Abstract

ABCF1 is an ABC (ATP binding cassette) transporter protein that lacks trans-membrane domains. Gene expression of ABCF1 has been shown to increase upon tumour necrosis factor alpha (TNFα) stimulation [1]. This is significant as TNFα is a pro-inflammatory cytokine produced by macrophages and T cells and has a number of functions in the immune response [2]. ABCF1 is thought to function in translational initiation by interacting with the eukaryotic initiation factor 2 (eIF2) [3]. We have determined that ABCF1 is an essential gene in development by the generation and characterization of mice that have a gene trap insertion in the ABCF1 gene, which terminates the expression of the ATP binding cassettes. Homozygous ABCF1 knock-out (ABCF1-/-) mice are embryonic lethal at 3.5 days post coitus (dpc) while heterozygous ABCF1 knock-out (ABCF1+/-) mice appear to be developmentally normal.

This thesis utilizes the ABCF1 gene trapped mouse model which contains a β-geo (β-galactosidase/neomycin) reporter gene in the ABCF1 gene to examine the endogenous ABCF1 promoter expression. This allows us to concurrently observe the activity of the ABCF1 promoter in all tissues through sectioning and X-Gal staining. This analysis provides further insight into the physiological function of ABCF1 by identifying the tissues and cell types that have the highest levels of promoter activity. Interestingly, ABCF1 appeared to be expressed in the marginal zone of the spleen and areas surrounding the lymphoid follicles.

Macrophages, which were isolated from spleens of ABCF1+/- mice, were found to be hyper-responsive to stimulation by TLR ligands, particularly LPS, while macrophages derived from the bone marrow were found to be hypo-responsive. When challenged with LPS, ABCF1+/- mice produced altered cytokine production compared to their wild-type controls.
Upon challenge with *Listeria monocytogenes*, ABCF1+/- mice succumbed to infection sooner than their ABCF1+/+ littermates. Taken together, these data indicate that ABCF1 is necessary for survival and development and likely has a role in the regulation of cytokines. Thus ABCF1 may play a significant role in regulating inflammation and pathogen induced “cytokine storm”.
Preface

The XKO97 ES cell was aggregated and injected into C57/B6 blastocysts by Jian Xin (Michael Smith Laboratories, University of British Columbia, Vancouver, BC). Blastocysts were isolated and Clone 1 was cultured by Lonna Munro (Michael Smith Laboratories, University of British Columbia, Vancouver, BC). The day 5.5-7.5 embryos were removed by Dr. Juan Hou (BC Cancer Agency, University of British Columbia, Vancouver, BC) Drs. Hebrit Adissu and Susan Newbigging (Toronto Centre for Phenogenoics, Toronto, Ont) helped analyse structures in the β gal stained tissues in chapter 3. Dr Hebrit Adissu took pictures 4.3 and 4.5 in chapter 4. Rayshad Gopaul (Department of Zoology & Michael Smith Laboratories, University of British Columbia, Vancouver, BC) managed one of the mouse colonies. UBC Animal care approval certificates A07-0270 and A09-0824 were acquired for this research. This thesis was written by Sara Wilcox and edited by Cheryl Pfeifer, Lonna Munro and Wilf Jefferies.
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<th>Description</th>
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<tbody>
<tr>
<td>5’ RACE</td>
<td>5’- rapid amplification of cDNA ends</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP binding cassette</td>
</tr>
<tr>
<td>ABCF1</td>
<td>ATP binding cassette, sub-family F, member 1</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>APCs</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ARE</td>
<td>(AU)-rich element</td>
</tr>
<tr>
<td>ATF</td>
<td>Activating transcription factor</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>βgal</td>
<td>β-galactosidase</td>
</tr>
<tr>
<td>βgeo</td>
<td>β-galactosidase and neomycin fusion protein</td>
</tr>
<tr>
<td>BHI</td>
<td>Brain heart infusion</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic local alignment search tool</td>
</tr>
<tr>
<td>BMMφ</td>
<td>Bone marrow derived macrophage</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>CBA</td>
<td>Cytometric bead array</td>
</tr>
<tr>
<td>CBC</td>
<td>Complete blood count</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>C/EBP</td>
<td>CCAAT/enhancer binding protein</td>
</tr>
<tr>
<td>Cfu</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>CHOP</td>
<td>CCAAT/enhancer-binding protein homologous protein</td>
</tr>
<tr>
<td>CIA</td>
<td>Collagen induced arthritis</td>
</tr>
<tr>
<td>CK2</td>
<td>Casein kinase 2</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CrPV</td>
<td>Cottontail rabbit papilloma virus</td>
</tr>
<tr>
<td>CSF-1</td>
<td>Colony stimulating factor-1</td>
</tr>
<tr>
<td>CSF-1R</td>
<td>Colony stimulating factor-1 receptor</td>
</tr>
<tr>
<td>C-terminus</td>
<td>Carboxy terminus</td>
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<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
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<tr>
<td>DB</td>
<td>Dilution buffer</td>
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<tr>
<td>DCs</td>
<td>Dendritic cells</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle media</td>
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<td>EAA motif</td>
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<td>EDTA</td>
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<tr>
<td>eIF</td>
<td>Eukaryotic initiation factor</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>Endoplasmic reticulum</td>
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<tr>
<td>ES cell</td>
<td>Embryonic stem cell</td>
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<tr>
<td>FDG</td>
<td>Fluorescein di-β-D galactopyranoside</td>
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<tr>
<td>FACS</td>
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FBS  Fetal bovine serum
FITC  Fluorescein isothiocyanate
GCN  General control nondepressible
GDP  Guanosine diphosphate
GEF  Guanine exchange factor
GTP  Guanosine triphosphate
GM-CSF  Granulocyte-macrophage colony stimulating factor
H&E  Haematoxylin and eosin
HCV  Hepatitis C virus
HcG  Human chorionic gonadotropin
HBSS  Hanks balanced salt solution
HEPES  4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
HLA  Human leukocyte antigen
HRI  Heme-regulated inhibitor kinase
Ht  Heterozygous
IFN  Interferon
IκB  Inhibitor of κB
IKK-β  IκB kinase β
IL  Interleukin 1
IL-1R  Interleukin 1 receptor
ip  Intraperitoneal
IRAK  Interleukin-1 receptor-associated kinase
IRES  Internal ribosome entry site
IU  International units
iv  Intravenous
Kb  Kilobase
LacZ  Lactose operon Z gene
LM  Listeria monocytogenes
LPS  Lipopolysaccharide
LTA  Lipoteichoic acid
Mφ  Macrophage
MAPK  Mitogen-activated protein kinase
MCP-1  Monocyte chemoattractant protein-1 (CCL2)
M-CSF  Macrophage colony stimulating factor
MEFs  Mouse embryonic fibroblasts
Met-tRNA  Methionyl-tRNA
Met-tRNAi  Methionyl-tRNA initiation complex
MHC  Major histocompatibility complex
miRNA  MicroRNA
MRCRB  Mouse red cell removal buffer
mRNA  Messenger ribonucleic acid
MyD88  Myeloid differentiation primary response gene 88
NBD  Nucleotide binding domains
NF-κB  Nuclear factor kappa-light-chain-enhancer of activated B cells
N-terminus  Amino terminus
NS  No stimulation
OVA  Ovalbumin
P-bodies  Processing bodies
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PERK</td>
<td>PKR-like ER-localized eIF2α kinase</td>
</tr>
<tr>
<td>PFU</td>
<td>Plaque forming units</td>
</tr>
<tr>
<td>PGN</td>
<td>Peptidoglycan</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PIC</td>
<td>Pre-initiation complex</td>
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<tr>
<td>PKR</td>
<td>Double-stranded RNA (dsRNA)-dependent protein kinase</td>
</tr>
<tr>
<td>PMS</td>
<td>Pregnant mare serum</td>
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<tr>
<td>PRR</td>
<td>Pattern recognition receptors</td>
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<tr>
<td>RACE</td>
<td>Rapid amplification of cDNA ends</td>
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<tr>
<td>RISC</td>
<td>RNA-induced silencing complex</td>
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<td>RNA</td>
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<tr>
<td>TCID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Tissue culture infectious dose affecting 50% of the culture</td>
</tr>
<tr>
<td>TIA-1</td>
<td>T-cell intracellular antigen-1</td>
</tr>
<tr>
<td>TIAR</td>
<td>TIA-1-related protein</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/IL-1 receptor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll/Interleukin-1 receptor</td>
</tr>
<tr>
<td>TMD</td>
<td>Trans-membrane domain</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF receptor associated factor</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer RNA</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase biotin-dUTP nick end labelling</td>
</tr>
<tr>
<td>TTP</td>
<td>Tristetraprolin</td>
</tr>
<tr>
<td>VSV</td>
<td>Vesicular stomatitis virus</td>
</tr>
<tr>
<td>VWM</td>
<td>Leukoencephalopathy with vanishing white matter</td>
</tr>
<tr>
<td>Wt</td>
<td>Wild-type</td>
</tr>
<tr>
<td>X-Gal</td>
<td>Bromo-chloro-indolyl-galactopyranoside</td>
</tr>
</tbody>
</table>
Acknowledgments

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Dedication

I would like to dedicate this thesis to my parents Mary-Anne and Barry Wilcox, my grandparents and my siblings.
Chapter 1: General introduction

1.1 Immunology

1.1.1 The innate immune system

The immune system is a complex biological system that protects organisms from infection. Historically, the immune system has been divided into the innate and adaptive immune systems. The innate immune system provides general front line defence against a wide variety of pathogens, while the adaptive immune system provides a more specific cellular response to a particular pathogen. Both systems are connected however, with the innate response being crucial for the development of an adaptive response to an infection [1]. The innate immune system is an evolutionarily ancient system that has developed in response to the constant threat of invasion by potentially deadly pathogens. The adaptive immune system evolved later, prior to the splitting of the vertebrate and plant kingdoms [2]. Innate immunity relies on the ability of a limited number of receptors to recognize conserved metabolic products from infectious agents [2]. Toll like receptors (TLRs) are key in the recognition of pathogens and are the gate keepers to the initiation of an immune response [2]. TLRs bind to their ligands (pathogen-associated molecular patterns) and initiate a signalling cascade that results in the upregulation of pro-inflammatory genes like TNFα and NF-κB (Nuclear factor of κB), as well as genes that signal for the other cells of the immune system [3]. The adaptive immune response then, through gene rearrangements and clonal selection, provides the pathogen specific cells necessary for the clearance of an infection [2]. The innate immune system is thus the key in determining whether something is dangerous or not. Defects in the innate immune
system are rare but are almost always lethal [1]. The adaptive immune response can also be problematic for an organism as it is also capable of acting against itself, thus being responsible for the symptoms that are associated with autoimmunity and allergy.

1.1.2 Innate immunity and macrophages

The innate immune system is made up of a variety of cells and it is important in patrolling the skin, airways and digestive tract. These tissues are normally populated with commensal microbes so it is important for the selective activation of the innate immune cells. Macrophages are resident phagocytic cells in tissues. Their primary roles are in the phagocytosis of old or damaged cells and the production of growth factors [4]. Macrophages also express various pattern recognition receptors (PRRs), like TLRs, which serve to recognize conserved features of pathogens. Signalling through PRRs leads to phagocytosis and the production of cytokines like TNFα [4]. Macrophages, lymphocytes, fibroblasts, neutrophils and endothelial cells are the primary producers of TNFα during an acute phase response [5].

TNF is composed of two related cytokines (α (cachectin) and β (lymphotoxin)) [6]. Each of these cytokines is encoded by a separate gene within the MHC locus [7]. The TNFα gene is driven by NF-κB-responsive elements in its promoter [8]. Activated macrophages only transcribe two fold more TNFα than unactivated macrophages and the majority of TNFα expression is regulated post translationally by miRNA and proteins such as TIA-1/TIAR (T-cell intracellular antigen-1/ TIA-1 related protein) [5, 9, 10]. In inactivated cells, transcripts are degraded, while in activated cells transcripts are
stabilized leading to a two hundred fold increase in TNFα mRNA and increased protein expression [5].

### 1.1.3 TLRs

The first TLR, Toll, was discovered in *Drosophila melanogaster* and was found to be required for proper dorso-ventral axis formation [11]. Human IL-1 receptor was later shown to share similar cytoplasmic domains as Toll along with the shared ability to induce NF-κB activation [12]. TLR4 is the human and mouse homologue of Toll [13]. TLR4, in mice, has been shown to be important for recognition and response to LPS (lipopolysaccharide), a component of the Gram-negative bacterial cell wall [14, 15]. TLR4 has also been shown to recognize and respond to lipoteichoic acid (LTA) from Gram-positive bacteria [16]. Upon binding its ligand TLR4 recruits MyD88 which engages IRAK at its Toll/IL-1 receptor (TIR) domain [17]. IRAK autophosphorylates and along with TRAF6 activates IKK-β which targets IκB (the NF-κB inhibitor) for degradation [18]. NF-κB is then free to translocate to the nucleus and transcribe target genes such as TNFα and genes important in inflammatory mRNA stabilization (Figure 1.1) [18-20]. The mitogen activated protein kinase (MAPK) pathway is also activated [19].
Figure 1.1. Signalling through the TLRs.

Most TLR signalling uses the adaptor protein MyD88 (Myeloid differentiation primary response gene 88) which interacts with IRAK (Interleukin-1 receptor associated kinase) [21]. IRAK interacts with TRAF6 (Tumour-necrosis factor receptor associated factor 6) leading to the activation of NF-κB and the MAPK (Mitogen-activated protein kinase) pathways (like p42/p44 MAP kinase and Jun N-terminal kinase (JNK)) [21]. MyD88 also activates IRF7 (interferon (IFN)-regulatory factor 7), which induces interferon-α [21].

Signalling through TLR2 and TLR4 recruits the adaptor, MAL, whose main function is as an adaptor for MyD88 [21]. TLR3 signals through TRIF (Toll/IL-1 receptor-domain containing adaptor protein inducing IFN-β), which interacts with TBK1 (TRAF-family-member-associated NF-κB activator-binding kinase 2), and leads to the activation of IRF3 [21].

The IFN-β gene is a target for this pathway. TRIF interacts with RIP1 (receptor-interacting protein 1) as well, which activates IκB (inhibitor of NF-κB) kinase 1 (IκK1)–IκK2–NEMO complex. AP1- activator protein 1, BTK- Bruton’s tyrosine kinase, ECSIT- evolutionarily conserved signalling intermediate in Toll pathway, ISRE-IFN-stimulated response element, MKK-MAP kinase kinase, TAB2- transforming growth factor-activated kinase (TAK)-binding protein-2, UEV1A and UBC13 -ubiquitin-conjugating enzymes. Diagram reproduced from Liew et al. with permission [21].
Since then, ten TLRs have been described in mice and humans (Figure 1.1). Each TLR has its own ligand specificity along with its own unique pattern of expression. TLR activation results in the up-regulation of host genes responsible for defence including but not limited to cytokines, MHC molecules and co-stimulatory molecules. TLRs have also been reported to recognize endogenous ligands which are released from necrotic cells [22, 23]. These endogenous ligands initiate inflammatory immune responses [19]. This results in a cycle of necrosis and inflammation which is thought to contribute to the chronic inflammatory state of rheumatoid arthritis (RA) [19].

### 1.1.4 Inflammation

Inflammation is the complex protective response by the body to invasion by pathogens or injury. It involves a series of changes in hemodynamics, caused by the release of signalling and effector molecules and the recruitment of effector cells [24]. A particular inflammatory response is tailored both in intensity and duration to the type of tissue and the nature of the injury [24]. The ideal inflammatory response is initiated upon insult and is terminated once homeostasis is achieved [24]. If, however, inflammation is not properly controlled, it can result in persistent tissue damage, as seen in patients with rheumatoid arthritis (RA), or it can lead to the development of neoplastic transformation [25].

Inflammation can be classified as acute or chronic. Acute inflammation differs from chronic inflammation by a shift in the types of cells and types of soluble mediators produced in response to the insult [7]. Cytokines involved in acute inflammation include
IL-1, IL-6, IL-8, TNFα, GM-CSF (granulocyte-macrophage-colony stimulating factor) and G-CSF (granulocyte-colony stimulating factor) [7].

The inflammatory response is controlled through cell differentiation, signalling cascades and ultimately the gene expression of the effector cells [24]. The transcription factor NF-κB is an activator required for the regulation of inflammatory genes in most types of inflammatory responses [24]. The synthesis of the acute response cytokines IL-1β, IL-6, IL-8 and TNFα are all mediated by the activation of NF-κB [26].

1.1.5 Cytokines

Cytokines are soluble proteins that are a principal means of cell communication during an infection [27]. The pattern of cytokine production defines the nature and magnitude of the immune response [27]. Different cell types produce specific arrays of cytokines which ultimately determine what cells are recruited to the site of infection and what action to take. Cytokines mediate their effects via binding to their cognate receptors [7]. This binding results in an intracellular signalling cascade that leads to the expression of genes which function in the cytokine-related responses [7].

Cytokines can enhance or inhibit immune responses. IFN-γ, IL-1, IL-2, IL-6, IL-8, IL-12, IL-18 and TNFα are pro-inflammatory cytokines [28, 29]. IL-4, IL-10 and TGF-β, on the other hand, are anti-inflammatory cytokines [28, 29]. Macrophages are capable of producing both pro- and anti-inflammatory cytokines in response to infection [30]. The combination of pro- and anti-inflammatory cytokine production is instrumental in determining the outcome of the immune response [30]. In diseases like RA, the
balance of pro-inflammatory to anti-inflammatory cytokines determines the extent of the inflammation and can lead to different clinical manifestations of the disease [29].

1.1.6 Autoimmunity

Autoimmunity is a condition caused by the immune response to self. There are over eighty autoimmune diseases that affect millions of people worldwide [31]. Autoimmune diseases are a major cause of morbidity and mortality in the industrial world [32]. Many factors are thought to contribute to autoimmunity, including environmental factors and genetic susceptibility [33]. Infection caused by viruses, bacteria and other pathogens appear to be the most important environmental factors that lead to autoimmunity [33]. Infectious agents initiate autoimmunity through mechanisms like molecular mimicry (by bearing elements with similar amino acid sequences to the self antigen), the production of super-antigens or bystander activation (activation of T-cells independent of TCR signalling) [33]. Viruses, such as vesicular stomatitis (VSV) have been shown to activate PRRs and induce IFN genes [34]. Apoptotic and necrotic cell debris is also capable of activating TLRs and inducing inflammatory gene expression [35]. Any long-term activation of the innate immune system has the potential of breaking tolerance to self, resulting in autoimmunity [36].

RA is an autoimmune disease caused by the chronic inflammation of synovial joints [37]. It is the most common form of inflammatory arthritis, primarily affecting the joints of the hands and feet [38]. In RA, immune cells invade the normally acellular synovium resulting in hyperplasia of the joint lining and destruction of the articular
Macrophage activation, along with the production of IL-1 and TNFα, in the rheumatoid synovium are characteristic of the disease [39].

The initial causes of RA are unknown, but they result in the activation of the innate immune response which initiates inflammation of the synovium [38]. Signalling through PRRs then lead to the activation of NF-κB (which is known to be activated in the joints of patients with RA) [40]. NF-κB is an important transcriptional regulator in chronic inflammation, mediating both the production of TNFα and IL-1 and their effects on target cells [41]. Target cells then express other cytokines, metalloproteinases and other small molecule mediators which can damage tissues and initiate the adaptive immune response [38]. The result is persistent disease (probably through the chronic activation of the innate immunity) and the associated disability caused by inflammation and joint destruction.

1.2 Eukaryotic translation

Eukaryotic translation begins with the assembly of the pre-initiation complex (PIC). This complex includes the 43S ribosomal subunit, methionyl-transfer RNA (Met-tRNA), ABCF1 and the eIFs (eukaryotic initiation factors): 1,1A, 2, 3 and 5 which are recruited to the 5’ capped end of the mRNA by eIF4: E, A and G [42, 43]. The PIC scans the mRNA until it reaches the AUG. The Met-tRNA is initially bound to the eIF2-GTP. Once the AUG sequence binds to Met-tRNA, GTP is hydrolysed to GDP triggering the arrest of the scanning mechanism and the release of the eIFs from the ribosome. The 60S subunit is then able to bind to form the 80S ribosome and peptide bond synthesis can proceed [42]. The eIFs are recycled and free to form a new PIC and eIF2-GDP is
switched back to eIF2-GTP by the guanine exchange factor (GEF) eIF2B (Figure 1.2) [44].
Figure 1.2. Eukaryotic translation initiation schematic for 5’ cap-dependent translation.
The translation of mRNA into protein begins with the assembly of the PIC. The PIC is composed of eIFs, mRNA, tRNA and the 43S ribosome. Once the initiation codon is recognised, eIF2-GTP is hydrolysed to eIF2-GDP, the 60S ribosome subunit binds and translation proceeds. eIFs are released upon 60S binding and are recycled for the next round of translation initiation [45, 46]. During this time the GEF eIF2B exchanges the GDP for GTP bound to eIF2 which is important for the interaction with Met-tRNA [47]. This schematic was adapted from Lopez-Lastra et al., Pestova et al. and Klann et al. with permission [46, 48, 49].
Several viruses can bypass this system through their use of internal ribosomal entry site (IRES) sequences. IRES sequences allow the virus to hijack the host ribosomes without the assembly of the eIFs [46]. Interestingly, there are several endogenous mRNAs which are also known to contain IRES sequences [42]. These endogenous mRNAs are involved in the regulation of apoptosis and mitosis when cap-dependent translation is inhibited [42].

1.2.1 Translation control

Translational regulation is important in many physiological processes in the cell including development, differentiation, immunity, and metabolism [50]. It allows cells to react quickly to changes in their environment by translating pre-existing mRNA. Translational control is normally exerted at the initiation step of translation, often through inhibition of the eIFs by phosphorylation or by the reduction of the amount of free mRNAs [42, 51]. The phosphorylation of the Ser51 residue on the α subunit of eIF2 is a key mechanism of translational control in times of stress [44]. Four kinases; PKR (Double-stranded RNA (dsRNA)-dependent protein kinase), PERK (PKR-like ER-localized eIF2α kinase), HRI (Heme-regulated inhibitor kinase) and GCN2 (General Control Non-depressible 2), are known to phosphorylate the eIF2 [52]. Phosphorylated eIF2-GDP can still bind to the GEF eIF2B, but the phosphorylated residue inhibits eIF2B from exchanging the GDP for GTP [53]. This prevents the recycling of the eIF2-GDP for eIF2-GTP, which is required for transporting the Met-tRNA to the pre initiation complex [52]. ATF4 (Activating transcription factor 4), ATF3 and CHOP (CCAAT/enhancer-binding protein homologous protein) translation are upregulated by phosphorylated eIF2,
which leads to the translation of genes involved in the stress response [54, 55]. NF-κB is one of the genes known to be transcribed in response to stress [52]. In normal conditions, NF-κB is bound to IκB (Inhibitors of κB). When cells are exposed to pro-inflammatory cytokines, IκB becomes phosphorylated by IKK (IκB kinase) which results in the targeting of IκB for degradation [56]. The release of NF-κB from IκB, allows NF-κB to translocate to the nucleus and regulate gene expression. The phosphorylation of eIF2α bypasses the IKK phosphorylation of IκB thus representing an alternative mechanism of activating NF-κB [57].

miRNAs (microRNAs) are also able to control translation through their association with the RNA-induced silencing complex (RISC) [58]. miRNAs base pair with complementary conserved AU-rich elements (ARE) on specific mRNAs and function to inhibit or stimulate their translation according to the cell cycle [10]. miRNAs can accumulate along with Argonaute (Ago) protein 2 (in vertebrates), accessory proteins and mRNAs in processing bodies (P-bodies) or stress granules [42]. P-bodies are sites within the cytoplasm where mRNA are degraded or temporarily stored; stress granules are cytoplasmic aggregations of mRNA and proteins that accumulate when cells are stressed (more for storage as opposed to degradation) [59, 60].

