FUNCTIONAL ANALYSIS OF EPITHELIUM-SPECIFIC ETS TRANSCRIPTION FACTOR-1 IN INFLAMMATION

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Laboratory Medicine and Pathobiology
University of Toronto

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Doctor of Philosophy
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Abstract

E26 transformation-specific (Ets) transcription factors are key regulators of hematopoiesis, tissue differentiation, and development, characterized by the presence of evolutionarily conserved DNA-binding domain (DBD) which recognizes a core 5’-GGAA/T-3’ sequence in promoter and enhancer regions of target genes. Epithelium-specific Ets transcription factor-1 (ESE-1), or E74-like factor 3 (Elf3) in mice, is a member of the Ets protein family which is known to be constitutively expressed in epithelial tissues. However, ESE-1 is highly and rapidly induced in non-epithelial cells such as endothelial cells, macrophages, and fibroblasts by pro-inflammatory cytokines, despite its lack of expression under resting conditions in these cell types. ESE-1 is also prominently expressed in various human inflammatory diseases including rheumatoid arthritis and vasculitis, and is known to regulate important genes involved in inflammation such as cyclooxygenase-2 (COX-2). Therefore, previous studies have focused on identifying the role of ESE-1 in inflammation using various cell lines and transfection tools as well as an ESE-1 knockout mouse model, which led to discovery of its many target genes and transcriptional regulation by nuclear factor kappa B (NF-κB), the central regulator of inflammation. To further enhance our understanding of ESE-1 in human inflammatory diseases, we hereby investigated the ESE-1 expression and function in human primary cells derived from patients suffering from sepsis or...
rheumatoid arthritis. We discovered that ESE-1 is constitutively expressed human neutrophils and overexpressed in those from septic patients, and that human and mouse neutrophils display distinct expression pattern of ESE-1/Elf3. Additionally, in contrast to earlier studies, we found that ESE-1 acts as a negative regulator of COX-2 in human rheumatoid arthritis synovial fibroblasts (RASFs). These findings, using new experimental approaches that take advantage of the increased availability of better analysis tools, provide new insights into the involvement of ESE-1 in human inflammatory diseases which were previously unrecognized.
Acknowledgments

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I dedicate this thesis to my late grandparents:

You have given me so much, and I hope I have been able to achieve some of your own dreams you never had the opportunity to pursue in life. Thank you for everything you did for me, both in the past and in the present. May God bless you always.
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<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>AA</td>
<td>arachidonic acid</td>
</tr>
<tr>
<td>7-AAD</td>
<td>7-aminoactinomycin D</td>
</tr>
<tr>
<td>ACC</td>
<td>Animal Care Committee</td>
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<tr>
<td>ACPA</td>
<td>anti-citrullinated protein antibody</td>
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<td>AD</td>
<td>transcriptional activation domain</td>
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<td>AID</td>
<td>autoinhibitory domain</td>
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<td>Ang-1</td>
<td>angiopoietin 1</td>
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<td>Ang II</td>
<td>angiotensin II</td>
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<td>ALI</td>
<td>acute lung injury</td>
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<td>AP-1</td>
<td>activator protein 1</td>
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<td>APRIL</td>
<td>A proliferation-inducing ligand</td>
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<td>ARDS</td>
<td>acute respiratory distress syndrome</td>
</tr>
<tr>
<td>ARE</td>
<td>AU-rich element</td>
</tr>
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<td>ATRA</td>
<td>all-trans retinoic acid</td>
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<td>AUF1</td>
<td>ARE/poly(U) binding factor 1</td>
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<tr>
<td>BAFF</td>
<td>B-cell activation factor member of the TNF family</td>
</tr>
<tr>
<td>BAL</td>
<td>bronchoalveolar lavage</td>
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<tr>
<td>BCR</td>
<td>B-cell receptor</td>
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<td>BM</td>
<td>bone marrow</td>
</tr>
<tr>
<td>BMDM</td>
<td>bone marrow-derived macrophage</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>C4HSU</td>
<td>control helper-dependent adenoviral vector</td>
</tr>
<tr>
<td>CAMKII</td>
<td>calcium-calmodulin-dependent protein kinase II</td>
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<tr>
<td>CAR</td>
<td>CXCL12-abundant reticular cells</td>
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<td>CARS</td>
<td>compensatory anti-inflammatory response syndrome</td>
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<td>Cas</td>
<td>CRISPR associated</td>
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<td>CBP</td>
<td>CREB binding protein</td>
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<td>CCR7</td>
<td>CC-chemokine receptor 7</td>
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<td>CD</td>
<td>cluster of differentiation</td>
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<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
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<td>C/EBP</td>
<td>CAAT enhancer binding protein</td>
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<td>CECAM1</td>
<td>carcinoembryonic antigen-related cell adhesion molecule 1</td>
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<td>CGD</td>
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<td>CLP</td>
<td>cecal ligation puncture</td>
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<td>COL2A1</td>
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<td>COPD</td>
<td>chronic obstructive pulmonary disease</td>
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<td>COX-2</td>
<td>cyclooxygenase-2</td>
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<td>CMP</td>
<td>common myeloid progenitor</td>
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<td>CRE</td>
<td>cyclic AMP response elements</td>
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<td>Abbreviation</td>
<td>Definition</td>
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</tr>
<tr>
<td>CRISPR</td>
<td>clustered regularly interspaced short palindromic repeat</td>
</tr>
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<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CUGBP2</td>
<td>CUG triplet repeat, RNA-binding protein 2</td>
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<td>CXCL12</td>
<td>CXC-chemokine ligand 12</td>
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<td>DAF</td>
<td>decay accelerating factor for complement</td>
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<td>DBD</td>
<td>DNA binding domain</td>
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<td>DC</td>
<td>dendritic cell</td>
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<td>DC-SIGN</td>
<td>DC-specific ICAM3-grabbing non-integrin</td>
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<td>DEAE-Dextran</td>
<td>diethylaminoethyl-dextran</td>
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<tr>
<td>DIC</td>
<td>disseminated intravascular coagulation</td>
</tr>
<tr>
<td>DMARD</td>
<td>disease-modifying anti-rheumatic drug</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>DP</td>
<td>PGD receptor</td>
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<tr>
<td>DTT</td>
<td>dithiothreitol</td>
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<td>EC</td>
<td>embryonic carcinoma</td>
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<td>EC</td>
<td>endothelial cell</td>
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<td>ECM</td>
<td>enhanced chemiluminescence</td>
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<td>EDTA</td>
<td>ethylene diamine tetra-acetic acid</td>
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<td>EES</td>
<td>ESE-1 enhancer sequence</td>
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<td>EGFP</td>
<td>enhanced green fluorescent protein</td>
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<td>Egr-2</td>
<td>early growth response-2</td>
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<td>EGTA</td>
<td>ethylene glycol tetra-acetic acid</td>
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<td>Ehf</td>
<td>Ets homologous factor</td>
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<td>Elg</td>
<td>Ets-like factor</td>
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<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<td>EP</td>
<td>E prostanoid receptor</td>
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<td>ERK1/2</td>
<td>extracellular signal-regulated kinase 1/2</td>
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<td>Erf</td>
<td>Ets repressor factor</td>
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<td>Erg</td>
<td>v-ets ovarian erythroblastosis virus E26 oncogene related</td>
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<td>Ets</td>
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<td>ETV</td>
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<td>FACS</td>
<td>fluorescence activated cell sorting</td>
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<td>FBS</td>
<td>fetal bovine serum</td>
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<td>Description</td>
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<tr>
<td>FEV</td>
<td>Fifth Ewing variant</td>
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<td>FGF</td>
<td>fibroblast growth factor</td>
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<td>Friend leukemia virus integration 1</td>
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<td>FLIP</td>
<td>FLICE inhibitory protein</td>
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<td>formyl-methionyl-leucyl-phenylalanine</td>
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<td>FP</td>
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<td>GABP</td>
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<td>glyceraldehyde 3-phosphate dehydrogenase</td>
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<td>growth factor independent-1</td>
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<td>green fluorescent protein</td>
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<td>G-CSF</td>
<td>granulocyte colony-stimulating factor</td>
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<td>GM-CSF</td>
<td>granulocyte/macrophage colony-stimulating factor</td>
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<td>GMP</td>
<td>granulocyte/macrophage progenitor</td>
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<td>HA</td>
<td>hemagglutinin (tag)</td>
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<td>histone deacetylase</td>
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<td>HEPES</td>
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<td>horseradish peroxidase</td>
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<td>heat shock protein 90</td>
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<td>human umbilical vein endothelial cells</td>
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<td>intercellular adhesion molecule-1</td>
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<td>interferon γ</td>
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<td>immunoglobulin</td>
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<tr>
<td>NOD1</td>
<td>nucleotide-binding oligomerization domain protein 1</td>
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<td>non-steroidal anti-inflammatory drugs</td>
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<td>recombinant TNF-related apoptosis-inducing ligand</td>
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<td>DNA synthesis phase</td>
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<td>signaling lymphocytic activation molecules</td>
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<td>SAM pointed domain containing Ets transcription factor</td>
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<td>T cell receptor</td>
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<td>TEL</td>
<td>Translocation, Ets, Leukemia</td>
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<td>Acronym</td>
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<tr>
<td>uPA</td>
<td>urokinase-type plasminogen activator</td>
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<tr>
<td>UDPDG</td>
<td>uridine diphosphoglucose dehydrogenase</td>
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<td>VCAM-1</td>
<td>vascular cell adhesion molecule-1</td>
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<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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<tr>
<td>VLA-4</td>
<td>very late activation antigen-4</td>
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<tr>
<td>WHIM</td>
<td>warts, hypogammaglobulinemia, infections, myelokathesis</td>
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<td>winged helix-turn-helix</td>
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<td>wild-type</td>
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Dissemination of Thesis Contents


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1 Introduction

Parts of this Chapter has been submitted to: Chan Mi Lee, Jing Wu, Emily Yi Xia, and Jim Hu. ESE-1 in early development: approaches for the future. Frontiers in Cell and Developmental Biology, 2016.

Parts of this Chapter has been published in: Chan Mi Lee and Rahul Kushwah. Making airway immunology disease-relevant. Jacobs Journal of Allergy and Immunology, 2014, 1(1):001.
1.1 The E26 transformation-specific transcription factor family

*Discovery of Ets protein family*

The first member of the Ets (E26 transformation-specific) transcription factor family to be identified was *v-ets* oncogene from E26 avian erythroblastosis transforming retrovirus, which induced erythroblastic and myeloblastic leukemias in chickens (Leprince et al., 1983). The *v-ets* expressed the oncogenic gag-myb-ets fusion protein that contained the 84 amino acid DNA binding domain termed the Ets domain, found to recognize a 5’-GGAA/T-3’ consensus sequence. Approximately 30 members of Ets transcription factors have so far been identified in mouse and human, and are known to play crucial roles in various physiological and pathological processes, such as embryonic development, differentiation, angiogenesis, inflammation, and oncogenic transformation (Hollenhorst et al., 2011; Oikawa and Yamada, 2003).

*The structure and function of the Ets domain*

The common characteristic feature of Ets family of transcription factors is the presence of the Ets DNA-binding domain, which is a conserved 84 amino acid domain that contains a winged helix-turn-helix motif. NMR analysis of the Ets domain delineated its composition of three α-helixes (α1-α3) and four-stranded β-sheets (β1-β4) arranged in the order α1-β1-β2-α2-α3-β3-β4 forming a winged helix-turn-helix (wHTH) motif (Donaldson et al., 1996; Kodandapani, 1996). The two alpha-helices are separated by a tight turn, and the extended loop that connects the two β-stands forms the ‘wing’ which, along with the third α-helix that contains major residues responsible for DNA binding (Shore et al., 1996), also contacts the DNA. Structural studies on Ets domains of Fli-1 (Liang et al., 1994), Ets-1 (Donaldson et al., 1996; Werner et al., 1997), PU.1 (Kodandapani, 1996), GABPα (Batchelor et al., 1998), SAP-1 (Mo et al., 1998), and Elk-1 (Mo et al., 2000) have revealed high degree of conservation among family members and a likely large overlap in binding specificities.

However, in addition to facilitating binding to DNA, the Ets domain is also involved in protein-protein interactions within self protein domains as part of auto-regulation, or with other co-regulatory proteins. In many Ets proteins, the DNA-binding domain is masked until activated by a trigger, such as phosphorylation or binding of a co-activator or co-repressor. For example, Ets-1
protein is autoinhibited by the intramolecular interaction between regions spanning the Ets domain, which is relieved upon unfolding of one of the α-helices in the inhibitory module by binding of co-activators such as AML-1/CBFα2 to Ets-1 (Jonsen et al., 1996; Kim et al., 1999; Petersen et al., 1995). In contrast, phosphorylation of Ets-1 by calcium-calmodulin-dependent protein kinase II (CAMKII) stabilizes the inhibitory conformation and thus the autoinhibition (Cowley and Graves, 2000). Similarly, members of the ternary complex factor (TCF) Ets-subfamily show regulation of activity by autoinhibition, which is relieved by phosphorylation of the carboxy-terminal domain of by MAPK (Yates et al., 1999) or interaction with other transcription factors such as serum response factor (SRF) (Dalton and Treisman, 1992; Hipskind et al., 1991) and Pax-5 (Fitzsimmons et al., 1996) to enable DNA binding.

Division of the Ets factors into sub-families

Ets family proteins have been divided into different subfamilies based on the homology of the Ets DNA-binding domain and other structural features (Figure 1.1). Most of Ets members carry the Ets domain in the C-terminal region, with few exceptions such as the TCF subfamily, which have the Ets domain near the N-terminus. Moreover, about a third of Ets transcription factors also contain another evolutionarily conserved domain called the Pointed (PNT) domain at the N-terminus, which forms a helix-loop-helix (HLH) conformation that mediate homotypic and heterotypic protein-protein interactions (Kim et al., 2001; Mackereth et al., 2004). Additionally, several Ets subfamilies, such as TEL, ERF, and TCF, contain a repressor domain, while the majority contain a transcription activation domain (TAD) (Seth and Watson, 2005; Sharrocks, 2001; Wasylyk et al., 1998). Unique pattern of expression in specific tissue types, such as epithelium-specific Ets factor (ESE) subfamily, also plays a part in sub-classifying Ets transcription factors.
**Figure 1-1 Ets transcription factors divided into sub-families.**

The E26 transformation-specific (Ets) family of transcription factors all contain the common Ets DNA binding domain. They can be further classified into subfamilies by the presence of distinct functional domains and their relative positions, such as the Pointed domain (Pointed), transcriptional activation domain (AD), and repressor domain (RD). Picture from (Gutierrez-Hartmann et al., 2007)
Figure 1-2 Summary of Ets factors involved in hematopoiesis.

