An immune modulatory role for the fes proto-oncogene

By

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Abstract

The Fes protein tyrosine kinase is abundantly expressed in phagocytic immune cells, including tumor associated macrophages. Fes knockout mice (fes⁻/⁻) display enhanced sensitivity to LPS, and this was shown to be associated with increased NF-κB signaling and TNFα production from fes⁻/⁻ macrophages. Interestingly, tumor onset in the mouse mammary tumor virus (MMTV-Neu) transgenic mouse model of breast cancer is significantly delayed in fes⁻/⁻ mice, and this was associated with increased frequency of CD11b⁺ myeloid and CD3⁺ T cells in the premalignant mammary glands. Recent studies have also implicated Fes in cross-talk between MHC-I and the NF-κB and IRF-3 pathways in macrophages. Signal 3, the production of inflammatory cytokines and Type I interferons downstream of NF-κB and IRF-3 pathways in antigen presenting cells, is considered an important component of T-cell activation, after engagement of T cell receptor by MHC presented antigen (Signal 1) and co-receptors by their ligands (Signal 2).

Using a lymphocytic choriomeningitis virus (LCMV) model of immune activation, I show that LPS stimulated fes⁻/⁻ macrophages promote more robust activation of LCMV antigen-specific CD8⁺ T cells than wild type macrophages (fes⁺/+). Furthermore, LPS stimulated fes⁻/⁻ macrophages showed increased phosphorylation of NF-κB and IRF-3. I also showed that Fes co-localizes with MHC-I in dynamic vesicular structures within macrophages. These observations are consistent with a model where Fes regulates Signal 3 in antigen presenting cells through roles in cross-talk between MHC-I and the NF-kB and IRF-3 signaling pathways. This suggests that Fes plays an immune checkpoint role at the level of Signal 3, and that Fes inhibition could promote tumor immunity through increased Signal 3 driven T cell activation.
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This thesis is dedicated to cancer patients
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List of Abbreviations

AA  Antibiotic-antimycotics
BMDM  Bone marrow-derived macrophage
β2m  β2 microglobulin
BFA  Brefeldin A
CAF  Cancer-associated fibroblast
CM  Condition medium
CC  Coiled-coil
DMEM  Dulbecco’s modified eagle medium
ELISA  Enzyme-linked immunosorbent assay
Eek  Extracellular regulated kinase
F-BAR  FCH-BIN/Amphiphysin/RSV
FCH  Fes-Fer CIP42 homology
FBS  Fetal bovine serum
Fes  Feline sarcoma
Fer  Fes-related
Fps  Fujinami poultry sarcoma
GFP  Green fluorescence protein
GM-CSF  Granulocyte-macrophage Colony Stimulating Factor
IgE  Immunoglobulin E
IKK  IκB kinase
IL  Interleukin
IL-4R  Interleukin-4 receptor
IFN  Interferon
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
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<tr>
<td>IRF</td>
<td>Interferon response factor</td>
</tr>
<tr>
<td>IMDM</td>
<td>Iscove’s modified dulbecco’s medium</td>
</tr>
<tr>
<td>Jak</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun N-terminal Kinase</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharides</td>
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<tr>
<td>M-CSF</td>
<td>Macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MMR</td>
<td>Macrophage mannose receptor</td>
</tr>
<tr>
<td>MMTV-LTR</td>
<td>Mouse mammary tumor virus long terminal repeat</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MD-2</td>
<td>Myeloid differentiation factor 2</td>
</tr>
<tr>
<td>N-terminus</td>
<td>Amino-terminus</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor kappa binding</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology 2</td>
</tr>
<tr>
<td>Shc</td>
<td>Src homology 2 domain-containing transforming protein</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducers and activator of transcription</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>TAM</td>
<td>Tumor-associated macrophage</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor α</td>
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<td>Tyr</td>
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Chapter 1

Introduction
1.1 Fes tyrosine kinase

Fps (Fujinami poultry sarcoma) and Fes (Feline sarcoma) are the founding members of a distinct subgroup of the non-receptor protein-tyrosine kinase family (reviewed in refs [1] and [2]). They were first identified as transforming viral oncoproteins encoded by retroviruses isolated from avian and feline sarcomas [3, 4]. The fps and fes proto-oncogenes, from which these retroviral oncogenes were derived are now recognized as being avian and mammalian orthologs [5]. The human fes proto-oncogene [6] displays a tissue-specific expression pattern, with highest levels seen in epithelial, vascular endothelial, neuronal and myeloid cells [7]. Gene targeting in mice has led to observations implicating Fes in the regulation of innate immune cells, such as mast cells, neutrophils and macrophages [8-10], which are important in recognizing and defending against pathogens and in tumor immunity.

In 1999 Senis and colleagues reported on mice in which the fes locus was targeted with a kinase-inactivating knock-in mutation [10]. Although Fes was known to be abundantly expressed in cells of the myeloid lineage, phenotypic analysis of these mice reveal only subtle differences in the number of circulating or bone marrow hematopoietic cells. In 2002, Zirngibl and colleagues reported a follow-up study with mice targeted with a null mutation in fes [11]. These fes−/− mice displayed subtle reductions in myeloid cells and an interesting corresponding slight increase in lymphoid and erythroid lineages. These phenotypes were corrected by crossing the mice with a transgenic line expressing human fes. While these observations did not support the initial expectation that Fes plays a role in myeloid differentiation, they revealed that Fes was involved in regulating innate immunity for the first time. However, these mice were viable and fertile and showed no significant abnormality in their hematopoietic cells [10, 11]. Moreover, when mice were challenged with LPS, the fes−/− mice displayed a significantly greater susceptibility to endotoxemia. A subsequent study by Parsons and colleague showed that this
LPS sensitivity was associated with elevated production of TNFα from LPS-challenged fes−/− macrophages and increases NF-κB activation [12]. Interestingly, this was also correlated with delayed internalization of LPS engaged TLR4 receptor in fes−/− macrophages. Those studies provided the initial evidence for a role for Fes in regulating TLR4 signaling.

1.2 Fes structure

Fes consists of an amino-terminal F-BAR domain, an FX domain, a central Src-homology 2 (SH2) domain, and a carboxyl-terminal kinase domain (Fig 1) [1, 13]. The F-BAR domain is about 300 amino acids long and is the defining structural feature of members of the Pombe 21 CDC15p homology (PCH) family of adaptor proteins, which typically consist of an amino-terminal F-BAR domain, a central monomeric G protein binding domain, and a carboxyl-terminal Src-homology 3 (SH3) domain. Fes and the homologous Fer kinase are the only members of the protein kinase superfamily which contain the F-BAR domain. PCH family adaptors have been shown to play roles in regulating cytoskeletal rearrangement, dimerization, endocytosis and vesicular transport [14-16]. The F-BAR domain also plays a critical role in regulating endocytosis and recruiting actin regulatory proteins to sites of membrane curvature [17, 18]. The FX domain of Fer has been reported to bind phosphatidic acid and mediate membrane binding [19]. Mutations in the F-BAR and FX domains of Fes and Fer have been shown to affect oligomerization [20]. The F-BAR domain in Fes was implicated in signaling from IgE receptors in mast cells [21].The SH2 domain, which is located between the FX and kinase domains, has the ability to bind to specific phosphotyrosine-containing peptide sequences, thereby mediating protein-protein interactions. SH2 is the prototypic example of a protein modules that has a role in directing protein interactions within the cell [22, 23]. Recent crystal
structure studies have shown that the SH2 domain of p130Gag-Fps can interact with the kinase domain and regulate kinase activity [24]. Lastly, the kinase domain mediates tyrosine phosphorylation activities. Structures of the SH2-kinase domains of p130Gag-Fps and the F-BAR domain of Fes have been solved (Ugochukwu et al. 2012). These support a model where the F-BAR domain mediates dimerization of the Fes kinase, and likely also mediates interaction with cellular membranes. This is consistent with previous reports that Fes co-localizes with monomeric proteins in the Rab family, which are known to associate with trafficking vesicles [25].
Figure 1. Structure of Fes protein. A. The Fes protein structure consists of F-BAR, FX, SH2 and Kinase domains [13]. B. The F-BAR domain which distinguish Fes and Fer from other non-receptor tyrosine kinases, plays a role in membrane binding and dimerization (Fes F-BAR structure from Ugochukwu et al, 2012.).
1.3 Fes in signaling

The Fes tyrosine kinase is a mediator of signaling transduction [26, 27]. Fes transfers the γ-phosphate group from ATP to specific tyrosine residues on substrate proteins which leads to the creation of binding sites for downstream signaling molecules [28]. Fes plays an important role in monocyte cells and may contribute to cell signalling associated with many growth factors and cytokines, including receptors for granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin 3 (IL-3) [29-31]. Fes can also promote the activation of signal transducer and activator of transcription factor 3 (STAT3) [32]. Moreover, \( fes^{-/-} \) mice have a greater sensitivity to lipopolysaccharide (LPS) and a high level of TNF-α was detected in their serum after LPS challenge [11]. This indicated that Fes is involved in immunity by regulating the inflammatory signaling pathway. A subsequent study showed that LPS induced greater activation of the NF-kB signaling pathway in \( fes^{-/-} \) macrophages and induced more TNF-α than \( fes^{+/+} \) macrophages [12]. A more recent study has independently validated that Fes can negatively regulate Toll-like receptor 4 (TLR4) signaling and therefore the production of proinflammatory cytokines [33]. This study also implicated Fes in cross-talk between MHC I and the TLR4 signaling pathway through a mechanism which involves Fes binding to MHC I and regulating the ability of protein-tyrosine phosphatase SHP-2 to bind to TRAF6 and TBK and inhibiting the activities of downstream proteins in their respective signaling pathways.
1.4 Fes in Breast Cancer

Breast cancer is the most common cancer among women around the world, about 1.7 million new cases were diagnosed in 2012 which represents almost 12% of all new cases of cancer and 25% of total cancer in women [34]. In Canada alone, breast cancer is the second leading cause of death from cancer among women [35]. Although it is uncommon, men can also develop breast cancer [36]. Even though it is an aggressive disease, early diagnoses can help treat the tumor and save lives. Patients with breast cancer are presented with treatment options such as radiation and chemotherapy, in addition to surgery. However, these treatment options are non-specific due to the fact that can target healthy cells as well. For the few past decades, treatment for breast cancer has improved become more specific as a result of its capacity to target cancer cells without harming the normal cells [37]. An example is reflected through targeting protein kinases. The majority of these proteins have been studied for cancer treatment purposes [38, 39]. It is interesting to note that in the field of cancer treatment, inhibiting the kinase activity even for a short period of time can result in apoptosis or necrotic death of cancer cells [40].

