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Impact of the Group IV Protein Tyrosine Kinase, Fer, on Intestinal Inflammation and Neutrophil Function

by

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Abstract

Fer kinase is a protein tyrosine kinase (PTK) belonging to the group IV family of cytoplasmic enzymes which consists of one other family member, Fps/Fes, and a truncated form of Fer (Fer^{T}). An important role for Fer has been proposed in modulating innate immune responses driven by bacterial antigen such as lipopolysaccharide (LPS). For example, data generated *in vivo* has shown LPS-induced epithelial barrier dysfunction is exacerbated in the absence of Fer kinase activity in mice. Increased damage was associated with enhanced neutrophil recruitment suggesting Fer may modulate neutrophil recruitment and/or function in the gut. Interestingly, both animal models and clinical studies have demonstrated a strong link between bacterial antigens and chronic inflammatory conditions such as inflammatory bowel disease (IBD). Therefore, we hypothesized that group IV PTK such as Fer could regulate intestinal inflammation by modulating neutrophil recruitment and/or function. In this thesis, we examined the role of Fer in two models of colitis using Fer null mutant mice ($Fer^{DR/DR}$). First, in a chemically-induced model of colitis (2, 4, 6-trinitrobenzene sulphonic acid (TNBS)) we demonstrate that $Fer^{DR/DR}$ mice develop an exacerbated and prolonged colitis compared to WT mice, which was associated with enhanced neutrophil recruitment to the gut. Next we generated double mutant mice to examine the role of Fer in a spontaneous chronic model of IBD- interleukin 10 deficient ($IL\text{-}10^{-\backslash-}$) model. A significant increase in macroscopic and histological inflammatory parameters was observed in $IL\text{-}10^{-\backslash-}Fer^{DR/DR}$ double deficient compared to single mutants. Using the under-agarose chemotaxis assay, we showed evidence for a stimulus specific role for Fer in restraining neutrophil chemotaxis. Enhanced chemotaxis was observed toward end target chemoattractants

fMLP-like peptide (WKYMVm) and C5a. Next the intracellular signalling pathways (PI3K and p38MAPK) used in chemotaxis to WKYMVm were examined. The PI3K pathway in neutrophils was shown to have prolonged activation after WKYMVm stimulation in the absence of Fer kinase. Finally, we demonstrated a role for Fer in modulating the rate of superoxide production using the cytochrome c reduction assay but show no role in neutrophil intracellular bacterial killing or apoptosis assays. Our studies indicate that Fer kinase regulates intestinal inflammation responses potentially through its ability to control neutrophil recruitment and superoxide production in response to bacterial peptide.

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Table of Contents

List of Tables

List of Figures and Illustrations

List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
3T3 cell line	Mouse fibroblast cell line
4G10 antibody	Anti-phosphotyrosine antibody
7AAD	7-Amino-Actinomycin-D
Ab	Antibody
Akt	Protein kinase B
A/L	Aprotinin (Anti-Trypsin)/Leupeptin (lysozyme neutraliser)
BSA	Bovine serum albumin
C5a	Complement split product 5a
CC	Coiled-coil motifs
CD	Cluster of differentiation
CD	Crohn`s disease
CGD	Chronic granulomatous disease
CHS	Chediak-hegashi syndrome
CO_2	Carbon dioxide
CR	Complement receptor
DC	Dentritic cell
DFP	Diisopropylfluoro-phosphate
DSS	Dextran sulphate sodium
ECL	Enhanced chemilumiscence
EDTA	Ethylene-di-aminetetraacetic acid

EGF	Epidermal growth factor
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FCH	Fer/Cdc42-interacting protein-4 homology
Fer	Fes-related protein
$Fer^{DR/DR}$	Fer kinase mutant
FGF	Fibroblast growth factor
FMLP	Formyl-met-leu-phe
FPR	Formyl peptide receptor
Fps^{Kr}/Fer^{Dr}	Fps and Fer kinase double mutant
GI	Gastrointestinal
GM-CSF	Granulocyte- macrophage colony stimulating factor
GPCR	G-protein coupled receptor
H_2O_2	Hydrogen peroxide
H&E	Hematoxylin and eosin
HBSS	Hanks balanced salt solution
HEPES	4-(2-hydroxylethyl)-1-piperazineethanesulfonic acid
IB	Immunoblotting
IBD	Inflammatory bowel disease
IFNγ	Interferon gamma
Ig	Immunoglobulin

IL	Interleukin
IL-10$^{-\backslash-}$	Interleukin 10 deficient
IL-10$^{-\backslash-}$Fer$^{DR/DR}$	Interleukin and Fer kinase double deficient
IP	Immunoprecipitation
IRS-1	Insulin receptor substrate-1
KC	Keratinocyte derived cytokine
LTB_4	Leukotrine B_4
LPS	Lipopolysaccharide
LY294002	PI3K inhibitor
MAPK	Mitogen-activated protein kinase
MAPKAPK2	Mitogen-activated protein kinase-activated protein kinase 2
MDP	Muramyl di-peptide
MIP-2	Macrophage inflammatory protein-2
Mm	Millilitres
MPO	Myeloperoxidase
Na_3VO_4	Sodium orthovanadate; inhibitor for protein tyrosine phosphatases, alkaline phosphatases and ATPases
NADPH	Nicotinamide adenine dinucleotide phosphate
NFκ-B	Nuclear factor kappa-light-chain-enhancer of activated B cells

NGF	Nerve growth factor
NKT	Natural killer T-cells
NOD	Nucleotide binding oligomerization domain
NRAMP-1	Natural-resistance-associated macrophage protein 1
NRTK	Non receptor protein tyrosine kinase
OH	Hydroxide
PAF	Platelet activating factor
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PDK1	Phospho-inositide-dependent-protein kinase
Pen/Strep	Penicillin/streptomycin
Pepstatin	Aspartyl protease inhibitor
PI3K	Phospho-inositide 3 kinase
PIP_2	Phosphatydilinositol 4-5-biphosphate
PIP_3	Phosphatydilinositol 3, 4, 5-triphosphate
PLC	Phospholipase C
PMSF	Phenylmethanesulphonylfluoride; serine protease inhibitor
PSGL-1	P-selectin glycoprotein ligand-1
PSI	N-carbobenoxy-L-isoleucyl-L-γ-t-butyl-L-glutamyl-L-leucinal
PTEN	Phosphate and tensin homolog deleted on

	chromosome 10
PTK	Protein tyrosine kinase
RA	Rheumatoid arthritis
RB68C5	Monoclonal anti-neutrophil antibody
ROS	Reactive oxygen species
RPM	Revolutions per minute
RT	Room temperature
RTKs	Receptor tyrosine kinases
SB203580	P38 MAPK inhibitor
SB239063	P38 MAPK inhibitor
SKF86002	P38 MAPK inhibitor
SDS	Sodium dodecyl sulphate
Sh2	Src homology 2 domain
SIRS	Systemic inflammatory response syndrome
SOD	Superoxide dismutase
STAT	Signal Transducers and Activators of Transcription protein
Th	T-helper cell
TGFβ	Transforming growth factor beta
TLR	Toll-like receptor
TNBS	2, 4, 6-trinitrobenzene sulphonic acid
TNF	Tumour necrosis factor
UC	Ulcerative colitis

VEGF	Vascular endothelial growth factor
WBCs	White blood cells
WKYMVm	fMLP-like peptide
Wortmannin	PI3K inhibitor
WT	Wild type

Chapter One: **INTRODUCTION**

1.1 Protein kinases

The specific reaction catalyzed by protein kinases modifies other proteins by chemically adding a phosphate group (via ATP) to serine, threonine, or tyrosine residues. This reaction usually results in functional changes of the target protein through changing enzymatic activities, cellular location, or association with other proteins. Approximately 30 % of all proteins can be modified by kinase activity, and their activity is known to activate a wide variety of signalling pathways that play a role in cell proliferation, differentiation, or metabolic changes [1]. The human genome contains about 518 protein kinase genes; they constitute about 2 % of all eukaryotic genes, and are classified into 20 different families based on amino acid sequence comparisons [2]. Disregulated kinase activity has been implicated in the pathogenesis of many disease states including cancer and diabetes [3, 4]. Drugs which inhibit specific kinases are being developed to treat several diseases, and some are currently in clinical use [5-7]. For example, Erlotinib inhibits the intracellular kinase activity of epidermal growth factor receptor (EGFR) and currently is used in the treatment of lung cancer [8].

Protein kinases are classified into different groups based on the residue they phosphorylate; mixed kinases, serine/threonine-, tyrosine-, histidine-, and aspartic acid/glutamic acid-specific protein kinases. Protein tyrosine kinases (PTKs) are enzymes that transfer phosphate groups to tyrosine residues on proteins.

1.2 Protein tyrosine kinases (PTKs)

There are about 90 PTKs in the human genome, which are classified into two major groups depending on whether they function as plasma membrane receptors (20 families), or are located in the cytosol (non-receptor; 10 families). Receptor tyrosine kinases (RTKs) are trans-membrane glycoproteins that are activated by the binding of their ligands to transduce the extracellular signal to the cytoplasm by phosphorylating tyrosine residues on the receptor themselves and on downstream signalling proteins. RTKs family include the receptors for insulin and different growth factors such as epidermal growth factor (EGF), platelet derived growth factor (PDGF), nerve growth factor (NGF), vascular endothelial growth factor (VEGF), and fibroblast growth factor (FGF). Non-receptor protein tyrosine kinases (NRTKs) are a large family which includes Src family kinase, Janus kinases (Jaks), FAK, Abl, TEC, SYK, Fps/Fes, and Fer. Non receptor PTK facilitate downstream signalling pathways of receptor TK or other cell surface receptors like G-protein coupled receptors (GPCR) or receptors of the immune system [9].

A unique group of cytoplasmic PTKs is group IV PTK which include two members; Fer and Fps/Fes. There is little known so far regarding their physiological and pathophysiological roles in the body, and the aim of my thesis was to determine if these kinases play a role in modulating intestinal inflammation specifically through neutrophil function.

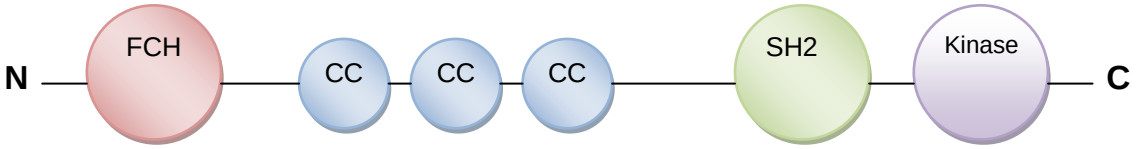
1.3 Group IV protein tyrosine kinases

Fer and its closely related family member Fps/Fes belong to a unique group of non-receptor protein tyrosine kinases (PTKs)- the group IV PTK [10]. Fps/Fes was first isolated as oncogene from tumour-causing avian and feline retroviruses in 1980 [11]. During initial attempts to identify Fps/Fes cellular location, another closely related protein was identified by chance which was subsequently cloned as Fes-related protein (Fer) in 1988 [12]. The close structural similarities between Fer and Fps/Fes kinases indicate that they might serve similar or even redundant physiological roles in the body. This redundancy has subsequently been demonstrated in hematopoiesis regulation and cytokine-induced signal transducers and activators of transcription protein-3 (STAT3) activation in macrophages [10, 13]. IL-10 is an important anti-inflammatory mediator which reduces the production of many pro-inflammatory cytokines [14]. The anti-inflammatory response of IL-10 in macrophages requires STAT3 activation [15]. It has been shown that IL-10 induced STAT3 activation was reduced in Fps^{Kr}/Fer^{Dr} double mutant macrophages which suggest a role for Fer and Fps/Fes PTK in regulating the inflammatory response [10].

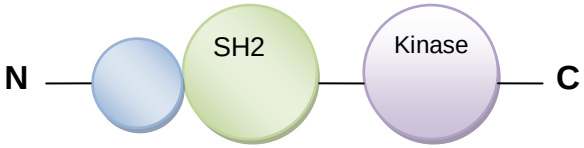
1.4 Structural components and expression profile of group IV PTK

Fer and Fps/Fes kinases share similar structural domains including an N-terminal FCH (Fer/Cdc42-interacting protein-4 homology) domain that distinguishes them from other members of Src, Abl, Jak, or Fak cytoplasmic tyrosine kinase subfamilies (diagram 1.1).

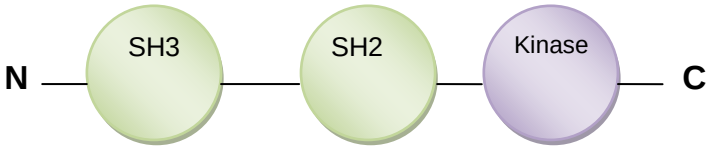
Fer and Fps/Fes structure



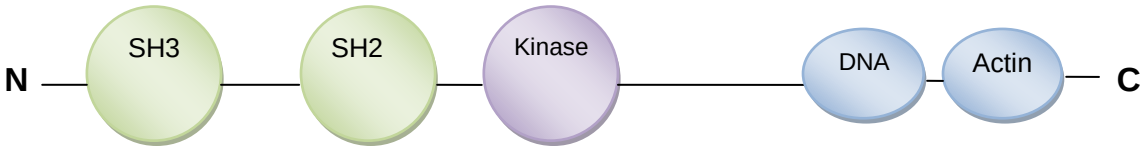
Fer ^{T}structure



Src structure



Abl structure



SH2- Src homology 2
SH3- Src homology 3
FCH- Fer/Cdc42-interacting protein-4 homology
CC- coiled-coil motifs

Diagram 1.1 Structural components of Fer and Fps/Fes PTKs in relation to other members of cytoplasmic PTKs

Group IV PTK contain an N-terminal FCH domain that distinguishes them from other members of cytoplasmic PTK subfamilies. They contain three predicted CC motifs which mediate their oligomerization followed by a SH2 domain which mediates protein-protein interactions. The C-terminal contains the catalytic domain. The testes-specific Fer^{T} lacks the N-terminal FCH and CC domains but retain the SH2 and the C-terminal kinase domain.

The N-terminal domain has been detected in numerous proteins, many of which are implicated in the regulation of cytoskeletal rearrangements, vesicular transports, and endocytosis [16-18]. Group IV PTK contains three predicted coiled-coil (CC) motifs that mediate the formation of homotrimers in the case of Fer, and pentamer or higher-order oligomers in the case of Fps/Fes. This oligomerization allows trans-auto-phosphorylation, which might modulate interactions between the SH2 domain and catalytic domain, or interactions with other coiled-coil domain containing proteins [19-22]. The central Src homology 2 (SH2) domain mediates protein-protein interactions, and the associations of Fer with several proteins like: p120 catenin, epidermal growth factor (EGF) receptor, platelet-derived growth factor (PDGF) receptor, phospho-inositide-3-kinase (PI3K), and insulin receptor substrate-1 (IRS-1) [23-26]. Both Fer and Fps/Fes form trimers mediated by their CC motifs, however heterotypic interactions were not detected between Fer and Fps/Fes. The C-terminal contains the catalytic domain [20, 27].

Group IV PTK are ubiquitously expressed. There is one other member of the group IV PTK family, a testes-specific Fer^{T} transcript truncated form which accumulates in primary spermatocytes during the mid- and late- pachytene stages of mitotic prophase suggesting a role for Fer^{T} in regulating spermetogenesis [28]. However, $Fer^{DR/DR}$ mice are fertile which suggest that Fer^{T} is not involved in reproduction.

1.5 Role of group IV PTK in inflammation

1.5.1 In vitro role

From *in vitro* studies, it has been shown that Fer is activated in fibroblasts downstream of epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) receptors [21, 25], and Fer has been postulated to mediate the phosphorylation status of proteins that play a role in cell-cell and cell-matrix interaction like β-catenin, p120 catenin, and cortactin upon activation with EGF and PDGF [29]. In mast cells, Fer is activated downstream of the high affinity receptor for immunoglobulin E (IgE), and this activation is needed for sustained p38 MAPK activity and increased antigen-mediated cell migration of sensitized mast cells [30, 31]. Recently, it has been demonstrated that Fer and cortactin are tyrosine phosphorylated in response to hydrogen peroxide (H_2O_2) and integrin engagement in different cell types which results in actin polymerization and fibroblast cell migration [32]. Fps/Fes is activated in hematopoietic cells in response to a wide variety of cytokines such as interleukin (IL)-3, IL-4, IL-6, erythropoietin, and granulocyte-macrophage colony stimulating factor (GM-CSF) [33-36]. It is possible that Fer kinase plays a role in the activation of cells through these stimuli because the antibody used in these early studies could have cross-reacted with Fer kinase due to the close similarities in the structure.

1.5.2 In vivo role

To study the physiological roles of Fer and Fps/Fes kinases *in vivo*, mice with kinase inactivation mutation in the Fer locus ($Fer^{DR/DR}$) [37] and Fps locus [38] have been generated by Peter Greer and Andrew Craig group at Queen`s university. $Fer^{DR/DR}$ mice were generated by targeting the *fer* allele with a kinase-inactivating mutation of aspartate 743 to arginine (D743R), which is predicted to disrupt the catalytic loop of the protein. These mutant mice are viable and fertile, and did not display any major defects in myeloid differentiation.

In 2002 McCafferty *et al* [39] demonstrated an important role for Fer in regulating innate immune response to LPS. Using intravital microscopy to study leukocyte recruitment *in vivo*, a fourfold enhancement in emigration was observed in the skeletal muscle microcirculation in response to local lipopolysaccharide (LPS) injection compared to wild type (WT) mice. These data suggest for the first time that Fer kinase may play an important role in regulating inflammation-induced leukocyte recruitment toward a bacterial antigen. Qi *et al* [40] extended this data to demonstrate a role for Fer kinase in leukocyte recruitment to the gut in response to LPS. In WT mice LPS recruited neutrophils to the gut which was associated with an increase in epithelial barrier damage measured by Cr^{51}-EDTA blood-to-lumen leakage. In the absence of Fer kinase, an exacerbated leukocyte recruitment and epithelial barrier damage was observed compared to WT leukocytes. The barrier defect was greatly attenuated in $Fer^{DR/DR}$ mice by depleting circulating neutrophils with monoclonal anti-neutrophil antibody (RB6 8C5) suggesting that neutrophils were responsible for dysfunction since RB68C5 prevented neutrophil recruitment and barrier damage. These data suggest that Fer regulates LPS-

induced neutrophil recruitment and epithelial barrier dysfunction *in vivo*. Subsequent bone marrow chimeric studies using irradiated WT mice which received bone marrow derived cells from either WT or $Fer^{DR/DR}$ mice were used to determine the role of hematopoietic components in this phenotype. Irradiated WT mice which received bone-marrow derived cells from $Fer^{DR/DR}$ donor mice experienced enhanced epithelial barrier dysfunction in the gut in response to LPS challenge which suggests that Fer within bone-marrow derived cells are depicting the phenotype. Taken together, these data suggest a role for Fer within the neutrophils in modulating epithelial barrier integrity in the gut in response to the bacterial antigen LPS.

Fer kinase may be acting to regulate neutrophil recruitment and/or function in response to LPS. LPS-induced inflammation results in the generation of a variety of chemoattractants. Loss of Fer activity within neutrophils may result in enhanced neutrophil recruitment to these chemoattractants. This enhanced recruitment would result in more damage. It is also possible that Fer kinase may regulate neutrophil function by modulating phagocytosis, degranulation and apoptosis at the site of inflammation which could result in an enhanced barrier defect.

Interestingly, Zirngibl *et al* [41] has recently demonstrated an important role for Fps/Fes kinase in regulating innate immunity *in vivo*. Fps null mice have an enhanced mortality in response to local LPS challenge compared to WT mice, and this response was rescued when Fps-null mice were crossed with a strain that carried a human Fps transgene. The mechanism for this enhanced mortality in Fps-null mice in response to LPS is thought to involve an enhanced tumour necrosis factor-α (TNF-α) production, prolonged NFκ-B activity, and a defect in TLR4 internalization in peritoneal

macrophages isolated from mutant mice [42]. Furthermore, a role for Fps/Fes in leukocyte emigration *in vivo* has recently been observed [43]. Parson *et al* demonstrated enhanced leukocyte emigration in the skeletal muscle in the Fps/Fes mutant mice in response to LPS challenge in a similar manner to Fer kinase deficiency. Neutrophil recruitment to the peritoneal cavity subsequent to thioglycollate challenge was also enhanced. These data may be explained by prolonged expression of the selectin ligand PSGL-1 on the neutrophil surface in response to LPS challenge which plays a role in leukocyte adhesion.

Taken together, these data suggest an important role for group IV PTK in modulating innate immunity in response to bacterial antigen possibly through an action within neutrophils. These observations may have a clinical relevance in IBD where neutrophils have a strong presence and activation of the immune response by bacterial antigen is thought to result in an abnormal chronic intestinal inflammation in genetically susceptible individuals.

1.6 Inflammatory bowel disease (IBD)

1.6.1 Epidemiology of IBD

Epidemiological data show the highest incidence rates and prevalence of IBD reported from northern Europe, the UK, and North America (USA and Canada). The rates continue to rise in low-incidence areas such as Southern Europe, Asia, and most developing countries [44-50]. It has been suggested that there are approximately 170,000 (or 1 in 180) Canadians with IBD [51]. IBD is a chronic condition that requires a lifetime

of care, and it can cause significant morbidity, but is not generally associated with increased mortality [52]. It costs the health care sector billions of dollars each year and accounts for significant physician visits and hospitalization [52].

1.6.2 Definition and types

IBD is a chronic inflammatory disorder of the gastrointestinal (GI) tract, which comprises two conditions: Crohn's disease (CD) and ulcerative colitis (UC) [53-56]. CD was first seen by German surgeon Wilhelm Fabry in 1623 [57], and was later described by and named after the US physician Burril B Crohn in 1932 [58]. It is generally a chronic condition with a relapsing nature, and there are often periods of remission, but it may also manifest as chronic continuous symptoms. CD can occur anywhere along the GI tract from the mouth to the anus (although it is more common in the ileocecal area), and the inflammation can extend to the all layers of the gut (transmural inflammation). UC was first described by the British physician Sir Samuel Wilks in 1859 [59]. In UC, the inflammation is limited to the rectum and colon, and it is a relapsing and remitting condition with disease-free intervals alternating with periods of symptomatic inflammation. The inflammation is continuous and limited to the mucosal and sub-mucosal layers.

Previous evidence was provided to demonstrate that the type of immune response in CD is typical of the T-helper (Th1) type, as assessed by elevated expression of interleukin (IL-12), TNF-α, and interferon gamma (IFN-γ) [60-62]. The immune response in UC is classically characterized by increased secretion of Th-2 cytokines like IL-5 and IL-13, but not IL-4 or IFN-γ [63]. However, several studies have recently lead to the

identification of more complex cytokine interaction network in IBD with the discovery of a distinct subset of Th cells-the Th-17 cell phenotype [64-66]. For a long time research in IBD focused on targeting the pro-inflammatory cytokine IL-12, which consists of two subunits p35 and p40. Neutralizing antibody to the p40 subunit was found to ameliorate established colitis in various animal models of IBD [67, 68]. In CD patients, the expression of the IL-12 p40 subunit was significantly enhanced, and a recent study by Mannon *et al* [69] demonstrated that a recombinant human full-length IgG1 antibody genetically designed to recognize human IL-12 p40 subunit was effective in the treatment of CD patients. With the discovery of IL-23 [70] which consists of p19 and a shared p40 subunit with IL-12, it became clear that more was going on and the clinical efficacy of anti-IL-12 p40 subunit in CD patients might be due to the inhibition of IL-23 rather than IL-12. IL-23 is a novel cytokine which belong to the IL-12 family required for effective priming of T-cell response. It is mainly produced by activated myeloid cells such as macrophages and dendritic cells [70]. IL-23 activates STAT3 and STAT4 and differentiates responsive CD4+ lymphocytes to a pathogenic subpopulation characterized by IL-17 production (Th17 lymphocytes) [70-73]. The IL-17 family consists of six members (IL-17A-F), and they play a major role in stimulating granulopoiesis and in mobilizing granulocytes to sites of inflammation thus linking myeloid and lymphoid host defence mechanisms [74]. IL-23/IL-17 has been shown to be elevated in animal models of IBD, and several studies suggest an important pro-inflammatory role [75-78]. Clinically, IL-23/IL-17 levels have been shown to be elevated in colonic tissues from CD patients [79]. In addition, IL-17 expression has been found to be enhanced in the serum and intestinal mucosa of both CD and UC patients [80]. Interestingly, a polymorphism in

the gene encoding the IL-23 receptor that confers strong protection against CD was recently discovered [66]. These data suggest an important role for IL-23/IL-17 signalling pathways in the development on colitis and serve as an important therapeutic target in drug therapy for IBD treatment.

Disregulated activity of immunoregulatory cytokines such as transforming growth factor (TGF-β) have also been found to be associated with disease pathogenesis. TGF-β is a cytokine produced by immune and non-immune cells and exerts a number of negative effects such as inhibition of T-cell proliferation and down-regulation of macrophages activation [81]. TGF-β initiates signalling cascade through the ligand-dependent activation of a complex of heterodimeric receptors and a family of proteins that serves as substrates for TGF receptors called Smads. TGF-β1 knockout mice spontaneously develop colitis and die early in life [82] and a defect in TGF-β signalling has been shown in CD and UC patients [83] . Furthermore, restoring TGF-β signalling through blocking the activity of the inhibitory Smad7 protein was associated with reduced colitis severity in models of IBD [84].

1.6.3 Role of bacteria in IBD

There are a number of factors which contribute to the development of IBD including genetic susceptibility, environmental triggers, impaired barrier function, and an over activated immune response toward commensal bacteria [85, 86]. Many studies have clearly addressed a role of commensal bacteria in the pathogenesis of IBD [87, 88]. For example, antibiotic pre-treatment can reduce the severity of inflammation in many models of IBD [89]. IL-10 deficient mice do not develop inflammation if they are kept in

a germ-free environment [14]. Furthermore, treating the mice with an antibiotic agent like metronidazole, which is active against anaerobic bacteria, before the onset of colitis can prevent disease development [90]. All of these data suggest an important role for bacterial antigens in the pathogenesis of intestinal inflammation.

Disregulation in innate immunity recognition is a component of IBD. Toll-like receptors (TLR) are trans-membrane receptors which bind distinct molecular signatures present on many pathogens. For example, TLR4 is the major receptor for LPS recognition [91]. TLR signalling can activate distinct intracellular signalling events via diverse adaptor proteins resulting in activation of NFκB and the subsequent transcriptional activation of genes encoding cytokines, chemokines, and costimulatory molecules (diagram 1.2). TLR expression levels have been shown to be modulated in the intestinal compartment of IBD patients and there is an association of $TLR4^{D299G}$-polymorphism with IBD in selective populations [92].

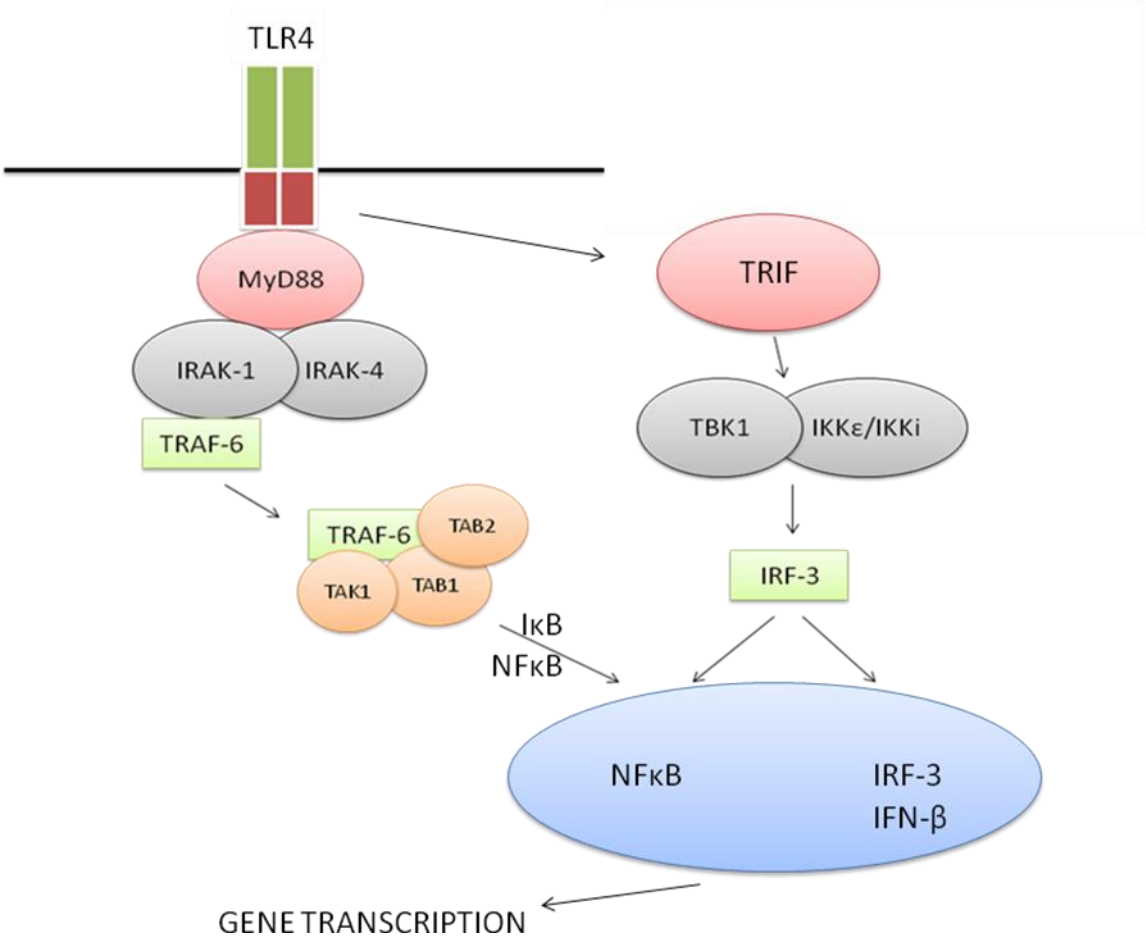


Diagram 1.2 Toll-like receptor (TLR) signalling cascade

TLR4 mediates signalling cascade through MyD88-dependent and independent pathway. MyD88 binds to the cytoplasmic portion of TLR then recruit and stimulate IL-1 receptor-associated kinase (IRAK). IRAK is activated by phosphorylation and associates with tumor necrosis factor receptor associated factor (TRAF6) leading to the activation of NFκB and the subsequent transcriptional activation of genes encoding cytokines, chemokines, and costimulatory molecules. MyD88-independent pathway leads to the activation of the transcription factor IFN-inducible gene (IRF-3) and association with IκB kinases IKKε/IKKi and TBK1 leading to induction of several IFN-inducible genes [93].

NOD2 (nucleotide binding oligomerization domain 2) is an intracellular sensor of bacteria-derived muramyl di-peptide (MDP). Ligand stimulation of NOD2 leads to NFκB activation and expression of several pro- and anti-inflammatory genes, and the augmentation of caspase induced apoptosis cascade [94]. Kobayashi *et al* [95] demonstrated that oral (but not systemic) infection of NOD2 deficient mice with gram-positive bacterium *Listeria monocytogenes* resulted in enhanced susceptibility to infection. Several other reports suggested a strong association between NOD2/CARD15 genotype mutation which correlates with the incidence of Crohn`s disease (CD) [96-98]. This mutation results in defect in defensin production from paneth cells found in the base of intestinal crypts which leads to defect in bacterial recognitions and clearance and results in chronic inflammatory responses in the gut.

1.6.4 Role of neutrophil in IBD

Neutrophils are key players in the innate immune response and are thought to play a role in the pathogenesis of this inflammatory condition. Although some reports did not demonstrate any benefit by depleting circulating neutrophils in acetic acid model of colitis [99], other studies strongly suggest a role for neutrophils in the pathogenesis of intestinal inflammation [100, 101]. Antibody treatment to deplete circulating neutrophils prior to colitis induction reduced the severity of dextran sulphate sodium (DSS)-induced inflammation [102, 103]. In addition, neutrophil-derived reactive oxygen metabolites have been shown to play a role in tissue damage in models of colitis, and treating the animals with antioxidants (such as superoxide dismutase; SOD) reduced colitis severity

[100]. These data suggest that neutrophil over activation secretion of mediators can lead to tissue damage and chronic colitis.

In addition, other data suggest that a defect in neutrophil function (recruitment and mediator release) toward bacterial killing is the early event in mediating chronic inflammation and IBD. Genetic disorders which result in defective neutrophil function include chronic granulomatous disease (CGD), Chediak-hegashi syndrome (CHS), and glycogen-storage disease type IIb have CD-like symptoms. Patients with these disorders can benefit from granulocyte macrophage colony stimulating factor (GM-CSF) therapy [104, 105]. For example, a daily subcutaneous injection of sargramostin (GM-CSF) for 56 days has been shown to reduce colitis severity and enhance mucosal healing and health related quality of life in CD patients. This is thought to be due to enhancing the innate immune response by increasing neutrophil numbers and function.

1.6.5 Medical therapy and its limitations in IBD

The current medical therapies available to treat IBD patients include aminosalicylates, immunosuppressant agents such as corticosteroids, azathioprine, and methotrexate, and anti-TNF therapy such as infliximab. The current known therapies for IBD provide therapeutic benefits and improvement in patient's quality of life, but they are associated with significant side effects. Moreover, significant proportions of patients are either steroid-resistant (15-20 %) or steroid-dependent (25-30 %) [106]. In addition, the existing therapies fail to induce and maintain remission in around 30 % of patients, and maintenance therapies have limited efficacy [107]. A high proportion of patients (20 % in UC, and 50 % in CD) require surgery during their lifetime. Although surgery for

UC is a cure, CD can reappear in previously unaffected parts of the colon [106-108]. The biological therapies, such as infliximab are currently used for the treatment of steroid-refractory CD patients, but this agent has its own disadvantages including restriction to non-oral routes of administration that requires multiple visits from the patient to the hospital, high cost of treatment, and immunogenicity of the product [107]. Despite the intense efforts in the field of medical research done so far, there is no medical therapy that provides a cure for this condition. The primary goals for therapy are to induce and maintain remision, ameliorate symptoms (reduction of abdominal pain, diarrhea, fatigue, anemia, extra-intestinal manifestations, hospitalization, and complications), and improve patient`s quality of life.

There is a need to define new therapeutic targets to offer alternatives to treat this chronic inflammatory condition and provide a cure. For many people who are refractory to standard therapy it may be that the inflammation they present is so disregulated and beyond control. TK are a potential target within the inflammatory response since they regulate many cellular functions. The discovery of small molecule inhibitors of specific TK in recent years has had a significant impact on the treatment of various inflammatory conditions such as rheumatoid arthritis (RA) and neurological diseases [109, 110]. In addition, these inhibitors have also been used to treat a wide variety of neoplastic conditions such as chronic myelogenous leukemia, non-small lung carcinoma, and breast cancer [111, 112] . Many TK have been targeted for inhibitory therapy. In this thesis, I will investigate the group IV PTK and their contribution in IBD like disease to evaluate their potential as targets for therapy. We hypothesize that these kinases play an important role in regulating the response in the gut to bacterial antigen such as LPS perhaps through

action within neutrophils which could potentially modulate intestinal inflammatory conditions like IBD.