Cytokines, particularly TNFα, are subject to post translational control by miRNAs and proteins such as TIA-1(T cell intracellular antigen), TIAR (TIA-1 related protein) and TTP (Tristetraprolin) [10, 61]. When growth is arrested, TNFα AREs increase translation which is important for inflammation and monocyte differentiation [10]. In unstimulated and stimulated macrophages, TIA-1 and TIAR are bound to TNFα mRNA. TIA-1 can negatively or positively regulate TNFα expression by forming a complex with other trans-acting ARE binding proteins which are known to either
stabilize or destabilize the TNFα transcript, depending on the dosage of the transcript and whether the cells are involved with differentiation or inflammation [61, 62]. When cells are stressed, such as during inflammation, the eIF2α becomes phosphorylated, and the TIA-1 and TIAR chaperone the PIC (which lack Met tRNA-eIF2-GTP) to stress granules where they are stored [63]. This process is important for halting the translation of normal housekeeping genes and for regulating the TNFα response [63].

1.2.2 Translational control and disease

A strong inflammatory response is important in order to quickly clear an infection. However, if the response is too strong or not curtailed quickly enough significant tissue damage can occur. Uncontrolled production of TNFα, for example, can induce gut and joint associated immune-pathologies [61]. Almost all of the animal models of RA can be cured by the administration of anti-TNFα antibodies [5]. One mechanism of TNFα induced arthritis is through the activation of PKR. TNFα stimulation in chondrocytes results in PKR activation which leads to eIF2 phosphorylation and NF-κB activation [64]. NF-κB activation then results in the activation of pro-inflammatory genes that have been implicated in articular cartilage degradation [64].

Dysregulation of eIF2 is also known to cause other chronic pathologies as well. eIF2 phosphorylation is important for endoplasmic reticulum (ER) homeostasis. Mice that have mutations in the Ser51 residue of the eIF2 or the PERK kinase (which phosphorylates eIF2) develop a type-2 diabetes phenotype. PERK normally functions in suppressing protein synthesis when cells have accumulated unfolded proteins [65].
Accumulation of unfolded proteins in the ER past a threshold limit lead to ER stress and causes cells to be committed to cell death [66, 67]. Loss of PERK causes cell death in pancreatic β-cells and thus results in diabetes [65]. It also affects other systems, causing osteoporosis and growth retardation [65]. Other mutations involving eIF2B have also been linked to ‘leukoencephalopathy with vanishing white matter’ (VWM). VWM is characterized by neurological deterioration caused by fever that can result in coma or death [65]. Finally, lack of translational control in the signal pathways associated with growth or proliferation have been implicated as major causes of cancer [42]. Changes in the expression of initiation factors, tRNA and translational regulators are associated with many types of cancer. For example, breast carcinomas, non-Hodgkin’s lymphomas and colon adenomas and carcinomas have been found to have increased eIF4E [65]. Constitutive activation of eIF2 through mutated phosphorylation sites can directly cause malignancy in cells in vitro [68]. Cancer cells are also frequently observed to have dysregulated eIF2 [69].

### 1.3 ABC transporters

#### 1.3.1 The ABC family of transport proteins

ABC transporters are a ubiquitous family of transport proteins which harness the energy of ATP in order to do work. Historically, ABC transporters are known for their ability to move substrates across biological membranes, but there are several members of this super-family that have functions in translation and DNA repair [70]. ABC transporters are found in all kingdoms of life. Humans have forty-eight different ABC transporters, separated into seven distinct sub-families [71]. These transporters are
involved with lipid metabolism, antigen processing, iron homeostasis, ion gradients and multi-drug resistance (Table 1) [72]. Many diseases have been associated with mutations in ABC transporters including cystic fibrosis (CFTR), diabetes (ABCG1), Tangier’s disease (ABCA1) and cancer (TAP transporters) [72-75]. ABC transporters have traditionally been characterised as having four core domains, including two trans-membrane domains (TMD), which transverse the membrane, and two nucleotide binding domains (NBD), which are involved in ATP binding (Figure 1.3). The TMD contain 6 helices each and are known to be highly variable in terms of their primary sequences, other than the EAA sequence motif located in the cytosolic compartment. The EAA motif is thought to connect the trans-membrane and cytosolic domains through the Q-loop on the cytosolic domain [76]. The NBD, on the other hand, contain the highly conserved Walker A and B motifs along with the ABC signature ‘LSGGQ’ motifs which are involved with ATP binding and hydrolysis.
**Table 1.1. The mammalian ABC superfamily members.**
The table indicates the individual ABC family members, their translated structures, their known functions and examples of each function. CFTR- Cystic fibrosis transmembrane conductance regulator.

<table>
<thead>
<tr>
<th>Family</th>
<th>Structure</th>
<th>Function</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCA</td>
<td>Full transporter</td>
<td>-Cholesterol transport[75]</td>
<td>-ABCA1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-N-retinylidiene PE efflux [77]</td>
<td>-ABCA4</td>
</tr>
<tr>
<td>ABCB</td>
<td>Full transporters and half transporters</td>
<td>-Multidrug resistance[77]</td>
<td>-ABCB1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Antigen presentation[73]</td>
<td>-TAP1 and 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Phosphatidylcholine transport[77]</td>
<td>-ABCB4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Iron homeostasis[77]</td>
<td>-ABCB7</td>
</tr>
<tr>
<td>ABCC</td>
<td>Full transporters</td>
<td>-Multidrug resistance[77]</td>
<td>-ABCC1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Ion homeostasis [78]</td>
<td>-CFTR</td>
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<td></td>
<td></td>
<td>-Nucleoside transport [77]</td>
<td>-ABCC5</td>
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<tr>
<td>ABCD</td>
<td>Half transporters</td>
<td>-Peroxisomal homeostasis [78]</td>
<td>-ABCD1</td>
</tr>
<tr>
<td>ABCE</td>
<td>NBDs only</td>
<td>-Translation control [79]</td>
<td>-ABCE1</td>
</tr>
<tr>
<td>ABCF</td>
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<td>Half transporters</td>
<td>-Cholesterol efflux [76]</td>
<td>-ABCG1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Sterol transport [76]</td>
<td>-ABCG8</td>
</tr>
</tbody>
</table>
Figure 1.3. Model of the structure of a typical ABC transporter protein. Typical ABC transporters are composed of four core domains: two TMD and two NBD. The TMD contains a minimum of 12 alpha helices and the nucleotide binding domain (NBD) contains the Walker A and B sequences along with the C loop or signature sequence. The NBDs are involved in the hydrolysis of ATP. The EAA motif is thought to interact with Q loop and links the ATP hydrolysis activity to conformational changes in the TMD. This diagram was modified from Abele et al. (in Physiology, 2004), Am Physiol Soc with permission. [73]
More recently, members of the ABC transporter super-family have been described where they are translated as full transporters (two TMD and two NBD), or as partial components, including half transporters (one TMD and one NBD), two ABC cassettes only or as individual components (Figure 1.4).
Figure 1.4. ABC transporter domain architecture.
ABC transporters can be translated as full transporters, half transporters, ATP binding cassettes only or as individual components. Examples are given of each different type of ABC transporter. Partial transporters are assembled post translationally into full transporters and the ABC cassette only transporters are thought to function in the absence of a TMD. This figure is adapted from Pohl et al. [81].
The ABCE and ABCF families are composed of proteins that only consist of the NBDs and are thought to have roles in translation, but to date, they have not been shown to participate directly in any known transport function. [82]. There are three ABCF class members (1, 2 and 3) and they all show strong conservation evolutionarily. GCN20, a *Saccharomyces cerevisiae* homolog of mouse and human ABCF1, is the best characterized ABCF member [77]. Its function is to regulate the phosphorylation of eIF2 which is involved in translation initiation [83].

### 1.3.2 TAP transporters

A number of transporters have been shown to be important for immune surveillance. One prime example are the transporters’ associated with antigen processing (TAP) proteins, which are members of the ABCB family [84]. They are translated as half transporters with one TMD and one NBD and come together to form heterodimers in the endoplasmic reticulum (ER), where they are responsible for the transport of peptides from the proteosome to the ER lumen. Inside the lumen, the transported peptides are loaded onto MHC class I and shuttled to the cell surface where they are exposed to the CD8$^+$ T cells involved in immunosurveillance (Figure 1.5).
Figure 1.5. The classical MHC class I presentation pathway. Endogenous peptides (self and those produced by viruses) are broken down to peptides by the proteosome. These peptides are transported through the TAP transporter into the ER and loaded onto MHC class I molecules where they are shuffled to the cell surface and presented to CD8\(^+\) T cells. \(\beta_2\)-microglobulin, TCR:T-cell receptor. Figure adapted from McMichael et al. with permission [85].
This peptide exposure to CD8\(^+\) T cells in the context of the MHC class I is important for the detection of aberrant proteins derived from virally-infected or from tumour cells [86]. However, most of the peptides that are presented to CD8\(^+\) T cells are endogenous peptides which do not elicit a T cell response. TAP 1 and 2 genes are located in the class II region within the MHC locus [87]. Both TAP genes and several components of the proteosome are regulated by IFN-\(\gamma\) and TNF\(\alpha\) through STAT1 [88].

### 1.3.3 ABCF1

ABCF1 (subfamily F, member 1) was the first mammalian ABC transporter found that lacked a TMD (Figure 1.6) [89]. The ABCF1 protein is composed of two NBDs connected through a linker region and is expressed ubiquitously in humans [1]. ABCF1 was originally discovered by differential display PCR in synoviocytes stimulated with TNF\(\alpha\) [89]. Synoviocytes from normal patients and patients with RA were both found to express ABCF1 mRNA, which was upregulated by treating cells with TNF\(\alpha\) [89]. Activated T cells were also found to upregulate ABCF1 protein expression upon stimulation with phorbol myristate acetate and ionomycin [43].
Figure 1.6. ABCF1 lacks trans-membrane domains.
a) Diagram of the results obtained when the ABCF1 peptide sequence NP_038882 was entered into the DAS Trans-membrane prediction server http://www.sbc.su.se/~miklos/DAS/. The X-axis represents the amino acid sequence of the protein while the y-axis represents strict (solid) and loose (dotted line) cutoff levels for trans-membrane prediction [90]. The DAS program uses a special scoring matrix to compare the test protein with a number of non-homologous membrane proteins in order to determine whether there are trans-membrane segments [90]. The strict cut-off indicates the number of matching trans-membrane segments while the loose cut-off is indicative of the actual location of the trans-membrane segment [90]. b) Diagram of the results obtained when the TAP 1 peptide sequence NP_000584.2 was entered into the DAS Trans-membrane prediction server. TAP 1 is an ABC transporter which is known to have a trans-membrane domain. c) Schematic representation of the protein structures of the ABCF1 and TAP 1 proteins indicating known domains. The numbers represent the amino acid sequence corresponding to the separate domains.
ABCF1 is thought to be the mammalian homolog of the yeast protein GCN20 [43]. GCN20 functions in the control of translation during stress responses linked to amino acid starvation [43]. When amino acids are scarce, GCN20 associates with GCN1 and GCN2 and promotes the phosphorylation of eIF2α [91]. Phosphorylated eIF2α competitively inhibits the GEF function of eIF2B, which is responsible for exchanging GDP for GTP on eIF2 [43]. Phosphorylated eIF2 is then unable to bind Met-tRNA and form the PIC [43].

ABCF1 was found to associate with eIF2 through five different chromatography procedures [43]. The N-terminal domain 1-42 residues are important for this interaction [92]. The presence of ABCF1 was determined to enhance the association of Met-tRNA with eIF2 possibly through conformational stabilization of the eIF2 but had no effect on the ability of eIF2 to bind GDP [43]. ABCF1 was also found to co-sediment with the 40S and 60S ribosome subunits [43]. This association is ATP dependent and require at least one NBD. However, functional Walker boxes were not required for ABCF1-ribosome-binding [43, 80, 92]. CK2 sites within the ABCF1 N-terminus were also shown to be important for the association of eIF2 with the ribosome [92].

siRNA studies showed that the knockdown of ABCF1 expression resulted in reduced 5’ cap-dependent and IRES-dependent reporter translation [80]. Mutations in the Walker A and B residues, however, only result in reduced 5’ cap dependent translation [80]. In contrast, cells that were transfected with either Walker box mutation were found to have augmented HCV and CrPV-IRES driven translation, indicating that ABCF1 is probably important in the negative regulation of IRES driven translation [80].

The *ABCF1* gene is located in the class I region of the MHC locus on chromosome 6p21 in humans and chromosome 17 in mice. This region is known to
contain several genes related to the immune system (like the TAP genes), as well as several genes implicated in diseases such as psoriasis, diabetes and RA [93]. ABCF1 in particular, is known to reside within a region known to be a susceptibility locus for autoimmune pancreatitis [94].

1.4 Rationale and hypothesis

Previously, the mRNA expression profiles of 46 ABC transporters were screened in resting and activated APCs (Macrophages (Mφs), Dendritic cells (DCs), B cells and T cells). APCs were activated by exposure to a variety of cytokines or soluble antigens. ABCF1 was found to be up-regulated in response to cytokines (IFNγ) in several APCs (Jefferies’ lab, unpublished data). Interestingly, the ABCF1 gene is known to reside within the MHC locus and could play a role in inflammation and immunity.

The goal of this thesis was to identify the in vivo function of ABCF1. Initially, upon starting this project, very little was known about the function of ABCF1. In 1998, ABCF1 was originally discovered by a differential display PCR screen in synovioocytes and was shown to be upregulated by TNFα stimulation [89]. Two years later, it was shown by a different group that ABCF1 could associate with the eIF2 and may be important for translational initiation [43].

ABCF1, unlike most other members of the ABC super-family, lacks functional TMDs. This precluded ABCF1 from having a TAP-like role as a transport protein unless it was able to bind to an unknown orphan TMD. The yeast homolog of ABCF1, GCN20, lacks TMDs and has been shown to function in the regulation of the eIF2 [95]. There are no known binding partners containing TMDs that associate with GCN20. Prior in vitro
investigations indicated that ABCF1 also interacts with eIF2 [43]. ABCF1 was found to directly bind to the eIF2 through residues in the N-terminus [92]. Interestingly, ABCF1 expression is known to be regulated with TNFα, and up-regulated in activated T cells, suggesting that ABCF1 may have regulatory roles outside of normal 5’cap-dependent translation [43, 89]. The hypothesis of this thesis is that ABCF1 has a role in immunity. This thesis examines the in vivo function of ABCF1 focusing on the role of ABCF1 in immunity.

1.5 Thesis synopsis

Chapter 2 describes the creation of the ABCF1 deficient mouse from the XK097 cell line. Since ABCF1 is now known to be associated with translation initiation, it is not surprising that homozygous ABCF1 knockout mice (ABCF1-/-) are embryonic lethal. ABCF1-/- mice were found to exhibit embryonic lethality shortly after the blastocyst stage of development. Several ES cell lines were derived from blastocysts recovered from timed mated heterozygous ABCF1 knock-out (ABCF1+/-) mice. One of the ES cell lines is a homozygous ABCF1 knockout (ABCF1-/-) as shown by RT-PCR. This ES cell line grew slowly (compared to wild type or heterozygous ES cell lines) and displayed aberrant morphology.

Chapter 3 characterizes the endogenous ABCF1 promoter activity in developing embryos and adult mice. It was hypothesized that the ABCF1 promoter would be ubiquitously expressed similar to the mRNA expression. The ABCF1 promoter was found to be highly expressed during development in virtually all tissues particularly those actively differentiating and proliferating. In adult tissues, the ABCF1 promoter activity
appeared to be more cell specific in its activity. Although it was present in every tissue tested, different regions of the tissues appeared to have more activity than others for example in the marginal zone of the spleen. The ABCF1 promoter also appeared to be turned on during the maturation of CD8$^+$ T cells.

Chapter 4 examines the phenotype of ABCF1 +/- macrophages. Since ABCF1 is regulated by TNFα and the promoter is active in the marginal zone of the spleen, it was hypothesised that ABCF1 deficient mice would also have deficiencies in their macrophage populations. Surprisingly, ABCF1+/- mice were found to have tissue derived macrophages that were hyper-responsive to TLR ligands, indicating a role as a possible negative inhibitor of cytokine activity, similar to its actions in IRES translation. Conversely, bone marrow derived-macrophages from ABCF1+/- mice were found to be hypo-responsive to TLR ligand stimulus. When challenged with LPS, ABCF1+/- mice were not found to be hyper-responsive to LPS. Their cytokine profiles however, were different compared to their littermate controls. When challenged with a lethal dose of *Listeria monocytogenes* (LM), ABCF1+/- mice succumbed to infection more quickly than their wild-type littermates. ABCF1+/- mice were, however, equally able to clear an infection with LM expressing ovalbumin (LM-OVA), indicating that the macrophages were capable of trapping and killing bacteria. Taken together these data indicate that macrophages from ABCF1+/- are altered compared to ABCF1+/+ macrophages both in number and phenotype.
1.6 References


Chapter 2: Gene trap disruption of the ABCF1 gene causes lethality at early embryonic stages of development.

2.1 Overview

ABCF1 is an ABC transporter protein that lacks trans-membrane domains. It is thought to function in translational initiation by interacting with eIF2 [1]. ABCF1 has yet to be demonstrated as a protein essential for survival *in vivo*. We identified, through a Blast search of the BayGenomics databases, an ES cell line XK097, which has a single gene trap insertion in the ABCF1 gene. We used this cell line to create a mouse. Heterozygotes were produced and bred to obtain homozygous offspring. No live born ABCF1-/- progeny were ever generated and the lethality was not mouse strain specific. Thus, we have determined that ABCF1 is an essential gene in development. ABCF1-/- mice are embryonic lethal at day 3.5 dpc while ABCF1+/+ mice appear to be developmentally normal. ABCF1+/- are fertile and show no significant differences in their gross anatomy when compared to their wild-type littermates. An ES cell line that appears to be ABCF1-/-, Clone 1, was derived from the mating of two ABCF1+/+ mice. It grows slowly and appears to have abnormal morphology when compared to ABCF1+/+ or ABCF1+/+ ES cell lines.
2.2 Introduction

ABCF1 (also called ABC50) is a member of the ATP binding cassette (ABC) super-family of proteins [2]. ABC transporter proteins are present in all phyla and harness the energy from the hydrolysis of ATP in order to transport substrates across cellular membranes and/or power cellular machinery [3]. All members of the ABC super-family contain conserved Walker motifs along with a signature ABC motif which are responsible for the hydrolysis of ATP [4]. Members of the ABC super family can be grouped into three categories: importers, exporters or the third category which are involved in DNA repair and translation (these transporters lack trans-membrane domains) [3]. ABCF1 falls into this latter category as it lacks trans-membrane domains, along with any known binding partner [2].

ABCF1 was initially identified as a protein that was up-regulated in synoviocytes in the presence of TNFα [2]. Its genetic sequence indicates that it may be homologous to the yeast ABC transporter GCN20 [5]. GCN20, along with GCN1, are thought to coordinate the trafficking of uncharged tRNAs to GCN2 during the process of translation elongation [6]. GCN2, a protein kinase, is activated by binding to uncharged tRNA [5]. Upon activation, GCN2 phosphorylates the eIF2α subunit thus reducing protein synthesis [6]. This process regulates protein synthesis by coupling the availability of amino acids to the control of the eIF2α by GCN2 [5]. The protein sequence of ABCF1 differs from GCN20 at its N-terminal end, having only 20% identity and 30% similarity to GCN20 in this region [1]. The N-terminal sequence of GCN20 is responsible for binding to GCN1 and complementing the function of GCN2, thus indicating that ABCF1 has a function
that is distinct from that of GCN20 [5, 7]. Like GCN20, ABCF1 was found to be important in translation initiation in an \textit{in vitro} siRNA model [1].

ABCF1 was found to associate with eIF2 through 5 different chromatographic procedures and markedly enhance the binding of methionyl tRNA (Met-tRNA) to eIF2 [1]. The N-terminal sequence of ABCF1 was found to be important for its interaction with eIF2 particularly the first 42 residues [8]. When ABCF1 was knocked down in Hela cells through siRNA, both cap-dependent and cap-independent translation of reporter genes was inhibited [5]. Mutations in either of the ATP-binding Walker motifs were found to reduce reporter gene cap-dependent translation in Hela cells, with Walker B mutants showing greater inhibition [5].

\textbf{2.2.1 Gene trapping}

Since most work thus far has focused on the \textit{in vitro} function of ABCF1, we decided to develop an \textit{in vivo} model. The ability to manipulate the mouse genome by specifically modifying genes and producing lineages of animals with these genetic mutations has provided insight into the \textit{in vivo} function of many genes and pathological mechanisms of many diseases. The use of mouse models began in the 1900s when spontaneous mutations were found in mouse colonies [9]. Since spontaneous mutations in mice are relatively rare and non-specific, the ability to insert genetic material into the mouse genome revolutionized scientific research. Genes can be either inserted or deleted into mouse chromosomes. Gene-trapping is one way of deleting pre-existing genes by inserting gene trap vectors into genes in mouse embryonic stem (ES) cells [10]. Gene trap vectors contain slice acceptor sequences along with reporter genes and polyadenylation (pA) sequences (Figure 2.1) [11].
Figure 2.1 Gene trap vector schematic.

The gene trap vector is inserted into an intronic region within the endogenous gene. The β-geo fusion gene is immediately downstream of a splice acceptor site in the vector so that when the gene is transcribed, the endogenous splicing machinery creates a fusion transcript. This fusion transcript then ultimately leads to the production of a fusion protein. SA-splice acceptor site, pA-poly adenylation, β-geo-β-galactosidase/ neomycin fusion gene. This gene trap diagram was adapted from Stanford et al. with permission [11].