A recent study found that Fes can promote tumour growth through effects in the tumor stroma, rather than in the cancer cells [41]. In an orthotopic mouse model of breast cancer, the authors showed that loss of Fes in the stroma resulted in decreased engrafted tumour growth as well as metastasis. Moreover, the tumour microenvironment in engrafted $fes^{-/-}$ mice showed a lower number of tumor-associated macrophages in comparison to engrafted $fes^{+/+}$ mice. These observations suggest that Fes may regulate immune cell interactions with the tumor.
1.5 Fes in other types of cancer

Fes has been reported to be associated with different types of cancer other than breast cancer. For example, in lymphoid cancer, hyper activation of Fes has been shown to be an important factor in supporting the uncontrolled proliferation that was caused by active mutants of membrane receptors such as KITD816V, which is an inhibitor-resistant variant of c-Kit [26]. Moreover, down regulation of Fes using interference RNA has been shown to decrease tumorigenicity and proliferation of renal carcinoma cells [42]. Furthermore, it has been shown that in leukaemia cell models, Fes can promote tumour growth by sustaining cell viability during differentiation of leukaemia cells [43]. These observations were strongly argued that Fes might also contribute in cancer development through cancer cell intrinsic roles.

1.6 Classification of Immune system

The immune system protects organisms from infections and cancer through different mechanisms. It includes multiple cell types, including phagocytes which can target invading organisms and cancer cells, as well as immune cells that are more specific and have the ability to remember and recognize previous invading organisms called lymphocytes. Based on that, the immune system of mammalian is classified of two types: innate immunity and adaptive immunity (Fig 2). Failure in any of these types will increase the susceptibility for the body to get infection.

Innate immunity is the rapid first line of the body’s defense against an infection which can be mediated by phagocytes including macrophages and dendritic cells. Phagocytosis by these
cells involves the detection of pathogen-associated molecular patterns (PAMPs) or other danger signals produced by stressed and damaged cells. Unlike adaptive immunity, innate immunity does not form lasting protection to the body. Because the innate immune defenses are non-specific, their capacity in fighting an infection is general. Speed also is a critical characteristic of the innate immune system, it can generate a protective inflammatory response within minutes of pathogen exposure. Furthermore, innate immunity plays an important role in activating the adaptive immune response [44].
Figure 2. The major types of immune cells. Immune cells are divided into two categories, innate immune cells and adaptive immune cells. The innate immune cells include macrophages, dendritic cells, neutrophils and NK cells, and upon their activation they can activate adaptive immunity. The adaptive immune cells include T cells and B cells (Modified from Vivier, et al, 2012 [45]).
1.7 Macrophages in innate immunity

Professional antigen presenting cells (pAPCs) are important for initiating the activation of T cells and B cells. They recognize the pathogen and internalize it either through phagocytosis or by endocytosis. The antigen will then be expressed on their surface by MHC (either class I for CD8⁺ T cells or class II for CD4⁺ T cells). T cells will recognize the antigen-MHC complex on the membrane of antigen presenting cells via T cell receptors (TCR), which provide Signal 1 [46]. Next, co-stimulatory signaling will be provided by the antigen presenting cells as Signal 2, which then leads to the activation of T cells.

Macrophages (Mϕ) are pAPCs derived from monocytes. When monocytes immigrate to damaged cells, they undergo a process of differentiation into macrophages in the presence of specific cytokines such as M-CSF [47]. Moreover, macrophages have a role in innate immunity, as they defend against the pathogen as well as having facilitating role to initiate adaptive immunity by stimulating T and B cells. Furthermore, there are several types of macrophages that have been described in the literature [48]. M1 macrophages, which are also known as pro-inflammatory macrophages, are activated by IFN-γ and LPS, and can secrete high levels of IL-12 and low levels of IL-10. M2 macrophages, also known as anti-inflammatory macrophages, acts in tissue repair and wound healing, and secrete high levels of IL-10 and low levels of IL-12. In addition to M1 and M2 macrophages, it is now appreciated that a range of macrophage phenotypes exist displaying in some cases both M1 and M2 polarized characteristics [49]. Tumor-associated macrophages are usually characterized as M2, and they, play important roles in tumor growth and progression [50, 51]. They are an important source for many cytokines such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF),
transforming growth factor (TGF-β), macrophage colony stimulating factor (M-CSF/CSF-1), IL-10 and IL-17A [52-54]. These cytokines promote the survival and the polarization of the macrophages in addition to their tumor promoting roles [55]. They also have a role in T cell proliferation, survival and function [56].

Macrophages also express other important molecules, which are critical to activate adaptive immunity, called major histocompatibility complex (MHC) class I and II molecule. MHC class I is the key protein that activates immune surveillance of CD8+ T lymphocytes by presenting peptide at the cell surface, leading to a cascade of events which will be explained in further details in another section. It is located on almost all nucleated cells in mammals. MHC class I consists of a 45 kD glycosylated transmembrane heavy chain, and it is associated with a 12 kD soluble protein called β2 microglobulin (β2m). This receptor complex presents short peptides of 8 to 10 amino acids to TCR on CD8+ T cells. In murine cells, the heavy chains of class I are encoded at chromosome 17 by three loci, H-2K, H-2D and H-2L [57]. The heavy chain consists of a cytoplasmic domain, a membrane spanning domain, and three extracellular domains called α1 α2 and α3. This extracellular domain binds to β2m, and together they constitute the MHC I complex (Fig 3). An assembly of the α-chain with β2m is required for antigenic peptide loading in the endoplasmic reticulum (ER) and cell surface expression of MHC class I [54].

In general, it is believed that MHC class I presents peptides from endogenous antigens (direct-presentation pathway), which usually originate from cytosolic and nuclear proteins [58]. However, MHC class I can also present peptides that are derived from exogenous proteins by a process called cross-presentation [59, 60]. In the direct presentation pathway, antigens can be derived from endogenously synthesized proteins, defective ribosomal proteins and
inappropriately translated proteins [61, 62]. These proteins will then get degraded by the proteasome to generate short peptides; and these will then transported via the transporter associated with antigen processing (TAP) [63] into the endoplasmic reticulum (ER) and then move to cell surface through the Golgi complex [64] (Fig 4). In the cross-presentation, (exogenous) pathway [60, 65, 66], pAPCs uptake soluble or cell-associated antigens and then generate MHC class I-peptide complexes from proteins internalized into the phagosome.
Figure 3. The structure of the MHC class I receptor complex. MHC-I consists of a heavy chain with three α domain called α1, α2 and α3 which bind to the 12 kD beta 2 microglobulin subunit. Together this complex can present an 8 to 10 amino acids long peptide on cell surface.
Figure 4. **Antigen processing pathway.** (Route A) endogenously synthesized antigens get processed in the endosome by proteasome to generate short peptides (I). The short peptides then will be translocated into ER lumen by TAP (II). In the ER MHC class I (the heavy chains and β2M) get fold and assemble in the ER which by then will bind to the peptide. In the end peptide loaded MHC class I molecules transported from ER to Golgi (II) and then to the plasma membrane [Adapted with permission from Kim, *et al.*, 2010 [67]].
1.7.2 Toll-Like receptors

Toll-like receptors (TLRs) are an important component of innate immune cells, because they sense components of microorganisms known as pathogen-associated microbial patterns (PAMPs) such as lipopolysaccharide (LPS), bacterial DNA and viral double-stranded RNA [68]. TLRs can also sense endogenous danger signals known as danger-associated molecular patterns (DAMPs), such as heat shock proteins and protein fragments which are usually released from necrotic cells [69]. Once TLRs bind to PAMPs or DAMPs, they initiate signaling cascades which will lead to the activation of transcription factors such as NF-κB and interferon regulatory factors (IRFs). The activation of transcription factors results in cellular responses such as the production of pro-inflammatory cytokines and interferons (IFNs). TLRs are located on the plasma membrane of innate immune cells such as macrophages, with the exception of TLR3, 7, 8 and 9, which are found in the endosomal compartment [70]. In my study, the focus will be centered on TLR4, because it is the only TLR that uses both MyD88 and TRIF adaptors for signaling.

