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Exploring the Roles of Atypical MAP Kinases ERK3 and ERK4 During Inflammation

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Résumé

L'insuffisance rénale aiguë est un évènement complexe marqué par une lésion tissulaire causée par une ischémie ou une exposition aux néphrotoxines. Cette lésion est suivie par l'activation des cellules intrarénales qui déclenchent une réponse au stress provoquant l'infiltration de leucocytes. Ensemble, ils aident à éliminer les tissus endommagés pour ensuite jouer un rôle dans le processus de guérison du rein. Les mécanismes par lesquels le système immunitaire régule les évènements dans l'AKI ne sont pas entièrement compris. Des travaux récents ont révélé un rôle clé des MAP kinase atypiques ERK3 et ERK4 dans la promotion de l'inflammation, en régulant la synthèse des chimiokines CCL2 et CCL5 et en jouant un rôle dans la migration et l'invasion des cellules immunitaires aux tissue enflammés/endommagés.

On suppose que l'activité de ERK3 et ERK4 aide à la migration des cellules immunitaire par des mécanismes intrinsèques et favorise une cascade inflammatoire. Également, on pense que ERK3 et ERK4 jouent un rôle dans la maturation et l'activation des cellules immunitaire et que le blocage de leur activité peut inhiber ce processus. En utilisant des cellules rénales épithéliales murines, nous démontrons qu'une perte de ERK3 atténue la production de CCL2 et CCL5 *in vitro* dans les cas de stimulation avec TNFα et TWEAK. De plus, la perte de ERK3 n'affecte pas la maturation des cellules de la moelle osseuse en macrophages ou leur polarisation. Cependant, l'absence d'ERK3 dans les macrophages inhibe leur capacité à migrer.

En résumé, nous fournissons des preuves que les membres du TNFSF peuvent réguler l'expression de ERK3 et que ERK3 joue un rôle dans la production de chimioattractants des macrophages, qui aident à favoriser la cascade inflammatoire nécessaire a l'infiltration des macrophages. De plus, nous démontrons que ERK3 joue un rôle dans la capacité intrinsèque des macrophages à migrer dans les tissus. Ces résultats suggèrent que ERK3 a plusieurs fonctions biologiques durant l'inflammation.

Mots-clés: MAPK atypique; ERK3; ERK4; insuffisance rénale aiguë; inflammation; CCL2; CCL5; macrophages; migration

Abstract

Acute kidney injury is a complex event marked by renal tissue injury after ischemia or exposure to nephrotoxins. It is followed by the activation of intrarenal cells that trigger a stress response causing the infiltration of leukocytes that together help clear damaged tissue and later play a role in the healing process of the kidney. The mechanisms in which the immune system regulates events in AKI are not fully understood. Recent work has unveiled the key role of the atypical MAP kinases ERK3 and ERK4 in promoting inflammation, in part through regulating the synthesis of chemokines like CCL2 and CCL5 and playing a role in migration/invasion capabilities of immune cells to inflamed/damaged tissues.

It is hypothesised that ERK3 and ERK4 activity promotes immune cell migration through intrinsic mechanisms and by fostering an inflammatory cascade. It is also believed that ERK3 and ERK4 promote immune cell maturation and activation and that blocking ERK3 and ERK4 activity can inhibit these processes. Using murine tubule epithelial kidney cells and bone marrow derived macrophages; we demonstrate that ERK3 deficiency attenuates the production of CCL2 and CCL5 in tubule cells stimulated with TNFα and TWEAK. Additionally, ERK3 loss does not impair bone marrow to mature into macrophages or polarize into pro-inflammatory or anti-inflammatory phenotypes. However, the absence of ERK3 in BMDMs inhibits their basal migration abilities in a scratch assay.

Taken together, we provide evidence that TNFSF members may regulate ERK3 expression and play a role in the production of macrophage chemoattractant, which help foster the inflammatory cascade needed for macrophage infiltration. Moreover, we demonstrate that ERK3 plays a role in inherent ability for macrophages to migrate. All suggesting that ERK3 has multiple biological functions during inflammation.

Keywords: Atypical MAPK; ERK3; ERK4; AKI; inflammation; CCL2; CCL5; macrophages; migration

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List of Abbreviations

ACTB	β-actin
AKI	Acute kidney injury
APC	Antigen presenting cell
Arg1	Arginase-1
ASC	Apoptosis-associated speck-like protein
bFGF	Basic fibroblast growth factor
BMDM	Bone marrow-derived macrophages
BORGS	Binders of Rho GTPases
CARD8	Caspase recruitment domain family member 9
CCAC	Canadian Council on Animal Research
CCL2	C-C motif chemokine protein 2 (also, MCP-1)
CCL5	C-C motif chemokine protein 5 (also, RANTES)
CCR2	C-C motif chemokine receptor type 2
CD	Common docking
CSF-1	Colony stimulating factor-1
CX3CL1	C-X3-C motif ligand 1 (also, fractalkine)
CX3CR1	C-X3-C motif receptor 1
CXCL1	C-X-C motif ligand 1
DAMPs	Damage-associated molecular patters
DCs	Dendritic cells
DMEM	Dulbecco's modified Eagle's medium
DN	Double negative
DT	Dipheria toxin
DUSP2	Dual-specificity phosphatase 2
Elk-1	ETS domain-containing protein
ERK	Extracellular signal-regulated kinases
Fn14	Fibronectin 14
GFR	Glomerular filtration rate
HIF-1α	Hypoxia-inducible factor 1-α
HMBG-1	High mobility-group B1
HSP1	Heat shock protein 1
HSP27	Heat shock protein 27
HSPs	Heat shock proteins
HUVEC(s)	Human umbilical cord vein endothelial cell(s)
ICAM-1	Intracellular adhesion molecule 1
IFN	Interferon
IGF2BP1	Insulin-like growth factor 2 binding protein 1
IL-10	Interleukin-10

IL-12	Interleukin-12
IL-17	Interleukin-17
IL-17	Interleukin-17
IL-18	Interleukin-18
IL-1α	Interleukin-1α
IL-1β	Interleukin-1β
IL-23	Interleukin-23
IL-6	Interleukin-6
IL-8	Interleukin-8
IRI	Renal ischemic injury
JNK1/2/3	c-Jun amino (N)-terminal kinase 1/2/3
KIM-1	Kidney injury molecule-1
M-CSF	Macrophage colony stimulating factor
mAb	Monoclonal antibody
MAP-1	Microtubule associated protein 1
MAPK(s)	Mitogen activated protein kinase(s)
MAPKK(s)	Mitogen activated protein kinase kinase(s)
MAPKKK(s)	Mitogen activated protein kinase kinase kinase(s)
MCP-1	Monocyte chemoattractant protein-1 (also, CCL2)
MCT	Mouse cortical tubule
MEF	Mouse embryonic fibroblast
MEK(s)	Mitogen activated protein kinase kinase(s)
MHCII	Major histocompatibility complex class II
MIP-2	Macrophage inflammatory protein-2
miRNA	MicroRNA
MK	Mitogen activated protein kinase activated protein kinase
MKP	Mitogen activated protein kinase phosphatase
NF-κB	Nuclear factor κ light chain enhancer of activated B cells
NGAL	Neutrophil gelatinase-associated lipocalin
NK	Natural Killer
NKG2D	Natural killer group 2 member D
NLK	Nemo-like kinase
NLRPs	NOD-like receptor family protein
NODs	Nucleotide-oligomerization domain like receptors
NOS2	Nitric oxide synthase 2
PAK1/2/3	p21-activated kinase 1/2/3
PAMPs	Pathogen-associated molecular patters
PRRs	Pattern recognition receptors
Rae-1	Retinoic acid early inducible 1
RAG	Recombination-activating genes

RANTES	Regulated upon activation normal T cell expressed and secreted (also, CCL5)
RNAi	RNA interference
RPTECs	Renal proximal tubule epithelial cells
RSK1/2/3	Ribosomal s6 kinase
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
Sept7	Septin 7
SRC-3	Steroid coactivator 3
T regs	Regulatory T cells
TCR	T cell receptor
TCRα	T cell receptor α
TDP2	Tyrosyl DNA phosphodiesterase 2
TECs	Tubular epithelial cells
TGFβ	Transforming growth factor-β
TLR	Toll-like receptor
TNFR	Tumour necrosis factor receptor
TNFSF	Tumour necrosis factor super family
TNFα	Tumour necrosis factor α
Top2	Topoisomerase 2
TWEAK	Tumour necrosis factor like weak inducer of apoptosis
USP20	Ubiquitin specific protease 20
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
α-MSH	α-melanocyte stimulating hormone

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Introduction

Mitogen-activated protein kinases

Mitogen activated protein kinases (MAPKs) are serine-threonine kinases involved MAPK signaling pathways conserved in eukaryotic cells.[1] They convert extracellular signals into intracellular responses through MAPK activation modules. These modules consist of three sequentially phosphorylated, and therefore activated, kinases; the activation of MAP kinase kinase kinase (MAPKKK) followed by MAP kinase kinase (MAPKK or MEK) and then the activation of the final effector MAPK. Once activated, MAPKs then activate a wide range of substrates that coordinate and regulate gene expression, mitosis, metabolism, motility, survival, apoptosis, differentiation and immune responses. In mammals there are seven MAPK modules, four of which are considered conventional and three considered atypical (Figure 1).[2,3]

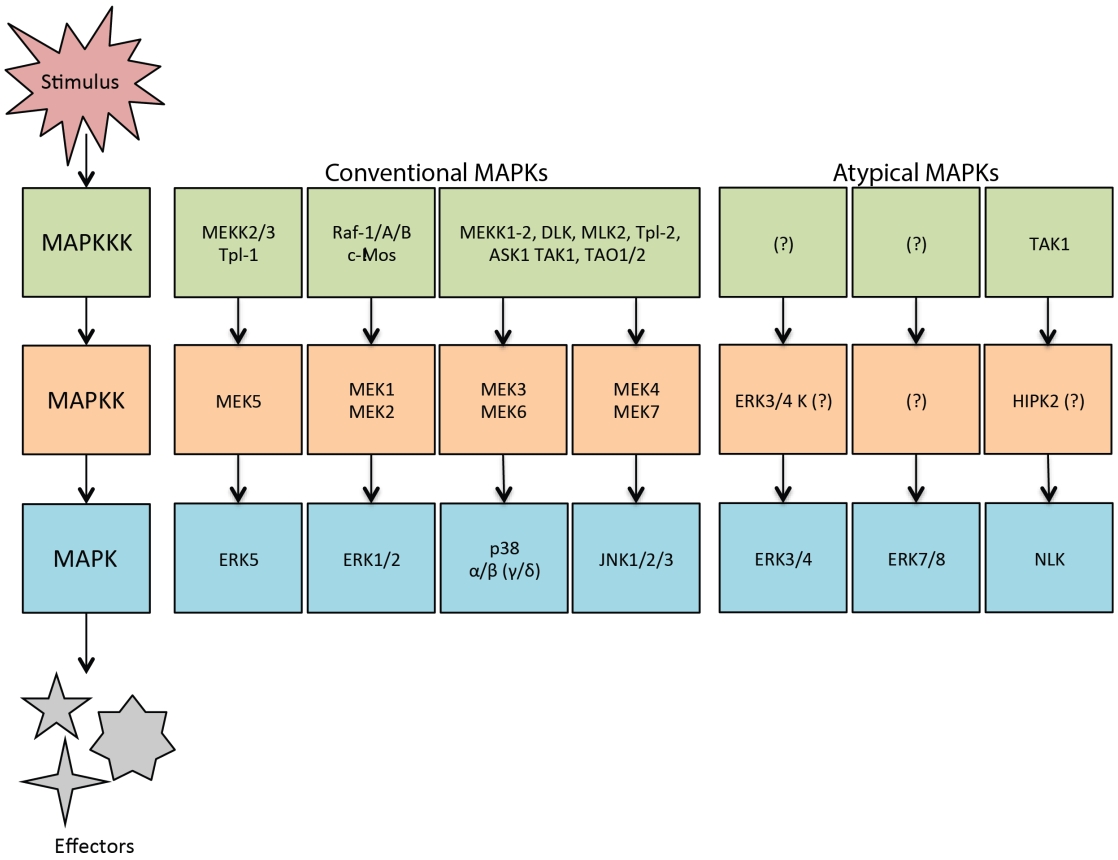

FIGURE 1. MAPK signaling modules. Adapted from reference 2.

Conventional MAPKs, including extracellular signal-regulated kinases 1/2 (ERK1/2), c-Jun amino (N)-terminal kinases 1/2/3/(JNK1/2/3), p38 isoforms (α, β, γ and δ) and ERK5, adhere to the descried three-tiered MAPK module. They are activated by MAPKKs through the dual phosphorylation on the threonine and tyrosine residues part of a conserved Thr-Xaa-Tyr motif in the activate loop of their kinase domain. Atypical MAPKs, ERK3/4, ERK7/8 and Nemo-like kinase (NLK), above all do not contain the Thr-Xaa-Tyr motif (with exception to ERK7/8) and are not activated by conventional MAPKKs; thus they do not conform to the three-tiered model (Figure 2).[2,3]

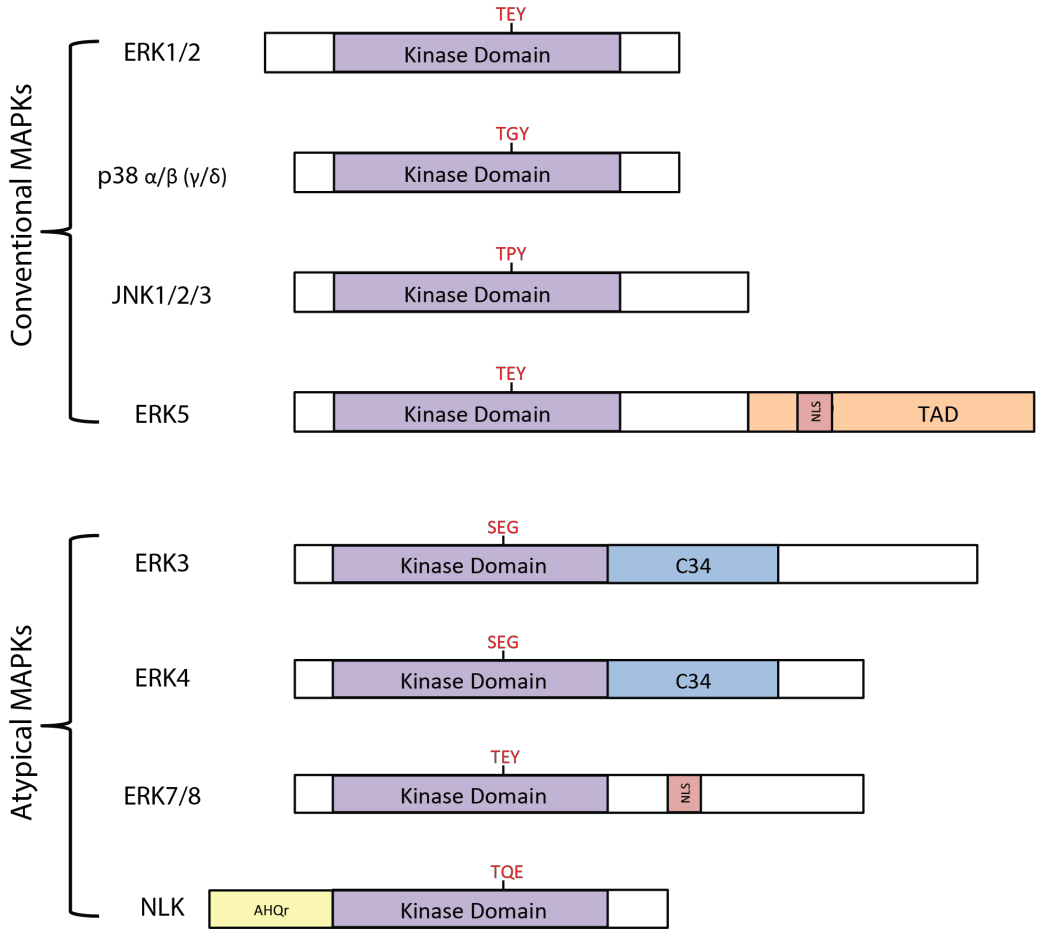

FIGURE 2. Structural representations of conventional and atypical MAPKs. Activation loop motif marked in red. Adapted from reference 2.

While conventional MAPKs have been well studied, their activators, substrates and regulators have been characterized; the activators, substrates and regulators of atypical MAPKs remain unclear.

This master's work focuses on the role atypical MAPKs, ERK3 and ERK4, during the inflammatory response following tissue damage, such as acute kidney injury (AKI). The next chapter aims at describing what is known about ERK3 and ERK4, in addition to events surrounding inflammatory responses during AKI.

Extracellular signal-regulated kinase 3 and 4

Identification and structure

The genes *Mapk6* and *MapK4* (coding for ERK3 and ERK4, respectively) were cloned by homology thanks to their similarity to MAPK ERK1 in 1991 and 1992, respectively.[4,5] At first, rat *Mapk6* was predicted to code a protein of 543-amino acids in length.[4] Later the sequences for human and mouse *Mapk6* were reported.[6–8] Sequence comparison of the three orthologs revealed 90% sequence homology over their shared length; human and mouse *Mapk6* being longer containing a c-terminal extension of 178 amino acids, which results in a larger predicted amino acid length of 721 and 720, respectively. Interestingly, closer inspection of rat *Mapk6* revealed a missing nucleotide between codons 502 and 503 that had resulted in shift of the reading frame and the introduction of a premature stop codon.[8,9] Reading frame corrected, rat *Mapk6* gene translates to a 720-amino acid protein with 94% homology to the human protein. Additionally, *in vitro* translation of rat, human and mouse *Mapk6* cDNA confirmed all three yield a protein which have a molecular mass of ~100kDa.[10]

Similar to *Mapk6*, the initial sequence for *Mapk4* had been mistakenly sequenced, missing five nucleotides that resulted in a predicted length of 557-amino acids.[5] The rectified *Mapk 4* sequence corrects four amino acids and adjusts the reading frame resulting in a predicted length of 587-amino acids and a molecular mass of ~70kDa.[5,11] Comparative genomic analysis of *Mapk6* and *Mapk 4* have revealed that the organization of exon/intron boundaries are similar, however differ from the genes that code for conventional MAPKs. Additionally, encoded proteins, ERK3 and ERK4, share 73% amino acid homology.[10] Interestingly, while conventional

MAPK orthologs have been found in invertebrates and plants, orthologs of ERK3 and ERK4 have not, suggesting that ERK3 and ERK4 MAPK genes are restricted to vertebrates.[12]

Similarities between ERK3 and ERK4 are not only restricted to their identification. Both contain a kinase domain the N-terminus and a C-terminal extension (Figure 2). Looking at the C-terminal, ERK3 and ERK4 share ~50% identity in the first 150 residues which then diverge at the C-terminus extreme. Nonetheless, the C-terminus of both MAPK are conserved throughout vertebrate evolution signifying probable important cellular functions.[13,14]

In relation to kinase domain of conventional MAPK ERK1, ERK3 and ERK4 display 45% and 42% amino acid identity respectively and yet still diverge from their conventional counterpart due to two structural differences. Conventional MAPKs have two phospho-acceptors in the activation loop, while ERK3 and ERK4 contain one. More specifically, ERK3 and ERK4 contain a Ser-Glu-Gly motif differing from the Thr-Xaa-Tyr motif typically found in conventional MAPKs; because of this, ERK3 is a poor substrate for dual-specificity-MAPKKs (Figure 2).[13,14] Secondly, all ERK3 and ERK4 orthologs have a Ser-Pro-Arg amino acid sequence instead of a Ala-Pro-Glu in subdomain VIII of their kinase domains – the only two MAPKs that have an arginine residue at this position.[15]

Expression

ERK3 messenger RNA (mRNA) is expressed ubiquitously in adult mammalian tissues.[3] The highest expression is found in the brain, skeletal muscle and the gastrointestinal tract.[8] ERK4 mRNA has a more restricted expression profile than ERK3. ERK4 is expressed in the brain, colon, eye, heart, kidney, lung, ovary, pancreas, placenta, prostate and skin. Similar to ERK3, ERK4 has the highest expression in brain tissue.[5,15,16]

Activators, Regulators & Substrates

The activation of MAPK is controlled by the phosphorylation by kinases upstream. However, they can also be regulated through dephosphorylation by phosphatases and other means like degradation. As mentioned, conventional MAPK, their activators, regulators and substrates, are well studied; those of ERK3 and ERK4 are more elusive.