Various Ets factors involved in different hematopoietic cell lineages are marked, with (+) to indicate low expression, (++) moderate expression, and (+++) high expression. Blue arrows with denoted transcription factors signify the requirement of the particular Ets factor in the commitment step of the differentiation step. Figure from (Maroulakou and Bowe, 2000)
1.1.1 E26 transformation-specific transcription factors in hematopoiesis and immune cell development

Hematopoiesis is the production of mature blood cells from pluripotent hematopoietic stem cells (HSCs) through a hierarchical series of differentiation into different cell lineages, such as granulocytes, monocytes, megakaryocytes, erythrocytes, and lymphocytes (Orkin and Zon, 2008). Aberrations in the activity or gene expression of regulatory factors in this process can lead to malignant transformation and various forms of leukemia (Look, 1997; Mueller et al., 2002; Okuda et al., 1996; Orkin et al., 1999; Tenen et al., 1997).

Ets family of transcription factors are characterized by the conserved Ets DNA binding domain. Members of the Ets protein family are closely involved in hematopoiesis and immune cell regulation (Gallant and Gilkeson, 2006) (Figure 1-2). For example, TEL1 is expressed in the bone marrow and required for normal adult hematopoiesis (Wang et al., 1997) as well as the maintenance of hematopoietic stem cells and multipotent progenitors (Wang et al., 1998). Elf-1 and PU.1 regulate the expression of stem cell leukemia (SCL or tal-1) gene in the hemangioblasts, which are pluripotent cells that develop from the mesoderm and generate blood and endothelium (Göttgens et al., 2002). Fli-1, another Ets family member, is essential in megakaryocyte differentiation where Fli-1 knockout mice exhibit a severe hemorrhagic phenotype and embryonic lethality (Hart et al., 2000; Spyropoulos et al., 2000). For the purpose of this thesis, we provide an overview of key Ets factors found to be involved in immune cell development and function based on knockout animal models, genetic human diseases, and other functional analysis:

1.1.1.1 ETS factors in myelopoiesis and myeloid cell function

Myeloid differentiation, which leads to the generation of granulocytes and monocytes, is controlled by a number of key transcription factors, such as PU.1, C/EBPs, AML1, IRF8, GFI-1, and others (Friedman, 2002; Rosenbauer and Tenen, 2007). PU.1, an Ets transcription factor, is a master regulator of myeloid lineage formation. Targeted disruption of PU.1 in mice leads to marked deficiency in common myeloid progenitors (CMPs) and mature macrophages, osteoclasts and functional neutrophils (Anderson et al., 1998; Iwasaki et al., 2005; McKercher et al., 1996; Scott et al., 1994). PU.1-/- hematopoietic progenitors also fail to respond to macrophage colony stimulating factor (M-CSF) or granulocyte- (G-) or granulocyte-macrophage colony-stimulating
factor (GM-CSF) (DeKoter et al., 1998), or to form myeloid colonies in culture (Iwasaki et al., 2005). Many myeloid-specific genes contain PU.1 and C/EBP binding sites in their promoter regions, including M-CSF receptor (c-fms), G-CSF receptor, CD11b/18 (Mac-1), myeloperoxidase (MPO), IL-1β, lysozyme, neutrophil esterase, and components of the NADPH oxidase such as gp91phox and gp47phox (Friedman, 2002; Oikawa et al., 1999). PU.1 also negatively regulates c-myb during terminal myeloid differentiation (Bellon et al., 1997) which may be related to cell cycle arrest, and physically interacts with the transcription factor GATA-1 to inhibit erythroid-megakaryocyte development (Cantor and Orkin, 2002; Nerlov et al., 2000).

Ets-1 and Ets-2 are critical regulators of macrophage differentiation as downstream activator of Ras signaling elicited by M-CSF. Ets-1/2 transactivate genes that are involved in both cell proliferation and differentiation, such as c-myc, c-myb, cdc2, and urokinase-type plasminogen activator (uPA), gelatinase B/MMP-9, and scavenger receptor-A (SR-A) genes, respectively. Many of the latter genes contain binding sites for AP-1/Ets ternary complexes in their promoters. The exit from cell cycle during macrophage terminal differentiation is mediated by METS (mitogenic Ets transcriptional suppressor), which is progressively induced during macrophage differentiation and specifically downregulates genes involved in cell cycle such as c-myc, cdc2, and p54 subunit of DNA primase gene by forming a complex with a co-repressor DR103. The METS/DR103 complex selectively replaces Ets activators from their monomeric binding sites on the cell cycle control genes, leading to inhibition of cell growth but continuation of differentiation (Klappacher et al., 2002).

1.1.1.2 ETS factors in B cell development and function

Similar to myeloid differentiation, PU.1 is one of the most important transcription factors for B cell development, where PU.1/- mice show complete lack of B cells in addition to macrophages (McKercher et al., 1996; Scott et al., 1994). PU.1 directly regulates the transcription of IL-7α gene, which is critical for B cell differentiation from common lymphoid progenitors (DeKoter et al., 2002). In pre-B and B cells, PU.1 regulates many B cell-specific genes such as immunoglobulin (Ig) chains. The immunoglobulin μ heavy chain gene enhancer, for example, contains μA, μE3 and μB sites that bind Ets-1, TEF3 and PU.1, respectively (Rao et al., 1997). PU.1 also binds to Igκ and Igλ enhancer and CD20 promoter region, forming a ternary complex with Pip (PU.1-interacting partner) (Eisenbeis et al., 1995; Himmelmann et al., 1997; Pongubala et al., 1992), as
well as regulating CD72 gene expression which is important for B cell activation, proliferation, and differentiation into plasma cells (Ying et al., 1998).

Spi-B is another member of the Ets transcription factors is closely related to PU.1, sharing 43% overall similarity and 67% homology at the amino acid level in the C-terminal region that includes the Ets DNA binding domain (Ray et al., 1992). Spi-B thus binds to many same sites as PU.1, albeit with different affinities (Rao et al., 1999; Ray et al., 1992; Su et al., 1996). However, unlike PU.1 which is expressed in multiple hematopoietic lineages, the expression of Spi-B is restricted to B cells and immature T cells, increasing during B cell maturation but decreasing during T cell maturation (Chen et al., 1995; Su et al., 1996). Spi-B/- mice produce mature B and T cells, but with severe functional abnormalities, such as defective antigen-dependent B cell proliferation, T cell-dependent antigenic responses, and defects in germinal center formation and maintenance in the spleen (Su et al., 1997). Therefore, it seems that PU.1 is required for B cell development, while Spi-B is required for normal B cell antigen-mediated signaling and function (Oikawa and Yamada, 2003).

Elf-1 is another Ets transcription factor which is highly expressed in B cells and is involved in the regulation of many B cell function-related genes, such as IgH, mb-1, lck, blk, lyn, TdT, and B29 (Akbarali et al., 1996; Bassuk et al., 1998). Another closely related Ets factor named new Ets-related factor (NERF), which shares 90% homology with Elf-1 for the Ets DNA binding domain, was also found to be co-expressed with Elf-1 in murine pre-B cell and mature B cell lines, BASC6C2 and A20, respectively, and both Ets factors bound to ets-related binding sites on promoters of target genes such as B-lymphocyte tyrosine protein-kinases lyn and blk with similar affinities (Oettgen et al., 1996). This may be an example of functional redundancy among related Ets factors, and other modes of functional regulation, such as differential phosphorylation or protein-protein interaction may also be involved to fine tune specific gene expression.

1.1.1.3 ETS factors in T cell development and function

Multiple Ets factors are expressed in lymphoid lineages, including PU.1, Spi-B, Ets-1, Ets-2, Erg, Elf-1, GABPα, and Fli-1, particularly in T cells where the Ets factors have been demonstrated to be expressed in step-wise, temporally regulated manner during T cell differentiation (Anderson et al., 1999; Bassuk and Leiden, 1997). Additionally, consistent with PU.1 being a central
transcription factor for myeloid cell development, T cell lineage commitment is associated with the down-regulation of PU.1 during the pro-T cell stage (Spain et al., 1999). Therefore while deletion of PU.1 delays T cell development, constitutive expression of PU.1 leads to growth arrest at the pro-T cell stage with repression of key genes such as pre-Tα, Rag-1, and Rag-2 (Anderson et al., 2002).

Mature T lymphocytes express a number of Ets transcription factors including Ets-1, Ets-2, Fli-1, GABPα and β, Tel, and Elf-1. A number of these Ets factors, especially Ets-1, -2, and Elf-1 were found to strongly transactivate human CD5 gene, which encodes for CD5 surface glycoprotein that acts as a dual accessory receptor that modulates antigen-specific receptor signaling (Lozano et al., 2000) and whose expression is tightly regulated by TCR signaling during T cell development in the thymus (Azzam et al., 1998). A number of T cell-specific genes, such as T cell receptor α (TCRα) (Ho et al., 1990; Mayall et al., 1997), TCRβ (Halle et al., 1997; Wotton et al., 1993), CD4 (Salmon et al., 1993), IL-2 receptor (Lin et al., 1993), and IL-5 (Blumenthal et al., 1999) contain Ets-binding sites on their promoter and enhancer regions, and were shown to be activated by key Ets factors such as Ets-1 and Ets-2. Ets-1 is also crucial for successful progression from double negative precursor (DN3) to double positive (DP) cells, where Ets-1-deficient DN4 cells are prone to apoptosis, as well as efficient inhibition of further rearrangement of the TCR β chain, referred to as allelic exclusion (Eyquem et al., 2004). Functional integrity of pre-TCR is critical for survival, proliferation, and maturation of T lymphocytes which express a unique TCR. More importantly, recent study has shown that Ets-1/- mice develop T cell-mediated splenomegaly and systemic autoimmunity, which was associated with reduced number and activity of Foxp3+ T reg cells. Ets-1 was found to interact with the Foxp3 intronic enhancer and necessary for the demethylation of the regulatory region (Mouly et al., 2010).

Elf-1 has also been shown to regulate a number of T-cell specific genes, such as CD4 (Sarafova and Siu, 1999; Wurster et al., 1994), IL-2 receptor (John et al., 1995; Serdobova et al., 1997), terminal deoxynucleotidyl-transferase (TdT) (Ernst et al., 1996), CD3ζ (Rellahan et al., 1998), LAT (Finco et al., 2006) and TCRα (Lacorazza and Nimer, 2003). Elf-1 is significantly upregulated in T cells stimulated with phorbol ester TPA (Leiden et al., 1992), suggesting that it plays a role in T cell proliferation and activation. However, no major defect has been observed in conventional T cell development in Elf-1/- mice (Garrett-Sinha et al., 2001), but instead a significant reduction
in invariant Natural Killer T (iNKT) cells in the thymus and in the periphery (Choi et al., 2011). iNKT cells express an invariant TCR α chain that pairs with specific TCR β chains (Vβ8, Vβ7, or Vβ2 in mice, and Vβ11 in humans) (Godfrey et al., 2004) and while originating from CD4+CD8+ DP progenitors, they undergo a very distinct positive selection by CD1d-expressing DP thymocytes that involve homotypic interactions with signaling lymphocytic activation molecules (SLAM) family receptors (Benlagha et al., 2005; Chung et al., 2005; Gapin et al., 2001). Therefore, these results indicate that Ets factors are intimately involved in various T cell development and function, both individually and in combination as a protein family.

1.1.2 Selectivity of Ets factors and cross-interaction

Identification Ets target genes has mostly been derived from gain or loss of function studies. Single knockout animal models have been useful in revealing function of individual Ets factors by distinct phenotypes (Bartel et al., 2000). However, more recent studies indicate that over two-thirds of Ets family members are co-expressed in most cell types (Hollenhorst et al., 2004). Notably, Ets factors are expressed temporally during differentiation, showing sharp transitions in expression that correlate with critical commitment (Anderson et al., 1999) or growth events (Bhat et al., 1987), and distinct pattern of co-expression during tumorigenesis (Galang et al., 2004).

Ets factors have been divided into four sub-groups by difference in preferred Ets-binding motifs, which were determined by variations in amino-acids that interact with the backbone of the core recognition sequence (Wei et al., 2010). Class I factors, for example, include at least 11 Ets factors and mainly recognize CCGGAA/T, while Class III factors, which include ESE-1 and at least 5 others, demonstrate strong preference for GCGGAAC (Wei et al., 2010). Therefore, other determinants of specificity, such as protein-protein interactions (Li et al., 2000b) with other transcription factors via distinct protein domains and additional regulatory sequences flanking the core Ets-binding site on target genes, are as important to guide correct DNA binding.

Ets factors are also known to physically and functionally interact with each other, such as the binding between Tel and Fli-1 (Kwiatkowski et al., 1998) and between Ets2 with Ets1 and Erg (Basuyaux et al., 1997), adding to the complexity of Ets function. These interactions tend to be tissue-specific, where in Ets2 knockout mice for example, Ets1 along with other gene targets such as MMP3, MMP9 and uPA are reduced in skin but unchanged in mammary gland (Yamamoto et
Therefore, recognizing this combinatorial nature of Ets family members would be essential in understanding the physiological function of Ets factors in vivo in future studies.

1.2 Epithelium-specific Ets transcription factor-1

1.2.1 Discovery and characterization of ESE-1

ESE-1, or epithelium-specific ETS transcription factor-1, is a member of epithelium-specific subfamily of Ets transcription factors, characterized by the epithelium-restricted pattern of expression. The mouse homolog of the human ESE-1 gene is called Elf3, and shares 89% homology with ESE-1 at the amino acid level (Tymms et al., 1997). Targeted deletion of Elf3 in mice leads to 30% of fetal lethality at around embryonic day 11.5 (E11.5), and severe defect in the formation and function of small intestine, with abnormal morphogenesis of enterocytes and goblet cells (Ng et al., 2002), implicating its role in tissue differentiation and embryogenesis. Our laboratory also had examined airway epithelial regeneration following Clara cell-specific injury in the Elf3 knockout (KO) mice and found that Elf3 regulated bronchiolar epithelial repair (Oliver et al., 2011).