1.7 Neutrophil biology

A great portion of bone marrow activity is dedicated toward the production of neutrophils. Around $0.9x10^9$ neutrophils are produced per kilogram body weight per day in an average person from the bone marrow [113]. In the blood circulation, there is an average of $4x10^6$ mature neutrophils per millilitre of blood, but large numbers of mature cells are also present in the bone marrow. During the inflammatory response or infections, the number of neutrophils in the circulation increase rapidly and dramatically, which results initially from the release of the mature cells stored in the bone marrow. Mature neutrophils recruited to the tissue will survive there for several days. However, if these cells are not recruited to the tissue, they are thought to exist in the circulation for about 12-24 hours before being cleared.

1.8 Neutrophil function

1.8.1 Chemotaxis

Neutrophils are the first line of the innate immune defence against insults to the body. They are very motile cells and have a powerful arsenal of weapons that enable them to destroy invading bacteria or inflammatory stimuli. They migrate from the blood stream to the site of inflammation in response to a wide variety of chemoattractive agents. Chemoattractive agents generated at the site of inflammation are classified into

two main groups: intermediate target chemoattractants [CXC chemokines like: keratinocyte derived cytokine (KC), macrophage inflammatory protein-2 (MIP-2), lipid mediator leukotrine B_4 (LTB_4) and platelet activating factor (PAF)], and end target chemoattractants [complement split product (C5a) and bacterial toxin formyl-met-leu-phe (fMLP)] [114]. When neutrophils are exposed to an attractant gradient, they will polarize and chemotax toward the source of the attractant at high speeds that can reach up to 20 μm/min. Neutrophils use different intracellular signalling pathways to migrate toward the source of the chemoattractant, and are dependent on the type of chemoattractant they encounter.

1.8.2 Pathogen recognition and uptake

Neutrophils can recognize different microorganisms through specific cell surface receptors. Microorganisms are recognized by neutrophils through toll-like receptors (TLRs) that bind to conserved molecular patterns, and whose engagement activate different signalling pathways resulting in new gene expression in the responding cell. Neutrophils express the entire repertoire of TLRs, with the exception of TLR3 [115]. Also, intact microorganisms or particles that are opsonized by immunoglobulin (Ig) or complement binding to Fc receptors and complement receptors will result in phagocytosis and engulfment [116, 117].

1.8.3 Oxidative and non-oxidative mechanisms of neutrophil killing

Neutrophils are efficient phagocytes that engulf and degrade microorganisms using a combination of oxidative and non-oxidative mechanisms. The oxidative arm of the

microbicidal action of neutrophils is through the synthesis and release of reactive oxygen metabolites. The interaction of certain proinflammatory mediators (LTB_4, PAF, immune complexes, and bacterial products like fMLP and LPS) with specific receptors on the neutrophil plasma membrane results in the dramatic increase in oxygen consumption due to the activation of nicotinamide adenine dinucleotide phosphate-(NADPH) oxidase system. Activation of this multi-component enzyme results in the production and release of large amounts of the superoxide anion radical [118]. Reactive oxygen metabolites can react with reactive nitrogen metabolites (eg nitric oxide) to generate highly toxic free radicals such as peroxynitrites [119] which can contribute to inflammation and potentially cancer development. Normally, most cells and tissues are protected from the dangerous effects of reactive oxygen metabolites by the action of anti-oxidant enzymes such as superoxide dismutase (SOD), catalase, and GSH peroxidase [120, 121].

The non-oxidative arm of neutrophil killing is through the action of anti-microbial peptides and proteases. The microorganism is detected and sequestrated in the neutrophil phagosome, and then neutrophil granules will fuse resulting in phagolysosome. After that, neutrophil proteases or anti-microbial peptides will be released from the granules into the phagolysosome resulting in microbial killing. Neutrophils contain the following four types of granules: azurophil (primary) granules, specific (secondary) granules, gelatinase granules, and secretary granules [122]. These granules are formed in different stages of neutrophil differentiation in the bone marrow, and they are classified according to their protein content and their ability to exocytose after neutrophil activation by inflammatory stimuli [123]. Azurophil granules undergo limited exocytosis, and they mainly contribute to the intracellular degradation of microorganisms in the

phagolysosome. These granules are defined by their high content of myeloperoxidase, defensin, and a family of structurally related serine proteases (Cathepsin G, neutrophil elastase, and proteinase 3) [124-126]. Specific granules contain different components such as lactoferrin, lysozyme, collagenase, and cytochrome b558. Gelatinase granules contain gelatinase, lysozyme, natural-resistance-associated macrophage protein 1 (NRAMP1), and leukolysin. Finally, secretory granules, which have the highest tendency for exocytosis after neutrophil activation, contain several components like complement receptor (CR)-1, CR3, CD14, CD16, and formyl peptide receptor (FPR) [123].

1.9 Hypothesis

I hypothesized that the group IV protein tyrosine kinase (PTK), Fer, plays a role in the pathogenesis of intestinal inflammation (such as IBD) through the regulation of neutrophil chemotaxis and/or function.

1.10 Objectives

1- Determining the role of group IV PTK in animal models of inflammatory bowel disease (IBD).

2- Determining the role of group IV PTK in neutrophil chemotaxis *in vitro* and in regulating the intracellular signalling pathways.

3- Determining Fer kinase activity in neutrophils.

4- Determining the role of Fer kinase in neutrophil superoxide production, bacterial killing and apoptosis.

Chapter Two: **MATERIALS AND METHODS**

2.1 Materials and methods

2.1.1 Reagents and materials

fMLP like peptide (WKYMVm) was purchased from Phoenix Pharmaceuticals. KC and MIP-2 were purchased from R&D systems. LTB_4, C5a, p38 MAPK inhibitors (SKF86002, SB239063, and SB203580), PI3K inhibitors (LY294002, and wortmannin), cytochrome c, and superoxide dismutase were purchased from Calbiochem. Percoll was purchased from Amersham Bioscience. RPMI 1640 culture medium, fetal bovine serum (FBS), Hanks balanced salt solution (HBSS), HEPES, and Penicillin/Streptomycin were purchased from GIBCO. IgG2b-FITC, IgG2a-PE, CD3-FITC, CD19-PE, CD11b-PE and Gr1-FITC, CD11c-FITC, and F4/80-PE antibodies were purchased from eBioscience. Collagenase II was purchased from Invitrogen. Phospho- or total- Akt, p38 MAPK, and PDK1 antibodies were purchased from Cell Signalling. Fer polyclonal antibody was kindly supplied by Dr. Andrew Craig, Queen`s University. Fer polyclonal antibody (affinity purified), and HRP-conjugated secondary antibody were purchased from Santa Cruz. Bovine serum albumin (BSA) and fMLP were purchased from Sigma. Enhanced chemilumiscence (ECL) solution was purchased from Pierce. AnnexinV-PE and 7-AAD were purchased from BD pharmingen.

2.1.2 Methods

2.1.2.1 Animals

Wild type 129SvJ (WT), $Fer^{DR/DR}$, and Fps^{Kr}/Fer^{DR} double mutant mice were obtained from Dr Peter Greer at Queen`s University and used between 6-10 weeks of age. Mice deficient in IL-10 on a 129Sv/Ev background ($IL\text{-}10^{-/-}$) generated by gene targeting in embryonic stem cells as described previously [14] were originally obtained from Dr RN Fedorak (University of Alberta, Edmonton, Canada) and were bred in house. All experimental procedures were approved by the University of Calgary Animal Care Committee and conform to guidelines established by the Canadian Council for Animal Care.

2.1.2.2 Generation of $IL\text{-}10^{-\backslash-}Fer^{DR/DR}$ double deficient mice

Mice were studied at 3 months and age matched 129Sv/Ev mice [wild-type (WT)] were used as non-inflamed controls for comparison. $IL\text{-}10^{-/-}Fer^{DR/DR}$ mice were generated by backcrossing the $Fer^{DR/DR}$ deficiency onto the $IL\text{-}10^{-\backslash-}$ background. We have previously used this method to generate $IL\text{-}10^{-\backslash-}iNOS^{-\backslash-}$ double deficient mice [127]. All mice were genotyped for Fer by polymerase chain reaction (PCR) analysis of genomic DNA purified from tail biopsies. The primers used for Fer genotyping were the following: (Intron 18 forward [TGGGGAAGGGAAGACATTTTGTAGC] and Intron 19 reverse [GGAAACTAGAAGCATTTTCACTTGG]) which span the D743 codon in axon 19. Age and sex matched double-deficient mice were compared directly with $IL\text{-}10^{-/-}$Fer-competent littermates.

2.1.2.3 TNBS colitis model

Wild type (WT), Fer null (Fer $^{DR/DR}$), or Fps/Fer double mutant (Fps^{KR}/Fer^{DR}) mice were used between 6-10 weeks of age. Colitis was induced by a single intrarectal injection of 0.1 ml TNBS (60 mg/ml in 30 % ethanol, or 40 mg/ml in 20 % ethanol) [128]. Healthy controls (saline treatment) were also studied. Animals were killed at 3, 7, 10, or 14 days post-induction of colitis. Macroscopic and microscopic (histological) inflammatory scores were quantified and colonic myeloperoxidase activity (MPO) as an indication of neutrophil recruitment was assessed.

2.1.2.4 Macroscopic assessment of colitis

Mice were killed by cervical dislocation, the colon excised, and the severity of colonic damage assessed using criteria previously established for the TNBS colitis model [129] (table 2.1) and the IL-10$^{-\backslash-}$ model (table 2.2) [127]. The scoring systems include features of clinical colitis including erythema, diarrhea, edema, stricture, haemorrhage, adhesions, and ulceration. The scores were combined with maximal bowel thickness in mm. Each variable was awarded 1 point if observed upon macroscopic examination of the gastrointestinal tissue, with the exception of the bowel wall thickness (value of which was added in millimetres).

Table 2.1 Criteria used to assess macroscopic damage in trinitrobenzene sulphonic acid (TNBS) induced inflammation in mouse colon	
Score	*Variable*
0	No damage
1	Hyperaemia without ulcers
2	Hyperaemia and thickening of bowel wall without ulcers
3	One site of ulceration without bowel wall thickness
4	Two or more sites of ulceration or inflammation
5	0.5 cm of inflammation and major damage
6-10	1 cm of major damage. The score is increased by 1 for every 0.5 cm of damage observed to a maximum of 10
0 or 1	Absence or presence of diarrhea
0 or 1	Absence or presence of stricture
0, 1, or 2	Absence or presence (mild or severe) of adhesions
mm	Maximum bowel wall thickness in mm
On examination of the colon, a score for the extent of ulcerative damage was awarded. Added to this value was the score for presence or absence of diarrhea, stricture, and adhesions, plus maximal bowel wall thickness in mm [128].	

Table 2.2 Variables used to assess macroscopic damage in IL-10$^{-\backslash-}$ and IL-10$^{-/-}$Fer$^{DR/DR}$ double-deficient mice
Erythema Diarrhea Edema Stricture Hemorrhage Adhesions Ulceration Mucosal hyperplasia Bowel wall thickness, mm
Each parameter was awarded 1 point if observed upon macroscopic examination of the gastrointestinal tissue, with the exception of the bowel wall thickness (value of which was added in millimeters) [130].

2.1.2.5 Microscopic (histological) assessment of colitis severity

Sample of colon tissue were fixed in formalin and processed for histological examination as previously described [127]. Briefly, tissue were dehydrated using graded alcohols and cleared (xylene) before being embedded in paraffin wax. Sections of tissue were cut 12µm thick and stained with hematoxylin and eosin (H&E) and scored using a standard histology scoring system [127]. Samples were coded a score in a blinded manner. Histological scoring was based on a semiquantitative scoring system where features were graded as follows: extent of destruction of normal mucosal architecture (0, normal; 1, 2, and 3, mild, moderate, and extensive damage respectively), presence and degree of cellular infiltration (0, normal; 1, 2, and 3, mild, moderate, and transmural infiltration respectively), extent of muscle thickening (0, normal; 1, 2, and 3, mild, moderate, and extensive thickening respectively), presence or absence of crypt abscesses (0, absent; 1, present) and the presence or absence of goblet cell depletion (0, absent; 1, present). The scores for each feature were summed with a maximum score possible of 11.

2.1.2.6 Histological analysis of neoplastic characteristics in IL-10- deficient mouse model

Neoplastic changes in the colon swiss rolls were determined histologically using a standard scoring system based on the following four criteria: Crypts (0, normal; 1, goblet cell depletion; 2, branching, irregular, dilate lumen or back-to-back glands; 3, complex budding). Epithelium (0, normal; 1, hyperplasia or aberrant crypt foci; 2, low-grade dysplasia: nuclear enlarge, mild hyperchromatism, nuclear crowding with stratification; 3, high-grade dysplasia: nuclear stratification, prominent hyperchromatism, pleomorphism,

loss of nuclear polarity). Crypt crowding and cribriforming, and submucosal invasion (0, absent; 1, present), and Serosal adhesions (0, absent; 1, present). The histological analysis was performed with the help of Dr. Stefan Urbanski, a pathologist at Foothills Hospital.

In some IL-$10^{-\backslash-}$ mice a mucosal hyperplasia is evident in the form of polyps. This is usually associated with the development of neoplasia. Polyp formation was assessed macroscopically and scored (0–3) according to the number of polyps observed (0, none; 1, one to three individual polyps; 2, four or less individual polyps; 3, merged polyps/raised plaques).

2.1.2.7 Myeloperoxidase (MPO) activity assay

MPO is an enzyme found in cells of myeloid origin, and has been used as a biochemical marker of granulocyte (mainly neutrophil) infiltration to the tissue. Samples of colon were weighed, frozen on dry ice and stored at -70^{o} C for no more than two weeks before the MPO assay was performed. MPO activity was determined using an assay described previously [40]. A kinetic microplate reader (Multiskan Ascent ®) was used to determine the rate of change in absorbance at 450 nm over a 3 minute time period. One unit of MPO activity was defined as that degrading 1 micromole of hydrogen peroxide per minute at 25 ^{o}C and values are expressed as units of MPO activity per milligram of tissue (U/mg tissue).

2.1.2.8 Circulating and differential white blood cell counts

Blood was drawn from mice by cardiac puncture and 50 µl of blood was added to 440 µl of 3 % acetic acid plus 10 µl of 5 % crystal violet solution. The number of cells was counted under the microscope using hemocytometer. To determine differential WBC count, blood was taken by capillary tube and a drop was placed on the slide surface and a second glass slide was placed on the edge of the blood drop and pushed forward. Blood smears were stained using hemacolor harleco kit from EM science.

2.1.2.9 Cytospin for bone marrow derived neutrophils

Bone marrow derived neutrophils were isolated by percoll gradient and the cells were transferred to a slide by cytospin technique. The cells were fixed by methanol and stained using hemacolor harleco kit from EM science.

2.1.2.10 Lamina propria cell isolation

To identify the cell populations present in the colon, lamina propria cells were isolated as previously described [131]. Briefly, mouse colon was dissected from cecum to rectum and radially cut into 5 mm pieces. The tissues were washed with 1xHBSS, 2 % HEPES and 1 % Pen/Strep solution 5 times. The tissues were transferred to a new flask containing 1xHBSS, 1 % EDTA, 1 % Pen/Strep, and 10 % FBS solution and stirred for 15 minutes at 37^{o}C and repeated 4 times. Tissues were transferred into a new flask containing RPMI + 100 U/ml collagenase type II solution and stirred at 37^{o}C for 1 hour. The flask contents were transferred through 100 µm cell strainer into a new 50 ml tube and the collagenase digestion was repeated for 3 times. Cells were washed with PBS and

counted by using hemocytometer. Cell viability was also determined by tryptan blue extraction under the microscope (x10 objective lens).

Cell population were identified using FACS analysis. Cells ($5x10^5$) were incubated with 2.5 µl of isotype controls (IgG2b-FITC, and IgG2a-PE), or specific cell markers [T-cell marker; CD3-FITC, B-cell marker; CD19-PE, neutrophil marker; Gr1-FITC, dendritic cell marker; CD11c-FITC, or macrophage marker; F4/80-PE antibodies] for 30 minutes at room temperature in the dark and washed once with PBS and fixed with 2 % formalin before FACS analysis.

2.1.2.11 Isolation of murine neutrophils

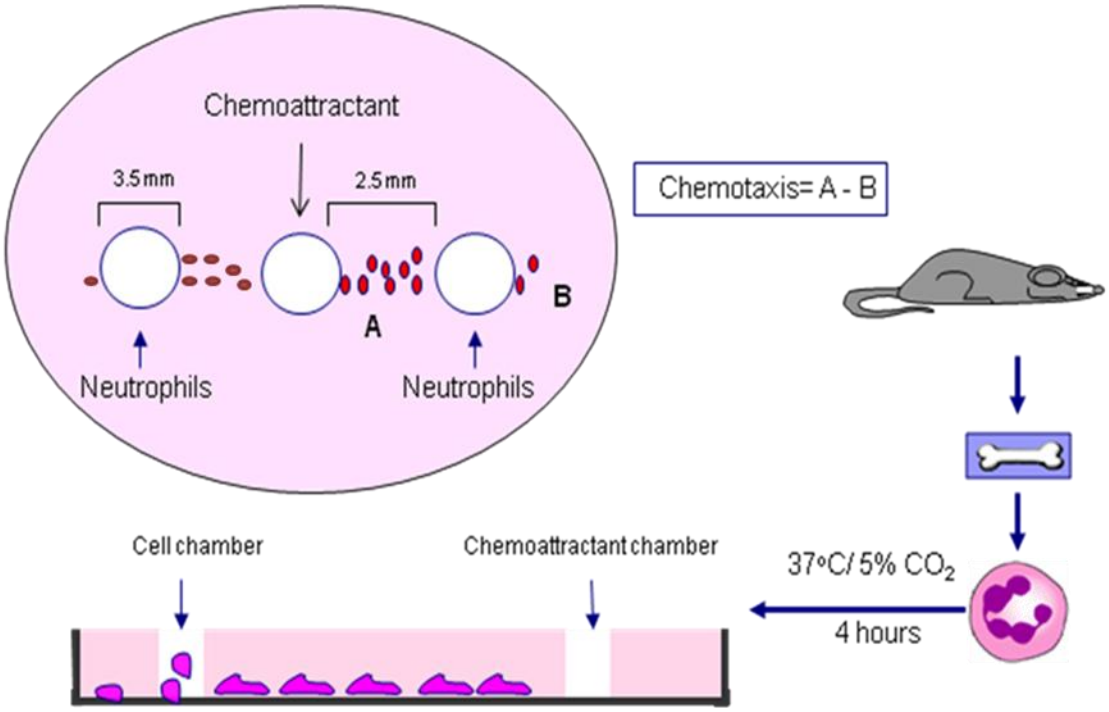
Neutrophils were isolated from mouse bone marrow as described in detail by Lieber *et al* [132]. Briefly, mice were euthanized and the femurs and tibias were dissected from the animal and the ends of bones were removed. The marrow flushed from the bone with ice-cold phosphate buffered saline (PBS, 50 ml) and was then centrifuged at 1300 rpm for 6 minutes at 4^oC. After re-suspension of cells in 3 ml of 52 % Percoll, the marrow was layered on a 3-step Percoll gradient (72 %, 64 %, and 52 % plus cells), and centrifuged at 2600 rpm for 30 minutes at 4^oC. Purified neutrophils were removed from the layer between the 64 % and 72 % Percoll and washed once with ice-cold PBS and then were suspended in RPMI culture media plus 20 % fetal bovine serum (FBS) at a concentration of 1 x 10^7cells/ml. Neutrophils were > 95 % viable using tryptan blue extraction under the microscope. Cytospin of the cell suspension after Percoll gradient purification was performed to identify the cell population, and were > 95 % neutrophils.

2.1.2.12 In vitro under agarose assay for neutrophil chemotaxis

The under-agarose assay was performed as described previously in detail by Heit *et al* [114]. In brief, 35 mm x 10 mm culture dishes were filled with 3 ml of a 1.2 % agarose solution and allowed to solidify. Three wells (3.5 mm) were created in the gel 2.5 mm apart in a horizontal line (diagram 2.1). The center well was loaded with 10 µl of chemoattractant (KC; 1.25 µM, MIP-2; 1.25 µM, LTB_4; 1 µM, WKYMVm; 0.01-100 µM, and C5a; 1 µM), and the outer wells were loaded with 10 µl ($1x10^7$ cells/ml) neutrophils. Once loaded, the gels were incubated for 4 h in a 37°C/5% CO_2 incubator. Control experiments were performed by loading 10 µl of PBS in the center well instead of the chemoattractants. Results were analyzed and recorded by using a video camera attached to a ZEISS Axiovert 135 microscope (x100). Chemotaxis was determined by counting the number of neutrophils which migrated toward the source of chemoattractant (area A) in equal size areas and subtracting the totals which migrated away from the source of the chemoattractant (area B, diagram 2.1). Data are expressed as A-B for the net chemotactic movement.

In separate experiments, we examined the intracellular signalling pathways used by WT or $Fer^{DR/DR}$ neutrophils during chemotaxis. The following p38 MAPK inhibitors were studied; SKF86002 (30 µM), SB239063 (10 µM), SB203580 (10 µM). These concentrations were previously shown to be the optimal inhibitory concentrations without nonspecific effects [114, 133]. PI3K inhibitors were LY294002 (50 µM) and wortmannin (100 nM) [114, 134]. The inhibitors were added to the neutrophils to give the desired concentration in ice for 30 minutes before loading them in the agarose wells. The methodology of this assay is simplified in diagram 2.1.

Diagram 2.1 The under agarose gel assay



Bone marrow derived neutrophils were isolated by Percoll gradient. The center well was loaded with 10 µl of chemoattractant, and the outer wells were loaded with 10 µl of neutrophils. Once loaded, the gels were incubated for 4 h in a 37°C/5% CO_2 incubator. Chemotaxis was determined by counting the number of neutrophils which migrated toward the source of chemoattractant (area A) in equal size areas and subtracting the totals which migrated away from the source of the chemoattractant (area B). Data are expressed as A-B for the net chemotactic movement

2.1.2.13 Western blotting

To determine the activation of p38 MAPK, Akt, and PDK1, western blots were performed. Briefly, neutrophils (5 x 10^6/condition) isolated from mouse bone marrow were untreated or stimulated with WKYMVm; 1 µM at 37^{o}C for the indicated time points. The reaction was stopped by adding equal volume of 2X SDS lysis buffer plus 100 µM PMSF, 100 µM Na_3VO_4, 10 µg/ml Aprotinin/Leupeptin, and 10 µM pepstatin. The samples were boiled at 90^{o}C for 10 minutes and lysates were loaded into 10 % sodium dodecyl sulfate-epolyacrylamide (SDS-PAGE) gel. After electrophoresis at constant voltage of 50 v overnight, proteins were transferred to a nitrocellulose membrane and were blocked with 2 % BSA for 1 hour. The membrane was incubated overnight at 4^{o}C with 1/1000 dilution of phospho- or total- p38 antibody, Akt antibody, or PDK1 antibody prepared in 2 % BSA. On the following day, the membrane was washed and incubated for 1 hr with 1/10,000 dilution of anti-rabbit HRP-conjugated secondary antibody. The membrane was developed with super signal ECL (enhanced chemilumiscence) and visualized with Kodak X- ray film.

2.1.2.14 Cytochrome c reduction assay

Superoxide production from neutrophils was determined by using the cytochrome c reduction assay as described in detail by Partrick *et al* [135]. Briefly, freshly isolated neutrophils (2.5x10^5 cells) were added to 96 well plates containing cytochrome c alone (1 µM) or cytochrome c plus superoxide dismutase (from bovine erythrocytes, 1500 units). Cells were activated by fMLP (8 mM) which was added to each sample. Other controls were also used in each experiment such as PBS alone and cytochrome c alone without

cells. Plates were read using a spectrophotometer (Spectra max) at 550 nm, and cytochrome c reduction was measured over 20 minutes at 37 ^{o}C.

2.1.2.15 Apoptosis assay

Freshly isolated bone-marrow derived neutrophils from WT and $Fer^{DR/DR}$ mice were re-suspended in RPMI + 20 % FBS culture media and plated in 35 mm x 10 mm Falcon culture dishes in a density of $1x10^6$ cells/ml. Cells were either incubated for 16 hours in a 37 ^{o}C/ 5% CO_2 incubator to determine the rate of spontaneous apoptosis, or were stimulated with different doses of WKYMVm (0.1-10 µM) for 1 hr at 37^{o}C . The cells were washed twice with ice-cold PBS and then washed once with 1x Annexin-V binding buffer [10x binding buffer; 0.1 M HEPES/NaOH (pH 7.4), 1.4 M NaCl, 25 mM $CaCl_2$]. Cell pellets were re-suspended with Annexin-V binding buffer stained for FACS analysis using the PE Annexin V apoptosis detection kit I from BD pharmingen. Cells isolated from each animal were stained in the following manner: 1- cells only (negative control), 2- 1 µl of Annexin V-PE, 3- 1µl of 7AAD, 4- 1µl of Annexin V-PE plus 1µl 7AAD. All incubations were performed at room temperature (RT) for 15 minutes in dark.

2.1.2.16 Neutrophil intracellular survival assay

To assess the ability of neutrophils to kill bacteria the neutrophil intracellular survival assay was performed as previously described by Liu *et al* [136]. In brief, mouse bone marrow derived neutrophils were isolated as previously described and re-suspended in RMPI culture medium plus 20 % FBS. S. aureus 2406 bacterial cultures were washed with PBS and diluted in RPMI plus 20 % FBS to a concentration of $4.5x10^6$ colony

forming units (CFU/ml) and mixed with $3x10^5$ neutrophils in the same media. The cells were centrifuged and incubated at $37^oC/5\%$ CO_2 incubator for 10 minutes. After 10 minutes, gentamycin (400 µg/ml) was added to kill any extracellular bacteria. At 15, 30, or 60 minutes, sample were taken and centrifuged to pellet the neutrophils, then washed to remove the antibiotic medium. Neutrophils were lysed (0.02 % Triton X) and the level of bacteria survived inside the neutrophils was assessed by the ability to form CFU. These set of experiments were performed by Kaiyu Wu.

2.2 Statistical analysis

Statistics were performed using Prism software (Graphpad). Data are expressed as the mean ± SEM. Analysis of the statistical significance for the indicated data was performed by using student t-test (two tailed, for two groups) or one-way ANOVA with Bonferroni`s correction (for three or more groups). Values of $P<0.05$ were considered to be statistically significant.

Chapter Three: **THE ROLE OF GROUP IV PTK IN ANIMAL MODELS OF INFLAMMATORY BOWEL DISEASE (IBD)**

3.1 Role of group IV PTK in models of colitis

Previous work published by our lab and by others has demonstrated an important role for group IV PTK in controlling (limiting) the innate immune response to bacterial antigen such as LPS [39-41, 43] in part through the down regulation of TLR4 and NFκB activation [42]. Furthermore, Fer kinase has also been shown to regulate LPS-induced inflammation in the intestine as demonstrated by enhanced neutrophil infiltration and epithelial barrier dysfunction in Fer kinase null mice [40]. In this chapter, we examine whether Fer kinase plays a role in models of IBD. A link between the presence of bacteria in the gut and the development of IBD has been established in both animal models [87, 88] and in clinical studies [137]. We examined two models of IBD; a chemically induced model using the hapten TNBS and a spontaneous chronic model which develops due to a gene mutation in interleukin 10- deficient mice (IL-$10^{-\backslash-}$). The TNBS model has been routinely used in gastrointestinal research and is an easily reproducible model of colitis which has been described to have both acute and chronic components [128]. The IL-$10^{-\backslash-}$ is a spontaneous model of colitis which develops from birth and results in an established chronic colitis which we study at 3 months of age.

3.1.1 Chemically induced colitis

Colitis was induced in wild type (WT), $Fer^{DR/DR}$, and Fps^{Kr}/Fer^{Dr} double mutant mice by a single intra-rectal injection of 2, 4, 6-trinitrobenzene sulphonic acid (TNBS) dissolved in ethanol. Ethanol acts as a mucosal barrier breaker and allows TNBS to penetrate colonic tissues, where it acts as a hapten and activates the immune system. The TNBS model of colitis has many histological and immunological similarities to human

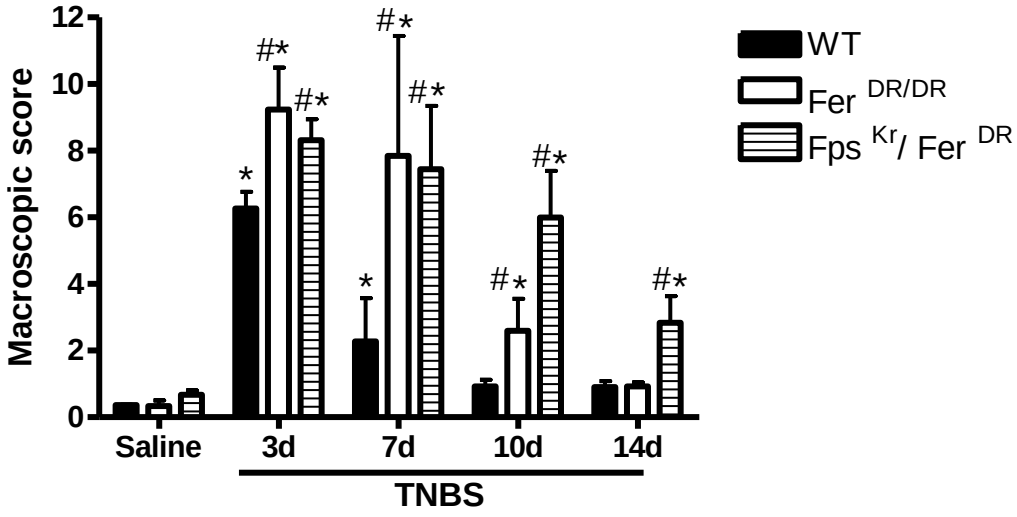
Crohn's Disease, which is manifested by an increase in T-helper-1 (Th1) type responses (increase in IFN-γ, TNF-α, IL-12, and IL-6) in the intestinal mucosa [138-140]. Mice treated with intra-colonic administration of TNBS experience weight loss, diarrhea, mucosal ulceration, influx of inflammatory cells to the colon, increase in transmural thickness, loss of goblet cells, and an increase in pro-inflammatory cytokines release from colonic tissues (IFN-γ and IL-6).

3.1.1.1 Results

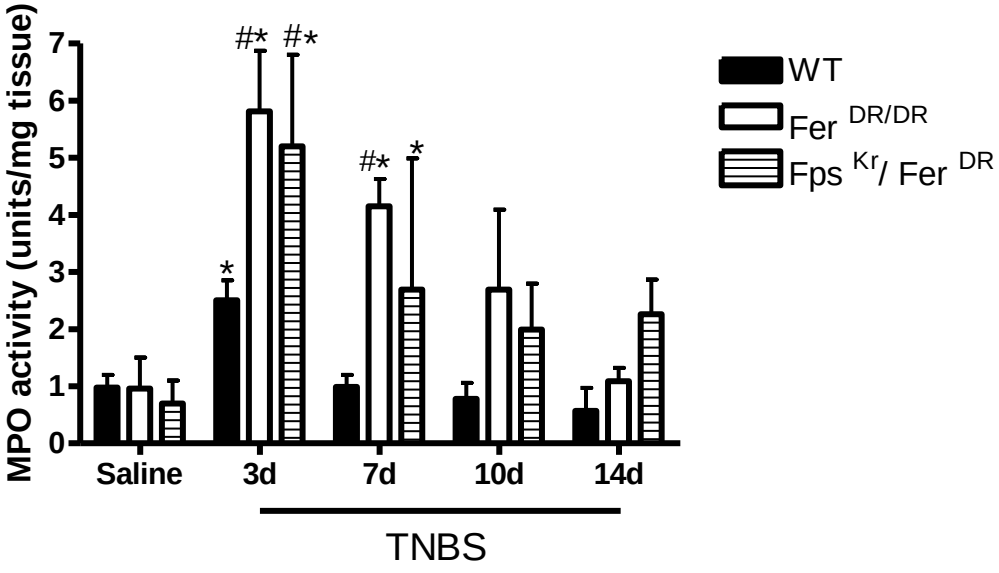
WT and $Fer^{DR/DR}$ mice were treated with TNBS (4 mg, 20 % ethanol) and euthanized after 3, 7, 10, and 14 days. Colons were scored macroscopically for colitis and samples were processed for histological examination. Tissue MPO activity was also assessed as previously described [40]. This dose of TNBS was used since preliminary experiments with 6 mg TNBS in 30 % ethanol resulted in significant mortality (36-40 %). Fps^{Kr}/Fer^{DR} double mutant mice were also studied to determine if there is any redundancy or synergistic effects between the two members of group IV PTK in this model of colitis. With this dose of TNBS, the mortality rate was reduced in all groups of mice. There was 24 % mortality in WT mice, 29 % in $Fer^{DR/DR}$ mice, and the mortality rate was significantly decreased to 10 % in Fps^{Kr}/Fer^{DR} double mutant mice. In WT mice, TNBS (4 mg, 20 % ethanol) induced a significant increase in macroscopic scores at day 3 (figure 3.1-A) over saline controls. Macroscopically these mice presented with colonic erythema, edema, and ulceration. Diarrhea was also evident in some mice. The inflammation was still evident at 7 days but had resolved to control levels by 10 days. Macroscopic colitis was associated with a significant increase in neutrophil infiltration by

day 3 which was back to control levels by day 7 (figure 3.1 B). A similar time course for inflammation was observed upon histological examination with a significant increase in neutrophil recruitment and destruction of mucosal architecture observed at day 3 and 7 and a return to control levels by day 10 (figure 3.1 C). Interestingly, an exacerbated colitis was observed in group IV PTK mutants. Macroscopic damage was significantly higher ($p<0.05$) in $Fer^{DR/DR}$ and Fps^{Kr}/Fer^{DR} mice compared to WT mice at 3, 7, and 10 days post injection. In the compound mutant mice, a significant macroscopic score was still evident at 14 days post-colitis induction compared to WT and Fer single mutant mice. Macroscopic colitis in Fps^{Kr}/Fer^{DR} mice at day 14 was associated with colonic erythema and ulceration, and diarrhea. Colonic MPO activity significantly increased in group IV PTK mutants compared to WT mice at day 3 and 7 post TNBS injection (figure 3.1 B). Histological examination of the colon sections of group IV PTK mutant mice revealed a significant increase in the microscopic scores at day 3, 7, and 10 compared to WT TNBS treated groups (figure 3.1 C). In the double mutant mice at 14 days post TNBS there was enhanced variability in the inflammation and neutrophil infiltration observed where mice presented with near control levels or presented with high MPO and histological scores. The reason for this variability is unclear but contributed to the lack of significance observed in this group from WT TNBS treated mice at 14 days.

