Green and Kerr *et al.* reported that epigenetic modifications control LPS-responsive genes, so primary macrophages may be primed *in vivo* or during isolation to express inflammatory genes[189]. PUER macrophages, in contrast, undergo minimal epigenetic programming compared to primary cells derived from animals. Gene expression in PUER cells may more closely reflect a truly naïve macrophage seeing polarizing stimuli for the first time, whereas gene expression in primary cells may be partially due to epigenetic programming from stimuli *in vivo,* during isolation, or during growth *in vitro.* On the other hand, PUER notably contain a modified PU.1 element: PU.1 attached to an estrogen receptor (ER) regulatory domain[190]. This artificial PU.1 may differ in remodeling ability; however, there has been no reason to suspect significant differences. PUER macrophages have been successfully used for prior studies and found to exhibit similar gene expression as macrophages from other models [129,132,191]. Therefore, Batf2 expression in other studies in response to LPS may result from external insults *in vivo* or during isolation. Further studies on LPS responsiveness of Batf2 are required to further define the regulatory pathways controlling Batf2 expression.

Post-translational modifications including phosphorylation and SUMOylation strongly regulate AP-1 family members[77]. M1-specific activating modifications may also

partially explain Batf2's M1-specific effects. cJun and cFos must undergo appropriate phosphorylation at specific residues for transactivation activity[64–66,144,145]. The antibody to Batf2 we used is a polyclonal antibody, raised to a peptide from human Batf2, and so the phosphorylation state of Batf2 remains unknown. AP-1 factors are also regulated through ubiquitination and SUMOylation[90,91]. These modifications tend to lead to degradation and thus reduce rather than enhance activity, and so are unlikely to mediate Batf2's M1-specific effects. Redox state also mediates cJun[92,93]. This regulation provides a mechanism for these proteins to mediate stress responses. Batf2, in contrast, is not associated with stress response, so is less likely to be influenced by oxidation. Of the post-translational modifications, phosphorylation state most likely regulates Batf2 function. Phosphorylation sites and their activities should be further explored in future studies to better understand post-translational regulatory mechanisms of Batf2 activity.

In summary, Batf2 likely undergoes regulation by a number of mechanisms including through cell-specific chromatin state and post-translational modifications. Thus, in contrast to our initial simple hypothesis, the mechanisms by which Batf2 influences macrophage functions is complex and will require in-depth mechanistic studies to fully understand its role in M1 macrophages. Our study did not explore these mechanisms, but they offer promising avenues of future research. Chromatin states differ between cell types and may mediate part of Batf2's cell specificity. PUER cells offer a good model for future Batf2 studies since they are relatively free of *in vivo* epigenetic modification. Phosphorylation very likely mediates Batf2 activity. Future studies could determine activating and repressing phosphorylation of Batf2, and the pathways mediating both.

5.5. Batf2 as a therapeutic target

One reason to define master transcription factors in inflammation is to manipulate them therapeutically. However, this study suggests that the effect of constitutively Batf2 expression alone such as could be delivered through adenovirus treatments may not produce changes robust enough for clinical benefit. For Cxcl10 in this study, the moderate enhancement of message failed to significantly increase protein levels ($p=0.204$). Functional studies are required to determine whether constitutive Batf2 expression alone has therapeutic applications. However, simultaneously inducing Batf2 and the limiting factor would likely induce more M1-specific genes than we detected in our study, including the genes inhibited by Batf2 knockdown. This remains to be tested. With our current knowledge, we can speculate that *in vivo* transduction of Batf2 with the required factor might enhance Cxcl10 significantly and suppress Il4-induced Arg1 significantly.

