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ATF3 regulates neutrophil migration in mice

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Abstract

Activating transcription factor 3 (ATF3) is a counter-regulatory immune transcription factor whose expression is induced by Toll-like receptor (TLR) signaling. In turn, ATF3 inhibits the expression of various TLR-driven pro-inflammatory genes. In response to low-dose or transient pro-inflammatory stimulation, ATF3 attenuates immune activation, yet ATF3 silences its own transcription after high-dose or persistent stimulation, facilitating immune activation under these conditions.

Given its counter-regulatory role in diverse innate immune responses, we defined the effects of ATF3 on neutrophilic airway inflammation in mice. Genetic deletion of *ATF3* was associated with increased lipopolysaccharide (LPS)-driven airway epithelia production of CXCL1, concordant with the presence of a consensus ATF3-binding site identified in the *Cxcl1* promoter. Unexpectedly, ATF3-deficient mice did not exhibit increased airway neutrophilia after LPS challenge. Bone marrow chimeras revealed a specific reduction in $ATF3^{-/-}$ neutrophil recruitment to wild type lungs, while *in vitro* studies revealed a profound $ATF3^{-/-}$ neutrophil-intrinsic chemotaxis defect. Global gene expression analysis identified an absence of *Tiam2* expression in $ATF3^{-/-}$ neutrophils. Kinetic mRNA analysis of human neutrophil development revealed *ATF3* and *TIAM2* expression patterns consistent with positive *TIAM2* regulation by ATF3 during neutrophil development.

TIAM2 regulates cellular motility by activating Rac1-mediated focal adhesion disassembly. Correspondingly, $ATF3^{-/-}$ neutrophils lacking TIAM2

exhibited increased focal complex area, along with excessive CD11b-mediated F-actin polymerization. The exact molecular mechanism(s) of ATF3 regulation of *Tiam2* expression and TIAM2-mediated cellular translocation are currently under investigation. Together, these findings implicate ATF3 as an important functional and developmental regulator of neutrophils. However, the effects of the ATF3/TIAM2 axis on chemotaxis may extend beyond neutrophils, as loss of ATF3 can confer metastatic potential on non-metastatic cancers cell lines.

Further, preliminary experiments have investigated the effect of ATF3 on immune responses in infectious models. ATF3$^{-/-}$ mice exhibit reduced *Toxoplasma gondii* cyst burden without the attendant increase in mortality seen with deletion of other counter-regulatory pathways. Additionally, ATF3 is induced by infection with persistent sub-strains of lymphocytic choriomeningitis virus (LCMV), and ATF3$^{-/-}$ mice exhibit modest reductions in viral burdens due to such strains. In total, this work reinforces the counter-regulatory nature of ATF3 in the setting of immune responses, and defines novel functions for ATF3 in neutrophil biology. This makes ATF3 and downstream effector molecules attractive potential therapeutic candidates for diseases associated with excessive or detrimental neutrophil recruitment, and/or chronic viral or parasitic infections.

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Glossary and Abbreviations

AA	Arachidonic acid
αDC	α-Dystroglycan
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
AP-1	Activator protein-1
APC	Antigen presenting cell
AR	Androgen receptor
Arm	LCMV Armstrong strain
ATF3	Activating transcription factor 3
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
Bcl-2	B cell lymphoma 2
BM	Bone marrow
BPI	Bactericidal/permeability increasing protein
BSA	Bovine serum albumin
bZip	Basic region leucine zipper domain
C/EBP	CCAAT/enhancer binding protein
CBP	CREB binding protein
CCL	CC motif-containing chemokine
CCR	CCL chemokine receptor
CD	Cluster differentiation antigen
cDC	Conventional dendritic cell
CDP	CCAAT displacement protein
CDS	Coding determining sequence
CE	Common era
CF	Cystic fibrosis
CFSE	Carboxyfluorescein succinimidyl ester
ChIP	Chromatin immunoprecipitation
Cl13	LCMV Armstrong sub-strain clone 13

CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CNS	Central nervous system
CoIP	Co-immunoprecipitation
COX	Cyclooxygenase
CRE	cyclic AMP response element
CREB	CRE binding protein
CTLA4	Cytotoxic T lymphocyte antigen 4
CXCL	CXC-containing chemokine
CXCR	CXCL chemokine receptor
DAG	Diacylglycerol
DAI	DNA-dependent activator of interferon regulatory factor
DAMP	Danger-associated molecular pattern
DAP12	DNAX activation protein of 12 kDa
DC	Dendritic cell
DH-PH$_c$	Dbl homology-Pleckstrin homology domains-carboxy terminus
DMEM	Dulbecco's modified Eagle's media
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DOCK2	Dedicator of cytokinesis 2
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EFNA2	Ephrin A2
ELISA	Enzyme-linked immunosorbant assay
ELR	Glutamate-leucine-arginine chemokine sequence
EMSA	Electromobility shift assay
ERDR1	Erythroid differentiation regulator 1
ERM	Ezrin-radixin-moesin
ESAM	Endothelial cell-selective adhesion molecule
F-actin	Filamentous actin
FA	Focal adhesion

FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FC	Fold change
FcR	Fc receptor
FDR	False discovery rate
fMLP	Formylated met-leu-phe
G-CSF	Granulocyte-colony stimulating factor
GAG	Glycosaminoglycans
GAP	GTPase-activating protein
GCP2	Granulocyte chemoattractant protein 2
GDI	GDP dissociation inhibitor
GDP	Guanine diphosphate
GEF	Guanine nucleotide-exchange factor
GFI-1	Growth factor independent protein-1
GFP	Green fluorescent protein
GM-CSF	Granulocyte/macrophage-colony stimulating factor
GMP	Granulocyte/monocyte progenitor
GP	LCMV glycoprotein
GPCR	G protein-coupled receptor
GRO	Growth related oncogene
GTP	Guanine triphosphate
HAT	Histone acetyl transferase
HBSS	Hank's buffered salt solution
HDAC	Histone deacetylase
HEK293FT	Human embryonic kidney 293 cell line fast growing, T antigen subclone
HEPES	2-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV/AIDS	Human immunodeficiency virus/Acquired immunodeficiency syndrome
HSC	Hematopoietic stem cell
HUVEC	Human umbilical vein endothelial cell

i.p.	Intraperitoneal
i.t.	Intratracheal
ICAM	Intercellular adhesion molecule
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IFN-I	Type I interferon
IgH-VJ558	Immunoglobulin heavy chain (J558 family)
IgJ	Immunoglobulin joining chain
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IP_3	Inositol 3-phosphate
IRF	IFN response factor
IRSp53	Insulin receptor substrate protein 53
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
JAM	Junctional adhesion molecule
JDP2	c-Jun dimerization partner 2
JNK	c-Jun N-terminal kinase
KC	Keratinocyte chemoattractant
KLRG1	Killer cell lectin-like receptor G1
LAG3	Lymphocyte activation gene 3
LBP	LPS binding protein
LCMV	Lymphocytic choriomeningitis virus
LFA-1	Lymphocyte function-associated antigen-1
LIX	LPS-induced CXC chemokine
LO	Lipoxygenase
LPS	Lipopolysaccharide
LRF/LRG	Liver regeneration factor
LSK	$Lin^{-}Sca^{+}$cKit+ bone marrow cells
LT	Leukotriene
LX	Lipoxin

Mac-1	Macrophage-1 antigen
MAL	MyD88-adapter-like, aka TIRAP
MAPK	Mitogen activated protein kinase
MCMV	Murine cytomegalovirus
MD-2	Myeloid differentiation protein-2
MDA-5	Melanoma differentiation-associated protein-5
MDGF	Macrophage-derived growth factor
MDM2	Mouse double minute 2
MEF	Mouse embryonic fibroblast
MEP	Megakaryocyte/erythroid progenitor
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MHCI	Major histocompatibility complex type I
MHCII	Major histocompatibility complex type II
MIP2	Macrophage inhibitory protein 2
MLC	Myosin light chain
MLE	Mouse lung epithelia
MMP	Matrix metalloprotease
MPO	Myeloperoxidase
MT	Microtubule
MTEC	Mouse tracheal epithelial cells
MTUS2	Microtubule associated tumor suppressor candidate 2
MyD88	Myeloid differentiation primary response gene 88
NADPH	Nicotinamide adenine dinucleotide phosphate
NALP	NACHT, LRR and PYD domains-containing protein
NAP2	Neutrophil activating protein 2
NBT	Nitroblue tetrazolium
NCI	National Cancer Institute
NE	Neutrophil elastase
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor-κB

NIH	National Institute of Health
NK cell	Natural killer cell
NKT cell	Natural killer T cell
NLR	NOD-like receptor
NOD	Nucleotide-binding oligomerization domain-containing
ODZ3	Odd Oz/ten-m homolog 3
ORF	Open reading frame
OVA	Ovalbumin
p-MLC	Phosphorylated myosin light chain
PAK1	p21-activated kinase 1
PAMP	Pathogen-associated molecular pattern
PARP-1	Poly (ADP-ribose) polymerase-1
PBS	Phosphate-buffered saline
PD-1/PDCD1	Programed death-1
PD-L1	Programed death-ligand 1
pDC	Plasmacytoid dendritic cell
PE-CAM	Platelet endothelial cell adhesion molecule
PFA	Paraformaldehyde
PFU	Plaque forming unit
PG	Prostaglandin
PGP	N-acetyl proline-glycine-proline
PI3K	Phosphatidylinositol 3-OH kinase
PIP	Phosphatidylinositol phosphate
PIXα	PAK-interactive exchange factor alpha
PK	Protein kinase
PL	Phospholipase
PPBP	Pro-platelet basic protein
PRG	PDZRhoGEF
PRR	Pattern recognition receptor
PSGL-1	P-selectin glycoprotein-1
PTEN	Phosphatase and tensin homolog

PTx	Pertussis toxin
PU.1	Transcription factor encoded by gene SPI1
qRT-PCR	Quantitative real time polymerase chain reaction
RIG-I	Retinoic acid-inducible gene-I
RLR	RIG-I-like helicase receptor
RMA	Robust microarray average
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SAHA	Suberoylanilide hydroxyamic acid
SAM	Significance analysis of microarrays
SAPK	Stress-activated protein kinase
SCF	Stem cell factor
SH2	Src homology 2
SHIP	SH2-containing phosphotyrosine phosphatase
STAT	Signal transducer and activator of transcription
SUSD4	Sushi domain containing 4
SYNPO	Synaptopodin
TEM	Transendothelial migration
TEpM	Transepithelial migration
TF	Transcription factor
TGF-β	Transforming growth factor- β
TIAM	T cell lymphoma invasion and metastasis protein
TIR	Toll-IL-1 receptor
TIRAP	TIR domain-containing adapter protein, aka MAL
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α
TRAM	TRIF-related adapter molecule
TRIF	TIR domain-containing adapter inducing IFN-β
VE-cadherin	Vascular endothelial calcium-dependent adhesion molecule
WASp	Wiskott-Aldrich syndrome protein
WAVE2	WASp-family verprolin-homologous protein-2

WT	Wild type

Chapter I: Background and Scope of Thesis Work

Paul Ehrlich first identified a neutral dye-retaining white blood cell type, which he named "neutrophil," over a century ago [1]. Elie Metchnikoff, a contemporary of Ehrlich, was studying the same cells and termed these phagocytes with multi-lobulated nuclei "polymorphonuclear" leukocytes [2]. Since then, the neutrophil has become recognized as an essential component of inflammation and innate immunity. The importance of neutrophil function is demonstrated by the numerous human diseases associated with dysregulated neutrophil production, regulation or function, including: congenital neutropenia (production defects leading to increased susceptibility to infection), leukocyte adhesion deficiency (dysregulation of molecules involved in neutrophil trafficking leading to increased susceptibility to infection), chronic granulomatous disease (neutrophil killing defects leading to increased susceptibility to infection), α_1 anti-trypsin deficiency (inefficient neutralization of neutrophil elastase leading to lung and liver destruction and fibrosis), cystic fibrosis (marked by dysregulated neutrophilic inflammation in the lung, leading to airway destruction), and diverse cancers, (with increased neutrophil recruitment being linked to a poorer prognosis [3, 4] and increased metastasis [5]).

While these diseases have varying etiologies, they all share one feature; neutrophil tissue recruitment, or lack thereof, is integral to disease pathogenesis. Understanding the factors that regulate neutrophil recruitment, both intrinsic and extrinsic, is of clear importance. This thesis focuses on the mechanisms by which the transcription factor ATF3 regulates neutrophil recruitment. An overview of the immune system and immune responses will be presented so as to appropriately place neutrophils within their functional niche. Then, neutrophil development and neutrophil

recruitment will be discussed, followed by an overview of the current understanding of ATF3, an adaptive-response transcription factor that drives innate immune counter-regulation.

1.1 The immune system and inflammatory responses

The immune system functions to defend the host from competing, non germline-encoded genomes [6]. The cellular barrier separating us from our environment is the first line of immunologic defense. Exposed exterior surfaces, composed of tightly apposed epithelia, are often coated in thick mucous secretions to inhibit pathogenic colonization, or are colonized by symbiotic microflora that compete for resources with pathogenic microbes [7]. If this perimeter is breached, essentially every nucleated cell contains machinery to recognize and respond to infection, often by initiating the physiologic components of inflammation. In 40 CE, Celcus described inflammation as 'rubor, calor, dolor and tumor.' These clinical findings of redness, heat, pain and swelling are indicative of increased blood flow and vascular permeability (redness, heat, swelling), and neurologic sensitivity (pain) that serve to increase recruitment of immune cells to, and limit use of, the inflamed site by the organism.

Inflammation must be tightly regulated: insufficient activation allows infections to spread unabated; over-exuberant inflammation result in immunopathology, which can cause morbidity or mortality exceeding the original stimulus [8]. Inflammation progresses as a series of go/no-go decisions through which information about the type and magnitude of the required immune response is communicated [9]. Tissue-resident sentinel immune cells effect the immediate vascular changes of inflammation by

secreting immunologic mediators such as histamine, eicosanoids, tryptases, as well as other cytokines and chemokines [9], in order to activate endothelial and immune cells. These soluble mediators, in addition to intracellular contents released by damaged cells, recruit and activate immune cells. Immediate responses are carried out by innate immune cells, which can activate and regulate later adaptive immune responses. Finally, long-lived adaptive memory cells can initiate a more immediate and robust immune response upon re-exposure to a previously encountered pathogen.

1.1.1 Innate immune cells

Immune cells are summarily classified as either innate or adaptive, although it is inappropriate to view these immunologic branches as hierarchically or temporally sequential [10]. The innate immune system is evolutionarily more ancient than the adaptive immune system. It is present in some form in all eukaryotes, while the adaptive immune system does not appear until the jawed fishes [11]. Innate immune cells include: (a) granulocytes, subdivided originally by their affinity for different dyes into neutrophils, basophils, eosinophils, and mast cells; (b) monocytes, which give rise to macrophages and myeloid dendritic cells in the tissues; (c) natural killer (NK) cells; and (d) other innate lymphocytes, including γδT cells, NKT cells, B1 cells and marginal zone B cells. Innate immune cells derive from different progenitors and have different functions and effector mechanisms, yet their prime immunological functionality derives from recognizing and responding to pathogens using germline-encoded surface, intracellular, and secreted receptors. Once activated, these cells can phagocytose pathogens and/or kill pathogens and infected host cells via antimicrobial proteins (e.g.,

proteases, perforins, defensins), or reactive oxygen and nitrogen species, and/or induction of apoptosis. Innate immune cells also release cytokines that recruit and regulate other immune cells. Certain innate immune cells, antigen presenting cells (APCs), present extracellular proteins on surface molecules (major histocompatibility complex; MHC) to adaptive immune cells to induce their activation.

1.1.2 Adaptive immune cells

Unlike innate immune cells, the receptors of adaptive immune cells (e.g., $\alpha\beta$T cells and B cells) undergo rearrangement of their germline receptor DNA during development [12, 13] and B cells can further fine-tune receptor specificity during a response through somatic hypermutation. This produces a staggering array of receptors (10^{16} to 10^{18}) [14]. B cells produce soluble forms of their antigen-specific receptors as antibodies. Antibodies of different isotypes neutralize microbes inside or outside of the body, target microbes for phagocytosis, and/or initiate innate soluble antimicrobial mechanisms such as the complement and coagulant cascades, leading to osmotic lysis and limiting pathogen spread. Antibodies are also an important component of immunologic memory and provide the basis for passive immunity wherein antibodies are transferred through breast milk to immunologically susceptible offspring. Antibodies also serve as important pharmacological treatments for diseases such as rheumatoid arthritis and numerous cancers.

The effector functions of T cells differ based on their co-receptor expression. Classically, CD4 T cells are restricted by MHC type II (MHCII) while CD8 T cells are restricted by MHC type I (MHCI). There is a broad and expanding stable of CD4 T cell

subtypes, classified by effector function, or increasingly, by the transcriptional markers dictating lineage fate decisions. Together, these subsets have diverse immunologic functions, including activation of different classes of innate immune cells, B cell isotype-switching, or inhibition or resolution of immune responses. CD8 T cells are also known as cytotoxic T lymphocytes. Once activated by APCs, CD8 T cells can kill cognate MHCI/protein complex-bearing cells through a variety of mechanisms and are an important source of cytokines that shape immune responses. A critical component of the adaptive immune system is the capacity for clonal expansion. Upon activation, antigen-specific T and B cells undergo clonal expansion, increasing the number of cells capable of responding to the stimulus. During resolution of an immune response, these clones largely undergo apoptosis, contracting the pool of antigen-specific T and B cells, except for a small population of long-lived memory cells that ensure a more robust and rapid immune response upon re-exposure.

While the adaptive immune system offers advantages in terms of pathogen specificity and memory, it requires innate immune cells to “educate” and activate naïve adaptive immune cells before they can function. Additionally, it takes time to mount an adaptive immune response, generally 5-10 days, during which time the host would succumb to most infections were it not for innate immune cells. Reciprocally, adaptive immune cells participate in complex homeostatic networks, modulating innate immune cell generation by producing soluble factors that regulate their production and release from the bone marrow [15]. Consequently, a close and cooperative relationship between innate and adaptive arms of the immune system is required for a successful immune response and host defense.

1.2 Neutrophils and the immune response

Neutrophils are short lived, continuously circulating innate immune cells. Tissue inflammation activates endothelial cells, which effectively pull unactivated or partially activated neutrophils out of the circulation and into inflamed tissues. Neutrophils must then integrate numerous extracellular stimuli in order to make critical cell fate decisions such as where to go, what to do when they get there, and how to determine where *there* is. Neutrophil adhesion to inflamed endothelium initiates the decision-making process by exposing them to chemoattractant cytokines (chemokines) held close to the luminal wall. Chemokines activate G-protein coupled receptors (GPCRs) and help induce cell polarity and guide directional migration towards increasing chemokine concentrations. Differential chemokine receptor affinities, chemokine concentration gradients, competition between intracellular pathways downstream of GPCRs, and even cross-sensitization and internalization by other receptors lead neutrophils closer to the infected or inflamed site and immobilize them nearest the epicenter. In addition to guiding neutrophils, chemokine gradients are converted by neutrophils into information regarding effector function decisions. Low concentrations of the potent human neutrophil chemokine, IL-8, upregulate adhesive molecules; moderate concentrations initiate the reactive oxygen species (ROS) generation; and high concentrations result in degranulation [16] and receptor desensitization [17], the latter functionally immobilizing neutrophils at the site of greatest need.

1.2.1 Neutrophil activation

Neutrophils express a comparatively limited number of pattern recognition receptors (PRRs) that signal the presence of pathogen-associated molecular patterns (PAMPs) [18, 19] and danger-associated molecular patterns (DAMPs) [20]. An expanding number of the latter have been recognized, including factors produced in response to pain [21] or are the extracellular presence of normally intracellular proteins such as heat shock proteins [22], transcription factors [23], and N-formylated mitochondrial proteins that can indicate necrotic rather than apoptotic cellular death [24].

Perhaps the largest and best-studied family of PRRs is the membrane-bound Toll-like receptors (TLRs). These receptors link recognition of PAMPs of various classes to diverse immune responses based on discriminative intracellular signaling cascades (detailed below for TLR4). In addition to the membrane-bound TLRs, innate immune cells express other surface PRRs such as C-type lectin receptors, which recognize repetitive microbial carbohydrate sequences, and phagocytic receptors that recognize microbial components or microbes decorated by components of the complement cascade and trigger uptake and neutrophil activation. While not directly microbicidal, phagocytosis can deliver ingested pathogens to highly microbicidal vacuoles within activated neutrophils where they are destroyed.

Essentially all nucleated cells are equipped with cytosolic PRRs that recognize damage or intracellular pathogens. The NOD-like receptors (NLR) are defined by their similarity to NOD-1 and NOD-2 [25]. NOD-1/2 specifically recognize cytoplasmic nucleotides. A NLR subfamily, the NALPs, recognize diverse intracellular PAMPs and DAMPs, to activate transcription of pro-inflammatory cytokines or induce formation of the inflammasome to proteolytically activate pre-produced pro-IL-1β or pro-IL-18. In

addition to NOD-1/2, there exist other cytosolic PRRs that recognize dangerous genetic material. Foreign RNA activates retinoic acid-inducible gene-I (RIG-I)-like helicase receptors (RLRs) and melanoma differentiation-associated protein-5 (MDA-5). Bacterial or damaged host genomes induce DNA-dependent activator of interferon regulatory factors (DAI). In addition, DNA can also be indirectly recognized via RNA polymerase III-dependent transcription into dsRNA, which activates RLRs. Together, these PRRs drive type 1 interferon (IFN) production through transcription factors NF-κB and IFN response factor 3 (IRF3) to induce pro-inflammatory mediator expression [26].

1.2.2 TLR signaling

Ligand binding to TLRs initiates complex intracellular signaling cascades, which activate cellular antimicrobial responses and produce chemical signals to recruit and activate other immune cells. TLR ligation can initiate signaling cascades through 4 adaptor molecules containing a Toll-IL-1 receptor (TIR) domain; MyD88 (myeloid differentiation primary response gene 88), TIRAP (TIR domain-containing adapter protein; or MAL, MyD88-adapter-like), TRIF (TIR domain-containing adapter inducing IFN-β), and TRAM (TRIF-related adapter molecule) [27, 28]. Different TLRs employ various combinations of these adapter molecules to generate stimulus-specific effector responses. TLR4 signal transduction will be used as an informative case study due to its importance in our model system, as well as the fact that it is the only TLR to utilize all 4 adaptor molecules.

TLR4 activates the immune system [29] upon recognition of a component of the gram-negative bacterial cell wall, lipopolysaccharide (LPS) [30]. Soluble LPS binding

protein (LBP) shuttles extracellular LPS to membrane-bound CD14, which hands it off to non-covalently bound TLR4/MD-2 [31] receptor complexes to initiate downstream signaling (reviewed in [27, 28]). LPS ligation induces oligomerization of TLR4/MD-2 and recruitment of TIR adapter proteins TIRAP/MyD88 and TRIF/TRAM. A full discussion of the downstream signaling intermediaries. MyD88 signaling ultimately activates the transcription factors AP-1, through the MAPK pathway, and NF-κB, to drive transcription of pro-inflammatory cytokines (e.g., TNF-α, IL-1β, IL-6, IL-12p40). TRIF signaling induces late phase NF-κB and AP-1, along with IRF3 and IRF7 to drive production of the type 1 IFNs α and β and later production of TNF-α. Limited numbers of TLRs can generate a diversity of transcriptionally regulated immune responses, in part through early and late phase transcription factor induction. Additionally, multiple TLRs, including non-TLR PRRs, can be simultaneously stimulated in the setting of an infection (e.g., TLR4 by LPS, TLR5 by flagellin, and DAI by bacterial DNA in *P. aeruginosa* infection) and the overall immune response is determined by integrating overlapping signaling cascades and communication between multiple cell types.

1.2.3 Neutrophil function

Perhaps the most basic neutrophil function, indeed that which drew Elie Metchnikoff to study these ‘microphages’ in the first place, is phagocytosis. Phagocytosis is an active process that delivers extracellular particles to intracellular phagosomes. While not immediately microbicidal, the phagosome can rapidly mature by fusing with various neutrophil granules. There are three different types of granules, primary, secondary and tertiary, as well as non-granule secretory vesicles. Primary, azurophilic, or peroxidase-

positive, granules principally contain myeloperoxidase (MPO), but also contain various defensins and other antimicrobial proteins (e.g., lysozyme, bactericidal/permeability increasing protein (BPI), neutrophil elastase (NE) and cathepsin G) [32]. Secondary, or specific, granules are characterized by the presence of lactoferrin, which is important in iron sequestration. Tertiary, or gelatinase, granules have fewer antimicrobial proteins and are primarily filled with matrix metalloproteases (MMPs) and other proteases necessary for remodeling the extracellular matrix (ECM) and extravascular migration. Secretory vesicles are not granules per se, yet they contain chemokine and chemoattractant receptors, and β_2 integrins, among other proteins necessary for neutrophil firm adhesion and transendothelial migration (TEM) and gradient sensing necessary for chemotaxis [33, 34]. Degranulation is not an all or none process; the threshold of stimulus necessary for granules subtype mobilization differs, increasing commensurate with the potency of the granule contents and inverse to their order of formation. Primary granules require the most potent and sustained stimulus, secondary granules less, and tertiary granules the least [33, 35-38].

Another component of mature phagosomes is the NADPH oxidase complex. The importance of the NADPH oxidase complex-driven respiratory burst is underscored clinically by the susceptibility to infection found in patients with chronic granulomatous disease due to mutations in NADPH oxidase proteins. The respiratory burst generates superoxide, which alone is not highly oxidative, but rapidly degrades to hydrogen peroxide, a powerful oxidizing agent. Degranulation of MPO into phagosomes then converts hydrogen peroxide into hypochlorous acid, which is highly damaging to microbes. In addition, there are numerous other ROS and reactive nitrogen species

generated by neutrophils with antimicrobial properties that differ in reactivity, localization, membrane permeability, and function [39].

Neutrophils are also a potent source of cytokines, chemokines, and chemoattractant molecules, either directly, or indirectly. Neutrophil enzymes proteolytically activate pro-chemokines, or degrade inhibitory chemokine binding proteins [40, 41], as well as generate chemotactic fragments from ECM proteins for subsequent neutrophils to follow [42]. Neutrophils produce and are the primary target of neutrophil chemokines such as IL-8 and Gro-α/CXCL1 (reviewed in [43]). Neutrophils also can produce TNF-α [41] and IFN-γ [44], which help to differentiate and activate macrophages and DCs. Importantly, neutrophil cytokine production can circumvent exogenous signaling requirements placed upon other cells, as in macrophage IFN-γ production [45, 46], making them uniquely able to initiate immune responses against intracellular pathogens. Finally, neutrophils possess the unique effector response of extruding their decondensed heterochromatin to trap and kill bacteria in neutrophil extracellular traps (NETs) [47, 48]. NET formation requires intact NADPH oxidase function as well as MPO [48], and neutrophil elastase translocation to the nucleus appears to play a role in chromatin decondensation [49].

1.2.4 Neutrophils and resolution of inflammation

Neutrophils play a critical role in recognizing the need for, contributing to, and amplifying an immune response. However, neutrophils also help to resolve inflammation. IL-10 is a potent anti-inflammatory cytokine and neutrophil production of IL-10 reduces inflammatory monocyte TNF-α production in a model of peritoneal sepsis [50].

Neutrophils also release arachidonic acid (AA) from membrane-anchored phospholipid moieties. AA is initially converted to the pro-inflammatory autacoid, leukotriene B4 (LTB_4), by 5-lipoxygenase (5-LO) and augments neutrophil recruitment and degranulation. As an immune response progresses and neutrophil numbers increase, AA, and another 5-LO metabolite, LTA_4, can be passed to 15-LO-containing tissue cells to be converted into anti-inflammatory lipoids (LXs), resolvins, and protectins (reviewed in [51]). Specifically, LXA_4 inhibits neutrophil function and neutrophilic inflammation in a variety of tissues [52], and stimulates uptake of apoptotic neutrophils [53], which further promotes the production of LXA_4 [52]. The clinical importance of anti-inflammatory LXs is clearly seen in the cystic fibrosis airway, which is associated with defective neutrophil recruitment and immunopathology [54]. Interestingly, neutrophil exposure to pro-inflammatory eicosanoids (prostaglandin E_2, PGE_2; produced by COX-2 activity on AA) can induce neutrophil 15-LO expression to make autacoid LXA_4 [52] and begin to restrain neutrophilic inflammation, thus ensuring an early feedback inhibition mechanism restraining excessive immune activation and immunopathology.

Continuous PAMP and DAMP exposure is necessary to sustain an inflammatory response and maintain high levels of pro-inflammatory and neutrophil life span-prolonging cytokines (GM-CSF or G-CSF). Through the course of the immune response, microbes are destroyed, their products cleared, and neutrophil life span-prolonging cytokines wane, resulting in neutrophil apoptosis. Further, neutrophil effector functions of degranulation or phagocytosis drive neutrophil apoptosis [55]. Macrophages clear apoptotic neutrophils, aided by LXA_4 and other lipid mediators of resolution, through numerous receptor-mediated pathways (reviewed in [56]), a redundancy that

highlights the physiologic importance of apoptotic neutrophil clearance. Ingestion of apoptotic neutrophils drives macrophage production of the anti-inflammatory cytokines TGF-β [57] and IL-10 [58]. Phagocytosis of apoptotic neutrophils also reduces phagocyte IL-23 production [59], and ultimately impacts neutrophil development and release from the bone marrow.

1.2.5 Granulopoiesis

The bone marrow (BM) is a highly specific anatomic niche that specializes in blood cell development. Numerically, neutrophil generation is the dominant function of hematopoiesis [60], with ~10^9 cells/kg released from the bone marrow daily in the adult human [61]. Hematopoietic stem cells (HSC) are long-lived cells capable of self-renewal and possessing the ability to divide and differentiate into all blood lineages [62]. HSC are maintained in a unique BM microenvironment by expression of CXCR4, which binds BM stromal cell-produced CXCL12 [63]. An early branch point occurs when multi-potent HSCs differentiate into oligo-potent, common lymphoid progenitors (CLPs) [64] and common myeloid progenitors (CMPs) [65], which lose the ability to generate cells of the other lineage. T and B cells derive from the lymphoid lineage while the myeloid lineage is comprised of monocytes, granulocytes, and platelet and red blood cell precursors.

Interplay between relatively few transcription factors has been shown to drive lineage differentiation (reviewed in [66]). PU.1 is pro-myelocytic, and expression is required to generate CMPs from HSCs. Concomitant expression of CCAAT/enhancer binding protein α (C/EBPα) is required for the development of granulocyte/monocyte progenitors (GMPs) rather than megakaryocyte/erythroid progenitors (MEPs) [67]. In the

absence of IRF8, GMPs commit to the myelocytic lineage, and ultimately GFI-1 and C/EBPε govern terminal neutrophilic differentiation past the promyelocyte stage [60, 66]. Although neutrophils are the most numerous granulocytes, their cousins, basophils, mast cells and eosinophils, are also derived from GMP. Basophil/mast cell progenitors [68] and eosinophil progenitors [69] have been described, yet identification of a committed neutrophil precursor linking GMP to myeloblast cell stages has remained elusive [70].

1.2.6 Neutrophil granule formation

Terminal neutrophil maturation past the myeloblast cell stage, while ultimately governed by transcription factor expression, can be appreciated microscopically through the formation of specific granules in morphologically distinct neutrophil precursors; myeloblasts, promyelocytes, myelocytes, metamyelocytes, band cells, segmented neutrophils, and mature neutrophils. Myeloblasts are the last neutrophil precursor with the capacity for mitosis, and signal the switch from proliferation to differentiation [71]. Granules begin to appear in post-mitotic promyelocytes and can be classified by their principle protein components. Granules are formed and filled sequentially [72]. Therefore, specific granule contents are regulated temporally rather than through protein targeting [73]. Primary granules are formed and filled during the promyelocyte stage, secondary granules in myelo- and metamyelocyte cell stages, and tertiary granules in the band cell stage [38]. Temporal gene expression analysis during granulopoiesis has confirmed the association between known granule contents and

protein localization [74], as well as allowing for the identification and localization of novel granule proteins [75-78].

1.2.7 Regulation of neutrophil production and clearance

Mature neutrophils survive for hours or days, depending on the species. Under homeostatic conditions, their apoptotic death and nonphlogistic clearance by liver, spleen, and BM macrophages [79, 80] is necessary to keep from overwhelming the vasculature with this ubiquitous cell type. At the same time, neutrophil extravasation in response to infection demands a rapid increase in circulating neutrophils to maintain immunologic competence. Appropriately then, the production of neutrophils is tightly linked to their consumption via an elegant feedback mechanism. Granulocyte-colony stimulating factor (G-CSF) drives neutrophil release, yet is dispensable for granulopoiesis [81]. Developing neutrophils express the chemokine receptor CXCR4, whereas CXCR2 is expressed by mature neutrophils [74]. CXCL12 is chemotactic to cells expressing CXCR4, while CXCL1 and CXCL2 induce chemotaxis through CXCR2. G-CSF promotes neutrophil release through modulation of chemokine production and chemokine receptor expression. G-CSF downregulates both CXCR4 expression on neutrophils [82], and CXCL12 production by BM stromal cells [63], while at the same time increasing endothelial cell production of CXCL1 and CXCL2 [83, 84]. The balance between neutrophil-retaining CXCL12-CXCR4 signaling and neutrophil-releasing CXCL1/2-CXCR2 signaling thus regulates neutrophil release versus retention.

Phagocytosis of apoptotic neutrophil by tissue macrophages regulates G-CSF production through suppression of IL-23 production [15]. Phagocyte-produced IL-23

stimulates T cell IL-17 production, which in turn promotes G-CSF production [85]. Therefore, apoptotic neutrophils signal the presence of sufficient neutrophils and reduce G-CSF production, leading to decreased neutrophil release from the BM. If neutrophil apoptosis is reduced, as occurs in the presence of pro-inflammatory cytokines during inflammation, G-CSF concentrations increase, resulting in greater neutrophil release. This system ensures that neutrophil production can dynamically meet (yet does not exceed) demand, and allows for the immediate escalation of an inflammatory response.

Only 1-2% of mature neutrophils are found freely circulating in the blood [86]. The remaining mature neutrophil pool is sequestered in the capillary networks of various tissues [87]. This marginated neutrophil reserve is rapidly, if not currently, available to respond to inflammatory stimuli. Tissues with high proportions of marginated neutrophils include the lung [88], liver, spleen, and BM [89]. Further homeostatic mechanisms exist to ensure that optimal numbers of neutrophils are optimally positioned to respond to infection. Frenette and colleagues have recently described diurnal oscillation of HSC and neutrophil recruitment to BM and tissues corresponding with increased tissue neutrophil localization during periods of activity [90].

Apoptotic neutrophils are not readily detectable in the circulatory system, yet 10^{11} neutrophils are replaced daily in the average human. Therefore, a highly efficient mechanism must exist to remove these cells. As neutrophils age, CXCR2 expression decreases and CXCR4 expression increases. Just as the dynamic balance between these receptors contributes to neutrophil bone marrow egress, increased CXCR4 helps drive senescent neutrophils back to the BM where they are phagocytosed by stromal macrophages [91]. Kupffer cell clearance of apoptotic neutrophils in the liver has also

been demonstrated, but rather than relying on differential CXCR4 expression, Kupffer cell-mediated clearance is P-selectin-dependent, and relies on recognition of phosphatidylserine, whose surface expression increases on senescent neutrophils [79].

1.3 Neutrophil recruitment

Neutrophils are critical early effectors of immune responses. Such responses occur in the tissues, whereas neutrophils circulate through the vasculature. Therefore, neutrophils must be recruited out of the circulation and through the extravascular tissues to the site of infection or inflammation. At the tissue level, chemokines and chemoattractant molecules serve to activate neutrophils and provide directional cues in the form of overlapping chemical gradients. Chemokine localization and potency, and chemokine receptor desensitization and internalization, help to guide neutrophil recruitment towards, and halt neutrophil recruitment at, sites of infection or inflammation. 'End-target' molecules are derived from bacteria and are more potent chemoattractants than tissue-derived chemokines[92]. They indicate the presence of bacteria in the immediate vicinity and, in conjunction with high local chemokine concentrations, trigger neutrophils to switch from migration to effector functions.

At the cellular level, circulating neutrophils begin rolling on inflamed endothelia-expressed selectins through expression of CD44, low affinity β_2 integrins, and P-selectin glycoprotein ligand-1 (PSGL-1). Slower, rolling neutrophils are exposed to and activated by chemokines, such that they firmly adhere to the endothelium via neutrophil β_2 integrin LFA-1 ($CD11_a$/CD18; $\alpha_L\beta_2$) and Mac-1 ($CD11_b$/CD18; $\alpha_M\beta_2$) interactions with endothelial intercellular adhesion molecules (ICAMs). Owing to vascular anatomy, neutrophil

adhesion usually occurs in the post capillary venule where blood flow is slow yet the vascular lumen is not too narrow as to be occluded by stationary neutrophils [60]. Adherent neutrophils then cross the endothelium through the process of transendothelial migration (TEM). Extravascular neutrophils sense chemokines using chemokine GPCRs and rapidly move up gradients.

At the molecular level, signals transmitted via GPCRs signal through cascades that activate neutrophil integrins to change into a higher affinity conformation state, called inside-out signaling. Neutrophil integrin binding to endothelial ICAMs causes further intracellular signaling (outside-in signaling) to maintain firm adhesion and induce cell spreading. GPCR signaling initiates cytoskeletal changes of adherent neutrophils through the Rho family of small molecular weight GTPases. Rho, Rac and Cdc42 GTPases are self-organizing and create a polarized cell morphology. Rac2 mediates filamentous (F)-actin polymerization at the leading edge (lamellipodium) [93, 94], and RhoA is localized by Rac1 [95, 96] to mediate actinomyosin contraction at the trailing edge (uropod). Together, these Rho GTPase activities generate sustained cellular movement, however, Cdc42 aligns the intrinsic activated neutrophil polarity with the extracellular chemoattractant gradient by localizing Rac2 to lamellipodia [97] and generating long-range signals in uropods [98].

Many immune cell types are recruited into the tissues and non-recruited blood cells, such as platelets, also express integrins. Consequently, much of our understanding of the molecular components of cellular activation, TEM and migration reflect a composite cell, with contributions from many cell types. Many individual proteins or protein isoforms critical to function in one cell type have yet to be evaluated

in neutrophils specifically. Further, focal adhesions (FAs), have been rigorously studied in slow moving cell types such as epithelial cells and fibroblasts. Neutrophils move much more rapidly and thus, cellular adhesion and de-adhesion is dynamic and cell contacts are transient in nature. As such, FAs do not have time to mature and technically do not exist in neutrophils. Immature FA-like focal complexes and more dense focal contacts are analogous in function to FAs, however, the molecules regulating the generation and disassembly of FAs have not been thoroughly investigated in neutrophil adhesive structures. The following sections will discuss the cellular and molecular mechanisms governing TEM and neutrophil migration. However, gaps in knowledge of neutrophil-specific proteins and function exist and will be indicated, while known proteins from other cell types will be presented discussed to physiologic processes generally.

1.3.1 Cellular components of transendothelial migration

Neutrophil recruitment is composed of rolling, adhesion, TEM and tissue migration, depicted as a schematic for the lung in Figure 1. In tissues such as the lung or intestines, neutrophils must further transit the epithelial barrier via trans*epithelial* migration (TEpM). Crawling, wherein cells adherent to the vascular endothelium migrate laterally prior to transiting the endothelium, is a recently recognized component of TEM preceding neutrophil extravasation, or diapedesis [99]. This process is uniquely dependent on the integrin Mac-1 and is readily revealed *in vivo* using intravital

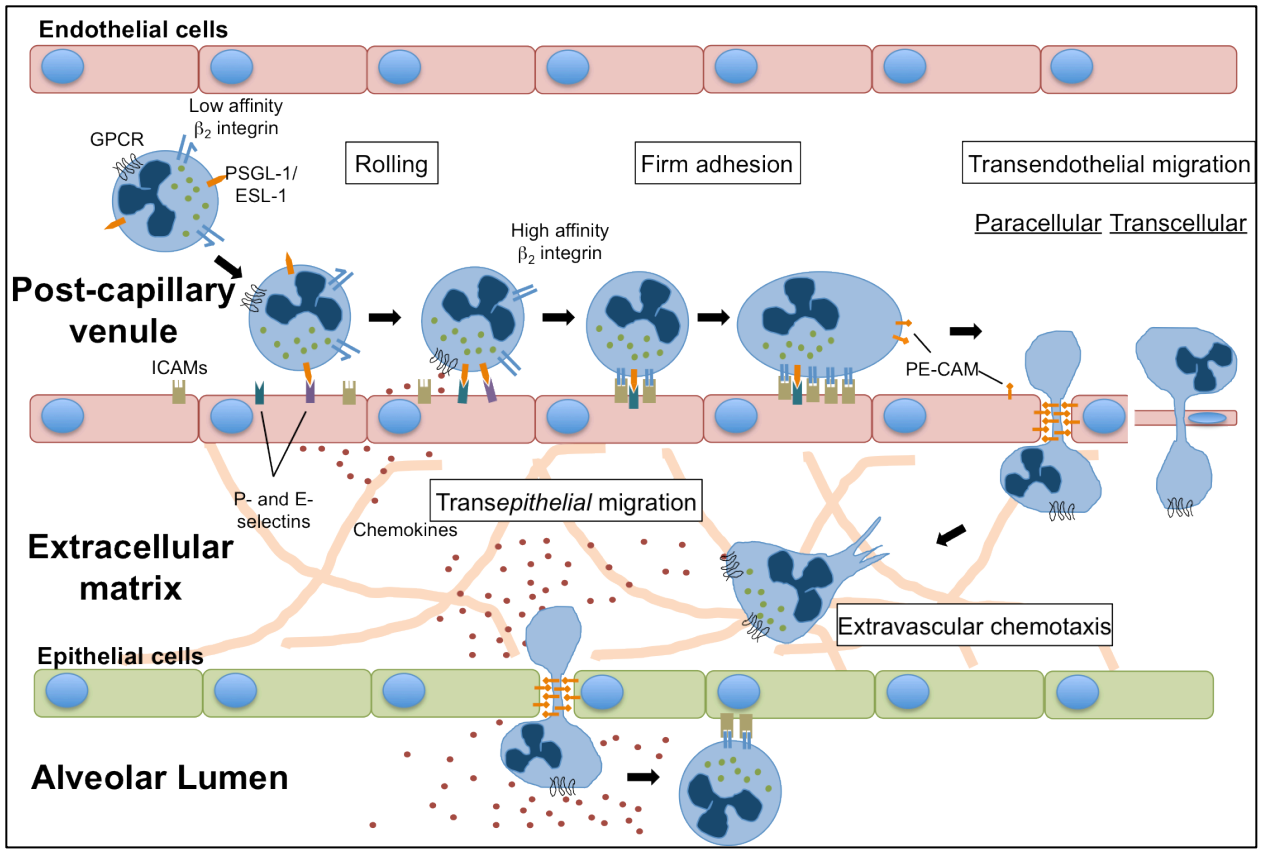

Figure 1. Sequence of events in neutrophil extravasation. *Neutrophil rolling, firm adhesion, transendothelial migration (TEM), and extracellular chemotaxis are depicted with examples of key molecular components of each step indicated. PSGL-1/selectin interactions and GPCR ligations activate integrin to high affinity states through inside out signaling. Integrin clustering further activates neutrophils through outside in signaling to activate cytoskeletal changes and induce cell spreading. Blood flow in this cartoon is to the right. Paracellular TEM occurs preferentially at endothelial cell junctions. Transcellular TEM involves penetration through endothelial cells and occurs where the endothelium is relatively thin. GPCR, G protein-coupled chemokine receptor; ICAM, intracellular adhesion molecule; PE-CAM, platelet endothelial cellular adhesion molecule; PSGL-1, P-selectin glycoprotein-1.*

microscopy [100]. Neutrophils display remarkable flexibility in their ability to be recruited to virtually any tissue, yet at the same time, they are selectively recruited only to inflamed tissues. This specificity is mediated by the endothelium, through upregulation of selectins and ICAMs in response to inflammation.