(A)


Macroscopic score
12
10
8
6
4
2
0
Saline
3d
7d
10d
14d
TNBS
WT
Fer DR/DR
Fps Kr/ Fer DR

(B)


MPO activity (units/mg tissue)
7
6
5
4
3
2
1
0
Saline
3d
7d
10d
14d
TNBS
WT
Fer DR/DR
Fps Kr/ Fer DR

(C)

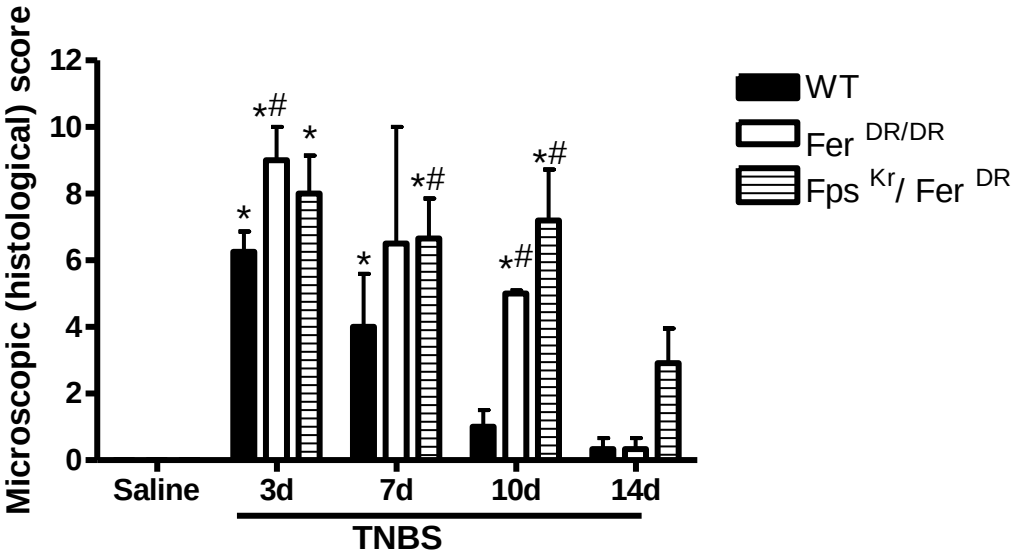


Figure 3.1 TNBS-induced colitis is exacerbated in Fer and Fps/Fer double mutant mice

Data illustrate (A) macroscopic score of colitis, (B) colonic MPO activity (U/mg tissue), and (C) histological score of colitis was determined in WT (solid bars), $Fer^{DR/DR}$ (open bars), and Fps^{Kr}/Fer^{DR} (hatched bars) mice in response to saline or TNBS injection (4 mg, 20 % ethanol). Mice were killed and colon tissue examined at 3, 7, 10, and 14 days post colitis induction. Saline treated mice were studied at all time points examined and the data has been pooled for each strain to simplify the graphs. Data are expressed as mean ± SEM, n= 5-15 mice, * $p<0.05$ from the saline treated group, # $p<0.05$ from TNBS-injected WT group.

Hematoxylin and eosin (H & E) stained colon sections from WT, $Fer^{DR/DR}$ and Fps^{Kr}/Fer^{DR} mice are shown in figures 3.2 and figure 3.3. Figure 3.2 represents colon sections taken from WT, $Fer^{DR/DR}$, and Fps^{Kr}/Fer^{DR} mice at Day 0 (saline treated: A, B and C) and 3 days post TNBS injection (D, E and F) respectively. Saline treated mice present with normal colon morphology with an intact epithelial layer, well organized crypts, and mucus containing goblet cells. No differences in normal colon architecture were noted between WT (A) and the mutant mice (B and C). TNBS treatment induced significant damage to the colon in WT mice at day 3 (figure 3.2-D) with loss of mucosal architecture, significant neutrophil infiltration, and goblet cell depletion. At day 3 post TNBS injection, $Fer^{DR/DR}$ (figure 3.2-E) and Fps^{Kr}/Fer^{DR} (figure 3.2-F) mice had a significant loss of mucosa architecture and neutrophil infiltration to the colon. Some mutant mice experienced complete loss of the mucosal layer indicating a severe inflammatory response toward TNBS injection at this time point. By day 10 post TNBS injection, the colitis had resolved in WT mice and a normal mucosal architecture was observed (figure 3.3-A). Interestingly, the colonic damage persisted in the mutant mice with significant immune cell infiltration and destruction of mucosal architecture at day 10 (figure 3.3- B and C) as reflected in the total microscopic scores (figure 3.1 C). In the double mutant mice significant mucosal damage and immune cell infiltration was still evident at day 14 post colitis induction as shown in figure 3.3-D, although this was more variable with some mice presenting with normal histology (figure 3.3-E).

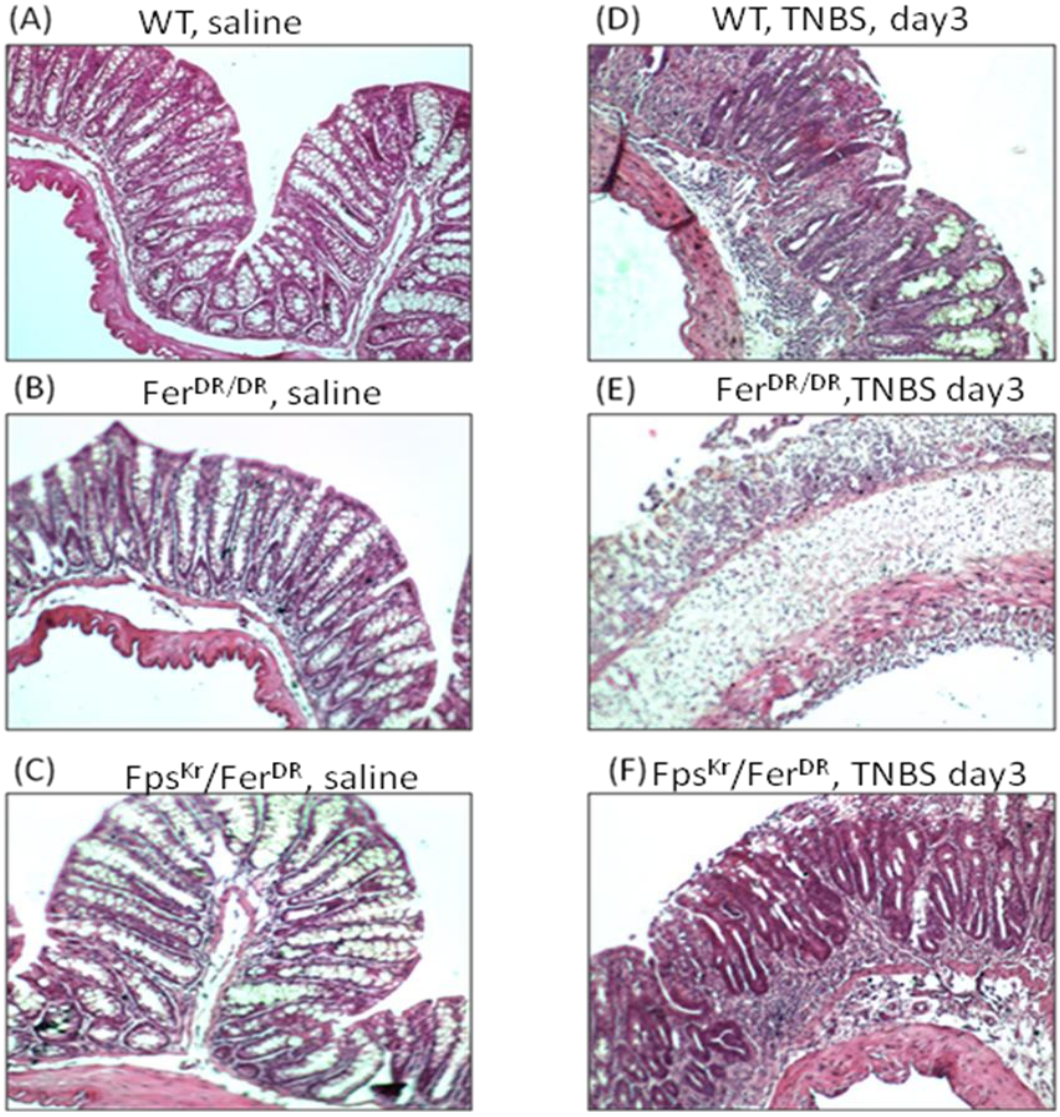


Figure 3.2 Representative hematoxylin and eosin (H & E) stained colon sections from the TNBS colitis is exacerbated in group IV mutant mice

WT, Fer$^{DR/DR}$, or Fps^{Kr}/Fer^{DR} mice were saline treated (A, B, and C respectively), or treated with TNBS for 3 days (D, E, and F respectively) prior to being euthanized. Colons were removed in formalin, processed and embedded in wax for histological examination. 12 µm sections were cut and stained with haematoxylin and eosin (H & E).

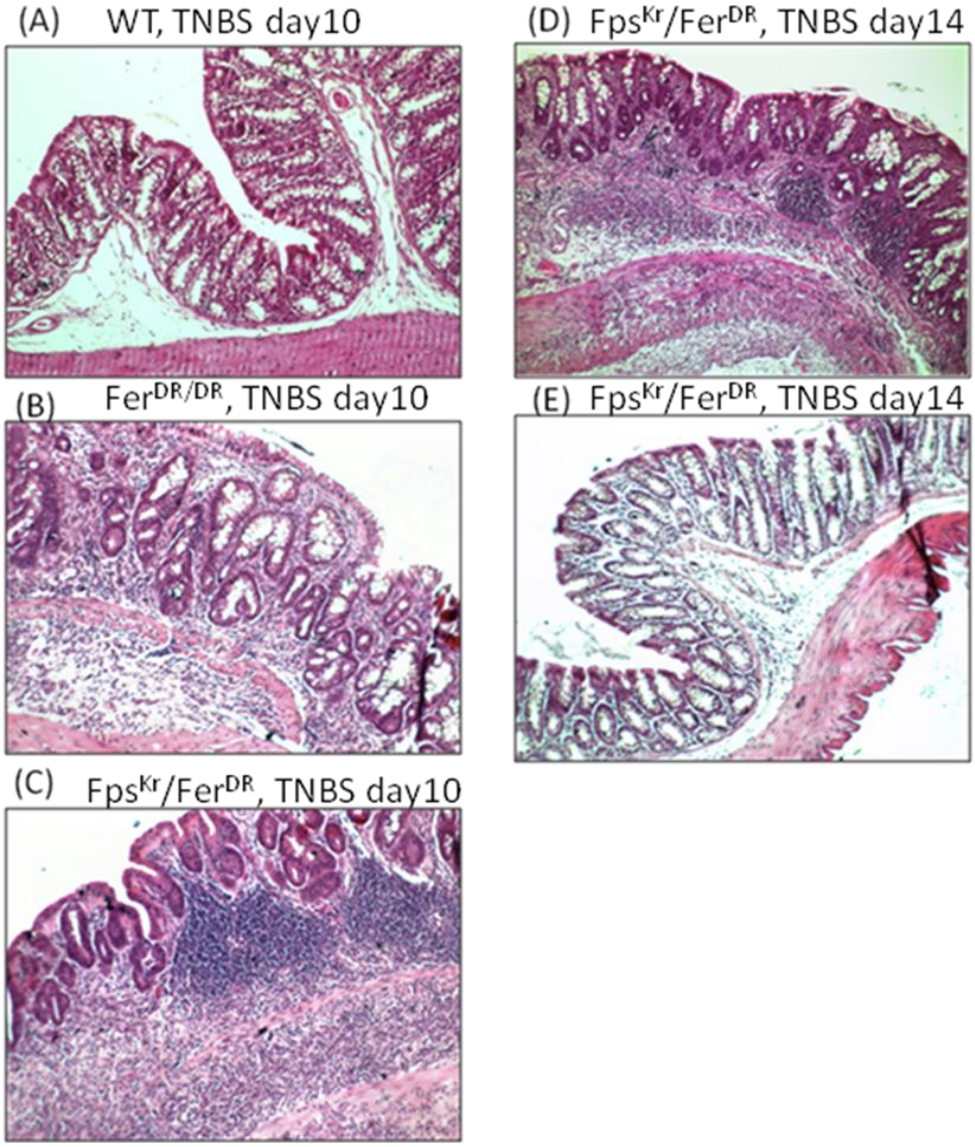


Figure 3.3 Representative H & E stained colon sections from the TNBS colitis is exacerbated in group IV mutant mice

WT, Fer$^{DR/DR}$, or Fps^{Kr}/Fer^{DR} mice were treated with TNBS for 10 days (A, B, and C respectively), or Fps^{Kr}/Fer^{DR} mice treated with TNBS for 14 days (D, and E respectively) prior to being euthanized. Colons were removed in formalin, processed and embedded in wax for histological examination. 12 µm sections were cut and stained with haematoxylin and eosin (H & E).

These data illustrate an important role for group IV PTK in this model of colitis. In the absence of Fer or both Fps/Fes and Fer, TNBS induced intestinal inflammation is exacerbated as measured by macroscopic and histological scores, and this is associated with increased MPO activity. These data suggest that group IV PTKs regulate intestinal inflammation perhaps through the regulation of the inflammatory infiltrate (eg neutrophils). In addition, there appears to be no redundancy between Fer and Fps/Fes in this type of inflammation as single mutants and double mutants have similar macroscopic and histological scores and neutrophil infiltration over the first 7 days. No synergistic effect was observed in the compound mutant in neutrophil infiltration over the time course studied suggesting no additional regulation of this parameter through Fps/Fes. However, both macroscopic and histological scores are enhanced at later time points in this model suggesting that Fps/Fes may play a distinct role in regulating TNBS-induced inflammation or resolution in the colon. This role may have been masked at earlier time points due to the severity of the inflammatory response. In fact, the role of group IV PTK, Fer, in epithelial cell migration and wound closure has been demonstrated *in vitro* [32] which may explain the delayed resolution in the mutant mice in the TNBS model.

In order to determine the influence of Group IV PTK on the overall leukocyte differential during intestinal inflammation, we examined isolated lamina propria cells as described in chapter 2. Isolated cells from the colon of WT and Fps^{Kr}/Fer^{DR} double mutant mice at day 10 post TNBS injection (4 mg, 20 % ethanol) were labelled with specific cell marker antibodies and analysed by FACS. This time point was chosen as a clear difference in macroscopic and histological damage was evident. Table 3.1 details the immune cell populations in saline- and TNBS-treated mice at day 10 post colitis

induction. No differences were observed between the WT and double mutant macrophages, B-cells, T-cells, dendritic cells and neutrophils under normal physiological conditions (saline treated). A significant increase in neutrophil population was observed in WT TNBS treated mice at 10 days. In group IV compound mutants neutrophil numbers were significantly elevated over TNBS-treated WT mice. Using the MPO assay no significance in neutrophil infiltration was observed between these two groups at this time point although the trend was there (Figure 3.1 B). These differences may reflect the sensitivity between the two techniques used to measure neutrophil levels. No increases in lamina propria dendritic cells (DC), macrophages, or B-cells population were noted after TNBS treatment in WT or mutant mice. Interestingly, a reduction in T-cells was noted in the TNBS treated WT mice compared to saline controls (table 3.1). One possible explanation might be due to an enhanced rate of T-cell apoptosis in the inflammatory environment compared to a healthy colon although other possibilities exist such as altered cytokine generation from macrophages or epithelial cells which are responsible for T-cell recruitment to the inflammatory site. Overall these data suggest that neutrophil recruitment is enhanced in the absence of group IV PTKs and other cell types are not modified. This suggests an important role for group IV PTK in modulating neutrophil recruitment to the inflammatory site which may result in enhanced tissue damage. Neutrophils are key players in this model which essentially demonstrates an acute intestinal inflammation in the gut. Next we examined the role of Fer kinase in a spontaneous chronic model of intestinal inflammation.

% Cell population	WT/Saline	Fps/Fer mutant/Saline	WT/TNBS	Fps/Fer mutant/TNBS
Macrophages	12.6±2.8	9.2±1.8	10.4±2.2	7.6±2.9
B-cells	8.9±1.5	6.0±2.5	15.1±6.8	13.2±4.1
T-cells	22.1±3.1	14.3±4.1	5.2±2.2*	5.9±3.7
Dendritic cells	1.5±0.3	1.5±0.5	1.1±0.5	0.5±0.2
Neutrophils	2.0±0.5	2.1±0.7	10.5±4.4*	22.0±5.4*#

Table 3.1 Enhanced neutrophil population in lamina propria of mutant mice after TNBS treatment

WT and Fps^{Kr}/Fer^{DR} mice treated with saline or TNBS (4 mg, 20 % ethanol) at day 10 post colitis induction. * Significance from saline group, # significance from WT-TNBS group, n= 5-7 mice. Cells ($5x10^5$) were incubated with isotype controls (IgG2b-FITC, and IgG2a-PE), or specific cell markers [T-cell marker; CD3-FITC, B-cell marker; CD19-PE, neutrophil marker; Gr1-FITC, dendritic cell marker; CD11c-FITC, or macrophage marker; F4/80-PE antibodies]

3.1.2 IL-10-deficient mouse model of colitis

IL-10 is an endogenous anti inflammatory and immune modulatory cytokine produced by a variety of cell types such as regulatory T-cells, $CD4^+$ Th1 cells, B-1 cells, macrophages, thymocytes, dendritic cells, and keratinocytes [141]. IL-10 is a potent suppressor of macrophage activation *in vitro*, and it inhibits the production of IFN-γ and other cytokines produced by Th-1 cells such as IL-1, IL-6, and TNF-α [142].

IL-10 deficient ($IL\text{-}10^{-/-}$) mice spontaneously develop chronic colitis with macroscopic and histological similarities to human IBD [14, 127, 143]. Bacterial antigens are the driving force for colitis development in this model, since mice will develop milder colitis or no inflammation if they are kept in a germ-free environment [14]. In addition, antibiotic treatment has been shown to reduce colitis severity in these mice [90]. An interesting characteristic of this model is that certain percentage of mice will develop colon cancer through the dysplasia sequence, a process which is similar to clinical IBD-associated cancer [144]. In order to examine the role of Fer kinase in this model of colitis, we developed $IL10^{-\backslash-}Fer^{DR/DR}$ double deficient mice and examined the colons at 3 months of age, a time point where colitis is established [130].

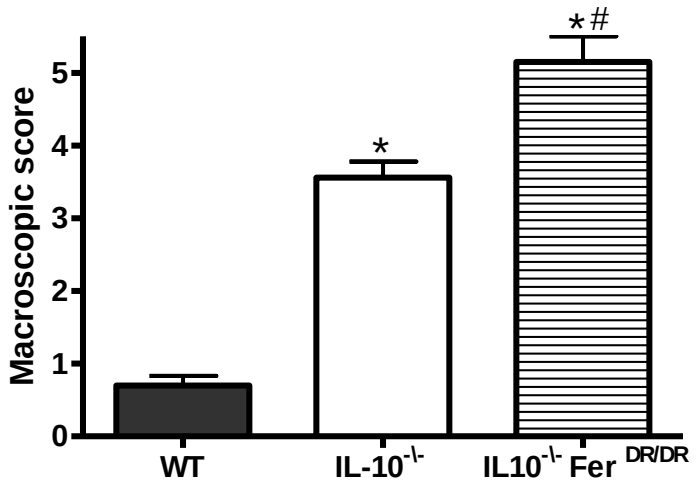
3.1.2.1 Results

Figure 3.4-A illustrates the macroscopic (gross) score of inflammation in WT (129 SvEv), $IL\text{-}10^{-\backslash-}$ and $IL\text{-}10^{-\backslash-}Fer^{DR/DR}$ mice at 3 months of age. In $IL\text{-}10^{-\backslash-}$ mice, a mild colitis was observed with evidence of erythema, edema, and diarrhea. A significant increase ($p<0.05$) in the macroscopic inflammation score (table 2.1, chapter 2) was observed in $IL\text{-}10^{-\backslash-}$ (3.2 ± 0.3) compared to WT mice (0.7 ± 0.1). In $IL\text{-}10^{-\backslash-}Fer^{DR/DR}$

double deficient mice, the macroscopic colitis score was significantly increased (4.9 ± 0.3) compared IL-$10^{-\backslash-}$ mice. It should be noted that approximately 80 % ($p<0.05$) of IL-$10^{-\backslash-}Fer^{DR/DR}$ double deficient mice compared to 15 % of IL-$10^{-\backslash-}$ mice developed rectal prolapse at 3 months of age, a sign which is not included in the macroscopic score. This condition may contribute to a slightly elevated mortality rate (11 %, $p<0.05$) in IL-$10^{-\backslash-}Fer^{DR/DR}$ mice compared to 0 % mortality either WT or IL-$10^{-\backslash-}$ mice.

Similar findings were obtained upon histological examination of H & E sections. Figure 3.4-B illustrates the mean microscopic score of colitis for IL-$10^{-\backslash-}$ and double mutants. No signs of inflammation were evident in WT mice, however, microscopic scores were significantly ($p<0.05$) increased in IL-$10^{-\backslash-}$ (3.0 ± 0.4) and double mutants (7.25 ± 0.7). The damage observed in the double mutant mice represented significant colonic epithelial destruction, immune cell infiltration and goblet cell depletion. Figure 3.5 illustrates neutrophil infiltration as measured by MPO activity in the colon. A significant increase in MPO activity was observed in both IL-$10^{-\backslash-}$ and IL-$10^{-\backslash-}Fer^{DR/DR}$ compared to WT mice, but no differences were observed between the two mutants. Total white blood cells (WBCs) count is shown in figure 3.6-A. A trend toward higher circulating WBC count is observed in IL-$10^{-\backslash-}$ and IL-$10^{-\backslash-}Fer^{DR/DR}$ compared to WT mice although this was not statistically significant. Additional analysis of blood smears revealed a significant increase in circulating neutrophils in IL-$10^{-\backslash-}$ and IL-$10^{-\backslash-}Fer^{DR/DR}$ mice and a significant reduction in lymphocytes in the double mutants (figure 3.6-B).

(A)



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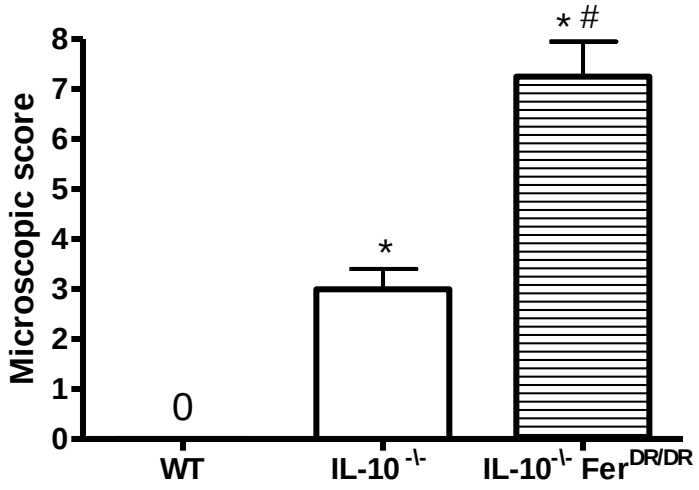


Figure 3.4 Fer deficiency in IL-10$^{-/-}$ model enhances colitis severity

(A) The macroscopic score, and (B) the microscopic score of colitis at 3 months of age in WT (solid bar, n=13 mice), IL-10$^{-/-}$ (open bar, n=23 mice), and IL-10$^{-/-}$Fer$^{DR/DR}$ (hatched bar, n=25 mice). Data are expressed as mean ± SEM, * significant increase from WT mice, # significant increase from IL-10$^{-/-}$ mice.

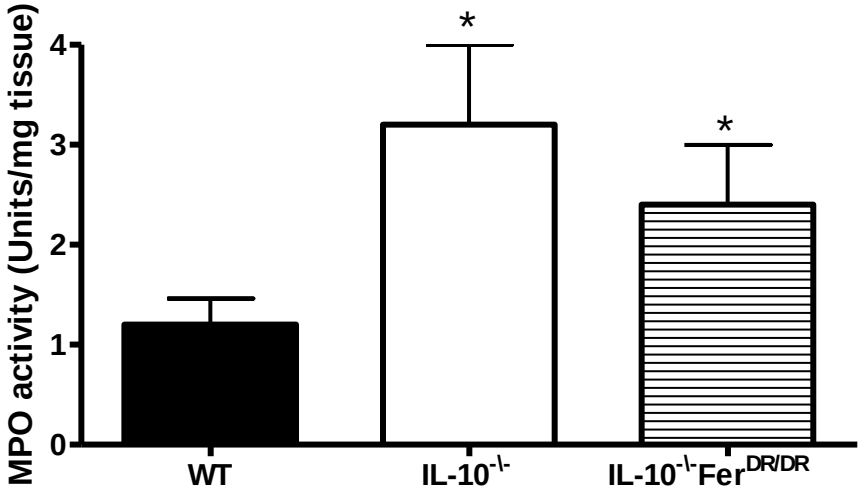
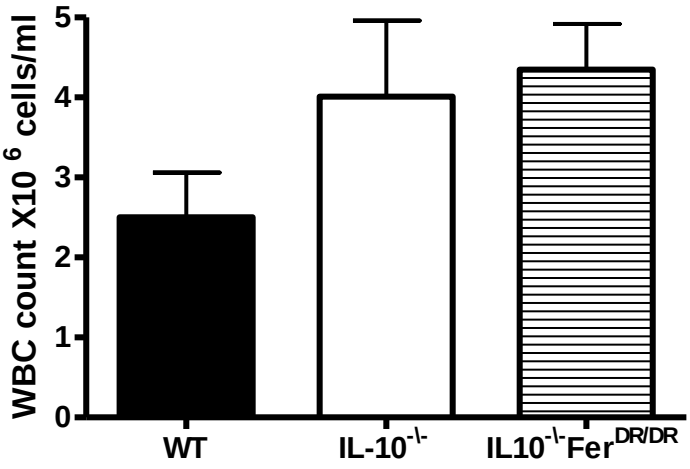


Figure 3.5 Colonic neutrophil infiltration

Neutrophil infiltration assessed by MPO activity was determined in WT (solid bar, n=8), IL-$10^{-\backslash-}$ (open bar, n=10), and IL-$10^{-\backslash-}Fer^{DR/DR}$ mice (hatched bar, n=12) at 3 months of age. Data are expressed as mean ± SEM, * Significant increase from WT mice.

(A)



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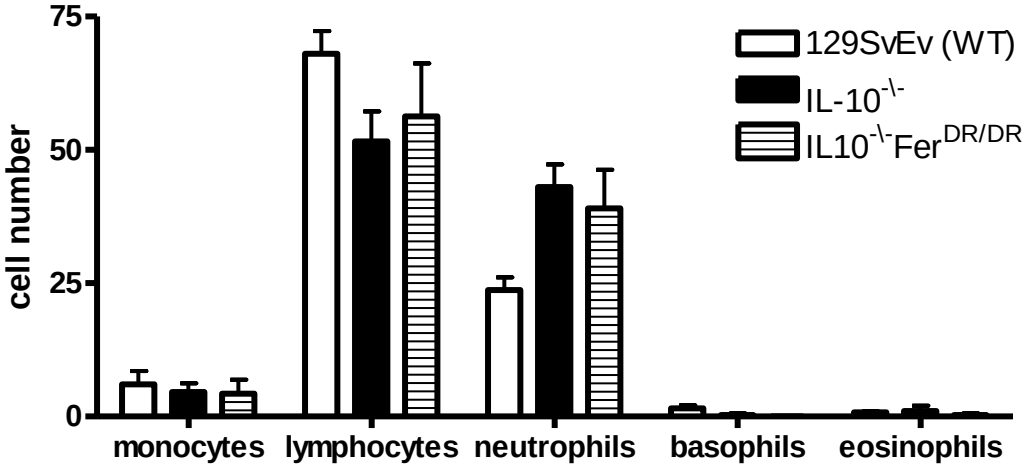


Figure 3.6 Total and differential WBCs count in the three groups of mice.

(A) Total circulating and (B) differential WBCs count in WT (solid bars, n=13), IL-$10^{-\backslash-}$ (open bars, n=23), and IL-$10^{-\backslash-}$$Fer^{DR/DR}$ mice (hatched bars, n=25). Data are expressed as mean ± SEM, * significant differences from WT mice.

Interestingly, upon histological examination mucosal hyperplasia (polyps) was observed in some mice in the ascending colon near the ileo-cecal region. IL-10$^{-\backslash-}$ mice had 34 % (8/23mice) polyps incidence and IL-10$^{-\backslash-}$Fer$^{DR/DR}$ mice had 52 % (13/25 mice) polyps incidence. Polyps which developed in IL-10$^{-\backslash-}$Fer$^{DR/DR}$ mice extended to greater length of colon (figure 3.7). No polyps observed in WT mice. IL-10$^{-\backslash-}$ mice had a mean of 2.5 millimetres (mm) polyps length, which was significantly increased ($p<0.05$) in IL-10$^{-\backslash-}$Fer$^{DR/DR}$ mice to 7.5 mm. Individual polyp scores are plotted against the extent of colon involved (figure 3.7) illustrating that double mutants have higher scores and more colonic involvement than single mutants. Preliminary data suggest polyps are present in the double mutant mice at earlier stage of life (2 months) but not in IL-10 deficient mice (data not shown). To determine if neoplastic changes were present in the tissue histological examination was performed.

Upon histological examination, no evidence of neoplastic changes was noted in IL-10$^{-\backslash-}$ mice with exception of one mouse (1/ 12) which had low grade dysplasia. In contrast, IL-10$^{-\backslash-}$Fer$^{DR/DR}$ mice had a 16 % incidence of low grade dysplasia, 11 % high grade dysplasia, 6 % intra-mucosal carcinoma, and interestingly 28 % incidence of adenocarcinoma (table 3.2). Two IL-10$^{-\backslash-}$Fer$^{DR/DR}$ mice had multiple adenocarcinomas in the colon. The histological scores for dysplasia are in figure 3.8. Representative tissue histology is illustrated in figure 3.9 (A, B, and C). The neoplastic and inflammatory scores were done in collaboration with Dr. Stefan Urbanski (Professor, Department of Pathology & Laboratory Medicine and Medicine, Foothills Medical Centre).

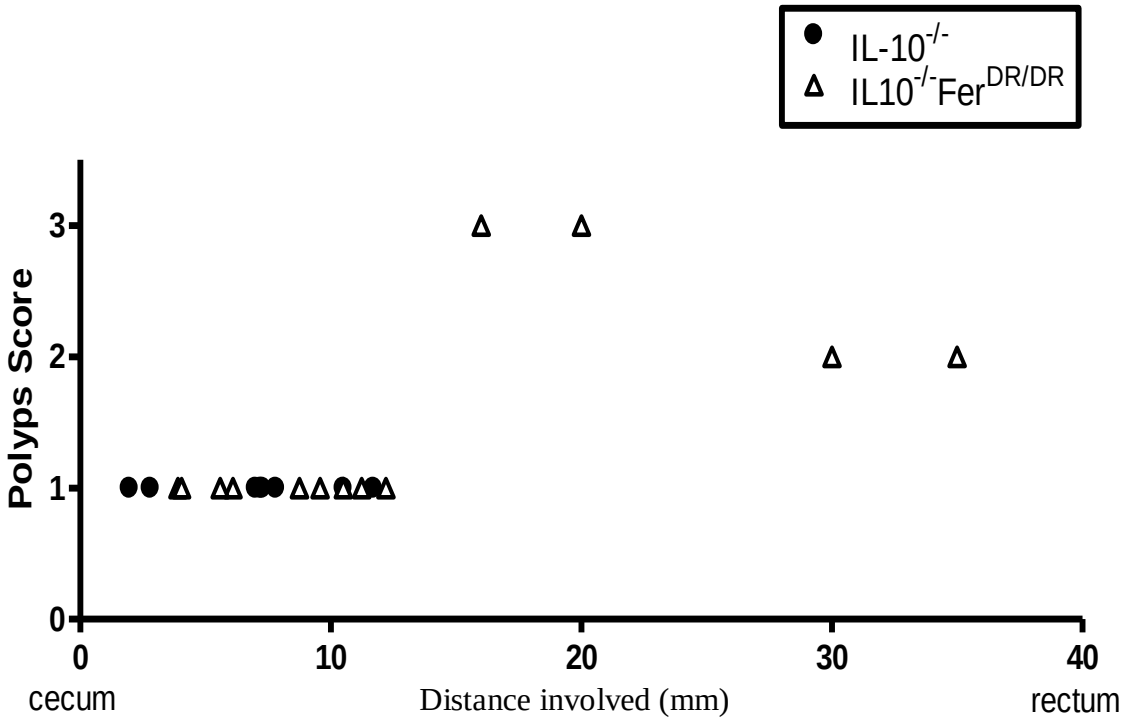


Figure 3.7 Macroscopic analysis for polyps formation was significantly increased in IL-10$^{-\backslash-}$Fer$^{DR/DR}$ compared to IL-10$^{-\backslash-}$ mice at 3 months of age.

Macroscopic analysis for mucosal polyps formation was determined in IL-10$^{-\backslash-}$ (solid circles, n=8), and IL-10$^{-\backslash-}$Fer$^{DR/DR}$ (open triangles, n=13) mice in relation to the distance involved in the colon.

	IL-10$^{-\backslash-}$	IL-10$^{-\backslash-}$Fer $^{DR/DR}$
Low grade dysplasia	1 (8%)	3 (16%)
High grade dysplasia	0	2 (11%)
Intra-mucosal carcinoma	0	1 (6%)
Adenocarcinoma	0	5 (28%)
Number of mice =	12	18

Table 3.2 Total incidence for neoplastic changes in IL-10$^{-\backslash-}$ and IL-10$^{-\backslash-}$Fer$^{DR/DR}$ mice at 3 months of age

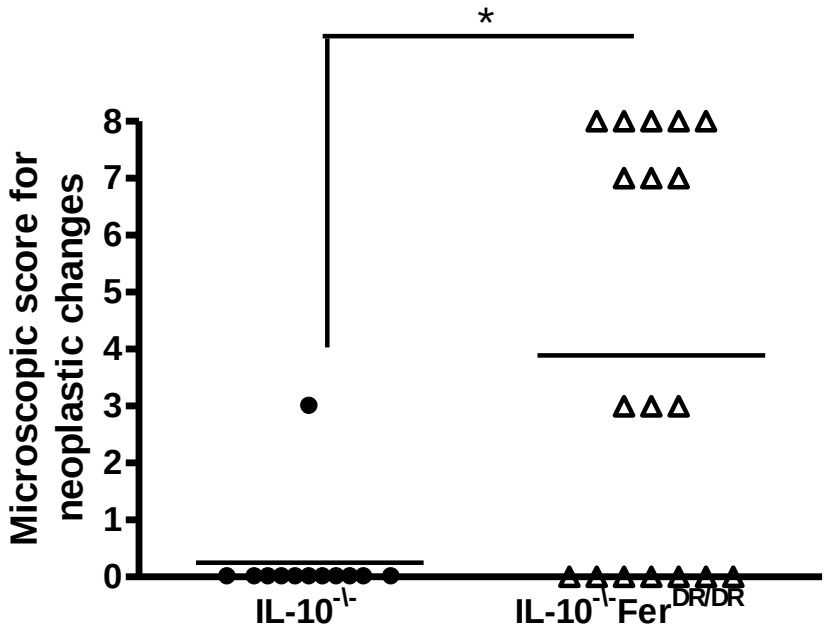
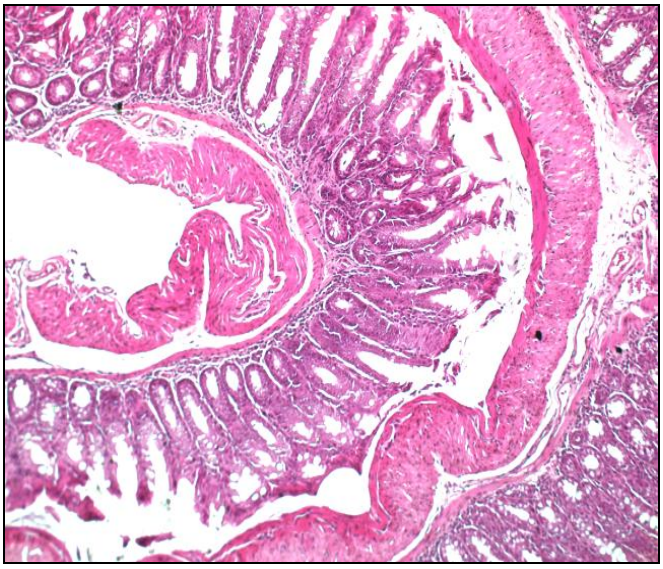


Figure 3.8 IL-10$^{-\backslash-}$Fer$^{DR/DR}$ mice had increased microscopic scores for neoplastic changes at 3 months of age.