Upon activation, the endogenous promoter transcribes the trapped gene and the gene trap vector. Splice acceptor sequences within the gene trap vector mimic an endogenous intron-exon boundary and results in a truncated mRNA fused to the trap vector sequence. Translation of this mRNA results in the production of a truncated protein fused to a reporter gene such as β-galactosidase (β-gal). 5’-RACE (5’ rapid amplification of cDNA ends) and DNA sequencing are used to identify the trapped gene using primer sequence unique to the gene trap vector [12].
2.2.2 Mouse embryogenesis

Mouse embryogenesis is dependent on a complex series of events involving gene regulation and cell signalling. Once the oocyte has been fertilized, the nuclear chromatin decondenses and leads to the formation of the pronuclei [13]. The pronuclei aggregate in the center of the zygote and then undergo the first cell division. During the two-cell stage, maternal control of the cell transitions into zygotic control and the majority of the mRNA that were maternally contributed are degraded to 30% of the maximum [14]. After the third division, the cells have maximized their inner cell contacts and they begin to condense [15]. Once the embryo has approximately 32 cells, a fluid filled cavity (called the blastocele) begins to appear and the embryo becomes a blastocyst [15]. During the formation of the blastocyst, there is an increase in embryonic protein synthesis due to an increase in mRNA stability [13]. Blastocysts are distinguished by the differentiation of the epithelial trophoectoderm cells which are responsible for implantation (and eventually become the placenta) and the pluripotent inner cell mass which develops into the embryo [15]. The blastocyst subsequently loses its zona pellucida (protective coating) in a process called hatching and the trophoectoderm cells are then free to attach and implant into the uterine wall (Figure 2.2) [15].
Figure 2.2. Preimplantation mouse development schematic.
Following fertilization the mouse embryo goes through a series of cell divisions until the formation of the 8 cell embryo. At this stage the embryo begins to compact [16]. Cells differentiation begins to occur resulting in the formation of the trophoectoderm and the inner cell mass [17]. At day 3 the embryo begins to form the fluid filled blastoceol which expands until the late blastocyst stage [16]. Once the blastocyst is formed, the zona pellucida is shed and the blastocyst is free to implant into the uterine wall. dpc-day post coitus. This diagram was adapted from Cockburn et al. [16].
1 dpc
1.5 dpc
2 dpc
2.5 dpc
2.75 dpc
3 dpc
3.25 dpc
3.5 dpc

Zona pellucida

4 dpc
(Late Blastocyst)

Uterus

Implantation

Zona Pellucida

Inner cell mass

Trophoectoderm
Growth factors that are maternally supplied are important for blastocyst development. TNFα is normally present in the female reproductive tract and is thought to regulate apoptosis in the blastocyst [18]. Pre-implantation embryos exposed to TNFα undergo inhibition of cell proliferation and apoptosis in their inner cell masses [19, 20]. It is thought that one of the physiological roles of TNFα in embryogenesis is in the elimination of embryos which are structurally or functionally damaged [21]. During normal development this stimuli is countered by the presence of survival factors which are not necessarily present in the correct quantities in abnormal embryos [21]. This shifts the balance in the abnormal embryos from pro-survival to pro-apoptotic leading to embryo death [21].

Here, we describe a mouse that was generated from the BayGenomics ES cell line, XK097. XK097 has a single gene trap vector inserted in the intronic region 31bp downstream of exon 7. We have determined that no live ABCF1-/- mice could be generated from ABCF1+/− matings and that the embryos of the ABCF1-/- are lethal shortly after implantation.
2.3 Materials and methods

2.3.1 XK097 and mice

The ES cell line XK097 (strain 129/SV), which contains a gene trap vector in the ABCF1 gene, was acquired from BayGenomics (baygenomics.ucsf.edu/). The XK097 ES cells were grown and aggregated for RT-PCR to confirm the presence of the vector sequence. ES cells were washed in PBS to remove media and RNA was extracted using Trizol reagent (Invitrogen, Burlington, ON, Life Technologies, Carlsbad, CA). cDNA was then created using Superscript II (Invitrogen, Burlington, ON) according to the manufacturers conditions. PCR was used to confirm the presence of the vector sequence using primers: forward (Ex7-f) 5’-GGCTCAGGAGTAAAAAGGGAA-3’ and reverse (BaycDNA) 5’-GACAGTATCGGCCTCAGGAAGATCG-3’. The control primers for the wild-type allele were forward Ex7-f and reverse (Ex-8-r) 5’-CCTGCTCAGCCTCCTTTTTTGT-3’. Other wild type allele control primers (F) were F 5’-AAAACAGCAGAAGTGCCGAC-3’ and 5’-ATCGGCTTCTTTAACCAGCA-3’ which represent a 345bp band further 3’ of the gene trap insertion. The conditions for the reaction were 95°C for 5 min followed by 40 cycles of 95°C for 30s, 60°C for 30s and 72°C for 30s followed by a 10 min 72°C elongation step. Genotyping of the XK097 cell line, blastocysts embryos and the ABCF1 knock-out mice was done by PCR using Platnium Taq polymerase (Invitrogen, Burlington, ON). The XK097 ES cells were then injected into C57BL/6 blastocysts in order to create chimeric mice. The injected blastocysts were implanted into the uterine horn of a 2.5 dpc pseudopregnant ICR female mouse (ICR, Harlan Laboratories). Pups were then born three weeks later and chimeras were identified by coat color and males were back-crossed with C57BL/6 females which
were obtained from The Jackson Laboratories (Bar Harbor, ME, USA). Progeny from these mice were then further back-crossed 12 generations onto the inbred C57BL/6 line, the inbred Balb/C line and the out-crossed ICR line. Mice were housed in the Biomedical Research Centres mouse facility (University of British Columbia, Vancouver, BC, Canada) and maintained according to the protocols and procedures of the Canadian Council on Animal Care. Animals were fed a standard diet and were routinely screened for pathogens.

2.3.2 Collection of mouse blastocysts

To isolate blastocysts, female ABCF1+/- mice were first superovulated (given an inter-peritoneal (ip) injection of 5IU Pregnant Mare Serum (PMS) followed 46 hours later with 5 IU Human Chorionic Gonadotropin (HcG) ip) and then mated with ABCF1+/- studs. Females were sacrificed using CO₂ asphyxiation 3.5 dpc and their uteri were dissected out and flushed with M2 media (Sigma, St. Louis, MO) into 6 mm plates (Falcon-Becton Dickinson, Oakville, ON).

2.3.3 Genomic DNA isolation

For genomic DNA isolation from blastocysts, the blastocysts were washed several times in M2 media and then transferred to PBS for one wash before placing them individually in Eppendorf tubes in 10 µL blastocyst lysis buffer solution (50 mM Tris-HCL (pH 8.0), 0.5% Triton X-100 and 200 µg proteinase K (Fermentas, Burlington, ON)) [22]. Blastocysts were then freeze thawed and incubated for 40 min at 55°C.
Proteinase K was inactivated by a 10 min incubation at 95°C. 1 µL of template was used per reaction.

For genomic DNA isolation from foetuses, female ABCF1 +/- were mated with ABCF1 +/- males. At various times of gestation, the females were sacrificed with CO₂ asphyxiation and their uteri were removed. Embryos were separated from their yolk sacs and genomic DNA was extracted from the yolk sac using the blastocyst lysis buffer and protocol described above.

For DNA isolation from adult mice, ear clips were placed in 20 µL of lysis buffer (50 mM Tris HCl (pH 8.0), 2 mM NaCl, 10 mM EDTA, 1% SDS and 1 mg/mL proteinase K (Fermentas)) and incubated for 60 min at 55°C. 300 µL of milliQ water was added to each sample which was then incubated for 10 min at 95°C to inactivate the proteinase K. 700 µL of milliQ water was added to each sample and 4 µL of this template was used in subsequent reactions.

2.3.4 Genotyping

The genotypes were determined by using two different PCRs. The first (1250 bp) which spanned the insertion site included primer int1-f which amplified intronic sequence 5’ of exon 7 (5’-CAGCCCGCACCTCTC-3’) and Bay cDNA which recognized sequence within the β-galactosidase reporter gene. The second PCR (1304 bp) which amplified the wild-type allele included primers for exon 3 (Ex3-f) 5’-AAGAGAGGAAATGGGGCAGT-3’ and exon 8 (Ex8-r). The conditions for both reactions were 95°C for 5 min followed by 40 cycles of 95°C for 30s, 55°C for 30s and
72°C for 1 min 30s followed by a 10 min 72°C elongation step. DNA from the reaction was separated and visualised by agarose gel electrophoresis (1% gel) and Sybr-Safe staining (Invitrogen, Burlington, ON).

2.3.5 ES cell line derivation

Blastocysts were recovered from ABCF1+/− knock-out females 3.5 dpc. Individual blastocysts were plated on a feeder layer of inactivated mouse embryonic fibroblasts (MEFs) in a 96 well plate. MEFs were originally obtained from Millipore (Billerica, MA) and expanded two passages prior to inactivation by 4,000 rads of gamma-irradiation. Blastocysts were cultured for 6 days on MEFs in Dulbecco’s modified eagle medium (DMEM) supplemented with 15% Fetal Calf Serum (FCS), 100 µM nonessential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin/streptomycin, 100 µM 2-(β)-Mercaptoethanol and 2000 IU/mL LIF. Inner cell mass outgrowths were disaggregated using trypsin and grown further on new MEFs changing the media daily. Cells exhibiting typical ES cell-like morphology (for example, with a well defined inner cell mass and blastocoel) were designated as ES cell lines and passaged every 2 days (or 4 days in the case of clone 1). ES cell-like cells were then genotyped as described above. To prepare ES cells for FACs, a 10 cm plate of ES cells was first trypsinised (using 3 mL of 0.25% trypsin (Invitrogen, Burlington, ON)), washed (once in ES cell media, described above, to inactivate trypsin) and resuspended in 5 mL of PBS. ES cells and were sorted from MEFs on the FACSVantage cell sorter based on their forward scatter (cell size) and side scatter profiles (cell complexity). ES cells were identified as the small compact cell population and the MEFs were the larger more
complex cells. Sorted cells were then genotyped (using the protocol above) and also subject to RT-PCR, as described above for the ES cell line. ES cell-like colonies were also tested for pluripotency by testing for Oct-4 expression by RT-PCR [23].
2.4 Results

2.4.1 The ES cell line XK097 from BayGenomics contains a gene trap vector in the ABCF1 gene.

The XK097 cell line was identified during a Blast search of the BayGenomics web site (http://baygenomics.ucsf.edu) using the ABCF1 gene (National Center for Biotechnology Information accession number NM_013854.1) as a template. BayGenomics randomly inserts one copy of the pGT0Lxf vector into the 129/SV mouse genome. pGT0Lxf, the gene trap vector, is designed to create a fusion protein between the 5’exon of the gene (where it is inserted) and its β-geo cassette (a fusion protein containing β-galactosidase and neomycin phosphotransferase II) by way of a splice acceptor sequence. ABCF1 is a gene encoded on chromosome 17 (the MHC locus) and contains 24 exons. The gene trap in XK097 was inserted into the intronic sequence between exon 7 and exon 8 thus creating a fusion protein containing exons 1-7-β-geo (Figure 2.3). To determine the position of insertion, BayGenomics used a 5’ RACE from the ES cell line mRNA to isolate the sequence downstream of the insertion which was later sequenced to determine its genomic location (Figure 2.4). RT-PCR was used on the XK097 cell line to confirm that the insertion was between exon 7 and exon 8 (Figure 2.5). The ATP binding cassettes begin translation at exon 10, so the fusion protein is most likely non-functional (in terms of its ability to hydrolyse ATP). We then used this cell line to produce a chimeric male mouse which was used to create ABCF1+/- knock-out mice (Figure 2.6). These mice were then backcrossed 12 generations to C57BL/6 females. The result was an ABCF1+/- mouse on the C57BL/6 line.
Figure 2.3. The ES cell line XK097 contains a gene trap insertion in the ABCF1 gene.
The genomic and mRNA location of the pGT0Lxf vector (gene trap) on chromosome 17
corresponding to the ABCF1 locus in the XK097 ES cell line. The locations and names
of the primers used for screening are in round brackets. The size of the PCR product is
indicated in square brackets.
Figure 2.4. XK097 contains the pGT0Lxf vector between exon 7 and 8 in the *ABCF1* gene.
Genomic DNA analysis of the XK097 cell line (left panel) compared to a wild type ES cell line (right panel). IB (Int1-f and BaycDNA (1250bp)) primer combination indicates the presence of the gene trap cassette in the ABCF1 locus. - is the water control and + is the positive control (Rps15 (350bp)) for the PCR.

Figure 2.5. XK097 produces a transcript containing exon 7 and the Baygenomics vector sequence.
RT-PCR analysis of the XK097 cell line (left) compared to a wild type ES cell line (right). 7-(Ex7-f-Bay cDNA primers), F- (downstream wild type control primers), β-β-actin primers.
Figure 2.6. The XK097 ES cell was used to create chimeric mice. XK097 ES cells were used to produce two chimeric mice as indicated by the brown coat color (red arrow). One male was capable of germ line transmission of the gene trap cassette.
BayGenomics sequencing indicated that the gene trap insertion was between exon 7 and exon 8 in the ABCF1 gene. The NCBI web site was then used to determine the genomic sequence of the ABCF1 gene on chromosome 17 and map the exact location of the insertion. We then used PCR to genotype the mice using a variety of primer sets both upstream and downstream of the insertion. Since the intronic sequences in this region of the genome are small, primers in the exons as well as in the introns could be used to map the insertion. The primer set that produced the most consistent positive results (Int1-f and BaycDNA) was then used to generate a fragment which was further cloned and sequenced. This 1.25 kb sequence confirmed the gene trap vector had inserted into the intronic sequence 31 bp downstream of exon 7. To differentiate the trapped from the non trapped genomic sequence, another set of primers (Ex3-f and BaycDNA) was generated to produce a 1.3 kb product which spanned the insertion site.

### 2.4.2 ABCF1 homozygous knock-out mice produced from the XKO97 cell line are embryonic lethal.

Mice positive for one copy of the gene trap cassette (ABCF1 +/-) were mated to produce homozygous knock-out mice. Ear clips were taken from 227 progeny and genotyped by PCR. None of the pups were positive for the gene trap cassette alone (Figure 2.7 and Table 2.1). ABCF1+/− males were also back-crossed several generations onto the inbred Balb/C strain and out-crossed ICR mouse lines. After several generations of back-crossing, the heterozygous pups were inter-crossed. No adult ABCF1−/− pups were identified on any background strain, indicating that the lethality associated with the lack of ABCF1 was not strain specific.
Figure 2.7. None of the progeny from intercrossed ABCF1+/- mice were ABCF1-/-.
ABCF1+/- intercrosses only ever resulted in the production of heterozygous knock-out (ht) mice or wild type (wt) mice. A representative agarose gel shows the PCR results that were used to distinguish between the ABCF1 +/- (1250 bp band) and ABCF1+/+ (356 bp band) mice.

Table 2.1. Genotypes of the offspring from ABCF1+/- intercrosses show that approximately 25% of blastocysts are ABCF1-/-.
Mice were either genotyped as 3 week old pups or embryos from pregnant females were excised at different dpc. Resorbed embryos were identified by the presence of uteri sacs containing no embryos. Genotyping was done by PCR. E-embryo.
The ratio of wild-type to heterozygous mice was 1:2 which is the expected ratio if the gene trap is embryonic lethal. Female ABCF1 +/- mice were then timed mated to ABCF1 +/- studs and dissected at 5.5-13.5 dpc. Embryos could not be recovered in approximately 1 out of 4 placentae which indicated that they had been resorbed. PCR genotyping also confirmed that none of the embryos were homozygous knockouts and the ratio of wild-type to heterozygous embryos was 1:2. Female ABCF1 +/- mice were superovulated then mated and sacrificed 3.5 dpc. Uteri were dissected out and flushed. Individual blastocysts were isolated and genotyped by PCR (Figure 2.8). ABCF1-/- blastocysts were identified at an expected 25% ratio. Since empty placentae were present, embryos were likely non-viable shortly after implantation.
Figure 2.8. ABCF1+/− mice are capable of producing homozygous knock out blastocysts. Individual blastocysts were genotyped by PCR (knockout allele (1250 bp) and wild type allele 1302 bp), representative homozygous knock out (−/−), heterozygous (+/−) and wild-type (+/+ ) blastocysts are indicated.
2.4.3 Clone 1, an ABCF1-/- knock-out ES cell line derived from blastocysts, is viable.

ABCF1+/- knock-out females were super-ovulated and mated to ABCF1+/- male mice. Uteri were dissected out and flushed to remove blastocysts. The blastocysts were then grown on feeder MEFs for six days. Inner cell mass outgrowths were disaggregated and further grown on new feeder cells. Cells that continued to grow in colonies were designated ES cells. Three ES cell lines resulted from 30 blastocysts. Individual colonies were then genotyped to determine whether any of the lines produced were ABCF1-/-.

None of the ES cell lines originally tested as ABCF1-/-.

For clone 1, the wild-type allele band was very faint suggesting that there may be some MEF cell contamination (MEFs were derived from a wild-type mouse) contributing to the weak signal. To reduce the MEF contamination, ES cells were sorted using a Fluorescence Activated Cell Sorter (FACs) as the ES cells are distinct in size and shape as compared to the fibroblasts. Individual cells were then genotyped using standard blastocyst genotyping or RT-PCR (this was also to ensure that the cell lines expressed Oct-4 indicating that they are still pluripotent). Clone 1 was shown to be homozygous knock-out by RT-PCR (Figure 2.9).

The fibroblast sample did show Oct-4 staining but these cells were not separated from the ES cells doublets in the sort. The fibroblasts were ABCF1+/+ so they provided a good control to show that there was no contamination of the wild-type allele cDNA in the clone 1 RT-PCR.
Blastocysts from time mated, intercrossed ABCF1+/- mice were used to produce an ABCF1-/- blastocyst line. ES cells were FACS sorted (first two FACs plots). The left plot indicates the two populations of cells that were isolated, the P2 population is the ES cell population (clone 1) and P4 is the MEF population (that does contain doublets and clustered ES cells). The plot on the right shows the purity of the sorted P2 population with virtually no fibroblast contamination. RT-PCR was then used to genotype this cell line using primers for Oct4 (to test for pluripotency) (310 bp), β-actin (as a control housekeeping gene) (400 bp) and primers for the knock out (631 bp) and wild type alleles (345 bp). C1 is clone 1, E is a wild-type ES cell, F is the fibroblast feeder cells and – is the water control.
2.5 Discussion

The mouse line created using the BayGenomics cell line XK097 has a gene trap cassette inserted in the intronic region between exon7 and exon 8 in the ABCF1 gene disrupting its ATP binding activity. Embryos homozygous for the gene trap cassette die shortly after implantation indicating that the loss of the ABCF1 protein is lethal in mice at a very early stage of development. Heterozygous mice however, appear to develop normally. These results are consistent with mutational studies done in Caenorhaditis elegans where ABCF1-/- mutants die as L1 larvae and ABCF1+/- mutants develop normally (unpublished data, Moerman lab, University of British Columbia, B.C.). The ES cell line Clone 1, a homozygous knockout ES cell line, was derived from blastocysts isolated from ABCF1+/- intercrossed females. Clone 1 appears to grows slowly and appears to have abnormal morphology (poorly defined colony boundaries) when compared to the wild-type or heterozygous ES cell lines. This observation could be due to reasons other than the lack of ABCF1 expression such as a chromosomal deletion or disruption in another gene which is important for growth. The fact that ABCF1-/- ES cells are viable indicates that an intact ABCF1 protein is not essential for survival. Since the gene-trap cassette creates a fusion protein with exons 1-7 of the ABCF1 protein it is impossible to say whether the N-terminal region contains a domain that is essential to survival. However, the ABC cassettes which are further down-stream of the gene-trap cassette are not expressed in clone 1 and are not responsible for the viability of the ES cell.

Previous studies show that ABCF1 interacts with the eIF2 protein which is a key member of the translation initiation machinery [5]. ABCF1 is thought to bind to eIF2
through residues located in the N-terminal region and enhance the binding of eIF2 to Met-tRNA [1]. RNA interference experiments in Hela cells showed that a reduction in ABCF1 protein correlated with a reduction in general protein synthesis as assayed through translation of reporter genes [5]. Interestingly, in the RNAi experiments, the Western blot data shown still contained a detectable ABCF1 signal [5]. Since mRNA translation is a fundamental process of life, any interference in this pathway should not be well tolerated in cells. This is also shown by the inability to produce ABCF1-/- mice. Provocatively, ABCF1-/- blastocysts can be produced and survive until implantation. This may be explained by the presence of long lived maternal ABCF1 which is present up to the post-implantation stage of development similar to the Argonaute 2 gene which is a member of the RNA-induced silencing complex (RISC) complex [24]. Maternally provided Argonaute 2 mRNA is known to be present in embryos at post-implantation stages of development [24]. Another explanation is that the N-terminal ABCF1-eIF2 association is sufficient for early embryo translation while the nucleotide binding domains have another distinct function, possibly in the control of translation. Since the XK097 gene trap is located between exon 7 and 8 of the ABCF1 gene in our knockout mice, the N-terminal 186 AA should be still expressed.

Mutations in either of the Walker boxes lead to slight inhibition of cap-dependent translation while they enhance the cap independent translation [5]. This would suggest that a functional ABCF1 is important in reducing the IRES cap-independent translation. GCN20 (an ABC protein thought initially to be a yeast homologue) along with GCN1 and GCN2 also function in the control of protein synthesis [1]. They phosphorylate the eIF2α thus preventing the GEF eIF-2B in times of amino acid starvation [1]. PKR (dsRNA protein kinase), HRI (Haem-regulated inhibitor) and PEK (pancreatic eIF2α
kinase) also control translation in times of stress through the phosphorylation of the eIF2α [25]. It is possible that ABCF1 also controls eIF2 through the phosphorylation of one of its residues. Interestingly, knockout mice are available for each of these proteins (PKR, HRI and PEK), which indicates that these genes are not required for survival and at least part of the ABCF1 protein has a distinct function that is essential to survival. Also, in the yeast *Saccharomyces cerevisiae*, GCN20 mutants are viable indicating that the lethality of ABCF1-/- is probably unique to multi-cellular organisms [7].
2.6 References


Chapter 3: The characterization of the ABCF1 promoter activity in tissues and the analysis of the ABCF1-deficient CD8$^+$ T cells.

3.1 Overview

ABCF1 is a member of the ABC transporter family. ABCF1 is thought to interact with eIF2 and plays an important role in transcription initiation. Several protein and RNA studies have shown that ABCF1 is expressed in various levels in all tissues however there has been no data on the ABCF1 promoter activity. In this study, we utilize a gene trapped mouse model which allows the endogenous ABCF1 promoter to drive the expression of a β-geo (β-galactosidase and neomycin fusion protein) reporter gene. This allows us to simultaneously examine the ABCF1 promoter activity in all tissues through sectioning and staining. Very high promoter activity was detected in the blastocysts and embryos, particularly in the differentiating tissues. The ABCF1 promoter was found to be active in all tissues in adult mice, but had varied promoter activity levels in different cell types within the tissues. In CD8$^+$ T cells, the ABCF1 promoter was turned on as the T cells matured. Functionally, the mature ABCF1+/- CD8$^+$ T cells appeared to produce less IFN$\gamma$, however they showed no difference in their ability to kill targets in a cytotoxic T lymphocyte (CTL) assay. These data confirm that ABCF1 is important for development and suggest that it may have a role in the regulation of cytokines, but does not function in the CTL-mediated killing of target cells.

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3.2 Introduction

Gene trapping is a high-throughput tool that can be used to generate knock-out mice [1]. Genes are trapped by the random insertion of a gene trap vector into an intronic sequence [2]. The gene trap vectors contain splice acceptor sites along with reporter genes, selectable markers and polyadenylation sequences. The endogenous promoter drives the expression of the gene trap vector which produces a fusion transcript [2]. The fusion transcript then results in a truncated or mutant cellular protein [2]. The β-galactosidase (β-gal) gene from *Escherichia coli* is a common reporter gene that is used in gene trapping [3]. β-gal expression is quickly and easily detected *in situ* by staining cells or tissues with bromo-chloro-indolyl-galactopyranoside (X-Gal) or by FACS analysis with fluorescein di-β-D galactopyranoside (FDG) staining [3]. X-Gal and FDG are both substrates for β-gal. X-Gal is cleaved into galactose and 5-bromo-4-chloro-3-hydroxyindole which is then oxidized into the blue insoluble 5,5'-dibromo-4,4'-dichloro-indigo product. FDG, which is colorless, is cleaved by β-gal resulting in the production of fluorescein which has an excitation wavelength of 490 nm and an emission of 513 nm [4]. Both reagents are useful in detecting β-gal expression which has previously been shown to be proportional to the endogenous promoter activity of the trapped gene if the fusion proteins are expressed [3].