1.7.3 TLR4 structure

TLR4 consists of a 608 residue extracellular domain, a single transmembrane domain, and a 187 intracellular domain. The extracellular domain of TLR4 is associated with MD-2, which forms a heterodimer complex known as TLR4-MD-2 that recognizes and binds to LPS derived from gram negative bacteria. Interestingly, it has been found that TLR4 alone is not enough to recognize LPS, and physical associations between TLR4 and MD-2 is required for ligand-induced activation. In addition to MD-2, two accessory proteins are involve to help TLR4
recognize its ligand, LPS is first bound by LPS-binding protein (LBP), which is present in blood and other fluid in tissue. LPS is then transferred to second protein called CD14, which then delivers it to the TLR4-MD-2 complex to activate the TLR4 pathway (Fig 5) [71, 72].
Figure 5. Structure of the LPS receptor TLR4 complex. The LPS receptor consists of TLR4, MD2, LBP and CD14. For TLR4 to recognize LPS derived from gram negative bacteria, the extracellular domain of TLR4 associates with MD-2, forming a heterodimer. CD14 together with the LPS-binding protein (LBP) deliver and load LPS onto the TLR-MD-2 complex which will then lead to activation of TLR4 signaling pathway [Modified from Kim, et al, 2007[73]].
1.7.4 TLR4 signaling pathway

The activation of TLR4 signaling pathways are initiated upon the recognition of LPS, which will lead to the initiation of a series of events that start from the TLR4 cytoplasmic TIR domain. Downstream of the TIR domain, an adaptor known as a TIR domain-containing adaptor MyD88, was first characterized to play an important role. Moreover, recent evidence indicates that the TLR4 signaling pathway uses MyD88-dependent, as well as MyD88-independent pathways [74]; which will be explained in further details below (Fig 6).

1.7.5 MyD88-dependent pathway

Upon LPS binding to TLR4, MyD88 activates IL-1 receptor-associated kinase-4 (IRAK-4), which is a member of the IRAK family. Studies have shown that IRAK-4 knockout macrophages have reduced production of pro-inflammatory cytokines after LPS stimulation [75]. Previous studies have used knock in mutation to deactivate IRAK-4 kinase activity and found that IRAK-4 is important for the TLR4 signaling pathway [76, 77]. Moreover, previous studies have provided evidence that suggests that IRAK-4 can activate and recruit IRAK-1[78]. Another critical adaptor protein for the MyD88-dependent pathway is the TNF receptor-associated factor 6 (TRAF6), which is downstream of IRAK-1 and IRAK-4. TRAF6 can bind to two ubiquitin-conjugating enzymes called UBC13 and UEV1A to form a complex to activate transforming growth factor-β-activated kinase 1 (TAK1) [79]. After activation, TAK1 will activate IKK (IκB kinase) and MAPK (mitogen-activated protein kinase) pathways [80]. As a result, the combination of IKKα, IKKβ, and IKKγ will form a complex and phosphorylate IκB proteins, which will lead to degradation of IκB proteins that bind to the transcription factor NF-κB in
(inactive form). This results in release of active NF-κB. Translocation of active NF-κB to the nucleus will lead to the production of pro-inflammatory cytokines and other immune effectors.

### 1.7.6 MyD88-independent pathway

The MyD88-independent pathway uses an adaptor protein called TIR-domain-containing adapter-inducing interferon-β (TRIF) instead of MyD88 proteins. Previous studies have used TRIF-deficient macrophages to show that TRIF has an important role in the activation of transcription factor IRF3, in addition to late-phase activation of MAPK and NF-κB [81]. Recent studies have shown how TRIF can activate IRF3. These researchers carried out experiments which suggested that TRAF3 will be recruited through TRIF adaptor protein after LPS stimulation. TRAF3 can then associate with TRAF family member-associated NF-κB activator (TANK), TANK binding kinase 1 (TBK1), and IKKi to mediate signalling downstream. Both TBK1 and IKKi are critical for the dimerization and translocation of IRF3. IRF3 can then activate the transcription of target genes. An example of that is found in type I interferons, which are important for anti-viral and anti-bacterial responses [82].
**Figure 6. Signaling pathways of TLR4 after LPS recognition.** LPS binds to TLR4 on the cell membrane, which will activate MyD88-dependent pathway and MyD88-independent pathway via different adaptor proteins. In the MyD88-dependent pathway, IKK and MAPK will get activated, which leads to activation of transcription factors NF-κB and activator protein (AP)-1 and controls the expression of pro-inflammatory cytokines. The MyD88-independent pathway is mediated by TRIF, which leads to activation of IRF3 and induce the expression of type I interferons.
1.8 CD8\(^+\) T cells and their role in Adaptive immunity

The adaptive immune system, which is also known as the specific immune system, is the second component of the immune system. Adaptive immunity consists of highly specialized cells which eliminate pathogens and create long term immunological memory. The cells that carry out the adaptive immune response are lymphocytes such as B cells and T cells which include CD4\(^+\) T cells, CD8\(^+\) T cells (reviewed in ref [83]).

CD8\(^+\) T cells, also known as cytotoxic T-lymphocytes (CTLs), are derived from naïve T cells. These cells express T cell receptors which binds to MHC class I molecules and also express a glycoprotein on their surface called CD8 which is co-receptor that also binds to MHC class I molecules [84], therefore, these cells are defined as CD8\(^+\) T cells. Along with TCR and CD8 expression, CD8\(^+\) T cells also express CD28 which is receptor for CD80 and CD86 that are expressed on antigen presenting cells. The major function of CD8\(^+\) T cells is to target and kill cancer cells and cells that are infected with viruses by releasing two types of cytotoxic proteins: the granzymes, which can induce apoptosis in target cell, and pore-forming protein perforin, which create holes in the membrane that allow granzymes to enter the cells [85]. CD8\(^+\) T cells can also produce cytokines such as IFN-\(\gamma\) which can inhibit the viral replication in the cells.

1.8.1 Signals required to activate naïve CD8\(^+\) T cells

Activation of naïve CD8\(^+\) T cells with Signal 1, T cell receptor binding to the antigen-MHC-I complex, and Signal 2, co-receptor CD28 binding to co-stimulatory molecules such as B7 that are expressed on antigen presenting cells, lead the cells undergo proliferation and weak clonal expansion. However, the cells fail to develop effector functions in the absence of an additional signal called Signal 3. Initiation of naïve CD8\(^+\) T cells to develop effector functions
and undergo strong expansion also requires Signal 3, which can be provided by interleukin-12 (IL-12) or interferon α (IFN α) [86] (Fig 7).

Although T cells can undergo proliferation, T cells that are lacking Signal 3 in their microenvironment failed to develop cytolytic effector functions [87, 88]. Moreover, a previous study has shown that some cytokines such as IL-12 and type I IFN can act as Signal 3 [89, 90]. These authors also demonstrated the importance of Signal 3 in vivo when they showed that Signal 1 and 2 in the absence of IL-12, resulted in CD8+ T cell proliferation and production of IFN-γ. However, these activities and cytokine production were limited. Furthermore, dendritic cell (DCs) have been shown to produce IL-12 in response to toll-like receptors and CD40 signals [91] which indicates that Signal 3 from antigen presenting cells can occur upon stimulation in TLRs [92].
Figure 7. Cellular and molecular signaling required to activate CD$^+$ T cells. Effector-CD8$^+$ T cells required at least two signals in order to be activated, the first signal is T cell receptor binding to MHC class I, the second signal is CD28 binding to B7. In order to generate an efficient activation of CD8$^+$ T cell, a third signal needs to occur, which is cytokines produced from antigen presenting cells such as macrophages [modified from Pacheco, et al, 2012 [93]].
1.9 LCMV as model to assist studying the role of Fes in immunity

Lymphocytic choriomeningitis virus (LCMV), a rodent virus from the Arenaviridae family, was first isolated by Charles Armstrong in 1933 when he studied epidemic encephalitis [94, 95]. LCMV is an enveloped virus which contains the RNA genome that consists of two single stranded RNA segments [96]. The large segment is about 7.2 kb and the small segment is about 3.4 kb (Fig 8). The Large RNA segment encodes a 200 kDa protein which has motifs that are conserved in all the viral RNA-dependent RNA polymerases, while the Small RNA encodes two mature virion glycoproteins, GP-1 (40 to 46 kDa) and GP-2 (35 kDa); and the nucleoprotein, NP (63 kDa) [97, 98]. GP-1 mediates the interaction between virus and receptor on the host cell surface, which has been identified as α-dystroglycan [99]. LCMV is one of the most popular model systems to study viral persistence, pathogenesis, the immune system and virus-induced immune disease, as the clearness of the virus is T cells dependent [100]. Here, we used LCMV strain WE to infect mice and explore the role of Fes in antigen presentation by macrophages to CD8⁺ T cells.
Figure 8. Illustration of LCMV structure. LCMV is an enveloped virus which contains an RNA genome that consists of two negative single-stranded RNA segments. The surface is covered with glycoproteins 1 and 2, and the inner envelop of membrane is coated with Z proteins. The viral ribonucleoproteins consist of small and large single stranded RNA segments coated with nucleoprotein, and both are associated with L polymerase [Adapted from LAPOŠOVÁ, et al, 2012 [101]].
1.10 Rational, hypothesis and aims

Previous studies from Dr. Greer’s lab have shown that the Fes tyrosine kinase plays a supportive role in tumour growth and metastasis in breast cancer. However, the specific roles of Fes in the immune system are still unclear and requires further investigation. In this project, I explored this using $fes^{-/-}$ mice and the LCMV mouse infection model in order to determine how Fes contributes to both innate immunity and adaptive immunity against viral infections in the presence of LPS to stimulate TLR4 signaling pathway. Thus, my hypothesis is that Fes deficiency in antigen presenting cells will correlate with increased TLR4 signaling during MHC-I based antigen presentation to CD8$^+$ T cells, and this will lead to enhanced T cell mediated immunity through enhanced Signal 3 mediated T cell activation. To test this hypothesis, my three specific aims were to:

1. Characterise macrophages from $fes^{+/+}$ and $fes^{-/-}$ mice and explore the role of the Fes tyrosine kinase in TLR-driven Signal 3 propagation.