Microscopic analysis for neoplastic changes was determined in IL-10$^{-\backslash-}$ (solid circles, n=12), and IL-10$^{-\backslash-}$Fer$^{DR/DR}$ (open triangles, n=18) mice. * Significant differences from IL-10$^{-\backslash-}$ mice.

(A)



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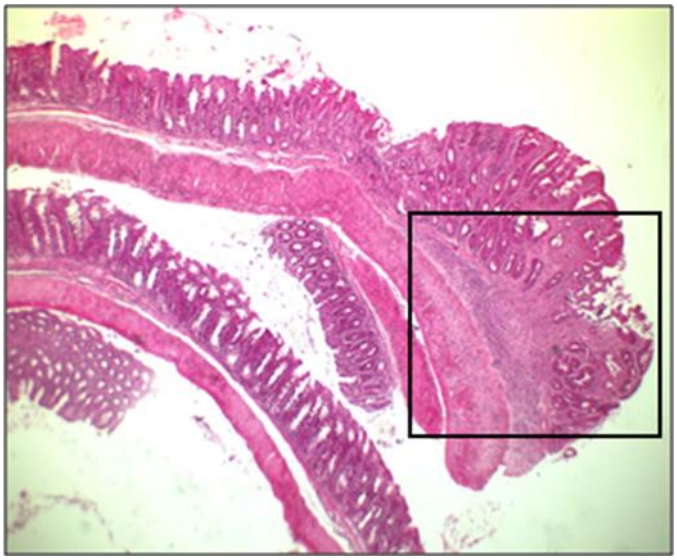

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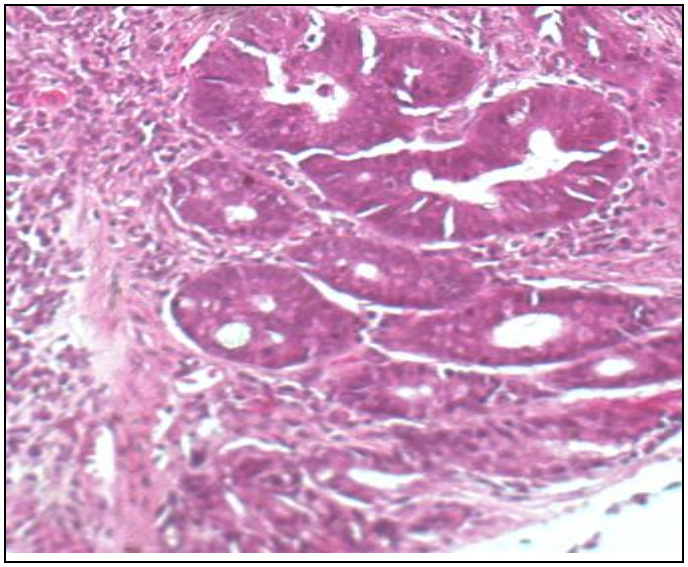


Figure 3.9 H & E colon section from IL-10$^{-\backslash-}$ and IL-10$^{-\backslash-}$Fer$^{DR/DR}$ mice at 3 months of age

IL-10$^{-\backslash-}$ mouse with mild inflammation (A, x40 objective lens), IL-10$^{-\backslash-}$Fer$^{DR/DR}$ mouse with adenocarcinoma (B, x10 objective lens) where malignant crypts break the muscularis mucosa layer and invade beyond this barrier (C, higher magnification of B, x100 objective lens).

These results are somewhat surprising in light of previous data which illustrate that Fer is highly expressed in different malignant cell lines and down regulation of Fer reduces the proliferation of prostate carcinoma cells and attenuates the development of prostate cancer [145]. However, our observation may be related to the enhanced inflammation which was observed in the double mutants, since epidemiological studies have demonstrated a link between inflammation severity and duration and IBD associated cancer [146].

3.1.3 Summary

In summary, $Fer^{DR/DR}$ and Fps^{Kr}/Fer^{DR} mice experienced enhanced and prolonged colitis severity compared to WT mice in response to TNBS injection in gross (macroscopic) and histological level. This was associated with enhanced MPO activity in the colon of the mutants compared to WT mice with TNBS injection. Lamina propria immune cell isolation from the colon with subsequent FACS analysis demonstrated an enhanced neutrophil population in response to TNBS treatment, and this population was significantly increased ($p<0.05$) in Fps^{Kr}/Fer^{DR} compared to WT mice at day 10 post colitis induction. A significant increase in inflammatory parameters (macroscopic and histological score, and MPO activity) was noted in IL-$10^{-\backslash-}$ compared to WT mice at 3 months of age. A moderate but significant increase in inflammation was observed in IL-$10^{-\backslash-}Fer^{DR/DR}$ double deficient compared to IL-$10^{-\backslash-}$ mice ($p<0.05$). In contrast to the TNBS model, IL-$10^{-\backslash-}$ mice did not have enhanced neutrophil recruitment to the colon (measured by MPO activity) in the absence of Fer. This may be due to differences between the two models. In our hands, the TNBS model is a relatively acute model

where neutrophils are recruited early and then the inflammation resolves. The IL-10$^{-\backslash-}$ model by contrast is a chronic model of colitis with significant neutrophil infiltration as well as many other immune cells (e.g. T-cells) which play an important role in its pathogenesis. Group IV PTK may play a more obvious role in the acute response to damage in the colon. Surprisingly, in the absence of Fer kinase, IL-10$^{-\backslash-}$ mice had a 52 % incidence of polyps with a 28 % incidence of adenocarcinoma. Multiple adenocarcinomas were noted in IL-10$^{-\backslash-}$Fer$^{DR/DR}$ mice. This enhanced adenocarcinoma development in the absence of Fer may be associated with a more severe inflammatory phenotype or the role of Fer within transforming cells (e.g. epithelial cells) and should be explored further. This might be done by generating chimeric mice from irradiated IL-10$^{-/-}$Fer$^{DR/DR}$ mice reconstituted with IL-10$^{-/-}$ bone marrow derived cells and vice versa.

Chapter Four: **ROLE OF GROUP IV PTK IN NEUTROPHIL CHEMOTAXIS IN VITRO**

Neutrophils play an important role during the inflammatory response. They are recruited early as the first line of defence during the innate immune response to bacterial antigen and also contribute to chronic inflammatory responses such as IBD [53, 122, 147]. An important property of neutrophils is their ability to chemotax to the site of injury. They do this by migrating along chemotactic gradients within tissue which are generated after chemokine release from various cell types including epithelial cells, mast cells, macrophages, and endothelial cells [114, 148-150]. This type of chemotactic gradient recruits neutrophils to the general areas which has been compromised and are known as intermediary chemoattractants. Chemokines classified into this group are keratinocyte-derived cytokine (KC), macrophage inflammatory protein (MIP-2), interleukin-8 (IL-8), and leukotrine B_4 (LTB_4) [114, 151]. Once recruited to the compromised tissue, neutrophils are directed towards their target by end-target chemoattractants (see diagram 4.1). Chemoattractants which fall into this category include complement peptide C5a and bacterial peptide such as formyl-met-leu-phe (fMLP) [114, 152, 153]. Chemotactic cells use a variety of intracellular signalling pathways which are activated after cell surface receptor (G-protein coupled receptor 'GPCR'). Chemoattractant binding promote the exchange of GDP with GTP in $G\alpha$, which leads to the dissociation of the $G\beta\gamma$ subunit. The activation of GPCR can initiate a number of effectors including: phospholipase C (PLC), phosphotadylinositide-3 kinase (PI3K), ion channels, and mitogen activated protein kinase (MAPK). These effectors then regulate cytoskeletal and adhesive elements that direct cellular shape change, polarization, and movement [154-156].

Studies have shown that neutrophils can prioritize between multiple signals and differentiate between intermediate and end target chemoattractants by differentially activating intracellular signalling pathways [114, 152, 153, 157]. For example in human and mice neutrophils p38 MAPK is important for chemotaxis in response to fMLP and PI3K is important for chemotaxis in response to IL-8.

Fer kinase has been shown to be associated with cytoskeletal protein components such as the actin binding protein cortactin [25, 37] and in the crosstalk between adherent junctions and focal adhesion [29], and could be involved in the intracellular regulation of chemotaxis. Furthermore, in mouse bone marrow-derived mast cells, Fer kinase is required for maximal p38 MAPK activation to promote chemotaxis of mast cells activated by cross-linking the high affinity IgE receptor (FcεRI) [31]. The aim of this chapter was to determine if Fer kinase is involved in the regulation of neutrophil chemotaxis through regulation of intracellular signalling pathways.

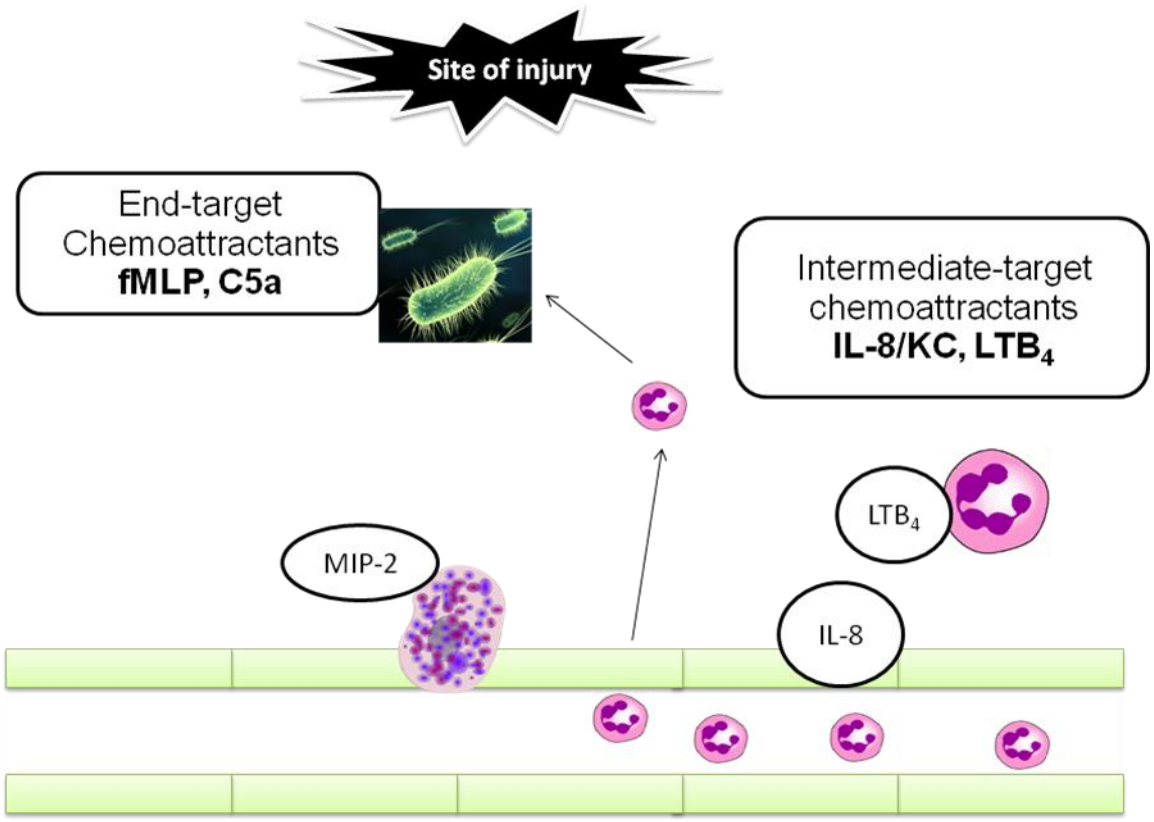


Diagram 4.1Neutrophil chemotaxis toward different chemoattractants

Neutrophils migrate from the blood stream to the surrounding tissue in response to the intermediate target chemoattractants (IL-8, MIP-2, and LTB_4) secreted from different cell types such as endothelial cells, neutrophils, and mast cells. Once there, neutrophils will ignore the intermediate targets and decide to move toward the end targets such as C5a and the bacterial peptide fMLP to fight and clear the inflammatory stimuli.

4.1 Results

Neutrophils were isolated from bone marrow of WT and $Fer^{DR/DR}$ mice and used in the under agarose gel assay to determine their chemotactic ability towards fMLP-peptide WKYMVm. WKYMVm was used as it is a potent activator of mouse neutrophil fMLP receptor (FPR) [158]

4.1.1 A stimulus specific role for Fer kinase in neutrophil chemotaxis in vitro

Figure 4.1 illustrates a dose-response curve for neutrophil chemotaxis toward different concentrations of WKYMVm studied. Using both WT and $Fer^{DR/DR}$ neutrophils, a bell-shape curve was observed in response to increasing concentrations of the chemoattractant. This is a characteristic of chemoattractant responses as stasis occurs at high concentrations. The optimal dose of WKYMVm in both groups for neutrophil chemotaxis was observed with 1 µM concentration. However at this dose 48.9 ± 8.3 WT neutrophils chemotaxed toward WKYMVm, and a 2 fold increase in chemotaxis was observed in $Fer^{DR/DR}$ neutrophil. A significant increase in $Fer^{DR/DR}$ neutrophil chemotaxis was observed towards all doses of WKYMVm compared to WT neutrophils. Figure 4.2 illustrates the number of neutrophils in each 200 µm segment as the cells move toward the WKYMVm (1 µM) well. A significant increase in $Fer^{DR/DR}$ neutrophil chemotaxis was observed compared to WT neutrophils in at each distance indicating more neutrophil WKYMVm-induced chemotaxis in the absence of Fer.

Phosphate buffered saline (PBS) was used as a negative control in each experiment and it induced no chemotaxis (data not shown). Our data for optimal WKYMVm concentrations for mouse neutrophil chemotaxis using the under agarose assay agrees with previously published concentrations [151, 159]. These data suggest that Fer kinase plays a role in damping neutrophil chemotaxis toward the end target peptide WKYMVm *in vitro*. Enhanced neutrophil chemotaxis in Fer null neutrophils might be due to increased fMLP receptor expression or Fer may play a role in modulating the signalling pathways used for chemotaxis towards fMLP peptide.

Next we examined the role of Fer kinase in chemotaxis towards the end target chemoattractant-complement product C5a. Figure 4.3 illustrates the number of WT or $Fer^{DR/DR}$ cells moving towards C5a after 4 h in the under agarose assay. Interestingly, the absence of Fer kinase in neutrophils enhanced C5a-induced chemotaxis observed in a similar manner as WKYMVm-induced chemotaxis. These data suggest that Fer kinase can modulate neutrophil chemotaxis towards end target chemoattractants.

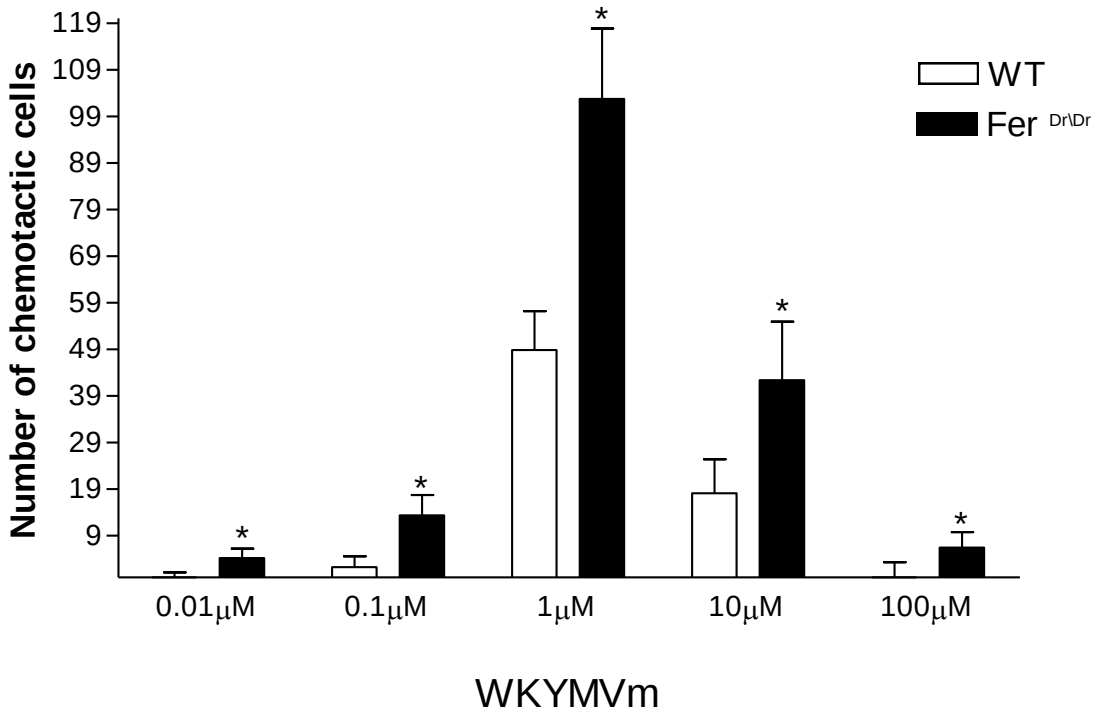


Figure 4.1 Neutrophil chemotaxis is enhanced in the absence of Fer kinase in vitro toward WKYMVm.

I*n vitro* total WT (open bars) and $Fer^{DR/DR}$ (solid bars) neutrophil chemotaxis toward different concentrations of WKYMVm (0.01-100 μM) was determined using the under-agarose gel assay. Data are expressed as mean ± SEM, n= 10-24 plates from 5 mice, * significant differences from WT neutrophils.

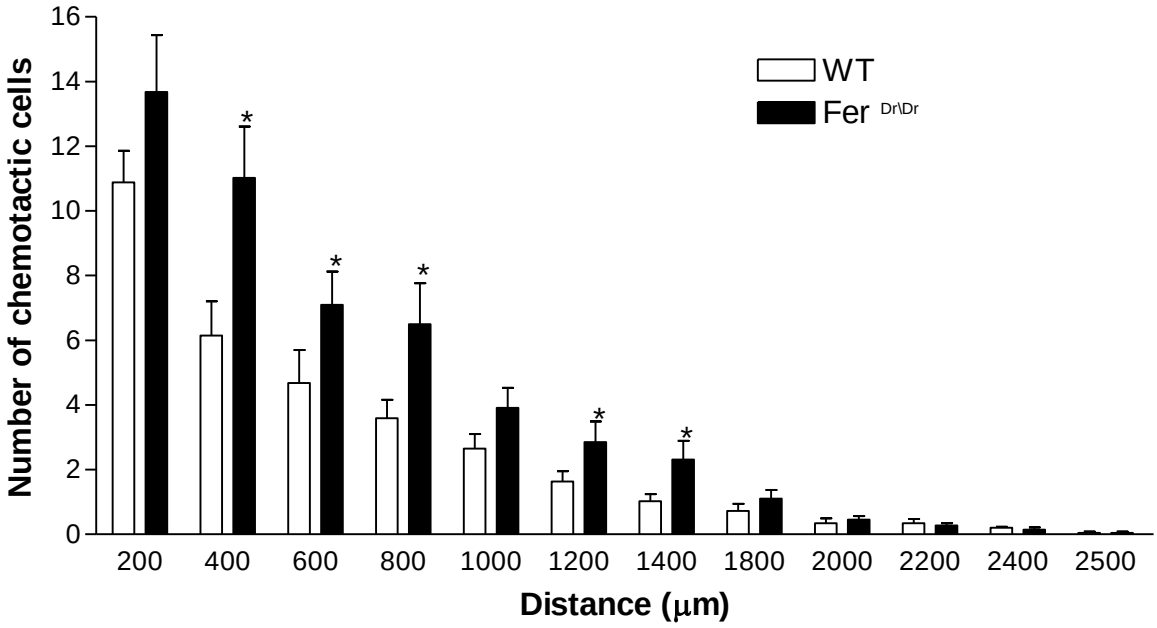


Figure 4.2 Neutrophil chemotaxis is enhanced in the absence of Fer in vitro in each distance segment toward WKYMVm.

The number of chemotacting cells expressed in each 200 μm segment in WT (open bars) and $Fer^{DR/DR}$ (solid bars) neutrophil toward WKYMVm (1 μM) are shown. Data are expressed as mean ± SEM, n=10-24 plates from 5 mice, * significant differences from WT neutrophils.

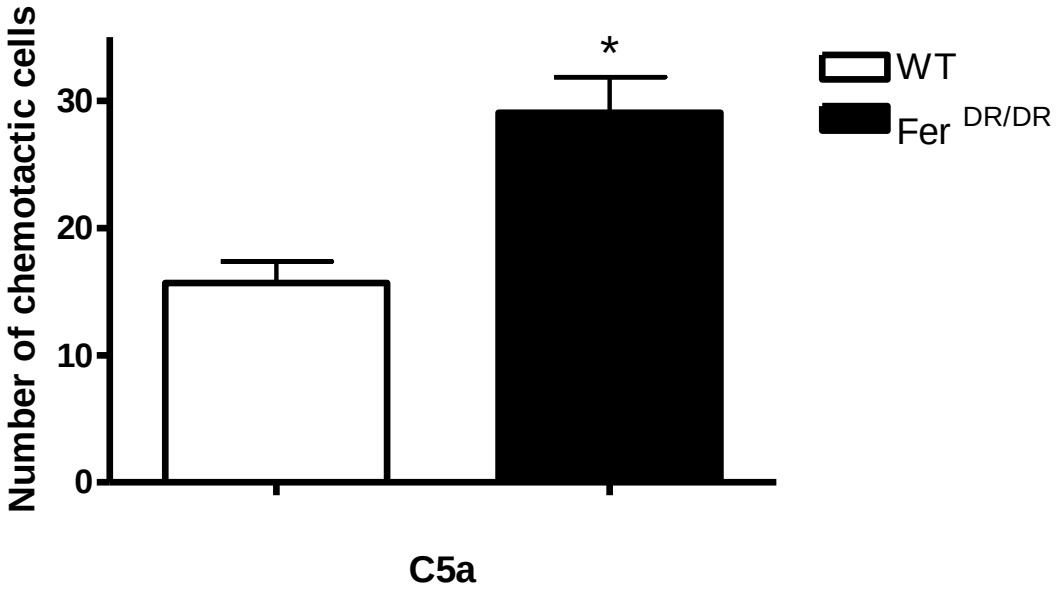


Figure 4.3 Fer kinase plays a role in neutrophil chemotaxis in vitro toward the end target chemoattractants.

In vitro total WT (open bar) and $Fer^{DR/DR}$ (solid bar) neutrophil chemotaxis toward C5a (1 μM) was determined by using the under-agarose gel assay. Data are expressed as mean ± SEM, n= 32 plates from 4 mice, * significant differences from WT neutrophils.

To determine if this regulatory role in chemotaxis was universal we examined intermediary chemoattractants. The following chemoattractants were studied using wild type or $Fer^{DR/DR}$ neutrophils; keratinocyte-derived cytokine; KC (1.25 µM), macrophage inflammatory protein-2; MIP-2 (1.25 µM), and leukotriene-B_4; LTB_4 (1 µM). These doses of the chemoattractants were chosen from previously published data obtained using chemoattractants dose response curve in mouse neutrophils [114, 151]. Figure 4.4 illustrates the number of cells moving towards each chemoattractant. In WT neutrophils KC induced around 25 cells to chemotax (PBS control < 1, data not shown). A similar level was observed in $Fer^{DR/DR}$ neutrophils indicating no role for Fer in chemotaxis towards KC. In a similar manner chemotaxis induced by MIP-2 and LTB_4 was not significantly different between WT and $Fer^{DR/DR}$ neutrophils. These data suggest a stimulus specific role for Fer kinase in neutrophil chemotaxis with a role toward end target but not intermediary chemoattractants.

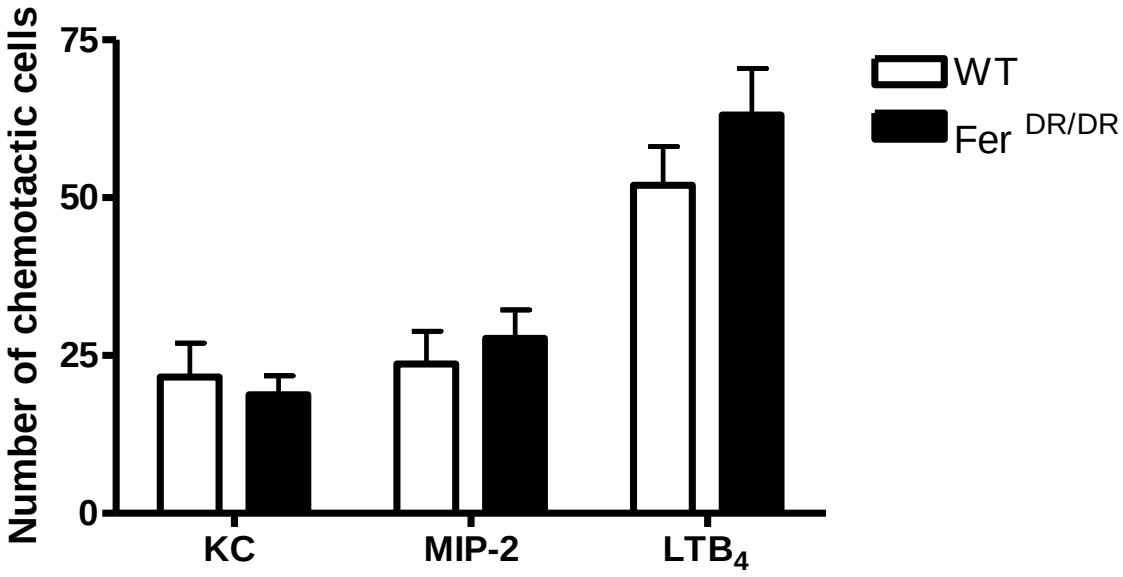


Figure 4.4 Fer kinase does not play a role in neutrophil chemotaxis in vitro toward intermediate target chemoattractants.

Numbers of WT (open bars) and $Fer^{DR/DR}$ (solid bars) neutrophil chemotaxis towards KC (1.25 µM), MIP-2 (1.25 µM), and LTB_4 (1 µM) *in vitro* was determined using the under agarose gel assay. Data are expressed as mean ± SEM, n= 26-37 plates from 3-9 mice. PBS negative control values were < 1.

4.1.2 Fps/Fes kinase does not account for the stimulus specific participation of Fer kinase in neutrophil chemotaxis

The possibility exists that in the absence of Fer kinase the closely related Fps/Fes protein tyrosine kinase compensates for Fer and regulates neutrophil chemotaxis towards intermediate chemoattractants such as LTB_4. To test this, in separate experiments we isolated bone marrow neutrophils from Fps^{Kr}/Fer^{Dr} double mutant mice and compared LTB_4-induced chemotaxis to that observed in neutrophils from wild type and $Fer^{DR/DR}$ mice. Figure 4.5 illustrates that LTB_4-induced chemotaxis in compound mutants at the level of chemotaxis observed using WT and $Fer^{DR/DR}$ neutrophils. These data suggest that Fps/Fes does not compensate for Fer during neutrophil chemotaxis towards intermediary chemoattractants. However, a trend towards increased WKYMVm-induced chemotaxis was observed in neutrophils from Fps^{Kr}/Fer^{Dr} mice although this was not significantly different from $Fer^{DR/DR}$ single mutant mice. These data may suggest that Fps/Fes kinase plays a similar role to Fer kinase in modulating neutrophil chemotaxis towards WKYMVm although the synergistic effect did not reach significance.

Next we investigated if the intracellular signalling pathways activated within neutrophils in response to chemotactic agents were modified in $Fer^{DR/DR}$ neutrophils. Two major pathways have been described to play a role in neutrophil chemotaxis- PI3K and p38 MAPK pathways [114, 159, 160]. Furthermore Fer kinase has been shown to have a role in p38 MAPK regulation in mast cells [31].

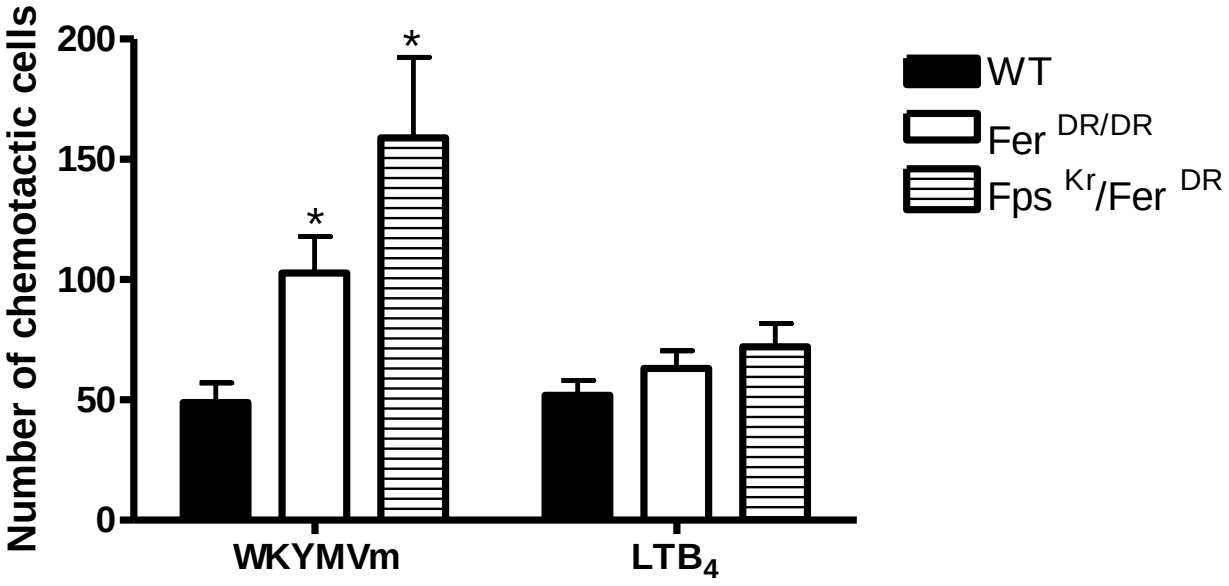


Figure 4.5 Fps/Fes kinase does not account for the stimulus specific participation of Fer kinase in neutrophil chemotaxis

Number of WKYMVm-induced (1 µM) or LTB4-induced (1 µM) chemotactic neutrophils observed using the under agarose assay. Neutrophils were obtained from WT mice (solid bars), $Fer^{DR/DR}$ mice (open bars), and Fps^{Kr}/Fer^{Dr} mice (hatched bars). Data are expressed as mean ± SEM, n= 23 plates from 3 mice, * significant differences from WT neutrophils.

4.1.3 The role of Fer in regulating intracellular signalling pathways

In this section we used pharmacological inhibitors of the p38 MAPK and PI3K pathway to investigate the contribution of these pathways in chemotaxis in WT and $Fer^{DR/DR}$ neutrophils. Three p38 MAPK inhibitors (SKF86002 ’30 μM’, SB239063 ’10 μM’, and SB203580 ’10 μM’) [114, 133], and two PI3K inhibitors (LY294002 '50 μM', and wortmannin '100 nM') [114, 134] were used at optimal concentrations based on previously published data.

Figure 4.6 illustrates WKYMVm-induced WT chemotactic cells expressed as percentage of untreated WT chemotaxis after p38 MAPK pharmacological inhibition. All of the p38 MAPK inhibitors used inhibited WT neutrophil chemotaxis significantly (71 – 85 %) suggesting WT neutrophils use p38 MAPK pathway to chemotax towards WKYMVm (1 μM). This is in agreement with Heit *et al* [114]. In contrast when we examined the same in $Fer^{DR/DR}$ neutrophil chemotaxis in response to WKYMVm, no inhibition was observed with SKF86002 and SB239063 and a moderate (31 %) inhibition was observed in SB203580 compound (figure 4.7). This is likely due to non-specific effects of SB203580 compound at the concentration used. These data suggest that p38 MAPK pathway is not the major pathway used in $Fer^{DR/DR}$ neutrophils to chemotax toward WKYMVm.

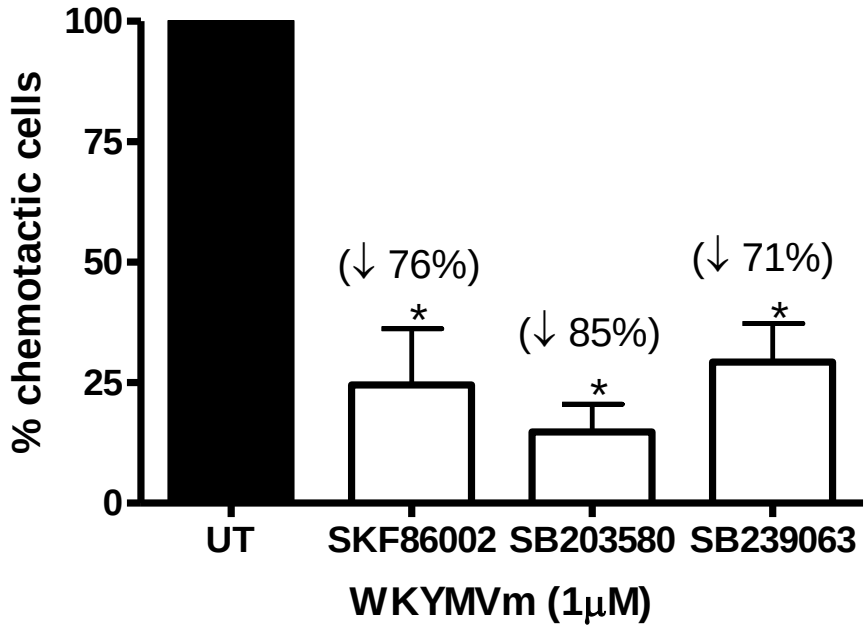


Figure 4.6 WT neutrophils uses p38 MAPK pathway in their chemotactic response toward WKYMVm

Percentage of WT neutrophil chemotaxis towards WKYMVm after pharmacological inhibition with p38-MAPK inhibitors [SB203580 (10 μM), SB239063 (10 μM), and SKF86002 (30 μM) (open bars)]. The number of untreated (UT) cells which moved toward WKYMVm was taken as 100 % (solid bar). Data are expressed as mean ± SEM, n=13-16 plates from 4 mice. * Significant differences from UT cells.