Initial analyses of ESE-1/Elf3 expression levels in human and mouse tissues revealed that, unlike other Ets family members, it is absent in cells or organs of hematopoietic origin, such as the adult spleen, thymus, peripheral blood lymphocytes or fetal liver under basal conditions (interestingly, ESE-1 is profoundly present in the adult liver) (Oettgen et al., 1997). In cell lines, ESE-1 expression measured by Northern blot is almost exclusively isolated to epithelial cells and is undetectable in mature cell lines of hematopoietic origin, such as K562 (erythroid), Jurkat (T-cell) or DU-528 (macrophage) (Tymms et al., 1997). Instead, ESE-1 is notably upregulated during fetal development, especially in the lungs (Oettgen et al., 1997), and is associated with tissue differentiation, such as in the intestines (Ng et al., 2002), mammary glands (Neve et al., 1998), skin (Brembeck et al., 2000; Oettgen et al., 1997) and the cornea (Yoshida et al., 2000), as well as in malignancies such as lung carcinoma (Tymms et al., 1997), prostate cancer (Longoni et al., 2013), and breast cancer (Chang et al., 1997; Coppe et al., 2010; Kaplan et al., 2004; Prescott et al., 2004; Walker et al., 2010).

Additionally, protein binding partners have been shown to be critical in regulating Ets factor function (Li et al., 2000a). ESE-1 protein harbors five distinct protein domains, including a pointed
domain (PNT) at the N-terminus which is believed to mediate protein-protein interaction; transactivation domain (TAD) which enables activation of gene promoters and interaction with co-activators such as TATA box-binding protein (TBP); a unique serine- and aspartic acid-rich (SAR) domain which was shown to be involved in cellular transformation when localized in the cytoplasm (Prescott et al., 2004); two AT-hook domains which may potentially enable ESE-1 to bind in the minor groove of AT-rich DNA and interact with other proteins, as well as carrying the nuclear localization signal (NLS); and lastly, the Ets DNA-binding domain near the C-terminus (Kopp et al., 2007). The exact involvement of some of the protein domains in ESE-1 function, such as the nuclear function of the SAR domain, however, is yet to be determined.

1.2.2 ESE-1 expression and function in inflammatory diseases

ESE-1 has been shown to play a role in epithelial tissue differentiation and pathogenesis of various diseases (reviewed in (Oliver et al., 2012) and was originally characterized as having an epithelial-restricted expression pattern (Oettgen et al., 1997; Tymms et al., 1997), hence placing it in the epithelium-specific ETS subfamily. However, several studies have shown that the expression of ESE-1 can be induced by proinflammatory cytokines, such as IL-1β, TNF-α, and LPS in non-epithelial cells. Various examples include synovial fibroblasts (Brown et al., 2004; Grall et al., 2003), chondrocytes (Grall et al., 2003; Peng et al., 2008), osteoblasts (Grall et al., 2003), monocytes/macrophages (Grall et al., 2003; Grall et al., 2005; Rudders et al., 2001), vascular smooth muscle cells (Grall et al., 2005; Wang et al., 2004), and endothelial cells (Rudders et al., 2001; Wang et al., 2004; Zhan et al., 2010).

Our laboratory have also demonstrated that ESE-1 regulates allergic airway inflammation by controlling dendritic cell-driven T cell differentiation (Kushwah et al., 2011). Another biologically interesting feature of ESE-1 is that it downregulates its own expression through inhibition of NF-κB activity (Wu et al., 2008), which may play an important role in protecting the host from tissue injury during inflammation. Additionally, proinflammatory cytokine-mediated induction of ESE-1 requires nuclear translocation and transactivation of the ESE-1 promoter by the NF-κB family members p50 and p65 binding to a high-affinity NF-κB binding site (Grall et al., 2003). However, despite the close association of ESE-1 with NF-κB, its role in many inflammatory disorders such as sepsis is yet to be established. We provide an overview of the involvement of ESE-1 in various inflammatory diseases below:
1.2.2.1 Vascular inflammation

There is significant evidence on the involvement of ESE-1 in vascular inflammation from a number of studies. ESE-1 has been shown to be upregulated human aortic smooth muscle cells (HASMCs) and human umbilical vein endothelial cells (HUVECs) in response to proinflammatory stimuli IL-1β, TNF-α, and LPS, and to regulate the expression of inducible nitric oxide synthase (iNOS/NOS2) gene by binding to the Ets binding site within the NOS2 promoter. This involved a synergistic interaction between ESE-1 and the p50 subunit of NFκB (Rudders et al., 2001). Additionally, ESE-1 was highly induced in the vascular endothelium and first medial layer of vascular smooth muscle cells of the aorta within few hours of endotoxin administration in a rodent model of acute endotoxemia (Rudders et al., 2001). ESE-1 has also been shown to transactivate the promoter region of the gene encoding angiopoietin-1 (Ang-1), which facilitates angiogenesis and stabilization of new vessels by recruiting surrounding mesenchymal cells and promoting their differentiation into vascular smooth muscle cells, as well as enhancing endothelial cell migration during many inflammatory response (Brown et al., 2004). Among a panel of Ets factors, only ESE-1 was able to transactivate the Ang-1 promoter (Brown et al., 2004).

Similarly, in angiotensin II (Ang II)-induced model of vascular inflammation and remodeling, Elf3−/− mice exhibited an exaggerated response demonstrated by increased inflammatory cell infiltration in the aortic vessel walls and increased aortic thickness and perivascular fibrosis (Zhan et al., 2010). This was accompanied by increased proliferation in the cells within the vessel adventitia in response to Ang II and elevated systolic blood pressure compared to the wild-type littermate controls (Zhan et al., 2010). Consistent with the previous finding on the regulation of NOS2 by ESE-1, Elf3−/− mice had a significantly reduced expression of NOS2 in the aorta upon infusion with Ang II, indicating that ESE-1/Elf3 is essential in the maintenance of NOS2 expression that in turn confer vascular protection (Zhan et al., 2010). These findings suggest that ESE-1 is closely involved in blood vessel homeostasis and dysfunction in vascular inflammatory diseases.

1.2.2.2 Rheumatoid arthritis and osteoarthritis

ESE-1 has long been implicated in the pathogenesis of rheumatoid arthritis and osteoarthritis by its high expression in the cells lining the synovial layer and in mononuclear and endothelial cells
in the inflamed synovial tissues of patients suffering from rheumatoid arthritis and osteoarthritis (Grall et al., 2003). ESE-1 is transcriptionally upregulated by proinflammatory stimuli such as IL-1β, TNF-α, or LPS in the resident cell types of the synovium in culture, including synovial fibroblasts, chondrocytes, osteoblasts, and macrophages, typically displaying a peak expression between 2-6 hours and dissipation by 24 hours in most cells (Grall et al., 2003). Analogous to vascular inflammation, ESE-1 is involved in the regulation of Ang-1 expression in human rheumatoid arthritis synovial fibroblasts (RASFs) in response to proinflammatory cytokines such as TNFα, which is mediated by synergistic transactivation of the Ang-1 promoter with AML-1 (CBP-α/CBFA2), which is constitutively expressed in RASFs. Ang-1 is expressed in the synovium of patients with rheumatoid arthritis with very similar expression pattern as that of ESE-1 (Brown et al., 2004).

In human chondrocytes, which are specialized stromal cells that produce cartilage matrix proteins, ESE-1 was shown to be induced by IL-1β in a NFκB-dependent manner, and to act as a potent transcriptional repressor of the type II collagen (COL2A1) gene (Peng et al., 2007). The suppression of COL2A1 gene by IL-1β was specifically mediated by ESE-1 among Ets family members tested, including Elf-1, TEL, PDEF, SAP-1A, NERF2, and ETS-2, which showed only minimal effect on the inhibition or activation of the COL2A1 gene promoter (Peng et al., 2007). However, since Ets-1 was found to increase COL2A1, ESE-1 may also be competing for the same Ets binding site in fine-tuning type II collagen expression (Peng et al., 2007). ESE-1 expression was detected in human osteoarthritis cartilage specimen by immunohistochemistry, where it was predominant in the deep zone and in the superficial and middle zones near the degraded cartilage lesion, but absent in normal cartilages derived from healthy donors (Peng et al., 2007). Therefore, these results suggest that ESE-1 plays a role in cartilage degeneration and defective repair in arthritic inflammation.

### 1.2.2.3 Airway inflammation

Our research group has shown that ESE-1 is rapidly upregulated in human bronchial airway epithelial cell lines by proinflammatory cytokines IL-1β and TNF-α, as early as 2 hours post-stimulation (Wu et al., 2008). The cytokine-mediated induction of ESE-1 required transactivation of the ESE-1 gene by the binding of NFκB to the ESE-1 promoter. Interestingly, ESE-1 downregulated its own expression within 24 hours following cytokine treatment, implying the role...
of ESE-1 in both elicitation and resolution of an inflammatory response. ESE-1 was also found to regulate the expression of another ESE subfamily member, ESE-3, as well as the expression of the proinflammatory cytokine interleukin-6 (IL-6) in the bronchoalveolar lavage (BAL) and serum of Elf3-/- mice intranasally instilled with lipopolysaccharide (LPS) (Wu et al., 2008). In another study, ESE-1 was also shown to transactivate human lysozyme gene (LYZ) in human pulmonary epithelial cells in both primary bronchial epithelial cells and cell lines, indicating that it plays a role in lung innate immunity (Lei et al., 2007).

As IL-6 is a key inducer of Th17 differentiation and Th17 cells are involved in airway hyperresponsiveness to inhaled antigen in models of allergic dermatitis-induced asthma (He et al., 2007; He et al., 2009; Ma et al., 2002), our laboratory also investigated the effect of ESE-1/Elf3 deletion in Th17-driven airway inflammatory response in Elf3-/- mice by epicutaneous sensitization with ovalbumin (OVA) followed by intranasal delivery of the same antigen (Kushwah et al., 2011). Consistent with the reduced production of IL-6 upon inflammatory stimulation, Elf3-/- mice demonstrated an impaired Th17 response and an exaggerated Th2 response indicated by higher titres of OVA-specific IgE and IgG1 and increased concentration of IL-4 and -5 in serum, as well as elevated number of eosinophils (Kushwah et al., 2011). Interestingly, Elf3-/- T cells had no intrinsic defect in their ability to differentiate to Th1, -2, or Th17, but Elf3-/- dendritic cells (DCs), which are responsible in priming T cell differentiation, were found to be hypermatured with increased expression of cell surface markers CD80, CD86, and MHC class II and show impaired ability to upregulate Th1-driving cytokine IL-12 in response to LPS or produce Th17-inducing cytokine IL-6 (Kushwah et al., 2011). Naïve T cells co-cultured with Elf3-/- DCs secreted significantly higher levels of IL-4 but lower levels of IFN-γ, indicating skewed T cell differentiation to Th2 phenotype (Kushwah et al., 2011). Therefore, these results suggest that ESE-1 is involved in both innate and adaptive immune responses in the airway, by regulating airway epithelial and dendritic cell function.

1.2.3 Regulation of ESE-1 gene expression

Promoter analysis of ESE-1 has revealed four main regulatory regions Ets, CAAT, TATA, and NFκB (Oettgen et al., 1999). However, different regions of the upstream segments of ESE-1 gene may be more critical than others depending on the context, such as during development and differentiation versus inflammation. For example, Hou et al demonstrated that in retinoic acid-
differentiated murine embryonic carcinoma (EC) cell line F9, there is a substantial increase in the utilization of an upstream regulator region approximately 2 kb upstream of the transcription start site that was responsible for the increase in the ESE-1 promoter site (Hou et al., 2004). While it is well-known that long-range enhancers have important role in mammalian gene regulation (Heintzman and Ren, 2009), factors which bind to the enhancer region of ESE-1 gene have not been well-defined. A conserved 30 base pair (bp) ESE-1 enhancer sequence (EES) was also shown to be essential in regulating ESE-1 transcription in response to epithelial differentiation signals, controlled by two EES-binding protein complexes that are of uncertain identity (Neve et al., 2006). The most notable regulator of ESE-1 so far known is NFκB, which is required for ESE-1 induction in response to pro-inflammatory cytokines and binds to the NFκB-binding site in the promoter region of ESE-1 gene (Rudders et al., 2001; Wu et al., 2008). Additionally, ESE-1 has been shown to downregulate its own cytokine-induced expression (Wu et al., 2008), possibly by auto-inhibition through intramolecular interaction between the N-terminal transactivation domain and the C-terminal ETS domain (Kopp et al., 2007). Other non-transcriptional mechanisms of regulation of ESE-1, such as through microRNAs (Di et al., 2014; Qin et al., 2015) has never been explored.

1.3 Sepsis

1.3.1 Clinical Manifestation and Pathogenesis

Sepsis refers to severe, exaggerated and systemic response to an infection, and is a leading cause of death in critically ill patients. In the United States, sepsis affects about 750,000 people per year with 28-50% chance of death, with calculated cost for treatment exceeding $17 billion per annum (NIGMS, 2015). These numbers exceed the number of deaths from prostate cancer, breast cancer and AIDS combined (NIGMS, 2015) and increasing over time (Lagu et al., 2012). In Canada, sepsis is responsible for 9,300 death annually with a six times higher per hospitalization cost ($31,200) than all other diseases at $5,200 per hospitalization (Press, 2009).

The pathological events leading to the onset of sepsis are very complex. It is often initiated with a breach in the epithelial barrier such as that of the skin, airway, gastrointestinal and reproductive tracts by various forms of tissue injury, burns, or the use of intrusive devices such as catheters, which cause local infection (Seam and Suffredini, 2007). Cancer patients who had undergone extensive chemotherapy and those under immunosuppressants or suffering from immunodeficiency disorders are also at an increased risk of contracting an infection and
developing sepsis (Seam and Suffredini, 2007; Stearns-Kurosawa et al., 2013). The clinical manifestation of sepsis drastically varies, depending on the initial site of infection, the causative organism, and the pre-existing conditions of the patient. Symptoms therefore range from fever (core temperature >38.3 °C) or hypothermia (core temperature <36 °C), increased heart rate (>90 beats per minute), hyperglycemia (plasma glucose >120 mg/dl; without diabetes), positive fluid balance (>20 mL/kg of body weight over 24 hours), to leukocytosis (white cell count >12,000/mL) or leukopenia (<4,000/mL), to indicators of organ dysfunction and defective tissue perfusion, such as arteria hypoxemia, acute oliguria, thrombocytopenia, hyperlactatemia and hyperbilirubinemia (Angus and Van Der Poll, 2013; Annane et al., 2005). Severe sepsis can lead to a state of shock due to life-threatening hypotension in spite of fluid resuscitation from circulatory failure, and resulting multiple organ failure especially that of the lungs (acute respiratory distress syndrome, or ARDS), kidneys, and the brain (Seymour and Rosengart, 2015).