2. Compare the ability of macrophages from $fes^{+/+}$ mice and $fes^{-/-}$ mice to present LCMV peptide antigen to and activate effector virus-specific CD8$^+$ T cells from LCMV infected mice, in the presence and absence of TLR4 ligand.

3. Compare *ex vivo* CD8$^+$ T cell activation of splenocytes from LCMV infected $fes^{+/+}$ mice and $fes^{-/-}$ mice.
Chapter 2

Materials and Methods
2.1 Mice

Fes knockout mice (fes−/−) and wild type mice (fes+/+) were generated in the 129Sv/J background [11] and were backcrossed through 10 generations into a C57BL/6 background. Mice were used at the age of 8-12 weeks in accordance with the guidelines of the Canadian Council of Animal Care with protocols approved by the Queen’s Animal Care Committee.

2.2 Cell culture and media

Dulbecco’s Modified Eagle Medium (DMEM), Iscove's Modified Dulbecco's Medium (IMDM) and Roswell Park Memorial Institute Medium (RPMI) were purchased from Gibco - Life Technology (ON, Canada). Fetal Bovine Serum (FBS) was purchased from Gibco - Life Technology (ON, Canada). L929 cells, which are mouse fibroblast cells [102], were used as source for Macrophage Colony-Stimulating Factor (M-CSF), which is an important cytokine for bone-marrow driven-monocytes to differentiate into macrophages. The cells were cultured with media containing DMEM, 15% FBS, 1% A.A, 1% glutathione, 20% L929 conditioned media and α-monothioglycerol (α-MTG) to 50 µM final concentration [103]. After week of culture media from L929 cells was collected and centrifuged for 5 min at 15000 rpm at room temperature to remove dead cells. The supernatant was filtered through a 0.45 µm filter and saved at -20°C to be used for preparing macrophage media.

The BMA cell line, a murine macrophage cell line, was obtained from Dr. K. Rock (University of Massachusetts Medical School, Worcester, MA) and were grown in media containing RPMI, 5% FBS, 1% A.A and 1% L-glutamine at 37°C in a CO2 humidified incubator. When BMA cells reached 90% confluence they were recovered by treatment with 0.5% trypsin
and either replated or collected for further experiments. Before experiments, cells were counted with a haemocytometer [American Optical, Buffalo, NY] and viability was determined by using the 0.3% (v/v) trypan blue exclusion method [Sigma-Aldrich, Oakville].

2.3 Preparation of bone marrow-derived macrophages

Bone marrow macrophages were generated from femurs of C57BL/6 mice at the age of 8-12 weeks. Bone marrows were flushed with media containing IMDM, 5% FPS and 0.5% AA using a 26 G 3/8 sterile needle [Becton-Dickinson, Rutherford, NJ]. Bone marrow cells were then centrifuged for 5 min in 1500 rpm and washed with sterile PBS. Next, bone marrow-derived macrophages were generated by culturing the cells in a 10cm petri dish plate in IMDM containing 20% L929-cell conditioned medium as a source of M-CSF, 15% FBS, 1% AA and α-monothioglycerol (α-MTG) to 50 µM final concentration [103]. The following day, nonadherent cells were collected by centrifugation for 5 min in 1500rpm and cells were resuspended and plated in new media and cultured in 10cm petri dish plates. This process was repeated after 2 days. On the 7th day the cells were harvested with trypsin for an experiment.

2.4 Preparation and measurement of effector T cells

Mice were immunized by s.c injection of 6 x 10⁴ PFU LCMV-WE (three mice each genotype). Six days post infection, splenocytes were isolated and used as effector cells for intracellular cytokine staining (ICS). Splenocytes were washed with sterile PBS and red blood cells were lysed by re-suspending in lysis buffer (1.66% w/v ammonium chloride) for 5 minutes. Splenocytes were re-washed with sterile PBS. Debris was removed by passing the cell suspension over a metal sieve [104, 105]
2.4.1 Intracellular cytokine staining (ICS) to measure CD8+ T cell activation *in vitro*

To measure the activation level of CD8+ T cells, ICS was used to detect IFNγ, which is produced upon activation, in CD8+ T cells. Splenocytes were isolated after 1 week of injection and prepared as previously described. Cell suspensions from the spleen were added into a 96 well plate which already contained antigen presenting cells (bone marrow macrophages or BMA) that are pulsed with gp33 epitope at ratio of 1 APC: 10 splenocytes and incubated at 37°C and after 2 hours. Brefeldin A (10 μg/ml) were added for restimulation. After 3-6 hours cells, were stained with PE-Cy5-conjugated anti-mouse CD8α (clone 53-6.7) antigen monoclonal antibody [Cedarlane] [105] (50μl, 1 μg/mL in PBS) on ice and incubated in the dark for 30 minutes. The samples then were fixed with 1% paraformaldehyde (50 μl) and washed twice. The samples were stained with Alexa Fluor® 488-conjugated anti-mouse IFN-γ (clone MG1.2) [106], as it was produced upon activation in CD8+ T cells, (1 μg/mL in PBS) [Biolegend, USA] diluted in 0.1 % saponin in PBS [Sigma-Aldrich, Oakville] and kept overnight in 4°C [105, 107]. The next morning, the samples were washed and harvested in FACS and buffered to acquire a Beckman Coulter Epics XL-MCL flow cytometer and analyzed with Flow-Jo software.

2.4.2 Measurement of activation level of CD8+ T cells *ex-vivo*

Mice were injected with s.c 6x10⁴ PFU LCMV-WE. Mice were sacrificed and spleens were harvested under sterile conditions after 0, 2, and 4 days post-injection. Splenocytes were prepared as previously described. Approximately 20 x 10⁶ or 60 x 10⁶ cells were obtained from spleens of naïve or infected mice, respectively. Cells were divided into a 96 well plate (2 x 10⁵ cells/well). The cells were washed and stained first with PE-Cy5-conjugated anti-mouse CD8α (clone 53-6.7) antigen monoclonal antibody [Cedarlane] [105] and with Alexa Fluor® 488-
conjugated anti-mouse CD3 (clone 17A2) [108], then with different activation markers such as PE-conjugated anti-mouse/human CD44 (clone IM7)[109], Biotin anti-mouse Ly6C (clone HK1.4)[110], FITC-conjugated anti-mouse CD69 (clone H1.2F3)[111], Alexa Fluor® 488-conjugated anti-mouse CD62L (clone MEL-14)[112] to assess the activation levels of CD8⁺ T cells ex-vivo [113]. The samples were washed and harvested in FACS and buffered to acquire a Beckman Coulter Epics XL-MCL flow cytometer and analyzed with Flow-Jo software.

2.5 Immunoblotting analysis

Macrophages were stimulated for time course studies with 1 μg/mL LPS. After stimulation, cells were lysed with RIPA lysis buffer (10mM Tris pH 7.2, 158mM NaCl, 1mM EDTA, 0.1% SDS, 1% sodium deoxycholate, 1% Triton 100), which was supplemented with protease inhibitors, including 10μg/mL aprotinin, 10μg/mL leupeptin, 100μM sodium orthovanadate, 100μM phenylmethylsulfonyl fluoride for 20 mins. Lysates were centrifuged at 13,000 × g for 15 min at 4°C. Protein concentration was measured using the Bradford assay. Samples were then diluted with a SDS sample buffer and heated for 5 minutes at 95°C. For SDS-PAGE, 30 μg protein per lane was electrophoresed on a 7% polyacrylamide gel and proteins were then transferred to a nitrocellulose membrane using a semi-dry transfer apparatus (Bio-Rad) and blocked with 5% skim milk in TBST for 1 hour. Membranes were incubated overnight with the primary antibody at 4°C. After washing three times in TBST, membranes were incubated with the appropriate secondary antibody-horse radish peroxidase conjugate for 1 hour at room temperature, and finally proteins were detected by using enhanced chemiluminescence reagents western lightning plus – ECL (Perkin Elmer, Inc Products). Primary antibodies were used to detect phosphorylated proteins including anti-phospho-SHP2, anti-phospho-TBK1, anti-
phospho-NFκB, anti-phospho-ERK, anti-phospho-p35 (Cell Signaling), and anti-tubulin (Sigma). Anti-mouse or anti-rabbit horse radish peroxidase antibody conjugates were purchased from cell signaling.