Endothelial P-selectin is immediately available as it is stored pre-formed in Weible-Palade bodies, and is involved in the initial tethering of neutrophils, while E-selectin is synthesized after a few hours [101]. Neutrophils express PSGL-1and CD44, which interact with endothelial P- and E-selectin [102] to initiate neutrophil tethering [103], and more stable rolling [104], respectively. Endothelial ICAM-1 and ICAM-2 are ligands for neutrophil LFA-1 and Mac-1, although these integrins have differing binding affinities depending on their conformation state. Rolling partially activates integrins [105] and slows circulating neutrophils so they may sample chemokines close to the apical endothelial surface. Chemokine receptor signaling fully activates β_2 integrins [106] and induces the surface expression of Mac-1 [33]. Fully active integrins then mediate firm adhesion through endothelial ICAM-1/neutrophil LFA-1 interactions and intraluminal crawling by endothelial ICAM-1 interactions with neutrophil Mac-1 [100].

Firmly adherent neutrophils have two methods for extravasation: (1) paracellular TEM, wherein neutrophils arrest at or crawl until they reach tri-cellular endothelial junctions [107]; and (2) the transcellular route, involving penetration directly through endothelial cells [108]. Vascular integrity is maintained by endothelial tight junctions, established through homotypic interactions between VE-cadherin; JAM-A, -B, -C; CD99; ESAM; and PE-CAM expression by endothelial cells. Paracellularly migrating neutrophils are able to penetrate into these junctions in part due to expression of all of

the above molecules except VE-Cadherin and ESAM. Endothelial ICAM-1 guides migration to the endothelia cell junctions, and neutrophil LFA-1/endothelial ICAM-1 binding results in intracellular destabilization of endothelial VE-cadherin interactions and the initial loosening of the endothelial junction [109]. Endothelial junction-enriched ICAM-2 guides neutrophils deeper into the widening endothelial junction [108]. Subsequent neutrophil LFA-1/endothelial ICAM-1-mediated loosening of endothelial JAM-A interactions then allows for the establishment of neutrophil/endothelial JAM binding [110]. Finally, neutrophil/endothelial PE-CAM interactions lead the neutrophil past the endothelial layer, while neutrophil PE-CAM ligation upregulates laminin receptor on neutrophils, aiding passage through the basement membrane [111]. It is possible that neutrophil proteases (e.g., MMP2, MMP9) are involved in degrading the basement membrane, but inhibition or genetic depletion of these molecules minimally impacts TEM [112].

Transcellular TEM is thought to comprise some 20% of neutrophil TEM [108]. It can increase under conditions wherein neutrophils are unable to migrate to endothelial junctions, such as in $CD11_b$ deficiency [113], or in tissues with particularly tight endothelial junctions such as the brain [114]. Endothelial cell ICAM-1 ligation by neutrophil LFA-1 induces active rearrangement of the endothelial cell surface to create an endothelial docking structure found in both trans- and paracellular TEM [114]. However, in transcellular TEM, neutrophils remain localized on the endothelium and the endothelial ICAM-1 docking structure progresses into a dome that engulfs the neutrophil in a process reminiscent of phagocytosis [113].

Neutrophil integrin function is necessary for TEM, although recent studies have suggested that, under certain circumstances, cells can migrate through integrin-independent mechanisms. Lammermann and colleagues found that integrins were required for extravasation, but were dispensable for migration in 3D matrices [115]. Under these 3D conditions, F-actin polymerization continuously propelled leukocytes through tight places, essentially allowing them to shoulder their way through the crowded extravascular environment by replacing integrin-mediated adhesive structures with friction against the ECM components [115]. This finding does not supplant the established roles of integrins in adhesion and migration, although it reinforces the context-dependent nature of neutrophil recruitment and migration.

1.3.2 Trans*epithelial* migration

Infections in the lumens of mucosal tissues such as the lung or the gut require neutrophils to travel across the epithelia in a basolateral-to-apical direction to reach the site of infection. However, TEpM is not as simple as TEM in reverse. Notable differences exist in the variable requirements for Mac-1 in TEM and TEpM. Mac-1 surface expression is increased through activation and is critical for TEpM in many tissues (reviewed in [116]), yet is dispensable for TEM [100]. Additionally, neutrophil firm adhesion to the epithelial basement membrane is Mac-1-dependent [117, 118], however its binding partner, yet to be defined, does not appear to be ICAM [116, 119].

TEpM often requires passage across a much thicker epithelial layer than is found in the endothelium, due to tall columnar epithelial cells. This results in a longer ‘interepithelial migration tunnel,’ something that may underlie the fact that there is no

evidence of neutrophils employing the transcellular route for TEpM [116]. However, similar to TEM, neutrophils undergoing TEpM utilize homotypic epithelial junction protein interactions, such as JAMs, to transverse the epithelium whilst preserving the tight junctions that maintain epithelial continuity and integrity. Specifically, epithelial JAM-C/neutrophil Mac-1 interactions are critical for successful TEpM [120].

Homeostatic and even most immune response-driven leukocyte recruitment through epithelial barriers does not damage the epithelium [116, 121]. Under non-pathogenic conditions, neutrophil binding to the basolateral epithelial surface can reversibly regulate tight junction loosening such that these complexes may reassemble after neutrophil passage, something that appears to happen under the direction of apical, neutrophil-derived factors [122]. However, pathologic conditions (e.g., inflammatory bowel disease, acute respiratory distress syndrome) demonstrate that excessive neutrophil recruitment and TEpM can have deleterious consequences. Large numbers of transmigrating neutrophils, and the attendant mechanical stresses involved in transmigration, may cause irreversible damage to the epithelial layer independent of the end effector functions of neutrophils localized in the tissue.

1.3.3 Unique features of neutrophil lung recruitment

Neutrophil recruitment to, or even *passage* through, the lung differs from that of other vascular beds. Anatomically, lung capillaries often are smaller in diameter than a spherical neutrophil [123]. Therefore, neutrophil deformability is requisite for physiologic transit. Microanatomically, the pulmonary vasculature is not homogeneous. On one side of the capillary wall, the endothelium is tightly apposed to the alveolar epithelium and

shares a common basement membrane. Conversely, the other endothelial wall is thicker, does not share a common basement membrane, contains fibroblasts and ECM within the interstitial space, and is highly enriched for endothelial tri-cellular junctions (reviewed in [123]). Pericyte contacts with endothelial cells disrupt the basement membrane near tri-cellular corners, although these basement membrane disruptions are plugged by (myo)fibroblasts.

Neutrophil migration in most tissues occurs at the post capillary venule, however, lung neutrophil recruitment occurs in the capillary bed. Stochastic neutrophil arrest would allow for ~10% of neutrophil adherence at endothelial tri-cellular junctions, however they are the preferred location of neutrophil TEM in alveolar capillaries (60%) [107]. At first glance, transit through a thicker, denser tissue and penetration of two basement membranes seems counter-productive to efficient neutrophil recruitment. However, endothelial tri-cellular junctions are located near pericyte-mediated, and myofibroblast-blocked holes in the basement membrane. Under inflammatory or stressful conditions, myofibroblasts reversibly contract, revealing preformed passageways for neutrophils to transit the basement membrane. Further, myofibroblasts serve as an endothelial-epithelial cellular bridge along which neutrophils may crawl to reach similar preferential passage sites in the epithelial basement membrane [123].

1.3.4 Molecular components of chemotaxis

The cellular components of neutrophil recruitment have attendant molecular mechanisms underlying their function. Rolling, firm adherence and TEM are dynamic processes with bidirectional molecular signaling between neutrophil integrins and

cognate endothelial ligands. However, this discussion will focus almost exclusively on the molecular components of neutrophil diapedesis and chemotaxis in the interest of space, and in light of a dense and ever-expanding literature. Further, chemokine-dependent adhesion and migration pathways also activate neutrophil effector functions such as degranulation or ROS generation, however this section will focus solely on the molecular components leading to chemokine-mediated adhesion and migration.

Chemotactic factors stimulate neutrophils through GPCRs. Ligand binding to GPCRs causes intracellular $G\alpha$ subunits to swap GDP for GTP and dissociate from the $G\beta\gamma$ complex. $G\alpha$ subunits, classified as G_s, G_i, and G_q isoforms, ultimately activate or inhibit protein kinase A (PKA; G_s or G_i) or activate phospholipase C (PLC; G_q). PKA regulates the activity of downstream proteins through phosphorylation, while PLC cleaves phosphatidylinositol 2-phosphate (PIP_2) into diacylglycerol (DAG) and inositol 3-phosphate (IP_3). IP_3 increases intracellular Ca^{2+} concentrations, which, in conjunction with DAG, activates protein kinase C (PKC). As depicted in Figure 2, the $G\beta\gamma$ complex can directly activate a number of proteins, including phosphatidylinositol 3-OH kinase (PI3K). PI3K phosphorylates PIP_2 into PIP_3, which can activate Akt. PIP_3 is converted back into PIP_2 by phosphatase and tensin homolog (PTEN) to downregulate Akt activity, although other molecules, such as SHIP1, have similar function and may play a more specific role in neutrophil chemotaxis [124]. PI3K exists as different isoforms. Class IA PI3K regulatory subunits contain SH2 domains, which localize PI3K near activating tyrosine kinases at the cellular membrane. Class IB, PI3Kγ, however, can be directly activated by the $G\beta\gamma$ complex [125].

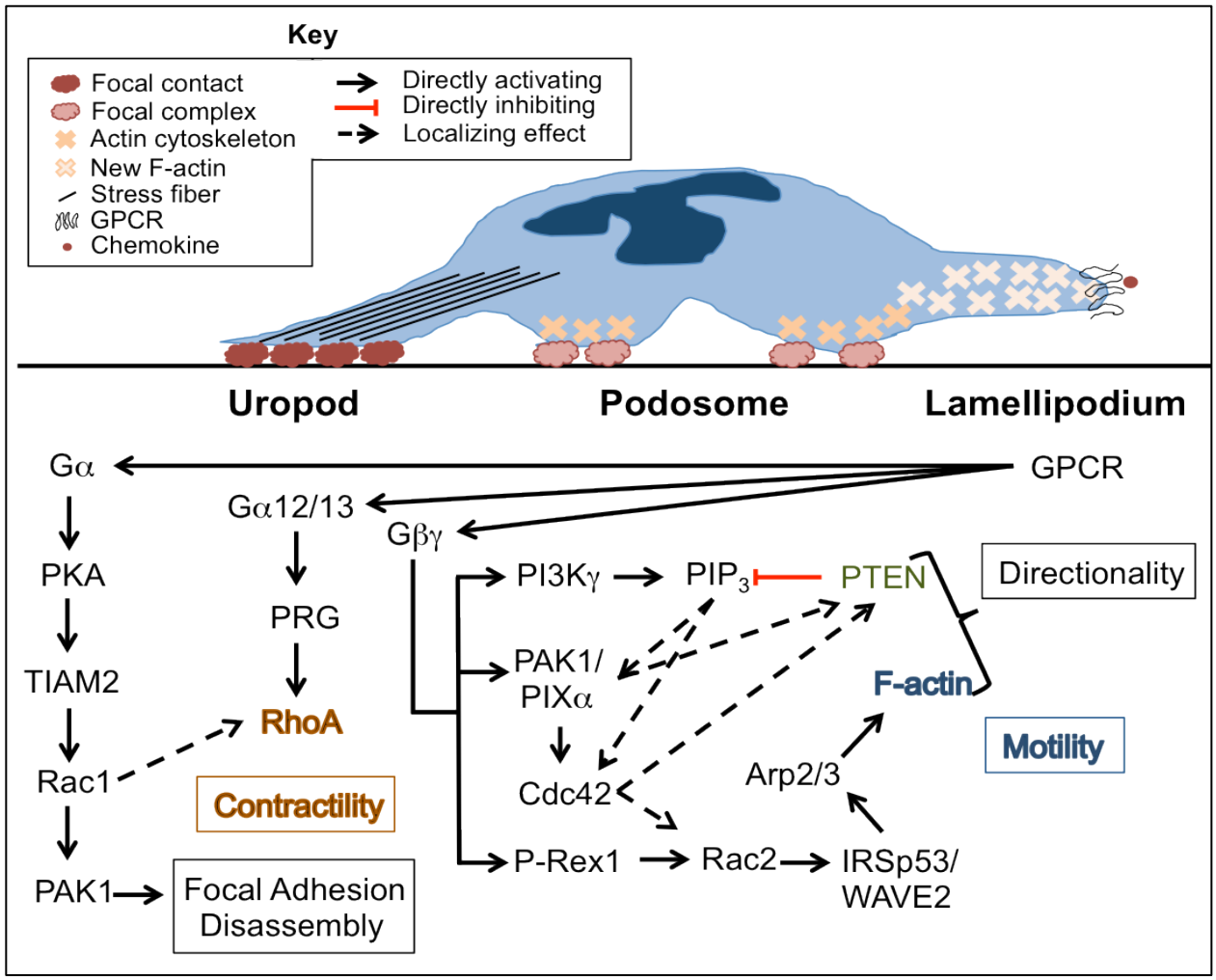

Figure 2. Molecular components of GPCR-induced neutrophil polarity and chemotaxis. *Neutrophils assume a polarized morphology aligned with the external gradients to ensure directional migration. Depicted are the molecules involved in motility, directionality and cellular contraction activated downstream of GPCR ligation. Activating (solid arrows) and inhibiting (red bar) cellular interactions are indicated along with those driving subcellular protein localization. GPCR, G protein-coupled receptor; IRSp53, insulin receptor substrate protein 53; PAK, p21 activated protein kinase; PI3K, phosphatidylinositol 3-OH kinase; PIP_3, phosphatidylinositol 3 phosphate; PIXα, PAK interactive exchange protein alpha; PKA, protein kinase A; PRG, PDZRhoGEF; PTEN, phosphatase and tensin homolog; TIAM2, T cell invasion and metastasis 2; WAVE2, WASp family verprolin-homologous 2.*

The Src family of kinases (Hck, Fgr, and Lyn) and Syk tyrosine kinase are also important in neutrophil function. These kinases are active in immunoreceptor signaling in T and B cells and drive analogous signaling cascades in outside-in integrin signaling. In neutrophils, kinases of the Src family bind to the cytoplasmic tails of activated transmembrane proteins and phosphorylate them and other proteins to effect downstream signaling pathways. Targets of Src family kinase proteins include adaptor molecules containing an immunoreceptor tyrosine-based activation motif (ITAM), such as DAP12 and the Fc receptor common γ chain (FcRγ). These phosphorylated adaptor molecules can then interact with the SH2 domain of Syk, localizing it to the membrane for phosphorylation and activation by Src family kinases. Phosphorylated, activated Syk can then phosphorylate downstream targets, including Rho-GTPases.

The Ras superfamily of small molecular weight GTPases comprises a vast interconnected intracellular network with important effects in cellular survival, function, migration, gene transcription and growth, among other processes. The Rho family GTPases, Rho, Rac and Cdc42, exist in two different conformations: inactive GDP-bound; and GTP-bound active states. This bi-molecular switch is extremely responsive and transient, due to: (1) inhibitory GTPase-activating proteins (GAPs), which enhance endogenous hydrolytic ability; and (2) activating guanine nucleotide-exchange factors (GEF), which catalyze the exchange of GDP for GTP. Additionally, GDP dissociation inhibitors (GDI) sequester and stabilize the inactive form to prevent GAP and GEF-mediated GDP/GTP cycling.

In the setting of neutrophil chemotaxis, Rho-GTPases have distinct functions, and are self organizing in response to stimuli. Rac2 is responsible for F-actin

polymerization and directional movement. If Rac2 is the engine of the cell, Cdc42 is the steering wheel, coupling directional movement to external gradients through gradient sensing. Cells defective in Cdc42 activation or localization generate comparable F-actin, but fail to move up chemoattractant gradients. Cdc42 also generates signals in the back of the cell, which control uropod function. Finally, Rac1 localizes RhoA at the rear of the cell where it mediates actinomyosin-based contraction. Additionally, Rac1 has been shown in epithelial cell lines to directly effect FA disassembly necessary for cellular chemotaxis.

1.3.4.1 *Rolling*

Unactivated, circulating neutrophils express surface proteins that are bound by proteins expressed by activated endothelium to initiate rolling. Endothelial selectins bind neutrophil-expressed PSGL-1 to induce intracellular signaling through Src kinases. Specifically, E selectin-activated PSGL-1 results in the phosphorylation of the Src kinase Fgr, and ultimately Syk, recruited through SH2 domain interactions with phosphorylated adapter proteins DAP12 and FcRγ [105]. Active Syk phosphorylates Bruton's tyrosine kinase, which regulates PLCγ_2 to activate the p38 MAPK pathway [126], and PI3Kγ [127]. PLCγ_2-driven p38 MAPK is necessary for partial LFA-1 activation, although the exact signaling cascade is unknown. PI3Kγ is induced through inside-out as well as outside-in signaling pathways and is critically important to align neutrophil polarity with external gradients.

Selectin/PSGL-1 binding also exposes intracellular PSGL-1 juxtamembrane binding sites for proteins of the ezrin-radixin-moesin (ERM) family. Similar to DAP12

and FcRγ, ERM proteins function as linking proteins, however, instead of recruiting tyrosine kinases, ERM proteins bind and link F-actin to the cytoplasmic tail of PSGL-1 [128] and are necessary for neutrophil rolling [129]. ERM proteins also contain ITAMs, which serve as scaffolding for Syk membrane localization and phosphorylation-based activation [128]. Activated L-selectin also binds to the cytoskeleton, although it accomplishes this through α-actinin, whose binding is increased by the presence of proteins talin and vinculin [130].

1.3.4.2 *Firm adhesion*

While neutrophil PSGL-1 can interact with endothelial selectins to slow circulating neutrophils through rolling, firm adhesion requires neutrophil integrin interactions with endothelial ICAMs. Integrins are heteromeric type I transmembrane proteins composed of α and β subunits. Leukocyte-restricted β_2 integrins [131] are constitutively expressed and, in the case of Mac-1, upregulated in response to chemotactic signals [33]. Unactivated integrins exist in a low affinity, 'bent' conformation, and are converted to an intermediate affinity 'extended' conformation by PLCγ_2 signals received by rolling [132] within as little as 0.4 seconds [106]. Extended, intermediate affinity integrin interaction with ICAMs and concomitant mechanical stress provided by circulatory shear forces mediate the transition to the high affinity conformation capable of firm adhesion [133-135]. Signals generated by rolling (Syk-mediated PLCγ_2 and p38 MAPK activation, and PI3Kγ activation) are able to induce the extended, but not the high affinity, integrin conformation [136, 137]. Signals downstream of GPCRs are necessary for full integrin activation [137].

GPCR-driven Gβγ signaling increases Ca^{2+}, and together with DAG, activates the appropriately named Rho-GTPase GEF, CalDAG-GEF. In lymphocytes and platelets, CalDAG-GEF activates and relocates Rap1 to integrin cytoplasmic tails [138, 139]. Rap1 activation recruits talin and kindlin-3 where they bind β chain subunits to facilitate breakage of αβ chain salt bridges and generate high affinity extended conformations (talins) and facilitate integrin clustering (kindlins). Salt bridge-disrupted αβ chains expose α-actinin binding sites on the β_2 subunit tail [140], allowing for α-actinin-mediated stabilization of active conformations. While CalDAG-GEF dysfunction in neutrophils is implicated in decreased neutrophil adhesion manifested by leukocyte adhesion syndrome type III [141, 142], and the recruitment of talin-1 and kindlin-3 to LFA-1 integrin clusters has been demonstrated [143], neutrophil utilization of Rap1 and the participation of talin-1 downstream of CalDAG-GEF in neutrophils remain unclear.

High affinity state neutrophil integrins cluster with endothelial ICAM to increase avidity and adherent neutrophil resistance to shear forces. Integrin/ICAM interactions also drive outside-in neutrophil signaling, which is critical for neutrophil and other myeloid cell functions such as adhesion, spreading, chemotaxis and granule release [144]. Src and Syk tyrosine kinase families are implicated in outside-in signaling, as deficiency of these proteins results in inhibition of critical neutrophil functions following integrin ligation [144, 145]. Further, these intracellular signaling molecules seem to be specifically essential to outside-in-mediated integrin signaling events, as Src family (Hck, Lyn and Fgr)-deficient neutrophils remain sensitive to fMLP-stimulated adhesion [146] yet integrin-mediated cell spreading and firm adhesion is lost [145]. There may also be a stimulus or tissue-specific use of different intracellular signaling adapter

molecules, as Syk-deficient mice show a defect in neutrophil lung recruitment [147] and adhesion in the vasculature of the cremasteric muscle [148], but neutrophils readily enter inflammatory sites in a model of hemorrhagic vasculitis [149]. Similar to their function in neutrophil rolling, integrin-localized Src family kinases phosphorylate and activate Syk as it is brought in range through associations between its SH2 domains and ITAM-containing adapter molecule(s) (possibly FcRγ or DAP12), which are membrane-localized next to integrin subunits [150].

1.3.4.3 *Polarization and chemotaxis*

Firmly adherent neutrophils spread out upon their substrate, polarize and begin migrating up chemotactic gradients. Neutrophils polarize in response to stimulation, with or without a gradient, however, their polarity aligns with external gradients as shallow as 2% across the width of the cell [151]. Polarized cells assume an elongated morphology with a blunted leading edge (or pseudopod) and narrow, contractile tail. At the leading edge, the pseudopod is composed of a rapidly polymerizing F-actin-filled lamellipodium, while more stable, actinomyosin structures are found along the sides and back (or uropod) of the cell. Some chemoattractant receptors are concentrated at the leading edge of the cell. However, polarization and directional migration occurs in response to ligation of receptors that are evenly distributed throughout the cell membrane [152]. Therefore, intracellular mechanisms must exist to establish asymmetrical intracellular protein localization independent of receptor distribution.

Chemoattractant GPCR-linked Gβγ complexes can directly activate PI3Kγ, and neutrophils from PI3Kγ-deficient mice show marked defects in migration [127, 153, 154].

However, PI3Kγ-deficient neutrophils *can* assume a polarized morphology, although they fail to align their polarity with external gradients [155]. Gβγ also directly activates Cdc42 through recruitment of the heteromeric GEF complex PAK1/PIXα, and PIP_3 localizes active Cdc42. Gβγ-mediated generation of PIP_3 additionally localizes PAK1/PIXα to the membrane and in this manner, PIP_3 determines where active Cdc42 is localized, even if PIP_3 is dispensable for Cdc42 activation and F-actin co-localization [156]. Cdc42 controls cellular polarity by localizing Rac2 activity to the leading edge [97], however, active Cdc42 also mediates long-range actions through the effector protein WASp [98].

PI3Kγ-activating signals generate PIP_3 to localize active Cdc42, yet PIP_3-degrading molecules are also important to restrict PIP_3 to a limited portion of the leading edge of the cell and thereby create an amplified intracellular polarity in response to a shallow external gradient. One of the first candidates for this counter-regulation of PIP_3 was PTEN, whose localization is also dependent on PIXα [156]. Yet some have questioned PTEN's necessity, and have identified another protein, SHIP1 that can also convert PIP_3 into PIP_2 in response to chemoattractant stimulation [124].

F-actin polymerization occurs in the absence of Cdc42 localization [127, 156] and is known to be dependent on Rac2 in primary neutrophils [93, 94, 157]. Rac activation in neutrophils is thought to predominantly be controlled by the Rac GEF P-Rex1, which is also activated by Gβγ [158], and by PIP_3-activated DOCK2. Rac-mediated F-actin polymerization occurs through an adapter protein, IRSp53, which recruits the WASp family protein, WAVE2. IRSp53-bound WAVE2 changes conformation to expose an Arp2/3 binding and activation site so that Arp2/3 can serve as a nucleation and

branching point for F-actin polymers (reviewed in [159]). Contrary to Rac2 localization, Rac1 is implicated in uropod formation and cell-body contraction through localization of RhoA and its attendant signaling cascade [95]. Rac1 also has recently been described to play a critical role in mediating FA disassembly, a necessary component of cellular motility [160].

Generation and restriction of PIP_3 to the leading edge can explain the spatial asymmetry of proteins involved in neutrophil polarity and chemotaxis, yet it provides no explanation for the amplification of shallow external gradients. Nearly 40 years ago, Gierer and Meinhardt proposed a theory that addresses the self-organized polarity found in stimulated cells [161-163]. In their theory, localized positive feedback loops amplify internal signaling cascades while global inhibitory mechanisms are instigated by the same stimulus, which serve to continually inhibit cascade spreading or initiation of multiple cascades at different locations. PIP_3 and F-actin polymerization form one such positive feedback loop as PIP_3 acts upstream of Cdc42 and Rac, while Rac-mediated F-actin polymerization can trigger PIP_3 accumulation [164]. However, PIP_3 generation is critical component of the positive feedback loop, as symmetrical PIP_3 generation is sufficient to establish polarity while uniform Rac activation is not [165].

Self-amplifying and -reinforcing signals generate the pseudopod through PTx-sensitive signaling pathways ($G\alpha_i$-linked $G\beta\gamma$). However uropod formation is unaffected in PTx-treated neutrophil cell lines, implicating PTx-insensitive $G\alpha12$ and $G\alpha13$ proteins in this pathway [166]. These uropod signaling pathways help to limit pseudopod signaling cascades from spreading, as indicated by uropod pathway-defective cell generation of multiple pseudopod formation, while cells with constitutively active uropod

pathways are unable to generate F-actin in response to point stimuli [166]. Gα12 and Gα13 sequentially activate the RhoA GEF PDZRhoGEF (PRG), RhoA, ROCK, phosphorylated myosin light chain (p-MLC), and ultimately activate myosin II, which results in contraction of the actinomyosin stress fibers to retract the rear of the cell [152, 166]. Finally, the downstream products of pseudopod (F-actin) and uropod (actinomyosin assemblies) are inhibitors of the opposing pathway at the level of the Rho-GTPase [166]. Thus, initial (within 30 seconds) stimulation-induced F-actin ruffling of the entire cell membrane collapses (within 2 minutes) into self-organized, segregated and mutually exclusive lamellar F-actin in the pseudopod and actinomyosin complexes laterally and in the uropod. These subcellular processes are manifest at the cellular level in that polarized neutrophils respond to changing chemoattractant gradients by following their leading edge, and will make a U-turn rather than reverse polarity as that would require *de novo* pseudopod formation.

Concomitant with F-actin protrusion and actinomyosin contraction, migrating neutrophils must form new cellular/extracellular pseudopod attachments while coordinately disassembling uropod attachments. FAs are large, heteromeric complexes that link the actin cytoskeleton to extracellular surfaces through integrin interactions. Unlike slower moving cells such as fibroblasts and epithelial cells, neutrophils do not develop true FAs. Rather, neutrophils generate more dynamic and short-lived focal complexes under the lamellipodium, which coalesce as the cell moves into focal contacts laterally and in the uropod. Talins and kindlins are important for the stabilization and clustering of high affinity conformation integrins, yet they are also implicated in FAs. Talin/integrin interactions recruit vinculin, which increases integrin

clustering [167] and links to actin via associations with the vinculin tail [168]. Talin-1 and kindlin-3 associated with LFA-1 additionally help to drive local Ca^{2+} release and cytoskeletal reorganization [143]. Maturing focal complexes incorporate more intracellular proteins and integrins, and transition into increasingly dense focal contacts. Focal contacts are dense, elongated structures associated with stress fibers that are controlled by myosin II [169].

Focal complexes/contacts are critical to provide the cellular attachment points necessary for polymerized F-actin to push against and extend the cell membrane at the lamellipodium. However, their disassembly at the rear of the cell is required for proper cellular translocation. While best studied in FAs, the following regulatory mechanisms of FA disassembly may be extrapolated to form a basis of understanding for possible focal complex regulation in neutrophils. A principal player in FA disassembly, PAK1, initially seems counterintuitive due to its essential role in establishing polarity at the lamellipodium. However, Rac1, which is localized to the uropod and implicated in cell tail retraction [95, 96], has a 5-fold greater ability to activate PAK1 than Rac2, which is localized to the lamellipodium [94, 157]. PAK1 localizes to FAs [170] and phosphorylates the integral FA protein paxillin, to increase disassembly rates [171]. Additionally, PAK1 phosphorylates and inactivates MLC kinase [172], reducing the myosin II-mediated pro-FA effect. Microtubules (MTs) also regulate FA disassembly by multiple MT targeting of FAs and their association with various proteins, including FA kinase (FAK) [173].

MT growth can also activate Rac1 in fibroblasts [174] and epithelial cell line cells through the Rac1 GEF, TIAM2 [160]. TIAM2 is a homolog of TIAM1, and, unlike TIAM1,

which can activate Rac1, Rac2 and Rac3, TIAM2 is specific to Rac1 [175-177]. Recently, Malliri and colleagues found that MT growth specifically activated Rac1 through TIAM2, and active Rac1 participates in a positive feedback loop to target MT to FA. This locally increases active Rac1 and effectors such as PAK1, and increases the rate of FA disassembly [160]. In the absence of coordinated FA disassembly, cells demonstrated larger FAs, decreased rates of disassembly, and reduced migration velocity [160].

1.4 Chemokines

Chemotactic molecules are required to fully activate integrins for neutrophil firm adhesion and extravasation. Additionally, chemotactic stimulation helps induce neutrophil polarization, and chemotactic gradients provide the directional cues that guide neutrophils to sites of infection or damage. Bacterial products (e.g., fMLP), components of the complement cascade (e.g., C5a), and even ECM degradation fragments (e.g., N-acetyl proline-glycine-proline (PGP) [42]) are highly chemotactic and indicate the presence of microbes, host damage, or inflammation. Various stimuli induce the production of endogenous chemoattractant cytokines, or chemokines, by different cell types with varying kinetics. Immune effector cells express numerous chemokine receptors, and this combination of chemokine receptor production and differential chemokine receptor expression shapes the nascent immune response.

Neutrophil function and recruitment are highly dependent on chemokines. However, numerous overlapping, and often non-congruent, chemotactic gradients are present under homeostatic conditions, and even more during inflammatory responses.

Neutrophils derive specific information from this complex chemical milieu. For example, LPS administration to the lung induces the production of CXCL1 and CXCL2, both chemotactic to neutrophils through binding to the receptor CXCR2. CXCL2 is retained in the tissue while CXCL1 is actively transcytosed into the vasculature [178], thus establishing long- and short-range neutrophil recruitment signals. Rolling neutrophils are activated by CXCL1 and extravasate, where they are now preferentially responsive to CXCL2 gradients, as CXCL2 has a higher affinity for CXCR2 than CXCL1. As neutrophils migrate into higher concentrations of CXCL2, CXCR2 becomes increasingly desensitized, and in the presence of LPS, CXCR2 is internalized, effectively blinding neutrophils to these chemokines and immobilizing them at the site of the needed effector response (reviewed in [179, 180]). Chemokines induce additional effector responses such as degranulation and NET production, however, this section focuses on their chemotactic function.

1.4.1 Nomenclature

Chemokines are small molecules (8-12 kDa) organized into families based on the sequence of a conserved 4-cysteine motif near the amino terminus: CXC, CC, C (or XC), and CX_3C. Grouping based on primary structure correlates well with function and receptor specificity, as competition for receptors between subclasses is rare. Chemokines across classes possess highly conserved tertiary structure, which in most cases, are required for function. In particular, disulfide bonds between the 1^{st} and 3^{rd} and 2^{nd} and 4^{th} cysteine residues (except in C chemokines, which lack the 2^{nd} and 4^{th} cysteine resides) help to stabilize the tertiary structure. Chemokines can form higher

order quaternary structures such as dimers and tetramers, although the physiologic role of these structures as they pertain to migration remains unclear. Monomer-only mutants of CXCL8 and CCL5 retain chemotactic ability *in vitro* [181, 182]. In contrast, monomer mutants of CCL2, CCL4 and CCL5 showed reduced *in vivo* chemotactic capacity, but this is likely due to oligomerization-dependent binding to glycosaminoglycans (GAGs) rather than inherent differences in receptor binding ability [182].

Due to their highly conserved tertiary structures, hetero-oligomerization within subclasses is perhaps not surprising, and is physiologically relevant given the simultaneous presence of numerous chemokines in immune responses. The exact effect of hetero-oligomerization depends on the assay used and receptor(s) studied. For example, CXCL4/CXCL8 heterodimers are fairly stable, and co-culture with both chemokines demonstrated attenuated CXCL8 signaling in HSC, enhanced CXCL8-induced chemotaxis in CXCR2-transfected cells, and enhanced CXCL4 anti-proliferative effects on endothelial cells [183, 184]. It is unclear if these effects are mediated at the level of hetero-oligomerization-induced differential receptor affinity, competing monomer receptor signaling, or sequestration of monomers available for signaling through modulation of GAG binding [185].

1.4.2 Neutrophil-specific chemokine receptors

A subset of CXC family chemokines possesses a conserved glutamate-leucine-arginine (ELR)+ sequence proximal to the amino terminus and prior to the first conserved cysteine residue. These have historically been viewed as neutrophil-specific chemokines. In reality, specificity is conferred by receptor expression, and it has since

been shown that a variety of cell types constitutively express ELR+ chemokine receptors in addition to neutrophils, including CD8 T cells [186], mast cells [187], intestinal epithelial cells [188], and endothelial cells [189], or have inducible expression (e.g., monocytes [190]). However, neutrophils are the predominant cell type that responds to ELR+ chemokines [191].

The human ELR+ receptors, CXCR1 and CXCR2, identified almost a quarter century ago [192, 193], differentially bind numerous ELR+ chemokines, summarized in Table 1. While both receptors engage similar G-protein subunits in response to ligation, there are differences in the downstream signaling cascades (recently reviewed in [194]). Neutrophils simultaneously express both receptors, and all CXCR2 chemokines also bind CXCR1. Thus, it is less informative to attempt to ascribe specific functions to either receptor. Nevertheless, *in vivo* studies suggest that CXCR1 exhibits slower internalization kinetics and more rapid recycling, and requires a more prolonged signal

Table 1: Human and Mouse ELR+ Chemokine Receptors and Ligands

Receptor	Sensitivity	Ligands	Human Common name	Mouse Common Name
CXCR1	Low	CXCL6 CXCL8	GCP2 IL-8	GCP-2
CXCR2	High	CXCL1 CXCL2 CXCL3 CXCL5 CXCL6 CXCL7 CXCL8	GROα GROβ GROγ ENA-78 GCP2 PPBP or NAP2 IL-8	KC MIP2 LIX CXCL7

Table 1 abbreviations: GCP2, granulocyte chemoattractant protein 2; IL-8, interleukin-8; GRO, growth related oncogene; KC, keratinocyte chemoattractant; MIP2, macrophage inhibitory protein 2; ENA-78, epithelial-derived neutrophil-activating peptide 78; LIX, LPS-induced CXC chemokine; PPBP, pro-platelet basic protein; NAP2, neutrophil activating protein 2.

to activate effector functions. Therefore, CXCR1 signaling likely indicates sustained, high concentrations of chemokine and therefore indicates the necessity of effector functions such as oxidative burst [195, 196], while CXCR2 mediates chemotaxis [194, 197].

Mice express orthologous receptors to both CXCR1 and CXCR2, although murine (m)CXCR1 was only recently identified, and is specific to mCXCL6 [198]. Prior to the identification of mCXCR1, mCXCR2 ablation was shown to abrogate neutrophil recruitment and intracellular Ca^{2+} flux in response to ELR+ stimulation, suggesting that this is the principle murine neutrophil chemokine receptor [199]. Mice have a more limited repertoire of ELR+ chemokines than humans; CXCL1 (keratinocyte chemoattractant, KC), CXCL2 (macrophage inhibitory protein 2, MIP2), CXCL5 (LPS-induced CXC chemokine, LIX), CXCL6 (granulocyte chemoattractant protein 2, GCP2) and CXCL7 (pro-platelet basic protein, PPBP; or neutrophil activating protein 2, NAP2), all of which are encoded by genes on chromosome 5. There is still some uncertainty as to which murine chemokines are homologous to which human chemokines; functional analogues are clear, however. Notably, mCXCL1 is orthologous to human paralogs CXCL1 and CXCL2, although it is analogous to CXCL8 in that it is the prototypical neutrophil chemokine [200]. Mice possess a unique ELR+ chemokine, CXCL15 (lungkine), not expressed in humans or rats, implicated in neutrophil recruitment and (aptly) lung defense, although it does not seem to bind CXCR1 or CXCR2 [201].

1.4.3 ELR+ neutrophil-recruiting chemokines in mice

CXCR2, essential for neutrophil recruitment in mice [199, 202], binds CXCL1, CXCL2, CXCL5, and CXCL7. Studies using blocking antibodies have revealed that neutrophil recruitment differentially utilizes specific ELR+ chemokines in a stimulus- and tissue-specific manner. In the setting of experimental neutrophilic lung inflammation, CXCL1, CXCL2 and CXCL5 are the principle CXCR2 neutrophil chemokines produced. Among these three ELR+ chemokines, CXCL1 is necessary to mediate neutrophil recruitment to the lung [203-206].

LPS stimulation leads to CXCL1 production by resident lung cells, including epithelial and Clara cells [204, 207, 208], alveolar macrophages [204, 209], and endothelial cells [210]. Neutrophils also make CXCL1 [211], and can augment production by other cell types [212]. LPS drives *de novo* [213] CXCL1 synthesis in a NF-κB-dependent manner [204]. NF-κB recruits CREB binding protein (CBP)/p300, which has intrinsic histone acetyl transferase (HAT) activity, to further expose the CXCL1 promoter to enhancing transcription factors such as C/EBP and poly (ADP-ribose) polymerase-1 (PARP-1) [213]. Transcriptional activation is balanced by inhibitory transcription factors such as CCAAT displacement protein (CDP), which recruits histone deacetylases (HDACs) that destabilize the promoter region. The role of other HDAC-associated, inhibitory transcription factors that regulate CXCL1 production has yet to be formally demonstrated.

In contrast to CXCL1, the CXCL2 promoter contains IRF binding sites and lacks some of the NF-κB sites identified in CXCL1. Consequently, TLR agonists such as LPS drive production of both CXCL1 and CXCL2 through MyD88- or TRIF-dependent, NF-κB or IRF production, respectively, while dsRNA-driven TLR3 signaling results in purely

CXCL2-mediated neutrophil recruitment [214]. This understanding helps to shed light on the varying reliance on CXCL1 or CXCL2 in neutrophil recruitment in response to different stimuli. It is important to remember however, that infections and inflammation often engage numerous TLRs (and other PRRs), driving sequential and overlapping antagonistic and/or synergistic intracellular and intercellular signaling cascades that rarely result in the preferential expression of a single chemokine or recruitment of a single cell type.

1.5 Activating transcription factor 3

Activating transcription factor 3 (*ATF3*) is a stress- or adaptive-response gene whose expression is induced by signals indicating a disruption of homeostasis. As an immediate-early gene, ATF3 encodes a transcription factor that regulates numerous downstream genes and effects downstream transcriptional cascades [215]. $ATF3^{-/-}$ mice are developmentally normal [216], supporting the original classification of ATF3 as an immediately and transiently expressed transcription factor that helps cells deal with stressful stimuli. Additionally, ATF3 mRNA expression is low in most tissues [217], presumably due to strict suppression by its homolog, c-Jun dimerization partner 2 (JDP2) [218, 219]. Despite its name, ATF3 most frequently acts as a repressive homodimer, repressing gene transcription through direct DNA binding and HDAC recruitment to condense promoter regions. ATF3 expression is immediate [215] and transient, due to transcriptional auto-repression [220]. However, ATF3 can also activate gene transcription through direct DNA binding, as well as regulate gene transcription indirectly, through protein-protein interactions [221, 222]. Furthermore, completely

belying its name, ATF3 can even regulate cellular processes by modulating protein stability and sub-cellular localization of target proteins [222].

In the immune system, ATF3 was recently described as a counter-regulatory transcription factor, induced by TLR signaling and negatively regulating TLR-induced pro-inflammatory signals [223, 224]. As the list of immunologic gene targets of ATF3 has expanded, it has become clear that ATF3 induction and regulation is highly cell type- and stimulus-specific. This is consistent with the conclusion of Hartman et al., that $ATF3^{-/-}$ mice were developmentally normal and required stimulus to observe ATF3-dependent effects [216]. However, closer inspection has revealed that ATF3 can serve a developmental role in mast cells [225], Th1 differentiating CD4 T cells [226], and neutrophils—the latter indirectly and in combination with JDP2 dysfunction [227]. Given both positive and negative effects, the overall effect of ATF3 regulation during an immune response can be positive or negative and must be carefully evaluated in specific situations.