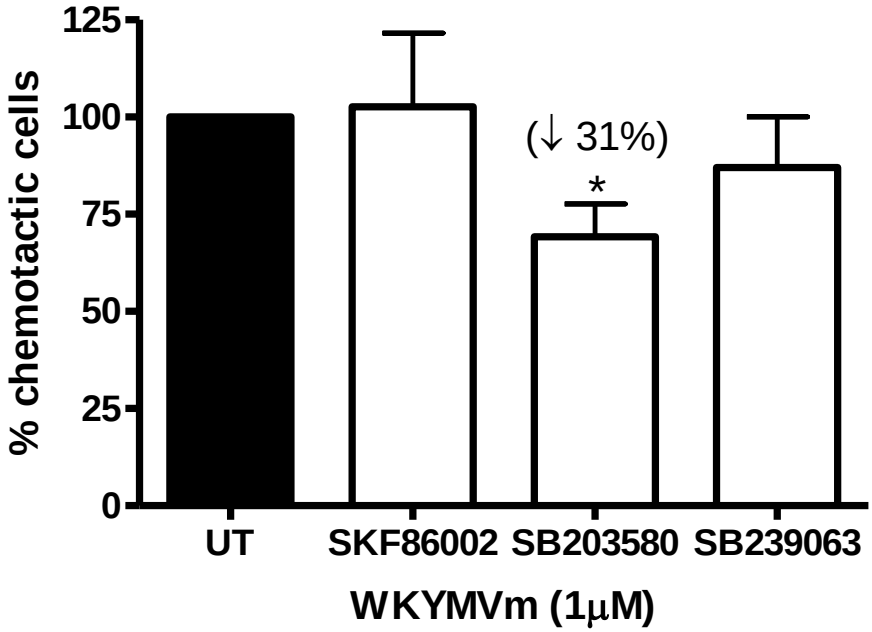


Figure 4.7 Fer$^{DR/DR}$ neutrophils do not use p38 MAPK pathway in their chemotactic response toward WKYMVm

Percentage of Fer$^{DR/DR}$ neutrophils chemotaxis towards WKYMVm after pharmacological inhibition with p38-MAPK inhibitors. The number of untreated (UT) cells which moved toward WKYMVm was taken as 100 % (solid bar). Data are expressed as mean ± SEM, n=13-16 plates from 4 mice. * Significant differences from UT cells.

Next we investigated the role of the PI3K pathway in WKYMVm-induced chemotaxis. A role for both pathways has been previously suggested by Heit *et al* [114]. Although the PI3K pathway is not the major route used by neutrophils to chemotax towards fMLP it is activated in neutrophils in response to fMLP stimulation. It has been suggested that there may be some crosstalk between these two pathways [114]. When neutrophils encounter both intermediate- and end target- chemoattractants they always choose to move toward the end targets using the p38 MAPK pathway, but when p38 MAPK activity is inhibited the preferential migration toward the end targets is lost and these neutrophils move toward the intermediate targets at greater numbers using PI3K pathway. In addition, neutrophils treated with p38 MAPK inhibitors show enhanced and prolonged PI3K activity in response to fMLP stimulation [114].

PI3K is a group of heterodimeric enzymes that phosphorylate plasma membrane phosphatydilinositol 4-5-biphosphate (PIP_2), yielding to phosphatydilinositol 3, 4, 5-triphosphate (PIP_3) upon exposure to chemokines and chemoattractants. PI3K is a very important component of cell signalling in neutrophil chemotaxis as well as other neutrophil functions like superoxide production. Studies using PI3Kγ-deficient mice showed impaired neutrophil chemotaxis in response to C5a and fMLP [161-163], while other studies showed that PI3K pathway does not play a role in human neutrophil chemotaxis in response to fMLP [114].

Using 2 inhibitors LY294002 (50 μM) and wortmannin (100 nM) in the under agarose assay we investigated the role of PI3K pathway in neutrophil chemotaxis towards WKYMVm in WT and $Fer^{DR/DR}$ neutrophils. Figure 4.8 illustrates the percent inhibition in chemotaxis toward fMLP peptide in WT neutrophils treated with two PI3K inhibitors.

Untreated (UT) neutrophils were taken as 100 %. These data demonstrate no reduction in chemotaxis with LY294002 and approximately 50 % reduction with wortmannin suggesting that PI3K plays a minor role in WT neutrophil chemotaxis towards WKYMVm. The significant inhibition of chemotaxis in WT neutrophils treated with wortmannin might be due to off target (non PI3K) effects of this compound at the concentration used. Interestingly LY294002 induced a 53 % and wortmannin > 75 % reduction in $Fer^{DR/DR}$ neutrophil chemotaxis toward WKYMVm (figure 4.9). These data illustrate that the PI3K pathway plays an important role in $Fer^{DR/DR}$ neutrophil chemotaxis toward WKYMVm. These data agree with previously published data which demonstrate a minor role for PI3K pathway in neutrophil chemotaxis toward fMLP [114, 159]. A complete inhibition of WKYMVm induced chemotaxis was observed in both WT and $Fer^{DR/DR}$ neutrophils with a combination of p38 MAPK and PI3K inhibitor (figure 4.10). Taken together, these data indicate that both p38 MAPK and PI3K are involved in WKYMVm induced chemotaxis in WT neutrophils and Fer kinase may regulate the extent to which each participate. Next we examined how p38 MAPK and PI3K pathway activation is altered in the absence of Fer kinase

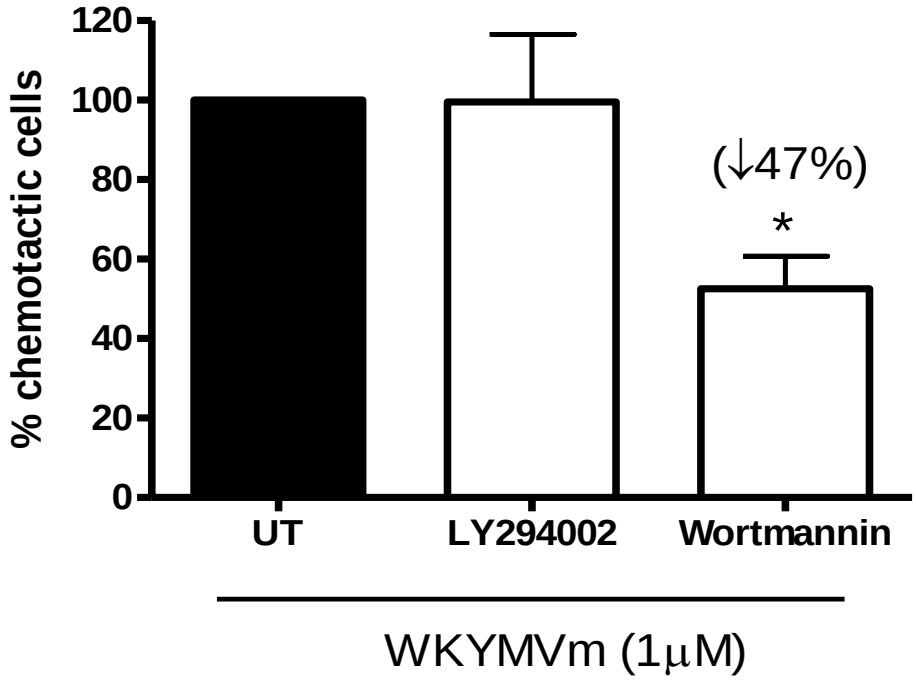


Figure 4.8 WT neutrophils uses PI3K pathway to a lesser extent than p38 MAPK pathway in their chemotactic response toward WKYMVm

Total WT neutrophils chemotaxis toward WKYMVm; untreated (UT) (solid bar), or treated with PI3K inhibitor (LY294002 and wortmannin) (open bars). Data are expressed as mean ± SEM, n=14-16 plates from 5 mice. * Significant differences from UT cells.

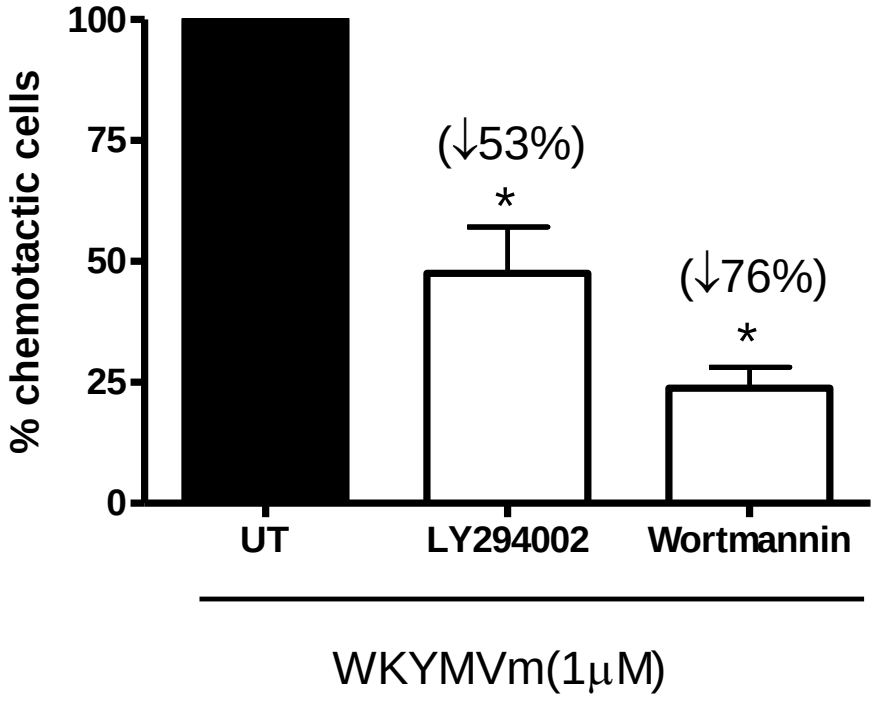


Figure 4.9 Fer$^{DR/DR}$ neutrophils uses PI3K pathway in their chemotactic response toward WKYMVm

Total Fer$^{DR/DR}$ neutrophils chemotaxis toward WKYMVm; untreated (UT) (solid bar), or treated with PI3K inhibitors (open bars). Data are expressed as mean ± SEM, n=14-16 plates from 5 mice. * Significant differences from UT cells.

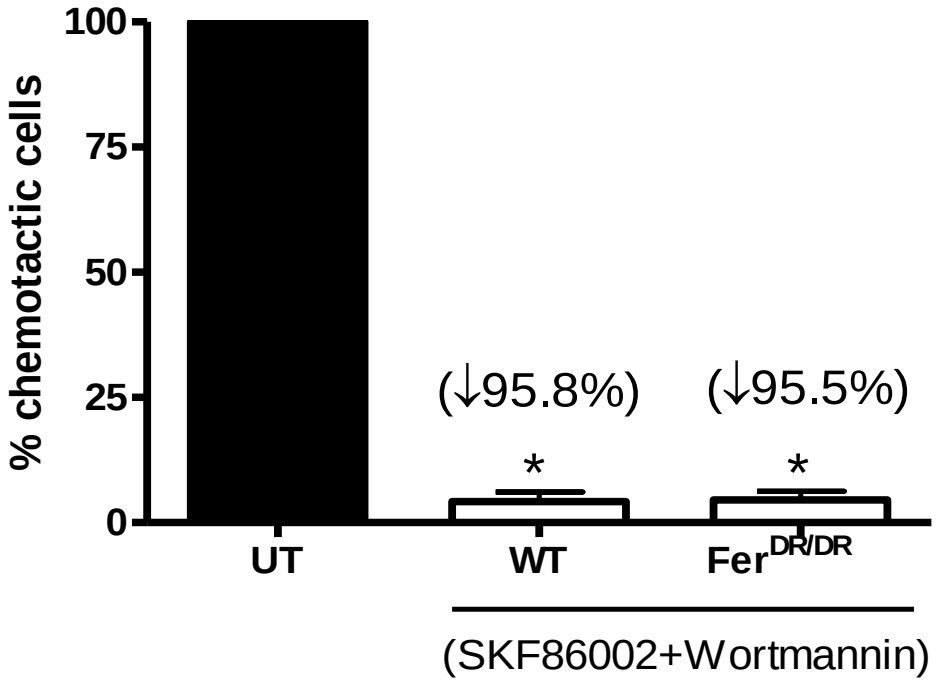


Figure 4.10 P38 MAPK and PI3K are the major pathways used by neutrophils to move toward WKYMVm

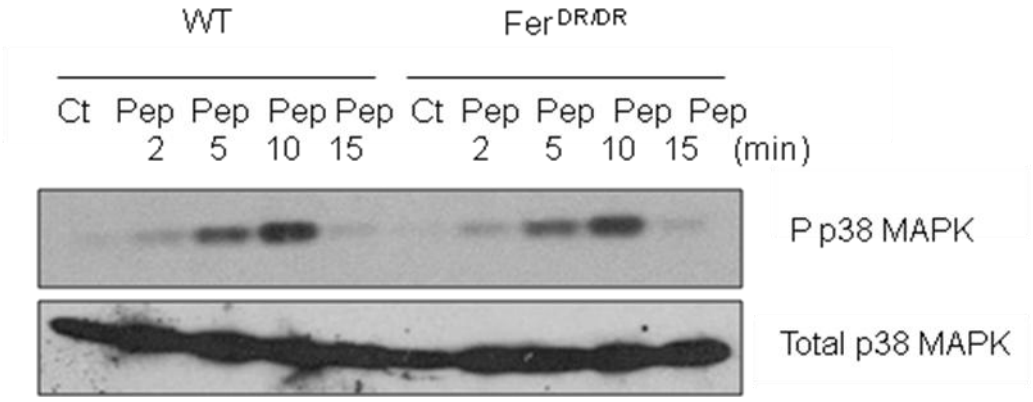
Total WT and $Fer^{DR/DR}$ neutrophils chemotaxis in response to WKYMVm; untreated (UT) (solid bar), or treated with a combination of p38MAPK and PI3K inhibitor (open bars). Data are expressed as mean ± SEM, n=14-16 plates from 3 mice. * Significant differences from UT cells.

4.1.4 Absence of Fer kinase does not alter WKYMVm-induced p38 MAPK phosphorylation in neutrophils

Our data suggest that Fer null neutrophil does not use the p38 MAPK pathway to chemotaxis toward WKYMVm. Therefore, we expected modulated degree of activation in $Fer^{DR/DR}$ neutrophils. Figure 4.11-A illustrates the degree of p38 phosphorylation in WT and $Fer^{DR/DR}$ neutrophils in response to WKYMVm (Pep; 1 μM) stimulation over 15 minutes. Phospho-p38 MAPK levels in WT neutrophils increased over baseline (Ct) and reached maximal levels at 10 min and returned to control levels by 15 min post WKYMVm stimulation. A similar time course was observed in $Fer^{DR/DR}$ neutrophils. No statistically significant difference was observed between WT and $Fer^{DR/DR}$ neutrophils over the time course of stimulation. Total p38 (figure 4.11-A-lower panel) show similar protein levels in all conditions suggesting equal loading. Figure 4.11-B demonstrates the densitometric measurements from three experiments for phospho p38 in both groups and illustrates a significant increase in p38 activation over 10 minutes post WKYMVm stimulation. No differences in activation are observed between WT and Fer null neutrophils. These data suggest that p38 MAPK activity is not altered in the absence of Fer kinase at the level of p38 MAPK in neutrophils in response to WKYMVm stimulation.

The possibility exists that Fer may activate the p38 MAPK pathway downstream of p38 MAPK level. A possible candidate downstream of p38 level is mitogen-activated protein kinase-activated protein kinase 2 (MAPKAPK2). Studies showed that p38 MAPK activates MAPKAPK2 in human neutrophils in response to fMLP and this activation is required for human neutrophil chemotaxis in response to fMLP [164]. Time did not permit further investigation of this pathway as we focused our attention on the role of Fer in modulating PI3K pathway as this pathway was shown to be the dominant pathway used for chemotaxis in $Fer^{DR/DR}$ neutrophils.

(A)



(B)

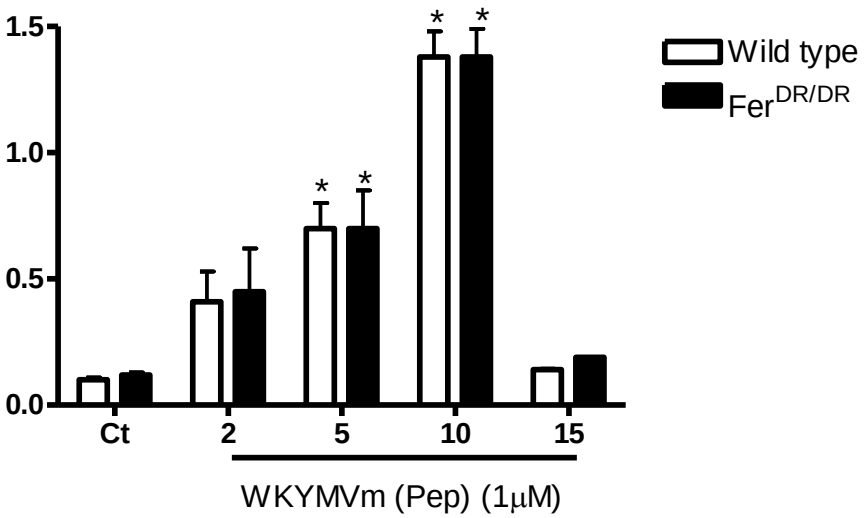


Figure 4.11 Absence of Fer kinase does not alter WKYMVm-induced p38 MAPK phosphorylation in neutrophils

Immunoblot of phospho and total p38 in WT and Fer$^{DR/DR}$ neutrophils challenged with WKYMVm at 2, 5, 10, and 15 min. Bone marrow derived neutrophils from wild type and Fer$^{DR/DR}$ mice were isolated and stimulated with WKYMVm. Lysates were resolved by SDS–PAGE and sequentially immunoblotted with anti phospho p38 antibody, and stripped/re-probed with anti-p38 antibody. A) representative immunoblot of phosphorylated p38 MAPK B) semi-quantitative analysis of p38 MAPK phosphorylation determined by densitometric analysis of the immunoblots. Data are expressed as the mean ± SEM. Data from wild type and Fer $^{DR/DR}$ neutrophils are shown in open or solid bars respectively, n=3. * $p<0.05$ significant difference from UT neutrophils.

4.1.5 PI3K activation is enhanced and prolonged in neutrophils stimulated with WKYMVm in the absence of Fer kinase

Our data suggest a couple of possibilities as to how Fer influences the signalling pathways responsible for chemotaxis. We showed previously that WT neutrophils use p38 MAPK to chemotax toward WKYMVm and enhanced chemotaxis was observed in $Fer^{DR/DR}$ neutrophils. We may have expected that in the absence of Fer an enhanced p38 MAPK activity would be observed. However, phospho-p38 levels were found to be the same in WT and $Fer^{DR/DR}$ neutrophils in response to WKYMVm stimulation. $Fer^{DR/DR}$ neutrophils use the PI3K pathway as illustrated with pharmacological inhibition. Fer kinase may therefore act on the PI3K pathway to inhibit/limit its normal activation allowing p38 MAPK to control chemotaxis. In the absence of Fer the PI3K pathway may be over-activated and become the dominant intracellular pathway for chemotaxis.

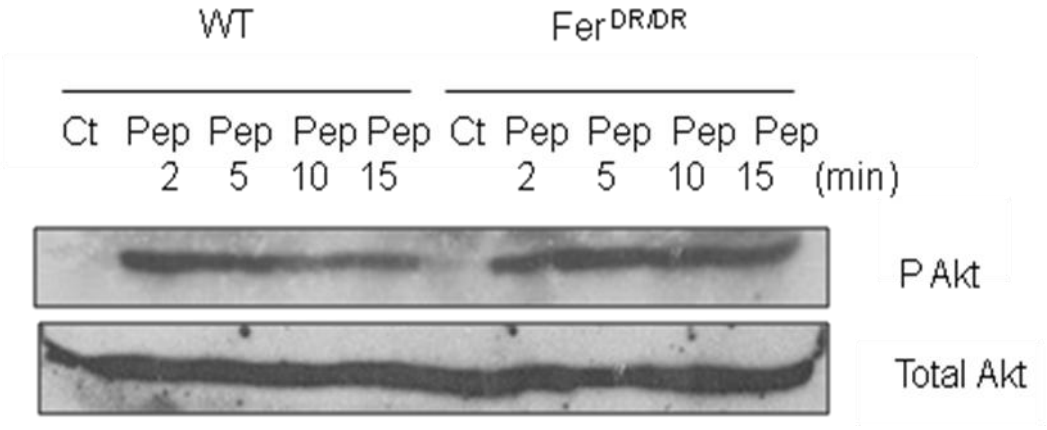
We measured Akt phosphorylation as an indication of PI3K activity in neutrophils downstream of WKYMVm stimulation. Figure 4.12-A demonstrates Akt phosphorylation (also known as protein kinase B), which is a downstream component of PI3K pathway in WT and $Fer^{DR/DR}$ neutrophils after WKYMVm (Pep) stimulation. Akt phosphorylation was significantly increased in WT neutrophils at 2 min and gradually decreases to base line levels by 15 min. The data show a similar Akt phosphorylation in $Fer^{DR/DR}$ compared to WT neutrophils at 2 min, but this activation was enhanced and prolonged in $Fer^{DR/DR}$ in response to WKYMVm stimulation. Total Akt (figure 4.12-A-lower panel) show similar protein levels in all conditions suggesting equal loading. Figure 4.12-B demonstrates the densitometric measurements from three experiments for phospho Akt in both groups. These data suggest that Fer kinase acts as an inhibitor of PI3K pathway

upstream of Akt and in the absence of Fer, this pathway has enhanced and prolonged activity in response to WKYMVm stimulation. These data suggest Fer may be involved in deactivating PI3K pathway upstream of Akt level.

One possible candidate that Fer kinase might act on upstream of Akt is phospho-inositide-dependent-protein kinase (PDK1). PDK1 is a 63 kDa serine-threonine kinase which consists of N-terminal kinase domain and C-terminal PH-domain, and it can be activated by phosphorylation in response to many agonists including insulin, oxidative stress, and peroxovanadate in the activation loop Ser^{241} for human, and Ser^{244} for mouse PDK1 [165, 166]. Several studies have shown PDK1 has a role in cell chemotaxis and migration in endothelial cell and in F-actin polymerization which are needed for the chemotactic response to occur [167, 168] .

To examine if PDK1 activation is altered in the absence of Fer kinase, we determined the degree of PDK1 phosphorylation in WT and $Fer^{DR/DR}$ neutrophils in response to WKYMVm stimulation (figure 4.13-A and B, representative of n=3). No difference in PDK1 phosphorylation level between WT and Fer null neutrophils was observed although increased Akt phosphorylation levels were observed in Fer mutant compared to WT neutrophils in the same neutrophil lysate (figure 4.13). This suggests that Fer kinase may modulate PI3K activity through other upstream targets excluding PDK1, or it can directly act at the Akt level. The possible targets of PI3K pathway that can be modulated by Fer kinase is illustrated in diagram 4.2.

(A)



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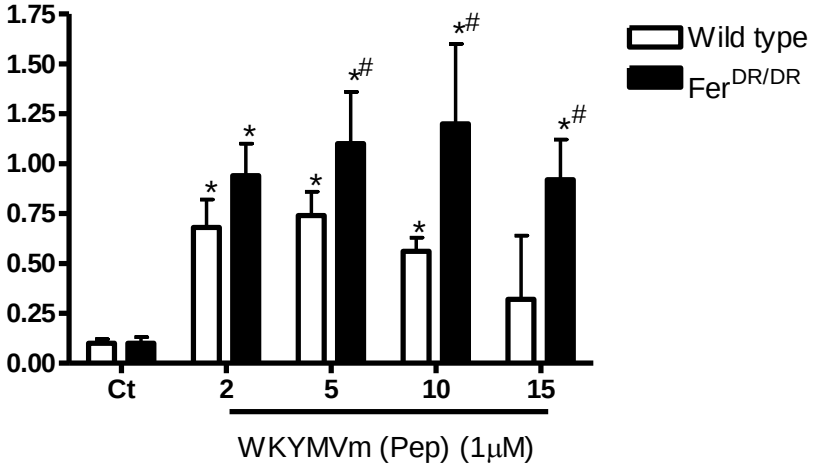
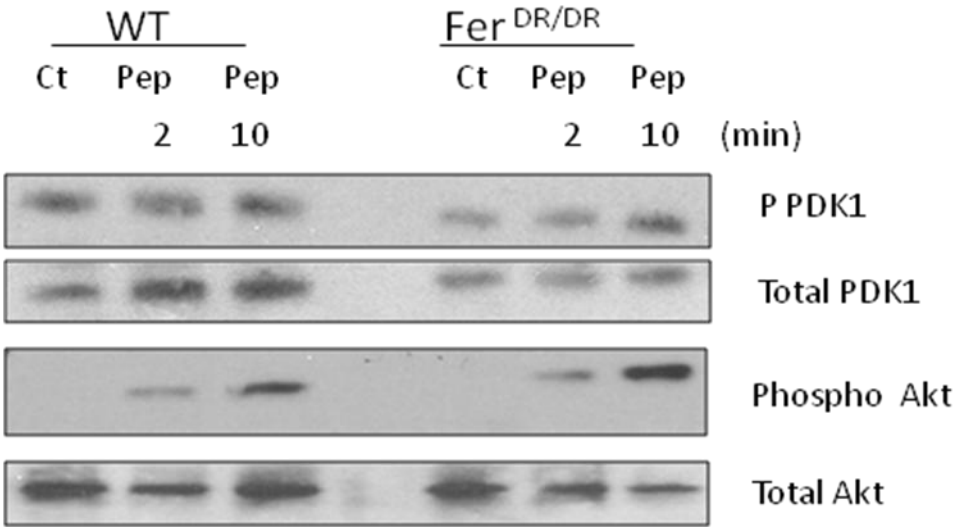


Figure 4.12 PI3K activity is prolonged in neutrophils stimulated with WKYMVm in the absence of Fer kinase

Bone marrow derived neutrophils from wild type and $Fer^{DR/DR}$ mice were isolated and stimulated with WKYMVm (Pep; 1μM) for 2, 5, 10 and 15 minutes. Lysates were resolved by SDS–PAGE and sequentially immunoblotted with anti phospho Akt antibody (Ser473) and stripped/re-probed with anti-Akt antibody. A) Representative immunoblot of phosphorylated Akt and total Akt in each condition B) Semi-quantitative analysis of Akt phosphorylation determined by densitometric analysis of the immunoblots. Data are expressed as the mean ± SEM. Data from wild type and $Fer^{DR/DR}$ neutrophils are shown in open or solid bars respectively, n=4, * $p<0.05$ significant difference from UT neutrophils. # $p<0.05$ significant difference from WT neutrophils.

(A)



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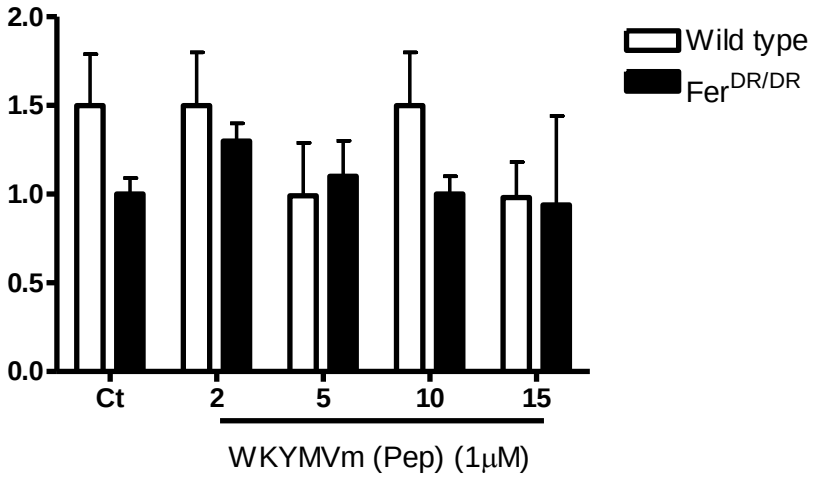


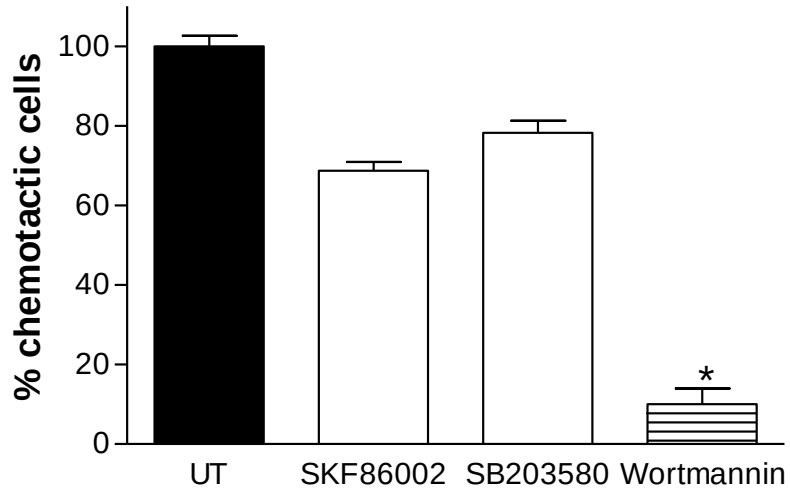
Figure 4.13 PDK1 activity is not altered the absence of Fer kinase

Bone marrow derived neutrophils from wild type and Fer$^{DR/DR}$ mice were isolated and stimulated with WKYMVm (Pep; 1μM) for 2, 5, 10 and 15 minutes. Lysates were resolved by SDS–PAGE and sequentially immunoblotted with anti phospho PDK1 antibody and stripped/re-probed with anti-PDK1 antibody. A) representative immunoblot of phosphorylated PDK1 in each condition B) Semi-quantitative analysis of PDK1 phosphorylation determined by densitometric analysis of the immunoblots.
Data are expressed as the mean ± SEM. Data from wild type and Fer $^{DR/DR}$ neutrophils are shown in open or solid bars respectively, n=3.

Interestingly when we used p38 and PI3K inhibitors in KC induced chemotaxis the signalling pathways were not altered in the absence of Fer. Figure 4.14 (A) illustrates the percentage of inhibition in WT neutrophil chemotaxis toward KC by using p38 MAPK and PI3K inhibitors. SKF86002 and SB203580 did not inhibit WT neutrophil chemotaxis significantly towards KC however wortmannin (PI3K inhibitor) inhibited this response by 91 % compared to untreated (UT) neutrophils. This is in agreement with previously published data which demonstrated a role for PI3K pathway in chemotaxis towards KC [114, 159]. Using Fer null neutrophils (figure 4.14-B), neither p38 MAPK inhibitors altered KC induced chemotaxis. The PI3K inhibitor wortmannin inhibited chemotaxis by 91%. These data demonstrate that the PI3K pathway is responsible for the chemotactic response of neutrophils toward KC in both WT and Fer null neutrophils and suggested that Fer kinase does not play a role in modulating the chemotactic behaviour or the signalling pathways used.

Using immunoblot we measured PI3K activity in WT and $Fer^{DR/DR}$ neutrophils in response to KC stimulation to confirm that Fer did not regulate PI3K activity toward KC. We showed that the degree of Akt phosphorylation was similar in $Fer^{DR/DR}$ compared to WT neutrophils in response to KC stimulation (data not shown). These data support data generated from the under agarose assay using chemical inhibitors of PI3K pathway. Taken together, these data suggest a stimulus-specific role for Fer kinase in modulating neutrophil chemotaxis and the signalling pathways utilized by neutrophils toward WKYMVm, but not toward KC.

(A)



(B)

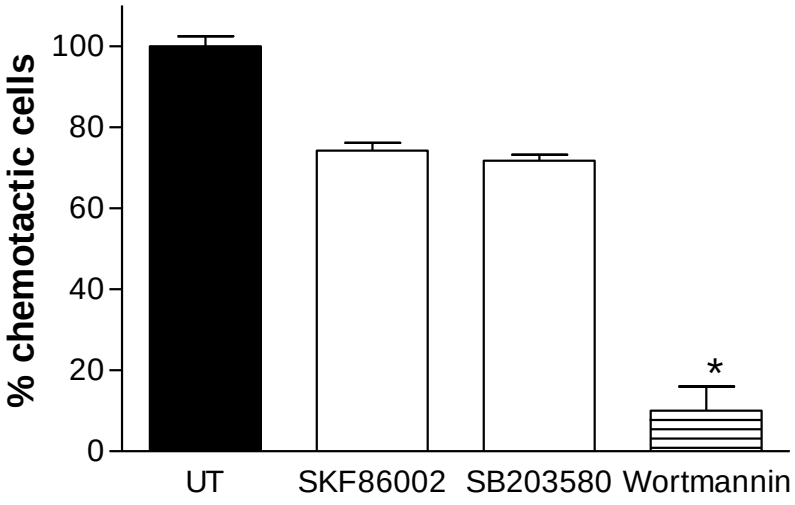


Figure 4.14 P3IK is the dominant pathway for WT and Fer mutant neutrophil chemotaxis toward KC

In vitro total (A) WT and (B) $Fer^{DR/DR}$ neutrophils chemotaxis in response to KC; untreated (UT) (solid bar), treated with p38-MAPK inhibitors (SB203580, and SKF86002; open bars), or treated with PI3K inhibitor (wortmannin; hatched bar). Data are expressed as mean ± SEM, n=10-15. * Significance from UT cells

4.2 Summary

We have shown here a significant increase in neutrophil chemotaxis toward end target chemoattractants WKYMVm and C5a in $Fer^{DR/DR}$ compared to WT neutrophils. Surprisingly, no role for Fer was observed for neutrophil chemotaxis toward the intermediate targets KC, MIP-2 and LTB_4. In addition we demonstrated that both p38 MAPK and PI3K pathways are activated in mouse neutrophils in response to WKYMVm with the p38 MAPK pathway being the dominant pathway in WT neutrophils which chemotax towards WKYMVm. Furthermore we demonstrated that although p38 MAPK is phosphorylated to similar levels and time course in Fer null neutrophils, the PI3K is the dominant pathway used for chemotaxis towards WKYMVm in the absence of Fer. PI3K pathway activation is enhanced and prolonged in the absence of Fer.

Fer may act upstream of Akt to regulate PI3K activity. Diagram 4.2 illustrates possibly targets. Our data suggest that Fer kinase regulates PI3K pathway directly through modulating Akt activity or indirectly through other regulators of PI3K pathway. Fer may interact with components of PI3K enzyme such as p85 and p110. Fer has been shown to directly interact with the p85 regulatory subunit of PI3K enzyme in adipocytes in response to PDGF stimulation and modulates PI3K activity [169]. Fer may modulate the activity of other phosphatases [PTEN, PH domain leucin-rich repeat protein phosphatase (PHLPP), or protein phosphatase 2A (PP2A)] which results in modulating PI3K activity. Fer may modulate the activity of kinases which act upstream of Akt such as integrin-linked kinases (ILKs) which couples integrins and growth factors to downstream pathways involved in cell survival, cell cycle control, cell-cell adhesion and cell motility [170]. Recently, Itoh *et al* [171] demonstrated that phospholipase D (PLD)

pathway is required for Fer activation and cell migration suggesting that Fer kinase may modulate this pathway to regulate neutrophil chemotaxis. Our data suggest Fer does not regulate PDK1 activation. Furthermore, Fer does not act at p38 MAPK level however it may have effects on downstream components such as MK2 to modulate this pathway. Overall our data illustrate an important role for Fer kinase in neutrophil chemotaxis towards the end target chemoattractants such as bacterial peptides and support a role for neutrophil chemotaxis to the gut.