ABCF1, like other members of the ATP binding cassette (ABC) super-family of transporters, possesses two nucleotide binding domains (NBD) which bind and hydrolyze ATP. The NBD contain conserved features including the ABC signature motif and the Walker motifs (A and B) [5]. Unlike other members of the ABC superfamily, ABCF class transporters have no known trans-membrane domains [6]. ABCF class transporters are highly conserved evolutionarily and are all thought to be involved in the regulation of translation initiation [7]. ABCF1 has been show to associate with eIF2 and is thought to be important in the transfer of
methionyl-tRNA (Met-tRNA) to the 40S ribosome [8]. The yeast homolog of ABCF1, GCN20, is known to be important for the regulation of eIF2 [9].

Until now, the only thing known about the ABCF1 promoter and its regulation is that it may be regulated by TNFα [10]. To examine the ABCF1 promoter in a physiological relevant system, we generated an ABCF1 deficient mouse containing a gene-trap cassette in the ABCF1 gene (Chapter 2). The gene-trap cassette insertion results in the production of a truncated fusion protein containing the N-terminal region of the ABCF1 gene fused to β-geo. This gene-trap insertion allows the expression of β-geo to be under the control of the endogenous ABCF1 promoter. Since β-geo expression can be visualized through staining, this system allows us to examine the physiological ABCF1 promoter activity in different tissues at different stages of development.
3.3 Materials and methods

3.3.1 Gene trapping and genotyping

The XK097 embryonic stem cell line (from Bay Genomics, San Francisco) contains the pGT0Lxf gene trap vector inserted into the ABCF1 gene between exon 7 and exon 8. The insertion of the pGT0Lxf vector results in a $\beta$-geo fusion cDNA which is under the control of the endogenous ABCF1 promoter. Vector insertion was verified by PCR and RT-PCR according to the protocol given by the Sanger Institute Gene Trap Resource (http://www.sanger.ac.uk/PostGenomics/genetrap/protocols.shtml). The XK097 ES cells were then used to create germline chimeras, which were further mated 12 generations onto a C57Bl/6 background (Chapter 2).

Offspring were genotyped using primer sets: for the wild-type allele (amplifying a 1304bp fragment) Ex3-f (5’-AAGAGAGGAATGAGGCGAGT-3’) and Ex8-r (5’-CCTGCTCAGCCTCTTTTGT-3’), for the gene trapped allele (amplifying a 1250bp fragment), Int1-f (5’-CAGCCCGGCACCTCTC-3’) and Bay cDNA (5’-GACAGTATCGGCCTCAGGAAGATCG-3’) (Chapter 2).

3.3.2 X-Gal staining and histology

In order to assess the $\beta$-geo activity in mouse embryos and whole organs, tissues were excised and fixed in X-Gal fixation buffer (0.1 M phosphate buffer, pH 7.3 (3.74g NaH$_2$PO$_4$;H$_2$O FW=137.99; 10.35 g Na$_2$HPO$_4$, FW=141.96; in 1 L H$_2$O), 5 mM EGTA, 2 mM MgCl$_2$ and 0.2%
glutaraldehyde) for 15-60 min depending on the size of the tissue. They were then washed three times for 5 min with X-Gal wash buffer (0.1M phosphate buffer, pH 7.3, 2 mM MgCl₂) and stained over night at 37°C in X-gal staining buffer (0.1 M phosphate buffer, pH 7.3, 5 mM K₄Fe(CN)₆·3H₂O, 5 mM K₃Fe(CN)₆, 2 mM MgCl₂, 1 mg/mL X-Gal). Stained tissues were then washed three times with X-Gal wash buffer.

Sectioned embryos and tissues were fixed, stained and washed using the same protocol. Sections were taken through entire embryos or tissues from multiple mice. After the final wash step, tissues were cryoprotected by incubation overnight at 4°C in a 20% sucrose solution. After incubation, tissues were immersed in Tissue-Tek O.C.T compound (VWR Scientific Bridgeport, NJ) and snap frozen, then stored at -80°C until sectioning. Sections of tissues 10-16 µm were cut onto Superfrost Plus Micro slides (VWR Scientific Bridgeport, NJ) and slides were then washed by carefully dipping them several times in X-gal wash buffer. Sections were mounted in Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL). For larger tissue sections (from organs) stained with X-gal alone, organs were immersed in Tissue-Tek O.C.T then snap frozen and stored at -80°C. Tissues were sectioned and mounted onto Superfrost Plus Micro slides then fixed for 15 min, washed, stained over night at 37°C and mounted as described above. For tissues that were also counter-stained with nuclear fast red (Sigma, MO); after the overnight incubation in X-gal staining buffer, the sections were incubated in nuclear fast red for 5 min. The slides were dehydrated (2 x 5 min in 90% ethanol, 2 x 5 min in 95% ethanol and 2 x 5 min in 100% ethanol), cleared with Xylene (Fisher, ON), then mounted with Permount (Fisher, ON). In order to ensure the specificity of β-geo activity, wild-type littermate controls were stained in tandem. Slides were sent to a veterinary pathologist for independent analysis (Drs. S. Newbigging and H. Adissu, Toronto Centre for Phenogenoics).
To isolate blastocysts, females were first superovulated to maximize the number of blastocysts recovered. Females were given an inter-peritoneal (ip) injection of 5IU PMS followed 46 hours later with 5IU HcG ip and then mated with males. Females were sacrificed by CO₂ asphyxiation 3.5 days post coitum (dpc) and their uteri were dissected out and flushed with M2 media. Individual blastocysts were isolated and washed several times in M2 media, then placed into fixation buffer for 2 min. The blastsocysts were then washed and placed in X-gal staining buffer. The blastocyst staining was apparent after 1 h and the cells were washed once with PBS and analysed.

3.3.3 ABCF1 Northern blot analysis.

An ABCF1 fragment was made through PCR amplification of a spleen cDNA library (made in the lab) using primers: forward 5’- AAAACAGCAGAAGTGCCGAC-3’ and reverse 5’ TGCTGGTTAAAGAAGCCGAT-3’. The conditions for the reaction were 95°C for 5 min followed by 40 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s followed by a 10 min 72°C elongation step. This fragment was cloned into the TOPO TA cloning vector (Invitrogen, Burlington, ON) and sequenced to identify the orientation. The ABCF1 fragment was then used to create a ³²P-labeled RNA probe using the Strip-EZ DNA Kit according to the manufacturer's instructions (Ambion, Huntington, UK). The FirstChoice Northern Blot Mouse Blot I (also from Ambion) was used with the Ambion wash solutions and Ultrahyb hybridization solution according to the manufacturer's recommendations.
3.3.4 β-geo expression analyzed by FACS

Spleens and thymi were removed from three ABCF1 +/- mice and three of their wild-type littermate controls and were mashed through a 40 µm cell strainer (Becton Dickinson, Oakville, ON) to produce a single cell suspension. Red blood cells were then lysed by incubating the splenocytes in 2 mLs of Mouse Red Cell Removal Buffer (MRCRB) (0.15 M NH₄Cl, 1 mM KHCO₃ pH 7.3) for 5 min at room temperature. The MRCRB lysis buffer was inactivated by the addition of 8 mL of complete RPMI-1640 (Invitrogen, Burlington, ON, Burlington, ON) supplemented with 10% heat inactivated fetal bovine serum (HyClone, Logan, UT, USA), 1 mM Na pyruvate, penicillin (100 IU/mL), streptomycin (100 µg/mL), 20 mM HEPES and 2 mM L-glutamine. Splenocytes and thymocytes were spun down then splenocytes were resuspended in 15 mL complete RPMI and thymocytes were resuspended in 10 mL of complete RPMI. 100 µL of the cell suspension was plated in wells of a 96 U-bottom plate (Falcon, Becton Dickinson, Oakville, ON) in duplicate. Cells were centrifuged at 200g for 5 min and the supernatant was removed. The cells were then resuspended in 100 µl of FACS buffer (PBS containing 2% FBS) containing a 1:100 dilution of PE-anti-CD8 antibody (Pharmingen, Oakville, ON) and incubated on ice for 20 min. After 20 min, the cells were washed 3 times in FACS buffer and resuspended in FACS buffer containing 1 mM FDG. The cells were incubated for 2 min at 37°C (heat shocked) then diluted 10-fold in cold PBS and incubated on ice for 1 h. A Becton-Dickinson FACSscan was used to analyze the cells using Cell quest software (Becton Dickinson, Oakville, ON). Results were analyzed using FlowJo software (Tree Star, Inc, Ashland, Or).
3.3.5 CD8⁺ T-cell CTL assay and immunostaining

The vesicular stomatitis virus (VSV), Indiana Strain, was given as a gift from Frank Tufaro (University of British Columbia, Vancouver, Canada). VSV was cultured on Vero cells (ATCC) and the titre was determined by plaque assays and reported as Tissue Culture Infectious Dose affecting 50% of the culture (TCID)₅₀ [11]. The CTLs were generated by inoculating ABCF1+/− mice and their littermate controls with 1x10⁵ TCID₅₀ of VSV. Their spleens were removed seven days later and mashed through a 40 µm cell strainer produce a single cell suspension. Splenocytes were then washed two times with PBS and incubated for 5 days at 37°C in 25 mL CTL medium (RPMI-1640 containing 20 mM HEPES, 1 mM Na-pyruvate, 0.1 mM non-essential amino acids, 100 IU/mL penicillin, 100 µg/mL streptomycin and 10% heat-inactivated FBS (Hyclone)) per spleen (approximately 10⁶ cells/mL) with 1 µM of VSV-NP52-59 peptide (RGYVYQGL) [12]. On day 5, the CTLs were harvested, washed three times, counted, resuspended (using the initial undiluted concentration of 5 x 10⁶ cells/mL) and mixed with RMA-S target cells at the indicated ratios. RMA-S is a thymoma cell line that presents the VSV-NP₅₂-₅₉ peptide in the context of H-2Kb [13]. RMA-S were counted and resuspended at 2 x 10⁶ cells/mL in CTL media for each sample. The RMA-S were then either pulsed by the addition of 1 µL with VSV-NP₅₂-₅₉ peptide or left unpulsed as a negative control. 10 µL (100 µCi) of ⁵¹Cr (sodium chromate, Amersham Biosciences) was added to each sample and incubated at 37°C for 1 hr. The cells were then washed three times in PBS, counted and mixed with the effector CTLs at the indicated ratios for 4 hr at 37°C. For maximal and minimal counts, RMA-S cells were either lysed with 5% Triton X-100 or incubated in media lacking the effector cells. Following the incubation, the cells were pelleted and 100 µL of supernatant was removed. The released ⁵¹Cr was then quantified in a gamma counter (LKB Instruments, Gaithersburg, MD). The specific
killing was quantified using the formula: \( \frac{((\text{experimental} - \text{minimum control}) \div (\text{maximum} - \text{minimum control})) \times 100\%} {\text{}} \).

100 µL of splenocytes from the expanded culture were plated per well into a 96 well U bottom plate and washed three times with FACS buffer. The cells were then stained for their expression of CD8, CD4, IFN-\( \gamma \) and H-2K\(^b\)-NP specific T cell receptors (using Phycoerythrin (PE) labelled-H-2K\(^b\)-NP tetramers ((immunomics-BeckmanCoulerTM)). For Fluorescein isothiocyanate (FITC)-\( \alpha \)CD8 (Pharmingen, Oakville, ON) and PE-\( \alpha \)CD4 (Pharmingen, Oakville, ON) staining, a 1:100 ratio of antibody to FACS buffer was used. For PE labelled-H-2K\(^b\)-NP tetramer staining, a 1:10 ratio of tetramer to FACS buffer was used. Cells were incubated with antibodies at 4\(^\circ\)C for 20 min on ice. For IFN-\( \gamma \) staining, the original 100 µL of expanded splenocytes were initially incubated in the presence of GolgiStop (Pharmingen, Oakville, ON), and then washed twice with FACS buffer. The cells were then fixed by incubating them in fix buffer (4% paraformaldehyde) for 20 min on ice. Once fixed, the cells were stained as described above with FITC-\( \alpha \)CD8. Cells were then permeabilized using a permeabilization buffer containing 0.1% saponin, 1% fetal calf serum, 0.1% sodium azide in PBS. PE-\( \alpha \)IFN-\( \gamma \) antibodies (Pharmingen, Oakville, ON) were diluted 1:100 in FACS buffer, added to the cells in permeabilization buffer and incubated at room temperature for 20 min. Finally, the cells were washed once in permeabilization buffer and resuspended in fix buffer. All of the stained cells were analyzed on the FACSscan (described above).
3.4 Results

3.4.1 Mice derived from XK097 contain a β-geo cassette that is under the control of the ABCF1 promoter.

ABCF1 deficient mice were derived from the BayGenomics XK097 ES cell line containing a gene trap vector, pGT0Lxf, inserted between exon 7 and exon 8 in the ABCF1 gene. The gene trap vector pGT0Lxf is convenient in that it contains a splice acceptor sequence and a β-geo cassette (http://www.sanger.ac.uk/PostGenomics/genetrap/vectors/). These features lead to the production of an ABCF1-β-geo fusion protein and thus put the expression of β-geo under the control of the endogenous ABCF1 promoter. The presence of the gene trap insert prevents the expression of the ABC cassettes which are located further downstream of exon 8 (Figure 3.1). X-gal staining was then used to examine the promoter activity in various tissues and cell types that express the gene trap cassette. Since no counterstaining was used to identify specific structures, the staining was evaluated based on morphology.
Figure 3.1. The insertion of the gene trap cassette results in a truncated ABCF1 transcript and protein fused to β-geo.

The location of the gene trap cassette is shown in reference to the wild-type ABCF1 protein. The gene trap vector contains a β-geo cassette which puts the expression of β-geo under the control of the endogenous ABCF1 promoter. The gene trap vector contains a polyA signal therefore eliminating the expression of the ABC cassettes of the ABCF1 gene. Both ABC cassettes are located downstream of the gene trap vector and are thus not transcribed when the gene trap insertion is present. The N-terminal 186 amino acids are still present in the gene-trapped protein which includes the residues that interact with the eIF2. Ex-exon.
3.4.2 The ABCF1 promoter is active during embryogenesis.

To examine the ABCF1 promoter activity in blastocysts, blastocysts were isolate 3.5 dpc and stained with X-gal to visualize the β-geo expression. Since the ABCF1 promoter drives the β-geo expression, blue staining is indicative of ABCF1 promoter activity. Given that ABCF1-/- embryos are embryonic lethal shortly after implantation (Chapter 2), two copies of the gene-trap were only expressed in ABC-/- blastocysts. Out of 15 blasts isolated, 2 were more darkly stained organized than the others indicating that they may be homozygous knockouts. Staining was present throughout the blastocysts with dark staining in the inner cell mass of both the ABCF1-/- and ABCF1+/- blastocysts and diffuse staining in the blastocoel (Figure. 3.2).
Figure 3.2. The ABCF1 promoter is active in blastocysts.
Blastocysts were recovered from intercrossed timed mated ABCF1+/- females and stained with X-gal. Three phenotypically different types of blastocysts were observed. The ABCF1-/- blastocysts were darkly stained, the ABCF1+/- blastocyst appeared intermediately stained and the ABCF1+/+ blastocysts lacked staining. Images were taken using a 20x lens. The bottom two images show the genotyping results of the blastocysts above (only the heterozygous and homozygous genotyping is shown). The top image shows the presence of the knock-out allele in both blastocysts. The bottom image shows the presence of the wild type allele only in the heterozygous blastocyst. The illustration has been adapted from Nishikawa et al. with permission[14].
To investigate the ABCF1 promoter activity in embryos, male ABCF1+/- mice were timed mated to ABCF1+/- females and their embryos were removed at various dpc. Whole embryos were stained and showed dark diffuse staining throughout, indicating the ABCF1 promoter is highly active in developing embryos (Figure 3.3). This result was not surprising as ABCF1 is essential in embryogenesis (Chapter 2).
Figure 3.3. The ABCF1 promoter is highly active in whole embryos.
Whole embryos were removed from intercrossed timed mated ABCF1+/- females at various dpc and stained with X-gal. The embryos appeared to be uniformly darkly stained indicating high ABCF1 promoter activity during embryogenesis. Images were taken using 8x-magnification (top panel) and 3.5x magnification (bottom panel).
Cryosections of 11.5 dpc embryos confirmed that the ABCF1 promoter is also highly active in all tissues of the developing embryo (Figure 3.4). Very high activity was detected in the cranial nerves, optic cup, the liver, the heart, neural epithelium and the dorsal root ganglion (Figure 3.5 and 3.6). The skin was darkly stained as well. All of these tissues at this stage of development are known to be actively proliferating and differentiating [15].
Figure 3.4. ABCF1 promoter activity was elevated in stained and sectioned 11.5 dpc ABCF1+/- embryos.

11.5 dpc embryos were stained, snap frozen and sectioned. The ABCF1 promoter appears to be highly active throughout the embryo particularly in the regions which correspond to differentiating and proliferating tissues for this particular stage of development. There was intense staining in the developing eye, liver, cranial nerves and dorsal root ganglia. Images were taken using 5x magnification.
Figure 3.5. Higher magnification of the region corresponding to the developing spine in the 11.5 dpc ABCF1+/- embryos show differential staining within the tissue. Within the tissues, ABCF1 promoter activity was not found to be evenly distributed. Rather, certain cell types appeared to have higher activity than others. In the dorsal root ganglia of the 11.5 dpc embryos, the promoter activity appears quite strong at the outer boundary. Dorsal root ganglia are known to be differentiating at this stage of development and do so in a cranial-caudal fashion [15, 16].
Dorsal root ganglia
Figure 3.6. Higher magnification of the region corresponding to the developing eye in the 11.5 dpc ABCF1+/- embryos shows elevated promoter activity in the retina. Within the eyes of the ABCF1+/- embryos, the ABCF1 promoter was found to be active in the region of the retina (proximal to the developing pigment granules) [15].
Pigment cells of the retina
3.4.3 The ABCF1 promoter is active in adult tissues

Initially to determine RNA expression of ABCF1, Northern blot analysis was performed on adult mouse tissues (Figure 3.7). Northern blot showed that ABCF1 mRNA is expressed in all tissues particularly the spleen, thymus, testis and ovaries. This data was confirmed by RT-PCR (data not shown) which also showed differential expression of mRNA expression in different cell types within a tissue.

Figure 3.7. The Northern Blot indicates that ABCF1 mRNA is present in all tissues particularly the spleen, thymus, testis and ovary.
A $^{32}$P-labelled ABCF1-specific probe hybridized to the Northern blot containing mouse cDNA generated from different tissues at 3.2 kb (expected size of the ABCF1 transcript). GAPDH was used as the loading control.
To investigate the physiological ABCF1 promoter activity in adult mice, we examined X-gal staining in whole organs as well as cryosectioned tissues. Whole organ staining allowed us to identify general regions of interest while sectioning allowed us to determine cell and tissue specific expression. Of the whole organs stained with X-gal, lung, testis, liver and heart showed the most prominent staining in the ABCF1+/- mice (Figure 3.8). Kidneys, lymph node, thymus and eye showed intermediate staining while spleen showed very little staining. Certain tissues like liver, testis and thymus showed back-ground staining (staining in the wild-type organs) and thus were stained for several hours as opposed to overnight. Nonspecific staining was unable to be eliminated in the whole mount livers but the staining was not as intense as in the ABCF1+/- livers. It has been reported that liver does have endogenous galactosidase activity which is mouse strain specific [17].
Figure 3.8. The ABCF1 promoter is active in whole adult mouse organs.
Whole organs were excised from ABCF1+/- mice and their ABCF1+/+ littermate controls and stained with X-gal for 24 h. Brain, testis, lung and liver showed high promoter activity. There was also some endogenous X-gal staining in wild-type liver, testis and thymus so staining times for these tissues were for 4-12 h depending on the development of endogenous expression in the wild-type control. Spleen and lymph nodes appeared to show low promoter activity.
Upon sectioning, tissues from adult mice were found to be very differentially stained. In the brains; the meninges, the cerebrocortex and hippocampus showed the most intense promoter activity (Figure 3.9). There was light staining in the thalamus, mid brain and forebrain and very light staining in the cerebellum. Staining of sectioned eye revealed strong ABCF1 promoter activity in the extraorbital muscles, ciliary body, retina and conjunctiva (Figure 3.10). Within the retina, the ganglion cells appeared the most intensely stained. There was also moderate to strong staining in the ganglion cell layer, neuron nuclear layer and the nuclei of the rods and cones. The photoreceptor segments of the rods and cones, outer synaptic layer and inner synaptic layer had lighter more diffuse staining. There was virtually no staining in the cornea and sclera. The heart showed strong transmural staining through the epicardium, myocardium and endocardium (Figure 3.11). There was little staining throughout the cardiac muscle cells, rather it was limited to punctate vesicles. The glomerulus and both the proximal and the distal convoluted tubules were positive for ABCF1 promoter activity in the kidney (Figure 3.12). The proximal convoluted tubule appeared more intensely stained than the distal convoluted tubule. The liver had some background diffuse staining in the ABCF1+/+ sections. The nonspecific staining in the ABCF1+/+ sections, however, was light and diffuse and not specific to any particular anatomical region unlike the staining in the ABCF1+/- sections. In the ABCF1+/- liver sections, there was strong staining in the periportal hepatocytes and less intense staining in the periacinar and midzonal areas (Figure 3.13). Sectioning and staining of the lungs showed strong ABCF1 promoter activity in the respiratory epithelia of the bronchioli. There was also prominent staining in the blood vessels within the lungs and slight staining in the alveolar walls (Figure 3.14). The staining in the lymph nodes was faint and diffuse throughout the medulla with more intense staining at the cortex around the lymphoid follicles (Figure 3.15). There was diffuse staining in
the pancreas and prominent staining in the pancreatic islet cells (Figure 3.16). Staining of sectioned skin showed ABCF1 promoter activity in the epidermis, hypodermis as well in the sebaceous glands (Figure 3.17). There was little staining in the dermis. In the duodenum, there was prominent staining on the surface of the intestinal villi and crypts of Lieberkühn (Figure 3.18). There was prominent staining in the lacteal and punctate staining in the muscular layer of the small intestine and very little staining in the submucosa. The spleen showed punctate expression in the vascular wall, and prominent staining in the marginal sinuses (Figure 3.19). The seminiferous tubules of the testis showed strong promoter activity (Figure 3.20). There was less staining in the interstitial cells of Leydig. The staining in the thymus was fairly diffuse in both the cortex and medulla (Figure 3.16). Taken together, these data suggest that ABCF1 is involved in development and that ABCF1 expression is regulated in a tissue-specific manner in adult mice.
Figure 3.9. The ABCF1 promoter is active in the meninges, cerebrocortex and hippocampus in the brains of adult mice.

ABCF1 promoter activity was determined by cryosectioning (10 µm sections) and staining mouse brains. The top panel shows a horizontal brain section stained with X-gal and taken using a 1x objective. The bottom right and left panels show brain sections stained with nuclear fast red (a nuclear counter-stain) and X-gal while the middle panel shows a section stained with X-gal alone. Sections are from ABCF1+/- mice and their ABCF1+/+ controls. The bottom panels are taken using a 40x objective.
Figure 3.10. The ABCF1 promoter is active in the conjunctiva, ciliary body and the retina in the eyes of adult mice.