1.5.1 ATF3 and the ATF/CREB transcription factor family

ATF was originally identified in 1987 as a transcription factor bound to adenovirus early promoters E2, E3, and E4 [228]. In the same year, the cAMP response element (CRE) binding protein (CREB) was identified bound to the CRE site within the somatostatin promoter [229]. Later it was determined that the consensus binding sites for ATF3 [230] and CREB [231] were the same, TGACGTCA. Since then, membership in the ATF/CREB family has grown considerably. ATF/CREB family members exert their transcriptional effects as homo- or heterodimers with other ATF/CREB family members

or basic region leucine zipper domain (bZip)-containing transcription factors such as AP-1 and C/EBP protein families (reviewed by Hai et al. [217]).

ATF3 was identified shortly after ATF in tetradecanoylphorbol acetate-treated HeLa cells [232]. ATF3 is also highly expressed in the liver after partial hepatectomy—thus the historical murine designation of liver regeneration factor (LRF)-1 in rats, or LRG-21 in mice (95% homologous to human ATF3 at the amino acid level [233]). Originally, ATF3 was considered to be a stress-response gene [233] whose induction was dependent on cellular injury. More recently however, ATF3 induction has been reported in response to non-injurious stimuli, prompting a reconceptualization of the role of ATF3 as an adaptive-response gene; a gene whose product helps cells to deal with perturbations in homeostasis [215]. In addition to immune responses, ATF3 has been shown to play a role in neural development and axon (re)generation, apoptosis, metastatic transformation of cancer cells, and endoplasmic reticulum stress responses.

1.5.2 *ATF3* gene, and transcriptional and translational regulation

The gene encoding ATF3 resides on chromosome 1q in humans and mice. A cursory review of the literature reveals that ATF3 induction and transduction pathways are stimulus- and cell type-dependent. The JNK/SAPK pathway drives *Atf3* transcription in response to various stressful stimuli [234]. In fact, binding of ATF2 and c-Jun, both downstream of the JNK/SAPK pathway, increases *Atf3* promoter activity [235]. In immune responses, ATF3 is induced by TLR signaling, through TLR4 [223, 224], and TLR2/6, TLR3, and TLR9 [224]. TLR4 signaling drives MyD88-driven early phase NF-κB, and AP-1 through the MAPK pathway. Concurrent TRIF signaling drives late phase

NF-κB and AP-1 in addition to IRF3. TLR2/6 and TLR9 utilize MyD88 and drive NF-κB-regulated gene transcription, while TRIF-coupled TLR3 uses IRF-mediated type 1 IFN production. ATF3 is positively regulated by NF-κB [223], and additionally has numerous AP-1 sites [235], observations consistent with TLR2/6, TLR4 and TLR9-based induction [224]. TLR3 induction of ATF3 is not as immediately obvious as there are no reported IRF binding sites in the *ATF3* promoter, yet TLR3 stimulation induces ATF3 prior to STAT1 phosphorylation, indicating that induction is not secondary to IFN signaling [224]. Of note, ATF3 has also been shown to be induced by IFN-α [236], and TLRs that do produce type 1 IFNs, such as TLR3 and TLR4, show a strong, biphasic ATF3 induction, while TLRs that produce minimal type 1 IFNs show a weaker, monophasic induction profile [224].

Many of the studies evaluating the stress-response role of ATF3 have utilized cell lines, which may account for the utilization of different signaling pathways compared to TLR stimulation of primary mouse macrophages. However, differential promoter usage offers another explanation. An expanded inspection of the 5' flanking region to *ATF3* revealed an alternative promoter, P1 [237], approximately 40kb upstream of the originally described putative promoter [235]. Related studies determined that the P1 promoter is preferentially activated by serum exposure [238]. This could explain the preferential induction of ATF3 via JNK/SAPK pathways at P1 following stress, and through AP-1 and NF-κB regulation at the putative promoter following TLR stimulation [223], with type 1 IFN-driven STAT1 contributing at an as-of-yet undefined promoter.

ATF3 mRNA is derived from 4 exons, A (or A1 if the alternate promoter P1 is used [238]) B, C and E. Exons A and A1 both contain the 5'-untranslated region (UTR)

and share conserved linkage to exon B, which contains the translation initiation site [235]. Consequently, usage of either the alternate promoter, P1, or the putative promoter, results in generation of the same 181 amino acid protein product in humans and mice, although mRNA stability and translation efficiencies differ between transcripts [238]. Exon C contains the basic region while exon E contains the leucine zipper domain and 3'-UTR [235]. An isoform, ATF3ΔZip, exists in which an alternatively spliced exon D introduces a stop codon and results in a truncated protein lacking the leucine zipper [239]. Different inciting stimuli have resulted in identification of various ATF3 isoforms in addition to ATF3ΔZip, ATF3ΔZip2c and ATF3ΔZip3 [240], ATF3ΔZip2a and ATF3ΔZip2b [241], and ATF3b [242]. However, the signaling events that induce formation of ATF3ΔZip isoforms over full length ATF3 remain unclear.

ATF3 protein stability is regulated by mouse double minute 2 (MDM2) in response to DNA damage [243]. MDM2 is an E3 ubiquitin ligase and is necessary for ATF3 degradation. Interestingly, MDM2 binds to ATF3 in the basic region and adds ubiquitin moieties to the leucine zipper domain [243]. While all ATF3 isoforms retain the basic region, the various ATF3ΔZip isoforms lack the leucine zipper domain. Thus, ΔZip isoforms may escape regulation at the protein level, at least through MDM2. This is significant in that serum exposure induces ATFΔZip [239], as well as transcription preferentially through the alternative promoter P1 [238]. The alternative promoter P1 has also been identified as the target of epigenetic changes leading to increased ATF3 expression in human cancer [238]. Together, these observations suggest that ATF3 mRNA and protein expression must be tightly controlled.

1.5.3 Regulatory functions of ATF3

ATF3 is predominantly considered to be an adaptive-response transcription factor, given its expression profile and leucine zipper DNA binding domain. However, ATF3 has also been reported to exert regulatory action through direct protein-protein binding. ATF3 has also been reported to bind to the androgen receptor (AR) and inhibit AR-mediated signaling [221]. ATF3 also directly binds to and stabilizes p53 protein expression by preventing its ubiquitination and degradation [222]. ATF3/p53 interactions are complex. Each gene contains the other's binding site and post-translational interactions also modify function. MDM2 directs ATF3 degradation, however it also ubiquitinates p53, and it is thought that an ATF3/p53/MDM2 complex may regulate ATF3 and p53 protein function in addition to stability [243].

Transcriptionally, ATF3 regulates gene expression through both ATF3/DNA and ATF3/protein interactions. ATF3 dimerizes through bZip interactions in the basic region. Dimers then bind to and inhibit target promoters containing ATF/CREB binding sites through direct interactions with HDAC1 [223]. ATF3 transcriptional auto-repression is dependent, in part, on direct ATF3/HDAC3 and HDAC6 interactions, as well as associations with other HDACs [219]. ATF/CREB family members can also regulate transcription by occupying other transcription factor binding sites (notably AP-1 binding sites) to inhibit binding of activating transcription factors [217]. It is unclear if ATF3 specifically does this, as well as whether non-consensus site-bound ATF/CREB proteins retain their normal transcriptional functions or simply exclude other, activating transcription factors. ATF3 can also activate gene transcription as a heterodimer. ATF3/c-Jun or ATF3/JunD heterodimeric complexes activate artificial promoters [244,

245], and ATF3/C/EBP complexes activate the gadd153/Chop10 promoter, which contains a composite ATF3 and C/EBP binding site [246]. It is unclear mechanistically how heterodimerization alters transcriptional activity. A partial list of immune genes regulated by ATF3, presented in Table 2, reveals that while direct ATF3 binding to and positive regulation of genes occurs, the majority of the ATF3 effect is repressive.

ATF3 transcriptional regulation is not dependent on DNA binding. Full length ATF3 can repress artificial promoter constructs that lack ATF3 binding sites [239] and differentially spliced ATF3 isoforms exist that lack the DNA-binding leucine zipper domain. By definition, these cannot act as DNA-binding transcription factors, but they do negatively regulate gene expression [239-242, 247]. It is thought that ATF3ΔZip isoforms can sequester activating transcription factors away from the promoter region [239]. Additionally, the ATF3ΔZip2 have a novel C-terminal amino acid sequence that confers a unique ability to directly bind to NF-κB subunit p65 and inhibit p65-CBP/p300 interactions [247]. ATF3ΔZip2 retain their ability to interact with HDACs and therefore, ATF3ΔZip2 may more potently repress transcription than full length ATF3 by tipping the balance more significantly in favor of histone deacetylation and promoter condensation.

1.5.4 ATF3 regulation of immune responses

The role of ATF3 in immune responses was originally identified through two different approaches. Aderem and colleagues utilized a systems biology approach to interrogate transcriptional networks involved in the regulation of TLR-driven innate immunity. Following LPS stimulation of macrophages, they identified and subsequently

Table 2: Gene Targets and Effects of ATF3 Regulation

Gene	Effect	Cell Type	Stimulation	Regulation	Refs.
Il-1β	+	Pancreatic islet cells	Isolation	Direct/bound	[248]
Il-4	-	Th2 CD4 T cells	OVA	Direct/bound	[249]
		Mast cells	IgE receptor	Direct/bound	[225]
Il-5	-	Th2 CD4 T cells	OVA	Direct/bound	[249]
Il-6	+	Pancreatic islet cells	Isolation	Direct/bound	[248]
	-	MEF+adeno ATF3	Heat shock/LPS	Direct/bound	[250]
	-	Macrophage	LPS	Direct/bound	[223]
	-	Macrophage	TLR	Direct	[224]
	-	Macrophage	Heat shock/LPS	Direct	[250]
	-	Mast cells	IgE receptor	Direct/bound	[225]
	-	DCs	TLR	Direct	[224]
	-	Whole lung	Stretch injury	Indirect	[251]
Il-10	-	DC	TLR	Direct	[224]
Il-12β	-	Macrophage	LPS	Direct/bound	[223]
	-	Macrophage	TLR	Direct	[224]
	-	RAW264 cells	TLR	Direct/Bound	[224]
	-	DC	TLR	Direct	[224]
		Whole lung	Stretch injury	Indirect	[251]
Il-13	-	Th2 CD4 T cells	OVA	Direct/bound	[249]
Ifn-γ	-	NK cells	IL-12/α-CD28	Direct/bound	[252]
	+	Th1 CD4 T cells	Th1 cytokines	Direct/bound	[226]
	-	Whole lung	Stretch injury	Indirect	[251]
Tnf-α	+	Pancreatic islet cells	Isolation	Direct/bound	[248]
	-	Macrophage	LPS	Direct	[223]
	-	RAW264 line	LPS	Direct/bound	[253]
	-	DC	TLRs	Direct	[224]
	-	Splenocytes	i.p. CpG	Direct	[224]
	-	Macrophages	TLR	Direct	[224]
Tgf-β	+	Breast Cx cell line	TGF-β	Direct	[254]
Ccl2	+	Pancreatic islet cells	Isolation	Direct/bound	[248]
	-	Whole lung	OVA	Indirect	[249]
Ccl3	-	Macrophage	IFN-γ/+ iNOS	Direct	[255]
Ccl4	-	Macrophage	LPS	Direct/bound	[256]
	-	Whole lung	Stretch injury	Indirect	[251]
	-	Macrophage	IFN-γ/+ iNOS	Direct	[255]
Ccl5	+	MEF	Heat shock/LPS	Direct	[250]
	-	MonoMac 6 line	LPS	Direct/bound	[257]
Ccl7, 8, 11 *Cxcl1, 2, 5*	-	Whole lung	OVA	Indirect	[249]
Cxcl16 *Cx_3cl1*	-	MEF	Heat shock/LPS	Direct	[250]
iNos	-	Macrophage	LPS	Direct	[223]
	-	MEF	Heat shock/LPS	Direct	[250]
Adiponectin	-	3T3-L1 adipocytes	Thapsigargin	Direct/bound	[258]
Mmp-1	-	Monocyte cell line	IFN-γ/LPS	Direct/bound	[259]
Mmp-2	-	Prostate Cx line	IL-10	Direct/bound	[260]

verified *Atf3* as a key counter-regulatory transcription factor restraining immune activation [223]. Another group had been working on an alphavirus-vectored melanoma vaccine and identified simultaneous TLR3 and TLR9 stimulation as critical to the vaccine's effects [261]. To better understand how combined TLR3/9 signaling was contributing, they profiled transcriptional changes in stimulated macrophages and identified *Atf3* as an important immune regulator of TLR signaling [224]. However, Whitmore et al. only showed direct ATF3 binding to IL-12p40 in TLR4 or TLR9-stimulated transfected cell lines [224], while Gilchrist et al. were able to demonstrate direct ATF3 binding to the IL-6 and IL-12p40 promoters following LPS stimulation of primary cells [223].

These reports, coupled with the prior generation and generous distribution of the $ATF3^{-/-}$ mouse by Tsonwin Hai's group [216], have resulted an explosion in reported immunologic effects of ATF3. Reports of ATF3 regulation falls into three classes: (1) transcriptional profiling of tissues or cell types following *in vivo* stimulation; (2) measurement of mRNA and/or protein products following stimulation *in vitro*; and/or (3) promoter occupancy following stimulation using chromatin immunoprecipitation (ChIP), electromobility shift assay (EMSA), or promoter-reporter assays in primary cells, cell lines or artificial constructs. Clearly, the strongest case for ATF3 regulation is made by ChIP-determined promoter occupancy following stimulation in primary cells, coupled to ATF3-dependent modulation of target expression. However, many reports present direct evidence of altered production *in vitro* and/or *in vivo*, in conjunction with proof-of-principle of ATF3 regulation using promoter-reporter constructs transfected into cell lines. These studies show that ATF3 regulation occurs and is likely, but not

conclusively, mediated by direct binding. Reports of altered gene transcription by indirect ATF3 regulation must be interpreted with caution. As ATF3 acts as an immediate-early response gene, these reports may indicate transcriptional changes due to global changes in immune or stress responses rather than direct regulation by ATF3.

In keeping with the originally defined immune role of ATF3 as a counter-regulatory transcription factor, the majority of the gene targets listed in Table 2 are inhibited by ATF3. However, the inciting stimuli vary from allergens [249], to heat shock [250], to cytokines [226, 254, 255, 259, 260], to viral responses [252], to modifiers of intracellular calcium levels [258], to PAMPs [223, 224, 253, 256, 262]. A notable exception to ATF3 inhibition occurs during isolation of human pancreatic islet cells. Isolated islet cells exhibit elevated pro-inflammatory cytokine production and ATF3 was shown to positively regulate their production, associated with direct promoter occupancy [248]. These studies serve to reinforce how context-specific ATF3 function is. Yet ATF3 function can also vary between cell types within the same immune context. Murine cytomegalovirus (MCMV) is a potent inducer of IFN-γ production by both NK and CD8 T cells. Curiously, ATF3 directly binds to and negatively regulates NK, but not CD8 T cell, IFN-γ production [252]. Finally, the overall effect of ATF3 regulation during an immune response varies. LPS-induced peritonitis and febrile responses are increased in the absence of ATF3 [223, 253], however ATF3 hobbles the NK-mediated clearance of MCMV, and ATF3$^{-/-}$ mice suffer from greater virus-induced liver damage [252].

As a counter-regulatory immune transcription factor, ATF3 functions as an attenuator rather than a global inhibitor of gene transcription. Early on, it was demonstrated that IL-10 production was elevated in ATF3$^{-/-}$ DCs stimulated with low

concentrations of CpGs, yet ATF3$^{-/-}$ DCs produced amounts of IL-10 equivalent to WT DCs in response to higher concentrations of CpGs [224]. This effect is also found in macrophages in response to a different TLR ligand. ATF3 inhibits macrophage TNF-α production in response to low levels of LPS [223], yet this is overcome in response to high levels of LPS stimulation [224]. Further, ATF3 participates in a regulatory circuit that discriminates between transient and persistent TLR4 stimulation [262]. Transient stimulation results in NF-κB-mediated activation of pro-inflammatory mediators as well as ATF3, which binds to and inhibits their production. Persistent TLR4 stimulation however, drives NF-κB sufficient to also activate C/EBPδ transcription. C/EBPδ acts as an enhancer of NF-κB-mediated transcriptional activation, ultimately overcoming ATF3 inhibition [262]. Together, these reports suggest that ATF3 can function to help determine the necessity of an immune response. ATF3 restrains immune activation in response to innocuous, low-level, or transient insults, while it allows (or, in the case of islet isolation, promotes) immune activation when indicated by high levels of or persistent antigen stimulation.

1.5.5 ATF3 regulation of immune cell development

ATF3 can also function as a developmental regulator of immune cell types. While the ATF3$^{-/-}$ mouse was described as developmentally normal [216], later studies identified a defect in the number of peritoneal and resident skin mast cells [225]. ATF3 plays a recognizable role in mast cell function. It is induced in response to IgE crosslinking, and negatively regulates *Il-4* and *Il-6* transcription. However, Gilchrist et al. were unable to generate *in vitro*-differentiated bone marrow derived mast cells using IL-3 stimulation for

4 weeks from ATF3$^{-/-}$ mice [225]. It is unclear if this is truly a developmental defect or a proliferative defect, as ATF3$^{-/-}$ mast cells underwent increased apoptosis in response to proliferative stimulation [225], and ATF3 plays known roles in apoptotic regulatory pathways, namely through regulation of p53 [222, 263, 264] and/or Bcl-2 [227] expression.

Recently, ATF3 has also been implicated in neutrophil development. JDP2 is an ATF3 homolog and negatively regulates *ATF3* transcription by epigenetic modification of the promoter [219, 227]. JDP2 expression increases as neutrophils mature, with lowest expression in CD11b^{+}Ly6G^{lo} immature bone marrow neutrophils, increased expression in CD11b^{+}Ly6G^{hi} mature bone marrow neutrophils, and highest expression in circulating CD11b^{+}Ly6G^{hi} splenic neutrophils [227]. Correspondingly, JDP2$^{-/-}$ neutrophils have significantly increased ATF3 expression, correlated with reduced Ly6G surface expression, increased apoptosis, and decreased bactericidal ability, but normal morphology and no reported defect in neutrophil production or tissue distribution [227]. ATF3 is an effector of JDP2 dysfunction, as ectopic ATF3 expression similarly reduced Ly6G expression but preserved morphology in *in vitro*-differentiated neutrophils [227].

1.6 Summary and scope of thesis work

Neutrophils are important effectors of innate immunity. Their rapid recruitment and potent effector functions make them indispensible to host defense against bacterial and fungal infections. However, over-exuberant neutrophil recruitment and/or activation are significant causes of immunopathology, and host morbidity or mortality. Neutrophil recruitment is a highly coordinated process initiated by chemotactic signals, often

cytokines. Chemokine stimulation induces neutrophil cytoskeletal rearrangement and coordinated assembly and disassembly of adhesive structures within seconds, a time scale preceding stimulation-induced transcriptional changes. ATF3 is a counter-regulatory transcription factor in immune activation, induced by pro-inflammatory stimuli to inhibit pro-inflammatory mediator production. ATF3 attenuates immune responses to low dose or transient immune stimulation, wherein immune activation may be more deleterious than the inciting stimulus, but does not impair immune response to more robust stimuli. ATF3 expression is low/absent in most cell types, but developmental roles have been described for ATF3 in mast cells and neutrophils.

The work presented in this thesis describes regulatory roles played by ATF3 in inflammatory and infectious models. The weight of this work is presented in chapter 2, which describes the complex role played by ATF3 in regulation of LPS-driven neutrophilic airway inflammation. Given the considerations outlined above, we hypothesized that ATF3 restrains airway inflammation in responses to microbial product challenge. In fact, the role of ATF3 is much more complex. ATF3 does indeed inhibit CXCL1 production by airway epithelial cells in response to LPS; however, ATF3 was found to play an unexpected role in facilitating neutrophil chemotaxis. Chapter 3 investigates the role of ATF3 in regulation of the immune response to *Toxoplasma gondii* infection: $ATF3^{-/-}$ mice exhibit increased control of chronic cyst burden and reduced early intracellular parasite levels through undetermined, although neutrophil-independent, mechanisms. Finally, work presented in chapter 4 explores the effect of ATF3 on clearance of persistent infection with lymphocytic choriomeningitis virus (LCMV) clone 13: $ATF3^{-/-}$ mice exhibit reduced viral titers, associated with increased

virus-specific IFN-γ-producing T cells. The final chapter discusses the relationship of these data to each other and the literature generally, and explores future directions intended to clarify the role of ATF3 regulation in neutrophil development and function.

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Chapter II: ATF3 is a novel regulator of mouse neutrophil migration*

***ATF3 is a novel regulator of mouse neutrophil migration. Boespflug, N., et al., Blood, 2013, submitted.**

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ATF3 is a novel regulator of mouse neutrophil migration

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Running Title: ATF3 regulates mouse neutrophil recruitment

Key Points

1. ATF3 inhibits lipopolysaccharide-driven CXCL1 production by airway epithelia.
2. ATF3$^{-/-}$ neutrophils exhibit defects in recruitment to the WT lung *in vivo*, and impaired chemotaxis *in vitro*.
3. TIAM2, a Rac1 activator implicated in focal adhesion disassembly, is absent in ATF3$^{-/-}$ neutrophils.
4. Mouse and human *ATF3* and *TIAM2* expression kinetics are consistent with a role for ATF3 regulation of *TIAM2* during neutrophil development.
5. TIAM2-lacking ATF3$^{-/-}$ neutrophils exhibit increased adhesion structure size and excessive adhesion-dependent F-actin polymerization.

Abstract

The activating transcription factor 3 (*ATF3*) gene is induced by Toll-like receptor (TLR) signaling. In turn, ATF3 protein inhibits the expression of various TLR-driven pro-inflammatory genes. Given its counter-regulatory role in diverse innate immune responses, we defined the effects of ATF3 on neutrophilic airway inflammation in mice. ATF3 deletion was associated with increased lipopolysaccharide (LPS)-driven airway epithelia production of CXCL1, but not CXCL2, findings concordant with a consensus ATF3-binding site identified solely in the *Cxcl1* promoter. Unexpectedly, ATF3-deficient mice did not exhibit increased airway neutrophilia after LPS challenge. Bone marrow chimeras revealed a specific reduction in $ATF3^{-/-}$ neutrophil recruitment to wild type lungs. *In vitro,* $ATF3^{-/-}$ neutrophils exhibited a profound chemotaxis defect. Global gene expression analysis identified an absence of *Tiam2* expression in $ATF3^{-/-}$ neutrophils. Kinetic mRNA analysis of human neutrophil development revealed *ATF3* and *TIAM2* expression patterns consistent with positive *TIAM2* regulation by ATF3 during neutrophil development. TIAM2 regulates cellular motility by activating Rac1-mediated focal adhesion disassembly. Notably, $ATF3^{-/-}$ neutrophils lacking TIAM2 exhibited increased focal complex area, along with excessive CD11b-mediated F-actin polymerization. Together, our data describe a dichotomous role for ATF3-mediated regulation of neutrophilic responses: inhibition of neutrophil chemokine production, but promotion of neutrophil chemotaxis.

Introduction

Activating transcription factor 3 (ATF3) is a counter-regulatory immune transcription factor (TF). It is induced by TLR signaling and, in turn, inhibits the transcription of genes encoding diverse TLR-driven, pro-inflammatory mediators.[1,2] ATF3 is a member of the ATF/cAMP response element-binding protein family of basic leucine zipper (bZIP) TFs.[3] ATF3 homodimers inhibit gene targets directly through association with histone deacetylase 1.[1,4] Conversely, ATF3 heterodimers, with other bZip TFs, positively regulate gene expression.[5-8] Basal ATF3 expression is low in most cell types, yet is rapidly and transiently elevated in response to various stimuli,[9] in part due to an autoregulatory ATF3 binding site in its promoter.[10]

Transient TLR-driven signals induce NF-κB-mediated ATF3 transcription, which then attenuates the expression of diverse other NF-κB targets.[4] Persistent TLR stimulation, however, additionally transcribes the NF-κB enhancer, C/EBP-δ, which augments NF-κB signaling and overcomes ATF3-mediated inhibition.[4] ATF3 induction and function are highly cell type- and stimulation-dependent.[9] In murine cytomegalovirus (MCMV) infection, for example, IFN-γ production by NK cells, but not CD8 T cells, is increased in ATF3$^{-/-}$ mice.[11] The overall effect of ATF3 on immune responses is similarly complex. ATF3 is necessary to restrain over-exuberant immune activation and immunopathology in models of sepsis[1] and LPS-induced febrile responses;[12] yet attenuation of NK effector responses by ATF3 contributes to increased MCMV-mediated liver damage.[11] Therefore, understanding the immunoregulatory role(s) of ATF3 demands careful evaluation in specific immune contexts.

Lung challenge with diverse microbial products induces production of glutamate-leucine-arginine (ELR+) chemokine family members, including CXCL1, by resident parenchymal[13-16] and hematopoietic cells.[16-18] ELR+ chemokines are highly neutrophil-specific, thus, their transcription, production, tissue localization,[19] and neutralization[20] are tightly regulated. Circulating neutrophils sample chemokines close to the endothelial wall while rolling on inflamed endothelia. Chemokine receptor ligation induces conformational changes in neutrophil β_2 integrins, allowing firm adhesion to the endothelia and eventual diapedesis or extravasation. Extravasation and extravascular neutrophil chemotaxis require the coordinated assembly and disassembly of cytoskeletal and adhesive structures. In slow-moving cells, complex, multi-protein focal adhesions (FAs) link the dynamic intracellular cytoskeleton to static, integrin-mediated extracellular structures. Focal complexes in neutrophils are highly dynamic and transient. Nonetheless, the processes regulating coordinated assembly and disassembly of FAs are incompletely understood, yet required for proper cell motility.[21]

Herein, we describe a novel and complex role for ATF3 regulation of neutrophil migration. LPS challenge resulted in significantly increased production of the potent neutrophil chemoattractant, CXCL1 in ATF3$^{-/-}$ mice; yet, neutrophil recruitment was not increased in ATF3$^{-/-}$ lungs. *In vitro* studies revealed significantly impaired ATF3$^{-/-}$ neutrophil migration to chemokine gradients. Gene expression analysis revealed ablation of *Tiam2*, an FA regulator, in mature ATF3$^{-/-}$ neutrophils. This was associated with dysregulated adhesion structure and cytoskeletal organization in ATF3$^{-/-}$, compared to WT, neutrophils. Of interest, ATF3 expression was restricted to neutrophil precursors, suggesting that temporal regulation of ATF3 expression during neutrophil development

is necessary for the proper expression of genes important to mature neutrophil chemotaxis. Together, these studies describe a complex and previously unappreciated role for ATF3 regulation of neutrophil recruitment to the lung and neutrophil chemotaxis generally.

Materials and methods

Mice and *in vivo* studies

ATF3$^{-/-}$ mice,[22] backcrossed >10 generations to a C57Bl/6NJ background, and wild type (WT) C57Bl/6NJ mice (Taconic Farms), were maintained in the Cincinnati Children's Hospital Medical Center's (CCHMC) specific pathogen-free animal facility. Intratracheal (i.t.) challenge studies were performed as described[23] using PBS (Gibco), ultrapure LPS (Invivogen), recombinant (r)CXCL1 (PeproTech), and/or anti-CXCL1 or isotype control antibodies (R & D Systems). For bone marrow (BM) transplants, WT CD45.1^{+} mice (B6.SJL-Ptprc$^{a/BoyAiTac}$; Taconic Farms) were lethally irradiated (700 and 475 rads, separated by 4 hours), and rescued with 2 x 10^6 BM cells from CD45.2^{+} ATF3$^{-/-}$ or WT mice. Euthanasia was performed by intraperitoneal injection of sodium pentobarbital followed by terminal exsanguination. Bronchoalveolar lavage (BAL) was performed with 500 ul ice-cold HBSS (Gibco), and lungs were harvested into TRIzol (Invitrogen). BAL fluid (BALF) cytospins were analyzed by Diff-Quik staining, and cell-free supernatant was assayed for CXCL1, CXCL2 or CXCL5 (R & D Systems) by ELISA. Experimental procedures were approved by the CCHMC IACUC.

In vitro MTEC culture

Mouse tracheal epithelial cells (MTECs) were isolated and cultured as described.[24] Briefly, tracheas from 4-5 week old mice were harvested and disaggregated by 0.1% pronase (Roche) and DNase I (Sigma) digestion, followed by fibroblast removal by plastic adherence. MTECs were then cultured on type I collagen-coated, 0.4um pore transwell inserts (BD) until formation of tight junctions (R $\geq$1,000Ω; 10-14 days). MTECs

were stimulated apically with LPS. Basolateral cytokine production was quantified by ELISA.

mRNA analysis

Neutrophils were isolated as described,[25] and RNA harvested by TRIzol. *Cxcl1, Cxcl2, Tiam1*, and *Tiam2* mRNA, normalized to *β-actin* expression, were quantified by quantitative (q)RT-PCR (LightCycler 480; Roche) using the following primers: *β-actin,* 5'-GGCCCAGAGCAAGAGAGGTA-3', 5'-GGTTGGCCTTAGGGTTCAGG-3'; *Cxcl1,* 5'-ACCCAAACCGAAGTCATAGC-3', 5'-TCTCCGTTACTTGGGGACAC-3'; *Cxcl2,* 5'-TCCAGGTCAGTTAGCCTTGC-3', 5'-CGGTCAAAAAGTTTGCCTTG-3'; *Tiam1,* 5'-TCACTCAGGACTTGAGCAGC-3', 5'-TGGGAGAATGTGCCAGAAAC-3'; *Tiam2,* 5'-CAGGGAAAAGATGGAGCAGA-3', 5'-ATGGCTCTCTGTTGGTGCTT-3'. For microarray analysis, WT and ATF3$^{-/-}$ neutrophils were isolated by Ly6G^{+} immunomagnetic selection (Miltenyi) and RNA harvested by RNeasy Mini Kit (Qiagen). RNA was analyzed on an Agilent SurePrint G3 Mouse GE 8x60K microarray with an Agilent 2100 Bioanalyzer (data accession #).

Immunofluorescence and flow cytometry

Adherent neutrophils (on uncoated or fibrinogen-coated [25 ug/ml; Sigma] slides), or neutrophils suspended in HBSS + 0.1% BSA (Sigma), were stimulated with 10 uM fMLP (Sigma), 1 mM Ca^{2+}/Mg^{2+} for 10 minutes at room temperature, and fixed with 2-4% PFA for 20 min. Cells were permeabilized with 0.1% Triton X-100 or 0.03% saponin (ACROS Organics), and stained with rhodamine-phalloidin (1:40; Invitrogen) and/or α-vinculin

(1:50; Sigma). Adherent cells were mounted in SlowFade Gold (Invitrogen) and imaged on a Leica DMI6000 fluorescence microscope using a 63X/1.3 NA objective, with an ORCA-ERC4742-95 camera (Hamamatsu) driven by Openlab version 5.5.0 software (Perkin Elmer). Flow cytometric analysis of non-adherent neutrophils was performed by incubation with monoclonal antibodies to CD11a, CD11b and CD18 (1:100; eBioscience), followed by use of an LSR II flow cytometer (BD).

In vitro neutrophil migration assays

Time-lapse video microscopy was performed as described.[26] Using a Zigmond chamber (NeuroProbe), adherent neutrophils were imaged every 5 seconds using ImageJ 1.43J software on a Zeiss Axiovert 200 microscope at 10X/0.3 NA objective, equipped with an ORCA-ER-C4742-95 camera (Hamamatsu) driven by Openlab software version 5.5.0, in a gradient of 10 uM fMLP in HBSS+10 mM HEPES (Gibco) for 20 minutes in a 37°C chamber. Individual neutrophil paths were traced in ImageJ software version 1.45s.

Statistics

Prism GraphPad 5 software was used for statistical analysis using unpaired, two-tailed *t*, 1-way ANOVA, or 2-way ANOVA tests, as appropriate. Microarray gene targets were analyzed using two independent approaches: (1) a 10% false discovery rate (FDR)[27] cutoff was used for non-parametric significance analysis of microarrays (SAM)[28] performed in R with the Bioconductor Siggenes package; and (2) probe expression was normalized by robust microarray average (RMA) in GeneSpring (Agilent), and probes

with absolute signal fold change > 2 were evaluated by unpaired *t* test, corrected for multiple comparisons by Benjamini-Hochberg post-test.

Results

ATF3 regulates LPS-driven CXCL1 production in the airway

To evaluate the role of ATF3 in acute lung inflammation, we analyzed lung homogenates for ELR+ chemokine transcripts. Airway LPS challenge induced expression of both *Cxcl1* and *Cxcl2*, yet only *Cxcl1* transcripts were significantly increased in ATF3$^{-/-}$ lungs, compared to wild type (WT) lungs (Figure 1A). *Cxcl1* promoter analysis revealed a consensus ATF3 binding site (Figure 1B), absent from the *Cxcl2* promoter (data not shown), suggesting specific ATF3 regulation of *Cxcl1* expression.

ATF3 functions are complex and context-dependent. ATF3 inhibits immune responses to transient signals, yet, following persistent TLR signaling, ATF3 silences its expression via auto-inhibition.[4] ATF3 regulation is also stimulus dose-dependent, as in LPS stimulation of ATF3$^{-/-}$ dendritic cells.[2] We therefore challenged WT and ATF3$^{-/-}$ mice with increasing LPS doses. We found that ATF3 deficiency resulted in significantly increased airway CXCL1 production at low LPS doses (1 ng/ml and 10 ng/ml), an effect that was overcome at higher doses of LPS. LPS-induced ATF3 regulation was CXCL1-specific, as CXCL2 production did not vary by genotype at any LPS dose (Figure 1C). No genotype-specific differences were observed in the (very low) concentrations of CXCL1 and CXCL2 measurable following control PBS challenge (data not shown). Kinetic analysis of low-dose LPS challenge revealed that induction of CXCL1 and CXCL2 production in WT airways was rapid and transient, returning to baseline levels within 24 hours. ATF3$^{-/-}$ mice had a similar temporal profile of CXCL1 production, although concentrations were significantly elevated at 3 hours (2 fold) and 4 hours (1.3

fold), compared to WT controls (Figure 1D). Again, the ATF3 effect was CXCL1-specific, as there were no significant differences in the production of CXCL2 (Figure 1D) or CXCL5 (supplemental Figure 1). Together, these data suggest a previously unappreciated role for ATF3 in the regulation of LPS-induced CXCL1 production during acute lung inflammation.

Resident lung cells are the source of increased CXCL1 production in ATF3$^{-/-}$ mice

LPS stimulation induces CXCL1 production by many cell types in the lung, including endothelial cells,[13] type 2 epithelial and Clara cells,[14-16,29] and various resident and recruited bone marrow (BM)-derived cells, such as alveolar macrophages[16,17,29] and neutrophils.[18,30] To test whether recruited BM-derived cells are the major source for differential CXCL1 production, we adoptively transplanted BM cells from CD45.2^{+} WT or ATF3$^{-/-}$ mice into CD45.1^{+} WT recipients (Figure 2A). Reconstitution efficiencies and peripheral blood cell populations were equivalent between transplanted groups prior to LPS challenge (supplemental Figure 2). Intratracheal (i.t.) LPS challenge induced similar CXCL1 and CXCL2 production in both groups (Figure 2B), indicating that recruited BM-derived cells are likely not the source of increased airway CXCL1 in ATF3$^{-/-}$ mice.

Epithelial cells are among the first and most abundant cells exposed to microbial products in the lung, and play a critical role in initiating immune responses.[14-16,29] To determine the contribution of lung epithelium to ATF3-mediated CXCL1 regulation, we stimulated mouse trachea epithelial cells (MTECs) from WT and ATF3$^{-/-}$ mice with LPS. Apical LPS stimulation resulted in a significant increase in basolateral CXCL1 secretion

in ATF3$^{-/-}$, compared to WT, MTEC cultures. This regulatory ATF3 effect was again restricted to CXCL1, as CXCL2 secretion was equivalent between genotypes (Figure 2C). Together, these results suggest that resident lung epithelial cells, not recruited or resident BM-derived cells, are the likely locus of ATF3-mediated regulation of LPS-driven CXCL1 in the airway.

ATF3$^{-/-}$ neutrophils exhibit impaired lung recruitment

CXCL1 induces neutrophil chemotaxis, but CXCL2 and CXCL5, also induced by LPS challenge of the airway, are additional neutrophil chemoattractants. Therefore, we sought to better define the specific role of CXCL1 in LPS-driven neutrophil lung recruitment. I.t. administration of recombinant (r)CXCL1 was sufficient to recruit WT neutrophils to the lung, in a dose-dependent manner (Figure 3A). Co-administration of CXCL1-neutralizing antibodies with LPS significantly reduced bioavailable CXCL1 without affecting LPS-induced *Cxcl1* transcription or CXCL2 production (supplemental Figure 3). Remarkably, CXCL1 neutralization attenuated LPS-induced neutrophil recruitment by almost 75% (Figure 3B), indicating that CXCL1 plays a dominant role in neutrophil recruitment in response to low-dose LPS challenge of the airway.

Given the elevated CXCL1 levels observed after LPS challenge of ATF3$^{-/-}$ airways, and the importance of CXCL1 in this model, we expected increased LPS-driven neutrophil recruitment to ATF3$^{-/-}$ airways. Unexpectedly, we observed no differences between ATF3$^{-/-}$ and WT airway neutrophil numbers during the 24 hours after i.t. LPS challenge (Figure 3C). This could not be explained by compensatory increases in other ELR+ chemokines (Figures 1C-D, supplemental Figure 1), or reduced

numbers or altered tissue distribution of ATF3$^{-/-}$ neutrophils (supplemental Figure 4). We thus examined potential neutrophil-intrinsic effects of ATF3 on neutrophil recruitment, using WT recipients reconstituted with WT or ATF3$^{-/-}$ BM cells, as in Figure 2A. Interestingly, we observed a striking defect in the recruitment of CD45.2^{+} ATF3$^{-/-}$ neutrophils to CD45.1^{+} WT airways; recruitment was reduced by almost 50% compared to WT controls (Figure 3D).

ATF3$^{-/-}$ neutrophils exhibit impaired *in vitro* migration

As shown in supplemental Figure 5, CXCL1 receptor (CXCR2) expression was comparable between ATF3$^{-/-}$ and WT neutrophils, suggesting (albeit not proving) that ATF3$^{-/-}$ neutrophils can respond to CXCL1. This prompted us to hypothesize that the above *in vivo* recruitment defect might be due to defects in the migration machinery of ATF3$^{-/-}$ neutrophils. We therefore compared WT and ATF3$^{-/-}$ neutrophil migration *in vitro* using time-lapse microscopy. WT neutrophils readily assumed a polarized morphology (Figure 4A) and migrated up fMLP gradients (Figure 4B). ATF3$^{-/-}$ neutrophils were also able to polarize and generate lamellipodial protrusions (Figure 4A). Notably, however, ATF3$^{-/-}$ neutrophils exhibited a striking inability to translocate, as significantly fewer ATF3$^{-/-}$ neutrophils moved productively (movement >20 um) compared to WT neutrophils (88% WT vs. 10% ATF3$^{-/-}$; Figure 4A). Supplemental videos clearly demonstrate this ATF3$^{-/-}$ migration defect, as well as their preserved ability to generate a polarized morphology. Of the few ATF3$^{-/-}$ neutrophils that did translocate, their ability to migrate was severely impaired, with a significant reduction (80%) in the distance traveled compared to WT neutrophils (Figure 4B).

ATF3 induction during neutrophil development is necessary for TIAM2 expression

This cell-intrinsic ATF3 effect in neutrophil function was initially surprising due to two observations. First, stimulus-dependent induction of ATF3 mRNA takes about an hour[9] and ATF3$^{-/-}$ neutrophil migration defects occur within minutes; therefore, ATF3-dependent chemotaxis effects are likely not due to *de novo* ATF3 transcription and regulation of gene expression. Second, we detected neither ATF3 protein (data not shown) nor mRNA (supplemental Figure 6) in mature WT neutrophils, either unstimulated or following LPS stimulation. Thus, WT neutrophils are similar to ATF3$^{-/-}$ neutrophils in their ATF3 protein status, and neutrophil-intrinsic, ATF3-mediated effects on migration and chemotaxis are likely a function of events occurring prior to neutrophil maturation. Kinetic analysis of gene expression supported a role for ATF3 regulation during neutrophil development.[31] DMSO-differentiated HL-60 cells revealed that *ATF3* is induced then silenced during differentiation, (Figure 5A); differentiation using all *trans* retinoic acid yielded similar results (data not shown).

We next sought to determine potential gene targets regulated by ATF3 during neutrophil development by comparing global gene expression in unstimulated WT and ATF3$^{-/-}$ neutrophils. We detected significant differences in only 9 protein-coding genes; *Tiam2*, *Erdr1*, *Odz3*, *Igh-VJ558, Igj, Efna2, Synpo, Susd4* and *Mtus2*. *Tiam2* was the most robust and statistically significant differentially expressed gene, with a 284-fold reduction in ATF3$^{-/-}$ neutrophils as quantified by microarray (corrected p <0.0001; supplemental Table 1), and a 99% reduction by qRT-PCR (Figure 5B). *Tiam2* was also

the only microarray-implicated gene whose product is purported to affect cell migration.[21] Consistent with potential direct regulation of *Tiam2* by ATF3, both human and mouse *TIAM2* promoters contain consensus ATF3 binding sites (Figure 5C). TIAM1 is closely related to TIAM2, yet it does not compensate functionally for TIAM2 reduction,[21] nor does it contain an ATF3 binding site (data not shown). Additionally, TIAM1 was not differentially expressed in TIAM2-lacking ATF3$^{-/-}$ neutrophils as quantified by microarray (FC =-1.003, corrected p =0.9889) or by qRT-PCR (Figure 5D).

To further evaluate the relationship between ATF3 and TIAM2, we analyzed their expression in previously published data derived from human CD34+ stem cells, promyelocytes, and mature neutrophils.[32] In agreement with our mouse data and *in vitro* human cell line analyses, *ATF3* expression was highest in human neutrophil precursors and lowest in circulating neutrophil populations (Figure 5E). *TIAM2* expression was reciprocal, with expression appearing to be restricted to mature neutrophils, while *TIAM1* expression was stable in all of these populations (Figure 5E). Together, these studies suggest that ATF3 expression during neutrophil differentiation is required for proper expression of TIAM2 in mature neutrophils.