On the other hand, if our modified hypothesis is correct and Batf2 interacts with a cofactor, Batf2 knockdown with siRNA may prove effective for modulating M1 polarization. Roy *et al*.'s knockdown study demonstrating broad suppression of M1-associated genes supports this possibility[1,108]. Further support of the use of Batf2 suppression in inflammatory conditions arises from Tussiwand et al.'s report of Batf2-/- mice, which display abnormal phenotype after inducing IFNγ and IL-12-induced immune function after *T. gondii* infection[83]. These mice possessed both fewer lung-resident $CD103^{+}CD11b^{-}$ cDCs and $CD103^{-}CD11b^{-}$ macrophages after infection, but not during

homeostasis, suggesting that Batf2 knockdown might have minimal off-target effects when used as a target for excess inflammation. This study did not report macrophage responses in depth, so further analyses are required to define the macrophage inflammatory phenotype in Batf2$^{-/-}$ mice.

In spite of Batf2's associating with inflammatory stimuli IFN and LPS in multiple studies, it is possible that Batf2 plays a role in a response that occurs in IFNγ/LPS-activated M1 as well as some subsets of M2. Macrophages *in vivo* exist on a spectrum, so it is indeed likely that Batf2 is expressed in *in vivo* macrophages that have seen M2-associated stimuli such as IL-4. Batf2's expression in our BMDMs and IL-10 activated macrophages support this, as well as the highly variable expression of Batf2 isolated human cells and tissues in cancer studies[115,116,119,120]. As further studies elucidate the functions of Batf2, the significance of Batf2 expression *in vivo* will become clearer.

Batf2-mediated manipulation of Cxcl10 would most likely impact local inflammatory cell influx. Cxcl10, enhanced by Batf2 in our study, binds to CXCR3 that is found mainly on polarized Th1 cells and NK cells but also on macrophages, microglia, and dendritic cells[128,192]. Cxcl10 activates chemotaxis to attract cells to the origin of infection. To a lesser extent, it is associated with T cell development, T cell function, T cell adhesion to endothelial cells, NK cell cytolysis, angiogenesis inhibition, tumor growth inhibition, mitogenesis and chemotaxis on smooth muscle cells, inhibition of development of bone marrow progenitor cells, and apoptosis and cell growth [51,193]. Batf2-enhanced Cxcl10 production could therefore contribute to immune cell influx and function at sites of inflammation, and more rapid responses to infection. Conversely, Batf2 suppression could reduce immune cell influx and function, and mitigate damaging

inappropriate inflammatory responses. This might be useful in cases of high-pain fibromyalgia and common variable immune deficiency (CVID), where Batf2 is highly expressed but inflammation is inappropriate[123,167].

Argininase 1 (Arg1), in contrast to Cxcl10, generally associates with anti-inflammatory macrophages. Arginase activity shuttles L-arginine into the pathway for ornithine, a cell growth promoter and building block for tissue repair, and urea[54], away from its potential conversion to the inflammatory nitric oxide by iNOS in M1-polarized cells. Thus, it not only provides metabolites for growth and repair but also actively counters inflammatory responses. Arginase competes with iNOS for arginine, and low arginine pools do indeed decrease iNOS activity as well as arginase activity. During infection, arginase prevents excess inflammation and fibrosis in the case of some pathogens such as *S. mansonii*[56]. Batf2 transduction with the limiting cofactor may suppress Arg1 induction and thus increase inflammation by blocking the shunting of arginase into alternative pathways, allowing its use in the iNOS pathway. Alternatively, Batf2 knockdown could enhance Arginase 1 activity, promoting tissue repair with ornithine production and decreasing tissue damage from nitric oxide production. Thus, manipulation of Batf2 may have therapeutic potential.