ABCF1 promoter activity was determined by cryosectioning (10µm sections) and staining mouse eyes. The top panel is stained with X-gal and taken using a 5x objective. The bottom right and left panels show retinas stained with nuclear fast red and X-gal while the middle panel represents the retina stained with X-gal. The bottom 3 panels were taken using a 400x objective. Sections are from ABCF1+/- mice and their ABCF1+/+ controls. The illustration represents the indicated region and is modified from Dyer et al. with permission [18].
Conjunctiva
Ciliary body
Choroid
Lens
Retina

Rod
Pigment cell
Cone

Outer nuclear layer
Inner nuclear layer
Ganglion cell layer

ABCF1+/+(nuclear fast red/ X-gal)
400x

ABCF1+/-(X-gal)
400x

ABCF1+/-(X-gal/ nuclear fast red)
400x
Figure 3.11. The ABCF1 promoter is active in the epicardium, myocardium and endocardium in the hearts of adult mice.
ABCF1 promoter activity was determined by cryosectioning (10µm sections) and staining mouse hearts. The top panels is a section (the illustration indicates the architecture) stained with X-gal and nuclear fast red taken using a 1x objective. The bottom right and left panels show the region, indicated by the red square, which are stained with nuclear fast red and X-gal while the middle panel shows the region which is stained with X-gal. The bottom panels are taken using a 40x objective. Sections are from ABCF1+/- mice and their ABCF1+/+ controls. The illustration shows the heart wall structure [19].
Figure 3.12. The ABCF1 promoter is active in the glomerulus and the convoluted tubules in the kidneys of adult mice.

ABCF1 promoter activity was determined by cryosectioning (10µm sections) and staining mouse kidneys. The top panel is a section of kidney (the slice is shown in the illustration) stained with nuclear fast red and X-gal taken using a 1x objective. The bottom right and left panels show kidney sections that are stained with nuclear fast red and X-gal while the middle panel shows a section that is stained with X-gal. The bottom panels are taken using a 40x objective. Sections are from ABCF1+/- mice and their ABCF1+/+ controls. The illustration was adapted from Schedl et al. with permission [20].
Figure 3.13. The ABCF1 promoter is active in the periportal hepatocytes in the livers of adult mice.
ABCF1 promoter activity was determined by cryosectioning (10 µm sections) and staining mouse livers. The top panel is taken using a 1x objective and shows the liver stained with X-gal. The bottom panels show the region represented by the red square in the top panel and are taken with a 40x objective. The left and right panels show liver stained with nuclear fast red and X-gal while the middle panel shows liver stained with X-gal alone. Sections are from ABCF1+/- mice and their ABCF1+/+ controls. The top illustration was modified from Dane et al. with permission [21]. The bottom illustration was adapted from Adams et al. with permission [22].
Figure 3.14. The ABCF1 promoter is active in the bronchioles and alveolar walls in the lungs of adult mice.

ABCF1 promoter activity was determined by cryosectioning (10 µm sections) and staining mouse lungs. The top panel shows lungs that are stained with nuclear fast red and X-gal taken with a 1x objective. The bottom panels show regions containing bronchioles taken with a 40x objective. The bottom left and right panels show lungs stained with nuclear fast red and X-gal while the middle panel shows lung stained with X-gal alone. Sections are from ABCF1+/- mice and their ABCF1+/+ controls. The illustration has been modified from Rowley et al. with permission [23].
Figure 3.15. The ABCF1 promoter is active in the peri-follicular region in the lymph nodes of adult mice.

ABCF1 promoter activity was determined by cryosectioning (10 µm sections) and staining mouse lymph nodes. The top panel shows a lymph node stained with nuclear fast red and X-gal taken using a 5x objective. The bottom panels show lymph node follicles stained with nuclear fast red and X-gal (left and right panels) or X-gal alone (middle). The bottom panels were taken using a 40x objective. Sections are from ABCF1+/- mice and their ABCF1+/+ controls. The illustration was modified from Drayton et al. with permission [24].
Figure 3.16. The ABCF1 promoter is active in the pancreatic islet cells.
ABCF1 promoter activity was determined by cryosectioning (10 µm sections) and staining mouse pancreases. The top panel shows a pancreas section stained with X-gal taken using a 5x objective. The bottom panels show pancreatic islets taken using a 40x objective. The bottom right and left panels show pancreas sections stained with nuclear fast red and X-gal while the middle panel shows a section stained with X-gal alone. Sections are from ABCF1+/- mice and their ABCF1+/+ controls. The illustration was modified from Bardeesy et al. with permission [25].
Figure 3.17. The ABCF1 promoter is active in the sebaceous glands and the hypodermis of the skin.
ABCF1 promoter activity was determined by cryosectioning (10 µm sections) and staining mouse skin sections. The bottom panels show skin sections images taken using a 40x objective. The bottom right and left panels show skin sections stained with nuclear fast red and X-gal while the middle panel shows a section stained with X-gal alone. Sections are from ABCF1+/- mice and their ABCF1+/+ controls. The illustration was modified from Fuchs et al. with permission [26].
ABC71+/+ (nuclear fast red/ X-gal)  
ABC71+- (X-gal)  
ABC71+- (nuclear fast red/ X-gal)
Figure 3.18. The ABCF1 promoter is active on the surface of the intestinal villi and crypts of Lieberkühn as well as in the lacteals and muscular layer. ABCF1 promoter activity was determined by cryosectioning (10 µm sections) and staining mouse duodenal fragments. The top panel shows a duodenal section stained with nuclear fast red and X-gal taken using a 2x objective. The bottom panels show villi sections taken using a 40x objective. The bottom right and left panels show duodenal sections stained with nuclear fast red and X-gal while the middle panel shows a section stained with X-gal alone. Sections are from ABCF1+/- mice and their ABCF1+/+ controls. The illustration was modified from Ganz et al. with permission [27].
Figure 3.19. The ABCF1 promoter is active in marginal zone in the spleens of adult mice.

ABCF1 promoter activity was determined by cryosectioning (10 μm sections) and staining mouse spleens. The top panel shows a spleen section stained with nuclear fast red and X-gal taken using a 5x objective. The bottom panels show splenic follicles taken using a 40x objective. The bottom right and left panels show spleen sections stained with nuclear fast red and X-gal while the middle panel shows a section stained with X-gal alone. Sections are from ABCF1+/- mice and their ABCF1++ controls. The illustration was modified from Pillai et al. with permission [28].
Figure 3.20. The ABCF1 promoter is active throughout the testis in adult mice.
ABCF1 promoter activity was determined by cryosectioning (10 µm sections) and staining mouse testis. The top panel shows a testis section stained with X-gal taken using a 2x objective. The bottom panels show images of seminiferous tubules taken using a 40x objective. The bottom right and left panels show testis sections stained with nuclear fast red and X-gal while the middle panel shows a testis section stained with X-gal alone. Sections are of ABCF1+/- mice and their ABCF1+/- controls. The illustration was modified from Cooke et al. with permission [29].
Figure 3.21. The ABCF1 promoter is active throughout the thymus in adult mice. ABCF1 promoter activity was determined by cryosectioning (10 µm sections) and staining the mouse thymus glands. The top panel represents a thymic section stained with nuclear fast red and X-gal taken using a 5x objective. The bottom panels show sections taken using a 40x objective. The bottom right and left panels show sections stained with nuclear fast red and X-gal while the middle panel shows a section stained with X-gal alone. Sections are of ABCF1+/- mice and their ABCF1+/+ controls. The illustration was modified from Blackburn et al. with permission [30].
3.4.4 The ABCF1 promoter is active in CD8$^+$ T-cells

Since there was significant background staining in the X-gal stained tissue sections, we decided to analyse thymocytes using fluorescein di-β-D-galactopyranoside (FDG) staining by FACS to see if we could eliminate the background. Initially we chose to look at the ABCF1 promoter activity in CD8$^+$ T-cells because we were particularly interested in whether ABCF1, like TAP 1, could play a role in MHC class I presentation. Also, ABCF1 mRNA expression is known to increase in proliferating CD8$^+$ T cells as assayed by microarray analysis [31].

The thymus is known to be a primary lymphoid organ where bone marrow derived progenitor cells undergo a series of differentiation steps to form mature CD8$^+$ T cells [32]. In the thymus, approximately 65% of the CD8 positive cells were fluorescein negative and 35% were positive (Figure 3.17). In the spleen in contrast, 84% of the CD8 positive cells were fluorescein positive and only 16% of the cells were fluorescein negative. These data suggest that the ABCF1 promoter could be turned on during the maturation of CD8$^+$ cells.
Figure 3.22. ABCF1 promoter activity in CD8⁺ T cells from the thymus and the spleen.
Spleens and thymi were dissected out of three ABCF1+/− (top panels) and three of their wild-type littermate controls (bottom panels). Cells were labelled with the PE-αCD8 antibody and then stained with fluorescein by heat shock. Data is representative of two separate experiments.
3.4.5 ABCF1+/- mice produce functional cytotoxic T lymphocytes (CTLs)

To determine whether ABCF1 is important for CD8+ T cell maturation, a CTL assay was used to assess whether ABCF1+/- mice can produce functional CTLs. ABCF1 +/- mice and their ABCF1+/+ littermates were infected with VSV for seven days. After seven days, their spleens were removed and the splenocytes were expanded in culture for five days in the presence of the VSV immunodominant peptide (VSV-NP52-59). Once expanded, T cell function was determined by staining cells with VSV-NP specific tetramers and antibodies to IFN-γ as well as with a CTL assay. After expansion, ABCF1+/- spleens had similar percentages of CD4+ cells but fewer CD8+ T cells (Figure 3.18). The tetramer and IFNγ staining also revealed that the ABCF1+/- mice had fewer tetramer-specific (Figure 3.19) and fewer IFNγ-producing CD8+ T cells (Figure 3.20).

For the CTL assay, ABCF1+/- and ABCF1+/+ splenocytes were incubated with 51Cr labelled target cells (RMA-S) at various ratios and killing was monitored by the release of 51Cr from the target cells into the supernatant. ABCF1+/- CTLs were found to be as good as ABCF1+/+ CTLs at killing their targets (Figure 3.21).
Figure 3.23. ABCF1 +/- mice produce fewer CD8⁺ T cells after infection with VSV.
ABCF1 +/- mice and their ABCF1 +/- littermate controls were injected with 1×10⁵ pfu of VSV ip. Seven days later mice were sacrificed and spleens were removed and mashed into single cell suspensions. Splenocytes were further incubated for five days with the H-2Kb restricted peptide VSV-NP. Cells were then stained with PE-αCD4 and FITC-αCD8 antibodies. Null represents wild type mice that were mock infected (injected with PBS). Figure shown represents the results of one experiment, which was later repeated seven times with similar results.
Figure 3.24. ABCF1 +/- mice produce fewer tetramer-specific CD8+ T cells after infection with VSV.

ABCF1 +/- mice and their ABCF1 +/+ littermate controls were injected with 1x10^5 pfu of VSV ip. Seven days later mice were sacrificed and spleens were removed and mashed into single cell suspensions. Splenocytes were further incubated for five days with the H-2K^b restricted peptide VSV-NP. Splenocytes were then stained with PE-VSV-NP tetramer and FITC-αCD8 antibody. Figure shown represents the results of one experiment, which was later repeated four times with similar results.
Figure 3.25. ABCF1 +/- mice produce fewer IFNγ positive CD8+ T cells after infection with VSV.

ABCF1 +/- mice and their ABCF1 +/+ littermate controls were injected with $1 \times 10^5$ pfu of VSV ip. Seven days later mice were sacrificed and spleens were removed and mashed into single cell suspensions. Cells were then stained with PE-αINFγ and FITC-αCD8. ABCF1 +/- splenocytes showed fewer IFNγ positive CD8+ T cells. Figure shown represents the results of one experiment, which was later repeated three times with similar results.
Figure 3.26. ABCF1+-/ CTLs were as efficient as ABCF1 +/- at killing of chromium loaded VSV specific target cells. ABCF1 +/- mice and their littermate controls were injected with VSV and treated as described above. A standard cytotoxicity assay with 51Cr labelled VSV-NP pulsed RMA-S (H-2Kb specific) was done and CTL activity was measured by the release of 51Cr into the supernatant. Figure shown represents the results of one experiment, which was later repeated three times with similar results.
3.5 Discussion

By taking advantage of gene trap technology, we provide the first detailed analysis of the ABCF1 promoter activity during embryogenesis and in adult mice. Gene trapping, through the random insertion of trap vectors into the mouse genome, is an invaluable tool for generating mutants. Not only does it disrupt normal gene expression, it has the added benefit of putting reporter genes under the control of the endogenous promoter. This allows the promoter activity to be examined under physiological conditions.

Here we show that the ABCF1 promoter is highly active during mouse embryogenesis. The ABCF1 promoter was found to be active in blastocysts and the expression of one copy of the wild-type ABCF1 gene was necessary for blastocysts survival to the post implantation stage (Chapter 2). The ABCF1−/− blastocysts had extensive promoter activity and morphologically appeared to have poorly organized inner cell masses. The ABCF1+/− blastocysts also had strong promoter activity but appeared to have well organized inner cell masses that were distinct from the blastocoel. The only known function of ABCF1 is in its interaction with eIF2 and its requirement in translation initiation [5, 8]. One interpretation of these data is that ABCF1, like its yeast homolog GCN20, also has a role in the regulation of the eIF2 [33]. It is possible that ABCF1 is necessary for translation through its regulation of phosphorylated eIF2. Since eIF2 phosphorylation is known to be involved in cell death, cells in ABCF1−/− may not be undergoing normal apoptosis (since the ABCF1−/− blastocysts appear as if they may lack a blastocoel). PPP1R15 genes are also known to be important regulators of eIF2 [34]. They are responsible for repressing eIF2α phosphorylation [34]. Interestingly, mice lacking PPP1R15 function also fail to form a blastocyst cavity and don’t survive past the preimplantation stage of development [34]. It
is thought that the increased phosphorylation of the eIF2α is responsible for cell death in the blastocyst [35]. Alternatively, if ABCF1 is important for canonical transcription, maternally derived ABCF1 may be present in high enough levels for survival until the blastocyst stage, but not high enough to allow differentiation. It is known that during development, gene expression in the zygote promotes the destruction of maternally derived transcripts but some maternal contributed genes are long lasting [36, 37].

We were unable to generate ABCF1-/- post-implantation stage embryos. However, we gained some insight into the function of ABCF1 by examining the ABCF1+/- embryos and mice. The ABCF1 promoter was highly active in the ABCF1+/- embryos throughout embryogenesis. Sectioning of 11.5 dpc embryos revealed strong staining in all tissues particularly in regions known to be actively proliferating and differentiating. Interestingly, although the promoter activity appeared strong, 100% normal ABCF1 expression was not required for development as ABCF1+/- embryos as well as adult mice appear phenotypically normal when compared to their wild-type littermates. One explanation for this phenomena is that the N-terminal region of ABCF1 (which was found to interact with eIF2) in the truncated protein is adequate for normal translation, but there are also developmental roles that require the full protein.

In adult mice, the ABCF1 promoter was found to be active in all tissues tested, which is supported by the Northern blot data. Some of the tissues exhibited background endogenous staining which has been observed in the literature by other groups investigating sectioned tissues which express β-gal [17]. The background staining in the tissues was diffuse and distinguishable in most cases from positively stained tissues. There were differences, however, in the promoter activity within different regions of the tissues. Within spleens and lymph nodes, for example, staining appeared to be primarily in the regions surrounding the white pulp and the lymph node
follicles. Both areas are known to contain specific macrophage populations along with many other cell types including B-cells [38]. The expression of ABCF1 mRNA in B cells, however, was found to be very low in comparison to other antigen presenting cells (Jefferies’ lab, unpublished data). Also, the staining in the thymus (organ responsible for T cell maturation) suggests that while T cells may express ABCF1, the most highly stained cells appear to be macrophage or fibroblast like cells (morphologically). Interestingly, mice which lack TNF cytokines, various transcription factors (NF-κB, rel) and chemokines tend to exhibit defects in marginal zone macrophage differentiation and positioning [39]. It is possible that ABCF1 could also be involved because its expression appears to be high in the marginal zone, ABCF1 is also regulated by TNFα and phosphorylation of eIF2α is important for NF-κB gene expression [40].

The ABCF1 promoter activity appeared to be prominent in the cells lining the blood vesicles in the lungs, the liver and the kidney. Intriguingly, these are sites where the organs contact potential foreign antigens from the blood stream [41]. Lung endothelial cells, periportal hepatocytes, renal podocytes and renal tubules are all known to express Toll-like receptors (TLRs) [42, 43] [44, 45]. Also, TLRs are expressed on Kupffer cells (in the liver) which represent 80-90% of the tissue macrophages in the body [46]. Kupffer cells adhere to the endothelial walls of the portal veins and provide a first line of defence against blood borne pathogens from the gastrointestinal tract [46]. The ABCF1 promoter was active in the epithelium of the skin, the bronchioles and the duodenum. These sites contact the external environment and provide a barrier from the outside world. The epithelial cells (which express TLRs) are in constant contact with commensal micro-organisms [47]. It is thought that TLR signalling is generally protective and contributes to homeostasis [48]. One mechanism is by down-regulating the pro-inflammatory response in the epithelial cells (through the participation of macrophages, DCs, and T and B lymphocytes) [49].
It is possible that ABCF1 functions in down-regulating the pro-inflammatory response. Sectioning of the pancreas showed high promoter activity in the pancreatic islet cells. Pancreatic islets are responsible for glucose regulation in mammals [50]. In diabetes mellitus, β-cells (within the islets) are destroyed by a T-cell mediated autoimmune response resulting in the dysregulation of blood glucose [51]. Diabetes occurs in genetically susceptible individuals who possess certain alleles of genes (both MHC related or non-MHC related genes), many of which are located within the MHC locus [51]. TLR pathways and the production of pro-inflammatory cytokines have been linked to the initiation of diabetes [52]. Interestingly, the ABCF1 gene locus (within the MHC locus) has been previously found to be a susceptibility locus in autoimmune pancreatitis [53]. In various studies, 42-76% of patients with autoimmune pancreatitis also have been recently diagnosed with diabetes mellitus (within a year) [54]. Provocatively, the ABCF1 promoter was also found to be active in the eye, brain, heart and testis which are not known to be immunologically active tissues. They are tissues, however, that are actively growing or turning over cells in adult mice. The retina contains two different populations of monocyte derived cells which are important in normal tissue homeostasis as well as inflammation [55]. These monocytic cells are known to turnover completely in 6 months as shown by bone marrow transplant mice expressing EGFP [55]. In the brain, GCN2 through its control of eIF2α phosphorylation, is known to be important in regulating synaptic plasticity associated with learning and memory [56]. In the testis, spermatogenesis is known to be regulated by TNFα [57]. Finally, in heart, it is known that there is a three-fold increase in the diameter of myocytes from neonates to adult mice [58]. This cell growth is known to be dependent on signals derived from the endo- and epicardiums [59].
To further investigate the role of ABCF1 in CD8+ cells, we used FACS to analyse their expression in the thymus and the spleen. We found that in the thymus (which contains immature and mature T cells), the population of CD8+ T cells had variable promoter activity, with quite a large percentage of cells expressing very little β-geo. This suggests that either certain subsets of CD8+ cells don’t express ABCF1 or that maybe ABC is expressed upon maturation. In contrast, the spleen (which contains mature T cells), contained mostly β-geo positive cells, suggesting that ABCF1 may be involved in CD8 T cell maturation. To further test this hypothesis, FACS could be used to analyse the thymic T cell expression of fluorescein along with other markers of T cell maturation such as CD4, CD3, IL-2 receptor α, CD5, CD69 and CD44 [60]. The difference in the promoter activity in subsets of CD8+ T cells led us to ask whether ABCF1+/− CD8+ T cells could produce a functional CTL response to virus to VSV infection. Not surprisingly, ABCF1+/− mice produced fewer CD8+ T cells than their littermates. These cells produced less IFNγ than the wild-type cells, but were as efficient at killing target cells. Taken together, these data indicate that while ABCF1 may have a role in the development and cytokine producing ability of CD8+ T cells in response to a VSV infection. However, it does not appear to function in the CTL recognition or response to viral epitopes in the context of H-2Kb.

In conclusion, this study provides insight into the control of the ABCF1 gene during embryogenesis and in adult tissues. Identifying the compartments that contain the highest level of promoter activity gives us an indication of what cell or tissues types may be affected by deficiency in ABCF1. This data, however, should be interpreted with caution as it is possible that the truncated fusion proteins are unstable or lead to toxicity in certain cell types (maybe due to extremely high levels of expression of βgeo). These observations should be followed up with cell specific immunostaining and western blot to definitively identify the cell subsets that contain the
highest levels of βgeo expression. ABCF1 is known to associate with the eIF2 and is thought to be responsible for the initiation of translation. The fact that ABCF1 is preferentially expressed in functionally diverse tissues suggests that it may play a unique role in translation initiation.
3.6 References


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Chapter 4 Aberrant macrophage phenotypes and population dynamics in ABCF1+/- mice.

4.1 Overview

ABCF1 is an ABC transport protein that is involved in translation initiation and its control. The N-terminus of ABCF1 is thought to bind to eIF2 and stabilize the eIF2 Met-tRNA complex. ABCF1+/− mice were found to have differences in their macrophage phenotypes and population numbers when compared to ABCF1+/+ mice. They had fewer circulating monocytes and their bone marrow derived macrophages were hypo-responsive to stimulation with TLR ligands. Splenic macrophages from ABCF1+/− mice on the other hand were found to be hyper-responsive to stimulation with LPS and CpG DNA, producing augmented IL-6 and TNFα responses. When ABCF1+/− mice were challenged with LPS, they were found to have distinct cytokine profiles in both their sera and their peritoneal fluid. Challenge of ABCF1+/− mice with lethal doses of Listeria monocytogenes (LM) showed that ABC+/− mice succumbed to the infection earlier than their wild-type controls. These observations indicate that ABCF1 affects the regulation of cytokines in macrophage populations and the populations of macrophages in ABCF1+/− mice are different from the macrophage populations in the ABCF1+/+ mice.
4.2 Introduction

Macrophages are cells with high plasticity, which are capable of responding and reacting to changes in their environment [1]. This plasticity helps to give rise to populations of highly heterogeneous cells [2]. Macrophages, under normal physiological conditions, are responsible for the removal and remodelling of old or damaged cells, tissue and extracellular debris [1]. In times of stress or danger, macrophages are capable of changing their physiology in order to react to the stressor. Toll-like receptors (TLRs), for example, on the surface of macrophages can bind to a number of different conserved features of pathogens (Figure 4.1). This binding initiates a signal cascade which ultimately upregulates the genes involved in innate defence along with the genes involved in the recruitment of cells necessary for adaptive immune responses [3].