Activators

The study of ERK3 and ERK4 have revealed their phosphorylation sites within their respective activation loops; serine 189 on ERK3 and its homolog serine 186 on ERK4, leading to the discovery of their respective activators.[17–19] To date the only activator, hence the only kinases known to phosphorylate ERK3 and ERK4, are p21-activated kinases 1/2/3 (PAK1/2/3).[20,21] Interestingly, and contrary to conventional MAPK, ERK3 and ERK4 phosphorylation is thought to be constitutive.[19] Because the biological activity of ERK3 and ERK4 does not appear to be a dynamic function of its phosphorylation (due to the observation of it being constitutively active), looking at their regulation is important.

Regulators

While little work has been done on the regulator(s) of ERK4, it is believed that the regulation of ERK3 relies on the protein's stability.

In mice, ERK3 and ERK4 gene expression markedly increases between embryonic days nine and eleven of development coinciding with early organogenesis.[8,16,22,23] Numerous *in vitro* studies have shown that various stimuli are able to upregulate the mRNA expression of ERK3. Many of these show ERK3 upregulation during differentiation, namely the differentiation of P19 embryonic carcinoma cells into neuronal cells, C2C12 myoblast cells into muscle cells and the differentiation of Burkett's lymphoma Raji cells.[24–26] Of note, is the *in vivo* work that demonstrates that ERK3-deficient mice display impaired thymocyte development, suggesting ERK3 involvement. In a study, Marquis *et. al.* demonstrate the increased expression of ERK3 during thymocyte development from double-negative (DN) 1 to the DN4 stage which then decreases further on in development.[27] Interestingly, ERK3 is also upregulated when T lymphocytes are stimulated through their T cell receptor (TCR) dependent on the RAF-MEK-ERK conventional MAPK signaling cascade.[28] This observation is in agreement with the study conducted by Hoeflich, *et. al.* which demonstrates that an activating mutation in B-Raf coincides with an increased expression of ERK3.[29] It has also been shown that mRNA ERK3 expression is upregulated upon stimulation with the cytokines TNFα and IL-1β and the growth factor basic fibroblast growth factor (bFGF) in human umbilical vein endothelial cells (HUVECs).[30] The same study also suggests that the c-Jun transcription factor binds to the *Mapk6* promoter and regulates *Mapk6* transcription in response to TNFα stimulation.[30] Lastly, two microRNAs

(miRNA) have been shown to regulate ERK3 expression. The first, miR499a, negatively regulates ERK3 expression in an *in vitro* model of hepatitis B induced hepatocellular carcinogenesis (Figure 3).[31] While the second, let-7i, is involved in a BMI1-let-7i-ERK3 pathway whereby BMI1 upregulates ERK3 expression by suppressing let-7i which directly targets ERK3 mRNA (Figure 3).[32]

In contrast to ERK3, the context in which ERK4 expression is controlled is less known. However, it has been shown that IGF2BP1 (insulin-like growth factor 2 binding protein 1), a RNA binding protein, inhibits the translation of ERK4 mRNA (Figure 3).[33]

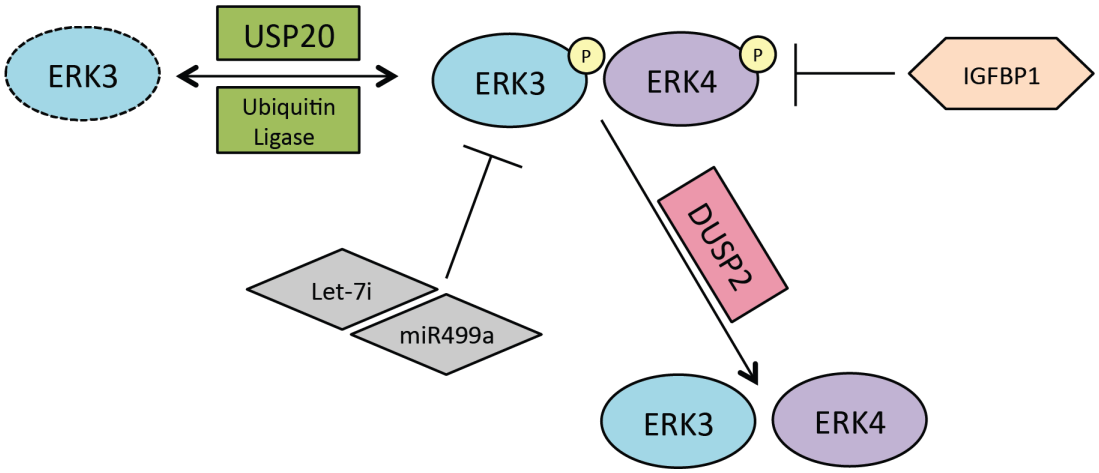

FIGURE 3. Regulators of ERK3 and ERK4.

ERK3 is a highly unstable protein with a half-life of 30-45mins in exponentially proliferating cells.[25,34] It is the first MAPK family member whose activity has been found to be linked to protein turnover.[25] In a study done by Zimmermann, *et. al.*, transcriptional profiling identified *Mapk6* as a gene significantly upregulated in response to treatment with a proteasome inhibitor.[35] Further investigation identified the proteolysis of ERK3 is executed by the proteasome and is dependent on the polyubiquination of the protein; proteasome inhibitor treatment or inactivation of the ubiquitin-activating enzyme (E1) was shown by Coulombe *et. al.* to result in the accumulation of endogenous or ectopically-expressed ERK3 protein.[25] In the same study, ERK3 turnover was found to be independent of its activation loop phosphorylation, enzymatic activity or its C-terminal extension. However, the analysis of a series of chimeras

made between stable ERK1 and ERK3 delimited two degrons in the N-terminal lobe of ERK3's kinase domain that are both necessary and sufficient to target ERK3 for proteasomal degradation.[25] Further investigation of the proteasomal degradation of ERK3 by our lab revealed that the deubiquinating enzyme, ubiquitin specific protease 20 (USP20) can regulate ERK3's stability and thus its biological activity (Figure 3).[36]

In contrast to ERK3, ERK4 is a highly stable protein.[16] In a recent study, the activity of ERK3 and ERK4 was shown to be regulated by the dual-specificity MAP kinase phosphatase (MKP) dual-specificity phosphatase 2 (DUSP2) (Figure 3). The interaction of DUSP2 with either ERK3 or ERK4 causes the dephosphorylation of serine 189 and 186, respectively, which also leads to the decrease in the phosphorylation of threonine 182 on MAP kinase-activated protein kinase 5 (MK5), a MAP kinase activated protein kinase (MK) and substrate of ERK3 and ERK4 (more on MK5 below).[37]

Away from specific regulators, cellular localization also appears to be important for ERK3 and ERK4 activity. While ERK3 is found in both the cytoplasm and in the nucleus, ERK4 is principally found in the cytoplasm in a large variety of cells types.[11,38,39] Likewise, the cytoplasmic localization of ERK3 is facilitated by active transport dependent on CRM1, for which the C-terminus of ERK3 is necessary.[38] However, in contrary to conventional MAPK, ERK3 and ERK4's localization within the cell does not change in response to stimuli investigated, and a few studies have suggested ERK3's localization changes during the cell cycle, yet they are not in agreement. Bind *et. al.* were the first group to suggest that ERK3 changes during mitosis by localizing to the Golgi apparatus in HeLa cells.[9] This is in conflict with Aredia *et. al.*'s study that demonstrates ERK3's homogenous distribution during mitosis.[40] In addition to the above, ERK3 and MK5 interaction is known to cause them to localize to the cytoplasm.[41,42]

Substrates

While ERK3 and ERK4's conventional counterparts, ERK1 and ERK2 are multifunctional and phosphorylate many different substrates, including microtubule associated protein 1 (MAP-1), c-Jun and ETS domain-containing protein (Elk-1), ERK3 and ERK4 appear to have a more restricted substrate repertoire.[17,43] Still, preliminary studies have shown ERK3

involvement in activating tyrosyl DNA phosphodiesterase 2 (TDP2), steroid coactivator 3 (SRC-3) and MK5 (Figure 4).[11,19,39,41,42,44–46]

In cancerous cells, TDP2 repairs topoisomerase 2 (Top2)-linked DNA damage protecting cancer cells against Top2 inhibitor-induced growth inhibition and apoptosis thereby conferring chemoresistance. While the regulation of TDP2 activity is largely unknown, a recent study by Bian, *et. al.* suggests that ERK3 phosphorylates TDP2 at serine 60. This promotes TDP2's phosphodiesterase activity, which upregulates TDP2-mediated DNA damage response and increases chemotherapeutic drug resistance.[44]

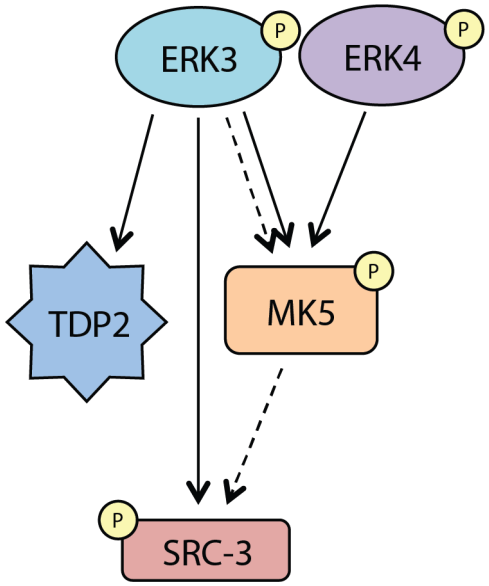

FIGURE 4. Substrates of ERK3 and ERK4.

SRC-3 acts as a coactivator of nuclear receptors and other transcription factors. It promotes cell proliferation, transformation and also cancer cell migration and invasion. Interestingly, when cells are depleted for ERK3, the ability of lung cancer cells to invade and form tumours is inhibited in a xenograft mouse model.[45] In the same study, it was shown that ERK3 promotes invasiveness by phosphorylating SRC-3 at serine 857 – phosphorylation site determined through immunoprecipitation of the kinase in 293T cells.[45] Conversely, unpublished data from our laboratory has shown that in 293T cells, the immunoprecipitation of ERK3 systematically yields the co-purification of MK5 (Déléris, P. and Meloche, S. unpublished data).

Interestingly, serine 857 on SRC-3 is situated in an Arg-Xaa-Xaa-pSer motif, the same motif recognized by MK5 and needed for phosphorylation by MK5, suggesting that SRC-3 activation by ERK3 likely passes through MK5. Additionally, it has been shown that ERK3 phosphorylates threonine 182 on MK5's activation loop, which is followed by a proline, leading to the belief that, like other MAP kinases, ERK3 is a proline directed kinase; serine 857 of SRC-3 is followed by a leucine.[19,45]

MK5 is the only *bona fide* substrate of ERK3 and ERK4 characterized by multiple groups. As mentioned, ERK3 and ERK4 phosphorylate MK5 on threonine 182 of its activation loop, this causes a nuclear-cytoplasmic translocation of a ERK3/4-MK5 complex and the activation of MK5 that also stabilizes ERK3.[11,39,41,42] Because of the stabilization effect, ERK3-MK5 involvement is more complex than the activation of MK5 by ERK4 phosphorylation. It has been proposed that, while ERK3 directly phosphorylates MK5, it's catalytic activity may be dispensable for MK5 activation as it can exert a scaffolding function needed for the auto-phosphorylation of MK5.[41,42] This said, *in vitro* kinases assays did not support the scaffold protein hypothesis; nevertheless, ERK3/4-MK5 interaction is complex.[19]

MK5 has low affinity for and interacts transiently with unphosphorylated ERK3/4. The physical interaction of ERK3/4 with MK5 promotes the recruitment and/or activation of ERK3/4 activation loop kinase, PAK1/2/3, catalyzing the phosphorylation of serine 189/186 of ERK3 and ERK4 respectively. The phosphorylation of ERK3/4 strengthens the interaction of the ERK3/4-MK5 complex subsequently phosphorylating MK5 on threonine 182 and activating it. Activated MK5 then phosphorylates ERK3/4 outside of their respective activation loops. It is believed that these further activated forms of ERK3 and ERK4 are competent to then phosphorylate additional physiological substrates to relay the signaling cascade further downstream (Figure 5). However, the identity and regulatory impact of MK5 phosphorylation of extra-activation loop sites on ERK3 and ERK4 remain elusive.[19,46]

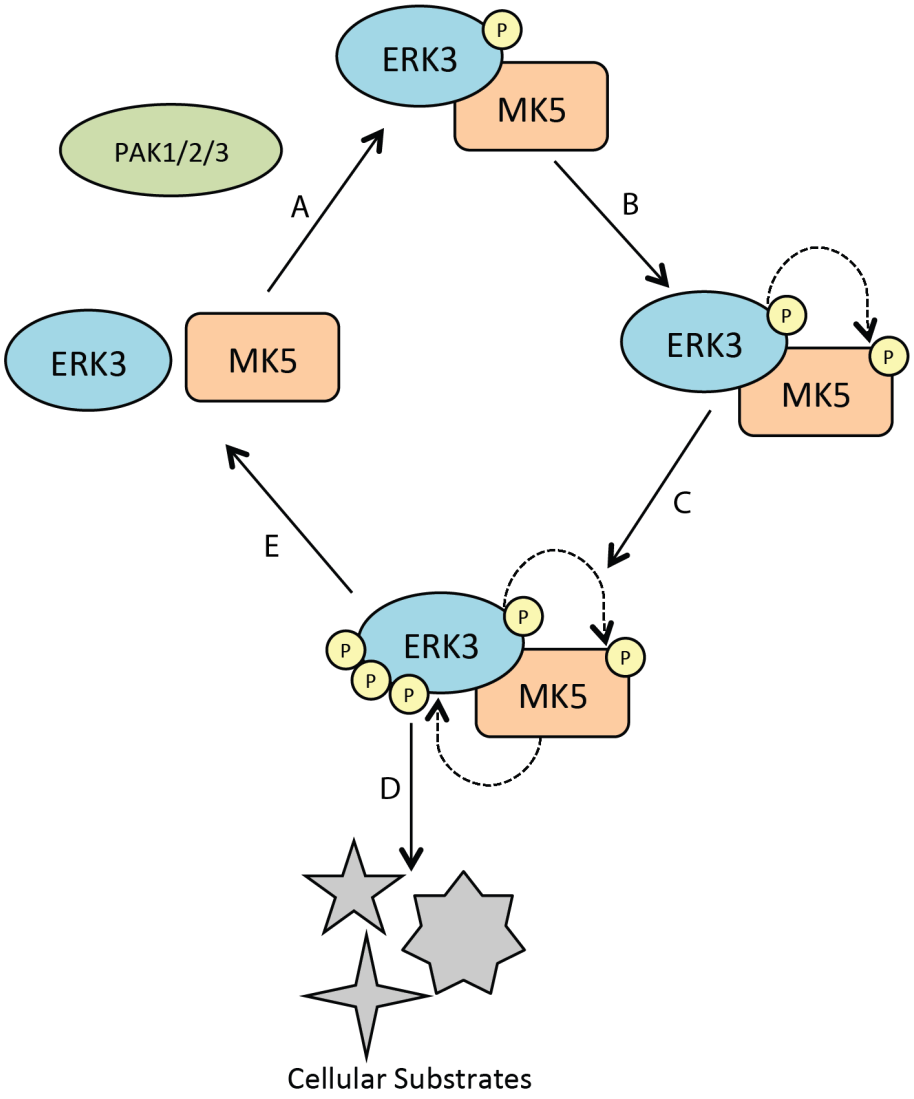

FIGURE 5. ERK3 and ERK4 interaction and activation of MK5. A) Unphosphorylated ERK3 interacts transiently with MK5 recruiting PAK1/2/3 that then phosphorylates ERK3. **B)** The phosphorylation of ERK3 strengthens the interaction with MK5 leading the phosphorylation of MK5 on threonine 182. **C)** Activated MK5 then phosphorylates ERK3 outside its activation loop. **D)** The additional phosphorylation of ERK3 allows activation of downstream cellular substrates. **E)** ERK3-MK5 complex disassembles. Note, ERK3 is used for simplicity, and can be replaced for ERK4. Adapted from reference 19.

Furthermore, the fact that ERK3 and ERK4 phosphorylation strengthens the interaction with MK5 differs from how conventional MAPK interact with their substrates. The classical MAPK

ERK1/2 and p38 interact with the downstream MK's, ribosomal s6 kinases 1/2/3 (RSK1/2/3) and MK2/3, through conserved clusters of acidic amino acids which form a common docking (CD) domain. This CD domain is dispensable for the interaction of ERK3 and ERK4 with MK5, in its place ERK3 and ERK4 interact with MK5 through a FRIEDE motif within ERK3 and ERK4 which is essential for ERK3 and ERK4 binding to the C-terminal region of MK5.[47]

Cellular functions

Development and Differentiation

Observations made through the creation of ERK3-deficient mice have led researchers to the idea that ERK3 is involved in development and differentiation.

Initially, the targeted disruption of the *Mapk6* in mice was accomplished by the insertion of the *LacZ* gene in-frame with the ATG initiation codon found in exon 2, creating $Mapk6^{LacZ}$ mice. The loss of ERK3 in $Mapk6^{LacZ}$ homozygous mice leads to intrauterine growth restriction, delayed lung maturation associated with defective type II pneumocyte differentiation, and neuromuscular abnormalities. Furthermore, 40% of $Mapk6^{LacZ}$ mice died after delivery from respiratory distress syndrome while the other 60% survived the initial neonatal interval but died with 24 hours from an unknown cause.[22] Hence the perinatal lethality of these $Mapk6^{LacZ}$ homozygous mice has stopped the analysis of ERK3 functions in post-natal development and growth.

To address the importance of ERK3 kinase activity and to study the role of ERK3 in post-natal development, two novel genetically engineered mouse models were generated. Mice that express a catalytically inactive (kinase dead) allele of ERK3 ($ERK3^{KI}$) are born at normal Mendelian ratios and do not exhibit signs of respiratory distress or neuromuscular abnormalities. Unexpectedly, mice with a conditional disruption of the ERK3 gene were crossed to obtain an ERK3 knockout ($ERK3^{\Delta/\Delta}$) also survived into adulthood, demonstrating that ERK3 expression and activity is dispensable for post-natal survival; however, kinase activity is necessary for optimal post-natal growth in mice (Soulez, M., Meloche, S., *et. al.* manuscript in preparation). These recent results dispute the original phenotypes of the original $Mapk6^{LacZ}$ mutant mice, and it is believed that the observed phenotypes of these mice were not directly due to the loss of

ERK3 but due to the *LacZ* construct used to target ERK3 (Soulez, M., Meloche, S., *et. al.* manuscript in preparation).