In 80% cases of sepsis, however, microorganisms (bacteremia) or LPS (endotoxemia) are not detected in the blood (Dejager et al., 2011), which indicates that it is the host’s immune response which result in an uncontrolled cytokine cascade, rather than the infection itself, that is responsible for the excess tissue damage in sepsis. At the cellular and molecular level, sepsis results from a widespread systemic inflammatory response to bacterial components, including endotoxins such as lipopolysaccharide (LPS) from Gram-negative bacteria, peptidoglycan and lipotechoic acid from Gram-positive bacteria, and mannan from fungi, which lead to the activation of neutrophils, macrophages and monocytes via pattern recognition receptors such as Toll-like receptors (TLRs). This leads to the secretion of large amounts of inflammatory cytokines and chemokines, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6, as well as reactive oxygen and nitrogen species, which in turn stimulate the synthesis of phospholipase A2, inducible cyclooxygenase, 5-lipoxygenase, and acetyl transferase, lead to the release of eicosanoids such as prostaglandins and leukotrienes, and platelet-activating factor. These mediators, through specific G-protein-coupled receptors, induce vasodilation and increased extravasation of immune cells, as well as elicitation of aberrant coagulation pathways which can lead to disseminated intravascular coagulation (DIC) and cause tissue hypoperfusion and hypoxia, and ultimately organ failure (Rice and Bernard, 2005; Sriskandand and Altmann, 2008).

In addition, the initial pro-inflammatory phase, which is referred to as systemic inflammatory response syndrome or SIRS, is often followed by an anti-inflammatory phase, or compensatory
anti-inflammatory response syndrome (CARS), which is associated with immune paralysis due to widespread apoptosis of peripheral white blood cells and refractory response of remaining immune cells to secondary stimulation (Carson and Kunkel, 2012). Within few hours or days following the onset of sepsis, peripheral tissue-resident immune cells such as splenic dendritic cells (DCs) are severely depleted due to cytokine-induced cell death (Tinsley et al., 2003), and cell density within primary and secondary lymphoid organs such as the thymus, bone marrow, and the spleen also significantly reduces (Riedemann et al., 2002; Wang et al., 1994). Worse, the excessive stimulation of immune cells renders them insensitive to further stimulus, resulting in a dulled response to secondary exposure to invading pathogens (Carson and Kunkel, 2012; Rice and Bernard, 2005). Therefore surviving sepsis patients become more susceptible to nosocomial or opportunistic infections, and with even higher mortality.

### 1.3.2 Role of neutrophils in immune response

Neutrophils play a central role in the initiation of a septic response, where overwhelming early activation of neutrophils lead to excess production of reactive oxygen species (ROS) and active proteases such as neutrophil elastases which can cause tissue damage (Fujimi et al., 2002; Sabroe et al., 2005). Tissue-infiltration of neutrophils is associated with organ dysfunction (Brown et al., 2006), and the increased number of peripheral immature neutrophils, or band cells, have long been used as a diagnostic tool for severe inflammatory responses, as they are indicative of deregulated rapid generation of new cells that are released before becoming full differentiated or functional, in response to widespread neutrophil apoptosis (Cavallazzi et al., 2010; Cornbleet, 2002). Neutrophils are the most abundant cell type in the human bloodstream, and are the major players in initiating and propagating the innate immune response (Amulic et al., 2012; Mocsai, 2013; Wright et al., 2010). Our body generates approximately 100 to 200 billion neutrophils from the bone marrow each day (Borregaard, 2010; Furze and Rankin, 2008). Neutrophils are terminally differentiated cells with usually a very short lifespan under steady state, ranging from 8-20 hours in circulation and 1-4 days in tissues (Luo and Loison, 2008). In response to an infection, they are rapidly recruited to phagocytose and kill invading microorganisms, and signal other immune cells by cytokine production (Amulic et al., 2012; Kumar and Sharma, 2010; Mocsai, 2013), consequences of which can lead to host tissue damage if excessive. Here I review neutrophil biology and its involvement in immune response in more detail:
1.3.2.1 Neutrophil differentiation and production

Ets and other key transcription factors involved in granulocytopoiesis

The production of mature neutrophils, also known as granulocytes is referred to as terminal granulocytopoiesis (Borregaard, 2010), and involves a number of transcription factors that are critical for myeloid lineage commitment as well as diversion from the monocyte lineage, such as C/EBPα (CCAAT/enhancer binding protein α) and PU.1. PU.1 is the master regulator of myeloid cell development and is essential for initial commitment to myelopoiesis (Iwasaki et al., 2005; Nerlov and Graf, 1998). The expression of PU.1 increases from the promyelocyte, myelocytes, and metamyelocytes, with concurrent increase in its coactivator c-jun and dimerization partner c-fos (Bjerregaard et al., 2003). PU.1 knockout mice lack terminally differentiated neutrophils and the small population of neutrophil-like cells fail to display functional competence, such as phagocytosis, superoxide generation, and response to chemokines (Anderson et al., 1998; Scott et al., 1994). The subsequent diversion from the granulocyte versus monocyte fate depends on the balance of expression in C/EBPα and PU.1 (Dahl et al., 2003; Laslo et al., 2006; Reddy et al., 2002). Maintained high expression of PU.1 leads to monocyte development, while high expression of C/EBPα drives granulocytopoiesis up to the myeloblast stage (Radomska et al., 1998; Zhang et al., 1997).

The transcription factor Gfi-1 (growth factor independent-1) is another necessary factor for neutrophil differentiation (Hock et al., 2003; Karsunky et al., 2002). Upregulation of Gfi-1 during stem cell commitment to granulocytic lineage represses genes for HoxA9, Meis1, and Pbx1, which curb stem cell proliferation and lead to progressive cell differentiation into granulocytes. Gfi-1 also inhibits the expression of monocyte-promoting transcription factor Egr2 (early growth response-2) (Laslo et al., 2006) and the monocytopoietic cytokine M-CSF gene Csf1 (Zarebski et al., 2008). C/EBPε peaks during the transition from the myelocyte to metamyelocyte, and partly regulates the exit from the cell cycle by binding to Rb and E2F1 (Gery et al., 2004) thus inhibiting their transactivation of genes involved in cell division. C/EBPε, as well as other C/EBP members C/EBPβ, C/EBPγ, C/EBPδ, C/EBPζ, are required for the production of granules and increases from the metamyelocyte stage to drive further granulocytic differentiation (Bjerregaard et al., 2003).
Production of neutrophils

Production of neutrophils constitutes approximately two-thirds of hematopoiesis in the bone marrow, generating 1 to 2 x 10^{11} neutrophils daily in healthy adult human. The key physiological regulator of granulocytopoiesis is granulocyte colony-stimulating factor (G-CSF), which promotes progenitor cell commitment to myeloid lineage (Richards et al., 2003), proliferation of the precursors (Lord et al., 1989) and release of the differentiated granulocytes from the bone marrow. Mice without G-CSF receptor (Lieschke et al., 1994; Liu et al., 1996) and humans with dominant negative mutations (Druhan et al., 2005; Sinha et al., 2003) are severely neutropenic. Plasma concentration of G-CSF is almost undetectable under normal conditions, but rises drastically during an inflammatory response. Many tissues and cell types, including endothelial cells, macrophages, epithelial cells and fibroblasts can produce G-CSF in response to inflammatory stimuli, such as LPS, TNF-α, IL-1β, vascular endothelial growth factor (VEGF), and IL-17 (Bendall and Bradstock, 2014). Neutrophil homeostasis is also regulated by the rate of apoptosis and neutrophil clearance in tissues, primarily in the liver, spleen, or bone marrow (Luo and Loison, 2008; Suratt et al., 2004; Suratt et al., 2001) by resident macrophages and dendritic cells. Phagocytosis of apoptotic neutrophils reduces their interleukin 23 (IL-23) production, which in turn decreases the interleukin IL-17A (IL-17A) secretion by specialized gamma-delta and NK-like T cells called neutrophil regulatory cells (Ley et al., 2006), which are mainly localized in mesenteric lymph nodes. IL-17A is a major stimulus for G-CSF (Schwarzenberger et al., 2000), and thus increased neutrophil clearance in tissues leads to decreased granulopoiesis (Borregaard, 2010).

1.3.2.2 Mobilization and recruitment

Neutrophil reserve in the bone marrow

The bone marrow contains a pool of mature neutrophils which reside in the hematopoietic cords separated from the blood by the sinusoidal endothelium (Furze and Rankin, 2008). The sinusoidal endothelium plays a critical role in regulating neutrophil egress, and constitutively expresses cell adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), P-selectin and E-selectin, unlike in other endothelial cells which only
express these adhesion molecules in response to an inflammatory stimulus (Schweitzer et al., 1996; Simmons et al., 1992). Additionally, when exiting from the bone marrow, neutrophils egress by transcellular migration through the sinusoidal endothelial cells rather than at cell junctions, as the underlying adventitial cells and basement membrane do not form a continuous barrier (Burdon et al., 2008; Summers et al., 2010).

Retention and release signals

Inflammatory triggers, such as leukotriene B4, C5a, bacterial products, platelet-activating factor, and chemokine interleukin 8 (IL-8) lead to a rapid and selective mobilization of mature neutrophils from the bone marrow (Jagels et al., 1995; Jagels and Hugli, 1992; Mollnes et al., 2002; Terashima et al., 1998). Neutrophils respond to the chemotactic gradient created from the blood across the sinusoidal endothelium, through surface receptors such as CXCRs, G-CSFR, and Toll-like receptors (Theilgaard-Mönch et al., 2005). CXCR4 plays a fundamental role in maintaining neutrophil reserve in the bone marrow. Specific deletion or inhibition of CXCR4 results in increased neutrophil release from the bone marrow but without affecting neutrophil life-span (Devine et al., 2008; Eash et al., 2009; Iyer et al., 2008; Ma et al., 1998; Pelus et al., 2005), and mutations in CXCR4 gene have also been associated with clinical syndrome of WHIM (warts, hypogammaglobulinemia, infections, myelokathesis), which is characterized by accumulation of mature neutrophils in the bone marrow and deficiency of neutrophils in circulation (Hernandez et al., 2003; Zuelzer, 1964). The CXCR4 ligand, chemokine stromal-derived factor (SDF)-1α (=CXCL12), is expressed on the surface of bone marrow stromal cells including osteoblasts and vascular endothelial cells, and acts as a retention signal for neutrophils (Martin et al., 2003; Suratt et al., 2004). The expression of SDF-1α and CXCR4 is downregulated by G-CSF, which leads to rapid neutrophil release from the bone marrow (De La Luz et al., 2007; Kim et al., 2006; Semerad et al., 2002). Other chemokines such as CXCL1 and CXCL2 (or KC and MIP-2, respectively) are also involved in neutrophil mobilization by binding to their receptor CXCR2 expressed on neutrophils. Mice pretreated with neutralizing monoclonal antibodies to KC and MIP-2, for instance, lead to 84% inhibition in neutrophil recruitment to the peritoneum in a model of acute peritonitis induced by intraperitoneal (i.p.) administration of thioglycollate (Wengner et al., 2008). Disruption of CXCR2 leads to a myelokathexis phenotype with mature neutrophils accumulating in the bone marrow, while CXCR2/CXCR4 double knockout shares similar phenotype as CXCR4 deletion (Eash et al., 2010). Therefore, neutrophil mobilization is broadly regulated by the
balancing act between CXCR4, which retains neutrophils, and CXCR2, which facilitates neutrophil release (Borregaard, 2010; Eash et al., 2010).

**Other surface molecules on neutrophils**

In rodents, transmigration of neutrophils is mediated by the interaction between the alpha 4 integrin CD49d expressed on the surface of neutrophils and VCAM-1, which is constitutively expressed on bone marrow stromal cells and sinusoidal endothelial cells (Issekutz et al., 1996a; Issekutz et al., 1996b). CD49d is highly upregulated in response to inflammation, and its blockage by specific neutralizing antibodies or antagonists inhibits neutrophil mobilization in response to chemokines such as MIP-2 (Burdon et al., 2005). However, whether this also applies to humans is under question. In addition, mobilized neutrophils express very low levels of L-selectin (CD62L), which is believed to act as a retention factor and that its shedding increases the velocity by which neutrophils roll along the endothelium (Hafezi-Moghadam and Klaus, 1999; Hafezi-Moghadam et al., 2001; Kassirer et al., 1999; Rogowski et al., 1998). Neutrophils also express high levels of CD18 integrins, in forms of CD11a:CD18 (leukocyte function-associated antigen (LFA)-1) and CD11b:CD18 (Mac-1), which mediate firm adhesion to ICAM-1 induced on endothelium during inflammation. However, CD18 neutralizing antibody had no effect on the rate or number of neutrophils released from the bone marrow in response to a CXC chemokine, indicating that it is not required for neutrophil mobilization during inflammation (Burdon et al., 2005).

**1.3.2.3 Neutrophil activation and function**

*Detection of invading pathogen by pattern recognition receptors*

Neutrophils express a wide variety of pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) (Hayashi et al., 2003; Kawai and Akira, 2011), C-type lectin receptors dectin 1 (also known as CLEC7A) (Greenblatt et al., 2010), CLEC2 (or CLEC1B) which is a phagocytic activation receptor (Kerrigan et al., 2009), and cytoplasmic RNA-sensors, RIG-I and MDA5 (Tamassia et al., 2008), as well as nucleotide-binding oligomerization domain protein 1 (NOD1), which recognizes bacterial peptidoglycan (Clarke et al., 2010). Neutrophils constitutively express all TLRs except TLR3, including that recognize bacterial lipopeptides (TLR2), LPS (TLR4), flagellin (TLR5), and double stranded DNA (TLR9) (Amulic et al., 2012). The sensing of invading pathogens and tissue damage activate neutrophils to produce ROS, antimicrobial peptides, lytic
enzymes, and proinflammatory cytokines. Activated neutrophils demonstrate enhanced phagocytosis and destruction of microorganisms, by the upregulation of additional receptors such as CD64, which is a high-affinity receptor for IgG induced by IFN\(\gamma\) and GM-CSF (Hoffmeyer et al., 1997; Turzanski et al., 1997) that leads to increased respiratory burst activity (Barth et al., 2002), and CD14, which is a co-receptor for LPS (Wagner et al., 2003). Other receptors involved in enhanced bacterial recognition and phagocytosis include CD16 and CD32, which similar to CD64 recognize the Fc sites of IgG, and the C3b receptor, which binds complement peptide C3b (Brown et al., 2006).