Adhesion structures and cytoskeletal organization are abnormal in stimulated ATF3$^{-/-}$ neutrophils

Focal complexes link static, integrin-mediated extracellular interactions to the dynamic cellular cytoskeleton, and proper assembly and disassembly of adhesive structures is necessary for cellular movement. Given the role of TIAM2 in focal adhesion (FA) disassembly and cellular motility,[21] and the lack of TIAM2 in ATF3$^{-/-}$ neutrophils, we

hypothesized that abnormal adhesive structure disassembly could underlie impaired ATF3$^{-/-}$ neutrophil chemotaxis. Indeed, fMLP-stimulated ATF3$^{-/-}$ neutrophils exhibited abnormally intense focal complex staining compared to WT neutrophils (Figure 6A). Adhesive structures were significantly larger (Figure 6B) and more numerous (Figure 6C) in ATF3$^{-/-}$ neutrophils. Adhesive structures can regulate F-actin polymerization.[33] Therefore, we also evaluated stimulation-dependent F-actin polymerization. When compared to WT neutrophils, ATF3$^{-/-}$ neutrophils displayed aberrant polymerized F-actin morphology (Figure 6A). ATF3$^{-/-}$ neutrophils exhibited broader and thicker polymerized F-actin-staining lamellipodia, resulting in significantly increased areas of polymerized F-actin (Figure 6D). Increased ATF3$^{-/-}$ neutrophil F-actin polymerization was adhesion-dependent, as there were no differences between ATF3$^{-/-}$ and WT neutrophils stimulated in suspension (supplemental Figure 7). Further, increased ATF3$^{-/-}$ F-actin polymerization was CD11b-mediated, as demonstrated using the CD11b-specific ligand, fibrinogen (Figure 6D). Expression of the principal neutrophil β_2 integrins CD11a, CD11b, and CD18 was normal (supplemental Figure 8). These data suggest that impaired TIAM2-mediated focal complex disassembly in ATF3$^{-/-}$ neutrophils is a potential mechanism of impaired ATF3$^{-/-}$ neutrophil chemotaxis. Together with inhibition of CXCL1 production in response to low-dose LPS challenge, these data identify ATF3 as a complex regulator of neutrophil recruitment and chemotaxis.

Discussion

ATF3 is a counter-regulatory immune TF[1,2] that acts as an immunologic rheostat, restraining immune activation in response to transient or low levels of stimulation, yet allowing immune activation by persistent or high doses of stimulation.[2,4] Here we report that ATF3 plays an unexpectedly complex role in acute neutrophilic lung inflammation. On the one hand, ATF3 inhibits LPS-dependent CXCL1 production by lung epithelia, which would tend to reduce neutrophil recruitment. On the other hand, neutrophils require ATF3 expression to migrate normally, likely through developmental regulation of TIAM2 expression, which would tend to facilitate neutrophil recruitment.

These studies are the first to demonstrate regulation of epithelial *Cxcl1* expression by ATF3. We have identified an ATF3 binding site unique to the *Cxcl1* promoter among ELR+ chemokines, and demonstrated dose-dependent LPS-driven CXCL1 transcript and protein levels in ATF3$^{-/-}$ airways and pulmonary epithelium. Specific regulation is likely due to direct *Cxcl1* promoter occupancy by ATF3, as others have immunoprecipitated ATF3 at the *CXCL1* promoter following ectopic overexpression in human cancer cell lines.[34] A previous study reported increased (mRNA) expression of the entire ELR+ chemokine family in ATF3$^{-/-}$ lung homogenates in an allergic asthma model, including members lacking consensus ATF3 binding sites.[35] We demonstrated ATF3-specific, LPS-dependent overexpression of CXCL1 mRNA and protein in airways and lung epithelia within 24 hours of challenge, while Gilchrist, et al.,[36] assayed transcripts in lung homogenates following a 4 week sensitization/challenge model. Therefore, their observation of globally increased ELR+

family expression in ATF3$^{-/-}$ lungs likely reflects global perturbations in the immune response in asthma.

In the airway, the kinetics of LPS-induced CXCL1 production and neutrophil recruitment did not overlap, although CXCL1 was the primary driver of neutrophil recruitment, as indicated by CXCL1 neutralization studies. The mechanism underlying these events is unclear. Unique among ELR+ chemokines, airway-produced CXCL1 is actively transcytosed into the systemic circulation.[19] Therefore, systemic CXCL1 could be the inciting event responsible for neutrophil recruitment by priming circulating neutrophils and inducing extravasation into the lung interstitium, while other molecules (potentially CXCL2 and/or CXCL5) contribute to the continued accumulation of neutrophils in the alveolar spaces independent of or in conjunction with CXCL1. This also suggests that increases in CXCL1 concentrations in bronchoalveolar lavage fluid may only represent a portion of the dysregulated CXCL1 response in ATF3$^{-/-}$ mice. Therefore, future kinetic experiments evaluating neutrophil accumulation in the pulmonary circulation, interstitium, and alveolar spaces,[37] as well as definition of systemic ELR+ chemokine production, is warranted. Nevertheless, the current studies uncover a fundamental role for ATF3-mediated regulation of CXCL1 in acute lung inflammation.

The most surprising result of our studies was the lack of increased ATF3$^{-/-}$ neutrophil recruitment to the lungs of ATF3$^{-/-}$ mice, despite significant increases in CXCL1. Time-lapse microscopy of chemotaxing neutrophils demonstrated a remarkable ATF3$^{-/-}$ neutrophil-intrinsic migration defect. Neutrophil migration necessitates rapid cell body reorganization and is commonly coordinated by signaling molecules leading to

rearrangement of diverse cytoskeletal structures. TFs are more likely involved in target gene regulation, which occurs on time-scales significantly longer than the immediate responses necessitated by cellular migration. Therefore, this observation raised certain questions: (1) Does ATF3 regulate neutrophil development by modulating the expression of genes necessary for proper mature neutrophil function? (2) Which proteins involved in neutrophil chemotaxis are regulated by ATF3 during neutrophil development? and (3) What is/are the mechanism(s) underlying ATF3$^{-/-}$ neutrophil-intrinsic chemotaxis defects?

Our data demonstrate that ATF3 is critical for generating fully functional mature neutrophils, likely by developmental regulation of effector proteins necessary for migration, such as TIAM2. The kinetics of *ATF3* and *TIAM2* expression in neutrophil development are particularly interesting. ATF3 expression peaks in neutrophil precursors but is absent in mature neutrophils, while *TIAM2* is expressed in mature neutrophils in which ATF3 is absent. Yet, *Tiam2* expression is ATF3-dependent. This suggests that ATF3 is necessary for subsequent *Tiam2* expression in the absence of ATF3. Further evidence for a regulatory role for ATF3 in neutrophil development has been described.[38] JDP2 is an important negative regulator of ATF3 expression[38-40] whose depletion results in elevated *Atf3* expression, decreased Ly6G expression (a neutrophil maturation marker), and defective effector function (e.g., superoxide production, NET formation).[38] Ectopic ATF3 overexpression throughout neutrophil differentiation recapitulated the immature phenotype of JDP2$^{-/-}$ neutrophils.[38] When considered in light of our findings, however, these data imply that ATF3 induction and subsequent down-modulation is necessary for terminal neutrophil development. Similar

to the waves of TFs integral to neutrophil development (i.e., PU.1, C/EBP-α, Gfi-1, C/EBP-ε), ATF3 induction may modulate subsequent waves of TF expression,[9] followed by ATF3 silencing necessary to release gene targets from transcriptional repression. These issues clearly deserve further exploration.

TIAM2 is a specific Rac1-activating guanine exchange factor (GEF)[41,42] that controls cellular motility by promoting FA disassembly in epithelial cells.[21] We observed significantly increased focal complex areas and numbers in ATF3$^{-/-}$ neutrophils, associated with an absence of *Tiam2* expression. Epithelial TIAM2 knockdown resulted in reduced rates of FA disassembly and consequently enlarged FAs, and reduced migratory speeds.[21] Therefore, TIAM2-mediated dysregulated focal complex disassembly is a plausible molecular mechanism for reduced ATF3$^{-/-}$ neutrophil chemotaxis. Conversely, ATF3 overexpression in breast cancer cell lines upregulates genes involved in cancer metastasis[43] and confers metastatic potential to prostate cancer cell lines.[44] Thus, ATF3-dependent migration effects may not be restricted to neutrophils, and may represent a broader ATF3 modulation of cellular adhesion.

In addition to increased focal complexes, we observed striking increases in F-actin polymerization in ATF3$^{-/-}$ neutrophils. It is unclear whether this is secondary to dysregulated focal complex disassembly, or represents an additional ATF3-dependent effect. In the migratory cycle, lamellipodia are stabilized to the substratum via adhesion, enabling cell body translocation and efficient migration. These adhesion points are then dissembled to complete movement. It is possible that increased adhesion complexes cause abnormal enlargement of the lamellipodia. Alternatively, Rac1$^{-/-}$ neutrophils display increased polymerized F-actin and aberrant lamellipodia morphology in

response to fMLP stimulation,[26] similar to that seen in ATF3$^{-/-}$ neutrophils. While the TIAM2-mediated effect on epithelial FAs was shown to be Rac1-dependent,[21] focal complexes were not evaluated in Rac1$^{-/-}$ neutrophils.[26] Therefore, ATF3-dependent *Tiam2* regulation may have multifaceted effects on neutrophil chemotaxis.

Overall, our study describes novel, complex roles for ATF3 in neutrophil recruitment: inhibition of epithelial CXCL1 production in addition to neutrophil-intrinsic promotion of chemotaxis, likely through developmental modulation of TIAM2 expression and focal complex and cytoskeletal regulation. Increased understanding of the developmental and regulatory ATF3 effects on neutrophil recruitment may identify novel therapeutic targets in disease marked by aberrant neutrophilic inflammation.

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Authorship

Contributions: N.D.B., M.-D.F and C.L.K. designed the experiments, interpreted the results, and drafted the paper. H.L.G. and K.H. aided in data interpretation and experimental design. N.D.B. performed the research with help from M.-D.F., J.W.M., and S.K.. J.D.P. provided expertise on microarray analysis. T.H. provided $ATF3^{-/-}$ mice and aided in editing.

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Figures

Figure 1

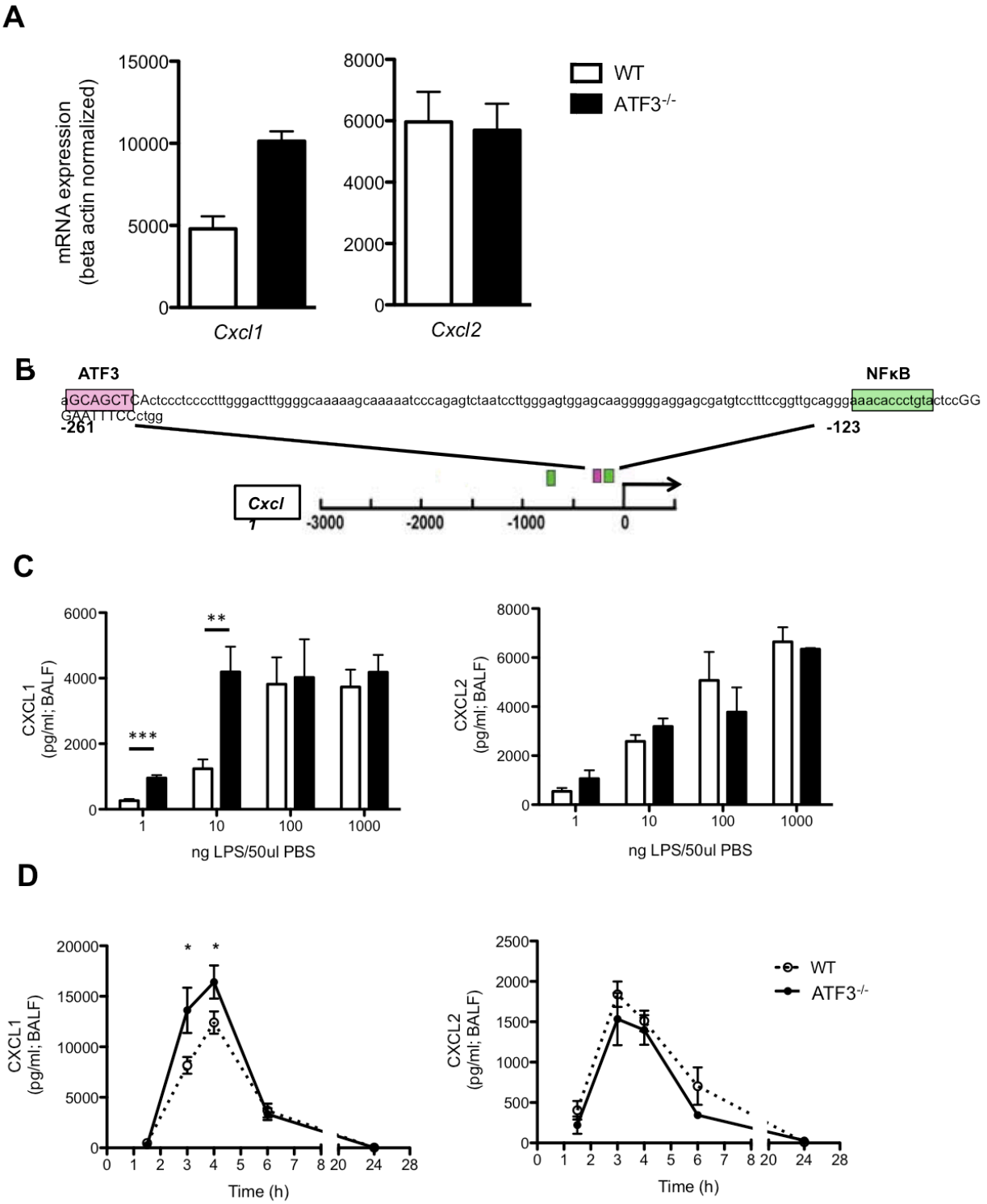


Figure 1. ATF3 regulates LPS-induced airway CXCL1 production. (A) WT (white bars) and ATF3$^{-/-}$ (black bars) mice were challenged i.t. with 10 ng LPS, and lavaged 4 hours later. Following lavage, the left upper lung lobes were removed for mRNA quantification by qRT-PCR for *Cxcl1* (left) or *Cxcl2* (right). Representative of a single experiment, N=3-5 mice/group. (B) The -3kb region of the *Cxcl1* promoter was analyzed using online TESS software to identify the putative ATF3 binding sequence GCA CGT CA (pink box) as well as known NF-κB binding sites (green boxes). (C) WT (white bars) and ATF3$^{-/-}$ (black bars) mice were challenged i.t. with increasing doses of LPS. After 2 hours, lungs were lavaged and cell-free supernatants analyzed by ELISA for CXCL1 (left) or CXCL2 (right). Representative of 3 experiments, N=2-9 mice/group. (D) WT (dotted lines) and ATF3$^{-/-}$ (solid lines) mice challenged i.t. with 10 ng LPS were lavaged at the indicated times to evaluate CXCL1 (left) or CXCL2 (right) production by ELISA. Representative of 7 experiments, N=3-7mice/group. Statistics are unpaired two-tailed *t* test compared to WT control. Data are mean ± S.E.M. **p* <0.05, ***p* <0.01. BALF, bronchoalveolar lavage fluid.

Figure 2

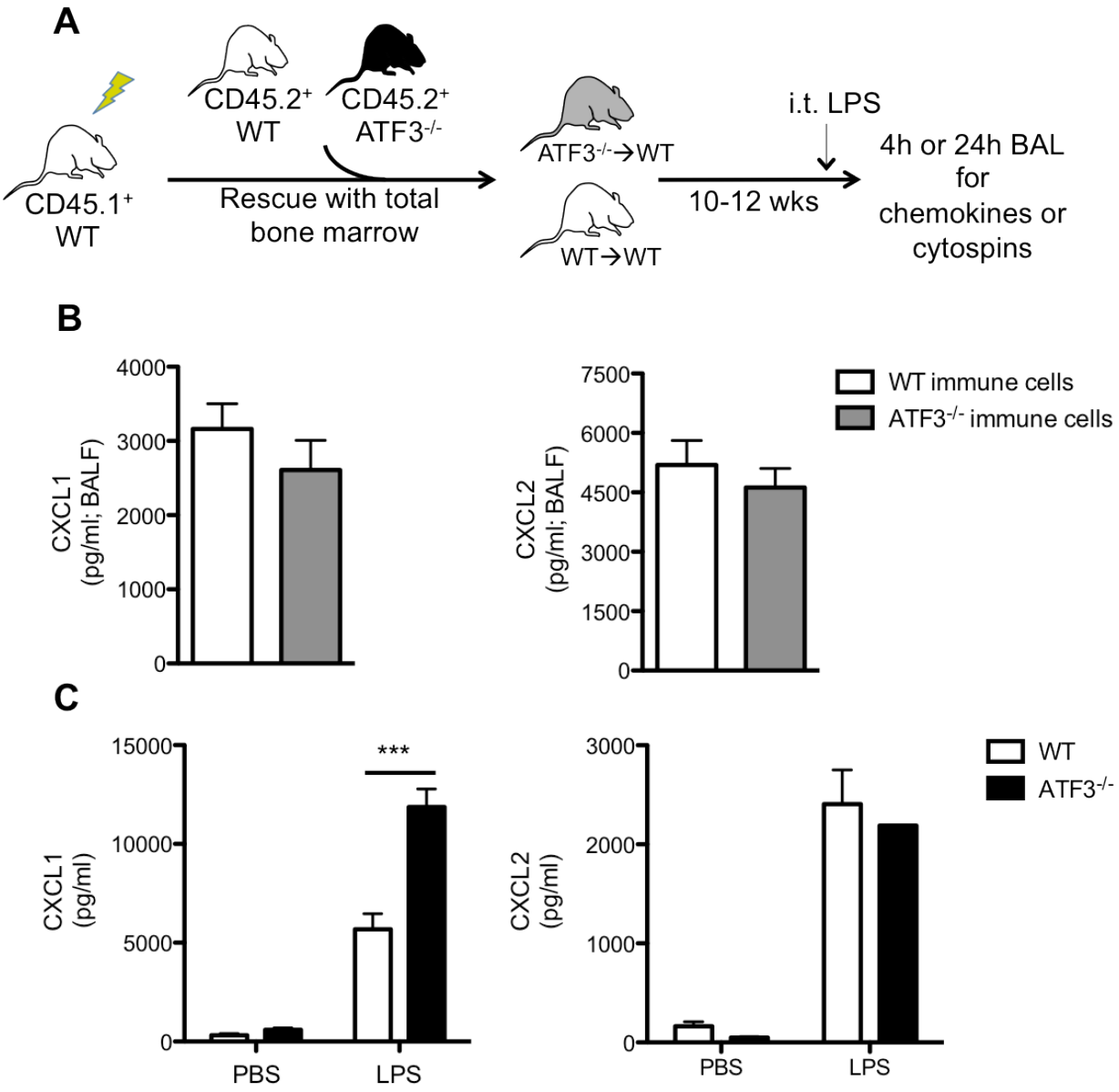


Figure 2. Lung parenchymal cells, not recruited hematopoietic cells, are essential to ATF3 regulation of chemokine production. (A) BM transplantation schema. CD45.1^{+} WT mice were lethally irradiated and reconstituted with BM from CD45.2^{+} WT (white) or ATF3$^{-/-}$ (black) mice. 10-12 weeks post transplant, CD45.1^{+} WT mice with CD45.2^{+} WT (white) or ATF3$^{-/-}$ (grey) BM were used for experimentation. (B) I.t.

challenge with 10 ng LPS and BAL fluid determination of CXCL1 (left) or CXCL2 (right) by ELISA at 4 hours in $CD45.1^{+}$ WT mice reconstituted with $CD45.2^{+}$ congenic WT (white bars) or $ATF3^{-/-}$ (black bars) BM cells. Pooled from 3 independent experiments, N=19-20 $ATF3^{-/-}$ donor conditions and 14-15 WT donor conditions. Statistics are unpaired, two-tailed *t* test. (C) Mouse tracheal epithelial cells (MTECs) from WT (white bars) or $ATF3^{-/-}$ (black bars) mice were challenged apically with 10 ng/ml LPS for 18 hours. Basolateral media was then analyzed for CXCL1 (left) or CXCL2 (right) production by ELISA. Representative of 3 experiments, N=2-3 wells/condition. Statistics are 2-way ANOVA with Bonferroni correction. Data are mean $\pm$ S.E.M. ***p <0.001. BALF, bronchoalveolar lavage fluid.

Figure 3

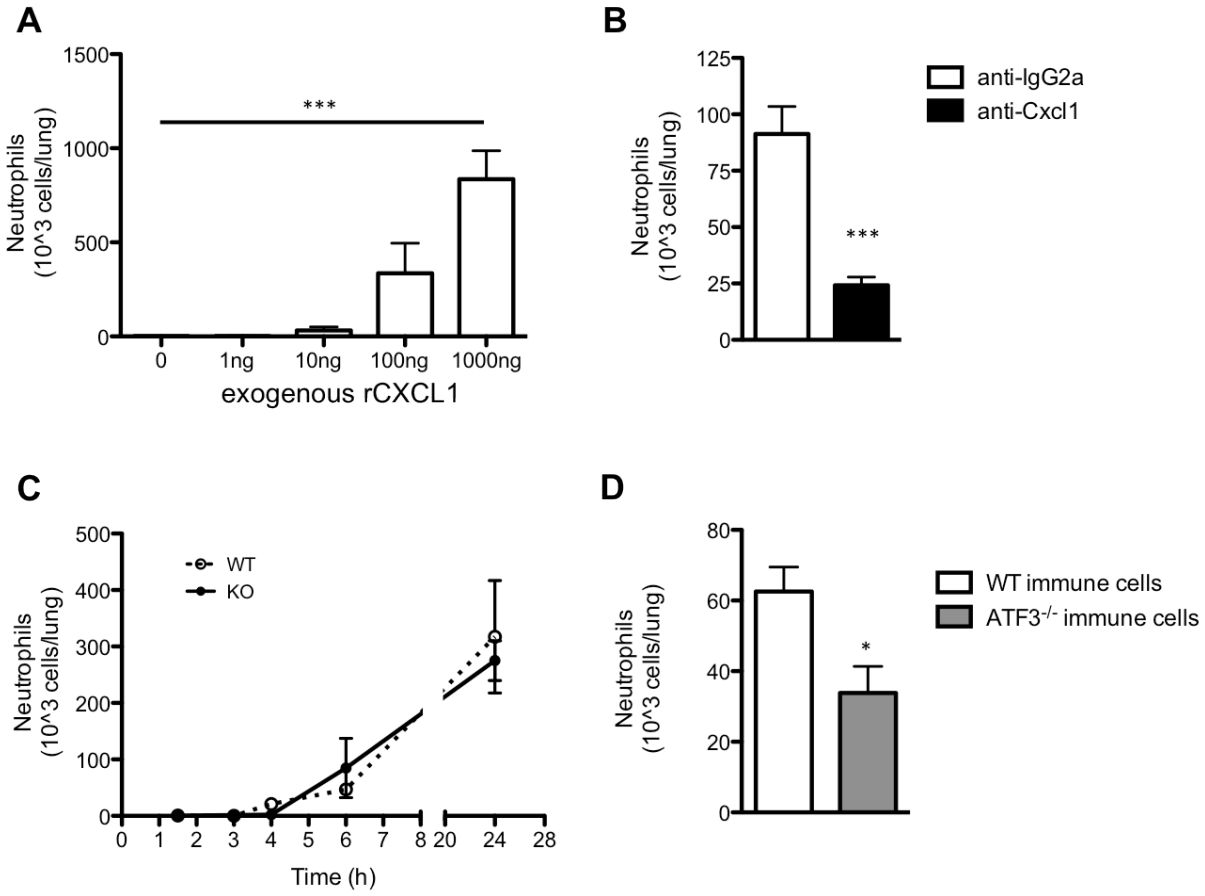


Figure 3. ATF3$^{-/-}$ neutrophil lung recruitment in impaired. (A) BAL fluid from WT mice challenged with increasing amounts of rCXCL1 i.t. were evaluated to determine neutrophil recruitment to the lung after 18-24 hours by microscopic evaluation of cytospins. Representative of 2 independent experiments, N=3-5 mice/group. Statistics are 1-way ANOVA. (B) 10 ng LPS, and 50 ug IgG2a control (white bars) or neutralizing CXCL1 antibody (black bars), were co-administered i.t. to WT mice. Neutrophil content of BALF was determined as in panel A. Representative of 2 independent experiments, N=3-5 mice/group. Statistics are unpaired, two-tailed *t* test. (C) WT (dotted lines) and *A*TF3$^{-/-}$ (black lines) mice were challenged i.t. with 10 ng LPS and neutrophils

enumerated as in panel A. Representative of 8 independent experiments, N=3-7 mice/group. (D) BM chimeras from Figure 2A were challenged and neutrophil recruitment determined as in panel A. Representative of 3 independent experiments, N=14-16 mice/group. Statistics are unpaired, two-tailed *t* test. Data are mean ± S.E.M. **p* <0.05, ****p* <0.001.

Figure 4

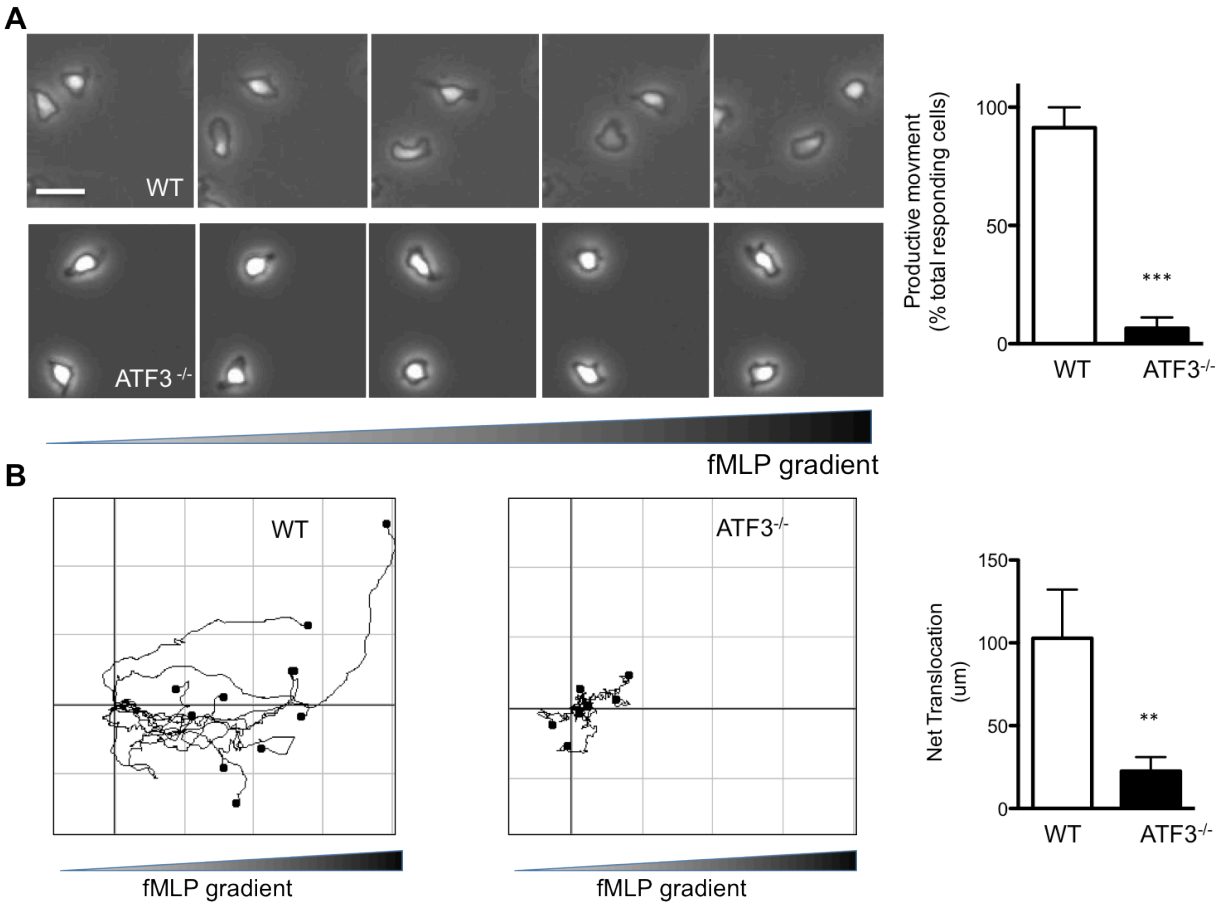


Figure 4. ATF3$^{-/-}$ neutrophils exhibit a profound defect in directional motion. (A) Representative images (1 minute between each frame) of migrating WT (top panels) or ATF3$^{-/-}$ (bottom panels) neutrophils in a gradient of fMLP on uncoated surfaces in a Zigmond chamber. fMLP concentration increases from left to right. Images were captured every 5 seconds for 20 minutes at 37°C. Original magnification X100. The number of neutrophils able to move productively (20 um from their starting position) is quantified at right. Images are representative of, and graph is pooled from, 4 independent experiments, N=6 WT, 9 ATF3$^{-/-}$ independent acquisitions/experiment and 20-100 neutrophils were evaluated/acquisition. Images were captured using a Zeiss Axiovert 200 microscope at 10X/0.3 NA objective, equipped with an ORCA-ER C4742-

95 camera driven by Openlab version 5.5.0 software. (B) Paths of migrating WT (left) and ATF3$^{-/-}$ (right) neutrophils were traced using ImageJ software. The schema represent cells moving in fMLP gradient over 20 minutes. The total distance traveled from the origin (net translocation) is quantified at right. Schema are representative of and graph is pooled from 3 independent experiments, N=4 WT, 9 ATF3$^{-/-}$ independent acquisitions/experiment. Statistics are unpaired, two-tailed *t* test. Data are mean $\pm$ S.E.M. $^{**}p < 0.01$, $^{***}p < 0.001$.

Figure 5

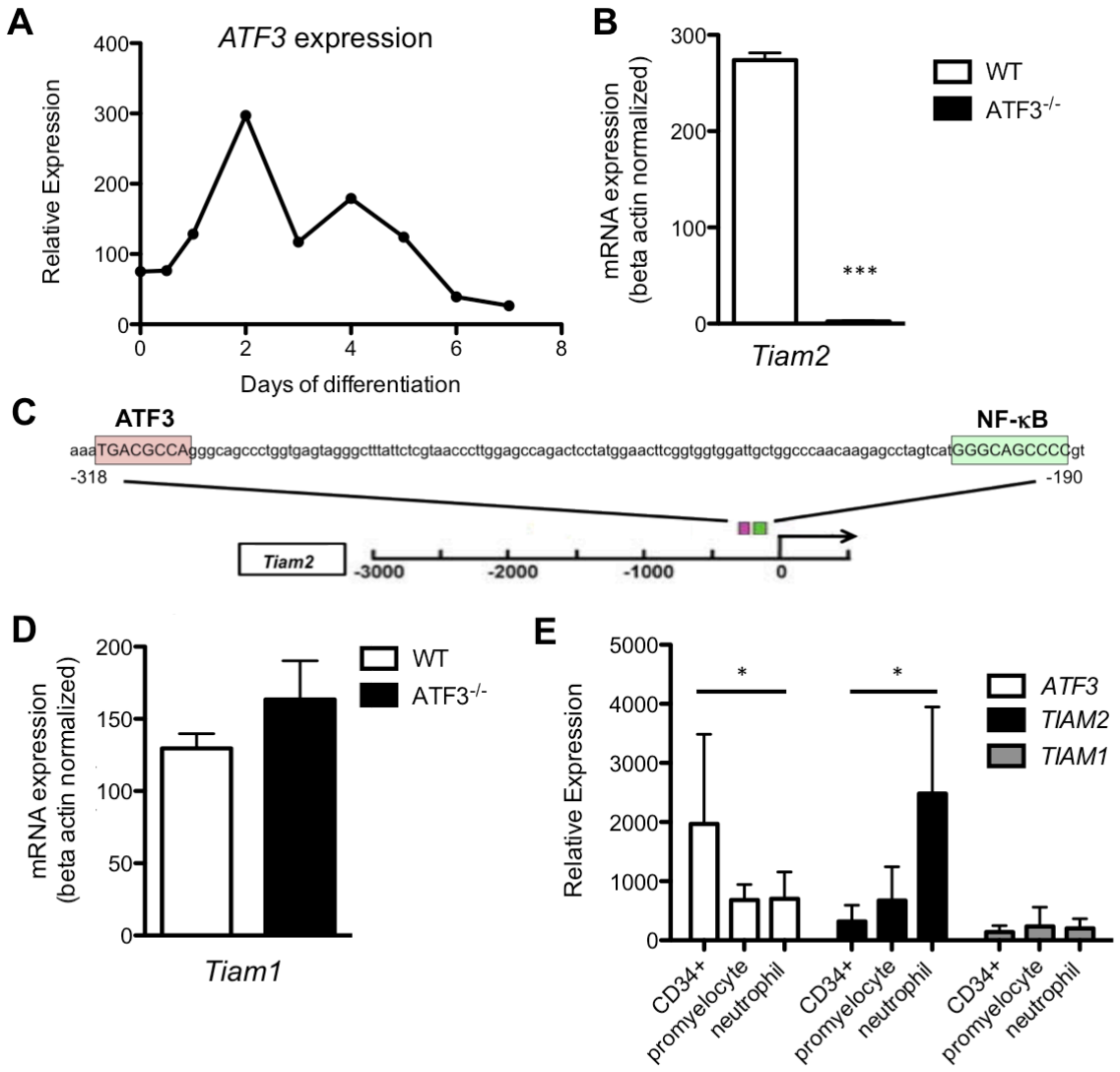


Figure 5. ATF3 regulates *Tiam2* expression during neutrophil development. (A) *ATF3* expression during HL-60 cell differentiation by DMSO as determined by microarray analysis (GSE14500). (B) RNA from WT (white bars) or ATF3$^{-/-}$ (black bars) neutrophils were analyzed by qRT-PCR for *Tiam2* expression. Representative of 3 independent experiments, N=2-6 mice/genotype. Statistics are unpaired, two-tailed *t* test. (C) The -3kb region of *Tiam2* variant 1 (NM_011878) shown with the consensus ATF3 binding site TGA CGC CA (pink box) indicated relative to the known

transcriptional activator NF-κB binding site (green box). (D) RNA from WT (white bars) or ATF3$^{-/-}$ (black bars) neutrophils were analyzed by qRT-PCR for *Tiam1*. Representative of 3 independent experiments, N=2-6/genotype. (E) *ATF3* (white bars), *TIAM2* (black bars), and *TIAM1* (grey bars) expression evaluated by microarray (GSE12662) in human primary cells along the spectrum of neutrophil differentiation. Statistics are Kruskal-Wallis test corrected by Dunn's multiple comparison test. N=5 donors/cell type. Data are mean ± S.E.M. *p <0.05, ***p <0.0001.

Figure 6

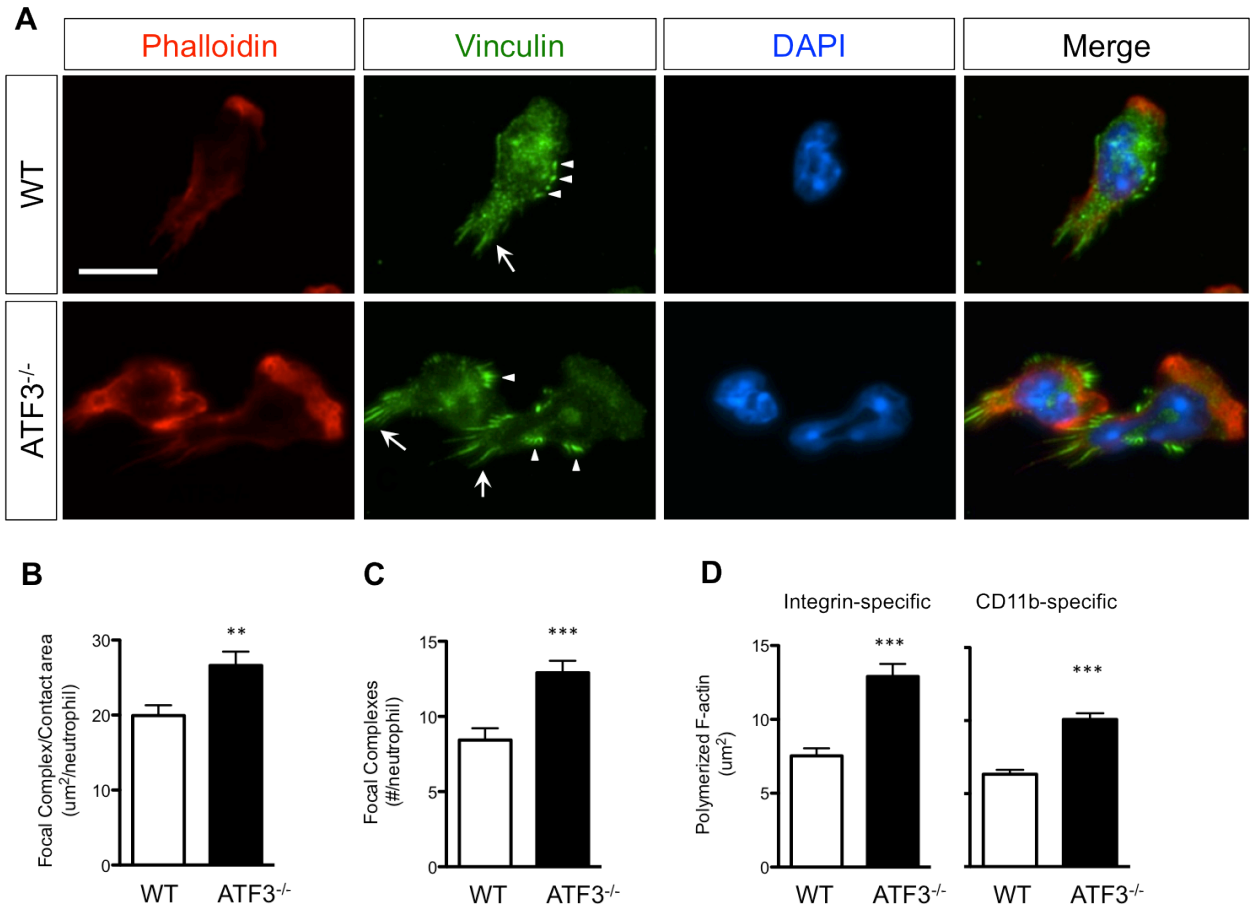
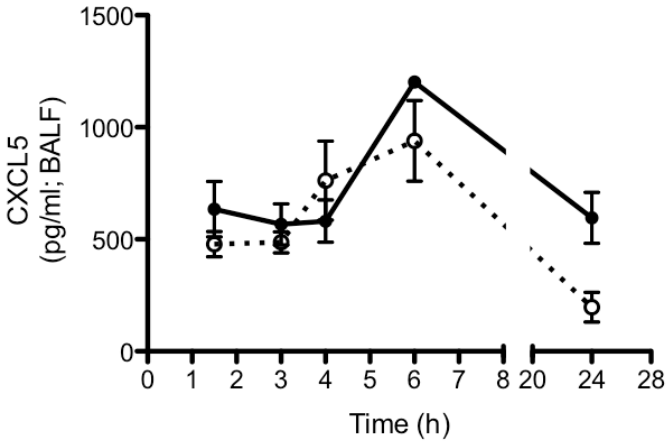


Figure 6. ATF3$^{-/-}$ neutrophils lacking TIAM2 exhibit dysregulated integrin-dependent cytoskeletal organization and adhesive structure regulation. (A) WT (top panels) or ATF3$^{-/-}$ (bottom panels) neutrophils were allowed to adhere to uncoated glass slides and then stimulated with 10 uM fMLP and stained for polymerized F-actin by phalloidin-rhodamine and focal complexes (arrows) and focal contacts (arrowheads) by anti-vinculin-Alexa Fluor 488. Representative focal complex/contacts (green), polymerized F-actin (red), DAPI-stained nuclei (blue) and merged immunofluorescent images are shown. Original magnification X630. Representative of 2 (vinculin) or 5 (phalloidin) independent experiments. Fluorescence images were captured at room

temperature using a Leica DMI6000 fluorescence microscope at 63X objective N/A1.3 with ORCA-ER C4742-95 camera driven by Openlab Version 5.5.0 software. (B-C) Total area, panel B, and numbers, panel C, of vinculin-containing focal structures quantified from panel A for WT (white bars) or ATF3$^{-/-}$ (black bars) fMLP-stimulated neutrophils. Representative of 2 independent experiments, N=52-83 polarized, nucleus-containing neutrophils/genotype. (D) The total area of F-actin polymerization quantified from A for WT (white bars) or ATF3$^{-/-}$ (black bars) neutrophils stimulated by fMLP on fibrinogen-coated (left) or uncoated (right) glass slides. Representative of 3 independent experiments, N=64-156 cells counted/genotype. Statistics are unpaired, two-tailed *t* test. Data are mean $\pm$ S.E.M. ** p <0.01, *** p <0.001.