2.6 Phenotyping analysis

Macrophages were plated at 1 x 10^6 cells/mL in 96 well plates (100 μL/well), then labeled with cell membrane markers such as; PE-conjugated anti-mouse MHC-I, H-2Kb/H-2Db (clone 28-8), Biotin anti-mouse CD80 (clone 16-10A1), FITC-conjugated anti-mouse CD86 (clone GL-1), and Alexa Fluor®488-conjugated anti-mouse F4/80 (clone BM8); or after cells were permeabilized with 4% PFA then stained with intracellular anti-macrophage markers (clone MOMA-2), anti-CD206 and anti-CD68 (clone FA-11) were purchased from AbD Serotec (NC, USA) [114].

2.7 Cell transfections and confocal microscopy

Macrophages were cultured on class coverslips in 6-well plates until they reached 70-80% confluence. Cells were transfected with pEGFP H-2kb, pWPXLD Fes dsRed and pWPXLD Fes KR dsRed using PolyJet reagent according to the manufactures directions (PolyJet™ DNA. SignaGen Laboratories). After 2 days, the live cells were observed using a Quorum Wave Effects Spinning Disc Confocal at 60X magnification and images were captured for quantitative analysis. In some experiments, cells were fixed with 1% paraformaldehyde prior to imaging.
2.8 ELISA

Macrophages were stimulated with 1µg/ml LPS for 4.5 hours in 6 well plates (2×10^6 cells/well), then culture supernatants were used to quantify cytokines expression as described [115] using the Mouse IL-6 ELISA Ready-SET-Go! ® kit (eBioscience) [116] which was used according to the manufacturer’s recommended protocol. Absorbencies of the samples were measured using BioTek Lex800 microplate reader (Fisher Scientific). Data are representative of the average of triplicate wells ± SD.

2.9 Statistics

Statistics were completed using Microsoft Office Excel 2013 software. Error bars represent standard error of the mean and P values were calculated by Student’s t-test. Data sets with P≤0.05 were considered statistically significant.
3.1 \textit{fes}^{+/+} \textit{macrophages and fes}^{-/-} \textit{macrophages express the same high level of F4/80 marker on their surface}

I first sought to develop a model to explore macrophages as an antigen presenting bone-marrow cells from \textit{fes}^{+/+} and \textit{fes}^{-/-} mice that were cultured in IMDM media containing macrophage colony-stimulating factor (M-CSF) for 7 days, as M-CSF helps induces monocytes to differentiate into macrophages [117]. When cells reached the maturation level from both genotypes, they were washed and stained with the macrophage-specific marker anti-F4/80, which is known to be expressed in macrophages [118]. Cells were then acquired using a flow cytometer. More than 95\% of the cells were positive for F4/80 which indicate the pure population of macrophages cells using this method of culturing (Fig 9). Moreover, F4/80 expression was indistinguishable between macrophage cultures from \textit{fes}^{+/+} and \textit{fes}^{-/-} mice. This finding is consistent with the previous observations using \textit{fes} targeted mice from the Greer lab, [10] [11], I concluded there were no effects of Fes disruption on differentiation of bone marrow progenitors to macrophages.
Figure 9. Indistinguishable levels of F4/80+ cells on fes−/− and fes+/+ macrophages. Histograms of anti-F4/80 antibody staining by flow cytometry. BMDMs were cultured for 7 days and then stained with anti-f4/80 for 30 mins. The cells then were washed and acquired using flow cytometry. Data representative from three independent experiments with similar results.
3.2 The absence of Fes does not affect common macrophage marker expression levels

After confirming the type of the cells that we generated from bone marrow using F4/80 staining, which is a macrophage specific marker, we looked at the level of other markers that are expressed on macrophages. We choose to look at extracellular markers that are expressed on macrophages, while simultaneously having a role in activating T cells. MHC-I was the first molecule that we looked at because of its role in presenting a peptide to T cells [119] in the context of Signal 1. We also tested the level of surface expression of CD80 and CD86 molecules which contribute to Signal 2 in the activation of T cells [120, 121]. Percentage of MHC-I$^+$ cells were slightly increased in $fes^{-/-}$ macrophages, but did not reach significant differences. In CD80 and CD86 expression there were no significant differences within the two genotypes which indicates that Fes do not contribute to the markers expression. The results indicate that Fes does not regulate the expression of these markers (Fig 10). We also investigated the level of expression of a number of intracellular markers: MOMA2 (a general marker for monocytes/macrophages) [122]; CD68 (a member of the scavenger receptor family that can recognize low-density lipoproteins) [123, 124]; and CD206 (a mannose receptor which has a role in phagocytosis of pathogens) [125, 126]. The data shows slight increase expression of CD206 and MOMA-2 in $fes^{-/-}$ macrophages. However, this increase did not reach the significant differences. CD68 markers expression was the same between the two genotypes, which suggests that Fes does not regulate the level of expression of these markers (Fig 11).
Figure 10. Flow cytometry analysis of MHC I, CD86 and CD80 expression on bone marrow derived macrophages. *fes*^{+/+} and *fes*^{-/-} macrophages were stained first with anti-F4/80 marker to gate on macrophages and then stained with surface markers anti-MHC-I, anti-CD80 and anti-CD86 antibodies (three mice each genotype). Data are from three independent experiments with similar results.
Figure 11. Flow cytometry analysis of CD206, MOMA-2 and CD68 expression on bone marrow derived macrophages. $fes^{+/+}$ and $fes^{-/-}$ macrophages were stained first with anti-F4/80 marker to gate on macrophages and then the cells were fixed, permeabilized and stained for indicated intracellular markers (three mice each). Data are from two independent experiments with similar results.
3.3 Fes does not down-regulate TLR4 after one hour of LPS stimulation

Parsons et al, 2006 [12] showed that Fes supresses the TLR4 signaling pathway and reduces the production of cytokines. We attempted to determine if Fes can regulate TLR4 surface expression, as TLR4 expression levels can determine the degree of LPS-susceptibility in macrophages [127]. The cells were stimulated for 1 hour with 1µg/ml LPS, which is a bacterial ligand for TLR4; and 1µg/ml R848, which is viral ligand for TLR7 and TRL8. Next, the cells were washed with PBS and TLR4 expression on macrophages was assessed by staining the cells with the PE-conjugated anti-mouse TLR4 (CD284)/MD2 in the presence or absence of ligands for TLR4 (LPS) and TLR7/8 (R848). Samples were then acquired using flow cytometry. The data showed no significant differences in the level of surface TLR4 expression between $fes^{+/+}$ and $fes^{-/-}$ macrophages in the absence or presence of LPS or R848 (Fig 12). However, this finding was different from previous finding from our lab, which showed that Fes down-regulated TLR4 expression on resident peritoneal macrophages after 5 and 15 min post 270 ng/ml LPS stimulation [12]. We were mindful of the fact that the inconsistence between these experiments can be due to experimental model, concentration of ligand treatment and even the time points of the treatment.
Figure 12. Similar TLR4 surface expression on fes"+/+ macrophages and fes"-/− macrophages. Cells were stimulated with or without LPS (TLR4 ligand) and R848 (TLR7/8 ligand) for an hour, then the cells were stained with PE-conjugated anti-mouse TLR4 (CD284)/MD2. This experiment was performed once with technical triplicates.
3.4 Increased LPS-induced activation of NF-κB and IRF3 in \( \text{fes}^{-/-} \) macrophages compared to \( \text{fes}^{+/+} \) macrophages

To determine if Fes was involved with TLR signaling in macrophages, I tested the activation status of mitogen-activated protein kinase (MAPK), transcription factor NF-κB in the p60 subunit, TANK-binding kinase (TBK-1) and IFN-regulatory factor-3 (IRF-3) pathways, which are all known to be engaged downstream of TLR receptors. The SHP-2 tyrosine phosphatase, which contributes to MAPK activation and has recently been implicated in signaling downstream of MHC-I [33]. Phosphorylation levels of NF-κB and IRF-3 were enhanced in LPS-stimulated \( \text{fes}^{-/-} \) macrophages (Fig 13). However, we did not find any differences in the phosphorylation levels of Erk, p38 or the SHP-2 tyrosine phosphatase between both genotypes (Fig 14). Furthermore, we tested another TLR ligand, R848, which activates the TLR7 and TLR8 receptors and investigated the phosphorylation levels of Erk, p38 and NF-κB and found no differences (data not shown). Collectively, these results indicated that Fes may have a role in suppressing TLR4 signaling pathway by inhibiting activation of MyD88-dependent pathways and MyD88-independent pathways in macrophages.
Figure 13. LPS-induced activation of NF-κB and IRF-3 was enhanced in \( fes^{-/-} \) macrophages. Macrophages were stimulated for the indicated times and lysates were prepared and assessed by immunoblotting for the indicated proteins. Data are from three independent experiments with similar results.
Figure 14. LPS-induce activation of SHP2, p38 and Erk was similar in $fes^{-/-}$ and $fes^{+/+}$ macrophages. Macrophages were stimulated with LPS for the indicated times and lysates were then assessed for the indicated proteins by immunoblotting. Data are from three independent experiments with similar results.
3.5 Fes co-localized with MHC-I in dynamic vesicles in the endosome