**Neutrophil arsenals for pathogen killing**

Neutrophils mediate killing of invading microorganisms by releasing antimicrobial molecules, such as proteases, lactoferrin, myeloperoxidase, and cationic peptides contained in their granules. Neutrophil granules can be divided into primary (or azurophilic), or secondary (or specific) and tertiary (or gelatinase) granules, depending on the presence or absence of myeloperoxidase (MPO), respectively (Bainton and Farquhar, 1968a; Bainton and Farquhar, 1968b). Azurophilic granules, which are the largest of neutrophil granules, are rich in serine proteases, including cathepsin G, azurocidin, neutrophil elastases and proteinase 3. Mice deficient in cathepsin G are susceptible to Gram-positive bacterial infections, such *Staphylococcus aureus* (Reeves et al., 2002), while mice deficient in neutrophil elastases are susceptible to several Gram-negative bacteria, such as *Escherichia coli*, *Klebsiella pneumonia* and enterobacteria (Belaaouaj et al., 1998; Weinrauch et al., 2002). Secondary (or specific) granules contain glycoprotein lactoferrin, as well as other antimicrobial compounds such as hCAP-18 and lysozyme (Faurschou and Borregaard, 2003; Faurschou et al., 2002), and tertiary (or gelatinase) granules, also MPO-negative, largely contain metalloproteases, such as gelatinase and leukolysin (Amulic et al., 2012). MPO is crucial in oxidative burst and the formation of neutrophil extracellular traps (NETs) (Metzler et al., 2010; Papayannopoulou et al., 2010; Winterbourn et al., 2006). NETosis, a unique form of cell death in neutrophils that involves the release of chromatin with concentrated bactericidal proteins (Brinkmann and Zychlinsky, 2007; Fuchs et al., 2007), is another mechanism by which neutrophils immobilize and destroy invading microorganisms.

**Interaction with cells of the adaptive immune system**
Despite previous beliefs of neutrophils as key players of innate immunity, increasing evidence suggests that neutrophils cross-talk with various cells of the adaptive immune system and modulate their function (Mocsai, 2013). For example, interaction between neutrophils and monocyte-derived DCs via the CD18 and CEACAM1 (carcinoembryonic antigen-related cell adhesion molecule-1) on neutrophils and DC-SIGN (DC-specific ICAM3-grabbing non-integrin) on DCs promotes the production of IL-12 and DC maturation, subsequently inducing T cell proliferation and maturation towards Th1 phenotype (Megiovanni et al., 2006; Van Gisbergen et al., 2005a; Van Gisbergen et al., 2005b; Van Gisbergen et al., 2005c). Additionally, neutrophils can express MHC class II molecules when stimulated by GM-CSF, IL-3, or IFN-γ, or by other activated immune cells such as macrophages, and interact with T cells to induce strong MHC class II-restricted T cell responses (Beauvillain et al., 2007; Gosselin et al., 1993; Mudzinski et al., 1995; Radsak et al., 2000). Human peripheral neutrophils also constitutively express B7-1-like molecules, which interact with CD28 on T cells to augment IFNγ secretion (Venuprasad et al., 2003; Windhagen et al., 1999). Furthermore, upon antigen capture at the periphery, neutrophils can migrate to the lymph nodes in a CC-chemokine receptor 7 (CCR7)-dependent manner like DCs (Beauvillain et al., 2011), and presumably compete with other antigen-presenting cells such as DCs and macrophages in the lymph node, whereby neutrophils lead to specific suppression of B cell and CD4+ T cell responses (Yang et al., 2010). Activated neutrophils also secrete Th1- and Th17-chemokines, such as CCL2, CXCL9 and CXCL10, or CCL2 and CCL20, respectively (Pelletier et al., 2010), to recruit T cells to the site of infection. Therefore, neutrophils are being newly found to undertake a complex immunomodulatory role beyond their previously established role as first responders of the innate immune system.

1.3.2.4 Neutrophil apoptosis in control of inflammation

Neutrophils are pre-programmed to undergo apoptosis

Neutrophils are pre-programmed to undergo spontaneous apoptosis by multiple mechanisms, including high basal levels of reactive oxygen species (ROS) and activated proteases such as cathepsins and calpains, which also act as arsenals to destroy microorganisms (Fox et al., 2010; Geering and Simon, 2011; Luo and Loison, 2008; Witko-Sarsat et al., 2011). Constitutive neutrophil death is not only essential to maintain the neutrophil homeostasis, but is also a critical modulator of immune response particularly during the resolution phase of inflammation (Fox et
They additionally have rapid turnover of a key anti-apoptotic protein Mcl-1, and an increased expression of Apaf-1 and procaspase-9 (Witko-Sarsat et al., 2011), which are involved in executing apoptosis. These mitochondrial and caspase-mediated apoptotic pathways can similarly be induced by the stimulation of external death receptors, such as Fas (a cell death receptor) (Croker et al., 2011) and TNFR1 (TNF-α cytokine receptor) (Cross et al., 2008; Geering et al., 2011a; Salamone et al., 2001) when engaged by their ligands (Fox et al., 2010; Geering and Simon, 2011), or conversely be inhibited via crosstalk with survival pathways, mainly the PI3K/Akt, p38MAPK and NF-κB (El Kebir and Filep, 2013; Geering and Simon, 2011), in response to external survival signals such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and lipopolysaccharide (LPS) bacterial endotoxin.

**Neutrophil apoptosis is delayed in septic patients and is a potential therapeutic target**

As mentioned above, timely resolution of inflammation is crucial to minimize tissue damage (Serhan et al., 2007). However, neutrophils continue to persist in non-resolving inflammation such as sepsis, due to delayed apoptosis or impaired removal of cellular debris by tissue macrophages (efferocytosis) (Nathan and Ding, 2010; Savill et al., 2002). Altered neutrophil apoptosis is also prominent in other human diseases, such as chronic obstructive pulmonary disease (COPD) (Brown et al., 2009), cystic fibrosis (McKeon et al., 2008; Watt et al., 2005), and rheumatoid arthritis (Wong et al., 2009). In sepsis, in particular, the degree of neutrophil apoptosis inversely correlates with disease severity (Fialkow et al., 2006), and systemic administration of human apoptotic neutrophils significantly improved survival in mouse models of sepsis (Ren et al., 2008). There thus has been a heightened interest of therapeutically targeting neutrophil apoptotic pathways as a potential treatment approach. In animal models of pneumonia (El Kebir et al., 2012), peritonitis (McGrath et al., 2011; Negrotto et al., 2006), and arthritis (Rossi et al., 2006; Sekine et al., 2008), apoptosis-inducing compounds such as R-roscovitine (Rossi et al., 2006), recombinant TNF-related apoptosis-inducing ligand (rTRAIL) (McGrath et al., 2011), and Resolvin E (El Kebir et al., 2012) successfully reduced inflammation and enhanced tissue repair.

Nonetheless, there are major challenges to exploiting the therapeutic potential of apoptosis-inducers. For example, in contrast to neutrophils, lymphocytes undergo increased apoptosis (Le Tulzo et al., 2002; Oberholzer et al., 2001; Unsinger et al., 2009), which likewise negatively impact a patient’s clinical outcome by instigating post-septic immunosuppression and leading to increased
susceptibility to nosocomial infections (Carson and Kunkel, 2012; Shubin et al., 2011; Vaki et al., 2010). Therefore, a better understanding of the molecular mechanisms of altered cellular apoptosis is critical to successfully target an appropriate signaling pathway in a specific cell population, especially given that early treatment of sepsis is necessary to maximize patient survival (Annane et al., 2005; Seam and Suffredini, 2007).

1.3.3 Animal models of sepsis

Animal models of sepsis have been established to study the pathobiology of human sepsis and to test therapeutic agents prior to human clinical trials. The main objective of animal models is to reproduce the disease pathogenesis which resemble human condition. However, in all sepsis models, there are several drawbacks in replicating the human disease, including the timing of the disease progression and lack of supportive therapeutic interventions in animals (Buras et al., 2005). The onset and progression of septic syndrome occurs within hours to days in most animals, while human patients develop sepsis over days to weeks. Additionally, human patients are promptly treated upon septic-like symptoms, such as removal of the source of infection (for example, contaminated catheter), fluid, antibiotics and vasopressor therapy, nutritional and oxygen support, and mechanical ventilation, which are often not fully employed in animal models (Buras et al., 2005). There are other limitations which are further discussed later in the chapter. However, animal models have immensely contributed to our understanding of the host response and the role of inflammatory mediators during the progression of sepsis. Sepsis animal models can be divided into three main categories (Buras et al., 2005; Doi et al., 2009; Rittirsch et al., 2007): (1) administration of exogenous toxin, such as LPS; (2) administration of viable pathogens, i.e. live bacteria; (3) disruption in the animal’s internal protective barrier, such as causing intestinal leakage by cecal ligation and puncture (CLP). Of these, LPS- and CLP-induced sepsis are the most common animal models of sepsis, which are further reviewed below:

1.3.3.1 LPS-induced endotoxemia model

LPS-induced endotoxemia model is one of the most widely used animal models for the study of sepsis. It offers advantages of specifically targeting a known signaling pathway through TLR4, and the ability to control the disease severity by titrating the amount of LPS that is administered, thus making it convenient and reproducible (Fink and Heard, 1990; Nemzek et al., 2008). LPS-
induced systemic inflammation mimics many of the initial clinical phase of sepsis, such as vast production of proinflammatory cytokines TNF-α and IL-1β in the absence of bacteremia (Michie, 1988; Remick et al., 2000; Wichterman et al., 1980). The animals respond well to neutralizing antibody against TNFα or IL-1β (McNamara et al., 1993; Tracey, 1987). However, clinical trials for anti-TNFα and anti-IL-1 therapies failed to improve survival in humans (Deitch, 1998; Fisher, 1996; Fisher et al., 1994). These may be attributed to the fact that rodents are a lot more resistant to LPS than humans and show much more rapid and intense cytokine responses. For example, while a single dose of approximately 2 ng/kg LPS was sufficient in humans to elicit profound physiological changes in humans, such as increased body temperature, systolic blood pressure and heart rate, mice required 500 ng/kg to show similar effects (Copeland et al., 2005). Mouse models often require 10 mg/kg or higher doses of LPS, depending on the LPS serotype, mouse strain and the age of the animals, to produce systemic hypodynamic cardiovascular state, and without the initial hyperdynamic cardiovascular phase seem in humans (Buras et al., 2005; Deitch, 1998; Fink, 2008; Nemzek et al., 2008). The dose of LPS can be reduced by sensitizing animals by co-injecting D-galactosamine (Buras et al., 2005); however, this can cause hepatotoxicity which may complicate the study (Galanos et al., 1979). LPS animal models exhibit rapid development of systemic clinical symptoms, such as lethargy, shivering, and scruffy fur, within hours of LPS administration, with cytokine production peaking between 1.5-4.5 hours and declining over 8 hour period (Remick et al., 2000). Therefore, LPS-induced endotoxemia models are particularly useful when studying the rapid innate immune and cytokine responses in the early phase of sepsis progression (Fink, 2014; Fink and Heard, 1990), but additional models are often used for the validation of clinical relevance.

### 1.3.3.2 Cecal-ligation and puncture (CLP) model

Cecal-ligation and puncture (CLP) model is now the most popular animal model of sepsis (Dejager et al., 2011; Doi et al., 2009; Nemzek et al., 2008). It involves surgically ligating the mouse cecum distal to the ileocecal valve and puncturing the ligated cecum with a needle to cause leakage of fecal contents into the peritoneum, which leads to polymicrobial bacteremia and sepsis (Dejager et al., 2011; Hubbard et al., 2005). Within a few hours following the surgery, animals show signs of illness, such as hunched posture, weight loss, and diarrhea (Ebong et al., 1999a; Ebong et al., 1999b; Nemzek and Remick, 2004). CLP models also demonstrate characteristics of early-stage sepsis, including increased blood flow, hyperglycemia and hyperinsulinemia, followed by late-
stage sepsis, such as decreased blood flow, hypoglycemia, increased serum lactate (Chaudry et al., 1979; Hubbard et al., 2005; Weighardt, 2002). The severity of inflammation and mortality can also be adjusted by the length of ligated cecum and the number of punctures. For example, in BALB/c mice, two punctures by 18-gauge needle leads to 100% mortality, but only 50% and 0% with 21-gauge or 25-gauge needles, respectively (Ebong et al., 1999a; Ebong et al., 1999b). The concentration of proinflammatory cytokines in the peritoneum and the plasma also correlate with the needle size (Ebong et al., 1999a; Ebong et al., 1999b), and higher proportion of cecum that is ligated leads to increased tissue necrosis, peritoneal inflammation, and mortality (Singleton and Wischmeyer, 2003). In addition, the genetic background, sex, and age of the animals influence the outcome in CLP models (De Maio et al., 2005; Dienstknacht et al., 2004; Turnbull et al., 2009a; Zellweger et al., 1996), and thus must be standardized to produce consistent results. Post-operative fluid supplements and antibiotic are often given following the invasive abdominal surgery, but this also differs among laboratories and can be a source of variation (Doi et al., 2009; Nemzek et al., 2008). Polymicrobial bacteremia induces progressive systemic inflammatory response syndrome that leads to multi-organ injury and septic shock. CLP-induced sepsis models demonstrate a cytokine profile which resemble that observed in human sepsis (Eskandari, 1992; Remick et al., 2000), and anti-TNF-α treatment fail to improve survival similar to humans (Echtenacher et al., 1990; Eskandari, 1992; Yasuda et al., 2006). Additionally, CLP-induced sepsis models show increased lymphocyte apoptosis which mimics the compensatory immunosuppressive phase of human sepsis that follows the initial pro-inflammatory phase (Ayala and Chaudry, 1996; Dear et al., 2006; Hotchkiss and Karl, 2003). Despite these advantages, however, the CLP model does have several limitations. Firstly, it does not reproducibly develop acute kidney or lung injury (Kuhlmann et al., 1994; Pedersen et al., 1989) as seen in humans, and the CLP procedure can cause abscess formation (Buras et al., 2005), which can be an added confounding factor to the immune response towards infection. Lastly, lack of standardization in the surgical techniques and postoperative care makes it difficult to directly compare studies or reproduce findings from the published literature (Nemzek et al., 2008).