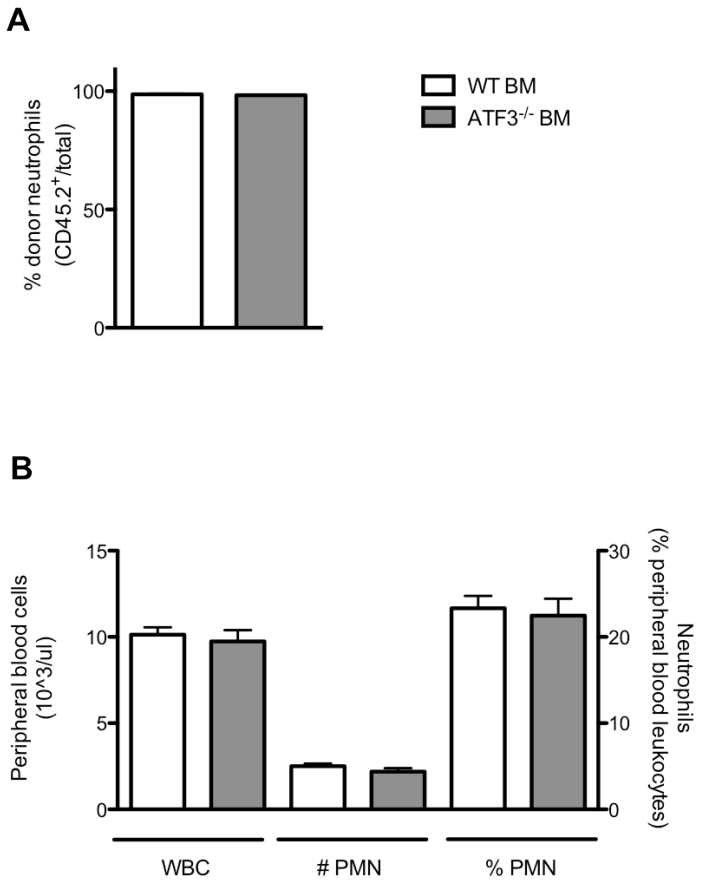
Supplemental Figures

Supplemental Figure 1



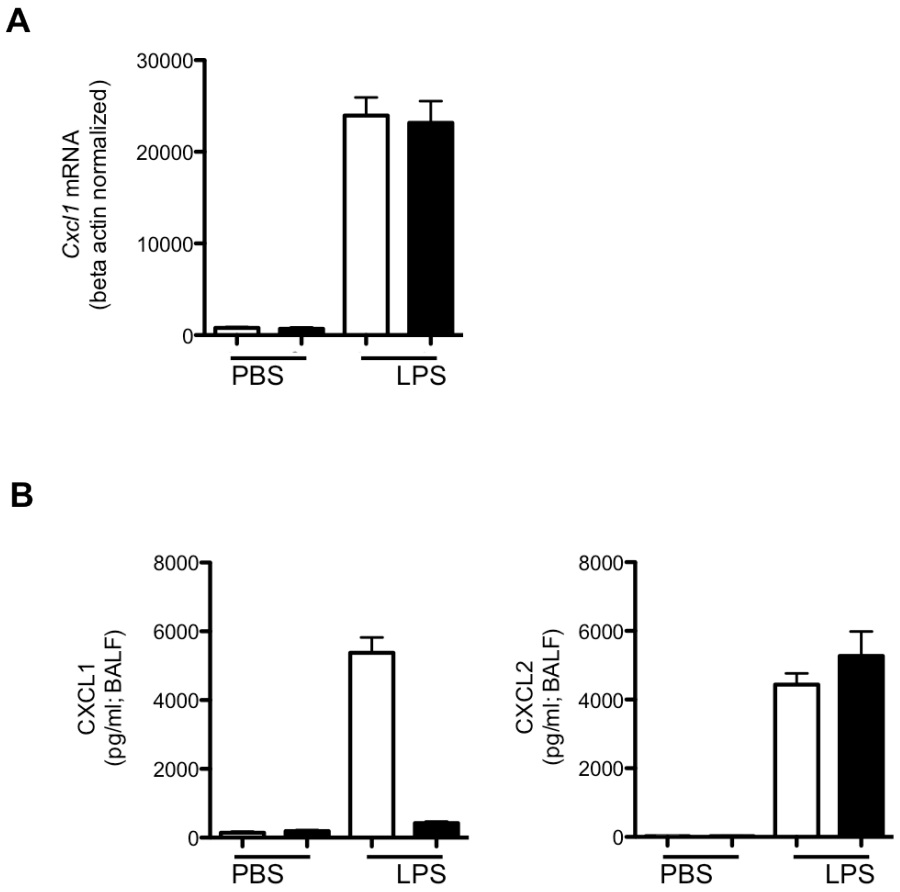
Supplemental Figure 1. LPS-dependent ATF3 regulation is CXCL1-specific. Temporal kinetics of airway production of CXCL5 in WT (dotted lines) or ATF3$^{-/-}$ (solid lines) mice following i.t. LPS challenge. Representative of 7 experiments, N=3-7 mice/group. BALF, bronchoalveolar lavage fluid.

Supplemental Figure 2



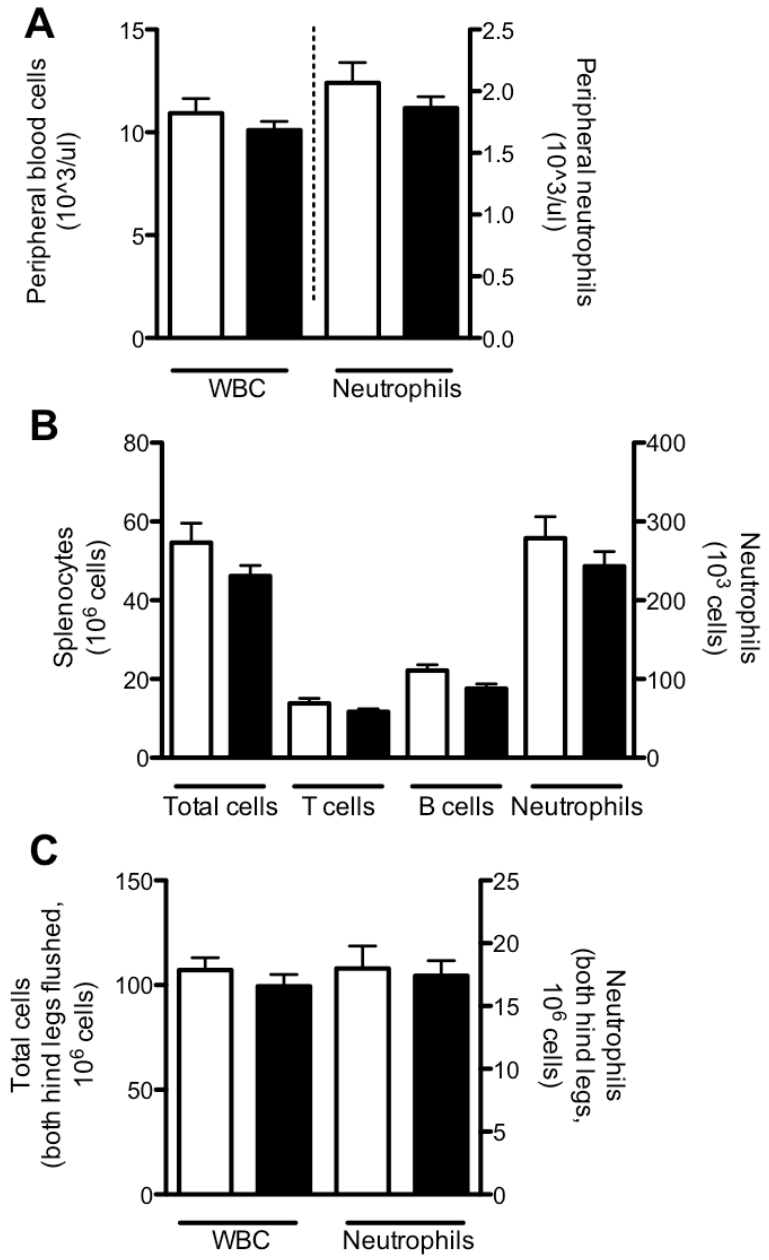
Supplemental Figure 2. WT- and ATF3$^{-/-}$-reconstituted BM chimeras are equivalent. Reconstitution efficiency (A) and leukocyte and neutrophil number and percent (B) in peripheral blood are equivalent between CD45.2^{+} WT (white bars) and ATF3$^{-/-}$ (black bars) BM-reconstituted CD45.1^{+} WT hosts. N=12 ATF3$^{-/-}$, 10 WT, representative of 4 individual experiments. WBC, white blood cells; PMN, neutrophils.

Supplemental Figure 3



Supplemental Figure 3. Co-administered anti-CXCL1 antibodies specifically neutralize LPS-dependent CXCL1 production. (A) WT mice challenged i.t. with PBS or LPS and IgG2a control (white bars) or anti-CXCL1 (black bars) antibodies were sacrificed after 4 hours and their lungs harvested into TRIzol after BAL. *Cxcl1* mRNA transcripts were determined as described in methods. (B) BAL from mice challenged in panel A were evaluated for CXCL1 (left) and CXCL2 (right) production by ELISA. Single experiment, N=6 mice/group. Statistics are unpaired, two-tailed *t* test. ***$p<0.0001$. BALF, bronchoalveolar lavage fluid.

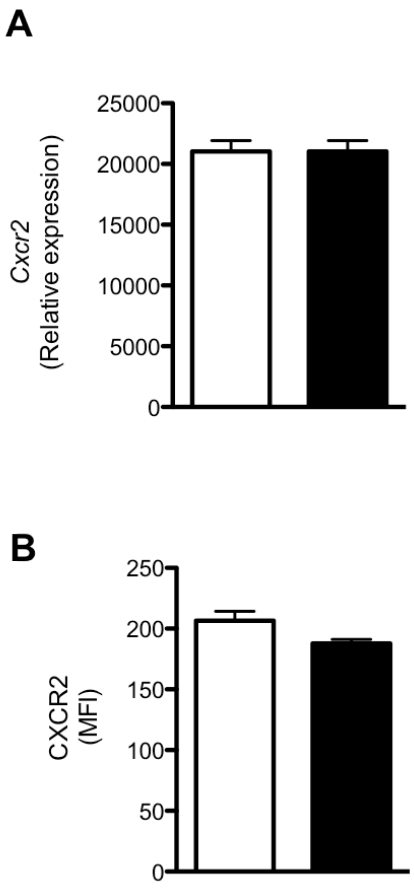
Supplemental Figure 4



Supplemental Figure 4. No differences in ATF3$^{-/-}$ neutrophil distribution. Neutrophils numbers were assayed by flow cytometry for neutrophil markers 7/4 or Ly6G in peripheral blood (A), spleen (B) or BM (C). Peripheral blood was drawn via tail bleeds into EDTA-chelated tubes. The spleen was harvested, disaggregated through a

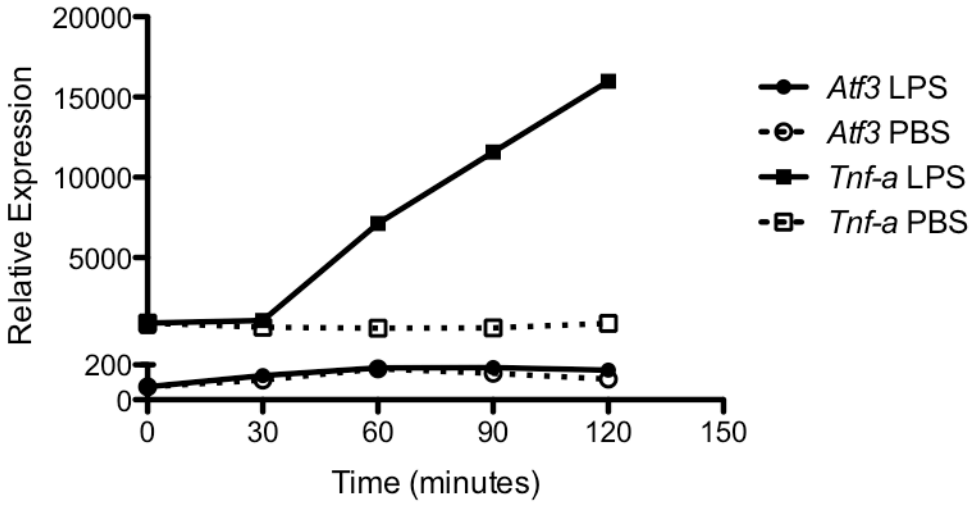
100um cell filter and treated with ACK to lyse red blood cells (RBCs). BM was flushed from hind leg bones and disaggregated through a 40um cell filter. Representative of 3 experiments N=8-11 mice/genotype. WBC, white blood cells.

Supplemental Figure 5



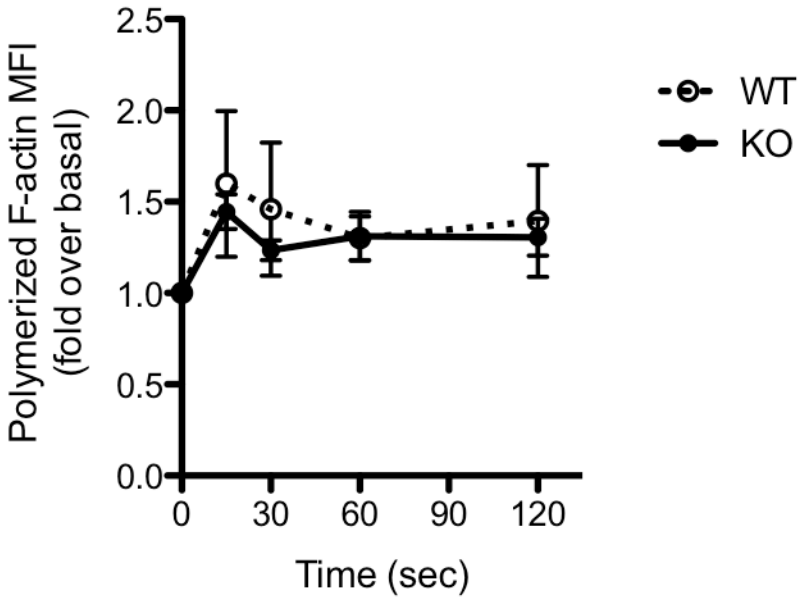
Supplemental Figure 5. ATF3$^{-/-}$ neutrophils show no differences in CXCR2 expression. CXCR2 expression analyzed at the mRNA level by microarray (A) and flow cytometry-based surface protein expression (B) on WT (white bars) and ATF3$^{-/-}$ (black bars) neutrophils. Representative of 1 experiment N=5-6 mice/genotype. MFI, mean fluorescence intensity.

Supplemental Figure 6



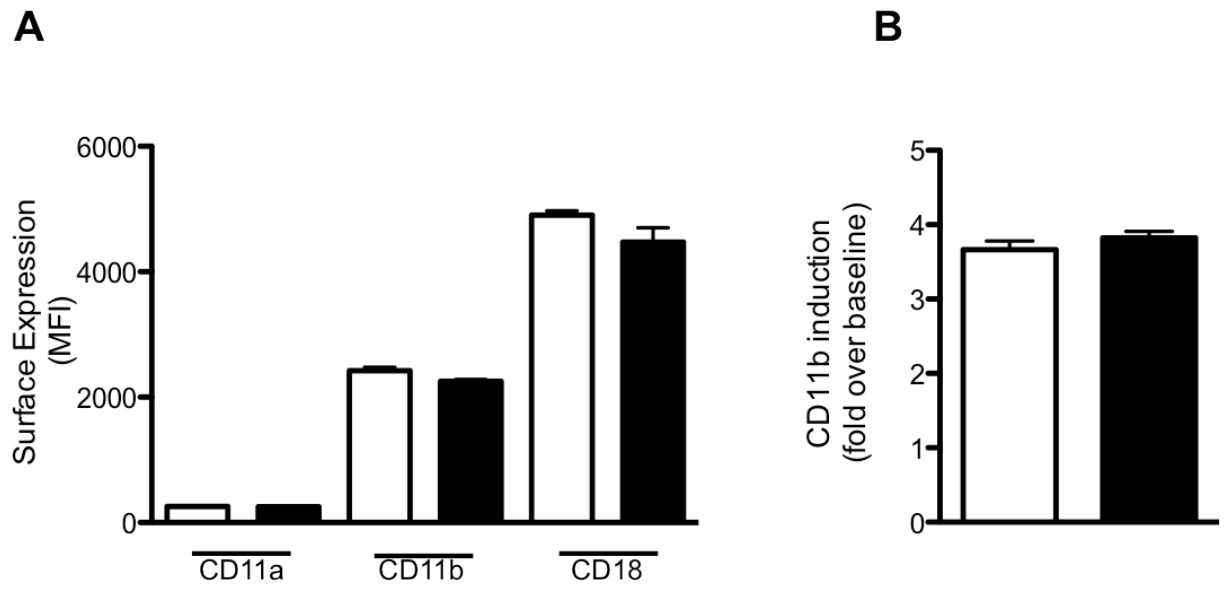
Supplemental Figure 6. *Atf3* is not expressed in stimulated mouse neutrophils. WT neutrophils stimulated *in vitro* were analyzed by qRT-PCR for *Atf3* (circles) and *Tnf-a* (squares) in response to 10ng/ml LPS (solid lines) or PBS control (dotted lines). Representative of a single experiment, N=2 wells/condition.

Supplemental Figure 7



Supplemental Figure 7. ATF3-mediated differences in F-actin polymerization are adhesion-dependent. Neutrophils were stimulated with fMLP for the indicated times in a 37°C water bath and quenched with 2% PFA. F-actin polymerization was determined by flow cytometry and reported as fold increase in phalloidin-rhodamine MFI over baseline. Results pooled from 3 independent experiments. 1 mouse/genotype/experiment. MFI, mean fluorescence intensity.

Supplemental Figure 8



Supplemental Figure 8. ATF3$^{-/-}$ neutrophils display normal β_2 integrin expression. (A) The major β_2 integrins CD11a (LFA-1), CD11b (Mac-1) and the common b_2 subunit, CD18, were quantified by flow cytometry on unstimulated neutrophils from WT (white bars) or ATF3$^{-/-}$ (black bars) mice. Data are representative of 2 experiments, N=4-6 mice/genotype. (B) WT (white bars) and ATF3$^{-/-}$ (black bars) neutrophils were stimulated with fMLP and CD11b surface expression enrichment compared to baseline was measured by flow cytometry. Data are from 1 experiment, N=4-6 mice/genotype. MFI, mean fluorescence intensity.

Supplemental Table 1

Gene ID	Gene Name	Accession #	Fold Change	Corrected *p* value
Tiam2	T-cell lymphoma invasion and metastasis 2	NM_011878	-284.039	0.000
Erdr1	Erythroid differentiation regulator 1	NM_133362	-24.121	0.004
Odz3	Odd Oz/ten-m homolog 3	AK050784	5.838	0.024
Igh-VJ558	Immunoglobulin heavychain (J558 family)	BC019425	5.544	0.033
Igj	Immunoglobulin joining chain	NM_152839	3.505	0.031
Efna2	Ephrin A2	NM_007909	2.498	0.046
Synpo	Synaptopodin	AK020250	2.493	0.024
Susd4	Sushi domain containing 4	NM_144796	2.433	0.020
Mtus2	Microtubule associated tumor suppressor candidate 2	NM_029920	2.083	0.027

Supplemental Table 1. Significantly differentially expressed protein-encoding genes identified by microarray analysis comparison of WT and ATF3$^{-/-}$ neutrophils. Table represents significantly differentially expressed genes independently co-identified by analysis of microarray data as described in "Methods." Indicated fold change and corrected *p* values are of ATF3$^{-/-}$ neutrophils relative to WT neutrophils from RMA-normalized probes and Benjamini-Hochberg post-test to correct for multiple comparisons.

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Chapter III: ATF3$^{-/-}$ mice exhibit reduced parasite burden without attendant increases in mortality following *Toxoplasma gondii* infection

3.1 Abstract

Toxoplasma gondii is a common human pathogen, the protective immune response to which is largely dependent on regulated activation of the IL-12/IFN-γ axis. ATF3 is a counter-regulatory transcription factor that suppresses NK cell IFN-γ production, and macrophage IL-12 production and iNOS activity, all of which are implicated in the immune response to *T. gondii* infection. We hypothesized that mice deficient in ATF3 would exhibit increased control of *T. gondii.* Indeed, ATF3$^{-/-}$ mice exhibited lower brain cyst burdens in response to low-, but not high-dose, infection. Unlike mice deficient in other counter-regulatory pathways important in toxoplasmosis, ATF3$^{-/-}$ mice did not suffer increased mortality. That said, ATF3$^{-/-}$ mice appeared to exhibit the sequelae of increased immune activation: when controlled for brain cyst burden, weight loss was more severe in ATF3$^{-/-}$ mice. Despite this, sustained increased systemic immune activation was not demonstrable in preliminary experiments in ATF3$^{-/-}$ mice. However, fewer ATF3$^{-/-}$ macrophages contained intracellular parasites at early time points after *in vivo* infection, suggesting the possibility that ATF3 regulates parasite entry and/or macrophage effector function.

3.2 Introduction

Toxoplasmosis is a common human disease with significant morbidity and mortality in select populations [1-3]. Epidemiologic data suggest that almost 10% of the US population 12-49 years old has evidence of *T. gondii* infection [2]. Acute toxoplasmosis in most individuals is asymptomatic. Chronic *T. gondii* infection is generally innocuous, however, studies have suggested links between chronic toxoplasmosis and human behavior and mental illness [4-6]. Primary *T. gondii* infection or reactivation of latent *T. gondii* has greater clinical implications in individuals with insufficiently functioning immune systems (e.g., AIDS, immunosuppression, developing fetuses). Congenital toxoplasmosis, leading to spontaneous abortion or miscarriage, retinochorioditis, cerebral calcifications, failure to thrive, or a seemingly normal infant with learning disabilities later in life [7], can occur via transplacental parasite transmission during acute toxoplasmosis occurring in pregnancy. In fact, most congenitally exposed infants are asymptomatic at birth but display ocular or neurological involvement later on [8]. Chronic *T. gondii* infection persists as latent, encysted parasites, often in the central nervous system (CNS) [9].

A successful immune response to *T. gondii* relies on complex orchestration of the innate and adaptive arms of the immune system, centered on the effector and counter-regulatory cytokines IFN-γ and IL-10, respectively. Mice deficient in IFN-γ die with high parasite loads [10-12], while IL-$10^{-/-}$ mice or mice lacking production of the pro-resolution eicosanoid lipoxin A_4 (LXA_4) have lower parasite burdens but die of immunopathology—albeit with delayed kinetics

relative to that of IFN-γ-deficient mice [13, 14]. *T. gondii* infection induces IL-12 secretion by macrophages [15], dendritic cells (DCs) [15-17], and neutrophils [18-20]. IL-12 stimulates NK cells to produce IFN-γ [11, 15, 21, 22], which activates macrophages to ingest and kill phagocytosed parasites, and produce TNF-α, IL-1β, and IL-12 [11, 15, 23-26]. Neutrophils contain preformed IL-12 [20] and, unlike macrophages and DCs, can further produce large amounts of IL-12 independent of IFN-γ priming [18-20]. Neutrophils are thought to be essential for the proper priming of the NK-mediated protective IFN-γ response [27, 28]. The combination of IL-12 and IFN-γ also produces a Th1-polarized T cell response that is critical for control of acute *Toxoplasma* infection and maintenance of chronic toxoplasmosis [29]. Among other effector pathways, these cytokines upregulate inducible nitric oxide synthase (iNOS) and indoleamine 2,3-dioxygenase (IDO) expression, both of which produce molecules that are toxoplasmastatic [23, 30, 31].

Neutrophils are recruited early in *T. gondii* infection [19] and are seen in the peritoneal cavity [19, 20, 27, 28, 32-34] or lamina propria [33] following intraperitoneal (i.p.) or peroral *T. gondii* infection, respectively. Human and murine neutrophils have been shown to respond to *T. gondii* or soluble *T. gondii* antigens *in vitro* by elaborating the cytokines TNF-α and IL-12 [18-20, 35], and the chemokines CCL3, CCL4, CCL5 and CCL20 [35]. Neutrophil depletion in experimental models of i.p. cyst infection is lethal [20, 27, 35]. Moreover, mice lacking the chemokine receptor CXCR2 have reduced neutrophil recruitment and elevated brain cyst burdens, without measurable decrements in neutrophil

effector function [28]. Murine CXCR2 binds CXCL1, CXCL2, and CXCL5, mediating neutrophil chemotaxis. *T. gondii* challenge induces the production of IL-8, among other chemokines, by human epithelia [36, 37] and fibroblasts [37], and CXCL1 by rat endothelial cells [38]. Further, i.p. infection of mice with *T. gondii* up-regulates CXCL1 and CXCL2 transcript and protein expression [39].

ATF3 is a member of the ATF/cAMP responsive element binding protein family of transcription factors, classified by shared possession of a basic region and leucine zipper domain. Immunologically, ATF3 acts as a counter-regulatory transcription factor, induced by pro-inflammatory stimuli and restraining transcription of further pro-inflammatory mediators [40]. ATF3 helps to discriminate between transient or low-dose stimuli and persistent or high-dose stimuli [41, 42]. In this manner, ATF3 restrains potential immunopathology resulting from innocuous stimuli but allows immune activation to occur when dangerous levels of microbes are indicated. ATF3 regulation has been described for numerous pro-inflammatory mediators including, IL-6 and IL-12p40 [40, 42], CCL4 [43], IFN-γ [44], iNOS and TNF-α [40]. Additionally, chapter 2 in this thesis describes the complex regulation by ATF3 of neutrophil recruitment through neutrophil-extrinsic inhibition of LPS-driven CXCL1 production and neutrophil-intrinsic promotion of chemotaxis.

The overlap between ATF3-regulated and *T. gondii*-induced cytokines suggested the possibility that ATF3 plays a role in regulating *T. gondii* infection. We hypothesized that ATF3$^{-/-}$ mice would exhibit increased parasite control, albeit at the expense of increased immunopathology similar to IL-10$^{-/-}$ mice or

LXA_4-deficient mice. Indeed, we found that ATF3 modulated *T. gondii infection*, with reduced brain cyst burdens in $ATF3^{-/-}$ mice. Unexpectedly, $ATF3^{-/-}$ mice did not exhibit increased mortality, although $ATF3^{-/-}$ mice lost more weight when controlled for cyst burden, a finding consistent with immune hyperactivity. That said, in preliminary experiments, we detected no sustained differences in plasma concentrations of IL-12 or IFN-γ in $ATF3^{-/-}$, compared with WT, mice. *T. gondii* infection drove neutrophil recruitment to the WT peritoneum, while $ATF3^{-/-}$ neutrophil recruitment was impaired. Nevertheless, fewer peritoneal macrophages in $ATF3^{-/-}$ mice contained intracellular parasites at the same time point, suggesting that ATF3 may regulate mechanisms of intracellular pathogen clearance in macrophages.

3.3 Methods

3.3.1 Mice and *in vivo* studies

$ATF3^{-/-}$ mice [45], backcrossed >10 generations to C57Bl/6NJ (generously provided by Tsonwin Hai), and wild type (WT) C57Bl/6NJ mice (Taconic Farms), were maintained in the Cincinnati Children's Research Foundation's specific pathogen-free animal facility. $ATF3^{+/-}$ heterozygous mice were generated by crossing $ATF3^{-/-}$ mice with WT mice. Mice were injected with 20 or 200 cyst-containing brain homogenate or control, uninfected brain homogenate in 400 ul PBS (Gibco) intraperitoneally (i.p.). Blood was drawn via submandibular vein bleeds into EDTA-chelated tubes to collect plasma. Peritoneal lavage was performed with 2 ml ice cold PBS. Peritoneal lavage fluid cells were counted and spun onto slides for Diff-Quik analysis of cell types and presence of intracellular parasites. Cell-free lavage supernatants and plasma were assayed for IL-12-p40 and IFN-γ (BD) by ELISA. All experimental procedures were approved by the Cincinnati Children's Hospital Medical Center IACUC.

3.3.2 *T. gondii* propagation and cyst determination

Toxoplasma gondii strain ME49, originally provided by Dr. A. Sher, was maintained by passage in WT mice for $\geq$ 28 days. To obtain cysts, infected mice were euthanized with CO_2 followed by cervical dislocation. Brains (or individual brain hemispheres) were harvested into 1 ml PBS (Gibco) and homogenized by sequential passage through a tuberculin syringe followed by 19, 20 and 21 gauge

needles. 20 ul of brain homogenate was counted by microscopy to quantify cyst concentration.

3.3.3 mRNA analysis

Brains (or individual hemispheres) were harvested into TRIzol for RNA analysis. *Ido1* and *Ido2* mRNA, normalized to *β-actin* expression, was quantified by quantitative (q)RT-PCR (LightCycler 480; Roche) using the following primers: *β-actin,* 5'-GGCCCAGAGCAAGAGAGGTA-3', 5'-GGTTGGCCTTAGGGTTCAGG-3'; *Ido1,* 5'-GTGGGCAGCTTTTCAACTTC-3', 5'-GGGCTTTGCTCTACCACATC-3'; *Ido2,* 5'-TGCCTGATGGCCTATAACCAGTGT-3', 5'-TGCAGGATGTGAACCTCTAACGCT-3'.

3.3.4 Statistics

Prism GraphPad 5 software was used for statistical analysis using unpaired, two-tailed *t* test or linear regression, followed by slope comparison by ANCOVA analysis, as appropriate.

3.4 Results

3.4.1 ATF3 attenuates parasite clearance in response to low-dose infection with *T. gondii*

To define the role of ATF3 in *T. gondii* clearance, WT, ATF3$^{+/-}$, and ATF3$^{-/-}$ mice were infected intraperitoneally (i.p.) with *T. gondii* cysts. A trend towards reduced cerebral parasite burdens was observed in mice lacking ATF3, with an apparent gene dosage effect (Figure 1A). Of note, ATF3$^{-/-}$ mice exhibited significantly reduced cerebral cyst burdens following low-dose (20 cysts), but not high-dose (200 cysts) infection (Figure 1B). The number of encysted bradyzoites in the central nervous system (CNS) is the result of both the tachyzoite burden that escapes early immune effector mechanisms to seed the brain with cysts, and the degree of sustained immune pressure against encysted parasites. Therefore, we first sought to determine the stability of chronic cyst burdens over time in WT and ATF3$^{-/-}$ mice. Despite significant differences in cyst burden between WT and ATF3$^{-/-}$ mice, we observed no significant differences in the slopes of the linear regression lines describing WT and ATF3$^{-/-}$ cyst burden relative to time in pooled data from 8 independent experiments comparing WT vs. ATF3$^{-/-}$ cyst burdens after 26 to 60 days of infection (slope =-29.21 ATF3$^{-/-}$, -22.42 WT, Figure 1C).

IDO-1 and/or IDO-2 are thought to be important in the immune response to *T. gondii* as pharmacologic inhibition of IDO leads to increased cyst burden and death [31]. In support of the apparent lack of demonstrable increased chronic immune pressure in ATF3$^{-/-}$ mice, *Ido1* and *Ido2* expression were not differentially regulated in chronically-infected ATF3$^{-/-}$ mice (Figure 1D), despite

the presence of an ATF3 binding site in the *Ido2* promoter (data not shown). Together, these data suggest that reduced brain cyst burdens in ATF3$^{-/-}$ mice are likely due to enhancement of early parasite clearance or reduced infectivity, rather than differential clearance in the chronic phase.

3.4.2 ATF3$^{-/-}$ mice do not exhibit increased mortality after *T. gondii* infection

ATF3$^{-/-}$ mice exhibit exaggerated immune responses to low-dose lipopolysaccharide (LPS) challenge [46], and increased mortality in sublethal LPS-induced peritonitis models, associated with increased IL-12 production [40]. Given the central role of IL-12 in *T. gondii* infection (and the reduced cyst burdens observed in ATF3$^{-/-}$ mice), we expected to see increased morbidity or mortality due to excessive immune activation in *T. gondii*-infected ATF3$^{-/-}$ mice. However, ATF3$^{-/-}$ mice exhibited no significant difference in mortality over 9 weeks of infection (Figure 2A), in marked contrast to findings in IL-10- and LXA_4-deficient models [13, 14]. Similarly, there were no differences in acute (first 2 weeks) or chronic (2-9 weeks) weight loss, although ATF3$^{-/-}$ mice tended to maintain a lower weight during chronic infection (Figure 2B). These data suggested that genotype-specific differences in systemic immune mediators might not exist. Indeed, there were no sustained differences in plasma IL-12 (Figure 2C) or IFN-γ (Figure 2D) concentrations over the first week of infection, although IL-12 levels were significantly increased in ATF3$^{-/-}$ mice on day 3.

3.4.3 ATF3$^{-/-}$ mice lose more weight than WT mice when weight loss is controlled for cyst burden

Linear regression analysis revealed that cyst burden was highly predictive of weight loss in both WT and ATF3$^{-/-}$ mice (WT, R^2 =0.7904, p <0.0001; ATF3$^{-/-}$, R^2 =0.9389, p <0.0001). However, weight loss in ATF3$^{-/-}$ mice was more sensitive to cyst burden than WT mice in response to low-dose (slope =-1/90.0 ATF3$^{-/-}$, -1/362.7 WT, p <0.0001 ANCOVA, Figure 3A) but not high-dose (slope =-1/249.3 ATF3$^{-/-}$, -1/161.5 WT, p =0.3925 ANCOVA, Figure 3B) infection. Therefore, while there were no significant differences in mortality or weight loss, ATF3$^{-/-}$ mice exhibited increased weight loss relative to parasite burden after inoculation with low infectious doses of parasites.

3.4.4 Cyst-induced neutrophil recruitment is inhibited in ATF3$^{-/-}$ mice

We next evaluated immunologic responses within the peritoneal cavity, the site of experimental *T. gondii* inoculation. In WT mice, we observed no differences in peritoneal lymphocyte numbers (data not shown) or monocyte/macrophage (Figure 4A) numbers between mice infected with cyst-infected brain homogenates or control, uninfected brain homogenates. However, i.p. injection of cysts resulted in neutrophil recruitment to the peritoneum, both immediately following injection and 3 days after injection (Figure 4B, black lines), concomitant with a significant increase in detectable IL-12p40 within the peritoneal cavity (Figure 4B, blue lines).

Given the important role of neutrophils in the immune response to *T. gondii* [20, 27, 35], we compared the peritoneal cell composition of WT and ATF3$^{-/-}$ mice 3 days after i.p. cyst injection (Figure 4C). We observed a trend towards reduced neutrophil numbers in ATF3$^{-/-}$ mice, but no differences in the numbers of monocyte/macrophages. Further, we found a significant reduction in mast cells, something that has already been reported in ATF3$^{-/-}$ mice at baseline [47]. We also detected a trend towards increased lymphocytes in ATF3$^{-/-}$ mice (Figure 4C), which is notable, given the lack of differences in peritoneal lymphocyte recruitment in cyst- or control-injected WT mice, and the absence of baseline differences in peritoneal cell composition between WT and ATF3$^{-/-}$ mice (data not shown). Despite equivalent monocyte/macrophage numbers between WT and ATF3$^{-/-}$ cyst-injected mice, there was a strong trend towards a decreased frequency of ATF3$^{-/-}$ monocyte/macrophages containing intracellular parasites 3 days after infection (Figure 4D), a time point coinciding with peak peritoneal IL-12 concentrations (Figure 4B). Together, these data suggest that *elevated* ATF3$^{-/-}$ neutrophil recruitment likely does not contribute to increased parasite control in ATF3$^{-/-}$ mice, but do not address the amount of IL-12 produced by ATF3$^{-/-}$ neutrophils (or macrophages) on a per cell basis. However, unknown mechanisms result in fewer infected macrophages in ATF3$^{-/-}$ mice as soon as 3 days after infection.

3.5 Discussion

Here we show that ATF3$^{-/-}$ mice have lower chronic cyst burdens than WT mice without attendant increases in mortality, in striking contrast to findings in IL-10- and LXA_4-deficient mice. The mechanisms responsible for these observations are unclear, however, ATF3$^{-/-}$ mice exhibited greater weight loss when controlled for cyst burden, potentially indicating increased immune activation, and had fewer peritoneal macrophages containing intracellular parasites early after infection. While preliminary, these findings suggest that ATF3 plays a role in restraining control of parasite replication or facilitating parasite entry into host cells.

Given the significant overlap between effector cells and molecules responsible for immune response to *T. gondii* that are also negatively regulated by ATF3, we were not surprised to see reduced cyst burdens in ATF3$^{-/-}$ mice. NK cell-produced IFN-γ stimulates macrophages to ingest and kill rapidly dividing tachyzoites during acute infection [11, 15], while T cell IFN-γ production at chronic time points is required to maintain control of encysted bradyzoites in the brain [29]. Reduced chronic cyst burdens, could therefore be due to increased acute immune clearance of tachyzoites and/or increased chronic immune pressure on encysted bradyzoites. However, we found that while chronic cyst burdens did decrease with time in both genotypes, the differences in cyst burden between WT and ATF3$^{-/-}$ mice remained constant. There are important caveats to this conclusion, as linear regression yielded relatively low R^2 values, suggesting that time alone is unlikely to account for the cyst burden changes. In fact, while

mouse age, sex, cyst type and number injected, and duration of infection were matched between genotype for each independent experiment, they varied significantly between the 8 pooled experiments. Importantly however, there were no sex-dependent differences within genotypes. Clearly though, future kinetic experiments quantifying chronic cyst burden longitudinally will be required.

Stable chronic differences in cyst burdens suggest that acute responses to *T. gondii*, or at least those occurring prior to 26 days post-infection (the earliest chronic time point we evaluated), are likely responsible for the altered cyst burdens observed in ATF3$^{-/-}$ mice, not increased effector mechanisms present during chronic infection. This supposition argues against differential regulation of important immune effector mechanisms critical to control of chronic *T. gondii* infection, such as IDO [31] and iNOS [26]. In fact, while the *Ido2* promoter contains an ATF3 binding site, we found that *Ido1* and *Ido2* expression were unaltered in chronically infected mouse brains. Conversely, iNOS activity is elevated in ATF3$^{-/-}$ mice responding to LPS-challenge [40], and iNOS-deficient macrophages exhibit defective microbicidal activity against *T. gondii in vitro* [26]. However, iNOS-deficient animals control *T. gondii* infection acutely and only experience parasite outgrowth in the CNS at chronic time points [26]. Nevertheless, the expression level of the iNOS-encoding gene, *Nos2,* remains to be determined in our model.

Perhaps the most interesting finding in our study was the lack of mortality in *T. gondii*-infected ATF3$^{-/-}$ mice. IL-10$^{-/-}$ mice or mice lacking production of LXA_4 demonstrate reduced parasite burdens at the expense of severe

immunopathology resulting in death [13, 14]. While survival kinetics differ depending on the model (10 to 20 days in IL-$10^{-/-}$ mice; 30 days in LXA_4-deficient animals), these animals display elevated IL-12 and IFN-γ production [13, 14] and significantly increased weight loss prior to death [14]. We did not observe sustained differences in plasma levels of IL-12(p40) or IFN-γ in $ATF3^{-/-}$ mice, despite a significant increase in IL-12 levels 3 days after infection. This could suggest that early killing of *T. gondii*, perhaps associated with early increases in IL-12 in $ATF3^{-/-}$ mice, contributes to lower $ATF3^{-/-}$ cyst burdens; that the increased immune activation occurs locally, prior to dissemination to the less host-tolerant environment of the brain; and/or that the degree of increased immune activation is meager. Elevated local cyst control is the most likely mechanism of reduced cyst burdens without increased systemic immune activation, given the dual findings of reduced frequency of intracellular parasites in $ATF3^{-/-}$ peritoneal macrophages and equivalent IFN-γ plasma levels. Further studies will be necessary to disentangle these mechanistic possibilities.

Neutrophils are critical to the early response to *T. gondii* [27, 34, 48, 49] and are specifically recruited in response to i.p. injection of cysts in our model. WT neutrophils appeared immediately following injection, likely due to injury incurred by i.p. injection. No IL-12 was detected at this time point, consistent with the fact that encysted *T. gondii* bradyzoites are immunologically relatively inert. Cyst injection resulted in a robust neutrophil recruitment 3 days after infection. This time point is consistent with cyst rupture and release of tachyzoites. It will be noted that this time point also coincides with the detection of IL-12 on day 3 in

peritoneal lavages, and the role of neutrophils as early producers of IFN-γ-independent IL-12 [18-20]. In comparison, cyst-induced ATF3$^{-/-}$ neutrophil recruitment was inhibited on day 3. This is not unexpected, given our identification of neutrophil-intrinsic migration and recruitment defects in ATF3$^{-/-}$ mice [Boespflug et al., 2013, submitted]. Therefore, ATF3$^{-/-}$ neutrophil recruitment (or lack thereof) likely does not contribute to increased parasite clearance as effector cells. Notably, this does not rule out the potential for increased IL-12 production at the cellular level in ATF3$^{-/-}$ neutrophils, as is seen in ATF3$^{-/-}$ macrophages [40], and *in vitro* neutrophil and tachyzoite co-culture experiments are envisioned to test this possibility. Alternatively, parasite-driven neutrophil apoptosis and phagocytosis by macrophages is a mechanism that can facilitate parasite replication in macrophages, as is the case in *Leishmania major* infection [50]. Therefore, reduced neutrophil recruitment in ATF3$^{-/-}$ mice could provide a mechanism for reduced parasite replication within macrophages by depriving parasites of neutrophils to subvert for entry into macrophages.

In addition to reduced cyst-induced ATF3$^{-/-}$ neutrophil recruitment, we also observed a trend towards increased lymphocyte numbers in the ATF3$^{-/-}$ peritoneum 3 days after *T. gondii* injection. This is interesting, as cyst injection did not induce differential WT peritoneal lymphocyte compositions, nor were there any differences between WT and ATF3$^{-/-}$ peritoneal lymphocyte numbers at baseline. The importance of this observation is unclear. Cytospin quantification of lymphocytes cannot distinguish between innate lymphocyte populations such as NK cells, NKT cells, B1 cells, and marginal zone B cells, and adaptive

lymphocyte populations. Phenotypic evaluation of the subpopulations within this group will therefore be required in order to determine the potential contribution to increased parasite control of different lymphocytic subtypes in ATF3$^{-/-}$ mice.

While we observed no genotype-specific differences in macrophage numbers, we observed a reduced frequency of macrophages in ATF3$^{-/-}$ mice that contained intracellular pathogens. The importance of this observation is also unclear. It is possible that a reduction in intracellular parasites indicates: (1), better clearance, through increased ATF3$^{-/-}$ macrophage sensitivity to activation by equivalent IFN-γ levels, or through increased killing effector mechanisms present in ATF3$^{-/-}$ macrophages; (2), reduced tachyzoite numbers, due to enhanced extracellular parasite killing, increased ingestion and/or killing by another cell type, or elevated infection of non-macrophage cell types; or (3) reduced uptake specifically by macrophages. It is unlikely that the observation of reduced intracellular tachyzoites was due to decreased macrophage ingestion as this would suggest impaired macrophage effector mechanisms while ATF3$^{-/-}$ mice have decreased cyst burdens chronically. Additionally, no differences were noted in extracellular parasite numbers in WT and ATF3$^{-/-}$ peritoneal lavages, nor were tachyzoites observed in other cell types. Use of *in vitro* killing assays will be needed to evaluate differential macrophage uptake and sensitivity to effector cytokines between genotypes. iNOS is a particularly interesting effector mechanism to evaluate in ATF3$^{-/-}$ macrophages, as it is elevated in ATF3$^{-/-}$ mice responding to i.p. LPS [40] and *Nos2*$^{-/-}$ mice demonstrate significantly reduced frequencies of peritoneal cells with intracellular tachyzoites [26]. Closer kinetic

analysis of tachyzoite numbers and peritoneal cell composition over the first week of infection in WT and ATF3$^{-/-}$ mice will be necessary to quantify infectious burden as well as to determine whether ATF3$^{-/-}$ neutrophil recruitment is inhibited or merely altered relative to WT mice.