5.6. Conclusions

Batf2, a bZIP transcription factor in the AP-1 family of proteins, is highly and rapidly upregulated in LPS/IFNγ-polarized macrophages. This dissertation initially hypothesized that Batf2 was a master regulator of LPS/ IFNγ-stimulated (M1)

polarization, driving expression of several M1-associated genes. However, constitutive high Batf2 expression did not induce or enhance expression of most of the M1-associated genes tested. Batf2 also did not broadly suppress IL-4-induced (M2-associated) genes, nor enhance or suppress potential downstream genes based on Batf2 activity in other cell types. Batf2 did mildly enhance LPS/IFNγ-induced M1 chemokine Cxcl10 mRNA and mildly suppress IL-4-induced M2 enzyme Arginase 1 mRNA expression. Based on this data and previous Batf2 studies, Batf2 likely requires a necessary cofactor for its role in M1 macrophages, which remains largely undefined. An Irf family members, especially Irf1, is one of the most likely candidates for a Batf2 interacting factor. More research is needed to determine the factors that interact with Batf2. Clearly the evidence from my studies and others suggest that Batf2 plays a role in M1 polarization. Defining the mechanisms of Batf2 induction and activity may offer in the future ways to approach therapies targeting macrophage inflammatory responses.

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Presentations
- **Marie A. Gehman,** Robert Hayman IV, Nichole S. Frantz and Joseph P. McGillis. "BATF2: A Master Switch for Inflammatory Macrophages?" Microbiology, Immunology and Molecular Genetics Retreat. University of Kentucky. 14 May, 2013.

- **Marie A. Gehman,** Nichole S. Frantz and Joseph P. McGillis."BATF2 promotes responses associated with classical macrophage polarization." Microbiology, Immunology and Molecular Genetics Retreat. University of Kentucky. 14 May, 2013.

- **Marie A. Gehman.** "Importance of cerebral IFN-γ production by non-T, non-NK cells for resistance against toxoplasmic encephalitis." Microbiology, Immunology and Molecular Genetics Seminar Series. University of Kentucky. 21 Feb, 2012.

- **Marie A. Gehman**, Tomoya Kudo, Qila Sa, Eri Ochiai, Sara Perkins, and Yasuhiro Suzuki. "Capability of microglia and cells other than T and NK cells in the brain to produce IFN-γ and IFN-γ-dependent immune responses to *Toxoplasma gondii*." Autumn Immunology Conference. Chicago, IL. 17 Nov 2012.

- **Marie A. Gehman.** "Polysaccharide Vaccines." Special Topics in Immunology. University of Kentucky. 23 Apr 2011.

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- **Marie A. Gehman,** Robert Hayman IV, Nichole S. Frantz and Joseph P. McGillis. "The Role of BATF2 in LPS/IFN-γ –Induced Macrophage Polarization." Poster Presentation. Microbiology, Immunology and Molecular Genetics Retreat. 13 May 2015.

- **Marie A. Gehman,** Nichole S. Frantz and Joseph P. McGillis."BATF2 as an important regulator of the pro-inflammatory macrophage transcriptome." Poster Presentation. MIMG Retreat. 14 May 2013.

- **Marie A. Gehman**, Tomoya Kudo, Qila Sa, Eri Ochiai, Sara Perkins, and Yasuhiro Suzuki. "Capability of microglia and cells other than T and NK cells in the brain to produce IFN-γ and IFN-γ-dependent immune responses to *Toxoplasma gondii*." Autumn Immunology Conference. Chicago, IL. 17 Nov 2012.

- **Marie A. Gehman,** Tomoya Kudo, Sara Perkins, Xisheng Wang, and Yasuhiro Suzuki. "Importance of IFN-γ production by cerebral non-T, non-NK cells for expression of chemokines and anti-Toxoplasma effector molecules during reactivation of infection with parasite in the brain." Poster Presentation. Microbiology, Immunology and Molecular Genetics Retreat. 27 Oct, 2011.

- Tomoya Kudo, **Marie Gehman**, Sara Perkins, Xisheng Wang, and Yasuhiro Suzuki.
- "Importance of IFN-gamma production by non-T, non-NK cells in the brain for induction of cerebral chemokine expression and prevention of reactivation of infection with Toxoplasma gondii." Poster Presentation. 11th International Congress on Toxoplasmosis Ottawa, Ontario, Canada. 27 Jun, 2011.

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