**Figure 4.1. TLRs and their ligands.**

TLRs recognize a range of different products derived from pathogens. LPS-lipopolysacharide, RSV-respiratory syncytial virus, polyI:C-double stranded RNA, ssRNA-single stranded RNA, HSP60-Heat shock protein 60, GIPL-glycoinositolphospholipid, VSV-vesicular stomatitis virus. Figure reproduced from Liew et al.[4].
ABCF1 is an ABC transporter protein encoded within the MHC locus (Figure 4.2). It is located within the class I region and is a susceptibility locus for autoimmune pancreatitis [5]. Gene expression of ABCF1 has been shown to increase when cells were stimulated with tumor necrosis factor alpha (TNF-α) [6]. This effect was initially discovered in synoviocytes, which are the cells that line synovial joints [6]. This is significant as TNF-α is a pro-inflammatory cytokine, produced by activated macrophages and lymphocytes, which has a number of functions in the immune response including initiating acute phase responses to infection [7, 8]. Dysregulation of TNF-α has been implicated in a number of pathologies, such as sepsis and rheumatoid arthritis (RA) [9]. TNFα production is under transcriptional and post transcriptional control in cells [10]. Post-transcriptionally, TNFα is regulated by (AU)-rich elements (AREs), mRNA stability and by controlling translational initiation [10, 11]. T-cell intracellular antigen-1 (TIA-1) and TIA-1-related protein (TIAR) are transacting factors that bind to AREs and are essential in controlling the expression of TNFα [10]. ARE binding proteins can be stabilizing or inhibitory and may differ in their regulation of TNFα in different cell types or at different stages of the cell cycle [12, 13].
Figure 4.2. A schematic of the mouse MHC locus on chromosome 17 including the location of the ABCF1 gene. 

ABCF1 is located in the MHC locus in the class I region in between the non-classical MHC class 1b genes $H2-Q$ and $H2-T$. Locations of the TAP and TNF genes are described as well. Diagram adapted from Nei et al. [14].
ABCF1 is known to associate with the eIF2, a protein involved with translational initiation [15]. Sequence homology shows that it may be related to the yeast protein GCN20 which exerts control over translation through its role in the phosphorylation of eIF2 [15]. Interestingly, eIF2α phosphorylation is important for the expression of Colony stimulating factor-1 receptor (CSF-1R) and instrumental in the activation of NF-κB and transcription of associated cytokines in response to stress [16, 17]. Phosphorylation of eIF2α also directs TIAR and TIA-1 to shuttle cytoplasmic mRNA to stress granules thus preventing translation and silencing mRNAs [18]. This combination of positive and negative regulation is important for fine tuning an immune response that is appropriate for fighting infection while preventing excess tissue damage [19]. siRNA experiments show that ABCF1 is important for normal translation, reducing the expression of reporter genes [20]. ABCF1 probably has more than one regulatory role in translation, as reporter genes driven by Cottontail rabbit papilloma virus (CrPV) and Hepatitis C virus (HCV) internal ribosome entry site (IRES) showed increased expression in cell lines which contained ABCF1 Walker box mutations (mutations in the ATP binding residues) [20].

Here we report the first in vivo investigation into the role of ABCF1 in macrophage development and activation. We provide evidence that ABCF1 deficient mice have populations of macrophages that are different from their wild-type littermates both in size and in their response to antigens. These data suggests that along with the previous known function of translation initiation ABCF1 also plays a key role in the regulation of inflammatory cytokines.
4.3 Materials and methods

4.3.1 Reagents

Toll-like receptor (TLR) ligands (Invivogen, San Diego, CA, USA) were prepared according to the package inserts. Ligand concentration was adjusted by diluting stock solutions with endotoxin free water. Aliquots of LPS (*Escherichia coli* 0111:B4), poly I:C, CpG oligonucleotides (Type C), peptidoglycan (*E.coli* K12), Flagellin (*Salmonella typhimurium*) were stored at -20°C. The stock solutions were either diluted using PBS for *in vivo* experiments or diluted with RPMI-1640 (supplemented with 10% heat inactivated fetal bovine serum (HyClone, Logan, UT, USA), 1mM Na pyruvate, penicillin (100 IU/ml), streptomycin (100 μg/ml), 20 mM HEPES and 2 mM L-glutamine) for experiments done *ex vivo*.

4.3.2 Mice

Gene trapped ABCF1 +/− mice were created from the Baygenomics ES cell line XK097 (Baygenomics, San Francisco, CA, USA) as described in Chapter 2. XK097 contains the gene trap vector pGT0Lxf, which is inserted between exon 7 and exon 8 in the ABCF1 gene. XK097 ES cells were aggregated and then micro-injected into the blastocele of C57Bl/6 blastocysts. Chimeric males (as determined by coat color) were mated to C57Bl/6 females and subsequent litters were further back crossed 10 generations onto the C57Bl/6 line. The XK097 mice were housed at either The Biomedical Research Center or the Zoology Small Mammal unit under specific-pathogen
free conditions and given free access to chow and water. All animal procedures were approved by the University of British Columbia Animal Care Committee.

### 4.3.3 Assessment of ABCF1+-/- mouse haematologic parameters and spleen counts

In order to perform the complete blood count (CBC) analysis, 100 µL of peripheral blood was collected from ABCF1+-/- mice and their wild-type littermates in EDTA coated microvette capillary blood collection tubes (Sarstedt, Germany) by saphenous vein puncture. Hematological parameters including haemoglobin, white blood cells, platelets and red blood cells were analyzed using the HemaVet 950 automated blood analyser ([Drew Scientific Inc., Oxford, CT](http://www.drewscientific.com)) according to the manufacturer’s instructions. To determine splenocyte numbers ABCF1+-/- mice and their wild-type littermate controls were killed using CO₂ asphyxiation. Their spleens were removed and immediately placed in complete RPMI 1640. The spleens were then mashed through a 40 µm cell strainer to produce a single cell suspension. The volume of the single cell suspension was brought up to 15 mL using complete RPMI 1640. 10 µL of the cell suspension was mixed with 90 µL of Trypan Blue (Invitrogen, Burlington, ON) and counted using a haemocytometer.

### 4.3.4 Histology

To assess splenic architecture, spleens from ABCF1+-/- mice and their wild-type littermate controls were removed and fixed in 10% neutral buffered formalin for 48h and
then embedded in paraffin. Sections from each of the spleens were stained with haematoxylin and eosin (H&E) and/or terminal deoxynucleotidyl transferase biotin-dUTP nick end labelling (TUNEL) (Wax-It Histology Services, University of British Columbia) for microscopic analysis. Slides were sent to a veterinary pathologist for independent analysis (Drs. S. Newbigging and H. Adissu, Toronto Centre for Phenogenoics). The number of TUNEL positive cells in the spleen was quantified by counting the dark brown TUNEL positive cells in 15 random fields of view in the ABCF1+/- and ABCF1+/+ sections using a 40x objective.

### 4.3.5 Immunostaining

Spleens from three ABCF1+/- and three ABCF1+/+ mice were removed and immediately placed in complete RPMI 1640 on ice. The spleens were then mashed through a 40 µm cell strainer to produce a single cell suspension. The volume of the single cell suspension was brought up to 15 mL using complete RPMI 1640. 100 µL of splenocytes were plated per well into a 96 well U bottom plate and washed three times with FACS buffer. The cells were then stained for their expression of annexin-V (Invitrogen, Burlington, ON), and propidium iodide (PI). For APC (allophycocyanin)-annexin-V staining, cells were suspended in 100 µL of annexin-V buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM CaCl2, pH 7.4) containing 0.625 µL of APC-annexin-V (Invitrogen, Burlington, ON) and incubated at room temperature for 15 min. After 15 min, 400 µL of annexin-V buffer was added to the cells which were then placed on ice. Just prior to FACs analysis, 1 µL of a 50 µg/mL PI (Sigma, St. Louis, MO) solution was added to cells and they were analysed on a Becton-Dickinson LSRII flow
4.3.6 Cytokine responses \textit{ex vivo} and \textit{in vitro}

To obtain splenic macrophages (spMØ), spleens from four ABCF1 +/- mice and their wild-type littermate controls were pooled and mashed through a 40 µm cell strainer to produce a single cell suspension. Red blood cells were lysed by incubating the splenocytes in 2 mL of Mouse Red Cell Removal Buffer (MRCRB) (0.15 M NH₄Cl, 0.001 M KHCO₃ pH 7.3) for 5 min at room temperature. The MRCRB lysis buffer was neutralized by the addition of 8 mL of complete RPMI 1640. Splenocytes were then stained in 1 mL of FACS buffer containing 5:1000 of FITC-αCD11b (Pharmingen, Oakville, ON) and 1:100 µL of PE-αF4/80 antibodies (eBioscience, San Diego, CA) on ice for 20 min. Double positive cells were sorted using the FACS Vantage (Becton Dickinson, Oakville, ON) and represents the macrophage portion of cells. Once sorted, 5x10⁴ macrophages (both ABCF1 +/- and ABCF1 +/+ ) were plated per well in a 96 well U bottom plate in triplicate and allowed to adhere for 3-4 hr. Once adhered, spMØ were washed several times with Hanks balanced salt solution (HBSS) (Invitrogen, Burlington, ON) then cultured 18 h in complete RPMI 1640 containing various concentrations of TLR ligands (as represented in the graphs). After 18h, supernatants were collected and stored at -80°C. Supernatants cytokine concentrations were measured using the Mouse IL-1β/IL-1F2 Immunoassay Quantikine kit (R&D Systems Minneapolis, MN, USA) and the Cytometric Bead Array (CBA) Mouse Inflammation Kit (Becton Dickinson). CBA beads were analysed using the FACSscan and Cell Quest software. The results were analysed using the FCAP Array software (Becton Dickinson).
To obtain bone marrow derived macrophages (BMMφ), femurs from three ABCF1+/- and three ABCF1+/+ mice were flushed with complete RPMI 1640, pooled and centrifuged for 5 min at room temperature. Bone marrow cells were resuspended in 500 µL of MRCRB lysis buffer and incubated at room temperature for 1 min to remove red cells. MRCRB was neutralized by the addition of 4.5 mL of RPMI. Bone marrow cells were then grown for 6 days in complete RPMI 1640 containing 10% L929-cell conditioned media. After 6 days, BMMφ were washed several times in HBSS and incubated for 5 min in 1 mL of Cell Dissociation Buffer (Invitrogen, Burlington, ON) at 37°C to dissociate the cells from the dish. The cells were then centrifuged and resuspended in 10 mL of complete RPMI 1640 media. After resuspension, BMMφ were counted and plated at 1 x 10⁶ cells per well in a 48 well plate (Falcon) and allowed to re-adhere for 24h at 37°C. Once adhered, the BMMφ were washed with HBSS and then 200 µL of complete RPMI with TLR ligands were added to each of the wells in duplicate. The supernatant was diluted 1/4 in dilution buffer (DB) (CBA Mouse Inflammation Kit (Becton Dickinson)) and the cytokine concentrations of IL-1β, IL-6, IL-10, MCP-1 (CCL2), TNFα, IFNγ and IL-12p70 were measured using the Mouse IL-1β/IL-1F2 Immunoassay Quantikine kit (as above) and the CBA Mouse Inflammation Kit (as above).

4.3.7 Cytokine responses \textit{in vivo}

To assess cytokine responses \textit{in vivo}, ABCF1 +/- mice and their ABCF1+/+ littermate controls were given an interperitoneal (ip) injection of either 10 or 100 µg of LPS. The mice were killed by CO₂ asphyxiation and their blood was obtained by cardiac puncture at 1, 3, or 6 h post LPS injection. The blood was allowed to clot for 2 h at room
temperature and then the serum was separated from the clotted blood by centrifugation for 20 min at 2500 rpm (revolutions per minute). Serum samples were collected and frozen at -80°C. Peritoneal lavage was also preformed on each of the mice by flushing the peritoneal cavity with 3 mL of PBS (containing 1mM EDTA). The serum and lavage supernatants were diluted $\frac{1}{2}$ in DB and the cytokine concentration of IL-1β, IL-6, IL-10, MCP-1, TNFα, IFNγ and IL-12p70 were measured using the Mouse IL-1β/IL-1F2 Immunoassay Quantikine kit (as above) and the CBA Mouse Inflammation Kit (as above).

4.3.8 *Listeria monocytogenes* infections *in vivo*

LM 10403s was originally acquired from Dr. John Priatel (UBC). Aliquots of bacteria were stored in BHI media (MSL laboratory media room, UBC) with 10% glycerol. For each experiment, aliquots of LM were grown in 5 mL of BHI media overnight. The next day, a 1/10 dilution of LM was transferred to a new tube with fresh BHI media and grown for a further 2 h to insure that the LM was in log phase growth. The number of LM were initially estimated by optical density measurements according to a standard growth curve and subsequently quantitated by colony counts of serial dilutions of the log phase stock. 2-5 x 10^5 CFUs/mouse of LM were calculated, centrifuged and resuspended in 100 µL of PBS. Sex-matched, age-matched ABCF1+-/- mice and their sex-matched, age-matched wild-type controls were inoculated by inter-venous injection with 2-5 x 10^5 CFU of LM 10403s. The mice were monitored daily for weight loss and signs of morbidity including ruffled fur and lethargy for 7 days.
4.3.9 Clearance of ovalbumin expressing *Listeria monocytogenes* (LM-OVA)

To assess whether ABCF1 +/- macrophages are capable of clearing LM bacteria from their spleens, LM-OVA was injected into mice and clearance of the bacteria was monitored. LM-OVA which is a recombinant form of LM containing a coding sequence for ovalbumin, as previously described, was acquired from Dr. Priatel (UBC) [21]. Cultures of LM-OVA were grown as described above and age matched, sex matched ABCF1 +/- mice and their wild-type controls were injected with 1 x 10^5 CFU in 100 µL of PBS iv. The mice were sacrificed by CO2 asphyxiation and their spleens were aseptically removed. The spleens were mashed through 40 µm cell strainers and the RBCs were lysed (as described above). The splenocytes were centrifuged and resuspended in 1 mL of PBS containing 0.1% NP-40 to lyse the splenocytes. After a 30 min incubation on ice, serial dilutions of splenocytes were made in PBS containing 0.1% NP-40. 100 µL of each of the splenocyte dilutions was plated in triplicate and incubated over night at 37°C. The next day the colonies of LM-OVA were counted and averaged (using plates containing between 30-300 colonies). The number of viable bacteria was then calculated for the entire spleen.

4.3.10 Statistical analysis

Statistical analysis of the spleen cell numbers, CBC data and the immunostaining was performed with GraphPad Prisim 5.00 (GraphPad Software, San Diego CA, USA) software for Windows using a Student’s t test. The statistical analysis of the ELISA, CBA cytokine data and LM-OVA colony counts was performed with GraphPad Prisim
5.00 using a two-way ANOVA with Bonferroni post-tests. Survival curves were created in GraphPad Prisim 5.00 and compared using a Log-rank (Mantel-Cox) test and a Gehan-Breslow-Wilcoxon Test. Error bars represent standard deviations.

The data were considered statistically significant if p< 0.05. *p<0.05, **p<0.01, ***p<0.001.
4.4 Results

4.4.1 ABCF1 deficient knock-out mice have fewer monocytes and splenocytes and more apoptosis in their spleens

Since very little is known about the effect of an ABCF1 deficiency on a living organism, ABCF1 +/- mice were created from the Baygenomics ES cell line XKO97 (previously described, Chapter 2). ABCF1-/- exhibited embryonic lethality while ABCF1+/+ mice appeared indistinguishable from their wild-type littermates in terms of outward appearance, life expectancy, nesting and nursing ability and complete mouse necropsies. To further characterize ABCF1 deficient mice at the cellular level, CBC analysis was performed on female and male ABCF1+/- mice. There were serological differences between sexes, therefore, females and males were analysed separately. Although there were mild variations amongst mice, ABCF1+/- had similar CBC populations for most of the parameters as their wild-type littermate controls. The only exception was in the blood monocyte population. Both female and male ABCF1+/- showed fewer monocytes in their blood compared to their wild-type controls (females p=0.049, males p=0.0311) (Figure 4.3).
**Figure 4.3.** ABCF1+/- mice have fewer monocytes in their blood.

Whole blood from ABCF1+/- mice or their ABCF1+/+ littermate controls was analysed in duplicate on the Hemavet 950 automated blood analyser. Females and males had differences in their complete blood counts therefore were analysed individually. Both female and male heterozygous mice showed fewer monocytes in their whole blood. *p<0.05.
Spleens were also dissected from ABCF1+/- mice and their wild-type littermate controls and their cell populations were analysed. While preparing splenocytes we observed that the spleens of ABCF1+/- mice contained 17% fewer cells than the ABCF1+/+ mice (p=0.0008) (Figure 4.4).

Figure 4.4. ABCF1+/- mice have fewer live cells in their spleens. Spleens from ABCF1+/- (n=5) or their wild-type littermate controls (n=5) were made into single cell suspensions and counted. ABCF1+/- mice consistently had fewer splenocytes than their littermates. *-p<0.001. Data was compiled from three different experiments.
To examine the histological differences in ABCF1+/- mice, spleens from ABCF1+/- mice and their wild-type littermates were aseptically dissected out, sectioned and H&E stained. ABCF1+/- spleens appeared to have a greater number of multifocal basophilic nuclear fragments in their follicles (observed by Dr. H. Adissu, Toronto Centre for Phenogenoics) (Figure 4.5). Increased apoptosis in the spleens of ABCF1+/- mice was further supported by TUNEL staining (Figure 4.6) and later confirmed by the FACs analysis of splenocytes stained with APC-annexin-V antibodies and PI (Figure 4.7). Some of the macrophages in the spleen of the ABCF1+/- mice were observed to contain phagocytosed cell debris thus indicating that the phagocytosis function of the ABCF1+/- macrophages was not impaired (Figure 4.8).
Figure 4.5. H/E staining of splenic follicles from ABCF1+/- mice and their wt controls.

H/E staining of paraffin embedded and sectioned spleens of ABCF1+/- mice and their ABCF1+/+ littermate controls showing multifocal deeply basophilic stained nuclear fragments in the splenic follicles. Black arrows indicate representative darkly stained nuclear debris. 40x objective.
Figure 4.6. TUNEL staining shows that there is more apoptosis in the spleens of ABCF1+/- mice.
A) Spleens from ABCF1 +/- and their ABCF1+/+ littermates were paraffin embedded and sectioned then stained with TUNEL. B) The graph represents the number of TUNEL positive cells in 15 random equivalent sized fields of view that were counted in the ABCF1+/- and ABCF1+/+ spleens using a 40x objective. Black arrows show representative TUNEL positive cells. *-p<0.001.
Figure 4.7. Annexin-V staining confirms that there is more apoptosis in the spleens of ABCF1+/- mice compared to their wt littermate controls. Splenocytes from three ABCF1 +/- and three ABCF1+/+ littermates were pooled and stained with APC-annexin-V antibodies and PI then analysed by FACs. *-p<0.05. The graph represents data compiled from two separate experiments which were repeated two times.
Figure 4.8. ABCF1+/- macrophage-like cells are able to phagocytose cellular debris. H&E staining of sectioned spleens of ABCF1+/- show macrophage-like cells with densely stained debris within their vacuoles as indicated by black arrows. 100x objective. Representative image.
4.4.2 Splenic macrophages from ABCF1+/- produced significantly more cytokines than than their wild-type littermate controls when stimulated with different TLR ligands

We next went on to evaluate the cytokine production profile of ABCF1+/- spMØ that were activated using TLR ligands. We analysed the production of IL-6 and TNFα (pro-inflammatory, acute phase response regulating cytokines), MCP-1 (pro-inflammatory chemokine involved in monocyte/macrophage recruitment), IFNγ (pro-inflammatory, intercellular bacteria and anti-viral response TH1 cytokine), IL-12p70 (pro-inflammatory TH1 cytokine) and IL-10 (anti-inflammatory cytokine) [22-24].

ABCF1+/- spMØ produced considerably more IL-6 (p<0.001) and TNFα (p<0.001) in response to LPS activation than their wt controls (Figure 4.9). There was no significant difference in the IL-10, MCP-1, IFNγ nor IL-12p70 production by ABCF1+/- spMØ compared to the ABCF1+/+ spMØ in response to LPS. ABCF1+/- spMØ produced less IL-6 (p<0.05) and TNFα (p<0.05) when stimulated with peptidoglycan (Figure 4.10). They produced similar amounts of IL-10, MCP-1, IFNγ, and IL-12p70 as the wild-type spMØ with a trend towards slightly higher concentrations of cytokines at the higher doses of peptidoglycan, although the results were not significant. The TNFα production with peptidoglycan challenge was only significantly lower at the lower doses. At the higher dose, the ABCF1+/- spMØ tended to produce more TNFα that their controls although the results were not significant. When stimulated with CpG DNA, ABCF1 +/- spMØ produced more IL-6 (p<0.01) and TNFα (p<0.05) than their controls
(Figure 4.11). They produced similar IL-10 and MCP-1 as the ABCF1 +/+ spMØ and neither ABCF1 +/- spMØ nor ABCF1 +/+ spMØ produced detectable IFNγ and IL-12p70 in response to CpG DNA. Stimulation of ABCF1+/− spMØ with flagellin produced an augmented IL-6 response (p<0.05) (Figure 4.12). The ABCF1+/− spMØ showed a similar trend (although not significant) in the production of TNFα particularly at the lower dosages. There was no difference in the production of IL-10, MCP-1, IFNγ, and IL-12p70 between the spMØ from the heterozygous mice as compared to their littermates. Finally, stimulation with poly I:C produced slightly more MCP-1 (p<0.05), IFNγ (p<0.01) and IL-12p70 (p<0.05) than the ABCF1+/+ controls (Figure 4.13). They also showed a trend towards slightly higher production of IL-6, IL-10 and TNFα, but the values were not significant. The cytokine production in response to poly I:C, however, was at the lower limit of the CBA kit with all of the cytokines other than TNFα. This suggests that splenic macrophages are not as sensitive to stimulation with poly I:C and the results represent a trend rather than a significant increase in cytokine production. Together these data suggests that ABCF1 is responsible for negatively regulating the TNFα/IL-6 response to TLR ligands in spMØs.
Figure 4.9. ABCF1 +/- spMØ produce significantly more IL-6 and TNFα than their wild-type controls when stimulated with LPS.