Xu, et al [33] showed that MHC class I binds to Fes in the plasma membrane through a mechanism involving a phosphorylated tyrosine residue on the cytoplasmic domain of MHC I and the Fes SH2 domain by using co-IP. To explore this proposed interaction further, I used different approach and transfected \textit{fes}^{-/-} macrophages with GFP tagged MHC-I together with DsRed tagged wild type or kinase-dead (K588R) Fes. Confocal microscopy showed that MHC class I interacted with wild type Fes and MHC-I GFP co-localized with Fes dsRed in a kinase-independent manner (\textbf{Fig 15}). This results suggested that Fes kinase activity is not required for the interaction with MHC-I. Other domains in Fes may be required for this interaction, such as SH2 or F-BAR. Moreover, the interaction between Fes and MHC I is not restricted to the plasma membrane, since I also observed colocalization in dynamic intracellular vesicles.
Figure 15. Co-localization of Fes wild type and MHC-I as well as Fes with dead kinase KR and MHC-I. Wild-type macrophages were co-transfected with MHC-I GFP and Fes kinase dead dsRed and Fes wild type. The cells were live and images were captured using Quorum Wave Effects Spinning Disc Confocal at 60X magnification. The arrows indicate endosomal co-localization. Data are from two independent experiments with similar results.
3.6 $fes^{-/-}$ macrophages show an enhanced ability to activate CD8$^+$ T cell after LPS stimulation

We further investigated the ability of Fes to regulate macrophage activation of CD8$^+$ T cells. To address this question, mice were injected s.c with $6 \times 10^4$ PFU LCMV-WE. Seven days after infection, splenocytes were harvested and co-cultured with bone marrow derived macrophages from $fes^{+/+}$ and $fes^{-/-}$ mice. These macrophages were pulsed with $10^{-7}$ M epitope to express it on their surface prior to co-culture with splenocytes. Activation of gp33 epitope-specific CD8$^+$ T cells were then measured using the intracellular staining assay (ICS) to detect level of IFN$\gamma$, which is an indicator of T cell activation [128]. We observed that both genotypes of macrophages activated epitope-specific CD8$^+$ T cells to the same level. However, this activation was significantly increased by $fes^{-/-}$ macrophages that were treated with LPS $P=0.0001$, which is known to stimulate TLR4 to produce pro-inflammatory cytokines. This LPS-promoted activity was specific to the $fes^{-/-}$ macrophages, suggesting a link between increased pro-inflammatory cytokine production in $fes^{-/-}$ macrophages and increased T cell activation. However, when macrophages were treated with R848, which is known to stimulate TLR7/8, the activation level of epitope-specific CD8$^+$ T cells remained the same for non-stimulated macrophages (Fig 16A). These data indicate that cytokines produced upon TLR4 activation may play an important role in CD8$^+$ T cells activation and may serve as “Signal 3”. Moreover, Fes may play a critical role in suppressing the TLR4 pathway, because LPS-stimulated $fes^{-/-}$ macrophages gave the highest activation level of CD8$^+$ T cells (Fig 16A).
Figure 16. Macrophages lacking Fes show an enhanced ability to activate CD8$^+$ T cell after LPS stimulation. Macrophages were stimulated with or without LPS and R848 for 2 hours and then pulsed with $10^{-7}$ [gp33] M epitope for an hour. Splenocytes were then co-cultured with these macrophages for 4 hours. Next, cells were stained with anti-CD8 and anti-IFN-γ analyzed by flow cytometry to assess the activation level of CD8$^+$ T cells. A. percentage of IFN-γ +ve cells. B. Mean fluorescence intensity analysis. Three mice were used for each genotype in each experiment. Error bars represent standard error of the mean. Data are from four independent experiments with similar results.
3.7 Evaluating the role of Fes in regulating CD8+ T cell *in vitro* after LCMV infection

We further investigated whether Fes has the same effect on CD8+ T cells as it has on macrophages and whether *fes*⁻/⁻ CD8+ T cells have an increased ability to be activated in comparison to the *fes*⁺/⁺ CD8+ T cells. We injected *fes*⁺/⁺ and *fes*⁻/⁻ mice at the age of 5-10 weeks old with s.c with LCMV-WE, and after 7 days splenocytes were then isolated and co-cultured with BMA cells which had been pretreated with different concentrations of gp33 peptide, ranging from $10^{-7}$ to $10^{-11}$, which enabled them to express it on their surface. Cells were co-cultured at a ratio of 1 BMA to 10 splenocytes. The activation level of CD8+ T cells was then measured using ICS by detecting IFN-γ in CD8+ T cells. Data shows that CD8+T cells from both genotypes have slight increase activation in high gp33 concentration, which are $10^{-7}$ and $10^{-8}$, but once we go lower there are similar levels of activation, this was also the same with the MFI data. This finding indicated that Fes has no significant impact on CD8+ T cells activation after culturing with antigen presenting cells *in vitro* (Fig 17).
Figure 17. In vitro assessment of CD8$^+$ T cell activation in co-cultures of splenocytes from \textit{fes}^{+/+} and \textit{fes}^{-/-} mice with BMA cells. BMA cells were pulsed with different concentrations of gp33 epitope for 1 hour. Splenocytes from LCMV infected \textit{fes}^{+/+} and \textit{fes}^{-/-} mice were co-cultured with these BMA cells for 4 hours. Cells were then stained with anti-CD8 and anti-IFN$\gamma$ antibodies and analyzed by flow cytometry to assess the activation level of CD8$^+$ T cells. \textbf{A.} percentage of IFN$\gamma$ cells. \textbf{B.} Mean fluorescence intensity analysis. Three mice of each genotype were used for each experiment. Error bars represent standard error of the mean. Data are from three independent experiments with similar results.
3.8 LPS treatment elicited increased levels of IL-6 from \(fes^{-/-}\) macrophages

To investigate what factors may have played a role in activating CD8\(^+\) T cells \textit{in vitro}, our attentions were focused on which cytokine is expressed higher in the \(fes^{-/-}\) macrophages and could play a role in CD8\(^+\) T cells activation as Signal 3. \(fes^{+/-}\) and \(fes^{-/-}\) macrophages were cultured in a 6 well plate (2× 10\(^6\) cells/ well). Cells were starved for 24 hours with media that contained no serum. On the day of the experiment, LPS (1\(\mu\)g/ml) was used to stimulate the cells for 4.5 hours, and non-stimulated cells were used as a negative controls. IL-12, IL-6 and TNF-\(\alpha\) were the main candidates to test, as they are known to play roles in T cells activation [129-131]. Using ELISA, we did not detect any IL-12 in macrophage culture media from either genotype (data not shown). This absence may be due to the fact that these cells were cultured in the presence of M-CSF, which has been shown in other studies to prevent IL-12 secretion [132]. We also looked at TNF-\(\alpha\) and found that \(fes^{-/-}\) macrophages secreted higher levels compared to \(fes^{+/-}\) macrophages (data not shown) which is consist with previous work from our lab [12]. The same finding was shown to be true with IL-6; \(fes^{-/-}\) macrophages secreted higher levels of IL-6 compared to \(fes^{+/-}\) macrophages (p=0.04), which suggests that IL-6 may promote CD8\(^+\) T cells activation after co-cultured with LPS-stimulated macrophages \textit{in vitro} (Fig 18).
Figure 18. LPS treatment elicited increased levels of IL-6 from $fes^{-/-}$ macrophages. ELISA analysis of IL-6 in culture supernatants from $fes^{+/+}$ and $fes^{-/-}$ macrophages 4.5 hours after 1µg/mL LPS challenge. Data is from one experiment with technical triplicates.
3.9 Increased early *ex vivo* activation of CD8⁺ T cells in LCMV infected *fes⁻/⁻* compared to *fes⁺/+* mice

To assess the effect of Fes on *ex vivo* activation of CD8⁺ T cells, *fes⁺/+* and *fes⁻/⁻* mice were infected with LCMV (s.c. injection with 6x10⁴ PFU LCMV-WE) and splenocytes were harvested from uninfected mice, or infected mice at 2 and 4 days post-infection for flow cytometry analysis. Splenocytes were stained with anti-CD3 and anti-CD8 antibodies to gate on CD3⁺CD8⁺ T cells. The cells were also stained with antibodies specific for CD44, Ly6c, CD62L, and CD69 to determine their levels of activation.

CD44 is a cell-adhesion molecule and expresses in cells that undergo the inflammatory phase (reviewed in ref [133]). CD3⁺CD8⁺CD44⁺ cell numbers increased on day 4 post infection, but there was no differences in the frequency of these cells between *fes⁺/+* and *fes⁻/⁻* genotypes. The MFI data also showed increase level of marker expression on CD3⁺CD8⁺ cells but with no significant different from infected *fes⁻/⁻* relative to *fes⁺/+* mice. (*Fig 19*). The high level of marker expression suggests that CD3⁺CD8⁺ T cells have the potential to attach and roll on endothelial cells in peripheral lymphoid organs, and finally to the sites of inflammation.

Lymphocyte antigen 6 complex (Ly6c) is a glycoprotein that express in hematopoietic cells and correlate with increased levels of IFN γ [134], which reflects the inflammation level in the microenvironment. In our experiment we found that CD3⁺CD8⁺Ly6c⁺ cell numbers increased to the same levels by 4 days post-infection in both genotypes, but this increase occurred earlier in the *fes⁻/⁻* cells, with a significant difference apparent at day 2 post-infection (*Fig 20A*, p=0.002). MFI analysis also showed significantly higher levels of Ly6c expression on *fes⁻/⁻* CD3⁺CD8⁺ cells at day 2 post-infection (*Fig 20B*, p=0.0001).
CD62L is a marker of naïve T cells and can also express on subset of memory T cells [135]. CD3+CD8+CD62L+ cell numbers and MFI was the same in both genotypes over the time, 0, 2 and 4 post infection (Fig 21).