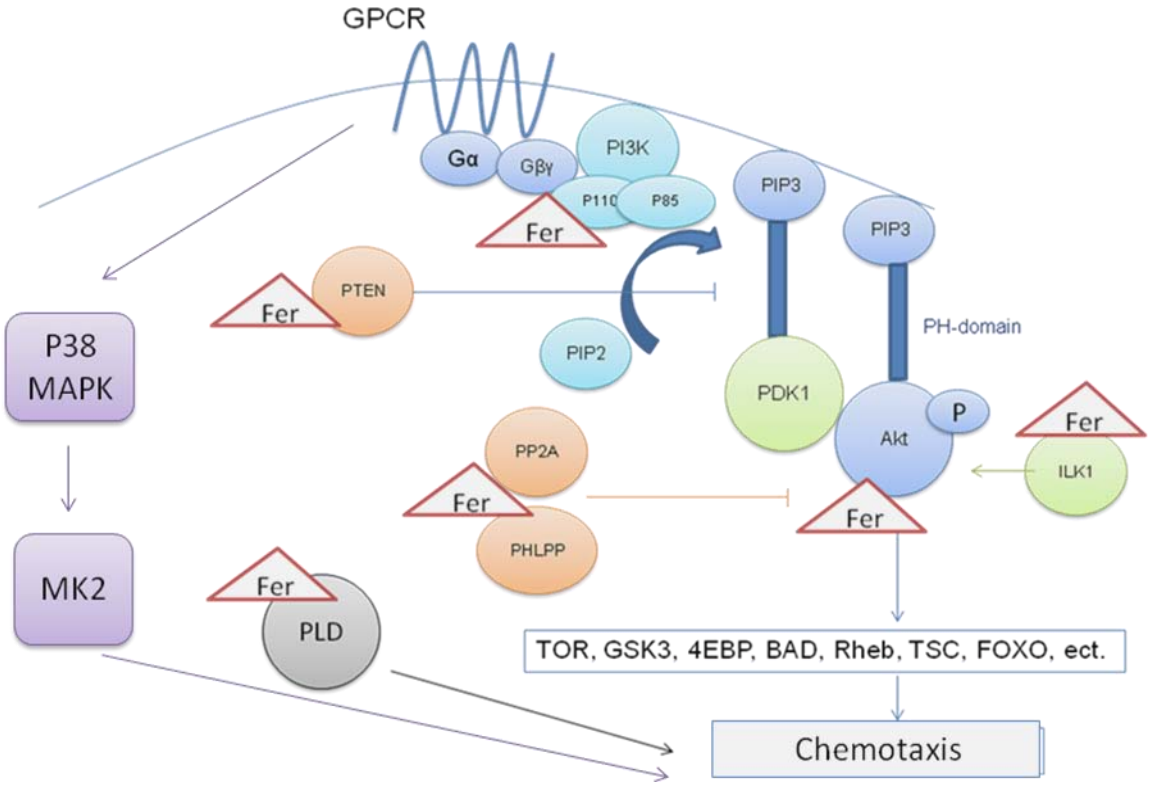


Diagram 4.2 Schematic diagram for the role of Fer kinase in modulating the signalling pathways used by neutrophils in their movement toward WKYMVm

WT neutrophils use p38 MAPK pathway in their chemotactic movement toward WKYMVm although both (p38 and PI3K) pathways are activated. The dominance of p38 pathway in WT may be due to Fer regulation of the PI3K pathway. Our data illustrate that Fer does not act at the p38 MAPK level to alter phosphorylation. In the absence of Fer Akt activation is enhanced.
PTEN; Phosphate and tensin homolog deleted on chromosome 10, PHLPP; PH domain leucin-rich repeat protein phosphatase, PP2A; protein phosphatase 2A, ILKs; integrin-linked kinases, and PLD; phospholipase D.

Chapter Five: **DETERMINING FER KINASE ACTIVITY IN NEUTROPHILS**

5.1 Determining if Fer is phosphorylated downstream of WKYMVm stimulation in neutrophils

In the previous chapter, we determined an important role for Fer kinase in modulating neutrophil chemotaxis and the signalling pathways used toward the bacterial peptide WKYMVm. In this chapter, our aim was to measure Fer kinase activation in neutrophils in response to WKYMVm stimulation. As will be discussed we encountered many issues in our attempts to measure Fer activation in neutrophils. We were able to demonstrate that Fer phosphorylation in neutrophils is increased upon WKYMVm stimulation (1 μM) but this result was not consistently reproducible. We have attempted to show Fer activation using 3 methodologies; (1) Immunoprecipitation (IP) of Fer protein and immunoblotting (IB) with 4G10 antibody which detects phosphorylated proteins only, (2) phospho-Fer antibody by direct IB, and (3) kinase activity assay using γ ^{32}P-ATP. Various issues were encountered in this aim and are detailed in diagram 5.1

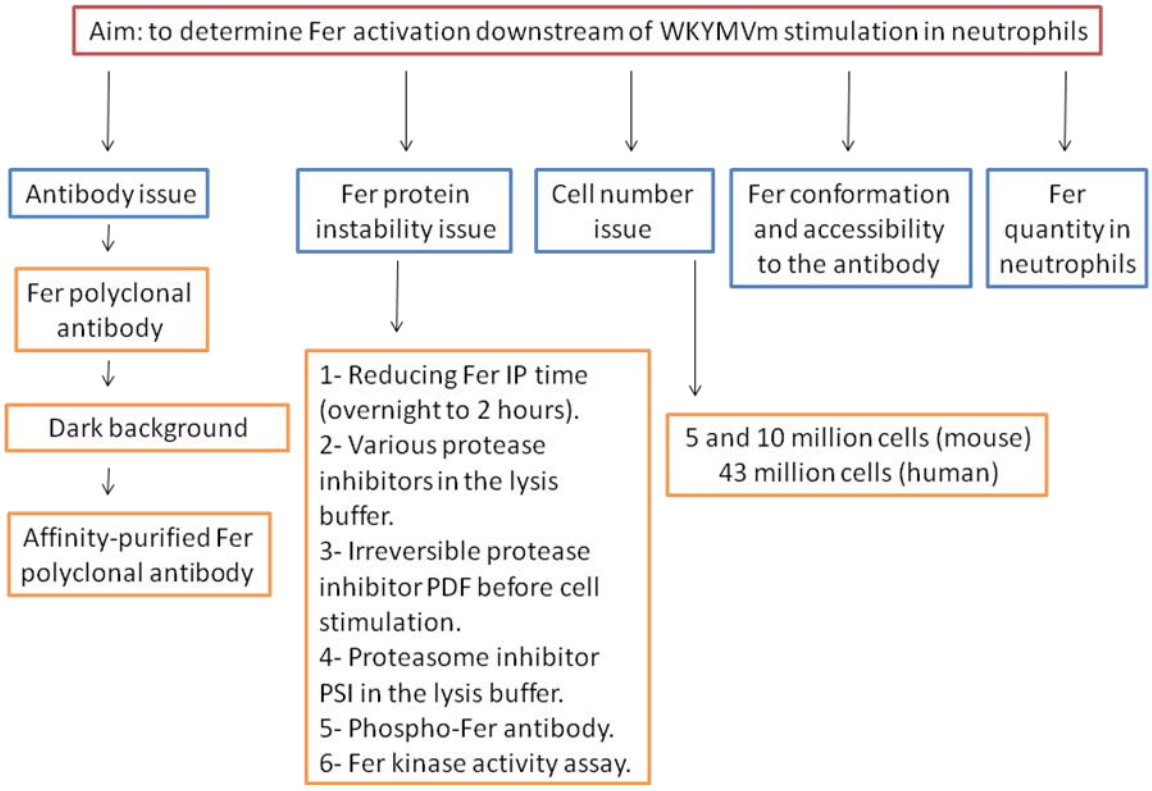


Diagram 5.1 Summary for the various problems we faced and how it was addressed to determine Fer activity in neutrophils.

Dark background using Fer polyclonal antibody provided by Dr. Craig was optimized by reducing the concentration of the primary and secondary antibodies used. Affinity purified Fer polyclonal antibody gave us a much cleaner background. To minimize Fer instability in neutrophils, we used different protease and proteasome inhibitors, reducing the IP time, and using phospho-Fer antibody provided by Dr. Craig by direct IB technique. In addition, we tried to measure Fer activity using other technique; Fer kinase assay. To optimize cell number issue, we used 5 and 10 million cells from bone marrow derived mouse neutrophils, and 43 million peripheral blood human neutrophils. Other factors which may play a role in difficulty to detect Fer in neutrophils are Fer protein conformation and accessibility to the antibody, Fer amounts in neutrophils, or other unknown factors.

5.1.1 Optimization of Fer immunoprecipitation

In the first set of experiments, we were using Fer polyclonal antibody which was kindly provided by Dr. Andrew Craig (Queen`s university). Using this antibody we performed immunoprecipitation (IP) as described in methods. On developing the blot high (dark) background was observed and we had difficulty observing the bands of interest in the blot. We used different concentrations of the primary (Fer) antibody (1/100, 1/500, and 1/1000 dilutions), different concentrations of the secondary antibody (1/10,000 and 1/20,000 dilution), and different washing buffers (TBS buffer + 0.1 % Tween20 or TBS + 0.1 % Tween20 + 0.1 % NP-40) aiming to reduce the background with no obvious success. We switched to use Fer polyclonal antibody which was affinity purified from Santa Cruz and this antibody gave us much cleaner background with no non-specific binding.

5.1.2 Fer protein levels and instability

These challenges were evident when we began our immunoprecipitation (IP) experiments and we attempted a series of changes to our methodology to optimize Fer IP and allow for reproducibility in our results. We would like to acknowledge Dr. Steven Robbins for his immense help in this process. The following parameters were investigated. We increased cell number from 5 to 10 million and extended the time for Fer IP from 3 h to overnight. In later experiments when we thought the degradation was an issue we reduced the IP incubation time to 2 h. Neutrophils contain high levels of protease which can degrade proteins therefore we incorporated pepstatin (1 mM) in the lysis buffer along with the other protease inhibitors (PMSF, aprotinin/leupeptin, and

N a_3VO_4). In separate experiments, we treated the isolated neutrophils with 1 mM DFP (Diisopropylfluoro-phosphate), which is a potent irreversible inhibitor of serine proteases and acetyl choline esterase at room temperature (RT) for 5 minutes before cell stimulation and lysis as suggested by Naccache group [172, 173]. Proteins can also be degraded through proteasomes. Proteasomes are large multi-subunit complexes localized in the nucleus and cytosol that selectively degrade intracellular proteins. A protein marked for degradation is covalently attached to multiple molecules of ubiquitin, and this ubiquitin-proteasome system plays a major role in the degradation of many proteins. We used a proteasome inhibitor PSI (N-carbobenoxy-L-isoleucyl-L-γ-t-butyl-L-glutamyl-L-leucinal) purchased from Sigma to incorporate it in the neutrophil lysis buffer along with treating the cells with PDF (irreversible protease inhibitor) and adding protease inhibitors in the lysis buffer. We used two concentrations of PSI; 30 µM and 60 µM as suggested by Mica *et al* [174]. Also we tried to IP all phosphorylated proteins from neutrophils by using 4G10 polyclonal antibody and immunoblot (IB) with Fer antibody (4G10 IP → Fer IP). After working through these issues we were able to IP Fer (figure 5.1). Fer controls for these experiments were used 3T3 mouse fibroblast cell line. We were able to successfully IP Fer protein from those cells reproducibility. Fer protein is very stable in 3T3 cells and can be detected up to 4 months in lysates stored at 4^oC. The conditions for figure 5.1 are detailed here.

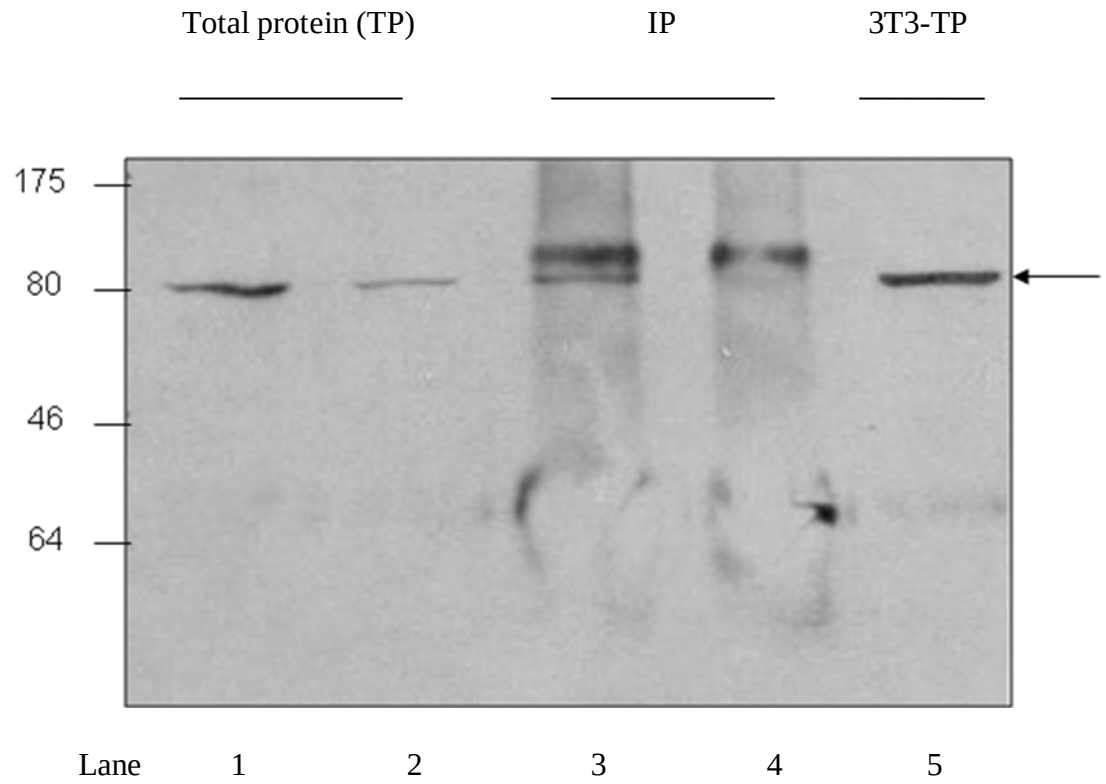
Conditions for figure 5.1:

WT and $Fer^{DR/DR}$ neutrophils (10 million cells per condition) were treated with DFP (Diisopropylfluoro-phosphate, Calbiochem) 1mM for 5 minutes at room temperature, and lysed with neutrophil lysis buffer (1 % NP-40, 20 mM Tric-HCl,137 mM NaCl, 1 mM

EDTA, 2 mM Na_3VO_4, 10 µg/ml aprotinin/leupeptin, 2 mM PMSF, and 1 mM pepstatin). 50 µl of gammabind sepharose beads (GE Healthcare) are added to each condition and incubated for 30 minutes on rotator at 4^oC (pre-clearing procedure). Next, 20 µl of Fer antibody (SantaCruz) plus the samples were incubated for 2 hours on rotator at 4^oC, then 50 µl of gammabind sepharose beads was added and incubated for extra 1 hour. The immune complex (in the beads) was washed three times with lysis buffer plus protease inhibitors (2 mM Na_3VO_4, 10 µg/ml aprotinin/leupeptin, 2 mM PMSF, and 1 mM pepstatin), and 30 µl of 2xSDS (Sigma) was added to the beads and boiled for 7 minutes. The gel run overnight at 1000v, then transferred to nitrocellulose membrane and blocked with 2 % BSA for 1 hour. Then, the blot was incubated with Fer antibody (1/1000 dilution) overnight at 4^oC and then incubated with secondary anti-rabbit-HRP conjugated antibody (1/10,000 dilution) for 1 hour. 3T3 cells were used as a positive control. These cells were lysed with 1 % NP-40, 2 mM Na_3VO_4, 10 µg/ml A/L, 2 mM PMSF.

Although Fer is a 93 kDa protein, in this experiment using the molecular weight ladder from Biolabs the bands appears around 83 kDa and is equivalent to 93 kDa identified with pre-stained ladder purchased from Sigma. Although we were able to measure Fer protein by IP-IB technique, the level of Fer protein was never impressive. It should be noted that the higher band in the IP conditions is related to Fer antibody used because we can detect this higher band when we add the antibody to the beads alone in lysis buffer

Fer IP → Fer IB



Lane 1: total protein (TP) from wild type (WT) neutrophils (5 million cells).
Lane 2: TP from $Fer^{DR/DR}$ neutrophils (3 million cells).
Lane 3: Fer IP from WT neutrophils (10 million cells).
Lane 4: Fer IP from $Fer^{DR/DR}$ neutrophils (10 million cells).
Lane 5: TP from 3T3 fibroblast cell line.

Figure 5.1 Fer protein immunoprecipitation from bone-marrow derived neutrophils

WT and $Fer^{DR/DR}$ neutrophils we either directly lysed in boiling 2XSDS lysis buffer (for total protein) or immunoprecipitated (IP) with Fer antibody. The gel was loaded with lysates as indicated and incubated with Fer antibody (primary antibody) and anti-rabbit HRP-conjugated secondary antibody.

These results indicate that Fer protein is detected in WT neutrophils either by immunoblotting (IB) or IP technique (figure 5.1, lane 1 and 3 respectively). Fer protein is present at reduced steady state levels in $Fer^{DR/DR}$ neutrophils (lane 2 and 4) because the mutation in $Fer^{DR/DR}$ mice is kinase inactivation not gene knockout. Fer is detected in total lysate from 3T3 cells (positive control, lane 5).

5.1.3 Fer phosphorylation levels

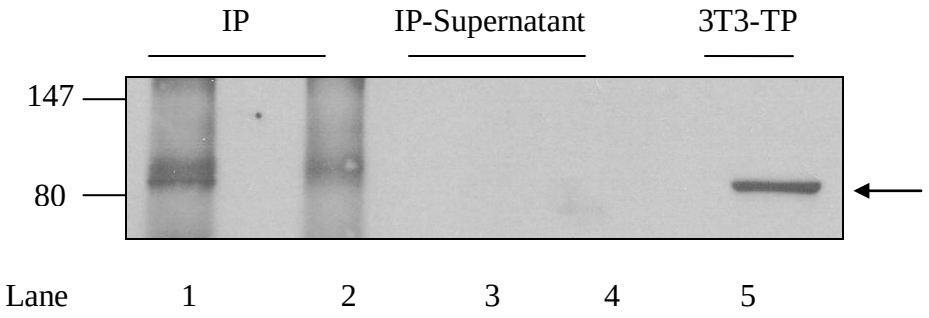
Next, we determined the degree of Fer phosphorylation by Fer IP followed by 4G10 IB. The methodology used in this blot was similar to figure 5.1. Since we thought that our protein is subjected to high risk of degradation during the IP process. We used another protease inhibitor cocktail [4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF, 416 mM), pepstatinA (6 mM), E-64 (5.6 mM), bestatin (16 mM), leupeptin (8 mM), and aprotinin (0.32 mM), Sigma] at Dr. Robbins suggestion. For these experiments cells were lysed with protease inhibitor cocktail in the lysis buffer. This is a high concentration for protease inhibitors cocktail since the product data sheet recommend 4 times lower concentration of those inhibitors. We used the recommended protease inhibitor concentrations [AEBSF (104 mM), pepstatinA (1.5 mM), E-64 (1.4 mM), bestatin (4 mM), leupeptin (2 mM), and aprotinin (0.08 mM)] for 3T3 cell lysis.

Figure 5.2 demonstrates Fer IP from bone-marrow derived neutrophils followed by Fer IB. Fer protein is detected in WT neutrophils (figure 5.2, lane 1) and in $Fer^{DR/DR}$ neutrophils in much reduced levels (lane 2). Fer protein was not detected in the supernatant from the IP lysate (lane 3 and 4). Fer is detected in total lysate from 3T3

cells (positive control, lane 5). These data suggest that Fer protein is detected in neutrophils by the IP technique using the protease inhibitor cocktail.

Next, we followed Fer IP with 4G10 immunoblot (IB) (figure 5.3) to show increased Fer phosphorylation in neutrophils in response to WKYMVm (1 μM) stimulation. Figure 5.3 demonstrates Fer IP from bone-marrow derived neutrophils followed by 4G10 IB. Fer phosphorylation was not detected in un-stimulated WT or $Fer^{DR/DR}$ neutrophils (figure 5.3, lane 1 and 3 respectively). Enhanced Fer phosphorylation was detected in WT neutrophils upon stimulation with WKYMVm (lane 2) but not in $Fer^{DR/DR}$ neutrophils (lane 4). Lane 5 represents total phosphorylated protein in 3T3 cell lysate. These data illustrate Fer phosphorylation in WT (but not in $Fer^{DR/DR}$) neutrophils in response to WKYMVm although low levels of protein were observed. Figure 5.3 is representative of 3 blots.

Fer IP → Fer IB

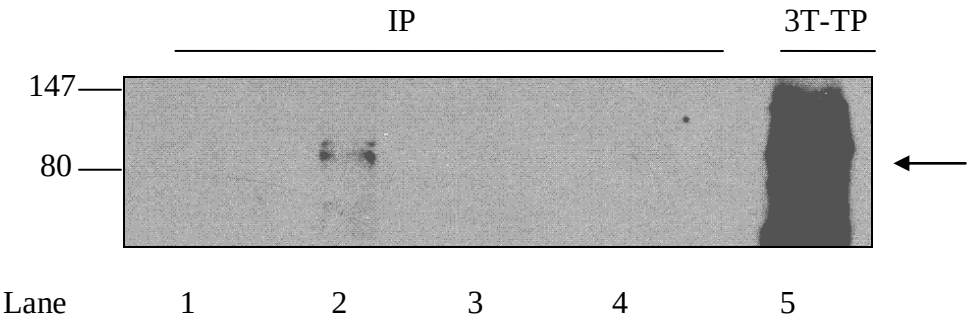


Lane 1: Fer IP from WT neutrophils.
Lane 2: Fer IP from $Fer^{DR/DR}$ neutrophils.
Lane 3: supernatant from WT neutrophils.
Lane 4: supernatant from $Fer^{DR/DR}$ neutrophils.
Lane 5: total protein from 3T3 fibroblast cell line.

Figure 5.2 Fer protein can be immunoprecipitated in neutrophils with the cocktail protease inhibitors from Sigma

New protease inhibitor cocktail was used to determine Fer expression in neutrophils by IP and IB technique. Fer protein was IP from WT or $Fer^{DR/DR}$ neutrophils. The gel was loaded with lysates as indicated and incubated with Fer antibody (primary antibody) and anti-rabbit HRP-conjugated secondary antibody.

Fer IP → 4G10 IB



Lane 1: WT neutrophils un-stimulated.
Lane 2: WT, stimulated with WKYMVm, 5 min (1µM).
Lane 3: $Fer^{DR/DR}$ neutrophils un-stimulated.
Lane 4: $Fer^{DR/DR}$, stimulated with WKYMVm, 5 min (1µM).
Lane 5: total protein from 3T3 fibroblast cell line.

Figure 5.3 Enhanced Fer phosphorylation upon WKYMVm stimulation in neutrophils using the cocktail protease inhibitors from Sigma

New protease inhibitor cocktail was used to determine Fer phosphorylation in neutrophils by IP and IB technique. Fer protein was IP from WT or $Fer^{DR/DR}$ neutrophils. The gel was loaded with lysates as indicated and incubated with 4G10 monoclonal antibody (primary antibody) and anti-mouse HRP-conjugated secondary antibody.

5.1.4 Can neutrophils affect Fer stability in 3T3 cell line?

We think that the difficulty to IP Fer protein in neutrophils comes from various issues; first, our protein is not stable due to high degree of proteolytic activity in neutrophils compared to other cells such as 3T3 cell line. Second, we might need to increase the number of neutrophils to start with to IP more than 10 million cells per condition. To address the first issue, we mixed neutrophils with 3T3 cell line which express Fer protein in high amounts to determine if the presence of neutrophils would affect the detected Fer protein levels. Figure 5.4 demonstrates Fer IP-Fer IB of 3T3 cells with or without neutrophils (lane 1 and 2). Fer protein could be measured in both conditions suggesting that the presence of neutrophils does not affect Fer protein levels in 3T3 cells. Even when neutrophils are activated with WKYMVm (lane 3) and mixed with 3T3 cells Fer protein can be still detected. Lane 4 represents total lysate from 3T3 cells (positive control).

These results may suggest that other factors contribute to our inability to detect Fer in neutrophils (low level of expression, instability, or conformational changes). It is also possible that the amount of Fer in 3T3 cells is very high which is not affected in the presence of neutrophils. Increasing the amounts of neutrophils may reduce Fer levels in 3T3 cells if neutrophil proteolytic activity is an issue.

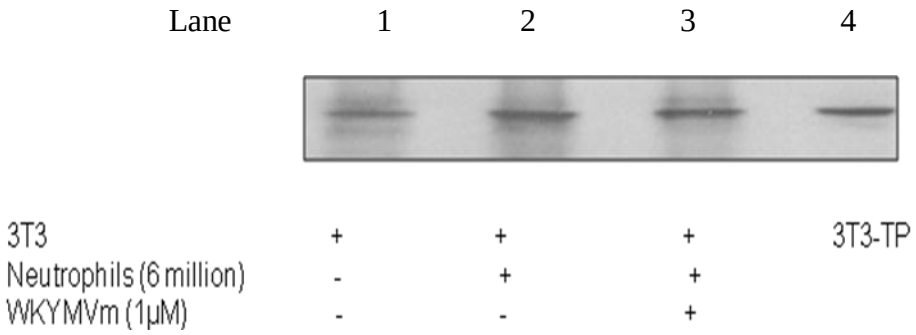


Figure 5.4 Fer expression is preserved in 3T3 cells in the presence of neutrophil lysate

Neutrophils (6 million cells) were either un-stimulated or stimulated with WKYMVm (1µM) for 5 minutes at 37^{o}C and mixed with 3T3 cells. Fer expression was determined by immunoprecipitation (IP) of Fer protein and immunoblot (IB) with Fer antibody. 3T3 total protein was used as a positive control for the experiment, n=2.

5.1.5 Fer IP from human neutrophils

We addressed the first issue as discussed previously. Moreover, the other issue in our experiments may be the number of neutrophils we use per condition. Other investigators used 40-80 million of peripheral blood human neutrophils per condition to IP different proteins [172, 173] and we are using a maximum of 10 million bone marrow derived neutrophils from mice per condition. It is not practical to use more than this number of neutrophils in our case, since we need around 3 mice to isolate 10 million cells which is suitable for one condition only, so we need to use approximately 24 mice to isolate 80 million cells for one IP condition. To overcome this issue, peripheral blood neutrophils were obtained from healthy volunteers with the help of Dr Patel`s lab. Fer has not been previously identified in human neutrophils, however using human neutrophils we will be able to use larger numbers of cells to increase our chances of measuring expression. The conditions used are as follows.

Conditions for figure 5.5

Human peripheral blood neutrophils ($43x10^6$ cells per condition as suggested by Dr Naccache, personal communication) were isolated and treated with DFP (1mM) at RT for 5 minutes. Cells were either un-stimulated (US) or stimulated with WKYMVm (1µM) for 2, or 5 minutes. After stimulation, cells were lysed with NP-40 lysis buffer plus cocktail protease inhibitors (same concentration for neutrophils lysis in figure 5.3, Sigma). After that, 50 µl beads were incubated with cells on rotator for 30 min at 4^oC, and then centrifuged. 20 µl of Fer antibody (Santa Cruz) was added to the cell lysate and incubated for 2 hours on rotator. After that, 50 µl beads were added and incubated for extra 1 hour. The immune complex (pellet) was washed 3 times with NP-40 lysis buffer

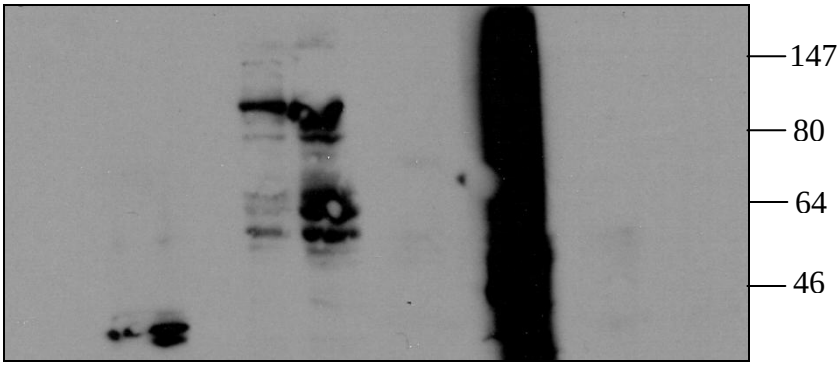
plus cocktail protease inhibitors. Then, 120 µl of 2xSDS buffer was added to cell pellet and boiled for 7 minutes. After gel transfer to nitrocellulose membrane, the membrane was blocked with 2 % BSA for 1 hour and incubated with 4G10 monoclonal antibody (1/1000 dilution) in 2 % BSA overnight at 4^{o}C. The membrane was washed and incubated with anti mouse HRP-conjugated secondary antibody (1/10000 dilution) for 1 hour and exposed to super signal ECL and developed using Kodak film. The membrane was stripped and re-probed with Fer antibody (1/1000 dilution) overnight at 4^{o}C in 2 % BSA, washed, and incubated with anti rabbit HRP-conjugated secondary antibody (1/10000 dilution) for 1 hour. The membrane was exposed to ECL and developed.

Fer protein was not detected in human peripheral blood neutrophils either un-stimulated or WKYMVm-stimulated for 2 and 5 minutes (figure 5.5, lane 1, 2, and 4). Fer expression was not detected in the supernatant from those IP conditions (lanes 7-9). Total phosphorylated proteins in neutrophil and 3T3 cells lysate was detected upon immunoblotting with 4G10 antibody (lane 3, and 5-6 respectively). When the blot was striped and re-probed with Fer antibody, Fer band is evident in 3T3 cells lysate only.

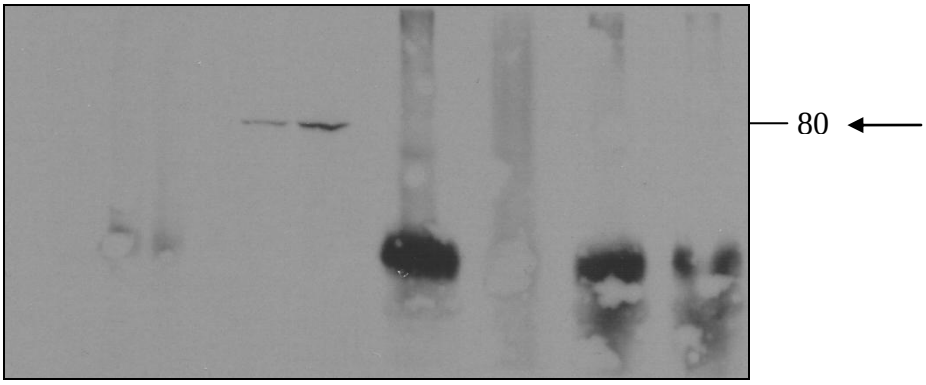
These data may indicate that Fer is expressed in very low levels, is unstable, or there is an issue with Fer protein conformation and accessibility to antibody in neutrophils.

(A) Fer IP → 4G10IB

IP-Supernatant 3T3-TP IP TP IP



(B) Fer IP → Fer IB (the blot was stripped and re-probed with Fer antibody)



9 8 7 6 5 4 3 2 1 Lane

Lane 1: human peripheral blood neutrophils (43 million cells) un-stimulated.
Lane 2: human neutrophils stimulated with WKYMVm (1µM) for 2 minutes at 37°C.
Lane 3: total protein from human neutrophils (43 million cells).
Lane 4: human stimulated with WKYMVm (1µM) for 5 minutes at 37°C.
Lane 5 and 6: 3T3 fibroblast cell line.
Lane 7-9: supernatant from lane 1-3 condition respectively.

Figure 5.5 Determining Fer phosphorylation in human peripheral blood neutrophils

Peripheral blood neutrophils we isolated and Fer expression and phosphorylation levels in response to WKYMVm stimulation was determined by the IP-IB technique.

5.1.6 Fer phosphorylation: alternative methodology

Since the immunoprecipitation (IP) technique to determine Fer expression and phosphorylation levels in neutrophils was not 100 % successful, we tried to determine Fer phosphorylation by using a newly developed phospho-Fer antibody. In this case, we directly lysed our cells after stimulation in boiling 2xSDS and direct immunoblotted (IB) to determine Fer phosphorylation with this phospho-Fer antibody without the immunoprecipitation (IP) step which may increase the risk of protein degradation. This antibody was kindly provided by Dr. Andrew Craig. The conditions and methodology used are described here.

Conditions for figure 5.6 and 5.7

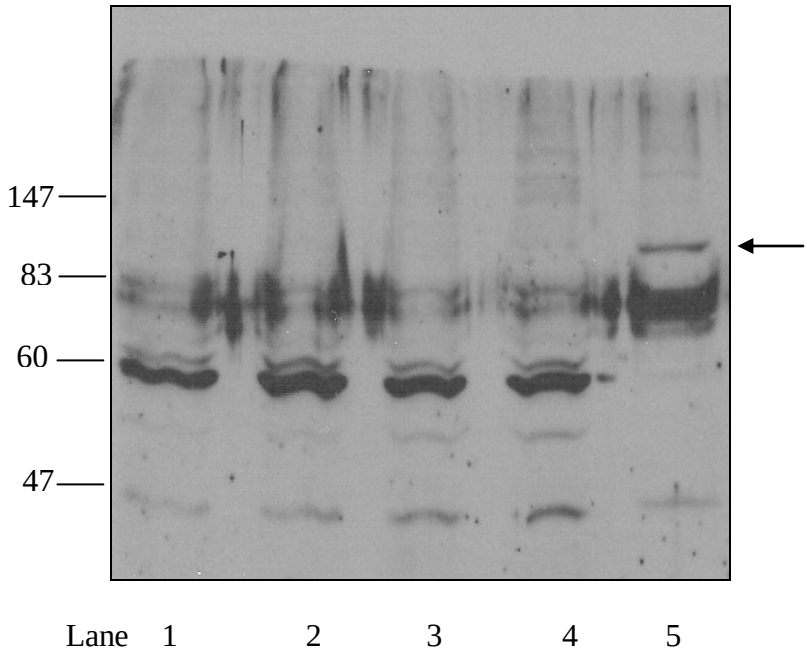
WT or $Fer^{DR/DR}$ neutrophils were either un-stimulated (US) or stimulated with WKYMVm (1 µM) for 5 minutes at 37^{o}C. After stimulation, cells were lysed with 2xSDS plus protease inhibitors cocktail [4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF, 416 mM), pepstatinA (6 mM), E-64 (5.6 mM), bestatin (16 mM), leupeptin (8 mM), and aprotinin (0.32 mM), Sigma] and boiled for 7 minutes. After protein transfer to nitrocellulose membrane, the membrane was blocked with 2 % BSA for 1 hour and incubated with phospho-Fer or affinity purified phospho-Fer antibody (1:500 dilutions, as suggested by Dr. Craig) in 2 % BSA overnight at 4^{o}C. Anti rabbit-HRP secondary antibody (1/10000 dilution) was incubated for 1 hour and the membrane was exposed to super signal ECL and developed. The membrane was stripped and re-probed with Fer antibody (1/1000 dilution) (Santa Cruz) overnight at 4^{o}C.