CD11b, F4/80 positive cells were sorted from four ABCF1+/- and four ABCF1+/-+ spleens. 5x10⁴ cells were plated per well in a 96 well, plated and stimulated for 18h with different concentrations of LPS. “0” represents the negative non-stimulated control. Cytokine concentrations were determined using the CBA Mouse Inflammation Kit. **p<0.01, ***p<0.001. Data shown represents the results of one experiment, which was repeated five times with similar results.
Figure 4.10. ABCF1 +/- spMØ produce less IL-6 than their wild-type controls when stimulated with peptidoglycan. Cells were stimulated for 18h with different concentrations of peptidoglycan. “0” represents the negative non-stimulated control. Cytokine concentrations were determined using the CBA Mouse Inflammation Kit. *-p<0.05. Data shown represents the results of one experiment, which was repeated three times with similar results.
Figure 4.11. ABCF1 +/- spMØ produce significantly more IL-6 and TNFα than their wild-type controls when stimulated with CpG DNA. Cells were stimulated for 18h with different concentrations of CpG DNA. “0” represents the negative non-stimulated control. Cytokine concentrations were determined using the CBA Mouse Inflammation Kit. *p<0.05 and **p<0.01. Data shown represents the results of one experiment, which was repeated three times with similar results.
Figure 4.12. ABCF1 +/- spMØ produce more IL-6 than their wild-type controls when stimulated with flagellin.
Cells were stimulated for 18h with different concentrations of flagellin. “0” represents the negative non-stimulated control. Cytokine concentrations were determined using the CBA Mouse Inflammation Kit. *-p<0.05 . Data shown represents the results of one experiment, which was repeated three times with similar results.
Figure 4.13. ABCF1 +/- spMØ produce slightly more MCP-1, IFNγ and IL-12p70 than their wild-type controls when stimulated with poly I:C. Cells were stimulated for 18h with different concentrations of poly I:C. “0” represents the negative non-stimulated control. Cytokine concentrations were determined using the CBA Mouse Inflammation Kit. *-p<0.05 and **-p<0.01. Data shown represents the results of one experiment, which was repeated three times with similar results.
4.4.3 ABCF1+/- mice produce unique responses to sublethal doses of LPS

The dramatic response to LPS by spMØ was striking. Therefore, we wanted to test whether LPS could have similar effects on cytokine responses in vivo. Serum and peritoneal cytokine responses to sub-lethal LPS challenge were evaluated. Based on experiments done by other groups, the LD$_{50}$ of LPS in C57/B6 mice was found to be between 1000 and 500 µg when injected ip [25]. ABCF1+/- mice were challenged with a range (0.1-500 µg) of sublethal LPS doses by ip injections. ABCF1+/- mice were found to be less active after challenge with as low as 100 µg of LPS. Mice receiving 10 µg doses could not be distinguished from the mock injected mice (PBS controls). Early cytokine responses after LPS challenge have been well documented and are known to occur hours after administration [25]. TNF$\alpha$, IL-1$\beta$, IL-6 have all been shown to increase shortly after the challenge and are thought to be imported mediators of endotoxin-related death [25]. Cytokine responses of mice receiving sub-lethal (either 10 or 100 µg) doses of LPS were evaluated. In this model, ABCF1+/- mice produced similar IL-6 (Figure 4.14), MCP-1 (Figure 4.16) and IL 12p70 (Figure 4.19) sera responses as their wild-type littermate controls. Peak serum concentrations of IL-6 were found to decrease incrementally with the dose of LPS. ABCF1+/- mice produced less IL-10 (only significantly less at the lower dose (10 µg) after 1h (Figure 4.15)) and less TNF$\alpha$ ((p<0.05 only significantly less at the higher dose (100 µg) after 1 h (Figure 4.17)) than their controls. Peak serum concentrations of TNF$\alpha$ and IL-10 were found to be independent of the dose of LPS as seen by other groups [25]. ABCF1+/- mice produced more IFN-$\gamma$ than their wild-type littermate controls though only significantly more
(p<0.01) at the lower dose of LPS (Figure 4.18). Finally, ABCF1 +/- mice produced significantly more IL-1β (p<0.01) than their littermate controls 6 h after receiving 100 µg of LPS (Figure 4.20). At the lower dose of LPS similar amounts of IL-1β were produced.
Figure 4.14. ABCF1 +/- and ABCF1+/+ mice produce similar serum IL-6 responses to LPS challenge.
ABCF1+/- mice and their littermate controls were challenged with 100 µg (left column) or 10 µg (right column) of LPS ip. Individual mice were sacrificed at various time points and their blood was extracted by cardiac puncture. Serum was isolated from whole blood and was analyzed for cytokine production by CBA Mouse Inflammation Kit analysis and Mouse IL-1β/IL-1F2 Immunoassay Quantikine kit analysis. Error bars represents the standard error of the mean of the cytokine concentrations from 3 mice for each group (ABCF1+/- and ABCF1+/+). Data represents the result of one experiment which was repeated at one time with similar results.
Figure 4.15. ABCF1 +/- mice produce less IL-10 in response to LPS challenge than their ABCF1+/+ littermates.

ABCF1 +/- mice and their littermate controls were challenged with 100 µg (left column) or 10 µg (right column) of LPS ip. Individual mice were sacrificed at various time points and their blood was extracted by cardiac puncture. Serum was isolated from whole blood and was analyzed for cytokine production by CBA Mouse Inflammation Kit analysis and Mouse IL-1β/IL-1F2 Immunoassay Quantikine kit analysis. Error bars represents the standard error of the mean of the cytokine concentrations from 3 mice for each group (ABCF1+- and ABCF1+/+). **-p<0.01. Data represents the result of one experiment which was repeated at one time with similar results.
Figure 4.16. ABCF1 +/- and ABCF1+/+ mice produce similar serum MCP-1 responses to LPS challenge.

ABCF1+/- mice and their littermate controls were challenged with 100 µg (left column) or 10 µg (right column) of LPS ip. Individual mice were sacrificed at various time points and their blood was extracted by cardiac puncture. Serum was isolated from whole blood and was analyzed for cytokine production by CBA Mouse Inflammation Kit analysis and Mouse IL-1β/IL-1F2 Immunoassay Quantikine kit analysis. Error bars represents the standard error of the mean of the cytokine concentrations from 3 mice for each group (ABCF1+-/ and ABCF1+/+). Data represents the result of one experiment which was repeated at one time with similar results.
Figure 4.17. ABCF1 +/- mice produce less TNFα in response to LPS challenge than their ABCF1+/+ littermates.

ABCF1+/- mice and their littermate controls were challenged with 100 µg (left column) or 10 µg (right column) of LPS ip. Individual mice were sacrificed at various time points and their blood was extracted by cardiac puncture. Serum was isolated from whole blood and was analyzed for cytokine production by CBA Mouse Inflammation Kit analysis and Mouse IL-1β/IL-1F2 Immunoassay Quantikine kit analysis. Error bars represents the standard error of the mean of the cytokine concentrations from 3 mice for each group (ABCF1+/- and ABCF1+/+). *-p<0.05. Data represents the result of one experiment which was repeated at one time with similar results.
Figure 4.18. ABCF1 +/- and ABCF1+/+ mice produce similar serum IFN-γ responses to LPS challenge.

ABCF1+/- mice and their littermate controls were challenged with 100 µg (left column) or 10 µg (right column) of LPS ip. Individual mice were sacrificed at various time points and their blood was extracted by cardiac puncture. Serum was isolated from whole blood and was analyzed for cytokine production by CBA Mouse Inflammation Kit analysis and Mouse IL-1β/IL-1F2 Immunoassay Quantikine kit analysis. Error bars represents the standard error of the mean of the cytokine concentrations from 3 mice for each group (ABCF1+/- and ABCF1+/+). Data represents the result of one experiment which was reproduced two separate times.
Figure 4.19. ABCF1 +/- and ABCF1+/+ mice produce similar serum IL-12p70 responses to LPS challenge.
ABCF1+/- mice and their littermate controls were challenged with 100 µg (left column) or 10 µg (right column) of LPS ip. Individual mice were sacrificed at various time points and their blood was extracted by cardiac puncture. Serum was isolated from whole blood and was analyzed for cytokine production by CBA Mouse Inflammation Kit analysis and Mouse IL-1β/IL-1F2 Immunoassay Quantikine kit analysis. Error bars represents the standard error of the mean of the cytokine concentrations from 3 mice for each group (ABCF1+-/ and ABCF1+/+). Data represents the result of one experiment which was repeated at one time with similar results.
Figure 4.20. ABCF1 +/- mice produce more IL-1β in response to LPS challenge than their ABCF1+/+ littermates.

ABCF1 +/- mice and their littermate controls were challenged with 100 µg (left column) or 10 µg (right column) of LPS ip. Individual mice were sacrificed at various time points and their blood was extracted by cardiac puncture. Serum was isolated from whole blood and was analyzed for cytokine production by CBA Mouse Inflammation Kit analysis and Mouse IL-1β/IL-1F2 Immunoassay Quantikine kit analysis. Error bars represents the standard error of the mean of the cytokine concentrations from 3 mice for each group (ABCF1+/+ and ABCF1+/-). **p<0.01. Data represents the result of one experiment which was repeated at one time with similar results.
The cytokine concentrations in the peritoneal fluid of mice receiving sub-lethal LPS injections were also evaluated. ABCF1+/- mice produced similar IL-6 responses to LPS as their ABCF1+/+ littermates (Figure 4.21). The IL-6 production at the higher (100 µg) dose of LPS was slightly higher in the ABCF1+/- mice than the control where as at a lower dose (10 µg) of LPS there appeared to be less of an IL-6 response to LPS. The IL-10 cytokine production in the peritoneal fluid showed that the ABCF1+/- mice produced significantly less IL-10 (p<0.05) at both doses of LPS (Figure 4.22). Like the serum, the peritoneal fluid IL-10 concentrations appeared to be independent of the dose of LPS administered. ABCF1+/- mice produced less MCP-1 in response LPS, though only significantly less at the lower dose (p<0.01) (Figure 4.23). ABCF1+/- mice produced statistically similar concentrations of TNFα in the peritoneal fluid (Figure 4.24). The trend however, matched the serum in that at both doses of LPS resulted in slightly lower TNFα concentrations in the ABCF1+/- mice when compared to the ABCF1+/+ mice. Similar to the serum, the INFγ concentration in the peritoneal fluid appeared to be higher in the ABCF1+/- mice when compared to the controls 6h after stimulation with both doses of LPS, however these results did not reach statistical significance (Figure 4.25). IL-12p70 and IL-1β were not detectable in the peritoneal fluid after LPS challenge and were probably secreted at very low concentrations. Together these data suggest that although ABCF1+/- tissue derived macrophages are hyper-responsive to LPS, ABCF1+/- mice do not produce an exaggerated TNFα and IL-6 response to LPS challenge. This suggests that either the recruitment of the macrophages from the bone marrow to the circulation or to the peritoneal cavity is inhibited or that the macrophages that are recruited aren’t properly matured.
Figure 4.21. ABCF1 +/- and ABCF1++/+ mice produce similar concentrations of IL-6 in their peritoneal cavity in response to LPS challenge.
ABCF1+/- mice and their littermate controls were challenged with 100 µg (left column) or 10 µg (right column) of LPS ip. Peritoneal washes were done on LPS challenged ABCF1+/- mice and their littermate controls. Cytokine production was analysed from the peritoneal fluid through CBA Mouse Inflammation Kit analysis. Error bars represents the standard error of the mean of the cytokine concentrations from 3 mice for each group (ABCF1+/- and ABCF1++/+). Data represents the result of one experiment which was repeated at one time with similar results.
Figure 4.22. ABCF1 +/- produce less IL-10 in their peritoneal cavity in responses to LPS challenge than their ABCF1+/+ littermate controls.

ABCF1 +/- mice and their littermate controls were challenged with 100 µg (left column) or 10 µg (right column) of LPS ip. Peritoneal washes were done on LPS challenged ABCF1 +/- mice and their littermate controls. Cytokine production was analysed from the peritoneal fluid through CBA Mouse Inflammation Kit analysis. Error bars represents the standard error of the mean of the cytokine concentrations from 3 mice for each group (ABCF1 +/- and ABCF1+/+). *-p<0.05. Data represents the result of one experiment which was repeated at one time with similar results.
Figure 4.23. ABCF1 +/- produce less MCP-1 in their peritoneal cavity in responses to LPS challenge than their ABCF1+/+ littermate controls.
ABCF1 +/- mice and their littermate controls were challenged with 100 µg (left column) or 10 µg (right column) of LPS ip. Peritoneal washes were done on LPS challenged ABCF1 +/- mice and their littermate controls. Cytokine production was analysed from the peritoneal fluid through CBA Mouse Inflammation Kit analysis. Error bars represents the standard error of the mean of the cytokine concentrations from 3 mice for each group (ABCF1 +/- and ABCF1+/+). **p<0.01. Data represents the result of one experiment which was repeated at one time with similar results.
Figure 4.24. ABCF1 +/- and ABCF1+/+ mice produce similar concentrations of TNFα in their peritoneal cavity in response to LPS challenge.

ABCF1+/- mice and their littermate controls were challenged with 100 µg (left column) or 10 µg (right column) of LPS ip. Peritoneal washes were done on LPS challenged ABCF1+/- mice and their littermate controls. Cytokine production was analysed from the peritoneal fluid through CBA Mouse Inflammation Kit analysis. Error bars represents the standard error of the mean of the cytokine concentrations from 3 mice for each group (ABCF1+/- and ABCF1+/+). Data represents the result of one experiment which was repeated at one time with similar results.
Figure 4.25. ABCF1 +/- and ABCF1+/+ mice produce similar concentrations of IFN-γ in their peritoneal cavity in response to LPS challenge.

ABCF1+/- mice and their littermate controls were challenged with 100 µg (left column) or 10 µg (right column) of LPS ip. Peritoneal washes were done on LPS challenged ABCF1+/- mice and their littermate controls. Cytokine production was analysed from the peritoneal fluid through CBA Mouse Inflammation Kit analysis. Error bars represents the standard error of the mean of the cytokine concentrations from 3 mice for each group (ABCF1+/- and ABCF1+/+). Data represents the result of one experiment which was repeated at one time with similar results.
4.4.4 Bone marrow derived macrophages are hypo-responsive to stimulation with TLR ligands

Since the *in vivo* response to LPS was not as robust as expected, cytokine profiles for TLR ligand stimulated ABCF1+/- BMMφ were also evaluated. ABCF1+/- BMMφ produced significantly less IL-6 (p<0.01 (lower dose) and p<0.05 (higher dose)) MCP-1 (p<0.001) and TNFα (p<0.05) than their wild-type controls in response to LPS (Figure 4.26). The production of IL-10 was statistically similar from the heterozygous and the wild-type BMMφ though the trend was that the ABCF1+/+ BMMφ produced higher concentrations of IL-10. When stimulated with peptidoglycan, ABCF1+/- BMMφ produced significantly less IL-6 (p<0.05), MCP-1 (p<0.001 (lower dose) and p<0.05 (higher dose)) and TNFα (p<0.05) than the ABCF1+/+ BMMφ (Figure 4.27). BMMφ from ABCF1+/- and ABCF1+/+ mice produced similar concentrations of IL-10 when challenged with peptidoglycan. Stimulation of ABCF1+/- BMMφ with CpG DNA resulted in the production of significantly less IL-6 (p<0.001), IL-10 (p<0.01), MCP-1 (p<0.001) and TNFα (p<0.05) that the ABCF1+/+ BMMφ (Figure 4.28). ABCF1+/- BMMφ produced less IL-6 (p<0.05), MCP-1 (p<0.01) and TNFα (p<0.05) than the ABCF1+/+ BMMφ in response to flagellin (Figure 4.29). The IL-10 production was similar from the ABCF1+/- and the wild-type BMMφ after challenge with flagellin. Finally, ABCF1+/- BMMφ produced significantly less MCP-1 (p<0.001) than the ABCF1+/+ BMMφ in response to poly I:C (Figure 4.30). They produced significantly less TNFα (p<0.05) at lower doses of poly I:C while at higher doses they produced similar TNFα. ABCF1+/- BMMφ produced statistically similar IL-6 and IL-10 concentrations as their controls with a trend to higher cytokine production with higher
doses of poly I:C. No detectable IFNγ or IL-12p70 was ever produced from the BMMφs even at high cell concentrations. Together this would indicate that the phenotype of the BMMφ is significantly different from the phenotype of the spMØ. Since BMMφ need to undergo differentiation in order to become mature macrophages. These data suggest that ABCF1+/− BMMφ are delayed in their ability to mature when compared to the ABCF1 +/+ BMMφ.
Figure 4.26. ABCF1 +/- BMMφ produce significantly less IL-6, MCP-1 and TNFα than their wild-type controls when stimulated with LPS.
Bone marrow was extracted from three ABCF1+/- mice and three littermate controls. The bone marrow was pooled and plated for six days in L929-cell conditioned media. The bone marrow was then counted and replated at 1 x 10^5 cells per well in a 48 well plate. After the cells were reattached to the plate, they were stimulated with various concentrations of LPS for 18 h. Supernatants were analysed by CBA Mouse Inflammation Kit analysis. Error bars represent replicate supernatants. *p<0.05, **p<0.01 and ***p<0.001. Data represents the results from one experiment which was done three separate times with similar results.
Figure 4.27. ABCF1 +/- BMMφ produce significantly less IL-6 and TNFα than their wild-type controls when stimulated with peptidoglycan.
Cells were prepared as described above and were stimulated with various concentrations of peptidoglycan for 18 h. Supernatants were analysed by CBA Mouse Inflammation Kit analysis. *p<0.05 and ***p<0.001. Data represents the results from one experiment which was done three separate times with similar results.
Figure 4.28. ABCF1 +/- BMMφ produce significantly less IL-6, IL-10 and TNFα than their wild-type controls when stimulated with CpG DNA. Cells were prepared as described above and stimulated with various concentrations of CpG DNA for 18 h. Supernatants were analysed by CBA Mouse Inflammation Kit analysis. *p<0.05, **p<0.01 and ***p<0.001. Data represents the results from one experiment which was done three separate times with similar results.
Figure 4.29. ABCF1 +/- BMMφ produce significantly less IL-6 and TNFα than their wild-type controls when stimulated with flagellin.
Cells were prepared as described above and were stimulated with various concentrations of flagellin for 18 h. Supernatants were analysed by CBA Mouse Inflammation Kit analysis. *p<0.05 and **p<0.01. Data represents the results from one experiment which was done three separate times with similar results.
Figure 4.30. ABCF1 +/- BMMφ produce less MCP-1 and TNFα when stimulated with poly I:C.
Cells were prepared as described above and were stimulated with various concentrations of poly I:C for 18 h. Supernatants were analysed by CBA Mouse Inflammation Kit analysis. *p<0.05 and ***p<0.001. Data represents the results from one experiment which was done three separate times with similar results.
4.4.5 ABCF1+/- mice have an impaired response to LM

Since X-gal staining indicated that the ABCF1 promoter appeared to be active in marginal zone of the spleen (Chapter 3), we were interested in determining whether the ABCF1+/- mice were immunocompromised in an LM infection model. Marginal zone macrophages and metalophillic macrophages have been found to be important in trapping LM and priming a T cell response to infection [26]. The LD\textsubscript{50} of LM 10403s was previously determined as $2 \times 10^5$ CFU in C57/B6 mice [27]. Groups of 7 or more mice (both ABCF1+/- and ABCF1+/+) were inoculated with $2-5 \times 10^5$ CFU of LM iv and monitored for 8 days. ABCF1+/- mice were found to be more susceptible to infection with LM compared to ABCF1+/+ mice (p=0.02) (Figure 4.31). ABCF1+/- mice also appeared more symptomatic in response to LM infection compared to the controls. They presented ruffled fur and eye discharge at earlier time points post injection. In many cases morbidity was determined before they dropped to their target weights (lost 20% of their original weight).
Figure 4.31. ABCF1 +/- mice succumb to infection by LM 10403 sooner than the ABCF1+/+ mice.
Male ABCF1 +/- mice were injected with 2-5 x 10^5 colony forming units of LM 10403 iv. Mice were monitored for 7 days. Criteria for euthanasia was weight loss in excess of 20% of their body weight or if they presented signs of illness (lethargy, discharge from their eyes or nose). ABCF1 +/- mice appeared much sicker than the wild-type controls and were more likely to show symptoms of illness early after infection. At the end of the experiment two of the wild-type mice survived the infection based on the fact that they didn’t lose 20% of their body weight and began to gain weight. The results are pooled from five separate experiments using 23 ABCF1+/+ and 26 ABCF1+/- mice and analysed using a Log-Rank test (p=0.244) and a Gehan-Breslow-Wilcoxon Test (p=0.102).
4.4.6 The effect of the ABCF1 deficiency on the growth of LM-OVA in the spleen

To determine whether the ABCF1 deficiency affected the ability of the spleen to clear bacteria, mice were challenged with LM-OVA and the numbers of viable bacteria in their spleens was monitored post-injection (Figure 4.32). LM-OVA bacteria (a less virulent strain of LM expressing ovalbumin antigen) were injected into ABCF1+/- mice and their wild-type littermate controls at $1 \times 10^5$ CFU per mouse. The mice were sacrificed at days 1, 2, 3 and 4 post infection and the numbers of viable bacteria were determined in their spleens. Splenic bacterial counts increased over the first 2 days then drastically fell by day 3, indicative of the attenuated strain of LM-OVA. The ABCF1+/- mice were found to be as effective as their wild-type littermates at clearing the bacterial load indicating that ABCF1 is not required for the killing and clearing of LM-OVA by macrophages. By day 2, ABCF1+/- mice infected with LM-OVA showed symptoms of sickness including eye discharge and ruffled fur while none of their wild-type littermates had any signs of sickness or discomfort.
Figure 4.32. ABCF1+/- mice as capable of clearing LM-OVA infections as their ABCF1 +/- littermates. ABCF1+/- mice along with their controls were injected with 1x10^5 CFU of LM-OVA iv. Mice were sacrificed 1, 2, 3 and 4 days after infection. Spleens were removed, mashed and plated on BHI plates at 1x10^1, 1x 10^2, 1x 10^3 and 1x10^4 dilutions in duplicates. Three mice were analyzed per group per day. Total numbers of bacteria were calculated based on the averaged numbers of colonies. Data represents the results of one experiment that was repeated three times.
4.5 Discussion

ABCF1 is ubiquitously expressed and plays a role in development as well as in inflammatory processes. The precise function of ABCF1 in these processes however remains unclear. Earlier work showed that the ABCF1 gene is upregulated by TNFα in synoviocytes [6]. In the present study we focused on the role that ABCF1 plays in the macrophage populations. We were initially interested in looking at the macrophage populations when we previously found that the ABCF1 promoter was active in the spleen marginal zone (Chapter 3). We went on to determine that ABCF1+/− mice have fewer blood monocytes and more apoptotic cells in their spleens, indicating that may be a macrophage deficiency in these mice (Chapter 3). H&E stained sections of spleens showed some macrophages which were in the process of phagocytosing dead cell debris demonstrating that the resident macrophages were capable of phagocytosis (Chapter 3). In this study we wanted to determine whether macrophages in ABCF1+/− are functionally able to respond to stimuli by analyzing the cytokine producing ability of the different macrophage populations.

We determined that ABCF1 plays an important role in the regulation of proinflammatory mediators by spMØ in response to TLR ligands. ABCF1+/− spMØs were found to be hyper-responsive to LPS and CpG DNA, producing far more IL-6 and TNFα (acute phase response regulating cytokines) than their wild-type controls. The ABCF1+/− spMØs produced similar concentrations of: TH1 cytokines (IL-12p70 and IFNγ), MCP-1 (monocyte/macrophage chemoattractant) and IL-10 (anti-inflammatory cytokine) than their ABCF1+/+ littermates in response to LPS, CpG DNA, flagellin, peptidoglycan and poly I:C (as the concentrations were barely detectable by the CBA
analysis). This is significant as TNFα expression is involved in upregulating IL-6 expression and both TNFα and IL-6 are responsible for local and systemic inflammation seen in autoimmune disorders particularly RA [28, 29].

The overexpression of TNFα is often due to a loss of negative regulation and an increase in mRNA stability [10]. ARE sequence elements in the mRNA along with TIA-1/TIAR have been shown to be important in the repression of TNFα expression in resting macrophages and in regulation of TNFα expression in activated macrophages [18]. Since eIF2 phosphorylation is important for the TIA-1/TIAR repression it is possible that ABCF1 is also involved in this translational control.