The last marker examined was CD69, which is an early activation marker [136]. CD69 levels on CD3+CD8+ splenocytes was increased two days post-infection, and had started to decline by 4 days post-infection (Fig 22A). There was no significant difference in this profile of CD69 expression between fes+/+ and fes−/− splenocytes, although MFI analysis did reveal that CD69 expression levels remained significantly higher in fes+/+ splenocytes at 4 days post-infection (Fig 22B). Indicate that fes−/− CD8+ T cells have the potential to become activated faster than wild type CD8+ T cells.

Taking all these observations together, we conclude that CD8+ T cells from LCMV infected fes−/− mice appear to initially have enhanced activation compared to the wild type mice, and this may be due to increased inflammation associated with viral infection in fes−/− mice. This suggests that the loss of Fes could promote increased inflammation at the infection site, therefore resulting in enhanced activation of immune cells to respond to the infection.
Figure 19. Enhanced CD44 levels on CD3+CD8+ T cells of LCMV infected fes-/- mice. fes+/+ and fes-/- mice were infected with LCMV or without as control. At two and four days post infection, mice were euthanized and spleens were collected for flow cytometry analysis. Splenocytes were stained with anti-CD3 and anti-CD8 antibodies to gate on CD3+CD8+ T cells and with anti-CD44 antibody to assess their activation. A. Percentage of CD44+ cells. B. Mean fluorescence intensity (MFI) of CD44 on CD3+CD8+ T cells. Data is from one experiment. n=3 mice for each genotype.
Figure 20. Enhanced Ly6c levels on CD3\(^+\)CD8\(^+\) T cells of LCMV infected \(fes^{-/-}\) mice at days 2 and 4 post infection. \(fes^{+/+}\) and \(fes^{-/-}\) mice were injected with s.c \(6 \times 10^4\) LCMV, two and four days post injection mice were euthanized and spleen were collected (n=3 each genotype). Splenocytes were stained with anti-CD3 and anti-CD8 antibodies to gate on CD3\(^+\) CD8\(^+\) T cells and with anti-Ly6c antibody to check their activation level. A. Percentage of Ly6c\(^+\) cells. B. MFI data of Ly6c expression on CD3\(^+\)CD8\(^+\) cells. Data is from one experiment. n=3 mice for each genotype.
Figure 21. Similar CD62L levels expression of CD8+ T cells in both genotype. *fes*+/+ and *fes*-/- mice were injected with s.c 6X10^4 LCMV, two and four days post injection mice were euthanized and spleen were collected (n=3 each genotype). Splenocytes were stained with anti-CD3 and anti-CD8 antibodies to gate on CD3+CD8+ T cells and with anti-CD62L antibodies to check their activation level. A. Percentage of CD62L+ cells, there is no difference in the marker expression between two genotypes. B. MFI shows no difference between two genotypes. Data is from one experiment. n=3 mice for each genotype.
Figure 22. Evaluation of early activation marker CD69 level of CD8+ T cells. fes+/+ and fes−/− mice were injected with s.c 6X10⁴ LCMV, two and four days post injection mice were euthanized and spleen were collected (n=3 each genotype). Splenocytes were stained with anti-CD3 and anti-CD8 antibodies to gate on CD3+ CD8+ T cells and with anti-CD69 antibody which is an early activation marker, it express fast and drops fast as well. A. Percentage of CD69+ cells. B. MFI data. Data is from one experiment. n=3 mice for each genotype.
Chapter 4

Discussion
This thesis assessed the effect of Fes tyrosine kinase on the ability of macrophages to activate CD8^+ T cells. Using and LCMV infection model system I showed that fes^-/- macrophages stimulated with LPS have enhanced ability to activate CD8^+ T cells. Moreover, LPS-stimulated fes^-/- macrophages showed enhanced activation of NF-κB and IRF-3, which suggests that Fes plays an immune regulatory role at the level of Signal 3.

Previously, our lab elucidated the role of Fes in mammary tumor progression, where they crossed fes^-/- mice with a mouse mammary tumor virus (MMTV)-Neu mouse model of breast cancer and examined multiple stages of tumor development to determine whether the loss of Fes function in MMTV-Neu mice could affect the tumor development (Connie Zhang, unpublished). MMTV-Neu female mice develop pre-neoplastic lesions, which progress to adenocarcinomas [137], and approximately 75% of tumor-bearing mice develop metastatic disease [138]. The average age of tumor onset in MMTV-Neu; fes^-/- mice was delayed by more than 100 days as compared to MMTV-Neu; fes^+/+ mice (Connie Zhang, unpublished). In order to investigate the underlying mechanism behind the delay of tumor onset in MMTV-Neu; fes^-/- mice, the pre-malignant mammary gland was assessed by immunoblotting and immunohistochemical staining. The results showed an increased number of monocytes and lymphocytes, as well as an increased level of NFκB activation in the premalignant mammary gland of MMTV-Neu; fes^-/- mice. These findings suggested that the loss of Fes tyrosine kinase can suppress tumor development through increasing the number of anti-tumor monocytes and lymphocytes in the tumor microenvironment [139].

From the earlier investigations of the role of Fes in immunity, we already knew that fes^-/- macrophages showed enhance levels of TNF-α when stimulated with LPS [12], literature also has reported a role of Fes tyrosine kinase in macrophages [33], and has investigated the
subsequent effects of the signaling on myeloid cells, specifically macrophages, and have provided an explanation of how MHC class I molecules on macrophages that are stimulated with TLR4 ligand may suppress the TLR signaling pathway via Fes/SHP2 pathway. The findings suggested that upon TLR4 stimulation, a Src family kinase will become activated and phosphorylate the intracellular domain of MHC class I, which will recruit Fes via its SH2 domain. Then Fes will phosphorylate the SHP-2 tyrosine phosphatase, leading SHP-2 to suppress the TLR signaling pathway, which will result in decreasing the production of pro-inflammatory cytokines and type I interferon [33]. Although a plausible mechanism of how MHC class I might suppresses the TLR signaling pathway via Fes/SHP2 is supported by the data in that paper, the effect of this mechanism on CD8+ T cells, and therefore adaptive immunity, remains unknown and further information is required. Going back to the MMTV model, Parsons’s paper [12] as well as Xu’s paper [33] may provide evidence that Fes may down regulate the tumor microenvironment and thereby, promoting tumor development in MMTV-Nue; fes+/+ mice. From this we generated our hypothesis that fes−/− macrophages can activate CD8+ T cells more effectively than the fes+/+ macrophages.

First, I studied the impact of Fes on differentiation of macrophages and confirmed that bone-marrow progenitor cells from fes−/− mice and fes+/+ mice can develop and differentiate into macrophages which have similar marker expression in vitro. By staining with anti-F4/80, we confirmed that bone-marrow cultured in the present of M-CSF can differentiate into mature macrophages. We also assessed the macrophages for expression of other markers which have an important impact on macrophages ability to activate CD8+ T cells. We observed no differences in the levels of expression of MHC class I which provides Signal 1 to CD8+ T cells; or CD80 and CD86, which provide costimulatory, Signal 2, to activate CD8+ T cells. We further tested the
other markers expression in these cells, which have level of expression on macrophages depend on differentiation stage of the cells [114]. MOMA2 (a general marker for monocytes/macrophages) [122]; CD68 (a member of the scavenger receptor family that can recognize low-density lipoproteins) [123, 124] and CD206 (a mannose receptor which has a role in phagocytosis of pathogens) [125, 126], were selected to examine if Fes has a role in their levels of expression. All markers show similar level of expressions in both genotypes. Therefore, this data suggests that Fes does not regulate MOMA1, CD68 and CD206 markers. We also investigated TLR4 surface expression after LPS and R848 challenge but found similar levels of expression on \( fes^{-/-} \) and \( fes^{+/+} \) macrophages. This data suggested that deficiency in Fes did not affect the endosomal distribution of TLR4 after activation with LPS and R848. Interestingly, in a different experimental model where the authors isolated resident peritoneal macrophages and treated them with 270 ng/ml LPS for 0, 5 and 15 min post stimulation, they found that ligand-induced internalization of TLR4 was suppressed in \( fes^{-/-} \) macrophages [33]. The inconsistent results between those published experiments and the one carried out here, might be due to differences in the experimental design, including the concentration of LPS and the time of treatment before assessing surface TLR4 levels.

In my study, I also tested downstream signaling in \( fes^{+/+} \) and \( fes^{-/-} \) macrophages after stimulation with LPS from 0 to 60 minutes. I found an increased activation level of NFκB as well as IRF3 in \( fes^{-/-} \) macrophages compared to \( fes^{+/+} \) macrophages. The observation with NFκB was consistent with previous findings from our lab [11, 12]. However, I did not observe any difference in LPS-induced phosphorylation of SHP-2 in \( fes^{+/+} \) and \( fes^{-/-} \) macrophages. This may be due to the presence of other protein tyrosine kinases such as Fes-related Fer kinase in the \( fes^{-/-} \) macrophages, because Fer may phosphorylate SHP-2 [140]. LPS-induced activation of TBK,
p38, and Erk was also similar in $fes^{+/+}$ and $fes^{-/-}$ macrophages. Other protein tyrosine phosphatases such as SHP-1 might also play a role in cellular signaling to MyD88 or TRAF6 [141]. Interestingly, SHP-1 can regulate the TLR signaling pathway differently than SHP-2. SHP-1 inhibits the production of proinflammatory cytokines and type I interferons that are induced by all TLR ligands, while SHP-2 inhibits only production of proinflammatory cytokines induced by TLR3 [142, 143]. These data (increased NFκB and IRF3 activation in $fes^{-/-}$ macrophages after LPS stimulation) suggested that Fes negatively regulates the downstream signaling pathways from TLR4, and might thereby suppress the production of pro-inflammatory cytokines and type I IFNs.