Unlike ERK3, the targeted disruption of the *Mapk4* gene in mice did not present with the same phenotypes. ERK4-deficient ($ERK4^{-/-}$) mice are viable, fertile and develop normally exhibiting no gross morphological or physiological irregularities. Additionally, ERK4 loss is not compensated by ERK3; there is no change in ERK3 catalytic activity or expression. Additionally, the loss of ERK4 does not exacerbate the phenotypes seen in the $Mapk6^{LacZ}$ mice nor does it compromise the viability of $Mapk6^{LacZ}$ neonates. Survival into adulthood of ERK4-deficient mice allowed for behavioral phenotyping revealing that they display depression-like behavior in a forced-swim test.[16]

Apart from development studies done through depletion of ERK3 and ERK4 in mice, studies have looked at its substrate MK5 as well. MK5-deficient mice display impaired spine formation of hippocampal neurons *in vivo*. It was found that ERK3 interacts with septin 7 (Sept7) to form an ERK3-MK5-Sept7 ternary complex that can then phosphorylate binders of Rho GTPases (BORGS). The same study showed that in transfected primary neurons, the ERK3-MK5 module stimulates Sept-7 dendrite development and spine formation, suggesting that the regulation of neuronal morphogenesis is a physiological function of ERK3-MK5 signaling.[48]

Classical MAPK are known to play a part in the signaling pathways activated during various steps of T cell development; for instance ERK1/2's essential role in β-selection and positive selection during thymocyte differentiation.[49] Studies have shown that ERK3 also plays a role in the development of thymocytes. A study conducted by Marquis, *et. al.* in collaboration with our lab has shown that ERK3-deficiency leads to a 50% reduction in $CD4^{+}CD8^{+}$ double positive (DP) thymocyte number. As a result, ERK3-deficient DP thymocytes have a decreased half-life associated with a higher level of apoptosis and their ability to make successful T cell receptor alpha (TCRα) gene rearrangement is compromised. These observations are linked to ERK3 catalytic activity as non-functioning ERK3 fails to rescue the reduction in DP thymocytes and establishes that ERK3 is essential to sustain DP survival during recombination-activating genes (RAG)-mediated rearrangements.[27] Further investigation has found that using a knock-in

mouse model in which the coding sequence for ERK3 is replaced by the gene encoding β-galactosidase reporter, that ERK3 is expressed by DP thymocytes undergoing positive selection. ERK3-deficient mice that have a polyclonal TCR repertoire have reduced positive selection that is also observed when ERK3-deficent mice are backcrossed to class I and class II restricted TCR transgenic mice; and TCR stimulation of DP thymocytes was strongly reduced in ERK3-deficient mice. These results show that ERK3 expression following TCR signaling is critical for accurate thymic positive selection.[28]

Cell Cycle and Proliferation

While indications in the literature have suggested that ERK3 plays a role in proliferation, it has not been well characterized.

Functional studies have shown that ERK3 expression is increased during the differentiation of P9 and PC12 cells into neurons and during the differentiation of C2C12 myoblasts into myotubes; differentiation of these cells are linked to a stop in proliferation.[4,25] Moreover, the overexpression of stable ERK3 inhibits fibroblast entry into the S phase.[25,38] Plus, in cellular models of hepatocellular carcinoma, it appears that miR499a promotes proliferation by inhibiting the expression of ERK3.[31] On the contrary to ERK3's suggested role as an inhibitor of proliferation, ERK3 appears to be a positive regulator of proliferation in endothelial cells and T cells in response to TCR simulation.[27,30,45] However, it has also been shown that the loss of ERK3 expression does not effect the proliferation of various cell lines, including mouse embryonic fibroblasts (MEFs) isolated from *Mapk6*^{LacZ} mice (Tanguay, P.L. and Meloche, S. unpublished data).

In an attempt to dissect a mechanism for ERK3's role in proliferation, molecular studies have suggested that ERK3 interacts with proteins cyclin D3 and Cdc14A/B which are cell cycle regulators.[50,51] In fact, ERK3 is phosphorylated on its C-terminal extension during mitotic entry and is dephosphorylated during the M/G1 transition.[50] However a defined role still remains elusive.

Very little is known about ERK4's involvement in cell cycle and proliferation; nevertheless, it has been shown that shRNA knock-down of ERK4 in HUVECs causes a

reduction in their proliferation.[52] Intriguingly, another study in the same cells has shown that an siRNA knock-down of ERK3 also causes a decrease in proliferation.[30] Sadly, a study was never conducted looking at the depletion of ERK3 and ERK4 on proliferation, to evaluate the redundancy between the two kinases.

ERK3, ERK4 and Cancer

The above roles of ERK3 and ERK4 in differentiation and proliferation have led many researchers to believe that both ERK3 and ERK4 may be involved in cancer.

Using patients cohorts, it has been found that ERK3 expression is increased in chewing tobacco associated oral squamous cell carcinoma.[53] The same study showed that those with elevated ERK3 in cancerous tissues also have a high expression of ERK3 in peripheral blood cells, which was absent in healthy individuals.[53] Moreover, Rai, *et. al.* suggested that an overexpression of ERK3 in normal healthy individuals may be indicative of increased risk of developing oral cancers.[53] Along the same line, 50% of individuals with colorectal or gastric cancers have increased ERK3 protein expression in cancerous tissue versus adjacent healthy tissue.[54,55] Plus, large-scale transcriptome studies have revealed that ERK3 expression is also increased in melanomas and cells derived from breast cancers.[56,57] Likewise, the study by Long *et. al.* that identified SRC-3 as a substrate of ERK3 also demonstrates that ERK3 is elevated in human pulmonary carcinomas.[45]

In models of oncogenesis, studies have suggested ERK3 involvement. In the Smgb-Tag transgenic mouse model, a tool that can identify genes that are deregulated in salivary gland carcinomas, ERK3 is elevated in dysplastic tissues and adenocarcinomas compared to healthy tissues.[58] Additionally, the oncogenic activating mutation of B-Raf, V600E, upregulates the mRNA and protein expression of ERK3 in cultured mouse fibroblasts.[29] As for ERK4, the profiling of pulmonary carcinomas induced by oncogene K-Ras in a transgenic K-Ras mouse model has shown elevated ERK4 mRNA expression.[59]

Apart from variances in expression, studies have found ERK3 and ERK4 mutations in cancer; however, none have shown the consequence of their mutation. Mutations found for ERK3 through the portal of the TCGA project show that a R399Q mutation is present in uterine,

breast and colorectal cancer tissues as well as melanomas.[60] The same portal also identifies the R114C/H mutation found in the kinase domain of ERK4, which has been found in colorectal carcinomas, gliomas, non-small cell pulmonary carcinomas and melanomas.[60] Other described mutations are H333D/N and E331D/D336N that appear in ERK3 and ERK4 respectively; and are important as they are required for the interaction with MK5.[47] More recently, Alsaran, *et. al.* described that L290P/V mutations in the kinase domain of ERK3 exist in several cancers.[61] Their work suggests that these mutations offer increased activity in promoting cancer cell migration and invasion but have little/no impact on ERK3's role in cell proliferation compared to wild type controls. Furthermore, while there is no clear effect on kinase activity, L290P/V mutations enhance ERK3's localization to the cytoplasm by increasing its integration with nuclear export factor CRM1.[61] Together, their findings suggest that ERK3 plays a role in cancer invasiveness.

Apart from looking at databases, experimental models and the mutations found, there is also work done in cancerous cell lines that further suggests that ERK3 (and therefore potentially ERK4) has a role in cancer, more precisely tumour progression via cellular migration and invasion.

Long, *et. al.*'s study, whereby SRC-3 is the downstream substrate of ERK3, suggests that the loss of ERK3 inhibits the migration of a pulmonary carcinoma cell line; additionally, the loss of ERK3 and therefore SRC-3 signaling, has been shown to inhibit the migration of human endothelial cells as discovered by Wang, *et. al* (Figure 6).[30,45] Also, pro-invasive studies through SRC-3 have been shown through *in vitro* and *in vivo* experiments.[45] To further these findings, another study demonstrated that the overexpression of ERK3 modifies the organization of the actin cytoskeleton and promotes the migration speed and adhesion of assorted breast cancer cell lines.[62] In a recent study, oncogenic polycomb group protein, BMI1, has been revealed to be a positive regulator of ERK3 levels in head and neck cancer cells.[32] Mechanistically, BMI1 upregulates ERK3 expression by suppressing the tumour suppressive miRNA let-7i that directly targets ERK3 mRNA. ERK3 can then act as an important downstream mediator of BMI1 in promoting cell cancer migration.[32] In terms of ERK4, it has been shown that ERK4 signals through a ERK4-MK5-heat shock protein-(HSP)-27 pathway

that controls the motility of U2OS cells, a human osteosarcoma cell line.[33] Furthermore, it is believed that IGF2BP1 inhibits ERK4-MK5-HSP27 pathway by inhibiting the translation of ERK4 mRNA thereby preventing MK5 activation and the phosphorylation of HSP27, which sequesters actin monomers available for F-actin polymerization (Figure 6). Likewise, HSP27-β-actin (ACTB) association is reduced, mobilizing cellular G-actin for polymerization in order to promote the velocity of cell migration.[33]

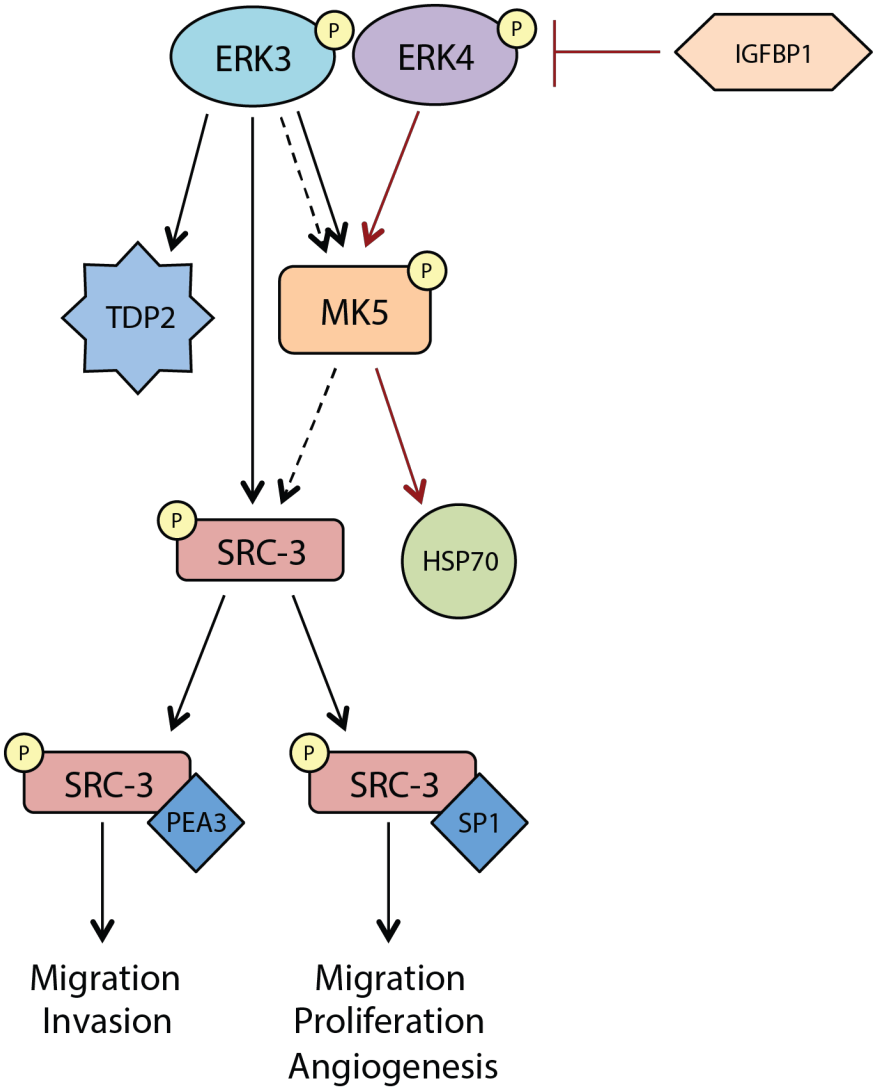

FIGURE 6. ERK3 and ERK4 signaling in cancer.

In other studies, in addition to ERK3's role in migration, ERK3 depletion inhibits the proliferation of HUVECs – further implicating ERK3 in tumour progression.[30] Additionally, HUVECs can form tube structures *in vitro* when stimulated with cytokines like TNFα, therefore they can be used as a model of angiogenesis. When HUVECs are depleted of ERK3 through siRNA, tube formation is inhibited and thus suggests that ERK3 plays a role in the progression of solid tumours through angiogenesis.[30] While the exact mechanism is not understood, the same study defines ERK3 involvement for the vascular endothelial growth factor (VEGF) receptor, VEGFR2, expression through SRC-3 signaling (Figure 6).[30] Although this study does not investigate ERK4, HUVECs appear to require ERK4 for normal proliferation *in vitro*.[52] Also, as previously mentioned, our lab believes that the activation of SRC-3 by ERK3 is not direct but through MK5 activation (Figure 6).

In other studies, apart for a direct role in tumorigenesis itself, ERK3 has been described as a regulator of TDP2, and its action confers chemoresistance to cancerous cells; ERK3's inhibition can therefore sensitize cancerous cells to chemotherapies involved in DNA damage (Figure 6).[44] There has been no work done on ERK4's involvement in DNA damage response and chemoresistance.

ERK3 and Immunity

While there has been work looking that ERK3's involvement during T cell development and that ERK3 expression is crucial for TCR signaling and thymic positive selection, little to no work has investigated the role of ERK3 and/or ERK4 in other areas of immunity; be it during the responses of the innate immune system and inflammation, or other dynamics of adaptive immunity.[27,28]

As described, MK5 is the only *bona fide* substrate of ERK3 and ERK4, and even its biological role remains largely unknown. However, there is some research that suggests that MK5 may be implicated in the inflammatory response, through the phosphorylation of $cPLA_2$ at serine 727 and hnRNAP at serine 84 in *in vitro* assays. However, their phosphorylation *in vivo* remains unclear and the biological significance in relation to immunity and inflammation and ERK3 and ERK4 are unknown.[63,64] Additionally, while we know there is ERK4-MK5-HSP27 (and potentially ERK3-MK5-HSP27) involvement in cell migration, MK5-HSP27 is

also activated in response to the activation of the cAMP-PKA (protein kinase A) pathway and there has not been a study looking at MK5-HSP27 or even ERK3/4-MK5-HSP27 involvement during cellular stress like that of inflammation.[65]

Finally, since ERK3 and ERK4 are understudied kinases there is a lot still to be learned about their biological functions and therefore it is advantageous to look at both in various contexts, including its potential function(s) in various immune responses to pathogens and tissue injury.

Acute kidney injury

Acute kidney injury (AKI) is defined as a rapid decrease in glomerular filtration rate (GFR) caused by vascular and tubular factors that include ischemia, hypoxia and nephrotoxicity.[66–68] Up to 7% of hospitalized patients develop AKI, this increases to 25% in intensive care unit patients, of which 5% will need renal replacement therapy.[69,70] It is usually diagnosed by increases in serum creatinine or blood urea nitrogen; however, with the increased study of AKI, biomarkers such as IL-18, kidney injury molecule-1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL) have been used for diagnosis.[71] While AKI incidence has decreased and the use of biomarkers contributes to earlier detection, AKI is still associated with a high mortality rate.[72] Additionally, it is now believed that inflammation plays a major role in the pathophysiology of AKI.[73,74] Endothelial injury increases macrovascular permeability leading to the recruitment of immune cells to the injured kidney during AKI; likewise damage to tubular cells produces pro-inflammatory factors that also result in immune cell recruitment.[75,76] This insight has led to the use of various anti-inflammatory therapies, which include the use of mycophenolate, α-melanocyte stimulating hormone (α-MSH), targeting pro-inflammatory pathways implicated in AKI, as well as lymphocyte or macrophage depletion.[77] To this end, further understanding of the inflammatory response in AKI can advance our understanding and ameliorate the treatment of AKI.

Pathophysiology of AKI

While the pathophysiology of AKI remains unclear, recent progress in identifying and elucidating the mechanisms and mediators of AKI can help identify a global definition of the disease progress and help reveal novel potential therapeutic targets for AKI prevention or early

treatment.[78,79] AKI can be broken down into three idealized phases: cell damage and cell death caused by kidney insult, acute inflammation and repair and regeneration of the kidney (Figure 7).[80]

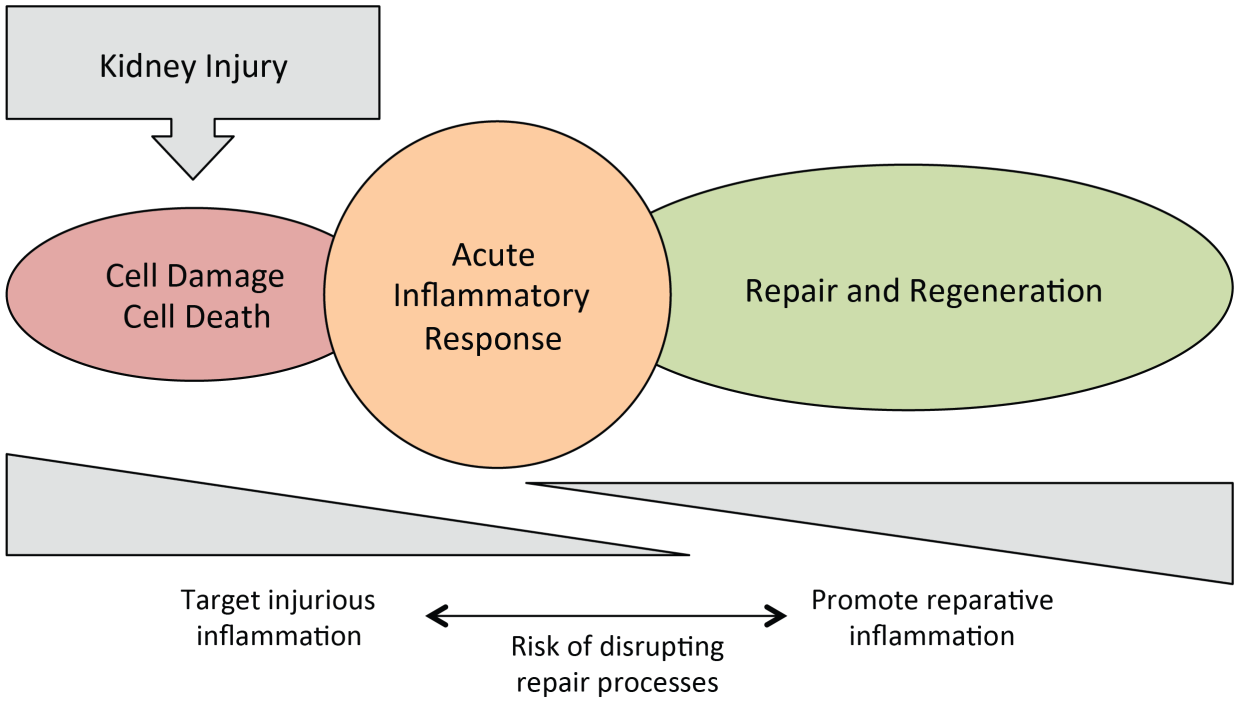

FIGURE 7. Idealized phases of AKI. After acute kidney injury, cell damage and death trigger activation of kidney resident cells causing an acute inflammatory response through the production of cytokines and chemokines and the recruitment of immune cells. If properly regulated, the acute inflammatory response evolves into repair and regeneration through the plasticity of immune cells, resolving AKI. Adapted from reference 80.