### 1.3.3.3 Accurately modeling human disease: disparities in animal models

Demographically, sepsis is a major disease of the aged, where over 60% of sepsis incidence and 80% of death arise from patients over the age of 65 (Angus et al., 2001; Martin et al., 2006). With aging population and increased life expectancy in many of the industrialized countries, burden of
disease is inevitably on the rise. However, despite extensive research and over 100 Phase II and III clinical trials (Marshall, 2014), there has been a disturbingly limited success in improving patient survival. While efforts continue to develop targeted immunomodulatory therapies (Hutchins et al., 2014), past failures signal a necessity to examine sepsis beyond the aberrant immune response. A closer look at the preclinical research studies reveals several discrepancies between the sepsis experimental approaches and the most-affected population, such as the use of young animals equivalent to under 20 years of human age to model endotoxemia, and inadequate optimization of animal models for respiratory microbial infection that more closely resemble the human condition. Additionally, human and mouse neutrophils differ quite significantly, where murine neutrophils lack defensins that are present in humans (Mestas and Hughes, 2004), and human and mouse neutrophil proteinase 3 and L-selectins have different substrate specificities (Hajjar et al., 2010; Zollner et al., 1997), thus leading to disparities in functional analysis and data interpretation when using animal models to study human sepsis. In fact, a recent study by Seok et al (Seok et al., 2013) systematically compared the genomics of peripheral leukocytes in human disease with mouse models of burn, trauma, and sepsis and demonstrated poor correlation in the ortholog genes, especially in the models of endotoxemia, and peripheral leukocytes are dominated by neutrophils in humans but by B lymphocytes in mice (Mestas and Hughes, 2004).

A number of recent studies have already demonstrated an age-dependent increase in mortality during systemic inflammation (Starr and Saito, 2014; Turnbull et al., 2009b), and age-specific factors involved are just starting to be uncovered (McConnell et al., 2011). However, another source of disparity is the means by which the causative agent, such as LPS, is administered in the animal models, which are often injected either intraperitoneally or intravenously. The respiratory tract infection is the primary source of infection in 47% of severe sepsis cases (Martin et al., 2009) and more than 50% of sepsis patients also develop acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) with higher disease severity compared to non-sepsis cases (Nomellini et al., 2009; Sevransky et al., 2009). Respiratory tract infection is also a leading cause of illness in the elderly (Meyer, 2005), implicating the lung as a central organ system associated with morbidity and onset of sepsis. Yet, unlike in polymicrobial peritonitis, systemic endotoxemia, or acute pancreatitis models, age-dependent increase in mortality has not been consistently observed in bacterial pneumonia models (Starr and Saito, 2014). Additionally, while differences in bacterial and animal strains and the inoculation techniques may largely be responsible for this variability, it
is likely that fundamental differences in the surrounding tissues, such as the airway structure, cell composition, and the process of airway epithelial senescence between mouse and human (Ware, 2008) are also accountable. Thus development of better animal models which are more representative of the human condition as well as greater understanding of age-related changes in the immune regulation is critical, to better replicate human disease in *in vivo* studies (Lee and Kushwah, 2014).

### 1.4 Rheumatoid Arthritis

#### 1.4.1 Pathogenesis of Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a systemic autoimmune inflammatory disease of the synovial membrane, characterized by hyperplastic and invasive transformation of the cells lining the synovium and infiltration of immune cells, which leads to chronic synovitis that cause pain, swelling, and destruction of cartilage and bone, as well as systemic effects on cardiovascular, pulmonary, and skeletal parts of the body (Choy, 2012; McInnes and Schett, 2011). While there are a number of putative factors which are believed to contribute to the onset of RA, the exact etiology and pathogenesis of RA remain unknown. Some patients show positive serotype for autoantibodies such as rheumatoid factor and anti-citrullinated protein antibody (ACPA), which recognize the Fc region of other antibodies or citrullinated self-proteins, respectively (Nielen et al., 2004; Strand et al., 2007). RA patients who are ACPA-positive have a less favorable prognosis than those who are ACPA-negative (McInnes and Schett, 2011). There is also genetic association to human leukocyte antigen (HLA)-DRB1*04 cluster to ACPA positivity (Gregersen et al., 1987; Smolen et al., 2007), as well as to single-nucleotide missense polymorphisms in PTPN22 gene, which encodes for a phosphatase involved in T-cell activation (Begovich, 2004). However, environmental factors may also play a role in inducing RA, as cigarette smoking has been associated with increased risk of developing RA (Klareskog et al., 2007; Padyukov et al., 2004). These environment-gene interactions are thought to trigger loss of tolerance to self-proteins, likely initiated in secondary lymphoid tissues or the bone marrow, through incompletely understood mechanisms (Mahdi et al., 2009; McInnes and Schett, 2011). In the diseased state, immune cells such as macrophages, B-, and T-cells infiltrate the synovial membrane, leading to the destruction of the articular cartilage and the underlying bone matrix by constant production of pro-inflammatory cytokines such as TNFα, IL-1β, and IL-17, and matrix metalloproteinases (MMPs)
including MMP-1, -9, and MMP-13, which is perpetrated by positive feedback (Choy, 2012; McInnes and Schett, 2011). The type B synoviocytes in RA, or rheumatoid arthritis synovial fibroblasts (RASFs), and osteoclasts also produce these stimulatory molecules involved in the RA pathology, while chondrocytes progressively undergo apoptosis in the highly inflammatory milieu (Choy, 2012; McInnes and Schett, 2011). Below we provide an overview of key cells and mediators involved in the RA pathogenesis, particularly on the role of RASFs and cyclooxygenase-2 (COX-2) signaling:

1.4.1.1 Altered phenotype of synovial fibroblasts

Normal function of synovial fibroblasts

In healthy individuals, the synovium is relatively acellular and quiescent, and produces synovial fluid which lubricates and nourishes the surrounding cells, mainly macrophage-like (Type A) and fibroblast-like (Type B) synoviocytes (Firestein, 2009; Smith, 2011). Type A synoviocytes have been shown to be true macrophages derived from bone marrow precursors, expressing surface markers such as CD68, CD163, and CD45, while type B synoviocytes show strong activity for uridine diphosphoglucose dehydrogenase (UDPDG, enzyme involved in haluronan synthesis), VCAM-1, and CD55, and are locally derived from perivascular fibroblasts (Barland et al., 1962; Bartok and Firestein, 2010; Edwards, 1994; Edwards and Willoughby, 1982; Smith, 2011). Type B synoviocytes are also referred to as ‘SFs’, or synovial fibroblasts, and are the dominant cell type of normal synovium (Smith et al., 2003). The synovial lining provides a non-adherent and lubricated interface which exhibit viscoelastic properties, to allow stretching, folding, and rolling of the joints (Smith, 2011). Synovial fibroblasts (SFs), under normal circumstances, provide cartilage lubrication and nutrition by secreting lubricants such as ‘lubricin’ and ‘superficial zone protein’ (Jay et al., 2000; Rhee et al., 2005), and prevent fibrosis and scarring by the production of plasminogen activator and DAF (decay accelerating factor for complement) (Firestein, 2009; Smith, 2011). SFs are also involved in continuous matrix remodeling by producing matrix-degrading enzymes and a number of matrix components such as laminin, collagen, fibronectin, and hyaluronan, which are critical for maintaining a constant synovial fluid volume and composition as well as for cushioning the synovial tissue and acting as a reservoir of lubricants and nutritious media for feeding chondrocytes (Bartok and Firestein, 2010; Smith, 2011). Interestingly, the normal synovium produces a basal level of inflammatory cytokines, including
IL-1, IL-6 and TNFα (Smith et al., 2003). However, their levels are far lower than that seen in RA, and are outweighed by the production of anti-inflammatory agents, such as IL-1 receptor antagonist and osteoprotegrin, which acts as a decoy receptor that inhibits receptor activator of nuclear factor kappa-B ligand (RANKL) required for the development and activation of osteoclasts (Bartok and Firestein, 2010; Gravallese et al., 1998; Smith et al., 2003). SFs can also express a number of adhesion molecules such as VCAM-1, ICAM-1, CD44, β1 and β3 integrins (Connolly et al., 2011), which may be important in cellular trafficking and recruiting immune cells to the synovial membrane.

**Alterations in rheumatoid arthritis**

In rheumatoid arthritis (RA), SFs become hyperplastic and demonstrate a hyperactivated phenotype, producing an enormous amount of cytokines, adhesion molecules, and matrix metallproteinases (MMPs). Normal synovial lining usually consists of 1-3 cell layers, but this increases to 10-15 layers in RA (Neumann et al., 2010). The hyperplastic tissue eventually overgrows the cartilage surface and causes cartilage destruction and bone erosion. RASFs synthesize a number of MMPs, particularly MMP-1, -3, -8, -13, -14, and -16, which are involved in the disassembly of the type II collagen network, while other matrix enzymes such as ADAMTS 5 degrade aggrecan (Stanton et al., 2005), thus altering both matrix content and water retention capability (McInnes and Schett, 2011; Sabeh et al., 2010). In addition to MMPs, RASFs also produce proteolytic enzymes cathepsin K and L, which degrade various types of collagens and proteoglycans (Schedel et al., 2004; Trabandt et al., 1990), and RANKL, which mature and activate osteoclasts (Lee et al., 2006), further contributing to the degradation process of the bone and cartilage.

The activation of RASFs seems to be both cytokine-dependent and cytokine-independent, and occur early in the RA development. The RASFs can act autonomously in the absence of immune cells, as demonstrated by the ability of implanted isolated human RASFs to invade co-implanted human cartilage in severe combined immunodeficiency (SCID) mouse model of RA which lacked any of the cellular and humoral immune responses (Müller-Ladner et al., 1996). This indicates that RASFs may both initiate and perpetuate RA. RASFs significantly contribute to the proinflammatory cytokine and mediator production in the joint space. In addition to well-known cytokines such as IL-1 β, TNF-α, and IL-6, RASFs are a major source of PGE2 in the synovial
lining (Kojima et al., 2003), and attract CD4+ T cells by producing CXCL16, IL-16 and SDF-1 (Müller-Ladner et al., 2007; Ruth et al., 2006), as well as macrophage inflammatory protein (MIP), monocyte chemotactic protein-1 (MCP-1), CCL5, IL-8 to attract other immune cell types such as macrophages and B-cells (Hanyuda et al., 2003; Müller-Ladner et al., 2007). Proinflammatory cytokines such as IFNγ, IL-1, IL-18, and TNF-α in turn upregulate the expression of ICAM-1 and VCAM-1 on RASFs, which enhances interaction with T lymphocytes, monocytes, and polymorphonuclear neutrophils via LFA-1 and VLA-4 (very late activation antigen-4), respectively (Müller-Ladner et al., 2007). Immune cell recruitment is further enhanced by the secretion of proangiogenic factors from RASF, such as angiopoietin-1 and -2 (Takahara et al., 2004). The importance of RASF-mediated vascularization in persistent inflammation was shown by RASFs transduced with antiangiogenic thrombosponin-2, which were able to inhibit local vascularization and inflammation in a SCID mouse model of RA (Yong et al., 2004).

Furthermore, accumulating evidence suggests that RASFs have prolonged survival due to an imbalance in the proapoptotic and anti-apoptotic factor expression. The primary growth stimulus for fibroblasts is the fibroblast growth factor (FGF) family, and RASFs not only respond to, but also produce FGFs, forming a positive autocrine loop. RASFs also overexpress a number of early response genes and proto-oncogenes, including egr-1, c-fos, myc, and ras (Müller-Ladner et al., 2007) which lead to increased proliferation. Ras, in particular, is predominantly expressed at sites of invasive growth, and is associated with the expression of cathepsin L (Pap et al., 2004). Similarly, RASFs often lack the expression of phosphatase and tensin homolog (PTEN) (Pap et al., 2000), which is a tumor suppressor that dephosphylates phosphatidylinositide 3 kinase (PI3K) and in turn inhibits the pro-survival Akt pathway. Resistance to apoptosis in RASFs can further be attributed to somatic mutations in p53 (Firestein et al., 1997) primarily localized in the intimal lining (Yamanishi et al., 2005; Yamanishi et al., 2002). This renders cells unable to induce p53-upregulated modulator of apoptosis (PUMA) required for p53-mediated apoptosis (Firestein et al., 1996; Tak et al., 1999). Other anti-apoptotic molecules involved in RASF survival include FLICE inhibitory protein (FLIP), which inhibits caspase-8 (Kataoka, 2005), and sentrin (SUMO-1), which protects cells from Fas- or TNF R1-induced cell death (Franz et al., 2000) by sequester pro-apoptotic molecules such as DAXX in promyelocytic leukemia (PML) nuclear bodies (Meinecke et al., 2007; Meinecke et al., 2009). RASFs also express high levels of anti-apoptotic members of
the Bcl family such as Bcl-2 and Mcl-1 (Korb et al., 2009; Kurowska et al., 2002; Liu et al., 2005), which contribute to their increased survival and persistence.