As shown in figure 5.6, the phosphorylated levels of Fer was not detected in bone-marrow derived WT or $Fer^{DR/DR}$ mouse neutrophils; un-stimulated or WKYMVm-

stimulated by direct IB using the phospho-Fer antibody (lanes 1-4). However, we were able to detect a band at the right molecular weight size for phosphorylated Fer in 3T3 cells (lane 5).

Dr. Craig affinity purified the antibody to improve specificity and reduce non specific binding. Unfortunately using this affinity purified phospho-Fer antibody, we were still unable to obtain clear blots (figure 5.7). As shown in figure 5.7, phosphorylated levels for Fer was not detected in WT or $Fer^{DR/DR}$ neutrophils either un-stimulated or WKYMVm-stimulated (lanes 1-4). In addition, no bands were detected in the 3T3 cells for phosphorylated Fer. By stripping and re-probing the blot with Fer antibody, Fer band can be detected in 3T3 cells (lane 5 and 6, lower figure).

Dr. Craig is in the process of improving his phospho-Fer antibody which might help us with future experiments to determine Fer phosphorylation levels in neutrophils by this direct immunoblotting (IB) technique.

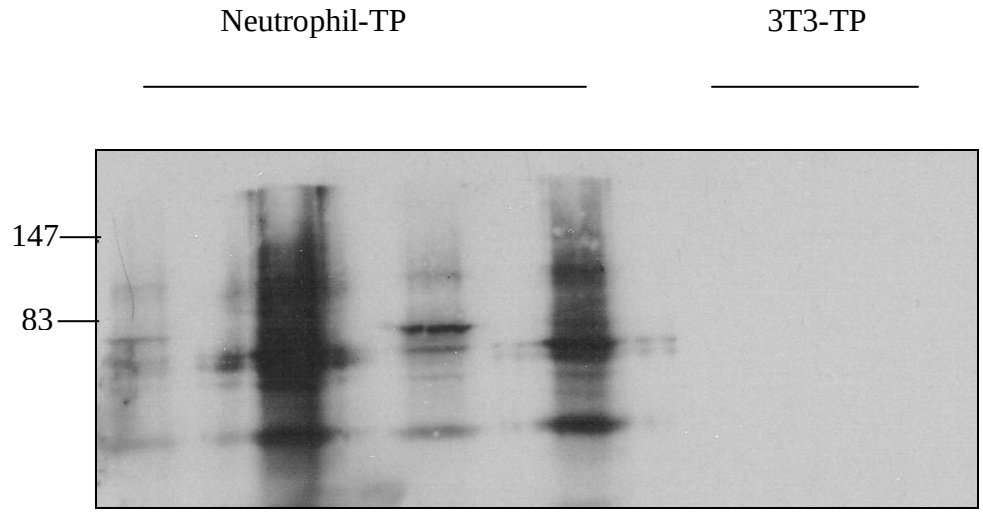


Lane 1: WT neutrophils un-stimulated (10 million cells).
Lane 2: WT neutrophils stimulated with WKYMVm (1µM, 5 min).
Lane 3: $Fer^{DR/DR}$ neutrophils un-stimulated.
Lane 4: $Fer^{DR/DR}$ neutrophils stimulated with WKYMVm (1µM, 5 min).
Lane 5: total protein from 3T3 fibroblast cell line.

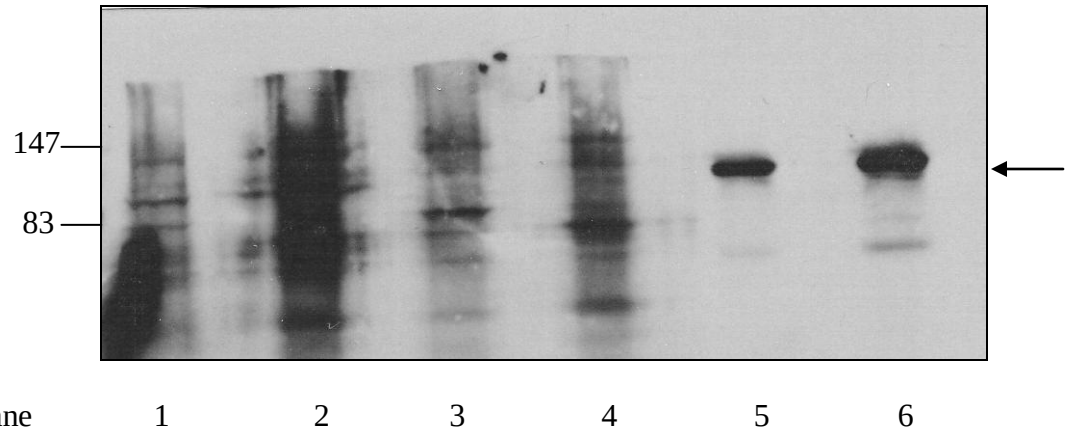
Figure 5.6 Determining Fer phosphorylation by direct IB technique using phospho-Fer antibody

Neutrophils were either un-stimulated or stimulated with WKYMVm and directly lysed with 2XSDS buffer with protease inhibitors. Fer phosphorylation levels in neutrophils were determined by direct IB with phospho Fer antibody. 3T3 cells were used as positive control.

Phospho-Fer antibody (affinity purified)



The blot was stripped and re-probed with Fer antibody



Lane 1: WT neutrophils (5 million cells) un-stimulated.
Lane 2: WT neutrophils stimulated with WKYMVm (1µM) for 5 minutes.
Lane 3: $Fer^{DR/DR}$ neutrophils un-stimulated.
Lane 4: $Fer^{DR/DR}$ neutrophils stimulated with WKYMVm (1µM) for 5.
Lane 5 and 6: total protein from 3T3 cell line.

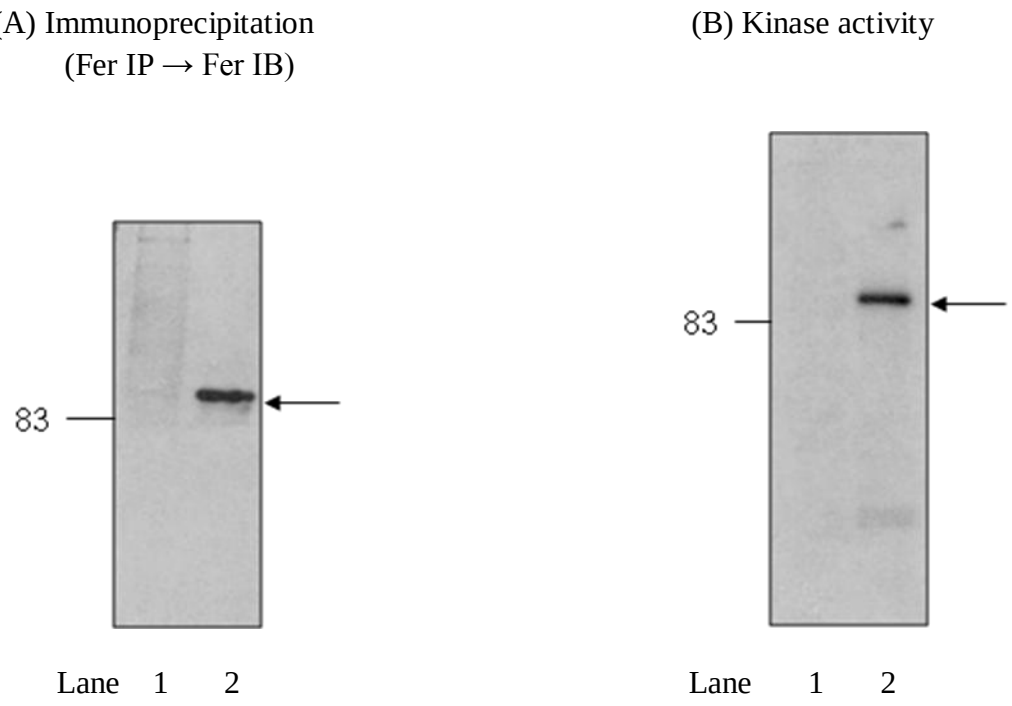
Figure 5.7 Determining Fer phosphorylation by direct IB using affinity purified phospho-Fer antibody

Neutrophils were either un-stimulated or stimulated with WKYMVm and directly lysed with 2XSDS buffer with protease inhibitors. Fer phosphorylation levels in neutrophils were determined by direct IB with affinity purified phospho Fer antibody. The blot was striped and re-probed with Fer antibody to detect Fer expression.

5.1.7 Fer kinase activity

When we had some success in Fer IP from neutrophils, we tried to determine Fer kinase activity in neutrophils in response to WKYMVm stimulation. 3T3 cell line was used as a positive control in each experiment. We performed Fer IP by using the same methodology discussed before, and then the immune complex was washed three times with lysis buffer plus protease inhibitors and one time with kinase reaction buffer. Kinase reaction was started by the addition of 10 μCi of γ ^{32}P-ATP with kinase reaction buffer (50 mM HEPES, PH=7.5, 10 mM $MgCl_2$, 1 mM $MnCl_2$) and incubated for 20 minutes at 37^{o}C. The reaction was stopped by the addition of 2X SDS lysis buffer and boiled for 5 minutes.

Figure 5.8 illustrates Fer IP and kinase activity in 3T3 cells. Fer expression is detected in 3T3 cells by IP technique as shown in figure 5.8-A, lane 2. Furthermore, Fer kinase activity (auto-phosphorylation) is detected in 3T3 cells (figure 5.8-B, lane 2). Pre-immune serum was used as negative control (lane 1). These results show that we were able to IP Fer in 3T3 cell line and show Fer kinase activity (auto-phosphorylation) of 3 separate experiments. However, we were not able to detect Fer activity in mouse neutrophils either auto-phosphorylation or phosphorylation of a downstream target; platelet endothelial cell adhesion molecule, pecam-1. We tried to measure Fer kinase activity in neutrophils for several times with no success. The difficulty of Fer IP in neutrophils most likely contributing to this assay also.



Lane 1: 3T3 cell line, Pre-immune serum IP (negative control)

Lane 2: 3T3 cell line, Fer IP.

Figure 5.8 Fer kinase activity (auto-phosphorylation) can be detected in 3T3 cell line.

Fer protein was immunoprecipitated from 3T3 cells and used for IB to detect levels of expression (A), or for kinase reaction to detect its kinase activity (B). Pre-immune serum was used as negative control, n=3.

5.2 Summary

In this chapter we have demonstrated Fer protein is expressed in mouse neutrophils using immunoprecipitation (IP) and immunoblotting (IB) techniques. In addition, we have some evidence to support the notion that Fer is activated downstream of WKYMVm (fMLP peptide) stimulation. However, we encountered many technical problems that resulted in dramatic inconsistencies which puts a severe cautionary note on our conclusions. This may be an issue with instability or degradation of Fer protein in neutrophils and/or protein concentration (or cell number). We attempted to minimize the degree of protein instability in neutrophils by using different combinations and concentrations of protease inhibitors, using an irreversible protease inhibitor (Diisopropylfluoro-phosphate , PDF) before cell stimulation, using a proteasome inhibitor (PSI), and reducing the IP incubation time from overnight to 2 hours. Fer kinase may be expressed in low levels within neutrophils therefore we increased the bone marrow derived neutrophil number from 5 to 10 million cells per condition to increase protein levels, and used neutrophils isolated from human peripheral blood (43 million cells per condition). Other contributing factors may be the conformation and accessibility of Fer protein to the antibody. It should be noted that Fer protein was consistently successfully IP from 3T3 fibroblast cell line (positive control in each experiment), and Fer kinase activity (auto-phosphorylation) was measured in 3T3 cell line. Finally, we tried to determine Fer phosphorylation by immunoblotting (IB) technique to overcome the problems we faced in the IP technique by using a newly developed phospho-Fer antibody provided by Dr. Andrew Craig. We were unable to

determine Fer phosphorylation in neutrophils by using this antibody although we still can detect Fer in 3T3 cell line.

One possibility is that Fer null bone marrow derived neutrophils are more mature than bone marrow derived wild type (WT) neutrophils perhaps in Fer null mice there is increased production of cytokines (eg GM-CSF) which allows them to mature. However, our analysis of bone marrow derived neutrophil maturity between WT and Fer null neutrophils using cytospin analysis did not identify differences in maturity levels (49 % band cells and 51 % mature cells in WT mice vs. 52 % band cells and 48 % mature cells in Fer null mice). Furthermore, chemotaxis (chapter 4) was similar in WT and Fer null bone marrow derived neutrophils in response to intermediate chemoattractants.

Chapter Six: **THE ROLE OF FER KINASE IN NEUTROPHIL PROPERTIES (SUPEROXIDE PRODUCTION, BACTERIAL KILLING, AND APOPTOSIS)**

6.1 Introduction

In chapter 4 we examined the role of Fer kinase in neutrophil chemotaxis demonstrating that in the absence of Fer kinase greater numbers of neutrophils are recruited toward certain chemoattractants *in vitro*. In this chapter we will investigate whether this PTK plays a role in modulating other functions of neutrophils which contribute to local tissue damage or resolution at the site of inflammation. We will first investigate if Fer PTK has a role in superoxide production. The generation of superoxide contributes to inflammation by killing the invading microorganism but if this process is not controlled it can lead to tissue damage and chronic inflammatory response [100]. Next we will examine if Fer plays a role in the ability of neutrophils to kill bacteria, and finally to examine if Fer plays a role in neutrophil apoptosis.

6.2 Determining the role of Fer kinase in superoxide production from neutrophils

6.2.1 Introduction

The bactericidal mechanism for neutrophils consists of phagocytosis of a pathogen, generation of anti-microbial products through oxygen dependent and oxygen independent mechanisms and the release of bactericidal proteins to the phagosome, which results in the killing of the invading organism. This process can also result in tissue damage from excessive release of these products from neutrophils. The oxygen dependent mechanism of neutrophil killing involves the transformation of molecular oxygen into highly reactive oxygen intermediates like hydroxide (OH), and hydrogen peroxide (H_2O_2), which are derivatives of superoxide generated by phagocyte nicotinamide adenine dinucleotide

phosphate (NADPH) oxidase [147, 175, 176]. In resting cells, the NADPH oxidase complex is disassembled with its components segregated into different parts of the cell. Upon stimulation, the cytosolic $p47^{phox}$ and $p67^{phox}$ proteins translocate to associate with the membrane-localized $p91^{phox}$ and $p22^{phox}$. The hematopoietic-specific GTPase Rac2 is also essential component of the oxidase machinery [175, 176].

6.2.2 Fer deficiency accelerates the rate of superoxide production in response to fMLP stimulation

Oxidative burst activity is a very potent mechanism for the neutrophils to kill invading microorganisms. The aim of these experiments was to determine the rate of superoxide production using the cytochrome c reduction assay described in methods. As shown in figure 6.1 WT neutrophils can produce superoxide 5 minutes after stimulation with fMLP. This time frame is needed to assemble the NADPH oxidase system at the plasma membrane [176]. Figure 6.1 illustrates the change in absorbance of cytochrome c as an indication of superoxide production. A gradual increase in absorbance is noted in WT neutrophils with the maximum rate of superoxide production noted from 5-10 minutes after fMLP stimulation. This response is abolished by treating the cells with superoxide dismutase (SOD) which is an antioxidant that neutralizes any superoxide anion produced. No change in absorbance was noted with neutrophils without fMLP stimulation or with vehicle alone (RPMI or PBS) (data not shown). In contrast, an accelerated rate of superoxide production was observed in $Fer^{DR/DR}$ neutrophils over the first 5 minutes period post fMLP challenge. The data was analyzed to determine the rate of production of superoxide over 0-5 min period post fMLP challenge and to calculate the

total amount of superoxide generated. Figure 6.2 illustrate an enhanced rate of production of superoxide in Fer mutant compared to WT neutrophils. The rate of superoxide production in both WT and $Fer^{DR/DR}$ neutrophils was similar over the 5-10 min period. These data suggest that Fer regulates superoxide production early in the process, possibly through an interaction with one or more components of the NADPH oxidase system. In the absence of Fer kinase, the NADPH oxidase system may be more rapidly assembled on the plasma membrane to generate superoxide. Overall the total amount of superoxide produced was not different between WT and $Fer^{DR/DR}$ neutrophils (figure 6.2).

In WT neutrophils, GM-CSF which is a well known priming agent for superoxide production [177] accelerated the rate of superoxide production (figure 6.2-A) to a similar level as observed in a Fer mutant unprimed neutrophils (figure 6.2-A). In addition, priming (100 ng/ml, 30 min, $37^{o}C$) significantly enhanced the total amount of superoxide anion produced (figure 6.2-B). Interestingly, in Fer mutant neutrophils, GM-CSF priming did not accelerate the rate of production further over the first five minutes period but enhanced the total amount of superoxide produced to similar levels compared to primed WT neutrophils (figure 6.2-A and –B).

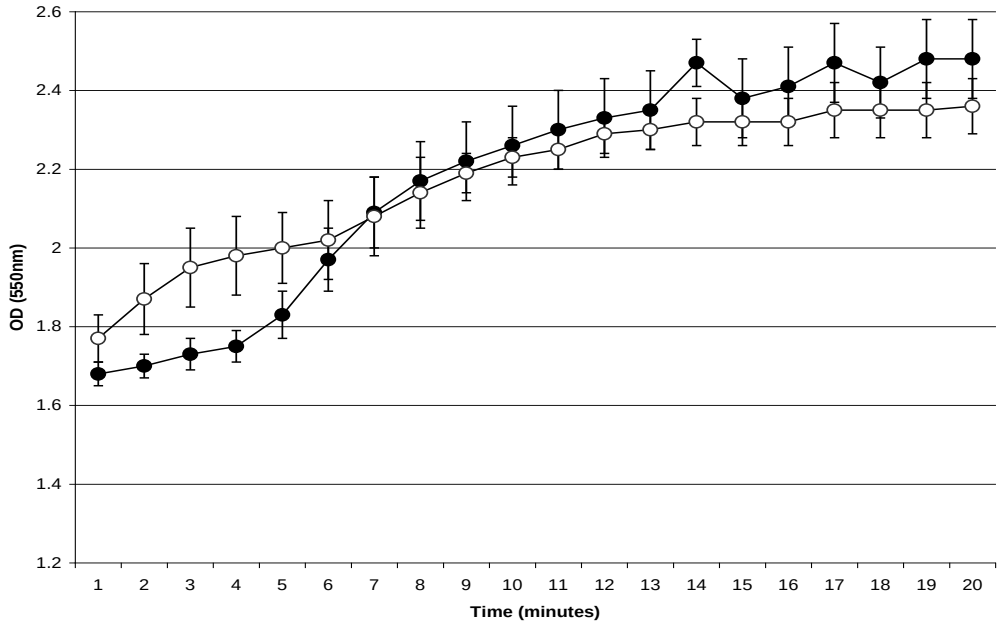
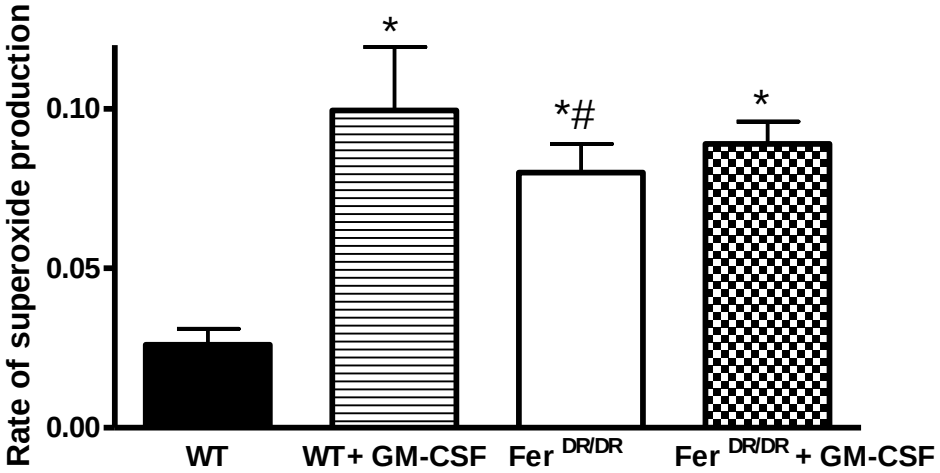


Figure 6.1 Fer deficiency accelerates neutrophil superoxide production in response to fMLP stimulation

Superoxide production as indicated by the change in absorbance in WT (solid circles) and $Fer^{DR/DR}$ (open circles) neutrophils in response to fMLP stimulation was determined using the cytochrome c reduction assay, n=4-7. Data are expressed as mean ± SEM. No change in absorbance was noted in neutrophils incubated with cytochrome c alone, or with SOD after fMLP stimulation.

(A)



(B)

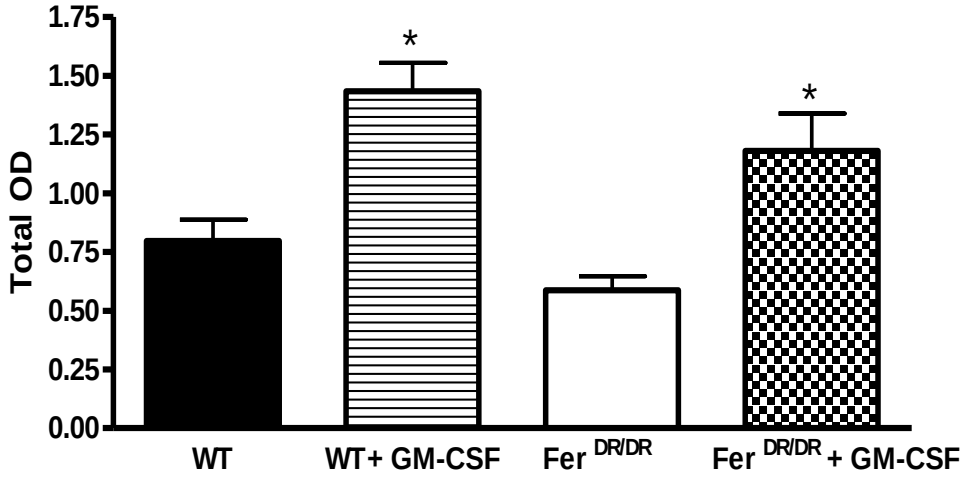


Figure 6.2 GM-CSF priming enhances the rate and the total amount of superoxide production in WT neutrophils and does not further enhance the rate of production in Fer$^{DR/DR}$ neutrophils.

(A) Illustrates the rate of superoxide production as measured by cytochrome c reduction assay from 0-5 minutes post challenge with fMLP, and (B) illustrates the total amount of superoxide production over 20 minutes. WT untreated neutrophils (solid bars), GMCSF-primed WT neutrophils (hatched bars), Fer$^{DR/DR}$ untreated neutrophils (open bars), and GMCSF-primed Fer$^{DR/DR}$ neutrophils (squared bars). Data are expressed as mean ± SEM, n= 4-7, * significant differences from unprimed neutrophils, # significant differences from WT unprimed neutrophils

Interestingly, there is some evidence suggesting an interaction between Fer and Rac. Fer has been shown to play a role in cortactin phosphorylation and lamellipodia formation [25, 37]. Over-expression of Fer in COS-7 cells induced lamellipodia formation and actin polymerization at the plasma membrane, and this response was blocked by coexpression with a dominant negative form of Rac suggesting that Fer acts upstream of Rac [171]. Rac2 is one of the cytoplasmic components of the NADPH oxidase system. To determine if Fer modulates the rate of superoxide production through modulating Rac2 activity, Dr. Andrew Craig`s lab in Queen`s university measured the activity of Rac2 GTPase by using the Cdc42/Rac-interactive binding (CRIB) domain pull-down assay [178]. However, no activity for Rac2 was observed in response to fMLP stimulation (1 µM) at 0, 5, 10, and 15 minutes time points either in WT or $Fer^{DR/DR}$ neutrophils (5 million cells per condition).

These data suggest that Fer is involved in physiological regulation (inhibition) of superoxide production such that in its absence superoxide production is briefly accelerated. This may have an influence on inflammation if superoxide is produced too rapidly it may contribute to tissue damage. The regulation of superoxide flux is important within the inflammatory environment as the rate of superoxide production is important in the generation of toxic reactive nitrogen metabolites [179]. In addition, these data suggest that Fer is not involved in GM-CSF priming in neutrophils as no synergistic effect was observed in the rate of production or total superoxide output.

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6.3 Role of Fer kinase in neutrophil intracellular bacterial killing

Next, we investigated the role of Fer kinase in the intracellular bacterial killing capacity toward staphylococcus aureus strain 2406 [136, 180]. Neutrophils were isolated from WT and $Fer^{DR/DR}$ mice. In addition, Fps^{Kr}/Fer^{Dr} neutrophils were also studied for their intracellular bacterial killing capacity as described in the methods. Figure 6.3 demonstrates the intracellular killing capacity in the three groups of neutrophils toward the S. aureus strain by counting the ability of lysed neutrophil contents to form colony-forming units per millilitre (CFU/ml) which survived inside the neutrophils as an indication of bactericidal ability. In wild type neutrophils 223 ± 56 CFU were generated after 15 min incubation time. This level decrease over time indicating that more bacteria were killed by the neutrophils with longer incubation periods. In Fer mutant neutrophils 209 ± 79 CFU were generated and this levels was decreased over time to similar levels observed with WT neutrophils. In the compound mutant neutrophils lesser CFU were generated initially (133 ± 31) but this differences was not statistically significant from the other groups of neutrophils, and this level decreased over time to similar levels observed in WT and Fer mutant neutrophils. In the three groups of neutrophils, the intracellular killing capacity was enhanced with longer incubation time as reflected by less CFU/ml formed. No statistical difference in the bacterial killing ability between Fer kinase and Fps/Fer compound mutant neutrophils was noted. These data suggest that group IV PTK does not play a role in modulating intracellular killing capacity of neutrophils toward S. aureus strain.

An important point to mention here is that this assay does not measure the phagocytic ability of neutrophils which may vary between the groups of neutrophils studied. A number of studies have suggested a role for group IV PTK in cytoskeletal remodelling [10, 21, 25, 181] which is an important component for phagocytosis [182]. Although the initial time point (15 min) of CFU formed (figure 6.3) is a rough estimate for the amount of bacteria engulfed, this should be confirmed by a phagocytosis assay. Future experiments should be performed to determine if group IV PTK modulates neutrophil phagocytosis for this strain of bacteria, and compare it with the degree of its intracellular killing which will highlight the role of group IV PTK in this function of neutrophils.

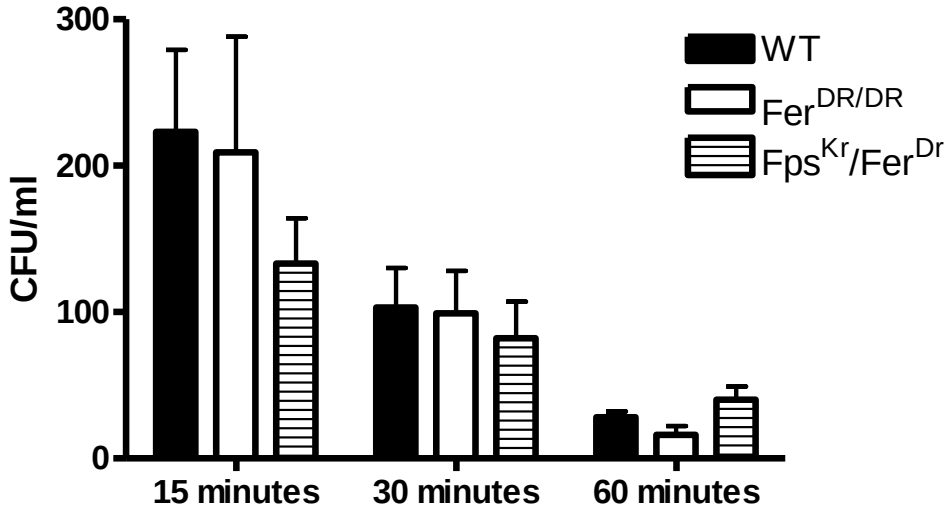


Figure 6.3 Intracellular bacterial killing capacities of WT, $Fer^{DR/DR}$, and Fps^{Kr}/Fer^{Dr} neutrophils toward S. aureus strain 2406.

The rate of intracellular killing of S. aureus strain was determined in WT (solid bars, n=7), $Fer^{DR/DR}$ (open bars, n=5), and Fps^{Kr}/Fer^{Dr} (hatched bars, n=6) neutrophils at three time points. S. aureus 2406 bacterial cultures were mixed with neutrophils and incubated at 37°C/5% CO_2 incubator for 10 minutes. After 10 minutes, gentamycin (400 µg/ml) was added to kill any extracellular bacteria. Neutrophils were lysed and the level CFU/ml remaining in neutrophils was counted. Data are expressed as mean ± SEM.

6.4 Determining the role of Fer kinase in neutrophil apoptosis

6.4.1 Introduction

In response to inflammatory stimuli, neutrophils migrate to the inflammatory site in response to different chemoattractants to fight and clear the infectious or inflammatory agent. After fulfilling their job, neutrophils become apoptotic and they are recognized, engulfed, and cleared by macrophages without releasing reactive oxygen species (ROS) or catalase from them to bring the body into resolution and prevent further tissue damage [183]. Neutrophil apoptosis is an essential mechanism to control the duration and the intensity of the inflammatory response and therefore the extent of neutrophil mediated tissue injury. In addition, apoptosis is essential for controlling neutrophil homeostasis. Accelerated neutrophil death can lead to neutropenia and increase the risk of bacterial or fungal infection. On the other hand, delayed neutrophil apoptosis leads to a chronic inflammatory conditions and tissue injury [184]. Disregulated neutrophil apoptosis has been implicated in different pathological conditions such as systemic inflammatory response syndrome (SIRS) [185, 186], rheumatoid arthritis [187, 188], and inflammatory bowel disease (IBD) [189]. Different intracellular and extracellular mediators control the rate of neutrophil apoptosis. Families of cystein proteases called caspases play a critical role in apoptosis. They can be activated via extrinsic death receptor pathway mediated by tumour necrosis factor receptor pathway (TNFR) and Fas, or via intrinsic mitochondria-mediated pathway [190, 191]. Calpains which are a family of non-caspase cysteine proteases also modulate the rate of neutrophil apoptosis [192]. Various extrinsic

inflammatory mediators also modulate neutrophil apoptosis such as LPS [193], type I and II interferons, GM-CSF, G-CSF, fMLP, C5a, LTB_4, and TNF [194-197].

A well established early feature of apoptosis is the externalization of the lipid phosphatidyl serine (PS) from the inner to the outer plasma membrane. Annexin V is a protein that specifically binds PS, which is used in conjugation with a nuclear dye; 7-Amino-Actinomycin-D (7AAD) that measure membrane integrity, a distinction between early and late apoptotic cells can be made. After FACS measurements, cells can be categorized as follows: 1- live/intact cells (Annexin V negative, 7AAD negative), 2- early apoptotic cells (Annexin V positive, 7AAD negative), 3- late apoptotic/necrotic cells (Annexin V positive, 7AAD positive).

In our IBD models we observed an increased number of neutrophils in the tissue of Fer null mice compared with WT animals. This difference may be due to enhanced chemotaxis and/or delayed apoptosis. Figure 6.4 illustrates the percentage of neutrophils which undergoing apoptosis in the absence of activation signals. Approximately 25 % of cells are in the early stages of apoptosis after 16 hours culture and around 50 % of cells are in the late stages of apoptosis. No statistical differences were observed in the rate of spontaneous apoptosis between WT and Fer null neutrophils.

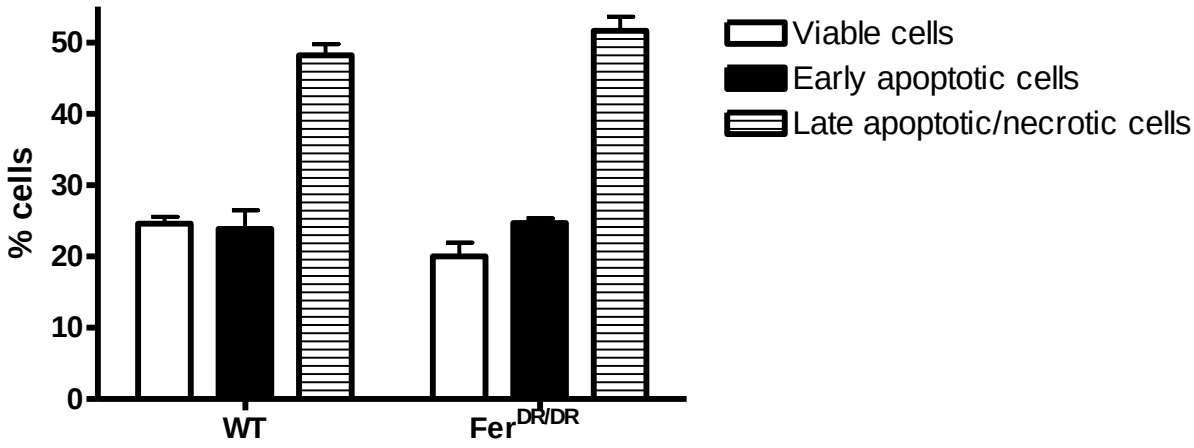


Figure 6.4 Fer kinase does not modulate the rate of spontaneous neutrophil apoptosis.

WT and $Fer^{DR/DR}$ neutrophils were cultured in RPMI plus 20 % FBS for 16 hours and then the rate of spontaneous apoptosis was determined by FACS analysis. Viable cells (open bars), early apoptotic cells (solid bars), and late apoptotic cells (hatched bars) were determined by Annexin-V and 7-AAD labelling. Data are expressed as mean ± SEM, n= 3.

Next, apoptosis was induced by stimulating WT and $Fer^{DR/DR}$ neutrophils with different doses of WKYMVm and observing 60 minutes later. As shown in figure 6.5, in WT neutrophils WKYMVm stimulation enhanced the percentage of early apoptotic cells by 9-11 % compared to unstimulated (US) neutrophils. In $Fer^{DR/DR}$ neutrophils, WKYMVm enhanced the percentage of early apoptotic cells by 12-17 % compared to US neutrophils. There was no statistically significant difference between WT and Fer null WKYMVm stimulated apoptosis. WKYMVm stimulation reduced the percentage of viable cells 5-9 % in WT and 9-13 % in Fer null neutrophils. The percentage of necrotic cells was not modulated by WKYMVm stimulation in both groups of neutrophils. These data suggest that Fer kinase does not modulate spontaneous or WKYMVm-induced neutrophil apoptosis.