The initial phase of AKI is governed by cell damage and cell death that varies in severity depending on the specific nature of the insult, be it nephrotoxicity, ischemia or sepsis. This phase can last minutes to hours and involves various cells types: epithelial, endothelial and other parenchymal cells in the affected zone or the entire injured kidney generating a broad range of factors that induce an acute inflammatory response.[78,79,81,82]

Acute inflammation the next phase, which overlaps with the previous, can last hours to days and is mediated primarily though immune cells. Important immediate inflammatory cell

responders are resident macrophages and dendritic cells.[83,84] Additionally, there are also rapid responses that occur in the bloodstream involving endothelial adherence and activation of monocytes and neutrophils which lead to the infiltration of neutrophils, macrophages, T cells and other myeloid and lymphoid effectors.[83,85–89] The activation of these resident and recruited cells are mediated by the production of classic pro-inflammatory mediators such as cytokines, chemokines, free radical species, enzymes and lipid mediators that have high cytotoxic potential, amplifying and extending cell damage and death while also signaling tissue injury.[81,83] Because of this, the inflammatory phase of AKI can be seen as both consolidating and worsening kidney dysfunction emphasizing the need for an improved understanding of cellular and molecular components of AKI in an attempt to limit organ damage in the early phases of AKI. Interestingly, recent studies have highlighted that the acute inflammatory response also incorporates counter-regulatory components, such as the production of anti-inflammatory cytokines (e.g. IL-10).[90–92]

These counter-regulatory mediators, while involved in during inflammation are key players in the third and final phase of AKI, repair and regeneration.[79,93,94] Experimental models have shown that programmed transition in the phenotypes of immune effect cells, especially macrophages, involves specific alternative intracellular signaling pathways and mediators.[83,84,93–95] Understanding phenotypic plasticity of these effector cells and their restorative mechanisms can help harness them to actively promote repair after AKI as well as acute organ injury in general.[72,79,96]

AKI and Inflammation

Renal vascular endothelium and tubular epithelium

The initial phase of AKI causes changes to the integrity to the endothelial cell layer leading to endothelial dysfunction causing the infiltration of immune cells into the injured kidney.[75,77,97] Brodsky *et. al.* show in a rat model of renal ischemic injury (IRI) that there is a loss of endothelial cells from afferent arterioles and an interruption of endothelial contacts, which can be reversed though endothelial cell transfer.[98] Interestingly, increased microvascular permeability after IRI was attenuated in mice deficient in $CD3^+$ T cells, suggesting that molecules like sphingosine-1-phospate (S1P, a regulator of both immune systems and vascular

function) and immune system effector, like T cells, can also be mediators of increased vascular permeability after renal injury.[99] In fact, treatment with activation of S1P with the pro-drug FTY-720 does attenuate renal injury in the mouse model of IRI.[100]

Additionally, IRI regulates the expression of adhesion molecules that facilitate endothelial-leukocyte cell interactions. In particular, the expression of intracellular adhesion molecule 1 (ICAM-1) increases after kidney IRI and mice lacking ICAM-1 are protected from renal injury attenuating leukocyte adhesion to endothelial cells reducing inflammation and the extension of cellular injury.[101] Moreover, renal endothelial cells upregulate the expression of C-X3-C motif ligand 1 (CX3CL1, also fractalkine), a substrate for the C-X3-C motif receptor 1 (CX3CR1) which is highly expressed on macrophages and acts as a mediator of macrophage recruitment during inflammation; pretreatment with a neutralizing CX3CR1 monoclonal antibody reduced the severity of AKI.[102]

In addition to regulating vascular permeability, renal endothelial cells along with renal tubular epithelial cells (TECs) express toll-like receptors (TLRs) that recognize and interact with microbial and non-microbial endogenous substances released by damaged cells to evoke immune responses.[103] For instance the release of high mobility-group B1 (HMBG-1) proteins that can activate inflammation in wild type mice, but not TLR4-deficient mice.[104,105] Similarly, mice that lack either TLR2 or TLR4 display blunted cytokine and chemokine production and reduced macrophage and neutrophil infiltration in response to models of IRI or cisplatin-induced AKI.[106–109] It is also known that both TLR2 and TLR4 are upregulated on TECs in response to kidney injury.[108,110,111] Additionally, it is believed that both MyD88-dependant and independent pathways of TLR activation are involved during injury (Figure 8).[112]

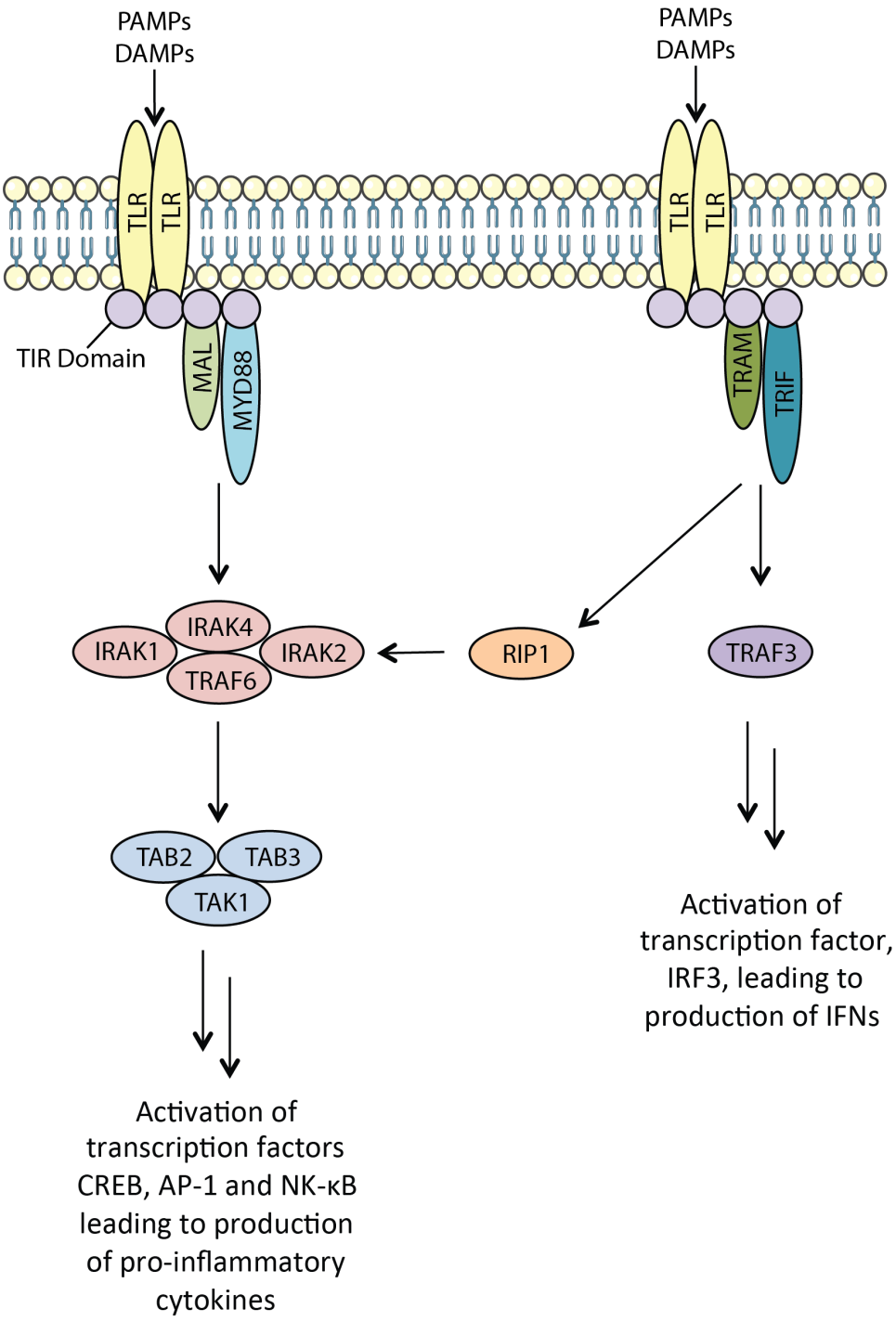

FIGURE 8. MYD88-dependant and MYD88-indepenant TLR signaling.

Taken together, these studies suggest that the renal endothelium and epithelium play roles in the inflammatory response to kidney injury by promoting the production of cytokines and chemokines thereby promoting the accumulation of immune cells.

Cytokines and Chemokines

Cellular damage/death and its associated molecular products are the key triggers for inflammation after kidney insult.[113] Once insult occurs, resident kidney cells release damage-associated molecular patters (DAMPs) such as HMBG-1, histones, heat shock proteins (HSPs), fibronectin, and biglycan into extracellular spaces. These activate pattern recognition receptors (PRRs) including TLRs, and nucleotide-oligomerization domain like receptors (NODs) that are found on epithelial, endothelial and resident leukocytes and thereby initiating a transcriptional response through transcription factors like nuclear factor κ light chain enhancer of activated B cells (NF-κB), HSP1 and hypoxia inducible factor 1-α (HIF-1α).[79,83,114–118] Their activation stimulates the synthesis of time-dependent pro-inflammatory and anti-inflammatory cytokines and chemokines throughout all stages of AKI such as IL-1β, Il-6, Il-18, IL-4, IL-10, TNFα, monocyte chemoattractant protein-1 (MCP-1, also known as CCL2), regulated upon activation normal T cell expressed and secreted (RANTES, also known as CCL5), CX3CL1, macrophage inflammatory protein-2 (MIP-2), ICAM-1, and transforming growth factor-β (TGF-β).[79,83,119–123] While there are a vast number of studies and reviews that investigate specific mediators of inflammation, be it a cytokine or chemokine, a few of interest will be further discussed here.

Interleukin-1β (IL-1β)

IL-1β cytokine levels are increased in mice following ischemic insult and recruits leukocytes to the area of injury.[101] TLRs expressed on intrarenal cells activated by DAMPs initiate a NF-κB transcriptional response producing immature forms of IL-1β and IL-18, termed pro-IL-1β as well as pro-IL-18. To mature these two cytokines, a second signal leads to the formation and activation of an intracellular multi-protein complex called the inflammasome that is comprised of a NOD-like receptor (NLR) family protein (NLRPs, the best characterized being NLRP3), apoptosis-associated speck-like (ASC) protein, caspase recruitment domain family member 9 (CARD9) and pro-caspase 1 enzyme. This process matures pro-caspase 1 into caspase 1 which then can proteolytically cleave pro-IL-1β and pro-Il-18 into their mature forms for secretion (Figure 9).[124–127]

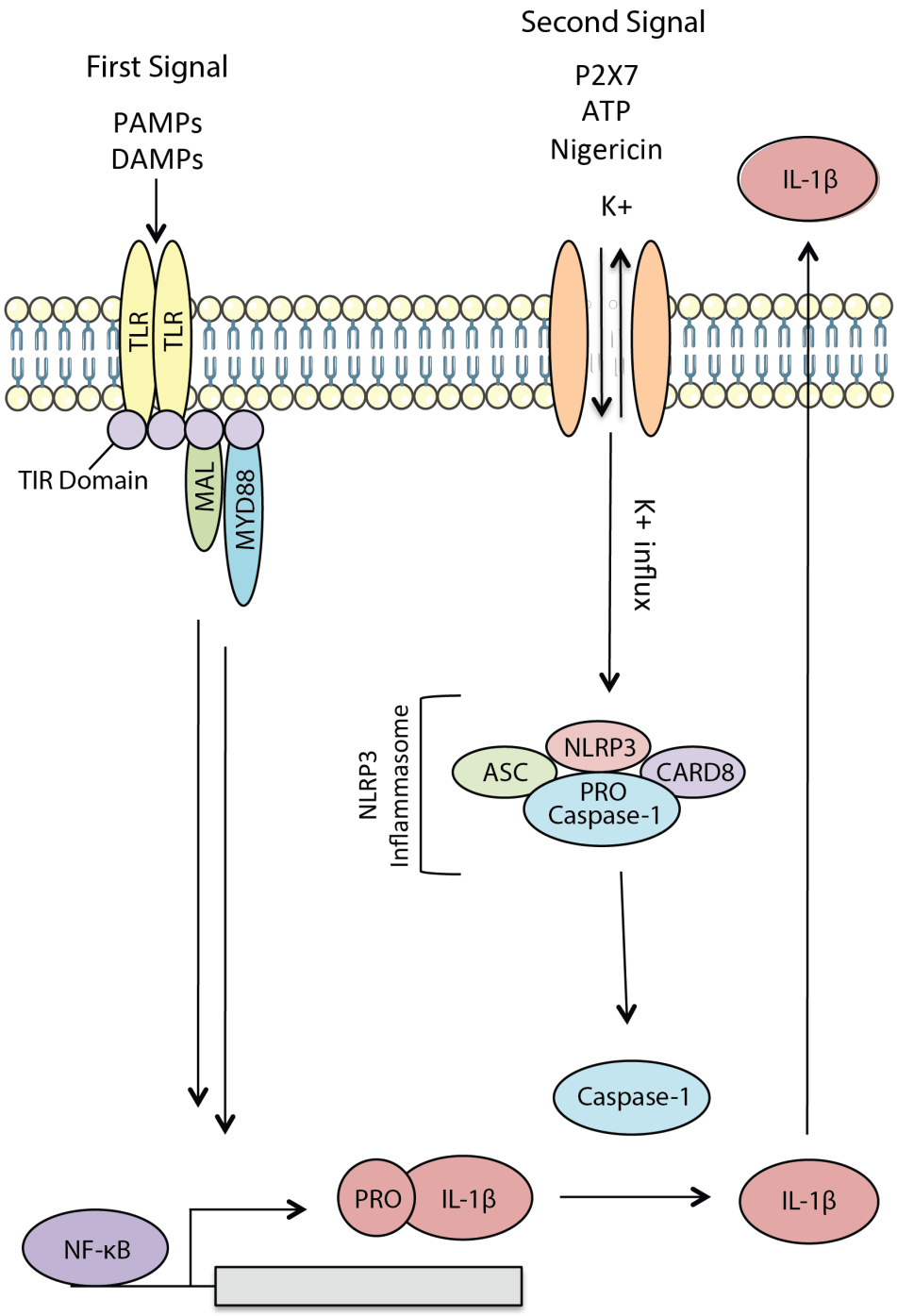

FIGURE 9. Two signal pathway and activation of the NLRP3 inflammasome.

Studies investigating the blockade of IL-1β signaling during AKI have revealed that using receptor antagonists or IL-1β-deficient mice reduces neutrophil infiltration into the kidney

following ischemic injury but had no effect on the resultant loss of renal function.[128] However, more investigation in required.

Tumour Necrosis Factor-α (TNFα)

TNFα is a potent pro-inflammatory cytokine and mediator of inflammatory tissue damage and is increased in both ischemic and cisplatin-induced AKI.[129–131] In rats, splenectomy attenuated renal injury by decreasing the production of TNFα and other pro-inflammatory cytokines.[129] Similarly, genetic or pharmacological inhibition of TNFα reduced the expression of other inflammatory cytokines, namely IL-1β, CCL2 and CCL5.[130] Moreover, TNFα knockout or inhibited mice were resistant to cisplatin nephrotoxicity.[130] A study by Zhang, *et. al.* using chimeric mice in which the bone marrow was ablated and replaced with donor bone marrow cells from wild type or TNFα knock-out mice determined that TNFα production was largely produced by kidney resident cells rather than infiltrating leukocytes.[132] Conversely, there is also support that infiltrating cells do contribute to TNFα production as the deletion of T cells protected against AKI and reduced levels of TNFα.[131] TNFα mediates biological activities through two different receptors, tumour necrosis factor receptor (TNFR) 1 and TNFR2. Interestingly, TNFR2-deficient mice had lower TNFα serum levels and developed significantly less severe renal dysfunction compared to TNFR1-deficient mice in a cisplatin-induced model of AKI; suggesting that TNFR2 is more important during AKI.[133]

Tumour Necrosis Factor-like Weak Inducer of Apoptosis (TWEAK)

Tumour necrosis factor-like weak inducer of apoptosis (TWEAK, Apo3L, TNFSF12) is a member of the TNF superfamily (TNFSF).[134] TNFSFs are widely expressed on many different cell types and play important roles in immune responses, inflammation, cell homeostasis and tissue repair.[135] TWEAK and its receptor, fibronectin 14 (Fn14), have multiple functions that depend on the microenvironment, the cell type and the state of activation; and therefore the cytokine is poorly understood. So far, TWEAK has been found to regulate cell proliferation, cell death, cell migration, cell differentiation, tissue regeneration, neo-angiogenesis and inflammation.[136–138] During tissue injury, repair and remodeling (events that occur in AKI), Fn14 expression is strongly unregulated and TWEAK expression, while not as strong as Fn14, is also increased.[137,139,140] TWEAK-induced inflammation is considered to be mediated by the canonical NF-κB pathway; numerous studies have confirmed NF-κB activation during AKI.[141–

[143] In addition, Sanz, *et. al.* have also described the activation of non-canonical NF-κB pathway through TWEAK involving NFκB2 and RelB during AKI.[144] Targeting TWEAK in a folic acid-induced model of AKI decreased TEC expression of NF-κB inducible CCL2 and CCL5, as well as NFκB2/RelB inducible CCL21 and interstitial inflammation by recruited macrophages and T cells.[141,144] Moreover, TWEAK-deficient mice showed a decrease in peak TEC apoptosis and a decreased peak of proliferation; likewise, Fn14 blockade reduced apoptosis in ischemic-induced AKI.[145,146] Interestingly, while it has been shown that TWEAK modulates IL-6 expression in cultured TECs, it is not translated to *in vivo* models, and therefore it is believed that there is another, more potent, modulator of IL-6 during AKI – thus adding to the complexity of AKI and identifying challenges between *in vitro* and *in vivo* investigation.[141] Because of this, and TWEAK's multifactorial biological functions, it is a promising cytokine for further investigation during AKI.

CCL2; Monocyte chemoattractant protein-1 (MCP-1)

MCP-1 is also known as C-C motif chemokine 2 (CCL2). CCL2 mRNA is upregulated during ischemic and nephrotoxic-induced AKI and enhances the infiltration and activation of macrophages thought its receptor, C-C motif chemokine receptor type 2 (CCR2).[147] Recently CCL2 has been used as a biomarker of the mononuclear inflammatory processes that occur in AKI.[148] In a setting of human kidney transplant (in which AKI is a common risk factor), donor kidneys with a TLR4 loss-of-function allele produce less TNFα and less CCL2, exhibiting a higher rate of immediate graft function.[105] Moreover, the delivery of truncated CCL2 protein into mice with ischemia-induced AKI also blocks the activation of CCR2 and thereby inhibits macrophage infiltration.[149,150] Interestingly, CCL2 deficiency alone does not have an effect on renal macrophage infiltration; suggesting ligand redundancy for CCR2 and further demonstrates the complexity of inflammation during tissue injury and AKI.[151] Furthermore, CCL2/CCR2 have been shown to guide regulatory T cells (T regs) in other pathologies and therefore may also drive T reg accumulation in AKI.[147,152,153] Taken together, CCL2/CCR2 axis plays a role in macrophage infiltration into the kidney as well as other immune cell types.