1.4.1.2 Critical role of COX-2 in rheumatoid arthritis

**COX-2 and Prostaglandin E\(_2\)**

Cyclooxygenase-2 (COX-2), also known as prostaglandin-endoperoxide synthase 2 (PTGS2), is one of the two COX isomers that catalyze the rate-limiting step of prostaglandin (PG) biosynthesis (Smith and Song, 2002). COX enzymes converts arachidonic acid, which is released from cell membrane phospholipids by phospholipase A\(_2\) in response to various triggers of inflammation such as C5a and thrombin, to prostaglandin H\(_2\) (PGH\(_2\)). PGH\(_2\) are further converted into a number of eicosanoid derivatives by cell-specific isomerases into PGE\(_2\), PGD\(_2\), PGI\(_2\) (also known as prostacyclin), PGF\(_{2\alpha}\), and thromboxane A\(_2\) (TXA\(_2\)), which mediate important biological processes including inflammation and its cardinal symptoms, and normal gastric and kidney function (Shimizu, 2009). The two isoforms of COX, though encoded by different genes, share 60% homology at the amino acid level and catalyze the same reactions (Rouzer and Marnett, 2009). However, unlike COX-1 which is constitutively expressed in many tissues and carry out “housekeeping” functions such as gastric epithelial protection, COX-2 is induced by inflammatory mediators such as cytokines, growth factors, and bacterial endotoxins (DuBois et al., 1998; Jones et al., 1993; Lee et al., 1992). The important role of COX-2 in arthritic inflammation has been demonstrated in a number of animal models (Ricciotti and FitzGerald, 2011). COX-2, but not COX-1, for example, is induced in the synovial tissues in rat adjuvant arthritis model, where COX-2 selective inhibitor (SC-58125) reduced paw edema and IL-6 production (Anderson et al., 1996). COX-2 is similarly induced in human RA and is strongly expressed in patients with inflammatory arthropathies, including RA, ankylosing spondylitis, or psoriatic arthritis (Crofford et al., 1994; Siegle et al., 1998). Other studies have shown that COX-2 is the primary source of PG during inflammation (Sundy, 2001).

Prostaglandins are small potent mediators of inflammation, and are recognized by G-protein coupled receptors (GPCRs), divided into subfamilies including DP (PGD receptor), EP (E prostanoid receptor)1-4, FP (PGF receptor), IP (PGI receptor), and TP (thromboxane receptor), which bind to PGD\(_2\), PGE\(_2\), PGF\(_{2\alpha}\), PGI\(_2\), and TXA\(_2\), respectively (Narumiya and FitzGerald,
Clinical efficiency of COX inhibitors are based on inhibiting PG production (Flower, 2003). PGE₂, in particular, is one of the most abundantly produced PG in the body, and by chondrocytes and RASFs in synovitis (Martel-Pelletier et al., 2003). PGE₂ is involved in eliciting cardinal signs of inflammation, such as fever, edema and pain, by acting as a pyretic agent (Cocceani and Akarsu, 1998), inducing arterial dilatation and increased microvascular permeability, and sensitizing peripheral sensory nerve endings to nociceptive mediators such as bradykinin and histamine, respectively (Funk, 2001). Consistent with this, selective neutralization of PGE₂ in rat model of adjuvant-induced arthritis using monoclonal antibodies significantly reverses edema in affected paws as well as reducing hyperalgesia and IL-6 production at sites of inflammation (Portanova et al., 1996). PGE₂ is synthesized by the conversion of PGH₂ by cPGES or mPGES-1 and mPGES-2, which are enzymes present in the cytosol or perinuclear membrane of various cell types (Samuelsson et al., 2007), and exerts its function by binding to one or more of its receptors referred to as EP1-EP4 (Trebino et al., 2003). Among these receptors, EP3 and EP4 are most widely expressed and have higher affinity to PGE₂ than EP1 and EP2, and are more closely implicated in RA pathology (Honda et al., 2006; Minami et al., 2001; Yuhki et al., 2004). EP receptors are present on many cell types including macrophages, dendritic cells, T and B lymphocytes, and can have both pro-inflammatory and anti-inflammatory effects depending on the context (Ricciotti and FitzGerald, 2011). PGE₂-EP4 signaling in DCs and T cells, for example, facilitates Th1 and IL-23-dependent Th17 differentiation (Yao et al., 2009), while PGE₂-EP2/4 signaling in microglial cells mediates bradykinin-induced neuroprotection and inhibited cytokine synthesis (Caggiano and Kraig, 1999; Noda et al., 2007).

**Regulation of COX-2**

The regulation of COX-2 expression is complex and occurs at both transcriptional and post-transcriptional levels. COX-2 promoter contains multiple cis-regulatory regions, including a canonical TATA element, cAMP response element (CRE), NF-IL6 (CCAAT/enhancer-binding protein-β), E-box, AP-2, SP-1, STAT, and NFκB-binding sites (Crofford et al., 1997; Kirtikara et al., 2000; Koon et al., 2006; Mestre et al., 2001; Saunders et al., 2001; Schroer et al., 2002; Tazawa et al., 1994; Zhu et al., 2002). The specific transcriptions factors that activate COX-2 are cell type- and stimulus-dependent. For instance, while COX-2 induction by IL-1β in RASF is mediated by NFκB (Crofford et al., 1997), the same induction in human microvascular endothelial cell line,
HMEC-1 involves AP2, NF-IL-6 and CRE elements (Kirtikara et al., 2000). COX-2 mRNA also contains a number of AU-rich elements (ARE) within its 3’ untranslated region (3’UTR), which is involved in controlling mRNA degradation and protein translation (Dixon et al., 2001; Sheng et al., 2000). ARE refers to a 116-nucleotide region that harbors a cluster of six AUUUA sequence elements located near the stop codon of COX-2 (Dixon et al., 2000). Post-transcriptional regulation of COX-2 through ARE is mediated by trans-acting ARE-binding factors (Cok et al., 2003; Cok and Morrison, 2001), such as HuR (Dixon et al., 2001), CUGBP2 (CUG triplet repeat, RNA-binding protein 2) (Mukhopadhyay et al., 2003), and Apobec-1 (Anant et al., 2004), which binds to the COX-2 ARE and stabilizes the COX-2 transcript, as well as ARE/poly(U) binding factor 1 (AUF1/hnRNP D) (Lasa et al., 2000), tristetraprolin (TTP) (Fenger-Gron et al., 2005; Phillips et al., 2004; Young et al., 2009), and T-cell intracellular antigen 1 (TIA-1) (Dixon et al., 2003) and CUGBP2, which enhance mRNA decay or inhibit translation, respectively. Up to 16 different proteins which bind to COX-2 mRNA 3’UTR have been identified (Young and Dixon, 2010). Therefore, the relative abundance of the ARE-binding proteins inside the cell can regulate COX-2 mRNA stability and efficiency of translation. COX-2 mRNA is also subjected to regulation by microRNAs, such as miR-16, miR-101a and miR-199a (Chakrabarty et al., 2007; Shanmugam et al., 2008; Strillacci et al., 2009).

5.2.2 **Current therapies for rheumatoid arthritis**

COX inhibitors, which are part of NSAIDs that also include common drugs such as ibuprofen and naproxen, have traditionally been important in RA disease management, to reducing pain, swelling, and inflammation. Over the past half-century, COX inhibitors have evolved to more specifically target COX-2, such as rofecoxib and celecoxib, given concerns over adverse effects of conventional inhibitors which target both isoforms of COX causing gastrointestinal ulcer and bleeding, kidney dysfunction, or issues with blood clotting. Although clinical responses vary, patients treated with NSAIDs demonstrate fewer tender or swollen joints and decreased morning stiffness (Flower, 2003; Sundy, 2001).

Unlike NSAIDs which can only relieve the symptomatic pain and swelling but not change the course of RA, however, disease-modifying anti-rheumatic drugs (DMARDs) are agents which can both inhibit and potentially prevent further inflammatory and destructive responses in RA (Smolen and Steiner, 2003). Both drug types are usually prescribed together for maximal benefit. The most
commonly used small-molecule DMARD is methotrexate (MTX), which is a folate analogue that blocks dihydrofolate reductase activity during folic acid metabolism required for the synthesis of purines and pyrimidines (Cutolo et al., 2001; Smolenska et al., 1999). Low dose of MTX leads to enhanced adenosine release (Cronstein et al., 1991; Cronstein et al., 1993), which inhibits lymphocyte and monocytic proliferation (Cutolo et al., 2000; Genestier et al., 1998), production of IL-8, IL-12, and TNF (Constantin et al., 1998; Link et al., 2000; Sajjadi et al., 1996; Seitz et al., 1995), prostaglandin and collagenase synthesis in synoviocytes (Mello et al., 2000; Vergne et al., 1998), as well as leukotriene B4 synthesis and chemotaxis in neutrophils (Kraan et al., 2000; Leroux et al., 1992), while increasing the expression of TIMPs (Seitz and Dayer, 2000). Monotherapy with methotrexate is effective in 1/3 of RA patients, and is often employed as a combination therapy which has been shown to be more effective (Boers et al., 1997; Möttönen et al., 1999; O'Dell et al., 1996). Other small chemical DMARD includes leflunomide (Smolen et al., 1999), which inhibits dihydro-orotate-dehydrogenase, a pivotal enzyme for de novo pyrimidine synthesis, which has shown to be effect in blocking TNF-dependent NFκB activation (Manna and Aggarwal, 1999) and IgG1 production (Siemasko et al., 1998). Leflunomide also inhibits the production of MMP-1, -3, and -13 in RASFs while increasing the synthesis of IL-1RA by downregulating the MAPK signaling (Migita et al., 2004; Palmer et al., 2004). Key biologic DMARDs include specific recombinant antibodies against tumor necrosis factor (TNF), such as infliximab, etanercept, and certolizumab; chimeric anti-CD20 monoclonal antibody (Rituximab) to block B cell function; anti-CTLA-4 fused with Fc portion of IgG1 (Abatacept), IL-1 receptor antagonist Anakinra, and more recently, tocilizumab which has been approved for severe RA and targets IL-6 (Kahlenberg and Fox, 2011). Biologic DMARDs are usually added to or substitute conventional DMARD treatment when common treatment options prove ineffective and depending on the disease progression (Kahlenberg and Fox, 2011).
Hypothese and Objectives

Hypotheses

ESE-1 was previously shown to be induced by proinflammatory cytokines and regulate gene targets involved in inflammation. However, most of the findings have been based on in vitro systems using cell lines, and there have been lack of tangible connections between the findings and human diseases. Therefore, in order to better understand the role of ESE-1 in complex human conditions, we decided to study the expression and function of ESE-1 using human patient tissue samples and Elf3-/- mice as an in vivo model. Sepsis and rheumatoid arthritis are serious systemic inflammatory diseases which have no cure and cause significant burden and morbidity on individual patients as well as the society as a whole. Both diseases have uncontrolled and hyperactive host immune responses as the underlying mechanisms of their pathogenesis. Based on previous findings on the involvement of ESE-1 in inflammatory responses, we therefore hypothesized that:

1. ESE-1 is highly upregulated in human neutrophils derived from patients suffering from sepsis to regulate important inflammatory genes. We also hypothesized that human inflammatory diseases can be successfully modeled in animals, and that Elf3-/- mice will provide an invaluable tool to study Elf3 in vivo, given the extensive use of murine models of endotoxemia to study the role of cytokines and innate immune response in human sepsis.

2. ESE-1 is rapidly induced in rheumatoid arthritis synovial fibroblasts (RASFs) by proinflammatory stimuli such as LPS and IL-1β, and it is stabilized in the cytoplasm by post-translational modification. Additionally, because ESE-1 was shown to positively regulate COX-2 expression in cooperation with NFκB in macrophages (Grall et al., 2005), we hypothesized that ESE-1 indirectly regulates COX-2 expression by affecting signaling pathways that occur in the cytoplasm, such as MAPK, which are known to target Ets factors (Selvaraj et al., 2015; Wasylyk et al., 1998).
3. *ESE-1/Elf3* plays an important role in macrophage function and differentiation, by acting as a downstream effector of differentiation pathways and by regulating key genes involved in macrophage function, such as *COX-2, iNOS,* and *LYZ.*

ESE-1 is known to play a dual role in squamous epithelial differentiation, where it suppresses the expression of basal keratin 4 (K4) that is important in mediating early proliferation, while concurrently activating the small proline-rich protein 2A (SPRR2A) promoter associated with late differentiation (Brembeck et al, 2000). Therefore, as macrophage differentiation is marked by similar distinct stages of early phase Ras-dependent cell proliferation and terminal phase METS-mediated growth suppression (Guidez et al, 1998; Klappacher et al, 2002), we speculated that *Elf3* will also play a likewise dual role in cell proliferation and differentiation pathways in macrophage development.

Therefore, to investigate the role of *ESE-1* in human health in relevance to the pathogenesis of two major human inflammatory diseases, sepsis and rheumatoid arthritis, and in macrophage development and function, we sought to test our hypotheses by meeting these specific objectives using human patient-derived primary cells and *in vivo* models using *Elf3/-* mice:

**Objectives:**

1. To investigate the expression of *ESE-1* in normal and septic human neutrophils, and relate its expression to neutrophil function using *in vivo* sepsis model in *Elf3/-* mice.

2. To investigate the role of *ESE-1* in human rheumatoid arthritis synovial fibroblasts.

3. To examine the effect of *ESE-1* deletion on myeloid cell development and function.
Chapter 2

2 ESE-1 is constitutively expressed in human neutrophils: implication of its role in neutrophil function and survival

A modified version of this chapter has been published in Genes and Immunity: Chan Mi Lee, Sahil Gupta, Jean Parodo, Jing Wu, John C. Marshall, Jim Hu. ESE-1 is constitutively expressed in human neutrophils: implication of its role in neutrophil function and survival. Genes and Immunity, 2015, 16(5):356-61.
2.1 Abstract

Epithelium-specific Ets transcription factor 1 (ESE-1) is a member of the ETS family of transcription factors that has an epithelial-restricted constitutive expression but is induced by inflammatory stimuli in non-epithelial cells. Here we report that ESE-1 is constitutively expressed in human, but not in murine, neutrophils, and that ESE-1 is modestly upregulated in septic patient neutrophils. In normal human neutrophils, ESE-1 was detected at both RNA and protein levels, but was found to be an unstable nuclear protein ex vivo. ESE-1 transcription was also induced during all-trans retinoic acid (ATRA)-mediated HL-60 differentiation, a human promyelocytic cell line often used as an in vitro model of human neutrophils. Elf3/− mice had normal neutrophils and no alteration in the disease manifestation of LPS-induced endotoxemia compared to wild-type littermate controls. These findings indicate a potential role of ESE-1 in regulating human neutrophil differentiation and function, and that it has different roles in the immune system of different species.

2.2 Introduction

The E26 transformation-specific (ETS) family of transcription factors includes approximately 30 members that are involved in immune regulation and hematopoiesis (Bartel et al., 2000; Maroulakou and Bowe, 2000). Key examples include PU.1, which regulates the fate of common myeloid progenitors (CMPs) and is important in the normal differentiation of B cell and T cell populations; Ets1/2, a downstream regulator of monocyte colony-stimulating factor receptor (M-CSFR) signaling which drives macrophage differentiation; Fli1 and Erg which are required for megakaryopoiesis and normal hematopoietic stem cell (HSC) homeostasis; and myeloid Elf1-like factor (MEF), also known as Elf4, which is involved in natural killer (NK) cell development (Bartel et al., 2000; Maroulakou and Bowe, 2000).