Here, we have shown that ATF3$^{-/-}$ mice have an increased ability to control low-dose *T. gondii* infections. We propose that this may be due to increased early control of tachyzoite replication due to macrophage-intrinsic effector mechanisms, or neutrophil IL-12 production. These data underscore the complex role of ATF3 in infectious disease, with overlapping and sometimes opposed immunologic effects. In response to *T. gondii,* ATF3 appears to restrain neutrophil recruitment yet promote macrophage effector responses, events that, together, avoid the increased mortality seen in ATF3$^{-/-}$ mice in LPS-induced peritonitis models [40] and in IL-10$^{-/-}$ mice infected with *T. gondii* [13]. Thus, understanding the exact molecular mechanisms of increased ATF3-dependent parasite control could provide insight into directed therapies to take advantage of ATF3 regulation of immune responses whilst avoiding ATF3-dependent immunopathology.

3.6 Figures

Figure 1

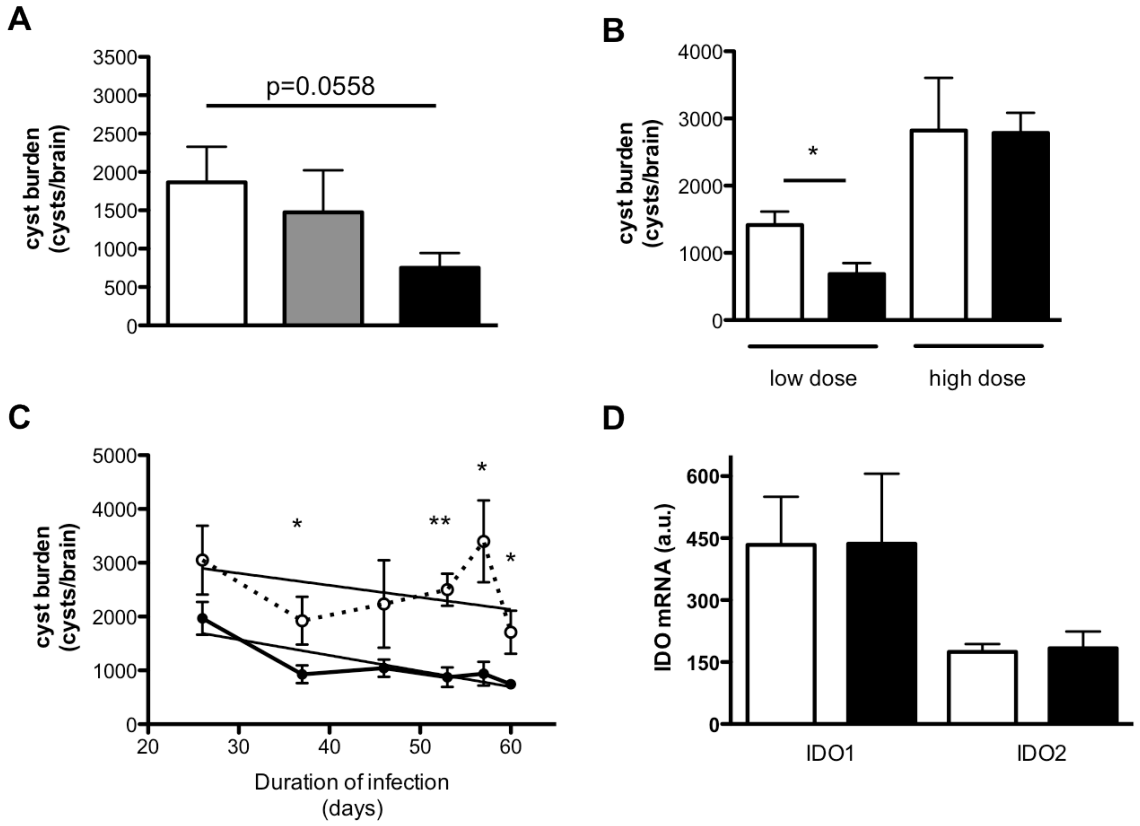

Figure 1. ATF3 inhibits control of *T. gondii* infection. (A) WT (white bars), ATF$^{+/-}$ (gray bars), and ATF3$^{-/-}$ (black bars) mice were challenged i.p. with 10 *T. gondii* cysts. 45 days later, mice were sacrificed, brains removed and homogenized for quantification of brain cysts. Data are an independent experiment, N=3-4 mice/group. Statistics are unpaired, two-tailed *t* test. (B) WT (white bars) and ATF3$^{-/-}$ (black bars) mice were challenged i.p. with low (20 cysts) or high (200 cysts) doses of *T. gondii* bradyzoite cysts. Brains were harvested 57 or 60 days post infection and homogenized for cyst determination. Data represent 1 (high dose) or 7 (low dose) independent experiments, N=5-7

mice/group. (C) Brain cyst burdens of WT (dotted line) and ATF3$^{-/-}$ (solid line) mice infected with 20 cysts i.p. were enumerated at indicated chronic time points. Lines are regression analysis (WT, m =-22.42, R^2 =0.026, *p* =0.2827; ATF3$^{-/-}$, m =-29.31, R^2 =0.3222, *p* <0.0001; slopes are not significantly different by ANCOVA. Each time point represents an independent experiment, N=4-14 mice/genotype/time point. (D) Brains from WT (white bars) and ATF3$^{-/-}$ (black bars) mice were harvested 26 days after infection and brain homogenates were analyzed for *Ido1* and *Ido2* expression by qRT-PCR. Data represent 4 independent experiments, N=8 mice/genotype. Statistics are unpaired, two-tailed *t* test with Welch's correction if variances were significantly different. Data are mean ± S.E.M. **p* <0.05, ***p* <0.01.

Figure 2

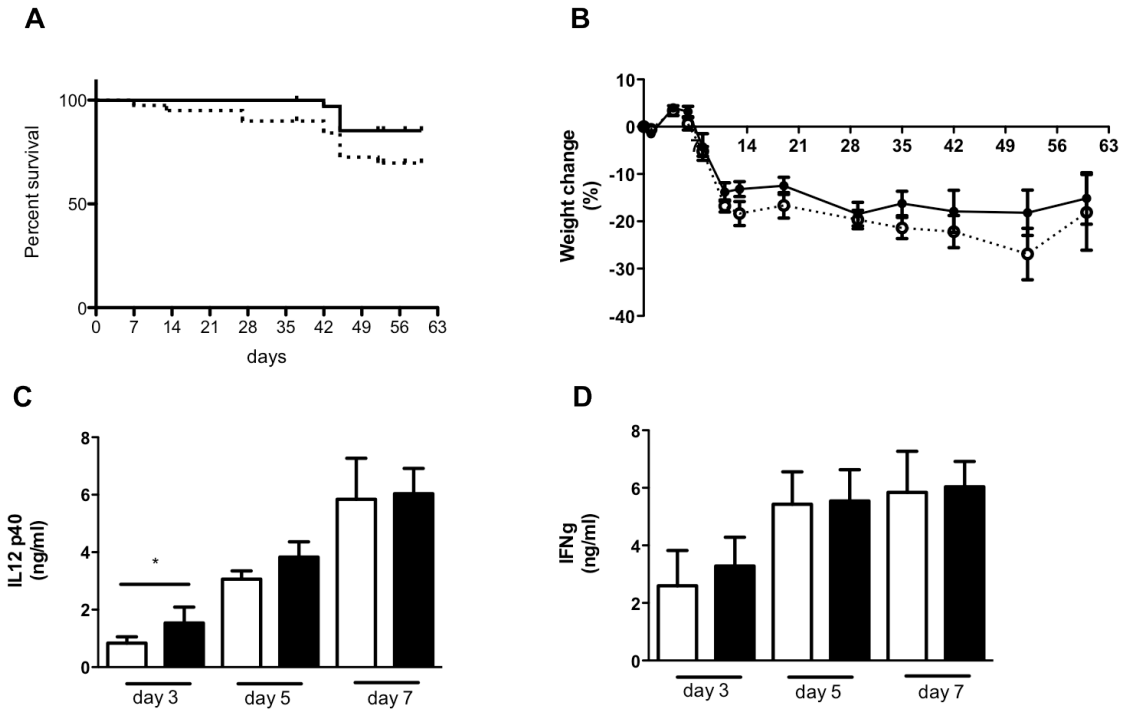

Figure 2. ATF3$^{-/-}$ mice do not exhibit increased mortality following *T. gondii* infection. (A, B) WT (dotted line) and ATF3$^{-/-}$ (solid line) mice infected with 20 *T. gondii* cysts and followed for survival (A), or weighed every 3-4 days for 9 weeks (B). Data from 6 independent experiments N=42 WT, 40 ATF3$^{-/-}$, are pooled for A and are representative of 7 independent experiments, N=4-13 mice/genotype in B. (C, D) Plasma IL-12p40 (C) and IFN-γ (D) levels were measured in WT (white bars) and ATF3$^{-/-}$ (black bars) mice after infection with *T. gondii* for indicated times. Mice were bled from the submandibular vein at the indicated times. Data are from 1 experiment, N=8-9 mice/genotype. Statistics are unpaired, two-tailed *t* test. Data are mean ± S.E.M. **p* <0.05.

Figure 3

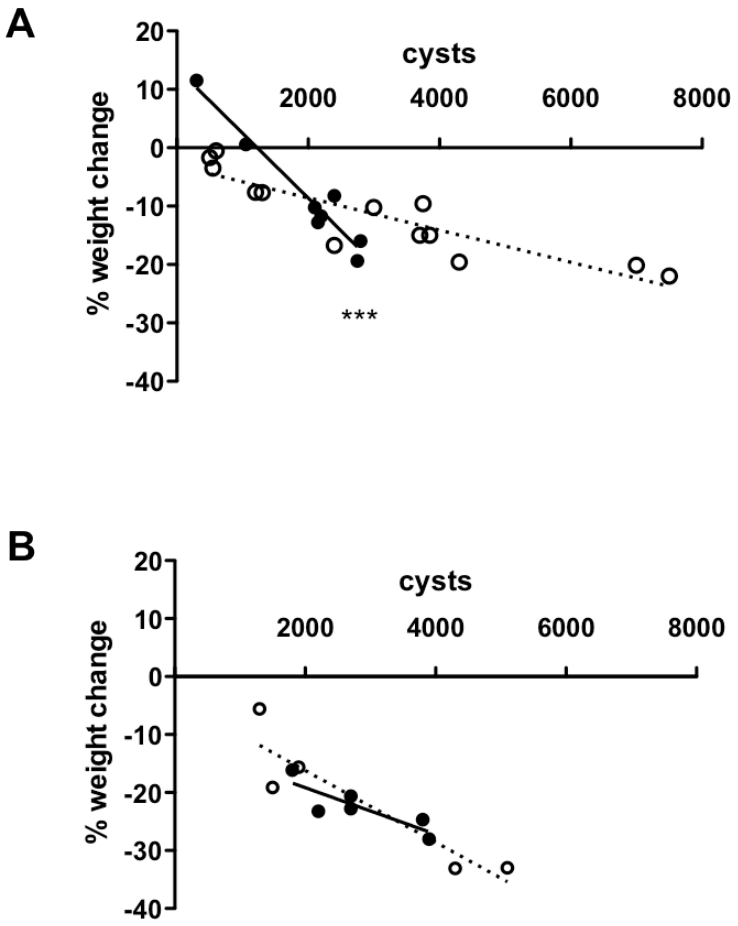

Figure 3. ATF3$^{-/-}$ mice lose more weight when controlled for cyst burden than WT mice. Linear regression analysis of weight change and cyst burden in WT (dotted line) and ATF3$^{-/-}$ (solid line) mice infected with 20 (A) or 200 (B) *T. gondii* cysts. A; WT slope =-1/362.7, R^2 =0.7904, p <0.0001; ATF3$^{-/-}$ slope =-1/90.0, R^2 =0.9389, ***p <0.0001. Slopes are significantly different by ANCOVA analysis. B; WT slope =-1/161.5, R^2 =0.8419, p <0.05; ATF3$^{-/-}$ slope =-1/249.3, R^2 =0.7187, p <0.05. Slopes are not significantly different by ANCOVA analysis. Data representative of 7 independent experiments, N=4-13 mice/genotype.

Figure 4

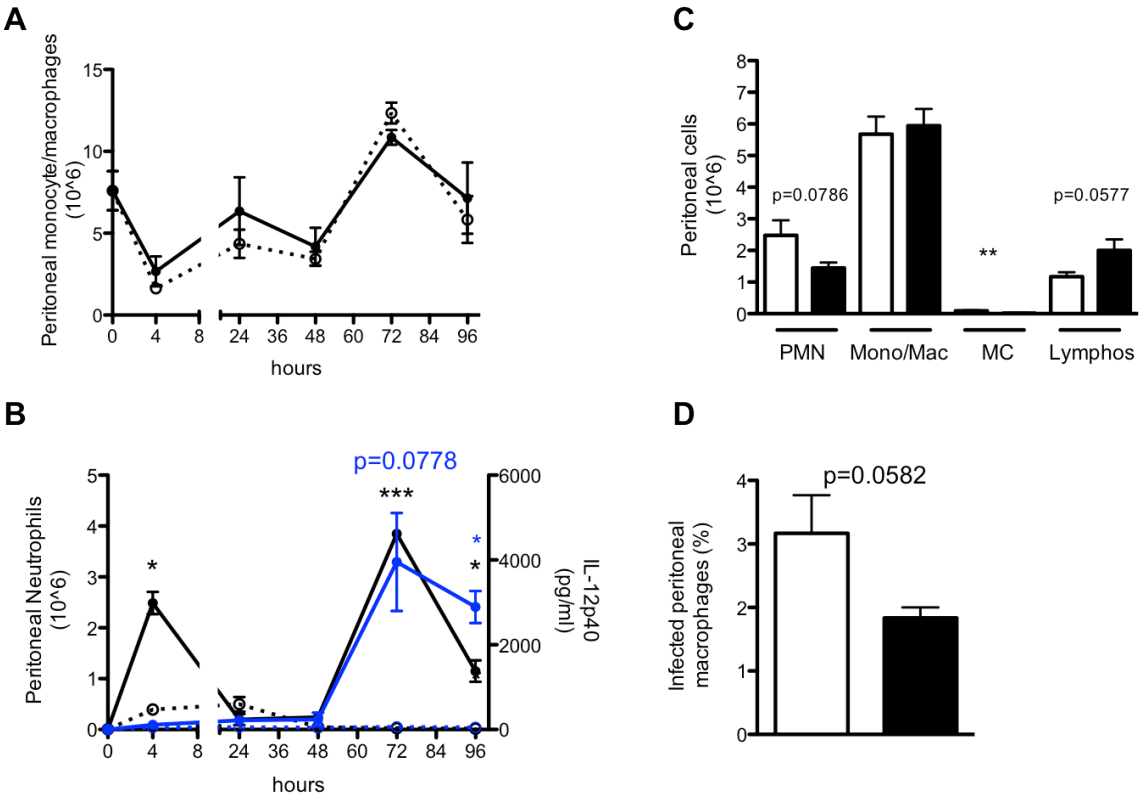

Figure 4. Neutrophil recruitment to the peritoneum is impaired in ATF3$^{-/-}$ mice. (A-C) WT mice were injected i.p. with *T. gondii* cysts (solid line) or control (dotted line) and peritoneal lavages were performed. Pelleted cells were analyzed by cytospin to determine macrophages (A), neutrophils (B, black lines), and the cell free supernatant analyzed for IL-12p40 production via ELISA (B, blue lines). (C) WT (white bars) and ATF3$^{-/-}$ (black bars) mice were infected with *T. gondii* i.p.. On day 3 after infection, peritoneal cavities were lavaged to determine cellular composition by cytospin. (D) WT (white bars) or ATF3$^{-/-}$ (black bars) mice were infected i.p. with *T. gondii* and peritoneal lavages were performed 3 days later. Cytospin analysis was used to determine the percentage of peritoneal macrophages infected with tachyzoites. Data are from 1 independent experiment,

N=2-3 mice/time point (A-B) or 6 mice/genotype (C-D). Statistics are unpaired, two-tailed *t* test. Data are mean ± S.E.M. **p* <0.05, ***p* <0.01, ****p* <0.001. PMN, neutrophils; mono/mac, monocyte/macrophages; MC, mast cells; lymphos, lymphocytes.

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Chapter IV: ATF3 regulates the immune response to persistent LCMV infection

4.1 Abstract

LCMV Clone 13 (Cl13) establishes persistent viral infection in mice, characterized initially by robust antiviral immune response followed by T cell exhaustion and deletion. In comparison to LCMV Armstrong (Arm), which is cleared within 10 days, Cl13 infection leads to the induction of numerous counter-regulatory pathways, blockade of which facilitates clearance of persistent Cl13 infection. Here, we report preliminary data indicating that the counter-regulatory immune transcription factor ATF3 is similarly induced by Cl13 infection and that genetic deletion of ATF3 modestly reduces viral burden. The mechanism(s) underlying increased viral clearance in $ATF3^{-/-}$ mice remain to be defined, however, $ATF3^{-/-}$ mice generate significantly more virus-specific IFN-γ-producing $CD8^{+}$ and $CD4^{+}$ T cells, associated with altered conventional dendritic cell subset composition and reduced regulatory T cell numbers. Together, these data implicate ATF3 in the immune counter-regulation associated with Cl13 infection, and suggest that ATF3 may modulate viral persistence through down-modulating the frequency of virus-specific IFN-γ-producing T cells.

4.2 Introduction

Lymphocytic choriomeningitis virus (LCMV), an enveloped, single-stranded RNA virus, was identified by Charles Armstrong in 1934. Adult mice infected with Armstrain strain (Arm) clear the virus within 7-10 days via potent, IFN-γ-dependent antiviral immune response [1, 2]. Infection with an Arm sub-strain, Clone 13 (Cl13), however, results in a persistent infection in adult mice. While the immune response to Cl13 is initially robust, $CD8^+$ effector responses decline during persistent viral infections and virus-specific $CD8^+$ T cells become exhausted and/or are deleted [3-7]. Dendritic cells (DCs) are critical antigen presenting cells (APCs) that prime antigen-specific $CD8^+$ and $CD4^+$ T cell responses in the setting of LCMV infection [8, 9]. Splenic DCs can be separated broadly into conventional (c)DCs and non-conventional, plasmacytoid (p)DCs. pDCs are potent producers of type I interferons (IFN-I) critical for limiting viral replication [10]. cDCs can be further subdivided into $CD8^+$ and $CD11b^+$ DCs. $CD8^+$ DCs are the primary DC subtype that primes $CD8^+$ T cell responses in LCMV infection [11], while $CD11b^+$ DCs activate $CD4^+$ T cells, which are critical to shaping and maintaining $CD8^+$ T cell responses against LCMV [12].

Cl13 differs from Arm by three amino acids, resulting in coding changes in the RNA polymerase and the viral spike protein GP1 [13-15]. Virus-specific $CD8^+$ and $CD4^+$ T cells react to identical epitopes in both strains. However, Cl13 induces significantly higher expression of counter-regulatory pathways involving programed death-1 (PD-1) [16] and interleukin (IL)-10 [17], and lymphocyte activation gene 3 (LAG3) [18]. Importantly, blockade of either the PD-1 ligand,

PD-L1, or the IL-10 receptor results in accelerated clearance of Cl13 infection [16, 17, 19]. Cl13 also appears to attenuate immune responses through a preferential tropism for DCs, while acute strains such as Arm preferentially infect macrophages [8, 15, 20]. Targeted DC infection might be associated with altered DC maturation or activation [21], decreased antigen presentation [22] with subsequent reductions in T cell priming and antiviral responses, and/or with differential generation of DC subpopulations [23].

Cl13 infection also directly subverts T cell responses. It can overstimulate $CD8^+$ T cells, resulting in an earlier expansion and faster contraction of $CD8^+$ T cell populations [24]. $CD8^+$ T cells from Cl13-infected mice also demonstrate impaired functionality, marked by a progressive inability to produce critical effector cytokines, including IFN-γ [16]. Additionally, Cl13 uniquely drives persistent IFN-I production [21, 25], which can impair dendritic cell maturation [21] and inhibit antiviral responses during persistent expression [25, 26]. In fact, blocking IFN-I signaling results in accelerated resolution of persistent infection through $CD4^+$ T cell-dependent elevation of IFN-γ production [25, 26].

Activating transcription factor 3 (ATF3) is a member of the ATF/cAMP responsive element binding protein (CREB) family of transcription factors. ATF3 acts as a counter-regulatory immune transcription factor, induced by TLR-signaling stimuli and restraining transcription of other TLR-driven pro-inflammatory mediators [27]. ATF3 helps to restrain excessive immune activation by transient or low-dose stimuli, yet allows immune responses to develop against persistent or high-dose immune stimulation [28, 29] [and Boespflug et al. 2013,

submitted]. ATF3 regulates the expression of diverse pro-inflammatory mediators, including IL-6, IL-12p40 [27, 29], CCL4 [30], IFN-γ [31], iNOS and TNF-α [27], and CXCL1 [Boespflug et al. 2013, submitted]. Additionally, ATF3 is induced following IFN-α stimulation [32], and its expression is increased during DC maturation [33-35]. Notably, ATF3 restrains NK cell IFN-γ production during infection with murine cytomegalovirus (MCMV), a persistent virus in its own right, leading to increased hepatic viral burdens and hepatotoxicity [36]. However, a role for ATF3 regulation in modulating LCMV infection has not been reported.

Here we report that $ATF3^{-/-}$ mice cleared Cl13 infection more efficiently than wild type mice, associated with an increase in virus-specific, IFN-γ-producing $CD4^{+}$ and $CD8^{+}$ T cells. The mechanisms responsible for increased anti-LCMV effector T cell responses in $ATF3^{-/-}$ mice remain to be defined. However, these data suggest a more general role for ATF3 in modulation of viral persistence, something with potential therapeutic implications in persistent viral infections marked by immune exhaustion such as HIV/AIDS.

4.3 Methods

4.3.1 Mice and *in vivo* studies

ATF3$^{-/-}$ mice [37], backcrossed >10 generations to a C57Bl/6NJ background, and wild type (WT) C57Bl/6NJ mice (Taconic Farms), were maintained in the Cincinnati Children's Hospital Medical Center's (CCHMC) specific pathogen-free animal facility. Mice were mock- or Cl13-infected under ketamine/xylazine anesthesia with 200 ul PBS (Gibco) or PBS containing $1.8x10^6$ PFUs (plaque forming units) Cl13 retro-orbitally. Blood was drawn from the submandibular vein on indicated days into serum or plasma tubes. Mice were euthanized by cervical dislocation, and blood, liver, and spleen were harvested. Experimental procedures were approved by the CCHMC IACUC.

4.3.2 Virus propagation and PFU determination

Virus was grown in BHK-21 cells (ATCC) and virus titers quantified by PFU assay on Vero cells (ATCC) [38]. Briefly, tissues (homogenized with Dounce homogenizer) or plasma were serially diluted and applied to Vero cell monolayers in DMEM (Media Tech) + 15 mM HEPES (Gibco) +5% FBS (Atlanta Biologicals) for 1 hr at 37°C, rocking gently every 15 minutes. 1% agarose in media 199 (Gibco) +10% FBS was overlaid, drop-wise, and allowed to cool for 1 hour at room temperature. 2x media 199 was added on top of solid agarose and plaque assays were then incubated at 37°C with 5% CO_2. To evaluate, media was aspirated and replaced with 10% acetate buffered formalin (Fisher) for 30 minutes. Formalin was then aspirated and the agarose plug gently scraped off.

Cells were stained with Crystal violet (Sigma) and PFU was quantified using a lightbox.

4.3.3 Cell isolation, *in vitro* cell culture, and flow cytometry

Spleens from mock- or Cl13-infected mice were homogenized through a 100 um cell strainer in PBS + 5% FBS, and red blood cells were lysed with ACK (Invitrogen). Total splenocytes were then counted by Coulter Counter (Beckman Coulter). Splenic leukocyte populations were analyzed by staining with the following monoclonal antibody panels (BD Pharmingen unless otherwise indicated; dilutions dependent on conjugated fluorochrome): CD4, CD16/CD32, CD127, CD44, CD8, KLRG1, CD122, and/or CD25. Virus-specific T cells were identified by staining with MHC class I or class II tetramers loaded with viral peptides gp31-44 (gp33; MHC class I, CD8 T cells) [39], gp61-80 (gp61; MHC class II, CD4 T cells) [40]. Intracellular staining for Foxp3 (eBioscience) was performed after surface staining, using eBioscience Fix/Perm solution as per manufacturer's instructions. For restimulation studies, splenocytes were incubated with viral peptides gp33-44 or gp61-80 in Brefeldin A (Sigma) for 4 hours at 37°C in DMEM (Gibco) + 5% FBS. Cells were surface-stained with monoclonal antibodies to CD4, CD8, and/or CD44 for the last 30 minutes of culture. Cells were then permeabilized with 0.03% saponin (ACROS Organics) and stained with monoclonal antibody to IFN-γ. Cells were analyzed using an LSR II flow cytometer (BD) and FlowJo software.

4.3.4 mRNA analysis

Gene expression arrays of splenic RNA isolated from mice infected with Arm or Cl13 LCMV strains [25] was analyzed using GEOR2 (NCBI). Livers of mock- or Cl13-infected mice were isolated and flushed with 5 ml PBS through the portal vein before harvesting into TRIzol for RNA isolation. *Atf3* mRNA, normalized to *β-actin* expression, was quantified by quantitative (q)RT-PCR (LightCycler 480; Roche) using the following primers: *β-actin,* 5'-GGCCCAGAGCAAGAGAGGTA-3', 5'-GGTTGGCCTTAGGGTTCAGG-3'; *Atf3,* 5'-AGCCTGGAGCAAAATGATGCTT-3', 5'-AGGTTAGCAAAATCCTCAAACAC-3'.

4.3.5 Statistics

Prism GraphPad 5 software was used for statistical analysis using the unpaired, two-tailed *t* test.

4.4 Results

4.4.1 Persistent LCMV infection induces *Atf3* expression with kinetics similar to that of *Pdcd1* and *Il10*

Infection with Cl13 causes significantly increased expression of PD-1 and IL-10, relative to infection with Arm [16, 17]. Blockade of PD-1 signaling with neutralizing antibodies to PD-L1 [16], or inhibition of IL-10 signaling with antibodies to IL-10 receptor (IL-10R) [17, 19, 41] or use of IL-$10^{-/-}$ mice [17], facilitates accelerated clearance of persistent Cl13 infection. In order to define other gene products with similar differential induction kinetics, we employed RNA microarray techniques to quantify splenic RNA from mice infected with Arm or Cl13: before, and 5, 9 or 30 days after infection [25]. As previously reported [25], Cl13 infection was associated with significantly increased expression of the PD-1 gene, *Pdcd1,* at all time points (Figure 1A), and *Il10* on days 9 and 30 (Figure 1B), compared to Arm infection. Similar to *Pdcd1* and *Il10, Atf3* was differentially (over-) expressed in Cl13, relative to Arm, infection. Such expression was most similar to *Il-10*, with significantly increased expression of *Atf3* seen on day 9 in Cl13, compared with Arm, infection (Figure 1C). Importantly, in this dataset (GSE44322) [25], Arm viral titers were significantly lower than Cl13 viral titers at all time points, and below the limit of detection by day 30 [25]. qRT-PCR analysis on day 4 confirmed that Cl13 infection induces *Atf3* expression in wild type (WT) liver (Figure 1D). These data demonstrate that expression of *Atf3* is significantly elevated by infection with the Cl13, as compared to the Arm, strain of LCMV.

4.4.2 Cl13 viral burdens are reduced in ATF3$^{-/-}$ mice

We next compared viral titer kinetics in WT and ATF3$^{-/-}$ mice that had been infected with Cl13 in order to determine the effect of genetic deletion of ATF3 on viral clearance. ATF3$^{-/-}$ mice exhibited significantly reduced virus concentrations in plasma, along with a trend towards decreased tissue viral burden in the liver and spleen, on day 4 after infection (Figure 2A). Kinetic analysis of liver viral burden in WT and ATF3$^{-/-}$ mice over 6 weeks of infection revealed that the trend towards decreased tissue viral burden seen in the liver of ATF3$^{-/-}$ mice on day 4 was not maintained by day 9 (Figure 2B). These data suggest that ATF3 plays a role in promoting Cl13 infectivity or replication, or in restraining viral clearance.

4.4.3 Cl13-specific T cell numbers are increased in ATF3$^{-/-}$ mice

Persistent viral infections are associated with reduced T cell expansion [24]. We thus quantified total splenocyte and virus-specific splenic T cell numbers in WT and ATF3$^{-/-}$ mice infected with Cl13. We observed no significant differences in total splenocyte numbers (as quantified by Coulter-Counter of undifferentiated homogenized spleen, and distinct from splenic leukocytes as determined by flow cytometry) between WT and ATF3$^{-/-}$ mice at any time point (Figure 3A). Determination of total splenic CD8^{+} (Figure 3B, top) and CD4^{+} (Figure 3C, top) T cell numbers by flow cytometry were also not significantly elevated in ATF3$^{-/-}$ compared to WT mice infected with Cl13, although CD8^{+} T cell numbers trended higher in ATF3$^{-/-}$ mice on day 10. However, virus-specific, gp33-41 MHCI tetramer^{+} (gp33^{+}) CD8^{+} and gp61-80 MHCII tetramer^{+} (gp61^{+}) CD4^{+} T cell

frequencies were significantly elevated in ATF3$^{-/-}$ mice on day 10, and continued to trend higher as a percentage of total CD8^{+} cells on day 52 (Figure 3B-C, bottom).

CD4^{+} T cell function [12, 25] and memory generation are defective in Cl13 infection [12]. Therefore, we next evaluated the composition of gp61^{+} CD4^{+} splenic T cells from Cl13-infected WT and ATF3$^{-/-}$ mice. While naïve, virus-specific splenic CD4^{+} T cell numbers were unchanged at both early (d10) and late (d52) time points, activated gp61^{+} CD4^{+} T cell numbers were significantly elevated 10 days after infection in ATF3$^{-/-}$ mice (Figure 3D, left). We also observed significant increases in gp61^{+} CD4^{+} memory T cell numbers early (Figure 3D, left), and a trend towards elevation later (Figure 3D, right) in ATF3$^{-/-}$, compared to WT, mice infected with Cl13. Together, these data suggest that induction of ATF3 during Cl13 infection results in fewer virus-specific T cell numbers, which is associated with increased viral burden.

4.4.4 ATF3$^{-/-}$ mice exhibit increased numbers of IFN-γ^{+} T cells after restimulation with viral peptides

As persistent viral infections are characterized by functionally exhausted T cells [16], we evaluated IFN-γ production by splenocytes from mice acutely- or chronically-infected with Cl13. WT and ATF3$^{-/-}$ splenocytes, harvested 10 or 52 days after *in vivo* infection with Cl13, were restimulated *in vitro* for 4 hours with viral peptides gp33-41 or gp61-80 (or media as a control). Restimulation resulted in significantly greater numbers of IFN-γ^{+} CD8^{+} (Figure 4A) and CD4^{+} (Figure 4B)

splenic T cells from ATF3$^{-/-}$ mice compared to WT mice in both acutely- (d10) and persistently- (d52) infected with Cl13. Of interest, we observed no differences in PD-1 expression [16] between WT and ATF3$^{-/-}$ CD8^{+} T cells (data not shown). These data suggest increased viral control is associated with increased numbers of virus-specific IFN-γ^{+} T cells in ATF3$^{-/-}$ mice, independent of altered PD-1 expression.

4.4.5 ATF3$^{-/-}$ mice exhibit altered splenic cDC subpopulations and decreased regulatory T cell frequencies compared to WT mice, during Cl13 infection

ATF3 induction occurs during DC maturation [33-35], and DCs are critical for LCMV-driven immune responses [9, 20] and are specifically targeted by persistent LCMV strains [21]. We therefore quantified splenic DC populations in WT and ATF3$^{-/-}$ mice infected with Cl13 for 10 days, in order to define the role of ATF3 in Cl13-dependent DC dysregulation. Our gating strategy, outlined in Figure 5A, was designed to enumerate pDC and cDC populations, as well as CD8^{+} and CD11b^{+} cDC subpopulations. Cl13-infected ATF3$^{-/-}$ mice exhibited no differences in the frequency of live splenic leukocytes (Figure 5B), pDCs or cDCs (Figure 5C), when compared to WT mice. However, within the cDC population, we found significant alterations in ATF3$^{-/-}$ cDC subpopulations; CD8^{+} cDC frequencies were significantly decreased while CD11b^{+} cDCs were significantly increased in ATF3$^{-/-}$ mice (Figure 5D).

Cl13 upregulates PD-L1 expression on Cl13-infected splenocytes [16] and Cl13 preferentially targets DCs [15]. We thus quantified PD-L1 expression on

splenic DC populations, and observed equivalent PD-L1 protein expression by all DC subsets, although we did not differentiate between infected and uninfected cells (data not shown). We also observed an unanticipated and significant reduction in the frequency of splenic regulatory T cells in ATF3$^{-/-}$ mice (Figure 5E). Together, these preliminary data suggest that Cl13 may mediate viral persistence through ATF3-dependent modulation of DC populations independent of PD-L1 expression, and/or through a reduction in regulatory T cell frequency.

4.5 Discussion

Persistent Cl13 infection is associated with robust induction of counter-regulatory pathways [16-18], associated with $CD4^+$ [12] and $CD8^+$ [16] T cell dysfunction. Interruption of such Cl13-induced pathways results in accelerated clearance of persistent infection [16-19, 25, 26]. Here, we implicate ATF3 as an additional counter-regulatory circuit induced by Cl13, one that appears to play a modest role in restraining early control of viral burden. Increased control of viral burden was associated with increased numbers of virus-specific, IFN-γ-producing $CD8^+$ and $CD4^+$ T cells. Additionally, $ATF3^{-/-}$ mice infected with Cl13 exhibited altered cDC subpopulations and reduced regulatory T cell populations in the spleen. The biological relevance of these observations remain to be defined.

Cl13 induces elevated *Pdcd1* [16] and *Il10* [17] expression relative to infection with Arm. In our analysis, significantly increased *Pdcd1* and *Il10* mRNA expression persisted until 30 days post-infection, a time point when Arm viral titers were undetectable [25]. In contrast, Cl13-dependent *Atf3* induction was only significantly elevated at day 10, while induction on day 30 was equivalent to that of Arm infection, and had returned to baseline despite the continued presence of Cl13 [25]. This suggests that Cl13-induced ATF3 modulates early immune responses or viral infectivity rather than regulating viral persistence through chronic effector or memory T cell mechanisms. In agreement with this assumption, $ATF3^{-/-}$ mice exhibited reduced Cl13 viral burdens at day 4 relative to WT mice, while genotype-dependent viral titer differences were not maintained at later time points in the liver.

It must be noted that the kinetics of Cl13 replication in WT mice in our experiments do not agree with other reports. While others demonstrate increased viral titers at 10 days relative to 5 days post-infection [25], in our hands, viral titers were highest in one experiment evaluating viral burdens 4 days post-infection. At 10 days post-infection, viral burdens were lower than the day 4 levels in three independent experiments. Obviously, these experiments must be repeated to determine viral titers kinetically in WT and ATF3$^{-/-}$ mice before one can confidently comment on viral burden differences between ATF3$^{-/-}$ and WT mice. However, early, but not sustained, differences in viral titers are consistent with the lack of differential *Atf3* expression in Cl13- relative to Arm-infected mice 30 days after infection. If future experimentation confirms our observed kinetics of viral burden differences (highest on day 4, reduced on day 10, with genotype-dependent differences restricted to day 4), they could represent the sequelae of early innate antiviral responses, or they could indicate reduced viral infectivity in ATF3$^{-/-}$ mice. Cl13 binds to the LCMV receptor, α-dystroglycan (α-DC), which is expressed at high levels on DCs [8, 42]. Future studies quantifying the expression of α-DG on DC populations would be necessary to evaluate this potential contribution to reduced viral burdens. However, increased virus-specific T cell generation in ATF3$^{-/-}$ mice makes decreased infectivity unlikely as the sole mechanism of reduced viral burdens.

Clearance of persistent Cl13 infection following IL-10 neutralization or genetic ablation, or PD-L1 neutralization, is associated with increased numbers of virus-specific T cells [16, 17, 19]. ATF3$^{-/-}$ mice generate significantly more IFN-

γ-producing virus-specific $CD8^+$ and $CD4^+$ T cells; however, it is unclear why they are significantly elevated 10 days after infection, when viral titers are equivalent between genotypes. This could be partly due to the fact that the viral titer kinetics presented here were pooled from 3 experiments, and in each experiment, $ATF3^{-/-}$ viral burdens trended lower than WT. This finding could also be due to inclusion of male and female mice. Viral burdens were consistently lower in females, yet insufficient numbers of mice were used in these preliminary experiments to be able to evaluate sex-specific ATF3-dependent effects. Finally, virus-specific T cell populations were not evaluated on day 4; therefore, we cannot rule out the possibility that T cell activation and expansion is more robust early on, and/or that increased T cell numbers on day 10 are a hold-over from early virus-specific immune responses. Clearly, closer kinetic evaluation of both viral burdens and T cell populations will be required to determine the association between viral burden and T cell responses.

It is unclear whether the increased numbers of virus-specific T cells observed in $ATF^{-/-}$ mice are due to T cell-intrinsic ATF3 effects, T cell-extrinsic ATF3 effects, or a combination of both. Further, it is unclear whether this increased number of virus-specific T cells is due to altered proliferation or apoptosis. The numbers of $ATF3^{-/-}$ $gp33^+$ $CD8^+$ T cells are markedly increased, despite the fact that the estimated apoptosis rates of $gp33^+$ $CD8^+$ are unchanged between Arm and Cl13 infection in WT mice [24]. This does not rule out T cell-intrinsic ATF3 effects on apoptosis and, in fact, ATF3 overexpression in $JDP2^{-/-}$ neutrophils is associated with increased *Bcl-2* expression [43]. Therefore, formal

evaluation of the rates of apoptosis in virus-specific ATF3$^{-/-}$ T cells is clearly warranted. ATF3$^{-/-}$ T cells might also have an intrinsically altered ability to proliferate. This is supported by the finding that ATF3 is differentially induced at day 10, which coincides with the peak expansion/early contraction phase of the CD8^{+} T cell response to Cl13 [24]. Experiments mixing Cl13 peptide-pulsed WT or ATF3$^{-/-}$ DCs with CFSE-stained WT or ATF3$^{-/-}$ T cells would provide insight into the cellular locus of the ATF3 effect as well as the proliferative capacity of ATF3$^{-/-}$ T cells. Finally, an *in vivo* T cell-intrinsic ATF3 effect could be investigated using reciprocal adoptive transfers of WT and ATF3$^{-/-}$ T cells in the setting of Cl13 infection in WT and ATF3$^{-/-}$ mice.

Within the virus-specific CD4^{+} T cell population, we observed increases in activated cells 10 days after infection, and memory cells 52 days after infection, in ATF3$^{-/-}$ mice. Importantly, there were no differences in naïve CD4^{+} T cell population numbers between genotypes at any time point, suggesting that the observed increase in T cell numbers is due to specific immune activation and not a larger or non-specifically hyper-responsive ATF3$^{-/-}$ lymphocyte pool. Oldstone and colleagues have reported that acute clearance of Cl13 in IL-10$^{-/-}$ mice results in increased development of memory T cells [17]. Our observations are in agreement with this finding. Further, the fact that splenocytes from ATF3$^{-/-}$ mice chronically infected with Cl13 (d52) generate significantly increased IFN-γ^{+} T cell numbers after only 4 hours of peptide restimulation suggest that a potent effector memory population exists in ATF3$^{-/-}$ mice. Future experiments will be necessary to better define memory responses by re-infecting WT and ATF3$^{-/-}$ mice

previously challenged with Cl13 and evaluating viral burden, cell population, and IFN-γ production kinetics to determine whether increased ATF3$^{-/-}$ memory T cell populations exert a functional effect.

CD8^{+} T cell IFN-γ production is necessary for successful immune response to LCMV [1, 2], and functionally exhausted T cells lose their ability to produce IFN-γ in Cl13 infection [16, 17]. Therefore, it is notable that we observed increased total numbers of IFN-γ^{+} virus-specific ATF3$^{-/-}$ T cells. This is a potential mechanism for lower viral burdens in ATF3$^{-/-}$ mice. In support of this, IFN-I blockade results in Cl13 clearance associated with increased IFN-γ [25, 26]. It does not appear that ATF3$^{-/-}$ T cells make more IFN-γ as measured by mean fluorescence intensity (MFI); however, this MFI analysis was based on very small populations and deserves repeating. An ATF3-dependent increase in IFN-γ^{+} T cell numbers without increased IFN-γ production on an individual cell basis is consistent with previous reports that ATF3$^{-/-}$ NK cells, but not ATF3$^{-/-}$ CD8^{+} cells, differentially regulate IFN-γ production during persistent viral infections [36].

DCs are the critical source of enhanced IL-10 production in Cl13-infected mice [17], and Cl13 is known to preferentially infect DCs [15, 20] and inhibit their maturation [21]. Given that ATF3 expression is induced during DC maturation [33-35], we hypothesized that ATF3 could effect increased T cell production through modulation of DC populations during Cl13 infection. The lack of differences observed in pDC or cDC population numbers suggests that pDCs are likely not responsible for the ATF3-mediated effect, in agreement with published reports that Cl13 does not differentially induce *Il-10* production in pDCs [17].

While there were no differences in the total percentage of splenic cDCs between genotypes, we observed significant decreases in $CD8^+$ cDC frequencies and increased $CD11b^+$ cDC frequencies in $ATF3^{-/-}$ mice. It must be noted that we did not specifically exclude TCR^+ cells during our analysis of cDC subsets and activated $CD8^+$ T cells can express myeloid markers, including CD11c [44]. However, errant inclusion of activated $CD8^+$ T cells in cDC gates would artificially inflate $CD8^+$ cDC populations in $ATF3^{-/-}$ mice, while we see the opposite effect.