CCL5; Regulated on activation normal T cell expressed and secreted (RANTES)

RANTES (also known as CCL5), is a member of the C-C motif chemokine family secreted by various tissues and is well known for its chemoattractant effect on immune cells

including neutrophils, monocytes and lymphocytes.[154–156] While the role of CCL5 during AKI is not well known, high levels of CCL5 have been found on interstitial cell surfaces and has been shown to act as a powerful activator of leukocytes during ischemic AKI.[157] Mice deficient in CCL5 show impaired recruitment of inflammatory cells to the injured kidney when compared to wild type mice as well as a reduction in IL-1β, TNFα and CCL2.[118] Furthermore, in other forms of ischemia (atherosclerosis, stroke, and myocardial infarction) CCL5 has been shown to play a role during inflammatory cell infiltration and the release of inflammatory cytokines during post-ischemic inflammation.[157–161] Interestingly, CCL5 antagonists reduced tissue damage in these ischemic settings by significantly reducing the infiltration of leukocytes and lymphocytes.[157,160] Furthermore, CCL5-deficiency or treatment with an anti-RANTES monoclonal antibody (mAb) leads to a significant reduction in inflammatory cytokines including IL-6, IL-10, TNFα, and CCL2.[118,157,159,160] Thus it can be hypothesized that CCL5 plays similar roles in ischemic-AKI as well as in tissue injury in general.

Inflammatory Cells

Neutrophils

Neutrophils are critical mediators of innate immunity and respond rapidly to invading pathogens and tissue injury by phagocytizing pathogens and particles, generating reactive oxygen species, and releasing anti-microbial peptides. Mouse models of IRI and cisplatin-induced AKI have confirmed neutrophil accumulation during the early phase of AKI.[87,101,162–164] Moreover the depletion or the prevention of neutrophil infiltration to the kidney reduces injury.[101,165] Some studies in other species (apart from mice) reported a lack of significant neutrophil infiltration into the injured kidney therefore conferring no benefit after neutrophil depletion; these results however, may be related to the differences in experimental models and limitations of various neutrophil depletion strategies.[123] Furthermore, neutrophils have been found on the biopsies of AKI patients.[74,166]

It is also known that many factors that affect neutrophil infiltration/activation, such as neutrophil elastase, tissue type plasminogen activator, hepatocyte growth factor and CD44 expression also contribute to injury during AKI.[167–170] Apart from releasing granules, neutrophils also can produce pro-inflammatory cytokines such as interferon (IFN)-γ, IL-17 and

the chemokine C-X-C motif ligand 1 (CXCL1), in the injured kidney.[87,165] All suggesting that neutrophils play a role in the pathophysiology of AKI.

Macrophages

Macrophages are phagocytic cells that arise from monocytes in the blood and are also present as resident cells in some tissues. They can act as both an effector cell and antigen-presenting cell (APC) thereby connecting the innate and adaptive immune systems. During AKI, macrophage numbers increase rapidly after insult and their infiltration is mediated by the CX3CL1/CX3CR1 and CCL2/CCR2 signaling pathways.[102,147,151,171] These macrophages have a distinct 'inflamed' F4/80^{low}Ly6C^{high}GR-1^{+}CX3CR1^{low} phenotype.[151] In both ischemia and cisplatin-induced AKI, CX3CL1 expression increases on endothelial cells facilitating the infiltration of monocytes and macrophages that express its receptor, CX3CR1. Blocking this pathway via a CX3CR1 antibody effectively reduced the severity of AKI while the adoptive transfer of activated RAW264.7 macrophages (a murine macrophage cell line) exacerbates injury.[102,151] Comparably, macrophages lacking CCR2 do not infiltrate the injured kidney and the resultant injury is less severe.[151] Similarly, depletion of macrophages by liposomal clodronate prior to kidney ischemia reduces renal injury and adoptive transfer of macrophages reverses the effect.[172,173] Conversely, while macrophage infiltration is seen in cisplatin-induced experimental AKI, blocking macrophage infiltration to the injured kidney did not reduce renal injury.[174]

Once in the kidney, macrophages are found to be key sources of many pro-inflammatory cytokines, including IL-6 and TNFα, as demonstrated by flow cytometry; as well as IL-1α, IL-8, and IL-12p40/70.[96,151,175,176] While the release of these cytokines promotes inflammation, an increased awareness of macrophage plasticity has arisen and has lead researchers to investigate the idea that macrophages can either promote inflammation by displaying a pro-inflammatory phenotype, termed M1 macrophages, or inhibit inflammation by exhibiting an anti-inflammatory phenotype, termed M2 macrophages throughout AKI.[177]

In a well-designed study by Lee, *et. al.* macrophages in the early stages of AKI display a M1 phenotype, however, several days later the same M1 macrophages adopt an M2 phenotype that is involved in subsiding inflammation and is vital to the repair process.[95] Therefore

suggesting that macrophages have a complex role in both injury-induced inflammation and in the subsequent repair process. Switching of macrophages from an M1 state to M2 appears to be caused by changes in the intrarenal microenvironment as well as the macrophage phagocytic uptake of apoptotic neutrophils.[178,179] Once in an M2 state macrophages can secret anti-inflammatory factors such as IL-10 and TGF-β among others, as well as secrete potential trophic growth factors and angiogenic factors.[180,181] The benefit of macrophage a M2 phenotype was demonstrated by Wang and Harris whereby *ex vivo* programming of macrophages towards the M2 phenotype improves chronic renal inflammation, suggesting possible amelioration in AKI.[181] Likewise, while macrophage depletion via liposomal clodronate lessened kidney injury, Jang, *et. al.* found that it also caused impaired recovery from ischemia-induced AKI; however, administration of macrophages during the repair phase increased renal cell proliferation and enhanced recovery.[172,173,182,183] Similarly, one study ablated macrophages using a transgenic approach whereby the dipheria toxin (DT) receptor is expressed on $CD11b^+$ cells. Administration of DT reduced macrophages in these mice which resulted in a longer and less successful recovery from AKI.[184] The same study also suggested that the reparative potential may be mediated by Wnt ligands expressed on the surface of M2 macrophages and can therefore interact with epithelial cells expressing Wnt-receptors; the Wnt pathway being involved during tissue regeneration.[184,185] Another study has shown that the treatment of macrophages with netrin 1 suppressed the inflammatory response by inducing a change towards an M2 phenotype, protecting against ischemic AKI.[186] Intriguingly, Zhang, *et. al.* demonstrate that an increased number of M2 macrophages is mainly caused by the *in situ* proliferation of resident macrophages stimulated by colony stimulating factor 1 (CSF-1).[93] The genetic or pharmacological inhibition of CSF-1 hindered intrarenal proliferation of macrophages and dendritic cells, reduced M2 macrophage polarization and attenuated renal recovery.[93] Moreover, it is believed that CSF-1 also plays a role in attenuating interstitial fibrosis.[187]

Although M2 macrophages have been reported to be beneficial to the repair process, other studies suggest that macrophages may influence the development of renal fibrosis during the recovery phase of AKI.[182,188] In attempt to prevent fibrosis, the macrophage specific deletion of TGF-β, a profibrotic cytokine, did not stop renal fibrosis following severe ischemic AKI.[189]

Furthermore, the repeated exposure to nephrotoxic agents such as cisplatin and a repeated administration increases renal fibrosis.[190]

Taken together, macrophages play an instrumental role during AKI from the initial stages of immune cell infiltration, inflammation and tissue regeneration. It is clear that further investigation is needed to dissect the roles of both M1 and M2 macrophages at all stages of AKI.

Dendritic Cells

Dendritic cells (DCs) are important messengers between the innate and adaptive immunity as they are able to present antigens to T cells. While their role in AKI is not entirely understood, there are several studies that show DC involvement.

Interestingly, $CD11c^{+}MHCII^{+}$ DCs are the most abundant leukocyte subset in the normal mouse kidney.[191] Upon kidney insult, DCs bind to the endothelium and migrate to the kidney increasing their numbers and their expression of major histocompatibility complex class II (MHCII) antigens.[111,192] Moreover, DCs migrate to the renal draining lymph nodes after ischemic-injury and induce T cell proliferation thus linking the adaptive immune response to AKI.[193]

Importantly, Dong, *et. al.* demonstrated that after ischemic insult, renal DCs produce the pro-inflammatory cytokines and chemokines, TNFα, IL-6, CCL2 and CCL5, and that the depletion of DCs in an ischemic model of AKI significantly attenuates TNFα production as well as reduce kidney injury and dysfunction.[194,195] Conversely, the depletion of DCs in cisplatin-induced AKI results in more severe renal dysfunction and inflammation, suggesting that DCs have renoprotective effects in cisplatin-induced AKI but not in ischemic AKI.[162,196] Furthermore, DC production of IL-10 is increased in response to cisplatin-induced AKI, which leads to decreased cisplatin nephrotoxicity and inflammation.[196] In addition to the pro-inflammatory factors mentioned, Li, *et. al.* also show that IL-12 and IL-23 are mainly produced from activated DCs during ischemic-AKI and their downstream cytokines, IFN-γ and IL-17, also contribute to the activation of macrophages, neutrophil recruitment and thus amplify the immune response.[165]

While these findings suggest that DCs play a role in orchestrating the immune response during AKI, additional studies are required to determine whether DCs are protective or injurious during the early stages of AKI.

Natural Killer Cells

Natural Killer (NK) cells are large granular cytotoxic lymphocytes that lack B cells and T cell receptors. They are able to kill infected cells directly and also produce cytokines like IFN-γ and TNFα. During experimental AKI, TECs upregulate retinoic acid early inducible 1 (Rae-1), a NK activating ligand, which promotes TEC killing through the release of perforins by activating the natural killer group 2 member D (NKG2D) receptor on NK cells; the same report also demonstrates that the depletion of NK cells attenuates renal injury both structurally and functuonally.[89] Also, it is thought that NK cells facilitate the inflammatory process secreting cytokines and activating macrophages and neutrophils, and therefore should be further investigated during AKI.[197,198]

Lymphocytes

Lymphocytes are major mediators of adaptive immunity and were not expected to participate in AKI as they respond later in the immune response to alloantigens or self-antigens; nonetheless many studies have revealed their role in AKI through models of ischemic and nephrotoxic AKI.

T cells

Direct evidence of the pathophysiological role of T cells in AKI has been demonstrated in mice knockout for either $CD4^+$ T cells or $CD8^+$ T cells or both. Double $CD4^+/CD8^+$ knockout mice are largely protected from AKI displaying reduced kidney injury and dysfunction after ischemia or cisplatin-induced AKI.[131,199,200] T cell-targeted medications such as tacrolimus and mycophenolate mofetil substantially attenuate early renal injury following ischemia.[201,202] Plus, adoptive transfer of $CD4^+$ and $CD8^+$ T cells restores AKI.[199] Interestingly, in single knockout studies, $CD4^+$ knockout mimics the results seen in double knockouts and $CD8^+$ knockout results in kidney injury similar to that of wild type mice in ischemic injury, suggesting that $CD4^+$ T cells have a larger contribution to AKI pathophysiology.[199,200] Surprisingly, in model of cisplatin-induced AKI, mice that lack either $CD4^+$ or $CD8^+$ T cells alone suffered less kidney dysfunction, signifying differences in the two mouse models.[131] Seeing T cell involvement,

further studies have demonstrated that TCR engagement contributes to the establishment of full renal injury.[203] Moreover, a study done by Satpute, *et. al.* using *Foxn1*$^{nu/nu}$ mice (lacking T cells) reconstituted with either polyclonal CD4$^+$ T cells with a diverse array of TCRs, or CD4$^+$ cells from DO11.10 mice which only recognize chicken ova antigens, demonstrated that both TCR-repertoire-dependent and TCR-repertoire-independent factors mediate the role of T cells in AKI.[204]

Interestingly, Day, *et. al.* demonstrated that *RAG-1*$^{-/-}$ mice (lacking both T cells and B cells) are also protected from ischemia-induced AKI, which is restored upon adoptive transfer of CD4$^+$ T cells from wild type mice but not from *IFN-γ*$^{-/-}$ mice suggesting a role of T cell produced IFN-γ during AKI.[172] However, other researchers dispute this finding by demonstrating that *RAG-1*$^{-/-}$ mice are not protected from AKI.[205,206] Moreover, it is the reconstitution of T cells or B cells alone which confer renal protection.[206]

B Cells

Few studies have investigated the role of B cells in AKI. Apart from the reconstitution of B cells in *RAG-1*$^{-/-}$ mice, a study by Burne-Taney, *et. al.* has demonstrated that B cell deficiency in mice (μMT mice) conferred renal protection in a ischemic model of AKI.[206,207] Moreover, post-ischemic kidneys void of B cells express higher IL-10 and VEGF levels and exhibit more tubular proliferation and less tubular atrophy during the repair and regeneration phase when compared to wild type mice.[208] This was reversed by adoptive transfer 24-hours after ischemic injury.[208]

With discrepancies regarded above, further studies are needed to elucidate the mechanisms underlying the role of T cells and B cells during AKI.

In all, under ideal conditions, there is a balance between pro-inflammatory and anti-inflammatory factors as well as immune cells that ensures clearance of damaged tissue, repair and regeneration and thus a return to homeostasis. AKI often results in abnormal repair as a result of prolonged hypoxia/inflammation and/or a sustained secretion of profibrotic cytokines; leading to post-AKI fibrosis and potentially chronic renal dysfunction.[79,83] Thus, further

investigation is required in order to further elucidate mechanisms (cytokines, chemokines and immune cell types) involved in the progression and resolution of AKI.

Rational and Objectives

Preliminary research from our lab has demonstrated that the loss of ERK3's catalytic activity and the loss of ERK4 attenuate the production of CCL2 and CCL5 in a folic acid-induced mouse model of AKI (Figure 10A). Furthermore, histopathology of the affected kidneys revealed a decrease in macrophage infiltration into the kidneys of $ERK3^{KI}$ and $ERK4^{-/-}$ mice via the macrophage specific marker F4/80 (Figure 10B).

The main goal of my master's project is to explore ERK3 and ERK4's potential role in the innate immune system, more specifically during inflammation. Given the preliminary findings, the objectives are: (1) investigate the effect of ERK3 and ERK4 loss through various immune stimulations in various cells types involved in AKI such as TECs and macrophages; and (2) investigate ERK3's and ERK4's potential role in the maturation, polarization and migration of immune cells.

It is hypothesized that ERK3 and ERK4 activity promotes immune cell migration through intrinsic mechanisms and by fostering an inflammatory cascade. It is also believed that ERK3 and ERK4 promote immune cell maturation and activation and that blocking ERK3 and ERK4 activity can inhibit these processes.

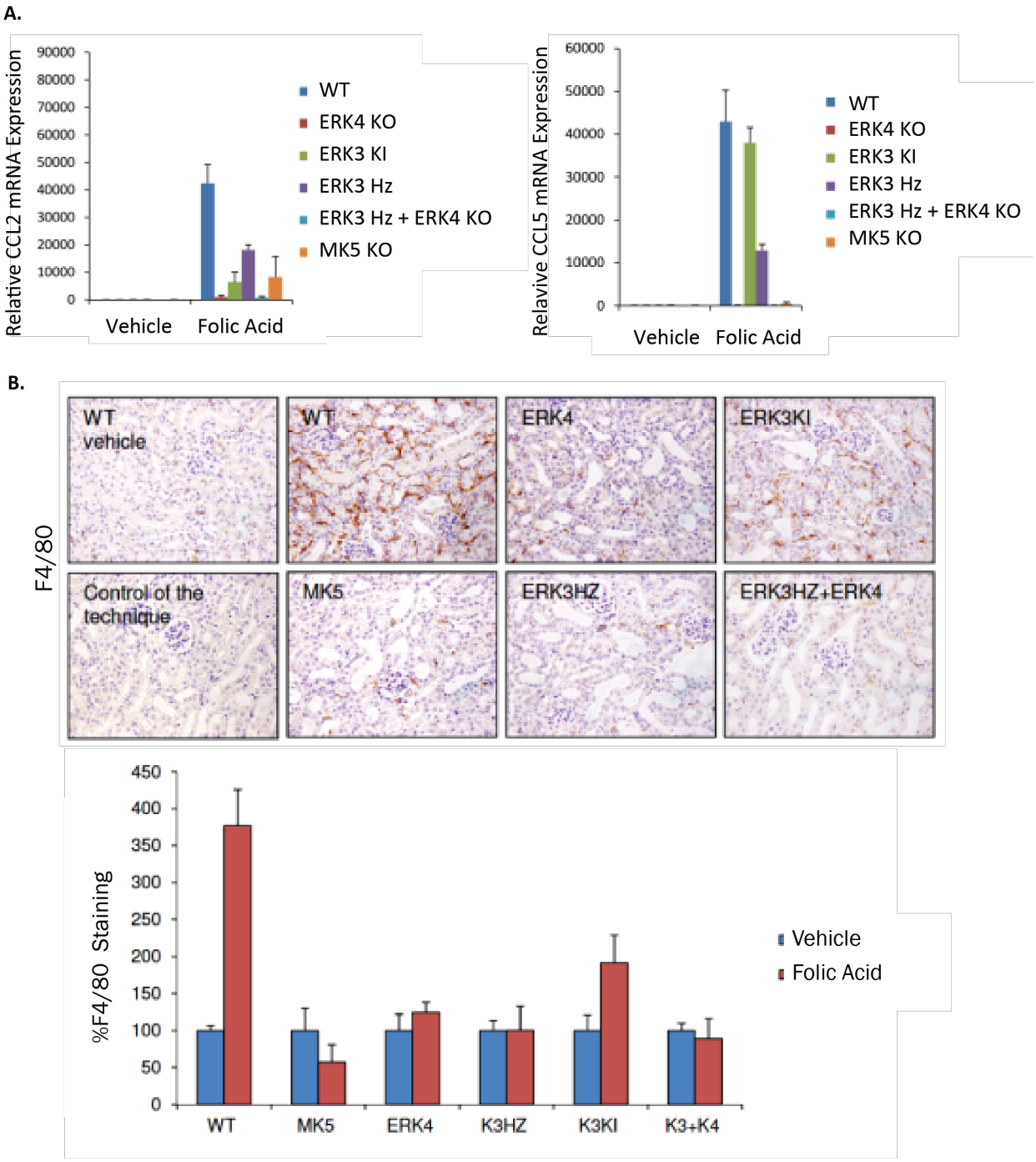

FIGURE 10. Potential role of ERK3 and ERK4 in AKI. A) Relative mRNA expression of CCL2 and CCL5 in whole kidneys of mice bearing a kinase-dead allele of ERK3 ($ERK3^{KI}$), ERK3 heterozygotes (ERK^{Hz}) and $ERK4^{-/-}$ (ERK^{KO}) mice after folic acid induced AKI. **B)** Histopathological analysis of

$ERK3^{KI}$, $ERK3^{Hz}$ and $ERK4^{-/-}$ mouse kidneys after folic acid induced AKI stained with macrophage marker, F4/80. Relative macrophage abundance is normalized to the wild type control using pixel density.

Materials & Methods

Reagents and antibodies

Recombinant mouse TWEAK protein was obtained from Kingfisher Biotech. Recombinant mouse TNFα and IFNγ proteins were obtained from ProspecBio. LPS was obtained from Invivogen. Nigericin was obtained from Invivogen.