In the study by Xu and colleagues [33], they stated that upon activation with LPS, the intracellular domain of MHC class I will recruit Fes through its SH2 domain [33]. Therefore, we attempted to validate this proposed SH2 domain role of Fes in binding to the intracellular domain of MHC class I; as well as investigate the subcellular localization of Fes and MHC I. We transfected macrophages with DsRed labeled wild type or kinase-dead Fes and GFP labeled MHC-I. We observed that these proteins co-localized in a dynamic endosomal compartment of the cells, as well as on the cell surface. Moreover, kinase-dead Fes showed similar co-localization with MHC-1, indicating that the kinase domain is not required for the co-localization and other domains such as F-BAR, FX, and SH2 may be required for the co-localization with MHC class I. One limitation of this study is that we did not investigate the other domain mutations; so further experiments need to be conducted in order to address this question.

To investigate the ability of macrophages to activate CD8$^+$ T cells in the presence and absence of Fes, we used the lymphocytic choriomeningitis virus (LCMV) model, which is a well-established model to study the regulation of MHC-I expression [144, 145]. In this model, we
have shown that when pulsed with gp33 epitope, \( fes^{-/} \) macrophages activated epitope-specific CD8\(^+\) T cells similar to wild-type macrophages. However, when we challenged \( fes^{-/} \) macrophages with TLR4 ligand for 2 hours, and then pulsed them with gp33, LCMV’s epitope, more robust activation of CD8\(^+\) T cells occurred in LPS-challenged \( fes^{-/} \) macrophages. However, this was not recorded with other TLR such as TLR7/8 ligands as representative of viral component, where we found that the activation level was similar to non-stimulated macrophages. These results suggested that Fes tyrosine kinase down-regulated the TLR4 signaling pathway which result in impairing the production of pro-inflammatory cytokines that can strengthen the activation of CD8\(^+\) T cells [90, 92, 129, 146-149].

According to our \textit{in vitro} data which showed that \( fes^{-/} \) macrophages have enhanced ability to activate CD8\(^+\) T cells in the presence of LPS, we tried to assess if expression of Fes in CD8\(^+\) T cells regulates their ability to get activated. This was explored by injecting \( fes^{-/} \) mice and \( fes^{+/+} \) mice with LCMV; and after a week post injection, the spleens were isolated to co-culture with BMA, which is macrophage cell line. The data shows that there were no differences in the activation level between \( fes^{-/} \) CD8\(^+\) T cells and \( fes^{+/+} \) CD8\(^+\) T cells. This observation suggests that Fes does not regulate the ability of CD8\(^+\) T cells to become activated.

As a part of the investigation to understand how Fes can mediate the ability of macrophages to activate CD8\(^+\) T cells, our data indicated that Fes does not affect the surface expression of MHC-I, and therefore it does not appear to regulate the signal one outcome; either surface expression of CD80 and CD86, which are required to activate CD8\(^+\) T cells. However, Fes may mediate signal three which is provided by cytokine expression from macrophages. A previous publication from our lab, showed that LPS challenged \( fes^{-/} \) macrophages secrete higher levels of TNF-\(\alpha\) compared with \( fes^{+/+} \) macrophages [12].
We went further to investigate other cytokines that can be lead to high secretions in \( fes^{-/-} \) macrophages, we stimulated these macrophages with LPS for 4.5 hours and then tested IL-6 level by ELISA. The data shows that \( fes^{-/-} \) macrophages secreted higher levels of IL-6 in comparison to wild-type macrophages. Previous studies have shown that IL-6 has indirect influence on CD8\(^+\) T cells, as it can expand CD4\(^+\) T cells \textit{in vivo} during immunization due to reduced apoptosis, which indicated that IL-6 may also increase both the effector and memory T cells population [150]. Also IL-6 has direct influence on the development of CD8\(^+\) T cells [151]. From this data, we concluded that \( fes^{-/-} \) macrophages upon stimulation with LPS have a higher ability to produce cytokines such as IL-6 which is important for T cells activation, hence this can explain the ability of LPS-stimulated \( fes^{-/-} \) macrophages to activate CD8\(^+\) T cells \textit{in vitro} [152]. We suggest that other cytokines need to be investigated. Such as type I interferons, in particular IFN-\(\beta\) which has been shown to act directly on CD8\(^+\) T cells to allow clonal expansion [149].

Finally, we tried to investigate the activation statuses of CD8\(^+\) T cells \textit{ex vivo}. The identification of surface markers that used to distinguish between naïve, activated and memory T cells has been involve in the characterization of T cell-mediated immune responses [153]. Mice were injected s.c with 6 \( \times 10^4 \) PFU LCMV and the spleens were isolated 0, 2, and 4 days post injection. Panel of activation markers were used to determine the level of CD8\(^+\) T cell activation; which includes CD44, Ly6c, CD62L and CD69 [154].

At day 0 and 2 post LCMV infection, only 40\% of CD8\(^+\) T is CD44\(^+\) In both genotypes and at day 4 post LCMV infection the percentage of CD44\(^+\) T cells increased up to 60\%. In the MFI at day 4 post infection the number of CD44\(^+\) in T cells increased in both genotypes. CD44 can be expressed in an active or inactive stage of T cells but it is higher in the active stage [155], hence, it is not always easy to make a clear distinguish between naïve, activated and memory T
cell cells based on CD44 expression [156]. In our data, we observed significant increase in the percentage and MFI of CD44+ CD8+ T cells but no differences between the two genotypes was observed. This increase indicates that CD8+ T cells at day 4 post infection are more susceptible to reach activation stage. Also, CD44 can mediate cell-cell interactions as well as cell-matrix interactions [157], therefore cells that express high levels of the marker may have the potential to localize within inflamed tissues.

*fes*−/− CD8+ T cells at day 2 post LCMV infection were 50% positive to Ly6c while in *fes*+/+ CD8+ T cells were only 20%. In the MFI data at day 2 post LCMV infection there were significant increase in the number of Ly6c+ in *fes*−/− CD8+ T cells. Ly-6 on T cells, including Ly6c, have been shown to affect T cells activation, inhibition of T cells activation have occurred when T cells are treated with Ly6-specific antibodies [158]. Furthermore, when naïve T cells become cytotoxic effectors will upregulate Ly6c and this will require T cells to rapidly secrete interferon γ [159]. Therefore, the high expression of Ly6c on *fes*−/− CD8+ T cells reflects that these cells have higher ability to secrete IFNγ* in vivo*. However, further experiments need to be done to measure the levels of IFNγ* in vivo* using ELISA.

The percentage of CD62L+ cell and their MFI were the same in both genotype as well as in the 0, 2 and 4 post LCMV infection, which is a naïve marker, and this could mean that these cells did not reach the complete effector stage yet [154, 160].

The last marker we tested was CD69 which is an early activation marker [136], CD69 is a differentiation marker and expressed shortly after T lymphocytes activation, and acts as a costimulatory molecule in proliferation [161]. This marker is detectable after 4 hours post activation and it drops down very fast after 2 days [162]. We observed at day 2 post LCMV infection, there were a slight increase in the percentage of CD69+ *fes*−/− CD8+ T cells and then it
decreased slightly with no significant difference. However, in the number of MFI at day 4 post infection was significantly reduced in $fes^{-/-}$ CD8$^+$ T cells. Indicates that the time kinetic of $fes^{-/-}$ CD8$^+$ T cells activation is faster than the $fes^{+/+}$ CD8$^+$ T cells.

**Significance and Future Directions**

Many studies have focused on studying oncogenes and the intrinsic signaling pathways which regulate cancer cells survival and proliferation. Moreover, other studies indicated that the tumor microenvironment plays a critical role in cancer development and metastasis. Here, our work provides evidence that Fes supresses the ability of macrophages to activate CD8$^+$ T cells. Although our study was conducted on bone-marrow derived macrophages, it may reflect and explain the delayed tumor growth in the $MMTV$-$Neu; fes^{-/-}$ mice. This work may also provide an opportunity to develop a new therapy such as Fes inhibitor drugs.

This work focused only on macrophages in innate immunity and their ability to activate CD8$^+$ T cells in the adaptive immunity. For the future, this projects can be extended to investigate the effect of Fes on other cells type such as dendritic cells as they are also known to express TLR4 [163] and understand how they can activate CD8$^+$ T cells. Moreover, it would be interesting to investigate the role of Fes in MHC class II in macrophages and dendritic cells and how they can activate CD4$^+$ T cells and to investigate which mice from both genotypes can clear viral infection faster using virus titration assay.

To summarize, this thesis suggests that Fes may suppress the ability of innate immune cells to activate T cells through inhibitory roles in signaling downstream of MHC I and TLR receptors. It follows that inhibiting Fes could increase the efficacy of immune cells to viral infection and possible tumor growth.
Chapter 5

Bibliography


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