To further assess the consequence of the dysregulation of cytokine production in the ABCF1+/- mice, we injected mice with LPS ip and analysed the sera and peritoneal fluid hours after challenge. We found surprisingly, that the ABCF1+/- mice were not hyper-responsive to LPS. They did however have slightly different cytokine profiles when compared to their wild-type littermates, with increases in sera and peritoneal IFNγ and sera IL-12p70 and decreases in sera and peritoneal IL-10 and TNFα. The peritoneal wash also revealed reduced MCP-1 and variable IL-6 concentrations depending on the dose of LPS administered. We previously showed that there was a reduction in the number of monocytes in the blood of the ABCF1+/- mice suggesting that the lack of response may be due to the lack of monocytes recruitment to the site of infection. Another possibility is that the monocytes that were recruited were not fully matured into functional macrophages. Microarray data has shown that ABCF1 mRNA increases during the differentiation of monocytes to macrophages [30]. Previous groups have also shown that although monocytes and macrophages produce similar TNFα mRNA, fully differentiated macrophages express and secrete significantly more TNFα protein [31].
One explanation for this is that ABCF+/- monocytes may express lower levels of CSF-1R like heme-regulated eIF2α kinase (HRI) deficient monocytes. HRI is a stress response gene that is known to phosphorylate the alpha subunit of the eIF2 (the GCN20 complex phosphorylates the same Ser 51 residue) when iron is scarce. Upon phosphorylation, the eIF2 halts cellular translation to reduce globin and heme synthesis. HRI -/- macrophages were also found to have impaired maturation along with reduced cytokine production in response to LPS in BMMφ and in LPS challenged mice [17]. The eIF2α phosphorylation thus was found to be important for the expression of the CSF-1R and thus maturation of BMMφ [17]. ABCF1 through its association with eIF2 could potentially be another regulatory point in this pathway. It is also important to note that CSF-1 plays an important role in determining the inflammatory response in phagocytes [32]. IL-6 expression is dependent on complex interactions between CSF-1, GM-CSF and TNFα so if the gene expression of these components is interrupted, IL-6 expression would be reduced as well [32]. Since ABCF1+/- is known to be associated with the eIF2 it is possible that ABCF1 supports or stabilizes the phosphorylated state of the eIF2 [15]. We determined that BMMφ from ABCF1+/- mice are also hypo-responsive to TLR stimulation by various TLR ligands. Together these data indicate that ABCF1 exerts different effects on the cytokine responses of different subsets of macrophages and may also be important for the maturation of macrophages from bone marrow (summarized in Table 4.1 and 4.2).
Table 4.1. Summary of the differences in the cytokine production from ABCF1+/- macrophages compared to their ABCF1+/+ littermate controls upon treatment with TLR ligands.

$p$ values are indicated as follows: *-$p<0.05$, **-$p<0.01$ and ***-$p<0.001$ along with whether the cytokine concentration is increased (+) or decreased (-) compared to the wild-type mice. 0 represents no significant differences and n/a represents cytokine concentrations that were too low to detect by the CBA kit. IL-6 and TNF$\alpha$ are pro-inflammatory cytokines responsible for the acute phase response, MCP-1 is a monocyte/macrophage chemoattractant chemokine, IFN$\gamma$ and IL-12p70 are cytokines that result in T$\text{H}_1$ responses and IL-10 is an anti-inflammatory cytokine.

<table>
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<th>TNF$\alpha$</th>
<th>MCP-1</th>
<th>IFN-$\gamma$</th>
<th>IL-12p70</th>
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n/a
Table 4.2. Summary of the differences in the cytokine production from ABCF1+/- mice in the serum and peritoneal fluid compared to their ABCF1+/+ littermate controls upon treatment with LPS.

P values are indicated as follows: *-p<0.05 and **-p<0.01 along with whether the cytokine concentration is increased (+) or decreased (-) compared to the wild-type mice. 0 represents no significant difference and n/a represents cytokine concentrations that were too low to detect by the CBA kit. IL-6, TNFα and IL-1β are pro-inflammatory cytokines responsible for the acute phase response, MCP-1 is a monocyte/macrophage chemoattractant chemokine, IFNγ and IL-12p70 are cytokines that result in TH1 responses and IL-10 is an anti-inflammatory cytokine.

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<th>TNFα</th>
<th>IL-1β</th>
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<th>IFN-γ</th>
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<td>** (-)</td>
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<td>* (-)</td>
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<tr>
<td>Peritoneal Cavity</td>
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<td>0</td>
<td>** (-)</td>
<td>0</td>
<td>n/a</td>
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To further characterize the phenotype of the spMØs we set up a LM infection model. ABCF1+/- mice were found to be more susceptible to a lethal challenge with LM compared to the ABCF1+/+ mice. When challenged with a similar number of the less virulent strain LM-OVA, ABCF1+/- splenocytes were surprisingly found to be as capable as their wild-type littermates at clearing the bacteria from the spleen. This suggests that ABCF1 is not required for the trapping and killing of the bacteria. This also suggests that the phagocytosis function in macrophages in the ABCF1+/- mice is normal, as the marginal zone macrophages are required for the initial trapping and killing of LM [26]. The ABCF1 +/- mice did however appear sicker than the ABCF1+/+ after
inoculation with both LM and LM-OVA. This suggests that the pathology associated with LM infection in ABCF1+/- mice has perhaps more to do with the cytokine response to the bacteria. It is well documented that lethality from sepsis requires functional TNFα signalling [8]. Signalling through TLRs is important for the activation of NF-κB and the transcription of cytokines responsible for the inflammation associate with innate responses [33]. The signal is then further modified and fine tuned through post-transcriptional regulation in order to achieve the appropriate protein synthesis [19]. Dampening of inflammation is important in preventing unintended tissue damage.

Tissue derived macrophages are known to be heterogenous, the tissue microenvironment is very important in influencing the phenotype of macrophages from different tissues [2]. Macrophages from the lamina propria for example tend to have increased phagocytic ability while low production of pro-inflammatory cytokines [2]. The differentiation of macrophages by different growth factors is thought to be partially responsible for the heterogeneity [34]. CSF-1 BMMφ are known to produce lower levels of TNFα and IL-12p70 than GM-CSF BMMφ which showed a stronger activation of the transcription factors NF-κB and AP-1 [35]. Op/op mice, which lack expression of M-CSF-1, are known to lack certain subsets of macrophages like sera monocytes and splenic metallophilic macrophages, but have other types of tissue derived macrophages [2]. Op/op mice, like ABCF1+/- mice, are more susceptible to bacterial infection with LM and they are more resistant to challenge with LPS [36, 37].

In conclusion, cytokine production and development in macrophages appear to be linked through the complex regulation of the translational machinery. ABCF1+/- mice have deficiencies in certain populations of macrophages and have dysregulation in cytokine production due to the apparent lack of post-translational control. ABCF1,
(possibly through an association with eIF2) appears to be a key player in the development and control of cytokine producing macrophages.
4.6 References


Chapter 5: General discussion.

5.1 Summary and conclusions

The role of ABCF1 in cell functioning and mouse development was examined in this thesis. Several observation and conclusions were made about the role of ABCF1 in development:

1) The XK097 ES cell was used to create ABCF1 +/- mice with a gene trap vector inserted between exon 7 and exon 8 in the ABCF1 genomic sequence. These mice were backcrossed for 12 generations onto the inbred C57Bl/6 mouse strain.

2) After extensive inter-crossing of ABCF1+/- mice, no adult ABCF1-/- mice were ever identified.

3) ABCF1+/- mice were back-crossed onto other strains including ICR and Balb/C. Inter-crossed progeny from each of these strains were still unable to produce ABCF1-/- adult mice indicating that the lethality was not strain specific.

4) No ABCF1-/- 5.5 dpc embryos were ever recovered from timed-mated ABCF1+/- females mated to ABCF1+/- studs. Upon dissection, approximately 25% of the uteri from the pregnant females were found to lack embryos. The presence of empty placentae in the uteri indicates that the blastocysts implanted into the uterine wall before dying.

5) Approximately 25% of blastocysts from supercycled ABCF1+/- females (mated to ABCF1+/- males) were found to be ABCF1-/-.

6) An ES cell line (Clone 1) was created that is ABCF1-/-.
Together this indicates that the lethality caused by the lack of ABCF1 occurs after the blastocyst stage of development and probably occurs shortly after implantation, prior to 5.5dpc.

Several observations were also made about the promoter activity of ABCF1:
1) The ABCF1 promoter is highly active in embryos in all tissue types at varying levels throughout development.
2) The ABCF1 promoter is active in all adult tissues tested. However, the promoter activity was not universal throughout the tissue with certain regions within the organs having more promoter activity than others.
3) In the spleen and lymph nodes, the ABCF1 promoter is most highly expressed in the regions surrounding the follicles
4) The ABCF1 promoter activity in CD8+ T cells is variable depending on the development of the T cell.

Numerous observations and conclusions were made about the role of ABCF1 in macrophage development and functioning:
1) ABCF1+/- mice had fewer monocytes in their blood compared to their wild-type littermates through CBC analysis
2) The spleens from ABCF1+/- mice contained more apoptotic bodies within their splenic follicles, which was analysed using H&E and TUNEL staining and FACs
3) CD11b/F4/80 positive splenic macrophages from ABCF1+/- mice produced different cytokine profiles compared to their wild-type littermate controls when stimulated with Toll-like receptor (TLR) ligands. SpMØ produced more IL-6 and TNFα than their
controls when stimulated with LPS (TLR4) and CpG DNA (TLR9). They produced more IL-6 when stimulated with flagellin (TLR5) and more MCP-1, IFNγ and IL-12p70 when stimulated with poly I:C (TLR3).

4) Bone marrow derived macrophages on the other hand were found to be hypo-responsive to stimulation with LPS (TLR4), peptidoglycan (TLR2 and 1), CpG DNA (TLR9), flagellin (TLR5), and poly I:C (TLR3) when compared to wild-type controls.

5) ABCF1+/- mice challenged with LPS produce less serum and peritoneal IL-10. They also produced more serum IFNγ and IL-1β and less serum TNFα than their wild-type littermates.

6) ABCF1+/- mice succumbed to infection with LM quicker than the controls

7) ABCF1+/- were as capable as their controls at clearing LM-OVA infections indicating that the macrophages are equally as capable of phagocytosing and killing bacteria.

These data suggest that ABCF1 has a role in the regulation of gene expression in macrophages. The ABCF1 deficiency possibly results in a delayed maturation of both BMMφ and monocytes resulting in a reduced ability of these cells to respond to stimuli. In a subset of matured spMØ however, ABCF1 seems to be important for negatively regulating the cytokine response. These processes may not necessarily be mutually exclusive as post-translational control governs both. Since ABCF1 associates with the eIF2 it is not surprising that it would be highly regulated and function in a number of ways, depending on the environment of the cell.
5.2 General model

A model of the function of ABCF1 given the data collected to date is summarized in Figures 5.1 and 5.2. ABCF1 is associated with eukaryotic initiation factor 2 (eIF2) though its N-terminal 1-42 residues [1]. During translation initiation, ABCF1 is responsible for forming the eIF2-GTP and Met-tRNAi ternary complex [2]. ABCF1 possibly functions to conformationally stabilize this complex. The 40S ribosome, eIF3, eIF1, eIF1a and eIF5 form the PIC on mRNA. eIF4A, B and F serve to unwind the terminal secondary structure on the mRNAs 5’ end so that the pre-initiation complex (PIC) can bind [3]. Once the PIC is formed, ABCF1 is associated with ribosomes through residues in its nucleotide binding domains (NBDs) [1]. The 43S complex is now free to scan the mRNA for the start codon. Upon start codon recognition, the GTP is hydrolyzed on the eIF2 by the GTPase activating protein eIF5. Upon P, release, the eIF1 dissociates from the complex along with the other eIFs [4]. The eIF5B then allows the 60S ribosomal subunit to bind and elongation commences [5]. Once released, the eIF2-GDP binds to the guanine exchange factor (GEF) eIF2B and GDP is exchanged for GTP so that the cycle of translation initiation can re-commence. Regulation of translation initiation can occur at any of the stages of initiation. Phosphorylation of the Ser51 residue on the eIF2α is known to competitively inhibit the eIF2B GEF function thus inhibiting canonical translation [6]. The yeast ABCF1 homologue, GCN20, is known to aid in this phosphorylation step thus suggesting that ABCF1 may also have a regulatory function as well. Phosphorylated eIFα can activate stress response genes like NF-κB which result in the transcription of inflammatory mediators like TNFα and IFNγ [7]. The cytokine response is regulated though post transcriptional control by proteins such as T-cell intracellular antigen-1 (TIA-1), TIA-1-related protein (TIAR) and miRNAs which
sequester TNFα in stress granules and processing bodies. ABCF1 also appears to be important in the regulation of TNFα and IL-6 possibly through its association with eIF2 or possibly through interactions with TIA-1/TIAR and miRNAs. In mature tissue macrophages, ABCF1 appears to negatively regulate the TNFα and IL-6 expression. TNFα is (along with IL-1) is one of the primary inducing soluble mediators of IL-6 thus the dysregulation of IL-6 is probably secondary to the dysregulation of TNFα [8]. miRNAs also play important roles in development, regulate TNFα and are known to associate with eIF2 [9]. It is possible that the phenotype of the ABCF1 +/- mice has to do with an interaction between ABCF1 and the miRNAs or other components of the RNA-induced silencing complex (RISC) which would explain both the development phenotype and the activation phenotype observed in the ABCF1 +/- mice.
Figure 5.1. ABCF1 associates with eIF2 in the translation initiation and regulates TNFα in mature cells.

ABCF1 is known to associate with eIF2 and eIF2 phosphorylation is known to activate NF-κB. ABCF1 spMØ were found to produce much more TNFα than their littermates in response to LPS indicating that ABCF1 may be responsible for TNFα regulation. This regulation probably involves other post-transcriptional modifiers like miRNA, TIA-1 and TIAR. PRK (Double-stranded RNA (dsRNA)-dependent protein kinase), PEK (PKR-like ER-localized eIF2α kinase), HRI (Heme-regulated inhibitor kinase) are mammalian proteins that phosphorylate the Serine 51 residue on eIF2 (this is similar to the action of GCN20 in yeast).
Figure 5.2. ABCF1 associates with e1F2 in the translation initiation and regulates the development of cells.
ABCF1 is known to associate with eIF2 and eIF2 phosphorylation is known to activate ATF4. ATF4 associates with other transcription factors like CCAAT/enhancer binding protein (C/EBP) and CCAAT/enhancer-binding protein homologous protein (CHOP) (also involved in the LPS response) in osteoclastogenesis and granulocyte differentiation [10-12]. C/EBP is known to activate TNFα, which is probably involved with development associated apoptosis [13]. ABCF1 may regulate the delicate TNFα balance in cells required for both development (positive regulation) and cytokine response (negative regulation) thus the role of ABCF1 is essential in normal cell physiology and functioning.
5.3 Closing thoughts and future directions

5.3.1 Embryogenesis

Most of the studies on the function of ABCF1 have been done in cell-line based systems. For our study, we generated a gene-trap mouse model from an ES cell line. This approach allowed us to determine whether ABCF1 had a role in development and where the expression of ABCF1 is most prevalent. Based on our results one functional copy of ABCF1 is required for embryo development past the blastocyst stage. This result is not surprising given that ABCF1 associates with the eIF2 and has been shown to promote translation initiation [2]. It was surprising, however, that adult heterozygous mice appeared phenotypically normal in terms of their outward appearance and fertility given that translation is such a fundamentally important process. It is possible that the truncated N-terminal region is sufficient for normal translation initiation so heterozygous mice continue to translate low levels of housekeeping genes through 5’ cap-dependent translation similar to their wild-type littermates. It is only when there is a stressor present and regulation of translation is needed that the differences in the phenotype can be observed. It would be nice to know the exact stage when the ABCF1-/- embryos are not viable. The first experiment that would help to determine the cause of the lethality would be to count the cells within the ABCF1-/- blastocysts by labelling their nuclei [14]. TNFα dysregulation often leads to fewer cells in the inner cell mass which could inhibit development after attaching to the uterine wall [15]. Blastocysts are also known to be phagocytic [16]. They are capable of uptaking latex beads as early as 3.5 dpc [17]. If ABCF1-/- blastocysts are incapable of phagocytosis it may signify an inability to differentiate the trophoectoderm. The blastocysts do appear to attach to and possibly
implant in the uterine wall, as observed by the presents of empty uteri. It should be also possible to look at the morphology and protein expression of the implanted blastocysts in sectioned uteri. If there are ABCF1-/- blastocysts present at later time points 4.5-5 dpc it would suggest that the embryo did implant properly but maybe failed to further differentiate. It would also be interesting to see if the Clone 1 ABCF1-/- ES cell line is capable of differentiating further by the addition of different growth factors to the media. For example, determine whether Clone 1 would be able to differentiate into macrophages through the addition of CSF-1 and IL-3 [18]. Alternatively, chimeras could be made using Clone 1 into a ROSA26-EGFP mouse. This way tissues and cells types that are contributed from Clone 1 can be differentiated from the ROSA26-EGFP tissues by the lack of GFP and the presence of β-geo. If only GFP positive cells are found in a tissue it would suggest that only the ROSA26-EGFP cells contributed to that lineage [19]. Also, it would be interesting to study the *C.elegans* ABCF1 knock out further and determine why the lethality is occurring in the L1 larval stage.

### 5.3.2 Promoter activity and expression

The ABCF1 promoter was highly active during embryogenesis and continued to be active in mature tissues. It would be useful to further characterize the specific subsets of cells in which the ABCF1 promoter is active in the different tissues. It would also be interesting to see what happens to the promoter activity when cells or mice are challenged with a pathogen. Infections with *Leishmania donovani* for example, lead to extensive TNFα dependent remodelling of the splenic architecture particularly in the marginal zone [20]. Since the ABCF1 promoter is highly active in the marginal zone of
the spleen it would be interesting to see if the spleen remodelling can occur in mice with ABCF1 deficiencies. Also, it would be interesting to make bone marrow chimeric mice. A competitive assay could be used (injecting equal numbers of ABCF1+/- bone marrow with wild-type bone marrow) to determine the ability of the ABCF1+/- monocytes to mature in response to stimulus compared to the wild-type cells in an in vivo model. This assay could also determine whether the monocytes (in the LPS model for example) are as efficient as the wild type cells at infiltrating the peritoneal cavity in response to the LPS challenge. It would also be interesting to look at the surface markers of the monocytes in the chimera and directly compare the maturation state of the ABCF1+/- bone marrow to the ABCF1+/+ bone marrow.

5.3.3 TNFα stability

Since post-transcriptional regulation can affect a number of different systems, the hyper-responsiveness of mature macrophages appears to be countered by the hypo-responsiveness of maturing macrophages. One explanation for this is that both cell stages are linked through post-translational control [21]. In mature macrophages ABCF1 negatively regulates TNFα production (Chapter 4), while in immature macrophages ABCF1 positively regulates the expression of genes involved with maturation like CSF-1R [22]. The CSF-1 gene is known to have (AU)-rich element (ARE) sequences in its mRNA which indicate that it is regulated by miRNAs similar to the way TNFα is regulated [23]. Alternatively, it’s possible that dysregulation of TNFα in the ABCF1 +/- bone marrow cells inhibits their maturation [24]. It would be interesting to see if there was a difference in the amount of TNFα expression in the ABCF1+/- bone marrow
throughout the process of maturation to BMMφ. It would also be interesting to see if there was a difference in the TNFα mRNA stability of the ABCF1-/+ BMMφ through a mRNA stability study [25].

5.3.4 Macrophage maturation

The mononuclear phagocyte lineage involves the maturation of cells through the monoblast, promonoblast, monocyte and macrophages stages of development [26]. Cells in all of these stages are known to express CSF-1R [26]. CSF-1R is activated by CSF-1 and this activation is known to be important for the proliferation and differentiation of cells through all of the mononuclear phagocyte stages [27]. Also, inhibitors of CSF-1R signalling are known to reduce the ability of macrophages to proliferate and differentiate, as well as reduce their ability to produce TNFα [28]. Monocytes are a heterogeneous immature population in the blood and bone marrow. Macrophages in the peritoneal cavity are known to be in different stages of maturation [29]. Together this suggests that the phenotype observed in the ABCF1+/+ mice and ABCF1+- BMMφ is possibly due to a delay in the ability to mature these cells. It’s interesting that the phenotype of the ABCF1+/- BMMφ and the ABCF1+- mice to LPS treatment is identical to the phenotype of the HRI-/- mice (which lack the ability to phosphoylate the eIF2α) [22]. This further strengthens the argument that ABCF1 and the eIF2α are important in the regulation of both development and inflammation. It would be interesting to monitor the cultured bone marrow for the expression of markers of monocyte and phagocyte development. CD31/ER-M12, Ly-6C/ER-MP20, ER-MP58 (immature), CSF-1R(intermediate), CD11b and ER-H3 (mature) could be monitored over 8 days in culture
to see if the difference in the ABCF1+/- BMMφ is actually due to the delayed maturation [29]. These cells could also be tested for the presence of CSF-1R on their cell surfaces. If ABCF1+/- bone marrow expressed less CSF-1 than the wild-type bone marrow cells, it would suggest a reason for the delay in maturation.

### 5.3.5 Infections

It was surprising that ABCF1+/- mice did not produce an augmented TNFα and IL-6 response when challenged with LPS since their splenic macrophages were hyper-responsive. The mice did, however, look more distressed in response to the LPS challenge than their littermates. The lack of TNFα/IL-6 response may have been due to the method of analysis (sera and peritoneal fluid). Spleens were removed from these mice and snap frozen so it is still possible to determine whether there was an increase in TNFα mRNA expression or NF-κB protein expression in the ABCF1+/- spleens compared to the wild-type spleens. The sub-lethal challenge of LPS also revealed a down-regulation of IL-10. IL-10 expression is important for antagonizing the actions of proinflammatory cytokines like TNFα [30]. It is known that IL-10 mRNA is also post-translationally regulated by ARE [31]. It is possible that this down regulation of IL-10 somehow contributes to the increased INFγ and IL-1β responses seen at later time points in the LPS model. This may provide a additional explanation for the fact that the ABCF1+/- succumbed to the LM infection sooner than their wild-type littermates from symptoms that were consistent with hypercytokinemia. Since there is such a difference in the phenotype of the ABCF1 +/- tissue derived macrophages and monocytes maybe a systemic infection model is not the best way to analyse the in vivo phenotype of these
mice. It would be interesting to see if the ABCF1+/− mice were more responsive to challenge of a particular subset of mature macrophages like in a collagen induced arthritis (CIA) study [32].

5.3.6 Conditional system

It is difficult to study just one system in this gene trap mouse model because of the requirement of one intact copy of ABCF1 for development. Our lab has recently obtained an ES cell line that has a conditional gene trap vector in the ABCF1 gene which will be used to produce mice. This will allow the ABCF1 gene to function throughout development and be knocked-out in the adult mouse in a tissue-specific manner. It will be exciting to see what happens when ABCF1 is completely knocked out in adult mice. If adult ABCF1 homozygous can be produced from the conditional system it will be also interesting to see if they are capable of producing mature macrophages from monocytes or bone marrow. If ABCF1 homozygous knock-out adult mice don’t survive it would still be possible to analyse the ABCF1−/− phenotype in macrophages in these mice by crossing them with mice that express Cre recombinase on a macrophage specific promoter.

5.4 Closing thought

In conclusion, this thesis gives the first insight into the role of the ABCF1 protein in an in vivo mouse model. ABCF1 has been minimally described up to this point thus there are a number of different possible directions for future study. From this thesis it
seems as though ABCF1 has two separate roles; one as a positive regulator in
development and another as a negative regulator of inflammation. These roles may be
connected through post transcriptional regulation of TNFα or could end up being
mutually exclusive. As the pathways controlling gene expression become clearer, so
should the role of ABCF1 in these processes and its regulation. Since ABCF1 is involved
in the regulation of inflammation it also makes for an attractive target for reducing
inflammatory related pathologies associated with diseases like RA.
5.5 References