The following commercial antibodies were obtained: anti-ERK3 (catalog number EP1720Y) from Abcam; anti-MK5 (catalog number M32220-050) from BD biosciences; anti-ERK4 (catalog number sc-68170) from Santa Cruz Biotechnology; anti-HSC70 (catalog number sc-7298); anti-GAPDH (catalog number sc-25778) from Santa Cruz Biotechnology; anti-F4/80-APC (catalog number 123116) from BioLegend; anti-CD11b-PE-Cy7-A (catalog number 25-0112-82) from eBioscience.

Plasmid constructs

plentiCRISPRv2 (Addgene plasmid #52961) was a gift from Feng Zhang.[209] plentiCRISPRv2-ERK3-Sg1-6 were generating by cloning sequence guides one through six into the plentiCRISPRv2 vector as previously described.[209]

TABLE 1. Forward and reverse RNA sequence guides.

No.	Forward	Reverse
1	CACCGCCATTGGGCTGCGGAGGCAA	AAACTTGCCTCCGCAGCCCAATGGC
2	CACCGGGCTGCGGAGGCAATGGCT	AAACAGCCATTGCCTCCGCAGCCC
3	CACCGAGCCCACATCGTCTGTTAAC	AAACGTTAACAGACGATGTGGGC
4	CACCGCTTTACTGGAAGAGCATGCC	AAACGGCATGCTCTTCCAGTAAAGC
5	CACCGCAAGGCGCAGATCGCGCTAG	AAACCTAGCGCGATCTGCGCCTTGC
6	CACCGCTGAATGGTCCCCGCGATGA	AAACTCATCGCGGGGACCATTCAGC

Mice

Wild type, *Mapk6*^{LacZ} heterozygous, ERK3 knock-in kinase dead (ERK3^{KI}), ERK3 conditional knockout (ERK3$^{\Delta/\Delta}$) and ERK4-deficient (*Mapk4*$^{-/-}$) mice were bred and housed

under specific pathogen-free conditions in filter-topped isolator cages under a 12/12-hour light/dark cycle with access to food and water *ad libitum* at the Institute for Research in Immunology and Cancer. Animals were handled in strict accordance with good animal practice as defined by the relevant local animal welfare bodies; the Canadian Council on Animal Care (CCAC) approved all experiments.

Cell Culture and lentiviral infections

293T cells were obtained from the American Type Culture Collection. The mouse cortical tubule epithelial (MCT) cell line was kindly provided by J. Poveda (Autónoma University). THP-1, RAW264.7 and L929 cells were obtained from M. Servant (Université de Montréal). 293T, MCT, L929 and RAW264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics at 37°C in a 5% CO_2 humidified atmosphere. THP-1 cells were cultured in RPMI supplemented with 10% fetal bovine serum and 0.05mM β-mercaptoethanol at 37°C in a 5% CO_2 humidified atmosphere.

293T cells were transiently transfected by using polyethylenimine. For lentiviral infections, 293T cells were transfected with pMD2.G/VSVG (Addgene plasmid #12259), pMDLg/RRE (Addgene plasmid #12251), pRSV-Rev (Addgene plasmid #12251) gifts from Didier Trono, and the indicated pLentiCRISPRv2 constructs.[210] After 48 hours, virus-containing culture media was filtered and used to infect cells. Polyclonal populations of infected cells were obtained by selection with puromycin. ERK3 knockout was confirmed by immunoblotting and polyclonal populations were used make monoclonal cell populations by limiting dilution.

Mouse Embryonic Fibroblasts (MEFs)

Mapk6^{LacZ} and *Mapk4*$^{-/-}$ embryos were dissected at embryonic day 14.5 (E14.5), and MEFs were prepared as described previously.[211] MEFs were cultured in DMEM supplemented with 10% newborn calf serum and antibiotics.

Inflammasome activation

Wild type, *Mapk6*^{LacZ} and *Mapk4*$^{-/-}$ MEFs were plated at 2.5×10^5 cells/ml in six-well plates. 18 hours after plating, the cells were stimulated with either vehicle, 1μg/ml lipopolysaccharide (LPS) alone for 3 hours, 20μM Nigericin alone for 30 minutes, or LPS for 3

hours followed by 30 minutes of Nigericin. Supernatants were then collected for ELISA and cells were washed with phosphate buffered saline (PBS) and used for quantitative PCR.

Enzyme-linked immunosorbent assay (ELISA)

Increased pro-IL-1β expression and maturation was stimulated through inflammasome activation with LPS and Nigericin in wild type, *Mapk6*^{LacZ} and *Mapk4*$^{-/-}$ MEFs as described above. IL-1β secretion was quantified by enzyme-linked immunosorbent assay (ELISA) using Mouse IL-1β/IL-1F2 DuoSet ELISA (R&D Systems) kit with the accompanying DuoSet ELISA Ancillary Reagent Kit 2 (R&D Systems) according to manufacturer's instructions.

Bone marrow derived macrophages

To generate bone marrow derived macrophages (BMDMs), the bone marrow cells from femurs from wild type and ERK3^{KI} and ERK3$^{\Delta/\Delta}$ mice were harvested and cultured as previously described.[212] Briefly, syringe isolated cells were incubated in DMEM supplemented with 10% fetal bovine serum, antibiotics and 20% L929 cell supernatant (containing macrophage colony stimulating factor (M-CSF)). On day 3 of culture, more media was added. On day 7 cells were washed, counted and either stained for flow cytometry or plated for polarization or migration experiments in DMEM containing 10% fetal bovine serum and antibiotics.

Flow cytometry

BMDMs from day 7 were harvested and stained with anti-F4/80-APC and anti-CD11b-PE-Cy7-A in FACS blocking buffer (2.4G2 cell supernatant diluted 10-fold with PBS) for 30 minutes. Then resuspended in FACS buffer (PBS with 1% formaldehyde) for acquisition and analysis on a FACSCanto (BD Biosciences).

TNFSF stimulation

MCT monoclonal cultures wild type (empty vector) and knockout for ERK3 were derived from plentiCRISPRv2 lentiviral infections and maintained as cell lines. To investigate the impact of ERK3 deficiency on TNFα and TWEAK stimulation, MCT cells where plated at 1×10^6 cells/ml in six well plates. 18 hours after plating, cells were stimulated with 100ng/ml TNFα or TWEAK at the indicated time points. Cells were then washed with PBS and collected for immunoblotting or quantitative PCR.

BMDM polarization

BMDMs derived from wild type and ERK3^{KI} were plated at 2×10^6 cells/ml in six-well plates. 18 hours after plating, the cells were classically activated (pro-inflammatory; M1 polarization) with 100ng/ml LPS and 20ng/ml IFNγ, or alternatively activated (anti-inflammatory; M2 polarization) with 20ng/ml IL-4 or received media alone (M0 condition) for 24 hours. Cells were then washed with PBS and collected for quantitative PCR.

Migration assays

Using a wound-healing scratch assay cell migration was assessed. The BMDMs from wild type and ERK3$^{\Delta/\Delta}$ mice were gown as a single monolayer in 6-well plates and then wounded by using a 200µl pipette tip. Phase contrast microscopy images of the wound area were taken at 0 and 6 hours, and wound healing was estimated by measuring the areas of the wound by using ImageJ software.

Immunoblotting

Cells were washed twice with cold PBS and lysed with Triton X-100 lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 50 mM sodium fluoride, 5 mM EDTA, 40 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10^{-4} M phenylmethylsulfonyl fluoride, 10^{-6} M leupeptin, 10^{-6} M pepstatin A, 1% Triton X-100) containing protease inhibitors for 20 minutes at 4°C. Lysed cells were then collected with a cell scraper and centrifuged. After centrifugation, the supernatant was collected, and protein levels were determined using BCA assay (ThermoFisher) as per the manufactures protocol. Samples were then further diluted and mixed with 5X Laemmli's buffer (60 mM Tris-HCL, ph6.8, 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, 0.1% bromophenol blue) and boiled at 95°C for 5 minutes before separated by electrophoresis on 10% polyacrylamide gels. Proteins were then electrophoretically transferred onto nitrocellulose membranes and blocked with 5% nonfat milk in TBS containing 0.1% Tween 20. Membranes were then incubated with primary antibodies overnight, washed, and incubated with HRP-conjugated anti-mouse or anti-rabbit. Prepared membranes were then incubated with enhanced chemiluminescence (ECL) solution and visualized by autoradiography.

Quantitative PCR analysis

Real-time quantitative PCR was performed on total RNA isolated with an RNeasy Mini Kit (Qiagen) as previously described using the PCR primers below.[213]

TABLE 2. Forward and reverse PCR primers.

Gene	Forward	Reverse
PRO-IL-1β	AGTTGACGGACCCCAAAAG	AGCTGGATGCTCTCATCAGG
CCL2	CTTCTGGGCCTGCTGTTC	GTGGGGCGTTAACTGCAT
CCL5	CCTACTCCCACTCGGTCCT	TTTCTTGGGTTTGCTGTGC
CCL7	AGGATCTCTGCCACGCTTC	TTGACATAGCAGCATGTGGAT
NOS2	CTTTGCCACGGACGAGAC	TCATTGTACTCTGAGGGCTGAC
IL-12B	TTGCTGGTGTCTCCACTCAT	GGGAGTCCAGTCCACCTCTA
IL-6	GCTACCAAACTGGATATAATCAGGA	CCAGGTAGCTATGGTACTCCAGAA
Arg1	GAATCTGCATGGGCAACC	GAATCCTGGTACATCTGGGAAC
RETNLA	CCCTCCACTGTAACGAAGACTC	CACACCCAGTAGCAGTCATCC
CHI3L3	GGTCTGAAAGACAAGAACACTGAG	GAGACCATGGCACTGAACG
GAPDH	TGTCCGTCGTGGATCTGAC	CCTGCTTCACCACCTTCTTG
HPRT	TCCTCCTCAGACCGCTTTT	CCTGGTTCATCATCGCTAATC

Expression of target genes was normalized to GAPDH/HRPT as a loading control and analyzed using the comparative method (ΔΔCt) or by looking at the normalized Ct values themselves.

Results

MEFs and the investigation of inflammasome signaling

The culture and use of MEFs represent a genetically clean and powerful system to test gene function due to their accessibility, rapid growth rates and ease to culture as compared to other types of primary cells. They represent a group of resident cells that have a ubiquitous distribution as tissue cells.[214] Moreover, because they are positioned to respond to newly activated innate immune cells, they are a useful tool to study inflammation and immunity.[214] Their nature, and the ability to retrieve *Mapk6*^{LacZ} embryos from mice despite neonatal lethality (note: at the time ERK3^{KI} nor ERK3$^{\Delta/\Delta}$ mice were available for use) and ERK4-deficient mice offered a starting point for the investigation of ERK3 and ERK4's potential roles during inflammation. We investigated the NLRP3 activation in the production of pro-inflammatory cytokine IL-1β, which plays a role during the AKI inflammatory process.[101,128] Here we activate the NLRP3 by first priming wild type, *Mapk6*^{LacZ} or ERK4-deficient MEFs with LPS thereby activating NF-κB and the transcription of cytokines including pro-IL-1β (Figure 9).[101,125,127,128] While there is no significant difference between the upregulation of pro-IL-1β mRNA between wild type and *Mapk6*^{LacZ} MEFs stimulated with LPS alone (Figure 11A), there appears to be a difference between ERK4 wild type and knockout MEFs (Figure 11B).

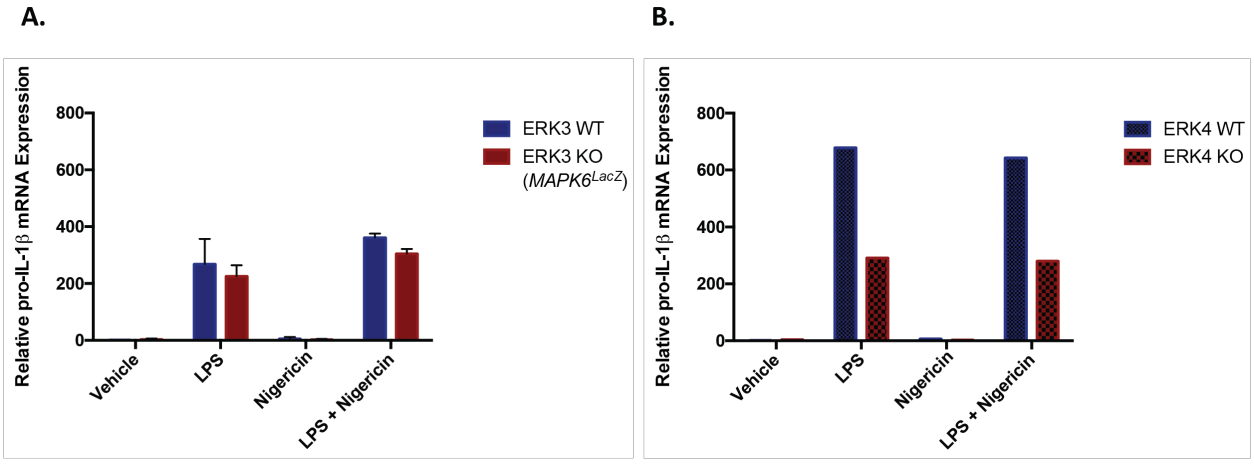

FIGURE 11. NLRP3 inflammasome regulation of pro-IL-1β mRNA in *Mapk6*^{LacZ} and ERK4$^{-/-}$ MEFs. A) Regulation of pro-IL-1β mRNA upon 3-hour stimulation with LPS (1μg/ml), 30-minute stimulation with Nigericin (20μM) or a combination of LPS followed by Nigericin in MEFs isolated from wild type and *Mapk6*^{LacZ} littermates. Values normalized to wild type vehicle (n=2). **B)** Regulation of pro-IL-1β mRNA upon 3-hour stimulation with LPS (1μg/ml), 30-minute stimulation with Nigericin (20μM) or a combination of LPS followed by Nigericin in MEFs isolated from wild type and ERK4$^{-/-}$ littermates. Values normalized to wild type vehicle (n=1).

Similarly, these differences are also observed when MEFs are further exposed to the second signal (Figure 11A and 11B), Nigericin is potassium ionophore that mimics the potassium efflux caused by the activation of the P2X7 receptor during NLRP3 inflammasome activation (Figure 9).[125,127,215,216] Nigericin alone does not effect the regulation of pro-IL-1β in wild type, *MAPK*^{LacZ} nor ERK4 knockout MEFs and appears not to potentiate the effect when used in conjunction with the TLR4 activator LPS (Figure 11A and 11B). This said, further investigation was halted as the production of mature IL-1β in the supernatant was below the limit of detection of the ELISA in all conditions (data not shown).

ERK3 is expressed in renal and macrophage cell lines

While ERK3 mRNA is known to be ubiquitously expressed, ERK4 expression is more restricted.[3,5,15,16] Thus we decided to analyze the expression of both kinases in the cell lines and primary cells used. ERK3 is expressed in murine cortical tubular epithelial (MCT) cell line, (Figure 12A). Interestingly, while ERK4 is reported as expressed in kidney tissues, MCT cells do not express ERK4 (Figure 12A).

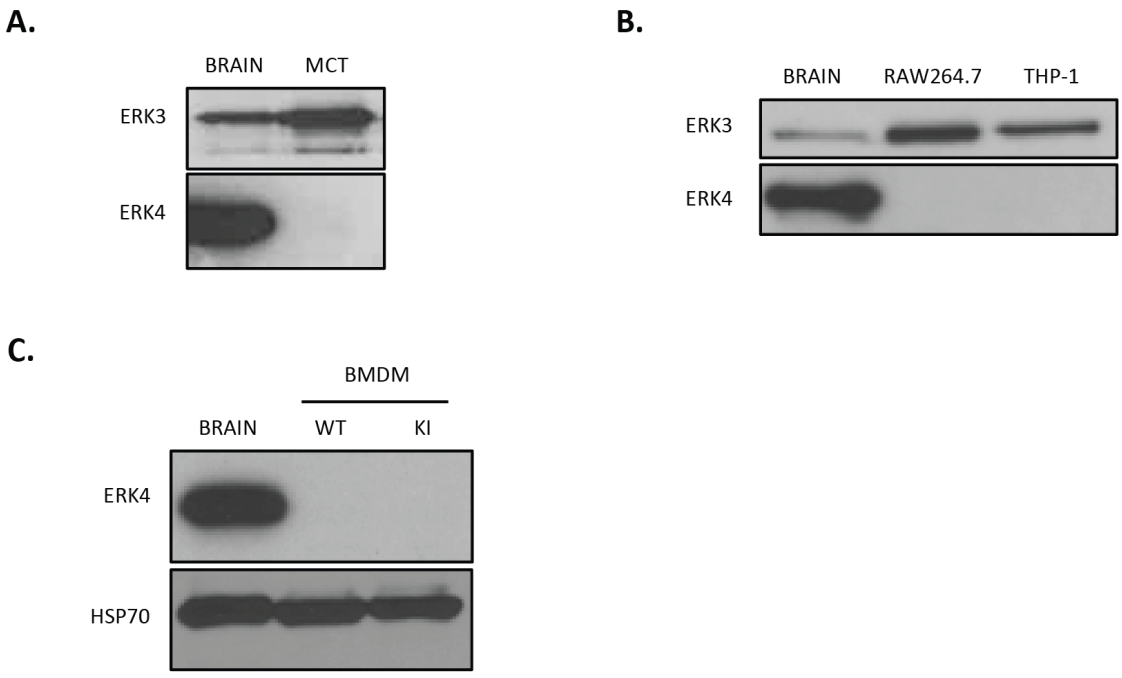

FIGURE 12. ERK3 and ERK4 expression in murine kidney and murine and human monocyte/macrophage cell lines. A) Expression of ERK3 and ERK4 in mouse cortical tubule (MCT) epithelial cell line. **B)** ERK3 and ERK4 expression in murine RAW264.7 macrophage cell line and human THP-1 monocyte cell line. **C)** ERK4 expression in BMDMs on day 7-post harvesting from wild type and $ERK3^{KI}$ mice. Brain is used as a positive control in all experiments for both ERK3 and ERK4.

Similarly, ERK3 is expressed in murine and human monocyte/macrophage cell lines, RAW264.7 and THP-1 and ERK4 is not (Figure 12B). Furthermore, ERK3 is expressed in BMDMs on day 7 (Mathien, S. and Meloche, S., unpublished data) and ERK4 is not expressed (Figure 12C). Importantly, optimization efforts for the silencing of ERK3 by RNAi or the knockout of ERK3 by CRISPR in RAW264.7 and THP-1 cell lines were unsuccessful, thus the implementation of BMDMs was necessary to investigate the effect of ERK3 loss on various biological functions of macrophages.

ERK3 expression is upregulated by members of the TNFSF family in tubule cells

It has been demonstrated that ERK3 is upregulated upon TNFα stimulation in HUVECs.[30] Furthermore, TNFSF members, TNFα and TWEAK, play roles in AKI that promote

inflammation and both have been linked to the production of CCL2 and CCL5 which were found to be markedly decreased in ERK3^{KI} and ERK4$^{-/-}$ mice (Figure 10A).[129–131,141–143] However, little is known about the effect of TNFα and TWEAK on ERK3 and ERK4 expression in other cell types. Thus, we examined the effect of TNFα and TWEAK stimulation on ERK3 and ERK4 expression on MCT cells *in vitro*. TNFα increases the expression of ERK3 over a time course of 24 hours that subsides by the 48-hour time point (Figure 13A). TWEAK also increases the expression of ERK3 over the 24-hour time period (Figure 13B).