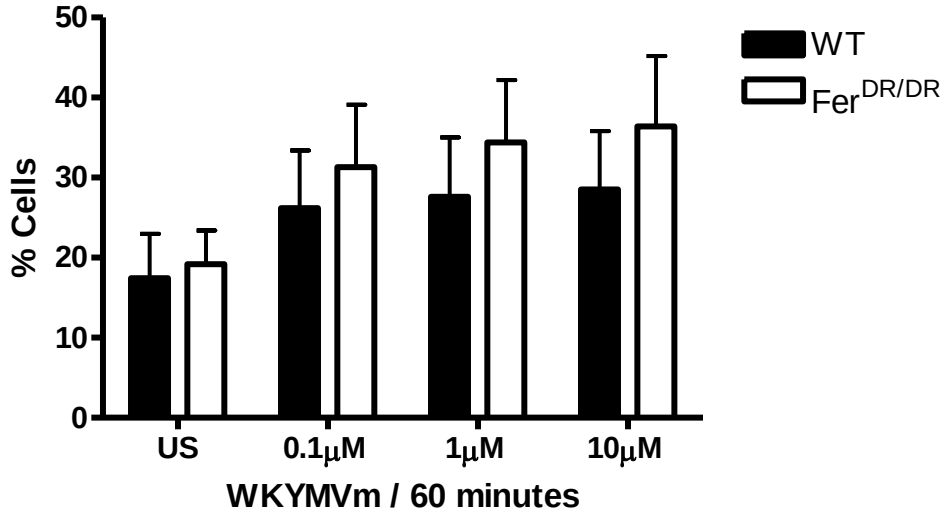


Figure 6.5 Fer kinase does not modulate the rate of WKYMVm-induced neutrophil apoptosis.

WT (solid bars) and $Fer^{DR/DR}$ (open bars) neutrophils were either unstimulated (US) or stimulated with different doses of WKYMVm (0.1-10 μM) and the rate of WKYMVm induced early apoptosis was determined by FACS analysis. Data are expressed as mean ± SEM, n= 5.

6.5 Summary

In this chapter, we examined the role of Fer kinase in various neutrophil properties. We showed evidence for the role of Fer in modulating the rate of superoxide production in response to fMLP stimulation however no role for Fer kinase in modulating neutrophil intracellular bacterial killing and apoptosis was observed. The enhanced colitis severity observed in $Fer^{DR/DR}$ mice *in vivo* was paralleled by enhanced neutrophil recruitment to the inflammatory site. Fer kinase plays a role in modulating (damping) neutrophil chemotaxis and the signalling pathways used in this process toward certain chemoattractants *in vitro* and the rate of superoxide production toward the bacterial peptide fMLP. These data may explain how Fer kinase modulates colitis severity *in vivo* which results in enhanced certain neutrophil functions in its absence which can lead to tissue damage.

Chapter Seven: **DISCUSSION**

Inflammatory bowel disease (IBD) is a chronic inflammatory condition of the GI tract which affects a large portion of people worldwide. The current medical therapies available to treat colitis do not provide a cure, have many limitations, and are associated with significant side effects [106-108]. Clearly, there is a need to define new therapeutic targets to offer alternatives to treat these chronic inflammatory conditions. There is good evidence for an important role of innate immunity in the pathogenesis of IBD [53]. In recent years protein kinase inhibition has been targeted as novel treatment options in various pathological conditions [110-112] Interestingly, previous work from our lab and others has linked Group IV PTK's with the innate immune response in the gut [40, 42]. For example, endotoxin challenge in Fer kinase null mice induced an exacerbated influx of neutrophils and epithelial barrier dysfunction. Therefore in this thesis we hypothesized that the group IV PTK's, specifically Fer kinase, may play a role in models of IBD through modulating neutrophil chemotaxis to the gut and potentially neutrophil function.

Our data demonstrate that the intestinal inflammatory response which develops in two models of IBD is significantly more severe in the absence of Fer kinase or both Fer and Fps/Fes. Colitis induced in WT mice by a single intra-rectal injection of TNBS developed rapidly over days 0- 3 post colitis induction and had resolved macroscopically and histologically by day 10. Fer mutant ($Fer^{DR/DR}$) mice experienced enhanced and prolonged colitis compared to WT mice and this was associated with enhanced neutrophil recruitment to the colon which was measured by MPO assay. When compound deficient (Fps^{Kr}/Fer^{Dr}) mice were studied a similar level of colitis was observed over day 0-7 to that observed in Fer single mutant mice. These data illustrate that Fps/Fes does not compensate for the role of Fer kinase in this inflammatory response and suggests that

Fps/Fes may not contribute to the inflammatory response over this time period. However, a synergistic effect on the inflammatory outcome was observed macroscopically and histologically in compound mutants at later time points. Therefore Fps/Fes may contribute to limiting the inflammatory response throughout the period of observation, for example by controlling TNFα production [42] but the effect is masked at the early time points due to the severity of the inflammation. Neutrophil influx however was not significantly enhanced in the compound mutants over the single mutants at any time point studied suggesting that Fps/Fes does not affect neutrophil recruitment to the gut. Alternatively these data may point to a distinct role for Fps/Fes in the resolution of inflammation in the gut. Interestingly, a link between mediators which are involved in the resolution of inflammation (e.g. lipoxins, resolvins) and IBD has been made. For example, Mangino *et al* [198] showed that lipoxin levels in the colonic mucosa are significantly reduced in ulcerative colitis (UC) patients compared to controls. Furthermore, exogenous administration of resolvin and lipoxin A4 has been shown to reduce leukocyte infiltration and resolve inflammation in various animal models of IBD [199, 200]. These data suggest that a failure of the gut mucosa to produce adequate levels of mediators involved in resolution may contribute to chronic IBD. The possibility exists that Fps/Fes contributes to the resolution phase of intestinal inflammation by controlling the level of mediators such as lipoxins and resolvins. These observations are worth further investigation. Interestingly, a role for group IV PTK in modulating epithelial cell migration and wound closure has been identified which could contribute to a delay in mucosal healing in the mutant mice [32]. Overall our data illustrate that Group IV PTK's, specifically Fer kinase, can regulate intestinal inflammation, acting to control

or limit the inflammatory responses in the gut potentially through regulating neutrophil infiltration.

In addition, we examined the role of Fer kinase in a spontaneously model of IBD; the IL-$10^{-\backslash-}$ model. At 3 months of age, there was a significant increase in the macroscopic and histological inflammatory scores in IL-$10^{-\backslash-}$ compared to WT mice. IL-$10^{-\backslash-}$Fer$^{DR/DR}$ double deficient mice experienced significantly enhanced inflammatory responses compared to IL-$10^{-\backslash-}$ mice. Neutrophil recruitment to the colon was enhanced in IL-$10^{-\backslash-}$ compared to WT mice, and a similar increase was observed in IL-$10^{-\backslash-}$Fer$^{DR/DR}$ mice. These data suggest an important role for Fer kinase in this chronic model of IBD.
At face value these data may suggest that Fer kinase does not contribute to neutrophil infiltration in intestinal inflammation, however the colitis in this model is significantly different from that observed in the TNBS-induced model. In the IL-$10^{-/-}$ mice, we are studying a well established chronic process compared with the rapid development of a relatively acute TNBS-induced inflammatory response. In the IL-$10^{-/-}$ model, Fer kinase may contribute more to the function of the neutrophils and/or through modulating other immune cell functions.

A surprising and interesting finding from the IL$10^{-/-}$Fer$^{DR/DR}$ mice is the development of an increased incidence of dysplasia and adenocarcinoma in the colon at 3 months of age. These data are somewhat surprising in light of previously published data on the role of Fer in other cancers. Tyrosine kinases (including Fer) can act as oncogenes when over-expressed or expressed as mutated forms of signalling molecules [10, 145, 201, 202]. Fer is highly expressed in different malignant cell lines and has been detected in high levels in malignant versus benign prostate tumours. Furthermore, down regulation

of Fer reduces the proliferation of prostate carcinoma cells and attenuates the development of prostate cancer [145]. Recent findings also suggest Fer is involved in the progression of breast cancer and plays a unique regulatory role during cell cycle progression in malignant cells [203]. All of these data support the role of Fer kinase as a potent proto-oncogene in different malignant cell lines. Our data, however, is the first study to demonstrate a role for Fer in cancer development *in vivo*. Using our *in vivo* model of chronic inflammation, we showed that the absence of Fer results in increased and accelerated cancer incidence. This may be due to the differences between the types of cancer studied and /or the environment in which cancer develops. Interestingly, our data from the *in vitro* studies in neutrophils highlight a role for this kinase in regulating PI3K activity in response to bacterial peptide. The PI3K pathway plays a very important role in cancer development and metastasis [204] and it is feasible that Fer kinase within the gut environment plays a role within other cell types, e.g. epithelial cells, in regulating the PI3K pathway to bacterial antigen. Alternatively the enhanced cancer phenotype may come from the increased severity of the colitis. A link between duration and severity of colitis and cancer development has been observed in epidemiological studies [146]. Our studies involved whole animal mutants; therefore it would be interesting to determine the contribution of Fer kinase within the non hematopoietic (e.g. epithelial cells) vs. hematopoietic (neutrophils) cell compartments to the development of cancer in this model. This might be done *in vivo* by generating chimeric mice from irradiated IL-$10^{-/-}$$Fer^{DR/DR}$ mice reconstituted with IL-$10^{-/-}$ bone marrow cells and vice versa.

Interestingly, the role of Fer kinase in controlling inflammatory responses is not universal. For example in the concanavalin-A (Con A) model of hepatitis Fer kinase did

not play a role in the severity of hepatitis 4 hours post Con-A administration in mice as measured by serum ALT levels (appendix C). Furthermore, neutrophil infiltration in the liver was not significantly different in Con A-treated WT or Fer null mice. These data suggest that Fer kinase may play a stimulus specific role during inflammation. When we examined Fer kinase in a model of septic peritonitis; the cecal ligation and puncture (CLP) model, a significant increase in the clinical score and neutrophil infiltration to the intestine and lung was observed in Fer null mice with CLP compared with the WT CLP mice (appendix B). These data may indicate that Fer kinase plays a role specifically in regulating inflammatory responses associated with bacterial antigen.

Interestingly, *in vitro* using the under agarose gel assay we identified a key role for Fer kinase in neutrophil chemotaxis towards bacterial antigen and complement factor 5a (end targets chemoattractants). We demonstrated that the enhanced chemotactic activity in the absence of Fer toward fMLP peptide was due to a role in modulation the signalling pathways used by neutrophils. Despite measuring Fer protein in neutrophils we were unable to consistently demonstrate Fer activation downstream of fMLP receptor activation. Another possibility is that Fer null neutrophils express increased fMLP receptors which results in a shift of the dose response curve to fMLP-induced chemotaxis. We did not quantify fMLP receptor in neutrophils therefore this possibility can not be discounted. Furthermore, no role was identified for Fer kinase in chemotaxis towards intermediate targets such as KC, MIP-2 and LTB_4. Interestingly MIP-2 has been shown to play a critical role in neutrophil recruitment to the liver during Con A hepatitis and these data may explain why Fer kinase does not play a role in that model [205, 206]. Our lab has confirmed this stimulus specific role of Fer in neutrophil chemotaxis *in vivo*

using intravital microscopy (appendix A). Furthermore, by generating chimeric mice we confirmed that the inhibitory role for Fer kinase in damping leukocyte recruitment toward WKYMVm was due to an action within the hematopoietic compartment (appendix A).

Structural similarities between Fer and Fps/Fes kinases strongly suggest that they could have similar or even redundant roles in the cells and in fact previous data suggest a redundancy exists between these two kinases in regulating hematopoiesis and STAT3 activation in macrophages [10]. Our data demonstrate that Fer kinase does not play a role in neutrophil chemotaxis toward LTB_4 (1 μM) and the possibility exists that Fps kinase may compensate for the loss of Fer in modulating chemotaxis toward LTB_4. However, LTB_4-induced chemotaxis was similar in WT, $Fer^{DR/DR}$, and Fps^{Kr}/Fer^{Dr} neutrophils demonstrating that Fps/Fes kinase does not compensate for Fer kinase. Furthermore, WKYMVm induced chemotaxis was similar in Fps^{Kr}/Fer^{Dr} double mutant neutrophils to that in $Fer^{DR/DR}$ neutrophils. These data suggest that Fps/Fes is not involved in neutrophil chemotaxis and supports our finding *in vivo* in the TNBS-colitis model.

Various signalling pathways have been demonstrated to play a role in neutrophil chemotaxis including PI3K and p38 MAPK pathway [114, 207]. Some evidence suggests a cross talk between p38 and PI3K pathway [114], and Fer kinase may control to this cross talk by inhibiting the PI3K pathway. In our studies we have demonstrated that both P38 MAPK and PI3K pathways are activated in neutrophils in response to bacterial peptide. Furthermore in the absence of Fer kinase, Akt phosphorylation is prolonged and phospho p38 MAPK unaffected. These data suggest that Fer kinase regulates PI3K upstream of Akt. We examined phospho-inositide-dependent-protein kinase (PDK1), a serine-threonine kinase which play a role in cell chemotaxis and in F-actin

polymerization [167, 168] and demonstrated this protein activation was not altered suggesting that Fer might modulate Akt phosphorylation through other upstream targets excluding PDK1. PI3K activation plays a critical role in neutrophil polarization resulting in chemotaxis in response to G protein coupled receptor activation [208-210] and Fer kinase may play a regulatory role in the localization pattern of specific proteins in migrating cells. This aspect is worth further investigation.

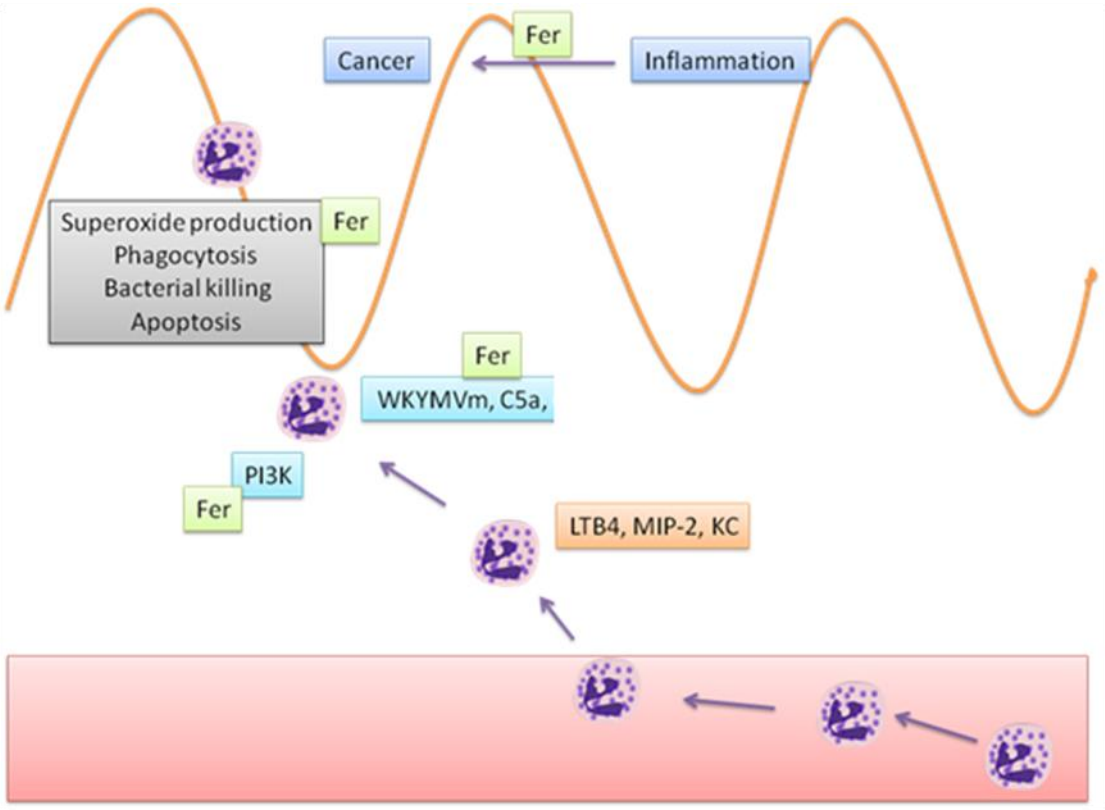
The role of Fer kinase in modulating the signalling pathways is cell type and stimulus specific. Fer kinase has been shown to enhance PI3K activity through an interaction with the p85 regulatory component of PI3K enzyme downstream of insulin and PDGF stimulation in adipocytes [169, 211]. In addition, Craig *et al* [31] demonstrated that Fer kinase is required for maximal p38 MAPK activation and chemotaxis of sensitized mast cells upon IgE cross linking. Fer null mast cells show reduced chemotaxis upon IgE cross linking and transfection of Fer protein into Fer null cells enhanced p38 MAPK phosphorylation and chemotaxis. A role for Fer in chemotaxis in other cell types was also demonstrated. Fer kinase phosphorylation downstream of hydrogen peroxide stimulation and integrin engagement is needed for cortactin phosphorylation and chemotaxis of fibroblasts and breast epithelial cell lines [32]. In addition, Fer activation downstream of phosphatidic acid(PA)/phospholipase (PLD) pathway in required for cortactin phosphorylation, lamellipodia formation, and chemotaxis of NRK52E kidney cell line [171]. Fer was also shown to be involved in enhancing hepatocellular cancer cell line metastasis *in vitro* and its levels were directly correlated with metastasis in patients with hepatocellular cancer [212]. These studies may indicate that the role of Fer

in chemotaxis as well as being stimulus specific may also be specific to the cell type under study.

Our data illustrating a role for Fer kinase in modulating neutrophil chemotaxis *in vitro* can explain the data generated from the TNBS colitis model. In addition to having enhanced neutrophil recruitment to the site of injury, Fer kinase may also modulate the function of neutrophils at the inflammatory site through regulating superoxide production. However no role for Fer in intracellular bacterial killing capacity of neutrophils toward S. aureus was observed. Furthermore, no role for Fer was observed in the rate of spontaneous or WKYMVm-induced apoptosis in WT and $Fer^{DR/DR}$ neutrophils. This latter data is in agreement with previous data published which did not detect any differences in apoptosis rate in Fps mutant neutrophils isolated from the peritoneal cavity of mice treated with thioglycollate [43]. In our *in vitro* functional assays, we used bone-marrow derived neutrophils which may function in a different manner compared to peripheral blood neutrophils which play a role in colitis severity in the TNBS model. Several studies suggested that neutrophils isolated from the mouse bone-marrow or peripheral blood is morphologically and functionally similar regarding superoxide production, granule release and cytokine generation [213, 214]. In this thesis, we provide evidence for the role of Fer kinase in neutrophil chemotaxis and superoxide production however we have not discounted the role of Fer in the function of other immune cells such as mast cells and macrophages which may enhance intestinal damage. In the absence of Fer, the endothelium or other immune cells may secrete increased levels of chemokines leading to enhanced neutrophil recruitment to the site of injury to cause more damage.

Therefore, in this thesis we have identified the Group IV PTK Fer kinase as a regulator of intestinal inflammatory conditions such as IBD. The mechanism involved may be through the regulation of neutrophil chemotaxis to the gut and in restraining the neutrophils ability to generate superoxide (diagram 7.1). The role of Fer kinase in chemotaxis and inflammation is not universal and we have identified Fer as an intracellular regulator of the PI3K pathway activation. Furthermore we have identified a surprising role for Fer kinase in inflammation associated carcinogenesis which should be explored further.

Diagram 7.1 Model for the thesis



In this thesis, we presented evidence suggesting an important role for group IV PTK in a chemically-induced (TNBS) and a spontaneous (IL-10$^{-\backslash-}$) model of intestinal inflammation which have the characteristics of human inflammatory bowel disease (IBD). We also showed data suggesting an important stimulus specific role for Fer kinase in damping neutrophil chemotaxis and modulating PI3K pathway in neutrophils in response to bacterial peptide WKYMVm. Other neutrophil functions at the inflammatory site can be modulated by Fer kinase eg. the rate of superoxide anion production. In the IL-10$^{-\backslash-}$ model, we demonstrated an important role for Fer kinase in inflammation-dysplasia-cancer progression sequence, and the mechanism of action needs to be further explored.

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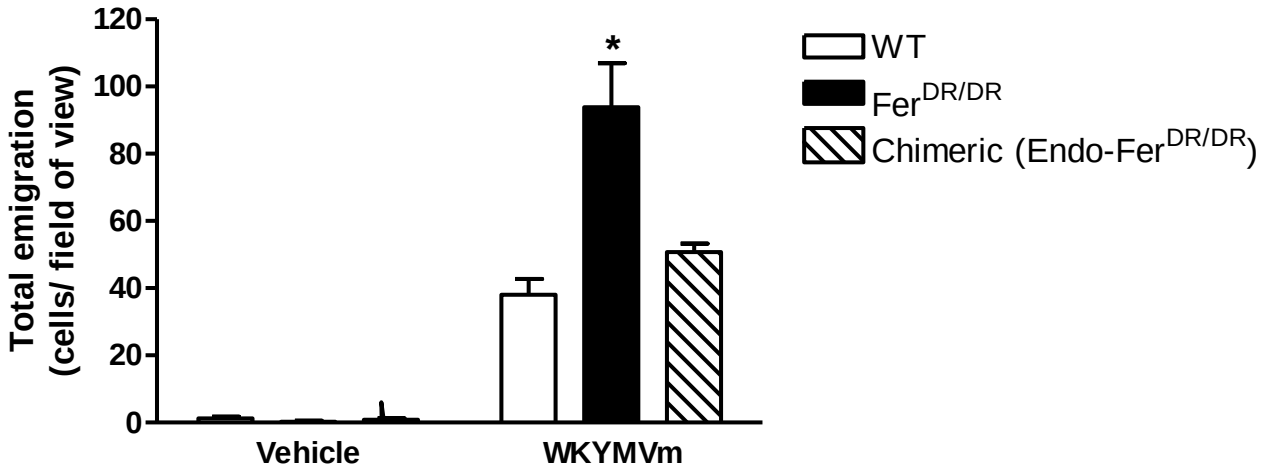
APPENDIX A: STIMULUS SPECIFIC ROLE FOR FER IN LEUKOCYTE CHEMOTAXIS *IN VIVO*

(A)



(B)

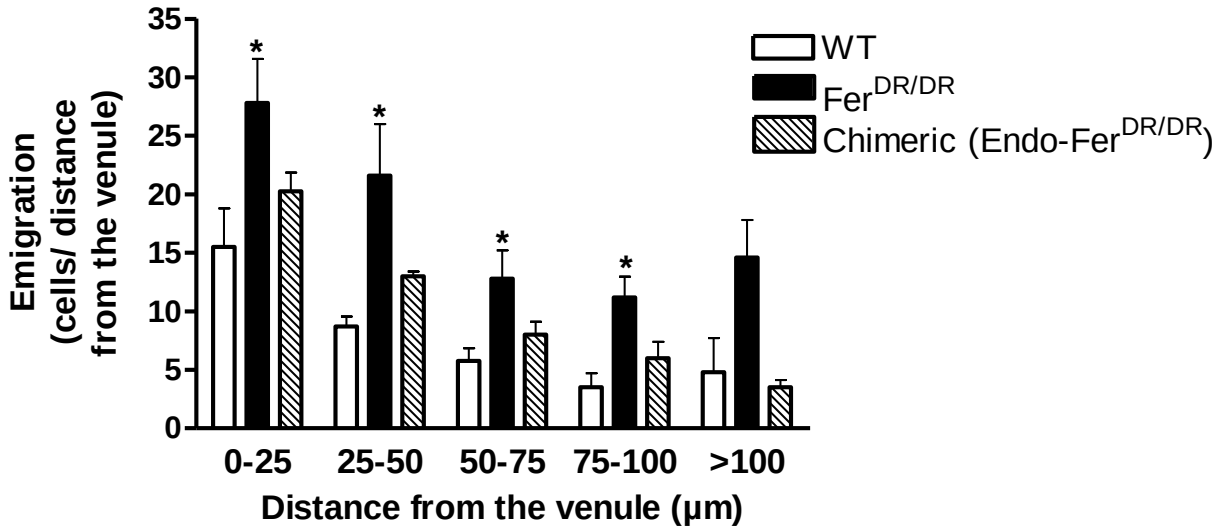


Figure 1: WKYMVm-induced leukocyte transmigration and chemotaxis are enhanced in the absence of Fer kinase *in vivo*

(A) Total leukocyte emigration *in vivo* (cells per field of view) observed in the extravascular space under control conditions (vehicle), or 60 min after WKYMVm gel placement in WT mice (open bars) or Fer $^{DR/DR}$ mice (solid bars). Leukocyte emigration was also determined in bone-marrow chimeric mice (Fer $^{DR/DR}$ endothelium and WT leukocytes) (hatched bars). (B) *In vivo* leukocyte chemotaxis towards the gel containing WKYMVm is illustrated as the number of extravasated cells in each 25 µm increment segment from the blood vessel. Data are expressed as mean ± SEM, n= 4 (WT), n=5 (Fer $^{DR/DR}$), n=4 (endothelium- Fer$^{DR/DR}$), * $p<0.05$ significant difference from WT mice group with WKYMVm.

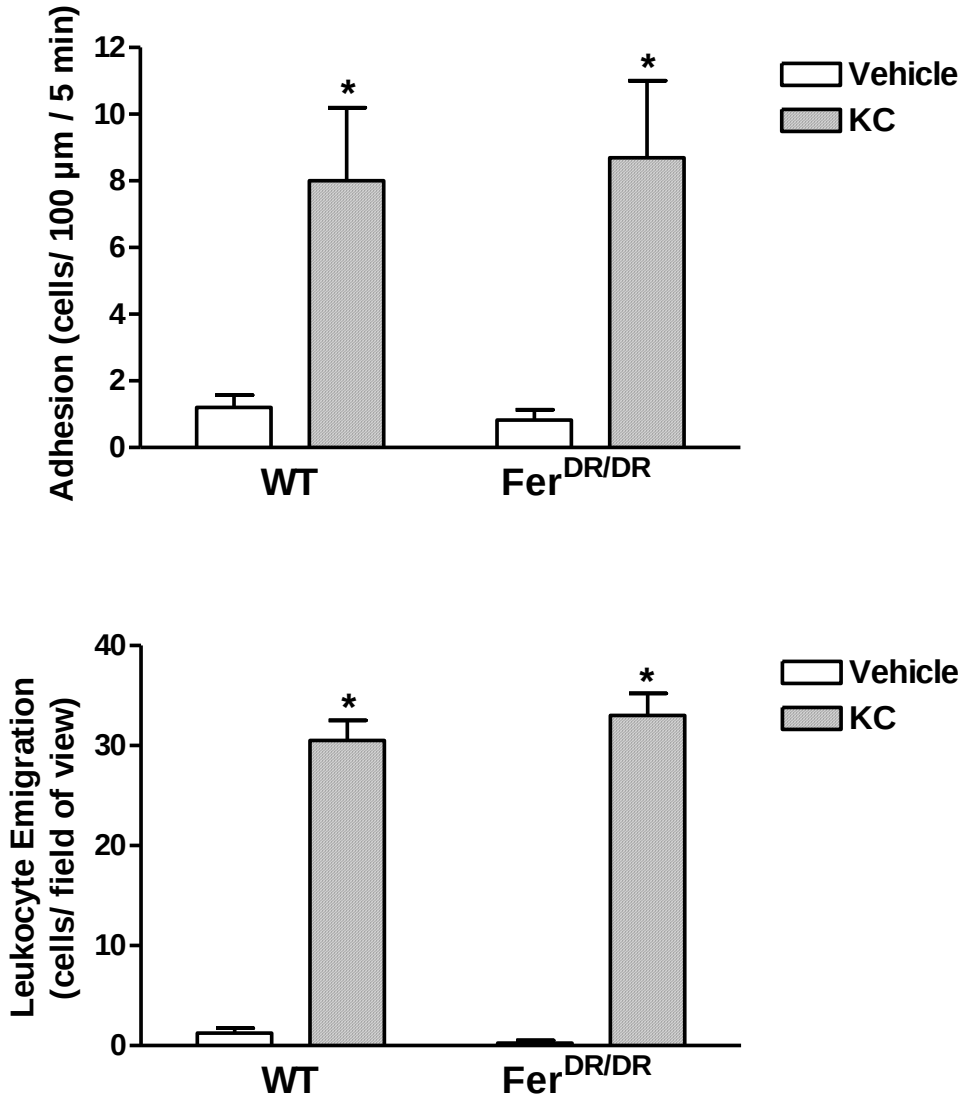
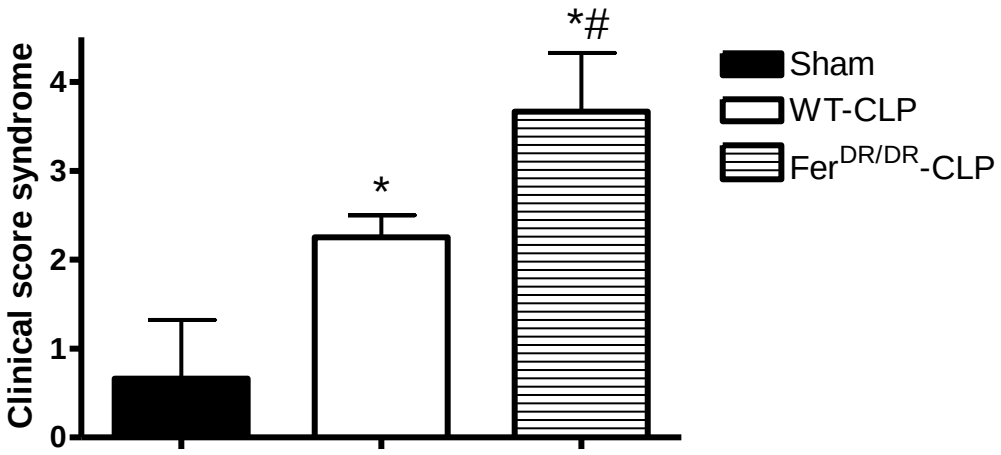


Figure 2: The role of Fer kinase in leukocyte transmigration *in vivo* is stimulus specific

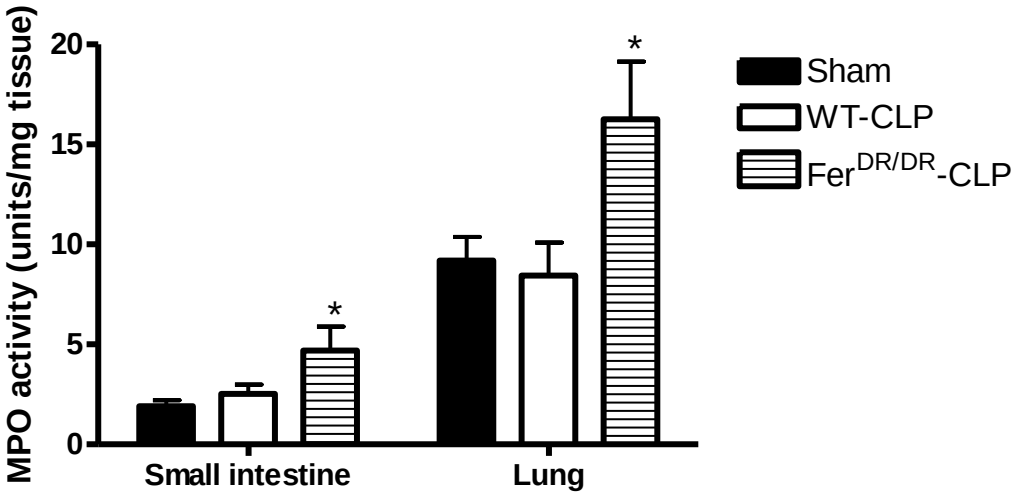
(A) Leukocyte adhesion (cells/100 µm/5 min) and (B) total leukocyte emigration (cells/field of view) are shown in wild type (WT) and $Fer^{DR/DR}$ mice. Leukocyte recruitment was induced by placing KC (5.2 µM) in an agarose gel in the extravascular tissue space 350 µm from the vessel. Data are shown during control conditions (vehicle) and at 60 min post gel placement. Data are expressed as mean ± SEM, n= 4 (WT) and n=5 ($Fer^{DR/DR}$), * $p<0.05$ significant difference from control group for each strain.
These data was generated by Dr. Grace Andonegui.

APPENDIX B: THE ROLE OF FER IN CLP MODEL OF SEPSIS

(A)



(B)

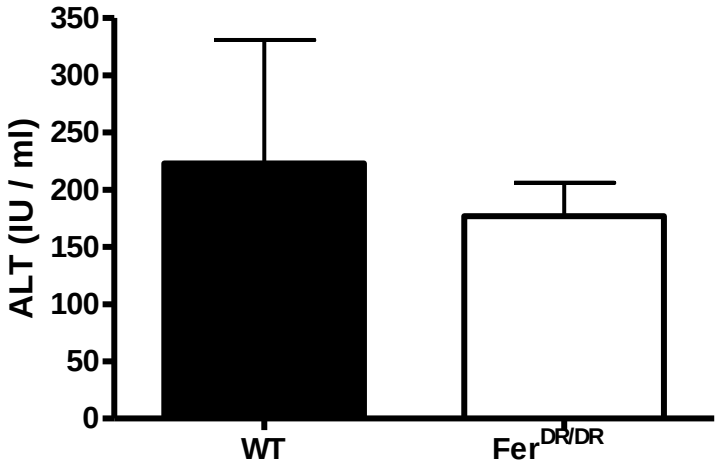


Fer mutant mice experienced enhanced morbidity in the CLP-model of sepsis

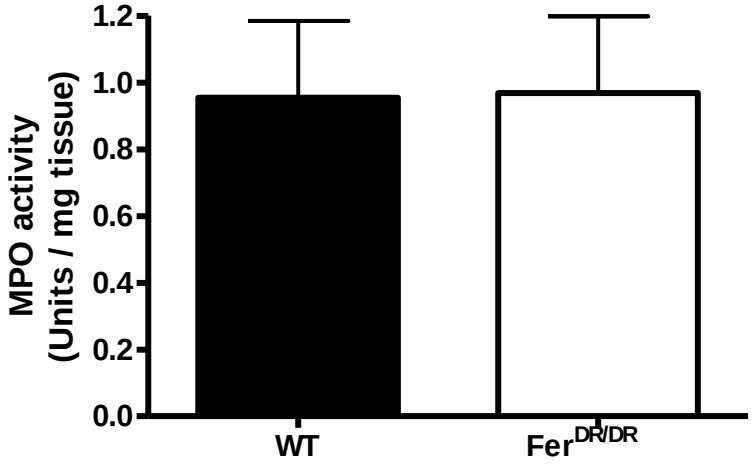
(A) Clinical syndrome score, and (B) small intestine and lung myeloperoxidase (MPO) activity in sham (solid bars), WT with CLP (open bars), and $Fer^{DR/DR}$ with CLP mice (hatched bars) was determined six hours after the operation. Data are expressed as mean ± SEM, n= 3-4 mice, * significant differences from the sham group, # significant differences from WT mice with CLP group.
These data were generated by Dr. Rui Zhang.

APPENDIX C: THE ROLE OF FER IN CON-A MODEL OF HEPATITIS

(A)



(B)



Fer deficiency does not alter the severity of hepatic injury in mice

Serum ALT levels (A) and liver MPO activity (B) were determined in WT (n= 9 mice, solid bar), or $Fer^{DR/DR}$ (n= 6 mice, open bar), following Con-A injection. Data are expressed as mean ± SEM.

These data were generated by Crystal Velichka.