The potential physiological impact of altered cDC subpopulations remains to be defined. $CD8^+$ cDCs are critical for antigen cross-presentation and activation of $CD8^+$ T cells in LCMV infection [11], and $CD8^+$ T cell responses are critical for control of LCMV infection [1, 2]. We observed striking increases in $gp33^+$ $CD8^+$ T cells, which, on face, seems to contradict reduced frequencies $CD8^+$ cDC populations. However, $CD11b^+$ cDCs prime $CD4^+$ T cell responses, which are implicated in sustaining $CD8^+$ effector functionality in persistent LCMV infection [12, 25]. Therefore, $ATF3^{-/-}$ mice may simultaneously experience reduced $CD8^+$ T cell priming by $CD8^+$ cDCs, but increased virus-specific $CD4^+$ T cell priming by $CD11b^+$ cDCs—with increased $CD4^+$ T cell support of fewer primed $CD8^+$ T cells predominating. Again, mixing experiments using T cells and viral peptide-pulsed DCs from WT and $ATF3^{-/-}$ mice would be informative in evaluating the potential mechanisms of DC subset-specific T cell priming and immune reactivity.

Previous reports have identified an increase in IL-10 production in $ATF3^{-/-}$ mice [29]. On face, this seems contradictory to our findings that ATF3 is

expressed similarly to IL-10 in the context of Cl13 infection. However, it is important to note that IL-10, like ATF3, is a counter-regulatory immune modulator and is induced in response to pro-inflammatory stimuli. Whitmore, et al., demonstrated that ATF3$^{-/-}$ DCs produced elevated IL-10 in response to low-, but not high-dose, TLR stimulation [29]. They did not, however, demonstrate that this was due to direct ATF3 regulation of IL-10 expression, yet they also found elevated expression of Il-12p40 in LPS-stimulated ATF3$^{-/-}$ DCs, indicative of enhanced pro-inflammatory production that could secondarily trigger enhanced IL-10 production. Therefore, the dual observations of Cl13 induction of both ATF3 and IL-10 to allow viral persistence and elevated IL-10 production by ATF3$^{-/-}$ DCs [29] are both consistent with the role of ATF3 as a counter-regulatory immune transcription factor.

Another cell population that was significantly altered at day 10 was regulatory T cells. This finding may contribute to the differential generation of DC subsets, as depletion of regulatory T cells can result in DC expansion [45]. However, blockade of CTLA-4, critical to regulatory T cell suppression of DC expansion and activation, has no effect on Cl13 viral persistence [16], and regulatory T cells do not mediate CD4^{+} T cell dysfunction in Cl13 infection [12]. Nevertheless, regulatory T cells are powerful immunosuppressive cells and depletion studies are needed to determine whether they play a role in increased T cell expansion and/or effector function specific to ATF3-dependent viral persistence.

We have shown here that ATF3 induction during Cl13 infection modulates this persistent viral infection by incompletely understood mechanisms. $ATF3^{-/-}$ mice have increased numbers of virus-specific IFN-γ-producing $CD4^+$ and $CD8^+$ T cells. This could be due to T cell-intrinsic, ATF3-dependent proliferative or apoptotic effects, which needs to be addressed. Reduced viral burdens and increased virus-specific T cell responses were also associated with altered splenic DC and regulatory T cell populations. While the direct effect of these observations is unknown, DCs have subset-specific effects on T cell priming and activation and could contribute to altered ATF3-dependent T cell responses and viral clearance. Understanding the molecular mechanisms of each of these findings will be critical to determine whether they are coincident, correlative or causal. Further, comparison of acute (Arm) and persistent (Cl13) LCMV infection in $ATF3^{-/-}$ mice would provide useful information as to the generality of a role for ATF3 in modulating the host response to chronic infections. Taken together, our data implicate ATF3 as playing a modest role in the modulation of immune responses that allow for persistent viral infection. Further investigation into the mechanisms by which ATF3 exerts these effects is warranted, and could result in the identification of novel therapeutic targets for treating or preventing persistent viral infections.

4.6 Figures

Figure 1

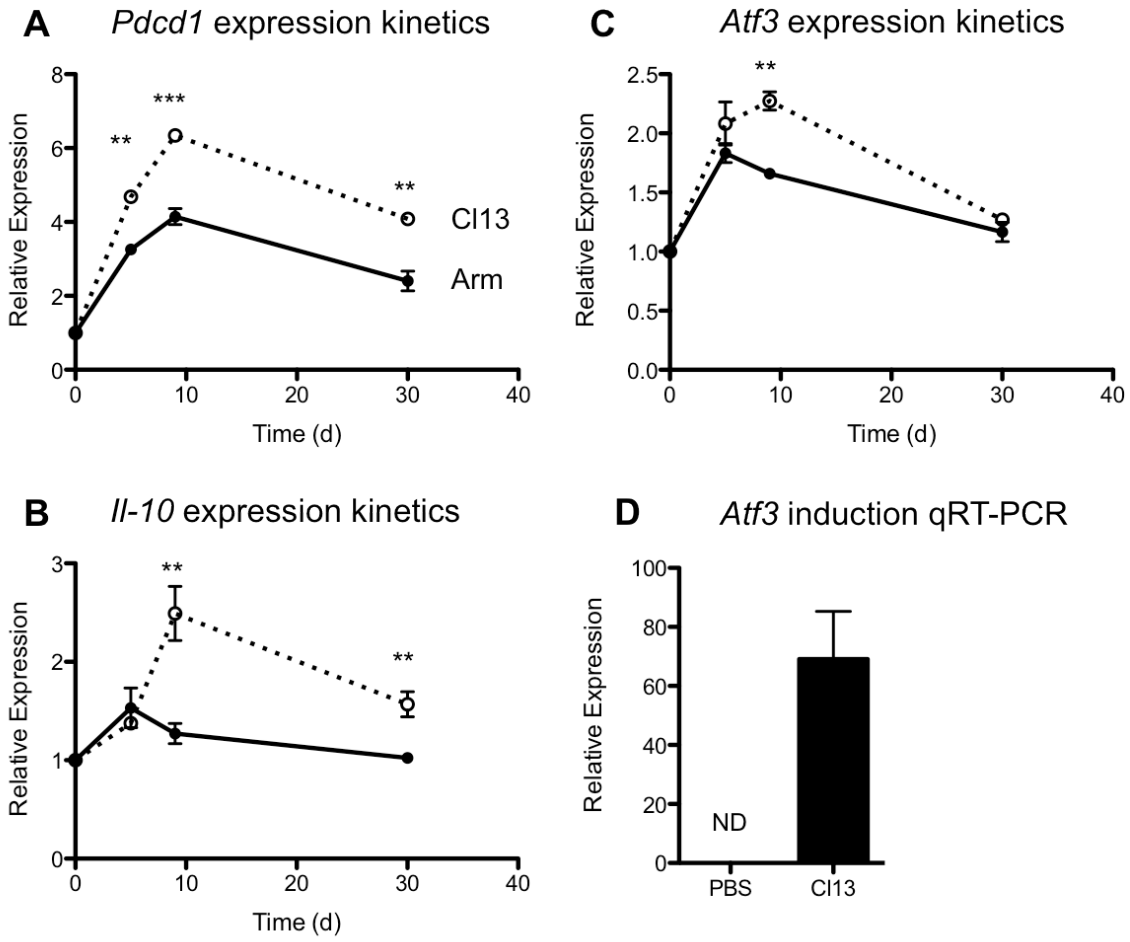

Figure 1. *Atf3* expression kinetics mirror those of *Pdcd1* and *Il10* in Cl13-compared to Arm-infected WT mice. Reanalysis of published gene expression studies (GSE44322) [25] to determine expression kinetics of *Pdcd1* (A), *Il10* (B), and *Atf3* (C) in total splenic RNA from WT mice infected with LCMV-Armstrong (Arm, solid line) or LCMV-Clone 13 (Cl13, dotted line) at indicated times. (D) WT mice were injected with PBS control (white bar) or Cl13 (black bar). Livers were harvested on day 4 and RNA isolated and evaluated for *Atf3* expression by qRT-

PCR. Statistics are unpaired, two-tailed *t* test. N=3-4 replicates/condition. Data are mean ± S.E.M. ** $p < 0.01$, *** $p < 0.001$.

Figure 2

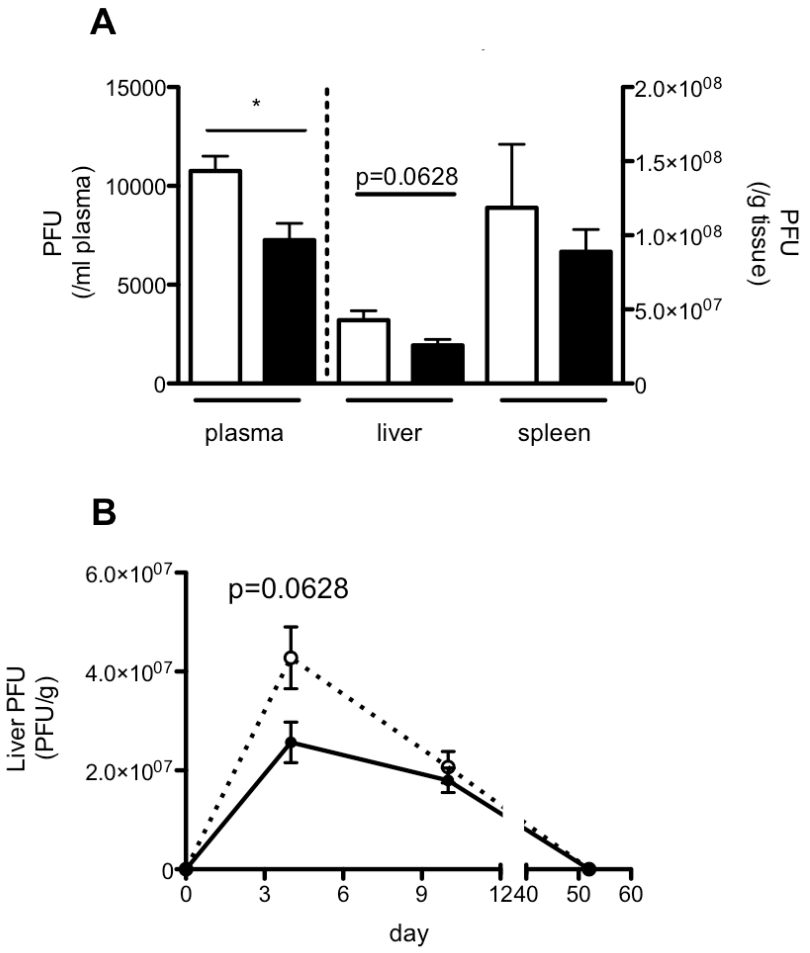

Figure 2. Early reductions of Cl13 viral burdens are not maintained over time in ATF3$^{-/-}$ mice. Viral PFUs from various tissues were enumerated in WT (white bars, dotted line) and ATF3$^{-/-}$ (black bars, solid line) mice on day 4 (A), or in liver homogenates at indicated times (B) after infection with Cl13. The liver data graphed in panel A is reproduced in the kinetic graph in panel B at day 4. Data are representative of 1 (4 day and 52 day time points) or 3 (10 day time point) independent experiments. N=9-10 mice/genotype. Statistics are unpaired, two-tailed *t* test. Data are mean ± S.E.M. *p <0.05.

Figure 3

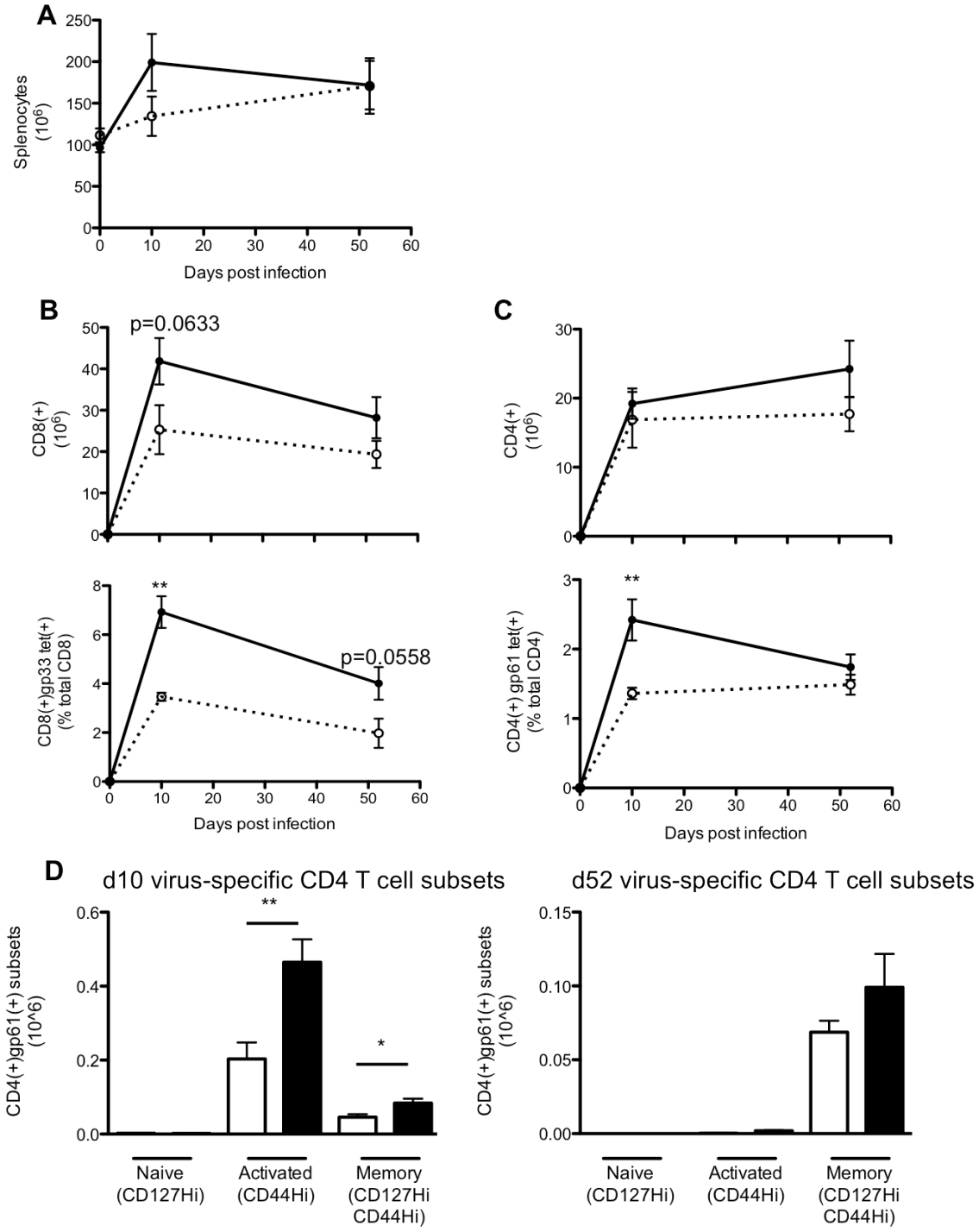

Figure 3. ATF3$^{-/-}$ mice exhibit increased numbers of Cl13-specific CD4^{+} and CD8^{+} T cells. WT (dotted lines) and ATF3$^{-/-}$ (solid lines) mice were infected with

$1.8x10^6$ PFU Cl13 and total splenocytes counted (A), and stained for flow cytometric analysis for total number (top) and % virus-specific (bottom) $CD8^+$ (B) or $CD4^+$ (C) T cells at indicated times. (D) Naïve ($CD127^+$), activated ($CD44^+$) and memory ($CD44^+CD127^+$) $CD4^+$ T cell subsets were also determined at d10 (left) or d52 (right). Data represent 1 (d52 time point) or 3 (d10 time point) independent experiments. N=9-10 mice/genotype. Statistics are unpaired, two-tailed *t* test. Data are mean ± S.E.M. **p* <0.05, ***p* <0.01.

Figure 4

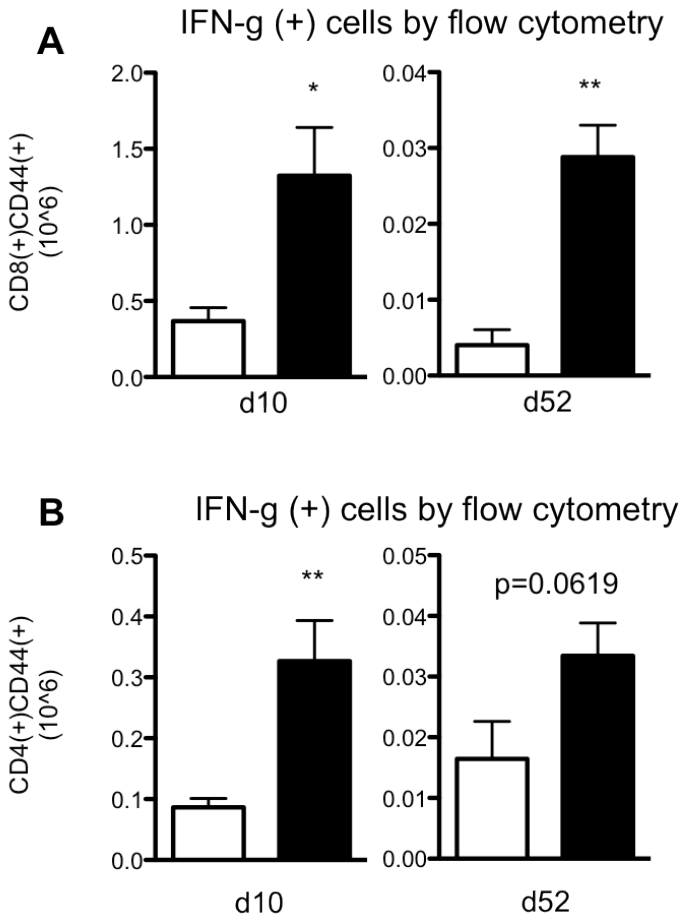

Figure 4. ATF3$^{-/-}$ mice exhibit increased numbers of IFN-γ-producing T cells. WT (white bars) and ATF3$^{-/-}$ (black bars) mice were infected with Cl13 and splenocytes harvested at the indicated times. Total splenocytes were then incubated with virus peptides gp33-44 or pg61-80 and IFN-γ(+) CD8^{+} (A) or CD4^{+} (B) T cells were quantified by flow cytometry. Data are mean ± S.E.M. and represent 2 independent experiments. N=8-10 mice/genotype/time point. Statistics are unpaired, two-tailed *t* test. **p* <0.05, ***p* <0.01.

Figure 5

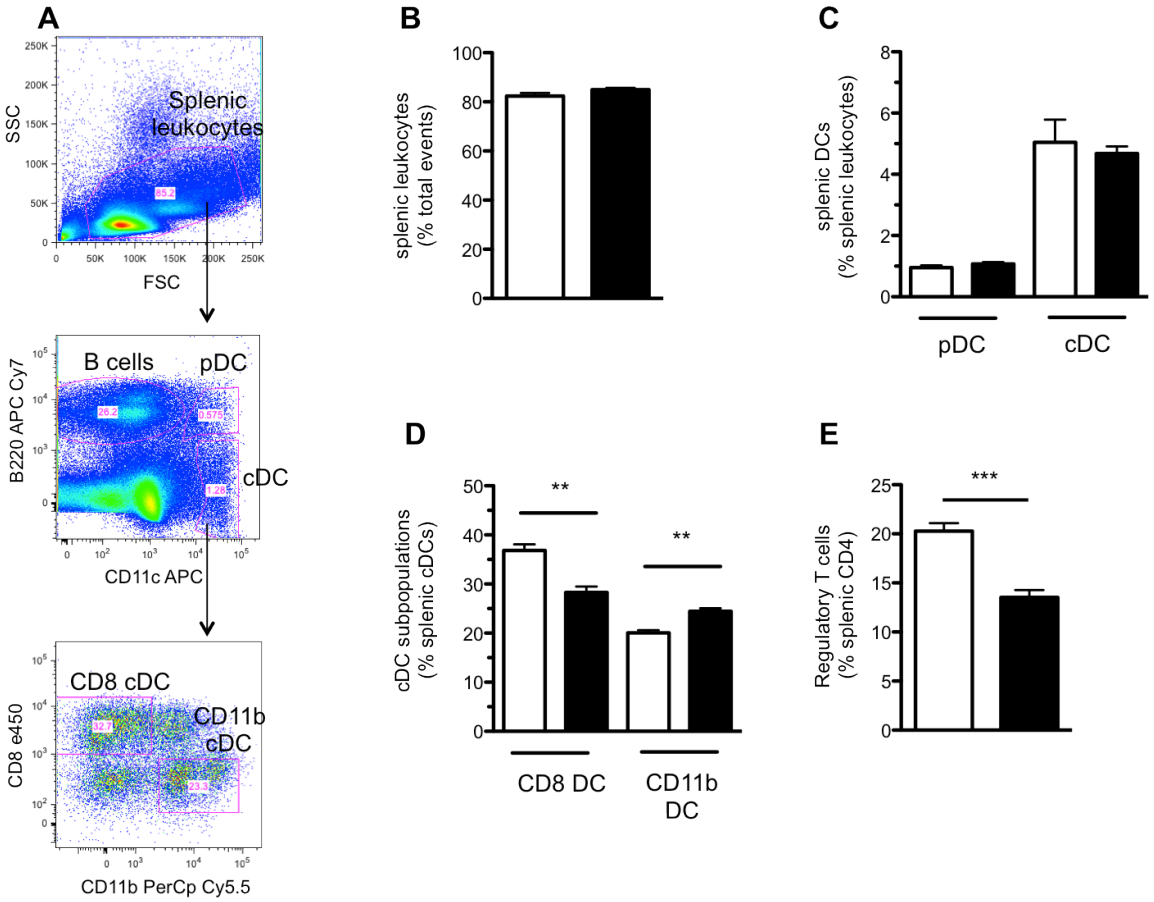

Figure 5. CI13-infected ATF3$^{-/-}$ mice exhibit altered splenic cDC subpopulations and decreased frequencies of regulatory T cells. (A) Representative flow cytometric analysis plots demonstrating the gating strategy used to define DC populations. Live cells (FSC x SSC) were gated on B220 and CD11c. CD11c^{+}B220^{-} cDCs were further subdivided into CD8^{+} and CD11b^{+} cDC subpopulations. (B) Spleens from WT (white bars) and ATF3$^{-/-}$ (black bars) infected with CI13 were harvested on day 10 and the relative percentages of live cells (B), DC populations (C-D) and regulatory T cells (E) were determined by flow cytometry. Markers used were as follows: pDC (CD11c^{+}B220^{+}), cDC (CD11c^{HI}B220^{-}) further subdivided into CD8^{+}CD11b^{-} or CD8^{-}CD11b^{+} DCs, and

regulatory T cells ($TCR\beta^{+}CD8^{-}CD4^{+}Foxp3^{+}CD25^{+}$). Data are mean ± S.E.M. and are from 1 experiment. N=9-10 mice/genotype. Statistics are unpaired, two-tailed *t* test. $^{**}p < 0.01$, $^{***}p < 0.001$.

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Chapter V: Discussion and Future Directions

ATF3 is a counter-regulatory immune transcription factor (TF) with stimulus- and cell type-specific effects. The overall effect of ATF3 induction on immune responses is highly context-dependent and can be beneficial or detrimental. Herein, we have identified novel and complex ATF3 regulatory functions in neutrophil recruitment. Consistent with its counter-regulatory role in immune activation, ATF3 discriminates between low- and high-dose lipopolysaccharide (LPS) challenge to attenuate CXCL1 production by lung epithelia. The physiologic impact of this in a setting of acute neutrophilic lung inflammation was initially obscured due to an unexpected and concomitant ATF3$^{-/-}$ neutrophil-intrinsic migratory defect *in vivo*, and an even more pronounced chemotaxis defect *in vitro.* This observation was unexpected as TFs are uncommonly responsible for chemotaxis defects, given the time it takes for induction and subsequent transcriptional regulation of targets, and the immediate chemotactic responses exhibited by neutrophils.

Gene array studies performed in our lab, and analysis of those from other labs, suggested that temporal regulation of ATF3 during granulopoiesis is required for proper neutrophil development through regulation of the expression of gene targets, notably *TIAM2*. These findings are novel in that they are the first to describe a developmental role for ATF3 in granulopoiesis. They also add to the limited studies describing ATF3 function during homeostatic rather than immediate-response events [1]. The discovery that TIAM2 was absent in ATF3$^{-/-}$ neutrophils was in agreement with the observed neutrophil-intrinsic chemotaxis defect: TIAM2 promotes disassembly of focal adhesions (FAs), and ATF3$^{-/-}$

neutrophils did not translocate, concomitant with increased numbers and areas of adhesive structures.

Several important questions related to ATF3-mediated regulation of neutrophilic inflammation remain to be addressed, however. These will be taken up in turn here: (1) As a counter-regulatory TF, what is/are the mechanism(s) of ATF3 regulation of *Cxcl1*?; and what is the biologic effect of ATF3-mediated modulation of CXCL1 production? (2) As a developmental regulator of neutrophils, what is/are the mechanism(s) of ATF3 regulation of *Tiam2*?; is there a larger ATF3-dependent neutrophil phenotype beyond migration/chemotaxis?; and what is the functional impact of changes in other microarray-implicated gene targets? (3) As for the $ATF3^{-/-}$ neutrophil migration defect, what are the underlying mechanisms of defective migration?; is TIAM2 responsible for the observed phenotype and if so, by what mechanism? Finally, the discrepancy between the severity of the $ATF3^{-/-}$ neutrophil migration defect *in vivo* (impaired) and *in vitro* (abrogated) will need to be addressed.

5.1 ATF3 regulation of *Cxcl1*

5.1.1 What are the immunobiological consequences of ATF3-mediated regulation of CXCL1?

The counter-regulatory role of ATF3 in immune function is well documented, and thus it was not surprising to observe attenuation of LPS-driven epithelial CXCL1 production by ATF3. Further, exogenous CXCL1 administration and CXCL1 neutralization during LPS challenge demonstrated the dominant role CXCL1

plays in neutrophil recruitment to the lung. Given these observations (prior to the identification of the intrinsic migratory defect of ATF3$^{-/-}$ neutrophils), we hypothesized that ATF3$^{-/-}$ mice would exhibit increased neutrophil recruitment to CXCL1-eliciting stimuli. Such aberrant neutrophilic inflammation is a pathogenic finding in important human diseases such as cystic fibrosis (CF) and rheumatoid arthritis.

CF, the most common, lethal autosomal recessive disorder in the United States, is marked by excessive neutrophil recruitment and IL-8 production in the airway when bacterial burden or LPS concentrations are normalized [2, 3]. Mice lack IL-8 and mouse models of CF do not recapitulate human CF lung disease; however, CXCL1 is considered to be the functional murine analog to IL-8 and is homologous to human (h)CXCL1 (also known as Gro-α), which contains a putative ATF3 binding site (data not shown). hCXCL1 is elevated in CF in some reports [4] but not others [5], and the human and mouse CXCL1 receptor, CXCR2, is a therapeutic target for neutrophil reduction in CF lung disease [6]. Therefore, the literature suggests that hyperproduction of CXCL1 could cause similar, detrimental, neutrophil recruitment in mice.

The concomitant defect in ATF3$^{-/-}$ neutrophil chemotaxis precluded direct testing of this hypothesis. Reciprocal bone marrow (BM) transplantation studies were therefore performed in order to determine whether increased CXCL1 production in the ATF3$^{-/-}$ lung resulted in enhanced wild type (WT) neutrophil recruitment. However, during the reconstitution period of 10-12 weeks, we observed an unexpected decrease in survival solely in the CD45.1^{+} WT (BoyJ)→

C45.2^{+} WT (B6) control group, all other transplant groups were equivalent (Figure 1A). Further, BoyJ→B6 controls also had elevated LPS-driven CXCL1 production, compared to the B6→BoyJ control group, and equal to the BoyJ→ATF3$^{-/-}$ experimental group (Figure 1B). ATF3$^{-/-}$ mice do not exist on a CD45.1^{+} background so, unfortunately, ATF3$^{-/-}$→ATF3$^{-/-}$ controls were not available.

The mechanism underlying either of these findings is unclear, although Van Zant and colleagues recently reported that the congenic CD45.1^{+} WT mice (BoyJ) have significant genetic differences from their CD45.2^{+} counterparts (B6) [7]. Specifically, they found that CD45.1^{+} BM-derived cells exhibit reduced fitness and are replaced by CD45.2^{+} BM-derived cells during mixed BM transplant into CD45.2^{+} recipients. Interestingly, this observation does not occur in the reciprocal mixed BM transfers to CD45.1^{+} recipients [7]. Our observations of decreased survival and increased immune response in the BoyJ→B6 control group would similarly suggest that BoyJ CD45.1^{+} WT mice are not appropriate for our studies and that some unidentified genetic incompatibility leads to increased immune activation and decreased fitness of CD45.1^{+} cells in a CD45.2^{+} host, be it WT or ATF3$^{-/-}$. Future BM transplant experiments using CD45.1^{+} WT mice available from NCI would be instrumental in determining whether these findings are specific to CD45.1^{+} WT mice from Taconic Farms, or represent occult genetic differences between strains. Additionally, identifying truly congenic BoyJ and B6 strains would allow for WT→ATF3$^{-/-}$ BM transfers that would enable interrogation of whether elevated LPS-induced CXCL1 in ATF3$^{-/-}$

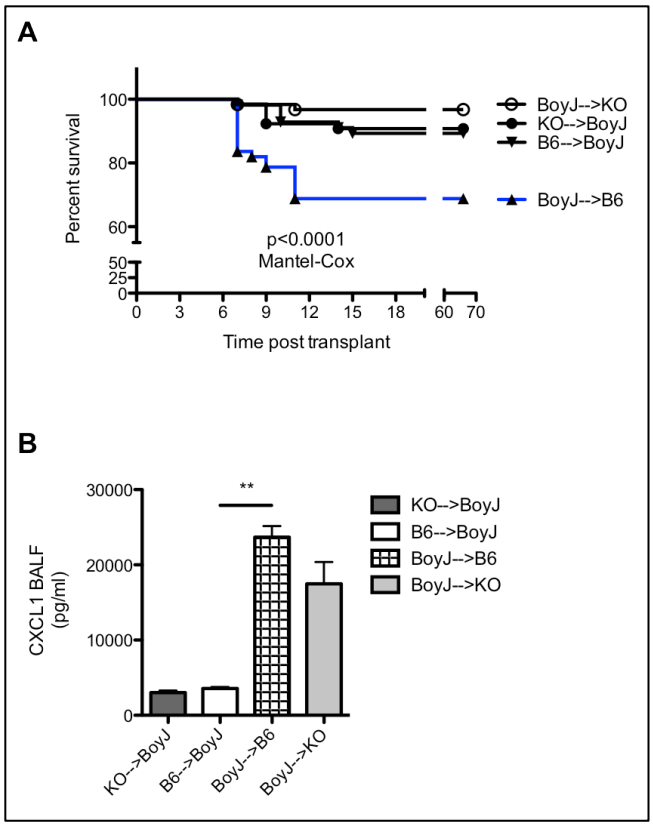

Figure 1. CD45.2^{+} WT (B6) recipients of CD45.1^{+} WT (BoyJ) BM are hyperinflammatory and exhibit reduced survival. *CD45.1^{+} WT BM was transferred into lethally irradiated CD45.2^{+} WT or ATF3$^{-/-}$ recipients, or vice versa. (A) Survival was followed during donor reconstitution of irradiated hosts for 10 weeks of engraftment. Data are pooled from 5 independent transplantation experiments, N=58-65 mice/group. Statistics are Mantel-Cox test between control groups. (B) Surviving mice were challenged with 10 ng LPS i.t., and BAL CXCL1 concentrations were measured by ELISA. Data are representative of 3 independent experiments, N=3-6 mice/group. Statistics are unpaired, two-tailed t test with Welch's correction for unequal variance. **p <0.01.*

mice results in increased WT neutrophil recruitment. Further, these models could be used to determine the physiologic impact of increased neutrophil recruitment by evaluating lungs for pathologic changes associated with aberrant neutrophilic inflammation or evaluating survival in mice challenged with live bacteria as described [8].

5.1.2 What is the mechanism of *Cxcl1* regulation by ATF3?

Transcriptional inhibition by ATF3 has been shown to involve association with histone deacetylases (HDACs) [9] and epigenetic modification, which reduces access to the transcriptional start site and impedes subsequent gene transcription. Others have demonstrated direct ATF3 binding to the *Cxcl1* promoter via chromatin immunoprecipitation (ChIP) using ectopic ATF3 expression in cancer cell lines [10]. Given the biological finding of CXCL1 regulation by ATF3, and the presence of an ATF3 binding site in the *Cxcl1* promoter, we hypothesized that ATF3 directly inhibited CXCL1 production through direct binding and inhibition at the *Cxcl1* promoter. We thus sought to demonstrate direct binding of ATF3 to the *Cxcl1* promoter in airway epithelial cells using ChIP techniques.

We utilized the mouse lung epithelial (MLE) cell line cells generously provided by Dr. Wikenheiser-Brokamp. We initially employed two clonal cell lines, MLE-12 and MLE-15, morphologically representative of the distal bronchiolar and alveolar epithelia, respectively [11]. After LPS stimulation, we found that both lines elaborated CXCL1 (Figure 2A). CXCL1 production by MLE-15 cells was

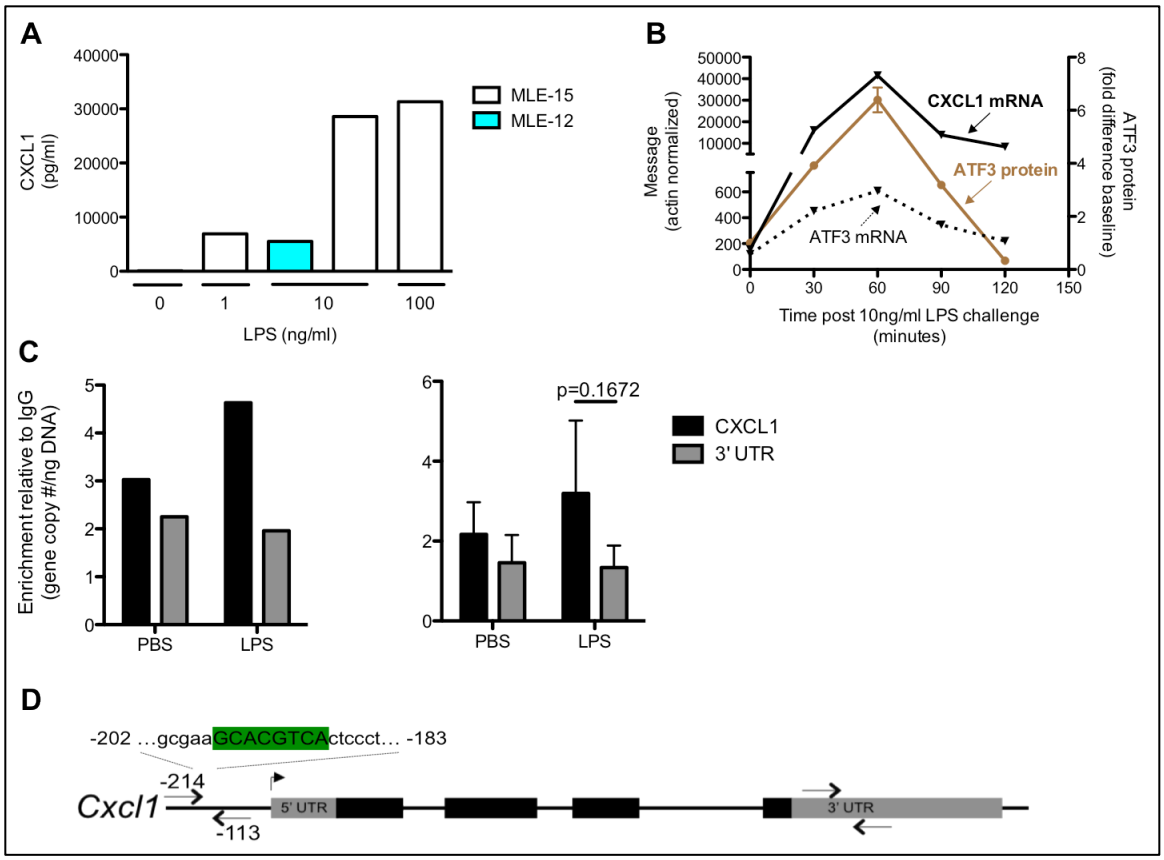

Figure 2. ATF3 kinetics and occupancy of the Cxcl1 promoter*. (A) MLE-12 (blue bars) and MLE-15 (white bars) cell line cells were stimulated with indicated amounts of LPS overnight and the supernatants were assayed for CXCL1 concentrations by ELISA. Data are from one experiment, with individual wells. (B) MLE-15 cell line cells were stimulated with 10 ng/ml LPS for the indicated times, and harvested for RNA or protein for kinetic analysis of CXCL1 (black solid line) or ATF3 (black dotted line) mRNA, or ATF3 protein (brown line) by qRT-PCR or western blot, respectively. Data are from a single experiment, with individual wells. (C) ChIP of MLE-15 cell line cells, stimulated with 10 ng/ml LPS or PBS for 1 hour, were fixed with PFA for 20 minutes and quenched with glycine. Cross-linked cells were sonicated into ~100 bp fragments, and immunoprecipitated with*

anti-ATF3 or isotype control antibodies and A/G beads. Immunoprecipitated DNA fragment/protein crosslinks were reversed by high salt incubation at 65°C overnight, and DNA was purified with Qiagen PCR purification kit. DNA was analyzed by qRT-PCR using primers specific to the Cxcl1 promoter region (black bars), or the 3' UTR (gray bars) as a negative control. Results are from one independent at left, or a combination of three runs on right. (D) A schematic of the Cxcl1 gene, with ChIP primer sites and the location of the putative ATF3 binding site (green box) indicated. Statistics are unpaired, two-tailed t test.

more robust, however, so we used these cells for future experimentation. Kinetic analysis in Figure 2B revealed that ATF3 protein peaked following 60 minutes of stimulation, coinciding with diminution of CXCL1 message. Therefore, attempts were made to ChIP ATF3 at the *Cxcl1* promoter at that time point.

Results were initially promising, but highly variable. In fact, when multiple runs on the same aliquots were combined, there was only minor enrichment of ATF3, compared to isotype control antibody immunoprecipitation, at the *Cxcl1* promoter in response to LPS, and relative to the *Cxcl1* 3' UTR (Figure 2C). There are several potential reasons for this observation. First, ATF3 may directly regulate *Cxcl1* as part of a multimeric protein complex that does not survive the ChIP process. Indeed, the variable nature of ATF3 enrichment observed when using identical aliquots suggests that lack of optimization of experimental conditions may have been contributory to our findings. To test this, future studies using known ATF3 targets in LPS-stimulated macrophages, *Il6* and *Il12b*, would be necessary to validate our techniques and reagents before repeat experimentation in MLE-15 cells.

Another possible explanation for the lack of robust ATF3 enrichment at the identified ATF3 binding site in the *Cxcl1* promoter is the potential use of a non-canonical binding site by ATF3 when regulating *Cxcl1.* Kim, et al., demonstrated functional repression of the adiponectin gene by ATF3 through an AP-1 binding site (TGACTCTC) [12], even though a canonical ATF3 binding site is present in the adiponectin gene promoter (data not shown). Subsequent reanalysis of the *Cxcl1* promoter revealed the presence of an AP-1 site almost 800 bp 5' to the

promoter region amplified by our ChIP primers (Figure 2E). Following validation of our reagents and techniques, it would be informative to compare ATF3 enrichment at both locations within the *Cxcl1* promoter to determine whether there is direct ATF3 binding to the *Cxcl1* promoter at either site.

Enrichment of ATF3 at either binding site, would demonstrate direct binding, but determination of the mechanism of ATF3 inhibition would require co-immunoprecipitation (CoIP) studies to identify binding partners (i.e., HDACs). Further, inhibition of HDAC activity (e.g., with suberoylanilide hydroxyamic acid [SAHA] or Trichostatin A) would allow determination whether ATF3 recruitment of HDACs, and their resultant chromatin remodeling activity, was the primary mechanism of *Cxcl1* regulation by ATF3. In the absence of any demonstrable binding following ChIP optimization and alternative binding site analysis, it must be considered that ATF3 does not exert regulatory control on CXCL1 through direct binding to the promoter, and may instead regulate *Cxcl1* expression by some other mechanism.

5.2 ATF3 as a developmental regulator of neutrophils

Perhaps the most intriguing data of this thesis are those describing an ATF3-dependent neutrophil-intrinsic migration defect. Signaling molecules are common causes of migration defects, not TFs, as the time scale of chemotaxis precludes TF induction and target gene regulation. Therefore, observing significant *in vitro* neutrophil migration defects within 20 minutes of stimulation strongly implied that ATF3 regulates gene targets prior to neutrophil maturation, during neutrophil

development, or granulopoiesis. Subsequent microarray analysis verified our hypothesis that, as an adaptive-response TF, ATF3-deficient, unstimulated neutrophils would have relatively few differentially expressed genes. In fact, only 9 protein-encoding genes were significantly different, and *Tiam2* was by far the most robustly different and statistically significant.

Apart from the novel identification of *Tiam2* regulation during neutrophil development, our data are among the first to implicate ATF3 involvement in neutrophil development. *ATF3* expression is negatively regulated by its homolog, Jun-dimerizing partner 2 (JDP2) [13, 14]. Consequently, $JDP2^{-/-}$ neutrophils have elevated *Atf3* expression, and impaired maturation (low Ly6G expression) with attendant reductions in bactericidal function [15]. Further, ATF3 overexpression during *in vitro* neutrophil development recapitulates the $JDP2^{-/-}$ neutrophil (im)maturity phenotype. Maruyama, et al., therefore concluded that ATF3 is an inhibitor of neutrophil functional development [15]. Our data in Chapter 2 demonstrated that in the absence of ATF3, neutrophil numbers and tissue distribution were normal (supplemental Figure 4), yet lung recruitment (Figure 3) and *in vitro* chemotaxis were severely impacted (Figure 4). Therefore, it is more likely that ATF3 is neither a pan-activator nor a pan-inhibitor of neutrophil development. Rather, tightly regulated temporal *Atf3* expression during neutrophil recruitment is likely required to initiate waves of downstream gene expression, similar to the waves of TFs known to regulate neutrophil development e.g., PU.1, GFI-1, C/EBP-α, and C/EBP-δ.