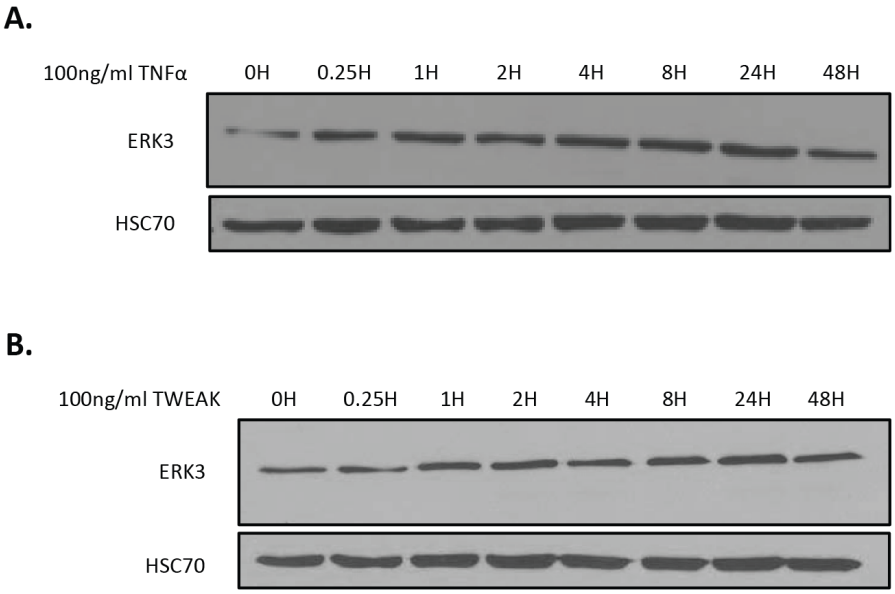

FIGURE 13. ERK3 expression upon TNFSF stimulation in MCT cells. A) ERK3 upregulation upon TNFα (100ng/ml) stimulation over a 48-hour time course. **B)** ERK3 upregulation upon TWEAK (100ng/ml) stimulation over a 48-hour time course. HSC70 was used as the experimental loading control. Representation of three independent experiments.

ERK3 deficiency reduces mRNA expression of CCL2 and CCL5 upon TNFSF stimulation in tubule cells

Seeing that TNFα and TWEAK stimulation upregulate the expression of ERK3, we next investigated the effect of ERK3 loss on the regulation of C-C motif chemokines, CCL2 and CCL5, induced by stimulation with either TNFα or TWEAK. To do so, clonal cell lines of MCT

cells deficient in ERK3 were generated by CRISPR (Figure 14B) and subjected to TNFα stimulation over a course of 4 hours and TWEAK stimulation over a time course of 24 hours.

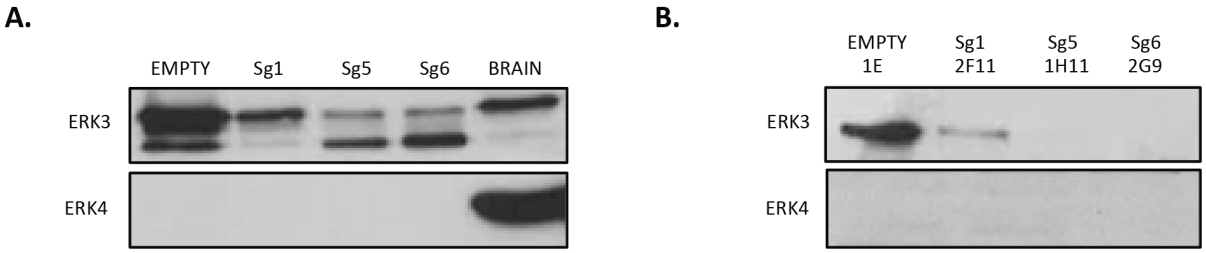

FIGURE 14. Lentiviral CRISPR knockout of ERK3 in MCT cells. A) ERK3 expression in polyclonal populations of MCT cells infected with either plentiCRISPRv2 empty vector or plentiCRISPRv2 plasmids encoding 3 distinct sgRNAs targeting ERK3. **B)** Confirmation of ERK3-deficiency in clonal cell lines generated from polyclonal populations in A.

TNFα induced an increase in CCL2 and CCL5 mRNA after 4 hours of stimulation (Figure 15A and 15B). ERK3-deficient clones, Sg5 1H11 and Sg6 2G9, show reduced increase of CCL2 and CCL5 when compared to their respective TNFα stimulated wild type empty vector control (EMPTY 1E1) (Figure 15A and 15B). Importantly, ERK3-deficient clone Sg5 1H11 produces significantly lower CCL2 and CCL5 than their empty vector counterpart (Figure 15A and 15B).

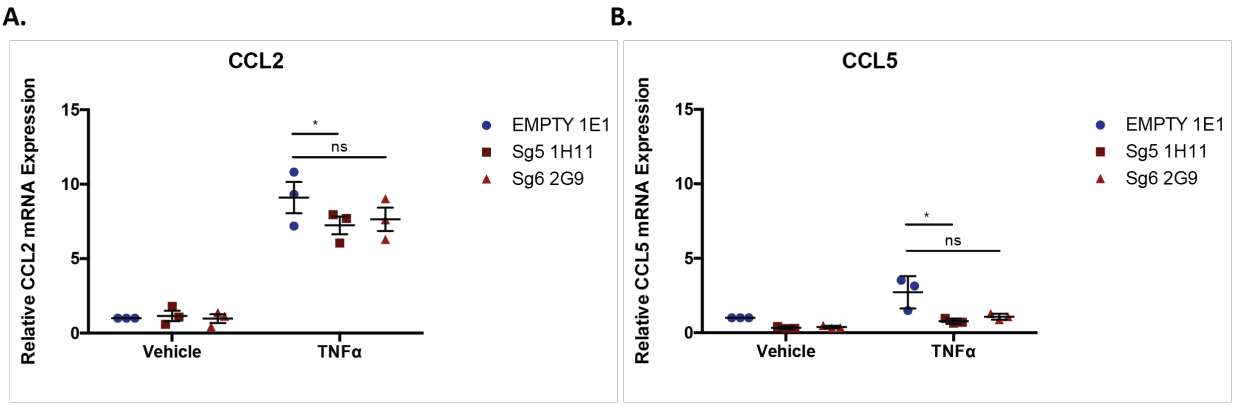

FIGURE 15. ERK3 regulation of CCL2 and CCL5 upon TNFα stimulation. A) CCL2 mRNA regulation upon 4-hour TNFα (100ng/ml) stimulation or vehicle in monoclonal MCT cell lines wild type or depleted of ERK3 by CRISPR/Cas9 gene editing. **B)** CCL5 mRNA regulation upon 4-hour TNFα (100ng/ml) stimulation or vehicle in monoclonal MCT cell lines wild type or knockout for ERK3. Results are expressed as means ± standard deviation of relative CCL2 or CCL5 expression levels normalized to values for the empty vector control treated with vehicle from data collected from three independent experiments (n=3). Statistical significance was determined by an unpaired *t* test. *, P≤0.05.

TWEAK induced a peak upregulation of CCL2 mRNA expression at the 4-hour time point (Figure 16A). At this time point ERK3-deficient clones, Sg5 1H11 and Sg6 2G9, show reduced CCL2 mRNA expression when compared to the wild type clone, EMPTY 1E1, expressing the empty plentiCRISPv2 vector (Figure 16B). Furthermore, ERK3-deficient clone Sg5 1H11 showed a statistically significant decrease in CCL2 at 4 hours (Figure 16B). Similarly, clone Sg5 1H11 showed a significant decrease in CCL5 mRNA expression at the peak of 24 hours when compared to the clone expressing the empty vector (EMPTY 1E1) (Figure 16C and 16D).

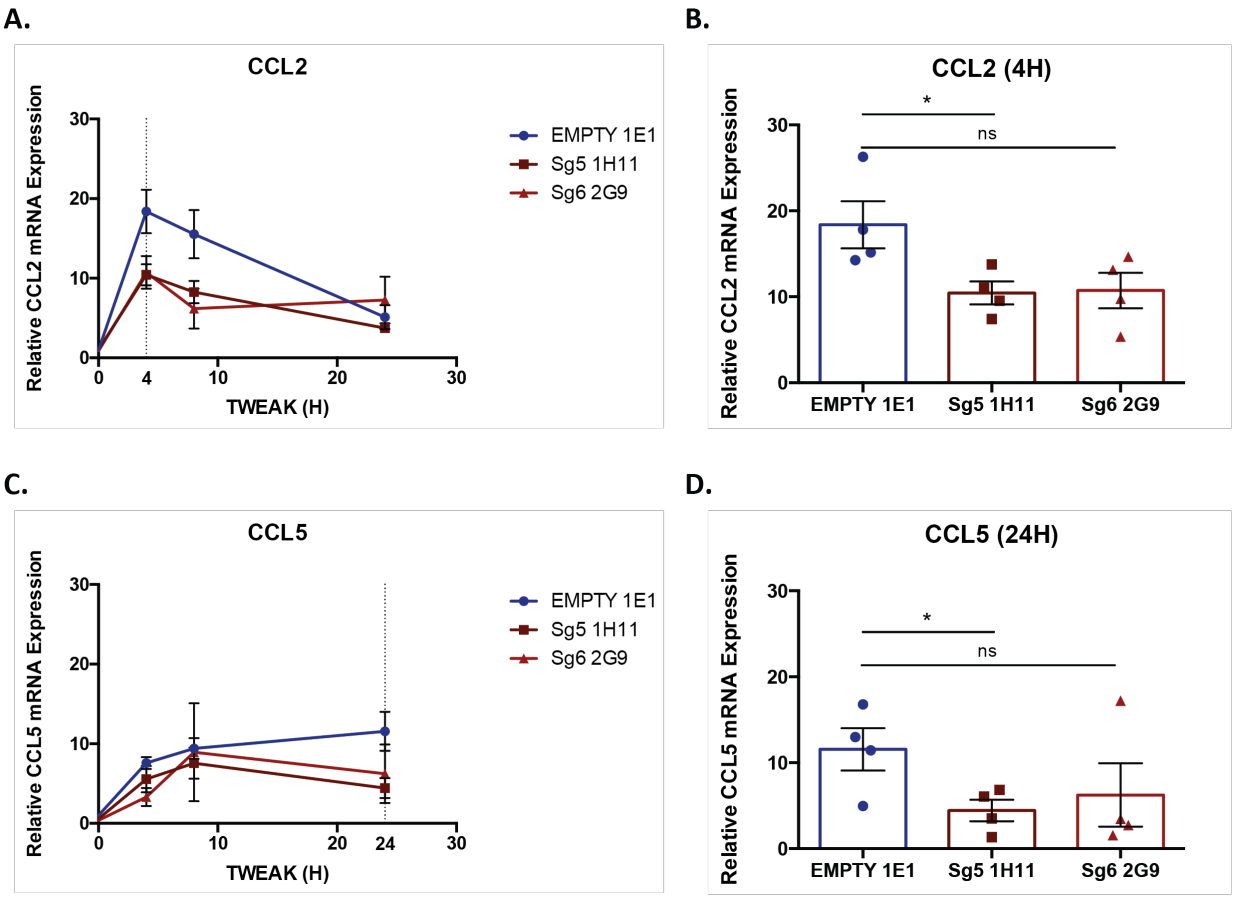

FIGURE 16. ERK3 regulation of CCL2 and CCL5 upon TWEAK stimulation. A) CCL2 mRNA regulation upon 24-hour TWEAK (100ng/ml) stimulation in clonal MCT cell lines wild type or knockout for ERK3. **B)** Peak CCL2 mRNA upregulation at 4 hours of TWEAK (100ng/ml) stimulation. **C)** CCL5 mRNA regulation upon 24-hour TWEAK (100ng/ml) stimulation in monoclonal MCT cell lines wild type or knockout for ERK3. **D)** Peak CCL5 mRNA upregulation at 24 hours of TWEAK (100ng/ml) stimulation. Results are expressed as means ± standard deviation of relative CCL2/CCL5 expression levels normalized to values for the vehicle 0-hour time point from data collected from four independent experiments (n=4). Statistical significance was determined by an unpaired *t* test. *, P≤0.05.

ERK3 loss does not effect monocyte to macrophage differentiation

Since ERK3 loss is suspected to attenuate macrophage migration to injured kidneys in AKI (Figure 10), we decided to look at the phenotype of BMDMs generated from mice that either have catalytically inactive ERK3 ($ERK3^{KI}$) or conditional ERK3 mice which are knockout for ERK3 ($ERK3^{\Delta/\Delta}$) by embryonic day 12.5 (Soulez, M., Meloche, S., *et. al.* manuscript

submitted). Thus, we studied the physiological importance of ERK3 during monocyte to macrophage maturation. While it has been previously reported that ERK3 is involved in the maturation/differentiation of other cell types, differentiation of P9 and PC12 cells into neurons, differentiation of C2C12 muscle cells and positive selection of T cells, its role during leukocyte maturation has not been investigated.[4,25,27,28] We observed no significant differences in the maturation of bone marrow derived macrophages, assessed by the macrophage specific markers F4/80 and CD11b, harvested from ERK3 knockout mice versus their wild type controls (Figure 17A and 17B).[217]

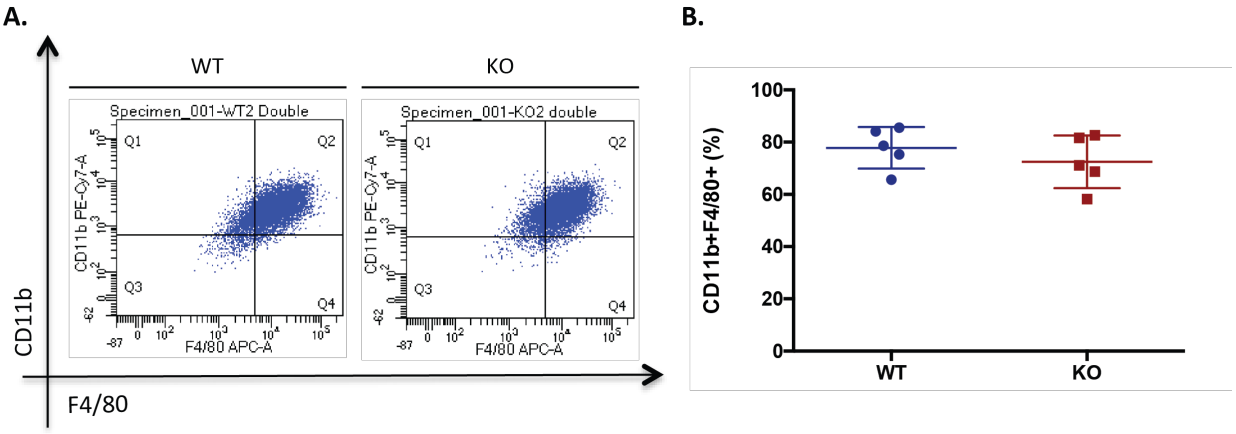

FIGURE 17. ERK3 deficiency does not impact BMDM maturation. A) Representative plots of BMDMs from one wild type and one ERK3 knockout mouse stained with macrophage specific markers F4/80 and CD11b after bone marrow harvesting and 7-day maturation. **B)** Cumulative representation of F4/80 and CD11b staining on wild type and ERK3$^{\Delta/\Delta}$ (knockout; KO) BMDMs after harvesting and 7 days of maturation. Results are expressed as means ± standard deviation of five independent experiments (n=5). Statistical significance was determined by an unpaired *t* test. ns, P=0.3765.

ERK3 loss does not effect macrophage polarization

Knowing the loss of ERK3 does not effect the maturation of harvested bone marrow cells into macrophages, we further explored the potential role of ERK3 in the polarization of macrophages towards a pro-inflammatory, M1, phenotype or an anti-inflammatory, M2, phenotype. We found that there are no significant differences between polarized M1 BMDMs (Figure 18A) or M2 BMDMs (Figure 18B) derived from ERK3^{KI} mice versus wild type mice by investigating the mRNA regulation of phenotypic specific markers: nitric oxide synthase 2

(NOS2), IL-6 and IL-12B, pro-inflammatory markers and Arginase-1 (Arg1), FIZZ-1 and YM-1, anti-inflammatory markers.[218] These results suggest that ERK3 is dispensable for macrophage plasticity. At the same time, we confirmed that the markers investigated are specific for their respective indications. NOS2, IL-6 and IL-12B gene expression is upregulated specifically during the M1 polarization (Figure 18A). The same can be said for M2 polarization and the upregulation of FIZZ-1 and YM-1 (Figure 18B); notably, a data point was removed from wild type and ERK3 knock-in M1 conditions as their respective Ct values were close to background. Moreover, while Arg1 was upregulated in both the M1 and M2 conditions, it is further increased in the M2 condition validating it as a marker of M2 polarization when used in conjunction with other markers (Figure 18B).

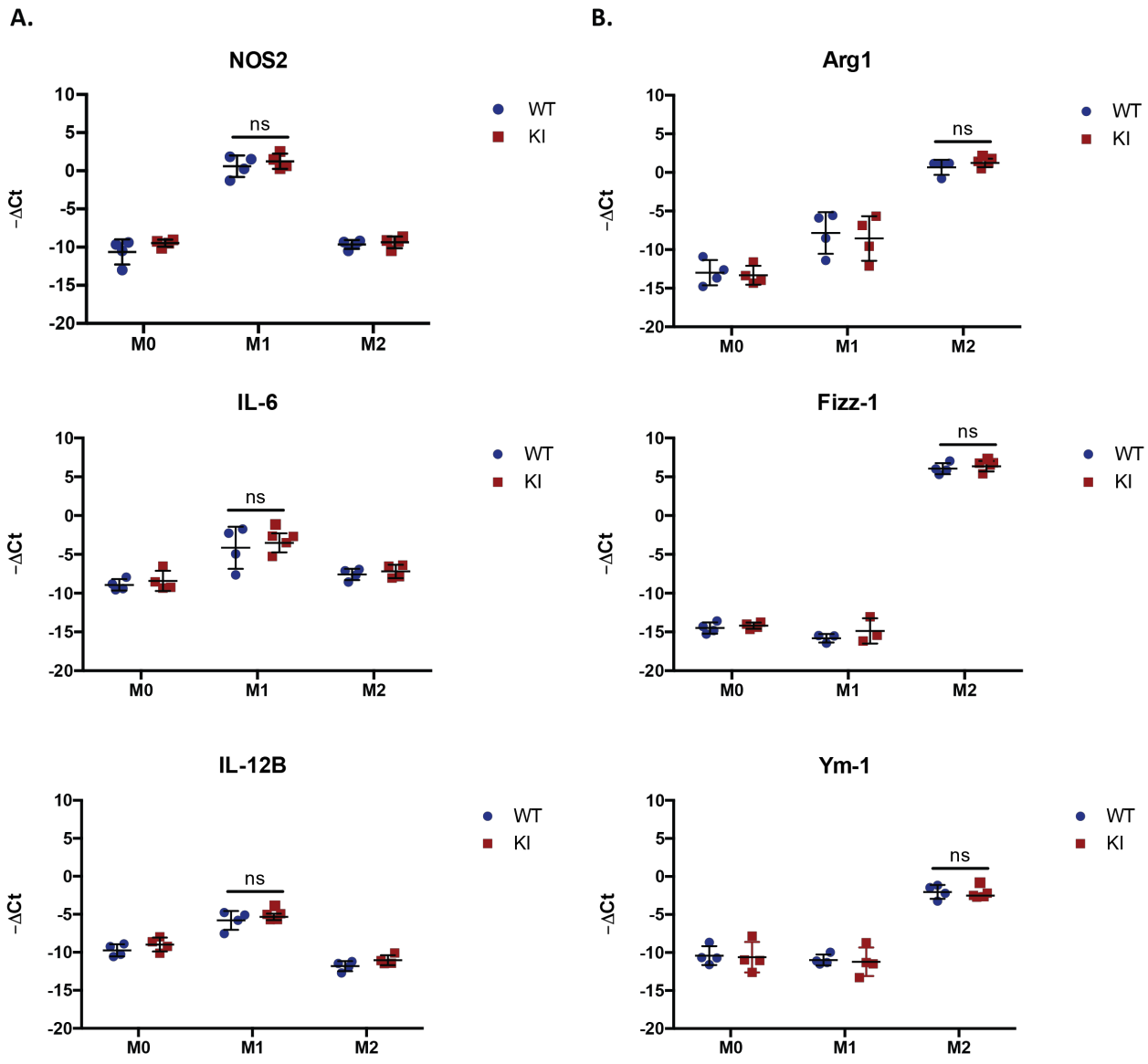

FIGURE 18. Impact of catalytically inactive ERK3 BMDMs on macrophage polarization. A) Regulation of macrophage pro-inflammatory markers from wild type and ERK3^{KI} (KI) BMDMs polarized with media alone (M0), LPS (100ng/ml) and IFNγ (20ng/ml) (M1) or IL-4 (20ng/ml) (M2) **B)** Regulation of macrophage anti-inflammatory markers from wild type and ERK3 knock-in kinase dead (KI) BMDMs polarized with media alone (M0), LPS (100ng/ml) and IFNγ (20ng/ml) (M1) or IL-4 (20ng/ml) (M2). Results are expressed as means ± standard deviation of -Ct values normalized to Ct values for control genes GAPDH and HRPT for the data collected from four independent experiments (n=4). Statistical significance was determined by an unpaired *t* test. ns, P≥0.05

ERK3 loss reduces basal macrophage migration

ERK3 has been proposed to promote cellular migration in various cell types.[30,32,36,45,62] Furthermore, macrophage recruitment was attenuated in mice with catalytically inactive ERK3 (Figure 10). Seeing that there is no difference in BMDM maturation or polarization upon ERK3 loss of function or expression, we next looked at the basal migration of BMDMs harvested from wild type and ERK3$^{\Delta/\Delta}$ mice. In agreement with previous observations, the loss of ERK3 expression decreased the migration rate of BMDMs harvested from ERK3 conditional mice compared to wild type control 6 hours after initial scratch (Figure 19).