5.2.1 How does ATF3 regulate *Tiam2* expression?

Our data to this point are highly suggestive, yet correlative, and future experiments will be necessary to define the role of ATF3 in regulation of *Tiam2* expression during neutrophil development. To this end, kinetic expression analysis of *Atf3* and *Tiam2* expression during *in vitro* neutrophil differentiation are underway. These studies will define the temporal expression profiles of ATF3 and TIAM2 at the mRNA and protein level necessary to determine whether ChIP assays are a tenable way to demonstrate ATF3 presence (or absence) at the putative binding site identified in the *Tiam2* promoter.

ATF3 functions predominantly as an inhibitory homodimer. However, *Tiam2* expression is almost completely abrogated in ATF3$^{-/-}$ mice, which leads us to hypothesize that ATF3 is a positive regulator of *Tiam2* expression during neutrophil development. In support of this hypothesis, microarray analysis demonstrates inversely related *ATF3* and *TIAM2* expression kinetics: *ATF3* is induced during neutrophil differentiation and basal in mature neutrophils, while *TIAM2* expression is undetectable in precursors and highly elevated in mature neutrophils. Therefore, *ATF3* induction prior to *TIAM2* is consistent with ATF3 protein translation and subsequent transcriptional activation of *TIAM2.* Conversely, the reduction in *ATF3* expression prior to induction of *TIAM2* could indicate that ATF3 inhibits *TIAM2* expression in order to restrict its expression to mature neutrophils. Due to the lack of *Tiam2* expression in ATF3$^{-/-}$ neutrophils, we believe that it is most likely that ATF3 *positively* regulates *TIAM2.* Luciferase reporter assays would be a useful tool to determine whether ATF3 binding to the

Tiam2 promoter results in transcriptional activation or repression *in vitro*. Further, *in vivo* CoIP studies of neutrophils at various stages of development will be necessary to determine the presence and identity of heterodimeric activating ATF3 binding partners.

5.2.2 Are there additional ATF3-dependent defects in other neutrophil effector mechanisms?

Studying JDP2$^{-/-}$ mice, Maruyama, et al., observed elevated *Atf3* expression associated with phenotypic immaturity (reduced Ly6G expression) and defective bactericidal effector mechanisms, including decreased reactive oxygen species (ROS) generation. Moreover, they demonstrated that ectopic ATF3 expression during neutrophil differentiation recapitulated the immature phenotype. However, they did not directly evaluate effector functions [15]. Therefore, we hypothesized that ATF3$^{-/-}$ neutrophils would exhibit enhanced effector functions. Using nitroblue tetrazolium (NBT) as an indicator of ROS generation, we found that ATF3$^{-/-}$ neutrophils had a modest but statistically significant increase in the percentage of NBT^{+} cells following low-dose fMLP stimulation (Figure 3A). This finding is consistent with the profile of *Cxcl1* regulation in that ATF3 again appears to attenuate, rather than globally inhibit, immunologic responses. Future studies evaluating the physiologic impact of this finding will be necessary to determine whether it affects the overall immune system's ability to appropriately respond to pathogens.

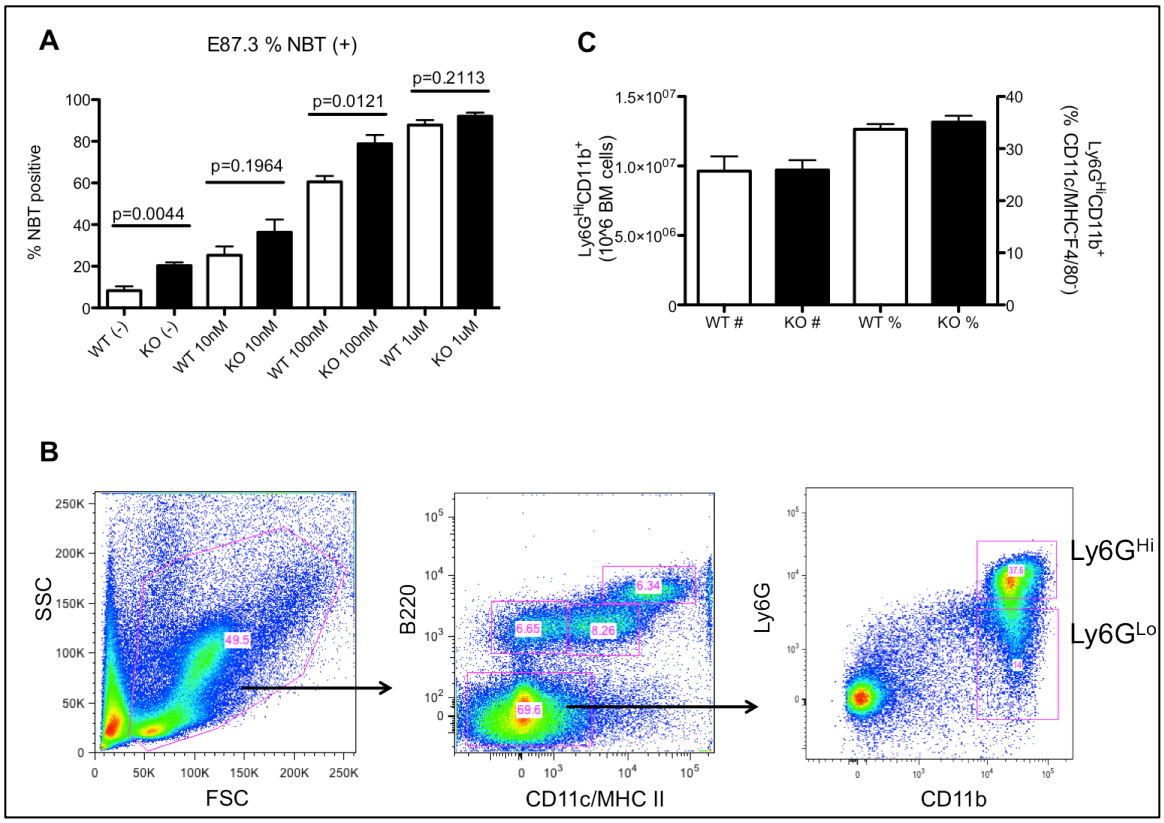

Figure 3. ATF3$^{-/-}$ neutrophils exhibit reduced ROS generation but normal Ly6G expression intensity. *(A) WT (white bars) or ATF3$^{-/-}$ (KO) neutrophils (black bars) were stimulated with media (-), or the indicated concentrations of fMLP, and ROS generation was quantified by nitroblue tetrazolium staining. Data represent 3 independent experiments, N=4 mice/genotype. (B) Flow cytometric gating scheme used to determine the intensity of Ly6G expression on BM neutrophils, defined as B220$^-$CD11c/MHCII$^-$ and CD11b$^+$Ly6G$^+$. (C) The total number (left axis) or percentage (of CD11cMHCII$^-$F4/80$^-$ cells; right axis) of Ly6G^{Hi} WT (white bars) or ATF3$^{-/-}$ (black bars) neutrophils quantified as in panel B. Data represent 3 independent experiments, N=6 mice/genotype. Statistics are unpaired, two-tailed t test. Data are mean ± S.E.M.. SSC, side scatter; FSC, forward scatter.*

We also quantified $Ly6G^{Hi}$ cells within the $B220^{-}CD11c/MHCII^{-}$ BM cell population (gating strategy shown in Figure 3B) and, in contrast to Maruyama and colleagues, found that the absence of ATF3 did not alter $Ly6G^{+}$ BM populations (Figure 3C). This observation could indicate that ATF3 does not mediate all of the neutrophil effects attributed to it by Maruyama and colleagues. In fact, Ly6G is a complex, and it is unclear whether ATF3-mediated (dys)regulation of one or more component(s) would effect expression of the overall complex. However, future studies will be necessary to define the effector capacity of $ATF3^{-/-}$ neutrophils by incubating isolated neutrophils with pathogens, e.g., *Staphylococus aureus* or *Candida albicans,* as described [15].

5.2.3 What is the impact of the other microarray-implicated ATF3 gene targets in neutrophil?

TIAM2 was, by far, the most statistically significant and robust differentially expressed gene product in unstimulated $ATF3^{-/-}$, compared to WT, neutrophils. However, microarray implicated 8 other genes that were also statistically significantly different in $ATF3^{-/-}$ neutrophils; *Erdr1*, *Odz3*, *Igh-VJ558, IgJ, Efna2, Synpo, Susd4* and *Mtus2.* One of these targets, *Synpo* (fold change [FC] 2.493, corrected *p* value 0.024) stood out due to known interactions with cytoskeletal proteins.

Synpo encodes the synaptopodin protein, which is critical for maintaining the structure and function of podocytes. Podocytes are highly specialized renal cells that support the glomeruli, which act as filters to prevent proteins and cells

from passing from the blood into the urine. Within the podocyte, synaptopodin is linked to stress fiber formation by blocking RhoA degradation, and to filipodial formation by blocking Cdc42 and Mena binding to insulin receptor substrate protein 53 (IRSp53) [16]. Further, synaptopodin was implicated in directional migration in podocytes using a wounding assay [17]. While it is unclear exactly how synaptopodin participates in cellular migration, the association with Rho GTPases and migration phenotypes makes it reasonable to evaluate synaptopodin function in neutrophils. It is unlikely, however, that synaptopodin is responsible for the migration phenotype seen in ATF3$^{-/-}$ neutrophils, as synaptopodin is thought to be a highly podocyte-specific gene. Studies to verify the array data using qRT-PCR are therefore required to determine if differences in *Synpo* expression are reproducible, or if the minor expression differences are artifactual.

If *Synpo* expression differences were verified, studies would then be necessary to determine the magnitude of its involvement in the ATF3$^{-/-}$ neutrophil migration phenotype. Given the role of synaptopodin in podocyte migration, we hypothesize that synaptopodin levels would be increased in ATF3$^{-/-}$ neutrophils, and would be associated with increased stress fiber content and filipodia formation. These findings would likely manifest as increased transcellular transendothelial migration (TEM), as filipodial microspikes are an integral component of that mechanism (reviewed in [18]). *In vitro* TEM studies of unstimulated neutrophils incubated on LPS-stimulated (activated) endothelial cell monolayers would be particularly informative to determine if ATF3$^{-/-}$ neutrophils

exhibit a disproportionately high frequency of transcellular migration. Further experiments providing insight into this possible phenotype are detailed below.

5.3 ATF3$^{-/-}$ neutrophil migration defect

Our data in Chapter 2 demonstrated that ATF3$^{-/-}$ neutrophils lacking *Tiam2* expression were almost completely unable to translocate *in vitro*, and that recruitment to the lung was significantly impaired *in vivo*. While it is highly likely that these observations were due to an absence of TIAM2, the mechanism(s) of impaired ATF3$^{-/-}$ neutrophil chemotaxis, the contribution of TIAM2 to impaired ATF3$^{-/-}$ neutrophil migration, and the mechanism(s) underlying TIAM2 effects on neutrophil migration are unclear. Additional experiments to define these issues are discussed, in turn, below.

Additional support for a role for ATF3 in adhesion/migration exists in studies of cancer metastasis. Ishiguro, et al., demonstrated that *Atf3* expression was higher in metastatic sublines of B16 melanoma cells than in the parental lines [19]. Further, they demonstrated that introduction of ATF3 into low metastatic potential B16 cells was sufficient to convert them to high metastatic potential cells [19]. Thus, multiple systems, immunologic and oncologic, suggest that ATF3 plays a role in cellular adhesion and/or migration.

5.3.1 What is/are the mechanism(s) underlying defective ATF3$^{-/-}$ neutrophil migration?

TIAM2 is a Rac1-specific guanine exchange factor (GEF) that regulates focal adhesion (FA) disassembly in epithelial cells through microtubule-dependent activation of Rac1 [20]. In response to knockdown of *Tiam2* in an epithelial cell line, cells demonstrated increased FA area due to a reduced rate of disassembly and reduced migration speed [20]. In our model, ATF3$^{-/-}$ neutrophils exhibited significantly greater reduction in TIAM2 expression (roughly 99%) than *Tiam2* knockdown in epithelial cell line cells (40%) [20]. Together, these findings make it highly likely that TIAM2 is the gene product targeted by ATF3 responsible for the neutrophil-intrinsic migration defect involving focal complex disassembly. However, the exact mechanism(s) underpinning defective neutrophil migration and direct TIAM2 involvement remains to be delineated. Given the similarities between our results in neutrophils and those of Rooney, et al. in epithelial cell lines [20], we hypothesize that ATF3-modulated *Tiam2* expression effects Rac1 activation and focal complex disassembly rates, resulting in the observed increased focal complex size and number and reduced transmigration. However, to test this hypothesis, we must first establish definitively whether TIAM2 is responsible for the ATF3-mediated neutrophil migration phenotype, ideally by restoring TIAM2 protein levels in ATF3-deficient neutrophils.

5.3.2 Is TIAM2 responsible for the observed ATF3$^{-/-}$ neutrophil migration defect?

It is tempting to attribute all of the observed ATF3$^{-/-}$ neutrophil migratory defects to TIAM2, given the striking reduction of expression in ATF3$^{-/-}$ neutrophils and the paucity of differentially expressed genes, compared to WT neutrophils. The

literature further supports this supposition. Gérard, et al., have reported that the Rac1-activating TIAM2 homolog, TIAM1, plays important roles in T cell migration. In fact, TIAM1$^{-/-}$ T cells exhibit defective chemotaxis *in vitro* and impaired homing, egress and contact hypersensitivity *in vivo* [21]. These findings are strikingly similar to our observations in ATF3$^{-/-}$ neutrophils lacking TIAM2, and provide further support to our hypothesis that dysregulated TIAM2 expression is a causative mechanism leading to impaired ATF3$^{-/-}$ neutrophil migration. However, definitive proof requires restoration of TIAM2 expression in ATF3-defficient neutrophils and/or TIAM2 ablation in ATF3-sufficient neutrophils.

5.3.2.1 *TIAM2 Overexpression*

Overexpression of proteins in primary neutrophils is technically difficult, yet has been successfully reported using by nucleofection of expression vectors [22-24] or direct entry of proteins fused to the TAT sequence from human immunodeficiency virus (HIV) [25-28]. However, the open reading frame (ORF) of TIAM2 is over 5 kb, and encodes a 1,700 amino acid, 190 kDa protein. In comparison, published reports of successfully expressed expression vector ORFs or introduced proteins are in the range of 85-130 nucleotides [22, 23] or ~80 amino acids [25], respectively. Nevertheless, we sought to overexpress TIAM2 by nucleofecting (Amaxa Nucleofector 4D system) the TIAM2 ORF within a pCMV6_entry vector (Origene; Figure 4A). As the GEF activity of TIAM2 is located at the C terminus, we chose an untagged construct and co-transfected GFP control vectors to determine nucleofection efficiency.

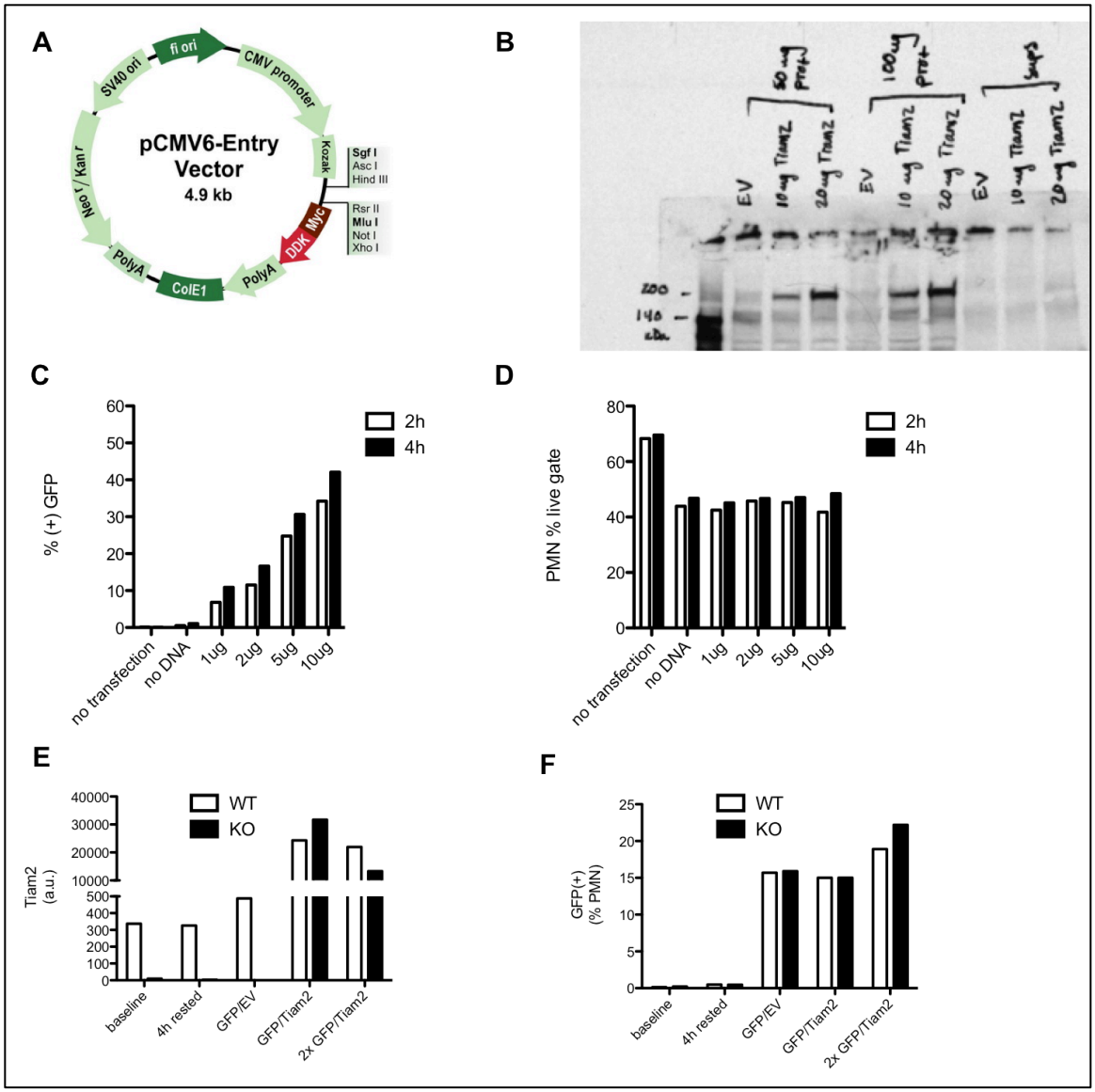

Figure 4. TIAM2 overexpression vector and nucleofection optimization. *(A) Schematic of TIAM2 overexpression plasmid. The CDS of TIAM2 (BC079600 nt 216-5363) was ligated into the pCMV6_entry vector using SgfI and MluI cloning sites. (B) Indicated amounts of TIAM2 overexpression vectors or empty vector control were transfected into HEK293FT cells using PolyFect (Qiagen) and incubated overnight. Supernatants or cell lysates were then evaluated by Western blot for TIAM2 expression at the predicted weight of 200 kDa. Data are*

from a single experiment. (C-D) WT neutrophils were mock-nucleofected or nucleofected with indicated amounts of control GFP plasmid, and incubated for 2 hours (white bars) or 4 hours (black bars) at 37°C to allow for protein translation. Cells were then analyzed for GFP expression (C) or percentage of live cells (D) by flow cytometry. Data are representative of 3 independent experiments, N=1 replicate/condition. (E-F) TIAM2 mRNA expression (E) and GPF expression (F) were determined in WT (white bars) or ATF3$^{-/-}$ (black bars) neutrophils at baseline, or after 4 hours of incubation at 37°C after mock-nucleofection or nucleofection with 1 ug GFP and 8 ug empty vector (EV) or TIAM2 plasmids, or 2 ug GFP and 16 ug TIAM2 plasmid (2x GFP/Tiam2). Data are from an independent experiment N=1 replicate/genotype/condition.

Preliminary experiments were promising. Transfection of HEK293FT cells with the TIAM2_pCMV6_entry vector resulted in identification of TIAM2 protein at the expected size of ~200 kDa by Western blot (Figure 4B). These findings were dependent on both the amount of plasmid nucleofected and protein loaded, and TIAM2 bands were completely absent in the supernatants, which is expected as TIAM2 is exclusively cytoplasmic. Nucleofection experiments of primary neutrophils were also initially promising. Using control, GFP-expressing plasmids, we observed a dose-dependent increase in GFP^+ neutrophils detected by flow cytometry in response to increasing amounts of input DNA (Figure 4C). The nucleofection technique moderately reduced neutrophil viability, however viability was not further reduced due to increased amounts of input DNA (Figure 4D).

Our TIAM2 overexpression vector lacked a tag, which added the unfortunate constraint of co-nucleofection of GFP control plasmids alongside TIAM2 plasmids. Thus, to ensure that all GFP^+ cells also were nucleofected with TIAM2, a minimum ratio of 1:4 GFP:TIAM2 vectors was required. Due to the difference in size between vectors, this entailed 8 ug of TIAM2 plasmid for every 1 ug of GFP plasmid (the lowest recommended amount per manufacturer's protocol). However, these transfection concentrations resulted in supraphysiologic amounts of *Tiam2* mRNA when compared to baseline or control GFP plasmid-nucleofected WT neutrophils (Figure 4E). Unfortunately, these plasmid ratios also resulted in relatively low percentages of GFP^+ neutrophils (Figure 4F).

These data could be interpreted in two ways. (1) if the GFP^+ cells represent the entire population of nucleofection-competent cells, then the supraphysiologic amounts of *Tiam2* mRNA are actually due to overexpression in only 15% of neutrophils. This would suggest that the *Tiam2* level in those cells are really 6X higher as they are diluted by the 85% GFP^-/*Tiam2*$^-$ neutrophils. (2) if 1 ug GFP vector limits the total number of cells that are nucleofected, which is more likely given our findings in Figure 1A, then there are many GFP^- neutrophils bearing *Tiam2* mRNA that we cannot identify immunofluorescently.

The inability to immunofluorescently detect successfully nucleofected neutrophils is problematic as we would be unable to sort or follow $TIAM2^+$ neutrophils, and thus, negative results could not confidently be used to disprove TIAM2 involvement in TIAM2-nucleofected $ATF3^{-/-}$ neutrophil chemotaxis. However, given the fact that effectively no $ATF3^{-/-}$ neutrophils translocated while essentially all WT neutrophils did, we hypothesized that even a 20% nucleofection efficiency with TIAM2 vector alone would likely be detectable as improved translocation if TIAM2 were responsible for the $ATF3^{-/-}$ neutrophil migration phenotype. Unfortunately, we found that migration differences were indeterminate due to a lack of adhesion in all nucleofected cells. Further investigation demonstrated that TIAM2 nucleofection resulted in a 5-fold decrease in adherent cells stimulated with PBS, and a 10-fold reduction in adherent cells when stimulated with fMLP, irrespective of genotype (Figure 5A). Closer inspection revealed that TIAM2 itself was not responsible for reductions in adherence as mock-nucleofected neutrophils as well as those nucleofected with

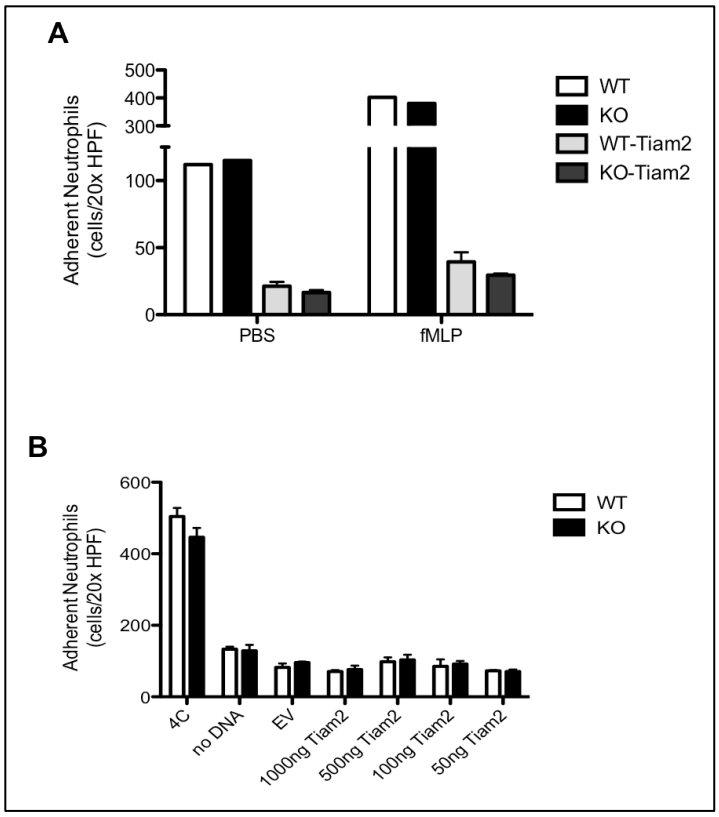

Figure 5. Nucleofection reduces adherence in WT and ATF3$^{-/-}$ neutrophils. *(A) WT (white and light grey bars) or ATF3$^{-/-}$ neutrophils (black bars) were mock-nucleofected or nucleofected with 1 ug TIAM2 plasmid and incubated for 2 hours at 37°C in glass chamber slides. Cells were gently washed with PBS and fixed with PFA for 20 minutes for quantification of adherent cells by microscopy. Data are from one experiment, N=1 mouse/genotype/condition. (B) Adherence was quantified as in panel A for WT (white bars) or ATF3$^{-/-}$ (black bars) neutrophils mock-nucleofected, nucleofected with indicated amounts of TIAM2, 1 ug empty vector (EV), or control neutrophils kept at 4°C. Data are an independent experiment, N=1 mouse/genotype/condition.*

empty vector controls similarly exhibited markedly decreased adhesion, with no further reductions seen in response to TIAM2 nucleofection (Figure 5B). Together these data suggest that while promising, TIAM2 overexpression by nucleofection is likely not a viable technique to evaluate TIAM2-mediated migration effects.

5.3.2.2 *TIAM2 inhibition*

The size of the *Tiam2* ORF makes overexpression through lentiviral transduction of neutrophil progenitors a very remote possibility. However, lentiviruses can easily accommodate short hairpin (sh)RNA sequences for transduction into neutrophil BM precursors, specifically $Lin^{-}Sca^{+}cKit^{+}$ (LSK) cells. Therefore, experiments are currently underway to quantify *Tiam2* and *Atf3* mRNA expression in WT neutrophils after shRNA-mediated *Tiam2* and *Atf3* knockdown. We hypothesize that *Atf3* knockdown will result in reduced *Tiam2* expression. To test this, LSK cells will be mock-transduced or transduced with shRNA-GFP lentivirus constructs for ATF3, TIAM2 and a scrambled shRNA control, and cells will be differentiated *in vitro* with a combination of G-CSF, MDGF, and SCF. RNA will be isolated over time to determine the efficiency of knockdown of various shRNA constructs, to define the kinetics of *Atf3* and *Tiam2* expression in mock-transduced controls, and to establish a causal relationship between *Atf3* knockdown and *Tiam2* expression.

In vitro experiments will identify the shRNA constructs that will then be used to transduce LSK cells for rescue of lethally irradiated WT mice by adoptive

BM transplantation. Following successful transplantation, neutrophils will be harvested from chimeric animals and evaluated for *in vitro* chemotaxis and immunofluorescence studies. We hypothesize that the ATF3 and TIAM2 shRNA knockdown animals will have similar phenotypes that recapitulate the $ATF3^{-/-}$ neutrophil phenotype, namely reduced translocation, increased focal complex size and number, and increased F-actin polymerization, similar to $ATF3^{-/-}$ neutrophils. While not as direct as TIAM2 overexpression studies, impaired neutrophil migration in TIAM2 knockdown animals, without reductions in ATF3 expression levels, would additionally implicate TIAM2 as the causative ATF3 gene target responsible for the $ATF3^{-/-}$ neutrophil phenotype.

5.3.3 What is the mechanism of TIAM2-modulated neutrophil migration?

If it can be demonstrated that TIAM2 is responsible for the ATF3-dependent neutrophil migration defect, studies are envisioned to evaluate the specific mechanism(s) underlying TIAM2-mediated control of neutrophil migration. Rooney, et al., demonstrated that TIAM2 activation of Rac1 was mediated by microtubule growth, which then targeted multiple microtubules to FAs, increasing their disassembly rates, and ultimately allowing timely cellular translocation [20]. Nocodazole is commonly used to arrest microtubule growth, and nocodazole washout results in microtubule regrowth and reduced FA size [29], which is dependent on TIAM2 [20]. However, nocadazole treatment directly polarizes and induces migration in neutrophils [30], and thus is unsuitable to evaluate microtubule growth in neutrophil migration.

The microtubule-mediated, TIAM2-dependent effect is also Rac1-dependent [20]. Therefore, we hypothesize that interrupting the TIAM2/Rac1 axis would recapitulate the ATF3$^{-/-}$ migration phenotype. Unfortunately, Rac1$^{-/-}$ neutrophils have significantly impaired chemotaxis and lung recruitment [31] and are therefore also not ideal for evaluation of ATF3- or TIAM2-dependent neutrophil migration effects involving Rac1. However, it is possibly to ablate TIAM2-mediated Rac1 activation using a TIAM2 mutant. Two point mutations have been described that abolish Rac1 activation by TIAM1, Q1191A and K1195A [32]. Homologous mutations in TIAM2 similarly abrogate Rac1 activation in a dominant negative fashion [20], although the specific TIAM2 mutations were not reported in the publication. Therefore, we used the recently reported comparative analysis of TIAM1 and TIAM2 protein structure and amino acid sequences [33], to identify the TIAM1-homologous DH-PD$_c$ domain region in TIAM2 (Figure 6). Site-directed mutagenesis of these sites to alanine, Q1271A and K1275A, should result in a dominant-negative TIAM2 variant (TIAM2QK→A) lacking Rac1 GEF function [20]. Overexpression of this protein would face the same challenges as discussed above, but would provide a useful model in which to test whether TIAM2-mediated Rac1 activation was required for neutrophil migration.

TIAM1
1171-AQNPRQQHSS TLESYLIKPI **Q**RVL**K**YPLLL RELFALTDAE SEEHYHLDVA IKTMNKVASH-**1240**

TIAM2
1251-ARNPTKQHSS TLESYLIKPV **Q**RVL**K**YPLLL KELVSLTDHE SEEHYHLTEA LKAMEKVASH-**1310**

Figure 6. The homologous Rac1-activating amino acid sequences in TIAM1 and TIAM2 proteins. *The Rac1-activating activity of TIAM2 was manually aligned to the homologous sequence of TIAM1 [33]. Mutation of specific glutamate (Q) and lysine (K) residues (bolded) to alanine abolished Rac1 activating ability of TIAM1 [32].*

5.4 Reconciling differences in magnitude of *in vivo* and *in vitro* migratory defects

While the experiments proposed in section 5.3 aim to determine the involvement and molecular mechanism(s) of TIAM2 in neutrophil migration, they will not reconcile the differences seen in the migratory capacity of $ATF3^{-/-}$ neutrophils when evaluated *in vivo* versus *in vitro.* 90% of $ATF3^{-/-}$ neutrophils are unable to move in fMLP gradients *in vitro*. In contrast, $ATF3^{-/-}$ neutrophils exhibit only a 50% reduction in recruitment to the WT lung *in vivo*, and no difference in recruitment to the $ATF3^{-/-}$ lung bearing increased concentrations of CXCL1. It is important to note that the *in vitro* studies interrogated a specific component of neutrophil migration, chemotaxis. Successful *in vivo* neutrophil recruitment however, requires sequential neutrophil arrest on inflamed endothelium, crawling along endothelial cells, TEM through or between endothelial cells, and finally chemotaxis up chemoattractant gradients through the extracellular space. Therefore, there are a number of possible explanations for relatively enhanced *in vivo* neutrophil migration.

Firstly, our *in vitro* experiments also tested neutrophil chemotaxis within a unidirectional gradient using a single chemoattractant, in 2 dimensions, under static fluid conditions. It is well known that fluid shear forces are a necessary component of integrin activation [18, 34, 35]. In the absence of these forces, we would hypothesize that $ATF3^{-/-}$ neutrophils are less adherent to the substratum, which could amplify chemotactic defects. To test this hypothesis, we envision

experiments utilizing intravital microscopy of neutrophil adhesion to, and transmigration across, the vascular endothelium in WT and ATF3$^{-/-}$ mice.

Another potential explanation for the differences in severity of migration defect observed *in vitro* vs. *in vivo* could be due to the use of a single chemoattractant agent, fMLP, when evaluating neutrophil chemotaxis *in vitro.* Following airway challenge with LPS, numerous chemokines, notably of the ELR+ family, were induced in both WT and ATF3$^{-/-}$ mice. While ATF3$^{-/-}$ neutrophils have an intrinsic defect in migration, activation through multiple pathways may partially overcome this defect *in vivo*. This hypothesis is supported by the fact that ATF3$^{-/-}$ neutrophil recruitment to the lungs are further increased by elevated CXCL1, as is seen in the ATF3$^{-/-}$ lung when compared to the WT lung. This would suggest that the ATF3$^{-/-}$ neutrophil-intrinsic defect is not total, but can be compensated for by increased chemoattractant gradients.

Another possible explanation for the increased severity of ATF3$^{-/-}$ neutrophil migration defect *in vitro* could be due to differential mechanisms of TEM. WT neutrophils adhere to inflamed endothelium and rapidly crawl to endothelial junctions to undergo paracellular TEM both *in vitro* [36] and *in vivo* [37]. As extracellular chemotaxis can be independent of integrin-mediated cellular adhesions [38], it is possible that ATF3$^{-/-}$ neutrophil migration could be preserved *in vivo* through mechanisms that circumvent luminal chemotaxis along endothelial cells. CD11b$^{-/-}$ neutrophils are unable to move along the vascular endothelium following firm adhesion and extravasate using transcellular, rather than paracellular, TEM [37]. Additionally, TIAM1$^{-/-}$ T cells preferentially undergo

transcellular TEM, in part due to an inability to crawl across endothelial surfaces and suboptimal activation of Rac1 [21]. Finally, transcellular migration can be very rapid [39], which could also compensate for the reduced chemotactic ability of ATF3$^{-/-}$ neutrophils in overall recruitment. Therefore we hypothesize that reduced endothelial chemotaxis by ATF3$^{-/-}$ neutrophils is mitigated by an increase in transcellular TEM and subsequent preserved ability to migrate extracellularly using integrin-independent mechanisms.

This hypothesis would ideally be tested using intravital microscopy *in vivo* as described [37]. However, in the absence of this technical ability, experiments are envisioned that evaluate WT and ATF3$^{-/-}$ neutrophil migration across human umbilical vein endothelial cell (HUVEC) monolayers overlaid on 3-dimensional gel matrices. A single preliminary study was performed that revealed a significant reduction in ATF3$^{-/-}$ neutrophil adherence (Figure 7A), yet preserved percentage of transmigrating neutrophils (Figure 7B). Repeat experiments using immunofluorescent staining with ICAM (to identify the transmigration pore), PE-CAM (to identify the endothelial cell perimeter), and LFA-1 (to identify the neutrophil) would more definitively determine the route of transmigration. While it is not entirely clear how these data fit with the rest of our results, it is possible that our observation of reduced ATF3$^{-/-}$ adherence is actually indicative of increased transcellular TEM driven by increased adherence and an inability to translocate on HUVEC monolayers rather than defects in adherence. Discrimination between these opposed hypotheses will require careful study of the route of TEM utilized by WT and ATF3$^{-/-}$ neutrophils.

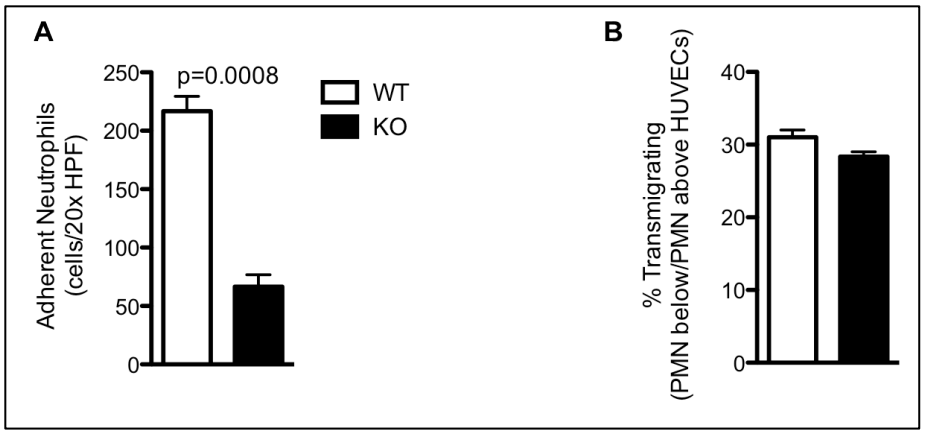

Figure 7. ATF3$^{-/-}$ neutrophils exhibit reduced endothelial adhesion yet preserved transendothelial migration. *Unstimulated WT (white bars) or ATF3$^{-/-}$ (black bars) neutrophils were incubated for 30 minutes at 37°C on human umbilical vein endothelial cells (HUVEC) that had been previously stimulated with 1 ug/ml LPS for 4 hours. Cells were gently washed and then fixed with PFA for 20 minutes. Adherent cells were counted (A) and transmigration was determined by counting cells above or below the focal plane of the HUVECs. Data are from one experiment, N=3 replicates/genotype. Statistics are unpaired, two-tailed t test. Data are mean ± S.E.M.*

5.5 Expanding the role of ATF3 in immune regulation: *T. gondii* and LCMV studies

Together the data presented in this dissertation describe a broad role for ATF3 regulation in immune responses, with specific novel effects identified in numerous immune cell types and inflammatory and infections settings. ATF3 plays a developmental role in neutrophils (Chapter 2), critical to proper mature neutrophil chemotaxis, while in epithelial cells, ATF3 attenuates LPS-driven epithelial CXCL1 production and presumably, subsequent WT neutrophil recruitment. In the setting of *T. gondii* infection (Chapter 3), the global ATF3 effect again appears to be that of attenuating immune reactivity to sublethal pathogen challenge. The direct mechanism, however, is unclear, but likely includes reduced macrophage effector functions with possible contributions of reduced neutrophil recruitment subverted by tachyzoites for macrophage entry. Finally, in response to persistent LCMV infection (Chapter 4), ATF3 reduces the production of virus specific T cells and alters the distribution of conventional DC subpopulations, associated with early and transient reductions in viral titers. In contrast to the rest of the dissertation, these preliminary studies did not identify a role for neutrophil recruitment in viral response, nor due they suggest that dysregulated neutrophil recruitment is a component of viral persistence. Rather, they implicate ATF3 in control of antiviral immune responses. While the studies described in Chapters 3 and 4 are preliminary, they strongly implicate ATF3 as a counter-regulatory molecule associated with increased pathogenic burden, likely through attenuation of immune responses, yet the exact mechanisms of these

effects are unknown. There are many potential experiments and future directions to be explored in these projects, which are outlined in Chapters 3 and 4.

5.6 Conclusions

In total, this thesis work reinforces the role of ATF3 as a counter-regulatory immune TF: ATF3 induction in response to low-level stimulation or infection attenuates host responses to limit immunopathology, albeit at the expense of increased pathogen burden. More importantly however, this work identifies ATF3 as critical to neutrophil chemotaxis, likely through developmental regulation of TIAM2 expression in differentiating neutrophils, although the involvement of other microarray-implicated proteins may contribute *in vivo*. This makes TIAM2 and downstream effector molecules attractive therapeutic candidates for clinical diseases associated with excessive or detrimental neutrophil recruitment, such as rheumatoid arthritis or CF. Additionally, aberrant ATF3 expression is associated with human malignancies [40], and ATF3 overexpression endows melanoma cell lines with metastatic potential [19]. Therefore, an ATF3/TIAM2 axis may exist in addition to neutrophil migration that plays a potential role in cancer metastasis. Obviously, further investigation into the biology of ATF3 and TIAM2 is needed and has the potential to broadly impact human disease.

5.7 References

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Appendix I: Publications list

1. **Nicholas D. Boespflug,** Sachin Kumar, Jaclyn W. McAlees, James D. Phelan, H. Leighton Grimes, Kasper Hoebe, Tsonwin Hai, Marie-Dominique Filippi, and Christopher L. Karp. *ATF3 is a novel regulator of mouse neutrophil migration*. Submitted. Blood. 2013.

2. Senad Divanovic, Jesmond Dalli, Lucia F. Jorge-Nebert, Leah M. Flick, Marina Gálvez-Peralta, **Nicholas D. Boespflug**, Traci E. Stankiewicz, Jonathan Fitzgerald, Maheshika Somarathna, Christopher L. Karp, Charles N. Serhan, and Daniel W. Nebert. *Contributions of the Three CYP1 Monooxygenases to Pro-Inflammatory and Inflammation-Resolution Lipid-Mediator Pathways.* Accepted. J. Immunology. 2013.

3. Cortez McBerry, Charlotte E. Egan, Reena Rani, Yanfen Yang, David Wu, **Nicholas Boespflug**, Louis Boon, Barbara Butcher, Julie Mirpuri, Simon P. Hogan, Eric Y. Denkers, Julio Aliberti, De'Broski R. Herbert. *Trefoil Factor 2 Negatively Regulates Type 1 Immunity Against Toxoplasma gondii.* J. Immunology. 2012. 189, 3078-3084.

4. Rajat Madan, Filiz Demircik, Sangeetha Surianarayana, Jessica L. Allen, Senad Divanovic, Aurelien Trompette, Nir Yogev, Yuayuan Gu, Marat Khodoun, David Hildeman, **Nicholas Boespflug,** Mariela B. Fogolin, Lothar Grobe, Marina Greweling, Fred D. Finkelman, Rhonda Cardin, Markus Mohrs, Werner Muller, Ari Waisman, Axel Roers, Christopher L. Karp. *Non-*

Redundant Roles for B Cell-Derived IL-10 in Immune Counter-Regulation. J. Immun. 2009 183, 2312 -2320.