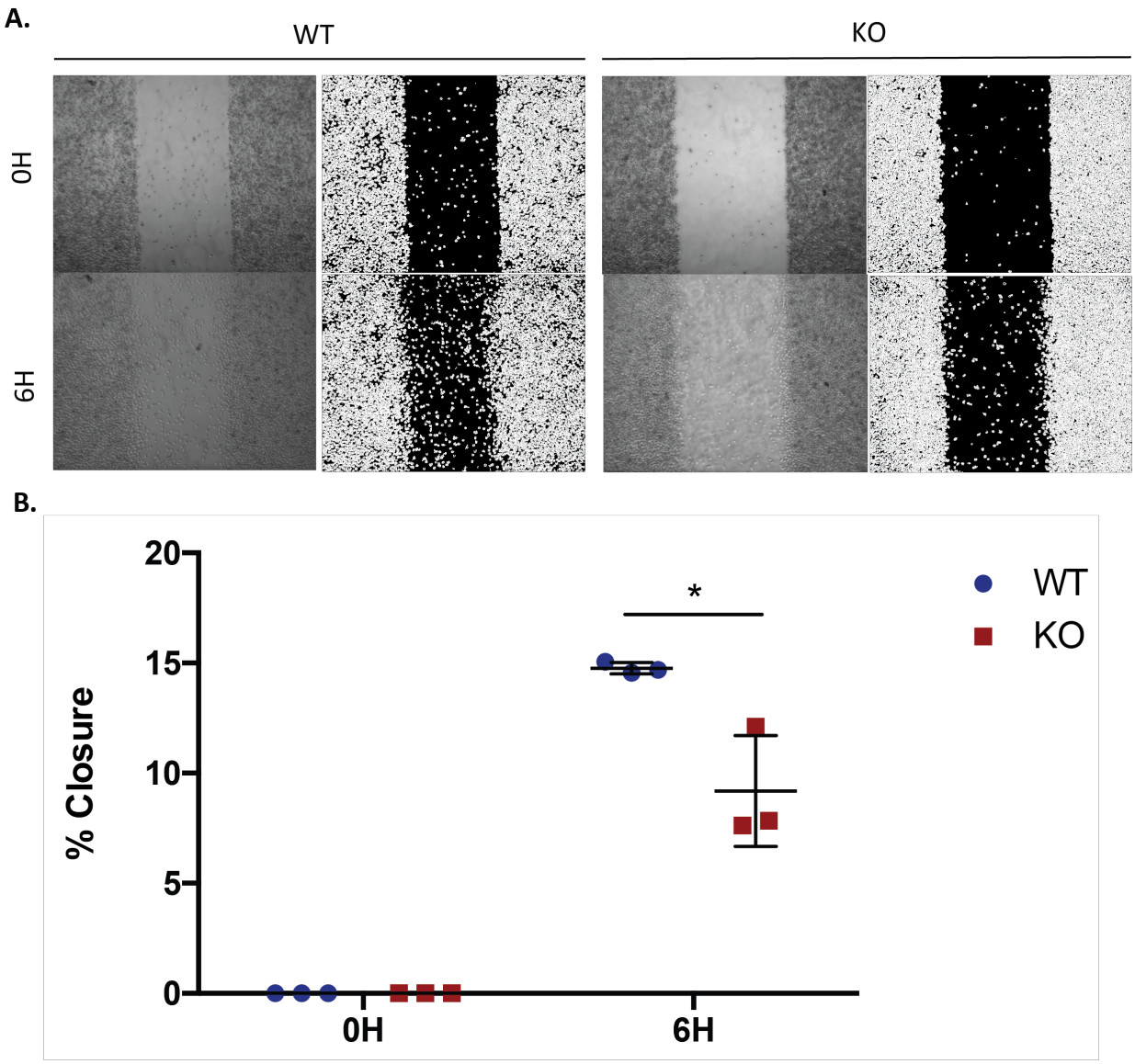

FIGURE 19. ERK3 regulation of BMDM migration. A) Representative images taken of pipette scratches at the 0 hour and 6-hour time point for wild type and ERK3$^{\Delta/\Delta}$ (KO) BMDMs. **B)** Percent closure of the scratched area of wild type and ERK3$^{\Delta/\Delta}$ (KO) BMDMs at the 0 hour and 6-hour time point. Results are expressed as means ± standard deviation of closure area normalized to area taken at the 0-hour time point for data collected from three independent experiments (n=3). Statistical significance was determined by an unpaired *t* test. *, P≤0.05.

Discussion

The discovery of ERK3 and ERK4 dates to the same time of the discovery of ERK1 and ERK2, however, much less in known about the atypical MAPKs ERK3 and ERK4 over their conventional counterparts.[2,3,24] To date, while there is a growing knowledge of these two kinases, their biological functions still remain elusive. Nonetheless, we know that ERK3 and ERK4 are not activated by conventional MAPKKs but through group 1 PAK members.[21] Additionally, MK5 is the sole *bona fide* substrate of ERK3 and ERK4 described by various groups including our laboratory, and the interaction between ERK3/4 and MK5 is complex (Figure 5).[11,21,39,41,42,46] Interestingly, other groups found additional possible substrates of ERK3, which include, TDP2 and SRC-3 - although our laboratory believes that the ERK3's phosphorylation of SRC-3 is in fact mediated by MK5 (Déléris, P. and Meloche, S. unpublished data).[30,44,45,62]

Long, *et. al.*, the group that first established ERK3 signals through SRC-3, were the first to suggest that ERK3 plays a role in cell migration using a pulmonary cancer cell line.[45] Thus the link between ERK3 and cell migration was further investigated and confirmed by other research groups, including our own, in breast cancer cell lines, head and neck cancer cell lines, HUVEC endothelial cells and HeLa cells.[30,32,36,45,62] Additionally, ERK3's role in migration is due to its regulation of the actin cytoskeleton.[30,36,45,62] Apart from cellular migration, work done in collaboration with our laboratory has revealed the role for ERK3 during the positive selection of thymocytes through the TCR signaling, demonstrating the first studies depicting ERK3 involvement in immune function.[27,28]

The above, taken together with the preliminary findings found in the folic acid induced AKI experiment whereby an attenuation of CCL2 and CCL5 was observed in catalytically inactive ERK3 and ERK4-deficient mice (Figure 10A), in combination with a reduced macrophage infiltration (Figure 10B), suggests that ERK3 not only plays a role in the adaptive immune system but may also play a role in the innate immune system. More specifically, during inflammation induced by tissue injury through the production of chemokines and the migration of immune effector cells; likewise, the preliminary findings also suggest a role for ERK4.

We hypothesized that ERK3 and ERK4 play roles in the acute inflammatory response through regulating the production of inflammatory factors such as cytokines and chemokines that are chemoattractant (ex. IL-1β, CCL2 and CCL5) and thus impact immune effector cell migration and infiltration to inflamed tissue. Likewise, we theorized ERK3 and ERK4 play inherent roles in macrophage migration and potentially in macrophage maturation and polarization.

Caspase-1 activation is a central event in innate immune responses to many pathogenic infections and tissue injury that requires the assembly of the NLRP3 inflammasome activated after two distinct signals.[124–127] It's activation is required for the processing of immature pro-IL1β into IL-1β for secretion; IL-1β being a key mediator in the inflammatory response and reported to be increased during AKI contributing to sustain inflammation and to the recruitment of leukocytes to the area of injury.[101,128] Thus we first investigated the potential role of ERK3 and/or ERK4 in the production and secretion of IL-1β in MEFs. MEFs represent a good initial starting point as they can be generated from genetically modified mice and thus have the same genetic mutation/manipulation, therefore do not need to be subjected to RNAi or CRISPR strategies to achieve knockout or catalytically inactivate the kinase of interest.[214] Moreover, they also can be isolated despite cases of neonatal lethality, as in the case of *Mapk6*^{LacZ} mice.

By performing inflammasome activation through priming of MEFs derived from wild type, *Mapk6*^{LacZ} and ERK4 knockout mice with LPS followed by Nigericin, we demonstrate that ERK3 deficiency does not significantly effect the mRNA expression of pro-IL-1β (Figure 11A), while ERK4 loss may impact pro-IL-1β expression (Figure 11B). Interestingly, the results for stimulation with LPS alone versus stimulation with LPS followed by Nigericin did not reveal significant changes. While changes were not expected at the mRNA level *per se*, analysis of IL-1β secretion in the cell supernatant should have revealed a marked increase with the addition of Nigericin compared to LPS alone; Nigericin being the second signal required for the maturation of pro-IL-1β into IL-1β for secretion.[124–127] Surprisingly, no IL-1β was detected in any conditions (values below the limit of detection), including that of LPS combined with Nigericin. Suggesting that IL-1β was not made available for secretion despite the upregulation of pro-IL-1β mRNA (Figure 11). This can be explained by the recent study by Conos, *et. al.* demonstrating

MEFs do not contain NLRP3, ASC or caspase-1, components of the NLRP3 inflammasome.[219] Thus further investigation was halted in MEFs; and while looking at ERK3 involvement during inflammasome activation could be done in monocyte/macrophage cell lines RAW264.7 and THP-1, investigation of ERK4 involvement could not as both cell lines do not express ERK4 (Figure 12B). Furthermore, optimization of either RNAi (both siRNA and shRNA) did not yield sufficient ERK3 knockout (data not shown); likewise, CRISPR strategies were unsuccessful (data not shown) and therefore implementation of primary cells, such as the use of BMDMs would be necessary. Unfortunately, like their cell line counterparts, BMDMs do not express ERK4 (Figure 12C), which suggests that ERK4-deficiency did not induce an inherent inability of macrophages to migrate, but that, perhaps the attenuation of macrophage chemoattractant signaling was efficient to reduce macrophage infiltration in the folic acid induced model of AKI (Figure 10). Furthermore it confirms the finding that ERK4 expression is more restricted than that of ERK3.[5,15,16]

Interestingly, while ERK4 is described as expressed in the kidney, mouse cortical tubule cell line, MCT, does not express ERK4 (Figure 12A).[5,15,16] Therefore cannot be used to study the loss of ERK4 on tubule cytokine/chemokine production. To surpass this, primary renal proximal tubule epithelial cells (RPTECs) isolated from ERK4-deficient (or ERK3-deficient mice) can be utilized – protocols for RPTC isolation are under optimization.[220] Meanwhile, although MCT cells do not contain ERK4, the lentiviral CRISPR targeting of ERK3 was successful (Figure 14A) and clonal cell populations were produced via limiting dilution confirmed by immunoblotting (Figure 14B). These clonal cell lines were then used for the investigation of ERK3 loss on the production of AKI implicated macrophage chemoattractants CCL2 and CCL5, via the stimulation by TNFSF members TNFα and TWEAK.[105,118,130,132,141,144,147,149,150,157] We show that ERK3 expression appears to be upregulated during TNFα stimulation of MCT cells (Figure 13A). This in conjunction with research done by Wang, *et. al.* which shows ERK3 mRNA upregulation during TNFα stimulation of endothelial cells suggests that TNFα regulates ERK3 mRNA and protein expression in multiple cell types.[30] Furthermore, we provide evidence of increased ERK3 protein expression upon TWEAK stimulation in MCT cells (Figure 13B), leading to the belief that other members of the TNFSF may regulate ERK3 expression.

Moreover, its regulation by TNFSF members may impact the secretion of cytokines and chemokines as demonstrated by the reduced production of CCL2 and CCL5 mRNA upon TNFα stimulation in ERK3 CRISPR knockout clonal MCT cell lines (Figure 15A and 15B). This is further supported by the attenuation of CCL2 and CCL5 production during TWEAK stimulation (Figure 16A and 16B). Nonetheless, while a reduction of CCL5 at 4 hours of TNFα stimulation is seen in ERK3 knockout clones (Figure 15B), the effect of TNFα stimulation over a longer period of time should be investigated as CCL5 peak mRNA upregulation was seen at the 24-hour time point during TWEAK stimulation (Figure 16C). Furthermore, a comment is needed on the difference between ERK3 knockout clones, Sg5 1H11 and Sg6 2G9, as only the Sg5 1H11 ERK3 knockout clone showed a significant reduction in CCL2 and CCL5 upon either TNFα or TWEAK stimulation (Figures 15 and 16). These difference are likely due to transcriptome variation across individual cells of the same cell type/line.[221] Because of this, additional investigation using a heterogeneous population of ERK3 knockout MCT cells is necessary to account for transcriptome differences between individual cells upon TNFα or TWEAK stimulation. Similarly, the use of RPTECs isolated from ERK3-deficient mice can support our findings.

Considering the preliminary results in the folic acid induced AKI model and within this thesis depicting TNFα and TWEAK induced stimulation of CCL2 and CCL5 and its attenuation by ERK3 loss, we investigated the effect of ERK3 deficiency in macrophages in order to clarify whether their attenuated infiltration into injured kidneys is solely due to the reduction in macrophage chemoattractants or an effect seen due to a combination of mechanisms. Thus we first investigated the effect of ERK3 loss on monocyte to macrophage maturation by investigating the ability of bone marrow cells harvested from wild type and ERK3$^{\Delta/\Delta}$ mice to mature into macrophages through the macrophage specific markers CD11b and F4/80.[217] We found that there is no significant difference between the maturation of BMDMs harvested from ERK3 conditional knockout mice compared to wild type (Figure 17B). Thus, the reduction of F4/80 stained cells in the histopathological kidney sections of ERK3 kinase-dead mice subjected to folic acid-induced AKI (Figure 10) is not due to a deficiency to mature. Interestingly, investigation looking at the specificity of cell markers of leukocytes resident and infiltrating the kidney has suggested that F4/80 stains both macrophages and dendritic cells.[222] Therefore it can

be hypothesized that the staining of F4/80 revealed not only reduced macrophage infiltration but also a deficiency in the infiltration of dendritic cells. Thus, revaluation of the histopathological kidney sections from the preliminary AKI experiment with other cell specific markers like CD11b for macrophages and CD11c for dendritic cells is necessary to discern cell infiltrating contributions.[217,222] Moreover, investigation in ERK3 (or ERK4) loss in dendritic cell maturation and function is necessary and this work is ongoing.

Observing no difference in maturation, we hypothesized that ERK3 may effect macrophage polarization to a pro-inflammatory M1 phenotype or an anti-inflammatory M2 phenotype. However, our findings revealed that there are no differences between M1 and M2 polarization in mice with catalytically inactive ERK3 versus their wild type counterparts (Figure 18). While this does not explain chemokine secretion or infiltration variances seen in the preliminary AKI findings (Figure 10), it suggests that future therapeutic targeting of ERK3 during the early phase of AKI may not disrupt the role played by cell mediators that are involved during the acute inflammatory phase that then transition and play a role in the repair and regeneration of the kidney (i.e. macrophages).[93]

Also, it is known that CCL2 deficiency alone does not have an effect on macrophage infiltration alone, and while we show data that suggests a reduction of CCL5 we also investigated the effect of ERK3 loss on the inherent migration abilities of macrophages as ERK3 has already been described as a regulator of cell migration in other cell types.[30,32,36,45,62,151] As found by other research groups, we demonstrate that ERK3 deficiency attenuates macrophage migration in a scratch assay (Figure 19A and 19B), however the mechanism remains elusive. Taking previous work, ERK3's role in modifying the organization of the actin cytoskeleton promoting cellular migration in addition to signaling through ERK3-MK5-SRC-3 can be offered as explanations, however, further investigation is necessary.[30,36,45,62]

Conclusion and Perspectives

In this thesis, we present the discovery that ERK3 plays a role during an inflammatory response though the secretion of pro-inflammatory chemokines and the migration of macrophages. We provide evidence that TNFSF members, TNFα and TWEAK, regulate ERK3 expression in the murine cortical tubular cell line MCT. Furthermore, upon ERK3 deficiency, TNFα and TWEAK induced upregulation of chemoattractants CCL2 and CCL5 is attenuated; suggesting that ERK3 has a role in chemoattractant mediated migration of immune cells to inflamed/damaged tissues. Interestingly, we also show reduction in the migration of ERK3-deficient BMDMs compared to wild type; thus, suggesting that ERK3 also plays a role in the intrinsic abilities of macrophages to migrate.

As per ERK4, we show that the tubule epithelial cell line, MCT, monocyte/macrophage cell lines RAW264.7 and THP-1, in addition to BMDMs do not express ERK4. Thus, the impact of ERK4 on tubule cell production of CCL2 and CCL5 and on BMDM migration was not investigated. This said, optimization of RPTEC protocols and their use can further the investigation of ERK4 and CCL2/CCL5 section. Importantly, RPTECs can be used to confirm ERK3 findings. Moreover, *in vivo* models of macrophage migration in wild type and ERK3-deficient mice can confirm our *in vitro* findings on cell migration.

Collectively the findings presented illustrate a new function for ERK3 during inflammation through two distinctive but connected roles: the production of chemokines that signal immune cell infiltration and the inherent ability, without stimulus, of macrophages to migrate.

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