Epithelium-specific Ets transcription factor 1 (ESE-1, or Elf3 in mouse) is a member of the ETS family which was originally characterized as having an epithelial-restricted pattern of expression (Oettgen et al., 1997; Tymms et al., 1997). ESE-1 is upregulated during fetal development, especially in the lungs (Oettgen et al., 1997), and is associated with epithelial tissue differentiation (reviewed in (Oliver et al., 2012)). It is also expressed in epithelial malignancies such as lung carcinoma (Tymms et al., 1997) and breast cancer (Chang et al., 1997; He et al., 2007; Kaplan et
al., 2004; Oettgen et al., 1997). Initial analyses of ESE-1 expression suggested that, unlike other ETS family members, it is absent in cells or organs of hematopoietic origin, including the adult spleen, thymus, peripheral blood lymphocytes and fetal liver (Oettgen et al., 1997; Tymms et al., 1997). Others have found ESE-1/Elf3 to regulate important genes involved in monocyte/macrophage function, including cyclooxygenase-2 (COX2), lysozyme (LYS), inducible nitric-oxide synthase (iNOS/NOS-2), and angiopoietin-1 (Ang-1). Recent studies from our laboratory have also demonstrated a role of ESE-1/Elf3 in both innate and adaptive immune responses in the airways, where it was found to be upregulated by pro-inflammatory cytokines in bronchial epithelial cells (Wu et al., 2008) and involved in airway Th2 and Th17 responses by controlling dendritic cell-mediated T-cell differentiation (Kushwah et al., 2011). Despite mounting evidence of the involvement of ESE-1 in myeloid cell function, however, no study has specifically looked at the presence or function of ESE-1 in neutrophils.

Neutrophils are the most abundant leukocytes in human peripheral blood (Bjerregaard et al., 2003). While they are well known to be phagocytic cells that destroy microbes by releasing lytic enzymes and reactive oxygen species (ROS), recent discoveries indicate that they also orchestrate the adaptive immune responses by activating dendritic cells and stimulating T cell responses, as well as playing a fundamental role in the resolution of inflammation (El Kebir and Filep, 2010; Mantovani et al., 2011). Neutrophils are involved in the pathogenesis of a number of inflammatory diseases including sepsis, rheumatoid arthritis, chronic obstructive pulmonary disease (COPD), asthma, cystic fibrosis (CF), and systemic lupus erythematosus (SLE) (Beyrau et al., 2012). Here we show that ESE-1 is constitutively expressed in human neutrophils and modestly upregulated in septic patient neutrophils, indicating its potential role in neutrophil function.
2.3 Materials and Methods

Reagents

DMEM, RPMI, fetal bovine serum (FBS), and L-glutamine were purchased from Gibco Life Technologies Ltd., Burlington, Ontario. Penicillin/streptomycin, phosphate buffered saline (PBS), and Hank’s balanced salt solution (HBSS) were from Wisent, St. Bruno, Quebec, and lipopolysaccharide (LPS) endotoxin (Escherichia coli, serotype O111:B4) endotoxin and all-trans-Retinoic acid were purchased from Sigma, Oakville, Ontario. Antibodies used for flow cytometry were: APC anti-human CD66 and APC Annexin V from BioLegend, San Diego, CA; anti-mouse PE CD11b (M1/70), APC Gr-1 (RB6-8C5), PE CD3e (145-2C11), and anti-mouse CD16/32 purified antibody from eBio science, San Diego, CA. 7-AAD for dead cell exclusion was obtained from BD Biosciences, Mississauga, Ontario. Antibodies for Western blot included monoclonal rabbit ESE-1 antibody produced from our laboratory in collaboration with Epitomics, Burlingame, CA; β-actin antibody was from Sigma, Oakville, Ontario; and SUMO (D-11) and LDH (H-160) antibodies from Santa Cruz Biotechnology, Dallas, TX.

HL-60 cell culture and ATRA treatment

HL-60 cells (American Type Culture Collection, Rockville, MD) were maintained in suspension in RPMI supplemented with 10% FBS, 2 mM L-glutamine, and 100 U/mL penicillin and 100 µg/mL streptomycin at 5% CO₂ and 37°C. For granulocytic differentiation, cells were seeded at 300,000 cells/mL in 0.1% ethanol (vehicle control), and 1 µM, 2 µM, or 5 µM all-trans-retinoic acid (ATRA) up to 6 days with media renewal every 2 days. The cells were counted on a hemocytometer chamber with trypan blue dye exclusion. HL-60 cells of passage 20 or less were used for all experiments.

Neutrophil Isolation and Culture

Neutrophils were isolated from the peripheral blood of healthy volunteers and septic patients admitted to St. Michael’s Hospital Intensive Care Unit (ICU). Procedures were approved by the St. Michael’s Hospital Research Ethics Board and performed upon written informed consent of all subjects. Briefly, blood was collected into syringes containing 100 U/mL heparin and red blood cells were sedimented in sterile 3% dextran 0.9% NaCl. Neutrophils were isolated by
centrifugation through a discontinuous Ficoll-Paque gradient (Amersham Biosciences). Following elimination of remaining erythrocytes with ammonium chloride hypotonic solution, isolated neutrophils were resuspended in 1 x 10⁶ cells/mL concentration in DMEM containing 10% FBS and streptomycin/penicillin and incubated in polypropylene tubes for indicated number of hours in 5% CO₂ and 37°C. Neutrophil purity was assessed by forward and side scatter on flow cytometry and was consistently >95%.

**Preparation of cytoplasmic and nuclear extracts and Western blot**

Nuclear and cytoplasmic extracts were prepared from 1 x 10⁷ human neutrophils, where pelleted cells were resuspended in 100 µL of hypotonic buffer (Buffer A: 10 mM HEPES pH 7.5, 10 mM KCl, 3 mM NaCl, 3 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, and 2 mM dithiothreitol) containing protease inhibitor cocktail (Roche, #11 836 170 001) and 1 mM Na₃VO₄ and 1 mM NaF phosphatase inhibitors and incubated on ice for 15 minutes. Following incubation, 0.05 volumes of 10% Nonidet P-40 were added and cells were vortexed for 10 seconds, and immediately centrifuged at 500 x g for 10 minutes at 4°C. The supernatant was collected and designated as the cytoplasmic extract, while the pellet was washed once with 200 µL of Buffer A containing protease inhibitors, re-centrifuged, and lysed in 100 µL 6xSDS sample buffer (2% (w/v) SDS, 58.3 mM Tris-HCl (pH 6.8), 6% (v/v) glycerol, 5% (v/v) 2-ME, 0.02% (w/v) bromophenol blue) and was designated as the nuclear extract. For the whole cell lysates, neutrophils (2 x 10⁶) were lysis in lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10 mM NaF, 1 mM PMSF, 1 mM Na₃VO₄, 10 µg/mL leupeptin, 10 µg/mL aprotinin). Protein concentrations were measured by BCA assay (Pierce) following trichloroacetic acid (TCA) protein precipitation. Denatured proteins were separated on 12% SDS-polyacrylamide gels and transferred to Immobilon-P (Millipore, Bedford, MA) membranes. Membranes were blocked with 5% (w/v) nonfat milk in TBST (50mM Tris-Cl pH 7.5, 150 mM NaCl, 0.05% Tween-20) for 1 hour at room temperature and probed for β-actin (1:4,000), ESE-1 (1:3,000), SUMO (1:200), or LDH (1:200) overnight at 4°C. Protein signals were detected with HRP-conjugated secondary antibodies at a dilution of 1:4,000 using ECL Western blotting detection system (Amersham Pharmacia Biotech).

**RNA Isolation and real-time quantitative PCR (RT-qPCR)**

To extract RNA from human neutrophils, 10 million cells were lysed in PureZOL RNA Isolation Reagent (Bio-Rad Laboratories, Mississauga, Ontario) and recovered from the aqueous phase of
the phenol-chloroform mixture following manufacturer’s protocols. Total RNA from HL-60 cells were isolated using GE Illustra RNA Spin Mini Kit (GE Healthcare Life Sciences, Baie-D’Urfe, Quebec) and RNA from the mouse cells and tissues were isolated using an RNeasy Micro Kit (Qiagen, Mississauga, Ontario). For quantitative real-time RT-PCR, total RNA (1 µg) was reversed transcribed using random hexamers and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) and the resulting cDNA template (50 ng) was used for qPCR reaction using Power SYBR Green PCR Master Mix from Life Technologies, Burlington, Ontario. ABI Prism 7700, from Applied Biosystems, Foster City, CA was used for the amplification and analysis. For relative ΔΔCt quantification, qPCR signals were normalized using GAPDH or TBP. The primers for human ESE-1 and GAPDH were purchased from ABI. The primer sequences used in this study were as follows: hESE-1 5′-GGCGTCTTTCAAGTCTCTCGG-3′ (Forward), 5′-CTCCCGTTTGTAGTACCT-3′ (Reverse); hTBP 5′-GGGCATTATTTGTGACTGAGA-3′ (Forward), 5′-TAGCAGCACGGTATAGCAACT-3′ (Reverse); hGAPDH 5′-GAAGGTGAAGGTCGGAGTC-3′ (Forward), 5′-GAAGATGGTGATGGGATTTC-3′ (Reverse); mElf3 primer set 1 5′-GAGATGGCTTTCCTGACTATAAG-3′ (Forward), 5′-GGTGGATTAGGATGTCTCGGAT-3′ (Reverse); mElf3 primer set 2 (from Harvard PrimerBank) 5′-GAAGGCAAGCTGGACTAGCG-3′ (Forward), 5′-GGAGCTGGCGTATACTTGTT-3′ (Reverse); mElf3 primer set 3 (also from Harvard PrimerBank) 5′-CTGACTCCGCTGGAAATGTATG-3′ (Forward), 5′-CGGGGTGGATTAGATGCTTC-3′ (Reverse); mGAPDH 5′-GTGGCAAAGTGGAGATTGCC-3′ (Forward), 5′-GATGATGACCCGTTTGGCTCC-3′ (Reverse).

Animals and murine neutrophil analyses

Elf3-/- mice on C57BL/6 background were maintained in pathogen-free conditions at the Toronto Centre for Phenogenomics (TCP), and wild-type littermates were used as controls. All experiments were reviewed and approved by the TCP Animal Care Committee (ACC) for the humane use of animals. To model the inflammatory response in vivo, 8-12 weeks old mice were intraperitoneally injected with 5 mg/kg LPS or equal amount of PBS and analyzed after 24 hours. For bone marrow analysis, tibia and femur from each mouse were flushed with 27G needle with RPMI media supplemented with 10% FBS and made into single cell suspensions by passing through the 70µm filter. Red blood cells were lysed with ammonium chloride lysis solution (155 mM NH₄Cl 10 mM KHCO₃, 1 mM EDTA) and remaining cells were stained for flow cytometry. For the analysis of
Peripheral neutrophils, blood was collected by cardiac puncture under isoflurane anesthesia with EDTA-coated syringes, and aliquots were run on the Hemavet 950FS Hematology Analyzer at the Centre for Modeling Human Disease (CMHD), TCP, Toronto.

**Flow cytometry and Fluorescence-Activated Cell Sorting (FACS)**

HL-60 cell granulocytic differentiation was quantified by flow cytometry with CD66 antibody staining and apoptosis by Annexin V antibodies using Annexin V-binding buffer (BioLegend). Bone marrow cells in single cell suspension were stained at 1 x 10^7 cells/mL concentration in staining buffer (2% FBS 1xHBSS 10 mM HEPES pH 7.2) with CD11b and Gr-1 conjugated antibodies for 30 min at 4 °C. Samples were acquired on LSRII (BD), and minimum 20,000 of live events were collected and analyzed using Flowjo (Tree Star). For FACS, bone marrow cells were likewise stained with CD11b and Gr-1 antibodies, and CD11b^{high}Gr-1^{high} cells were sorted using MoFlo Astrios (Beckman) with purity >99%.

**Peripheral cytokine measurement**

Following intraperitoneal administration with LPS (10 mg/kg), whole blood was collected by cardiac puncture into EDTA-coated tubes and centrifuged at 2,000 g for 15 min 4 °C to collect the plasma. Cytokines were quantified by Quantibody Mouse Cytokine Array from RayBiotech Inc, Norcross, GA following manufacturer’s instructions.

**Statistical Analysis**

Individual experiments were repeated minimum three times, and results are expressed as mean±SD unless indicated. Statistical analyses were performed using two-tailed Student’s t-test with Welch’s correction for unequal variances where appropriate, or ANOVA with Bonferroni’s post-test analysis for multiple groups. A P-value of less than 0.05 was considered as statistically significant.

### 2.4 Results

*ESE-I is expressed in human neutrophils and upregulated in sepsis*
Therefore, given the previously identified roles of *ESE-1* in inflammatory diseases such as rheumatoid arthritis and vasculitis, we investigated the expression of *ESE-1* in neutrophils from both healthy volunteers and sepsis patients. By qPCR and Western blot using the A549 human lung adenocarcinoma cell line as a positive control, *ESE-1* was detectable at both RNA (Ct of around 27) and protein levels in human neutrophils (Figure 2-1), and was modestly upregulated in septic patient neutrophils (Figure 2-2A,B). Consistent with previous findings of ESE-1 as a nuclear transcription factor, ESE-1 was found to be residing mostly in the nucleus with negligible amounts in the cytoplasm (Figure 2-1A-C). The expression of *ESE-1* transcripts, however, declined as early as 2 hours, and continued to drop over time (Figure 2-1D), in association with a delayed yet still rapid degradation of the ESE-1 protein (Figure 2-1A-C). Ectopically expressed EGFP-ESE-1 protein through nucleofection into HL-60 cells, was similarly rapidly downregulated within 24 hours, as detected by flow cytometry (Figure 2-3A) and Western blot (Figure 2-3B). LPS treatment did not rescue the rapidly dissipating ESE-1, and we observed a large variability of *ESE-1* expression in septic patient neutrophils (Figure 2-2A), likely reflective of the disease heterogeneity. Upon checking *ESE-1* protein levels in more than 20 patients, however, it proved very difficult to pinpoint any particular subgroup of patients that upregulate the protein. Also, *ESE-1* expression had no correlation with the degree of apoptosis of septic neutrophils (Figure 2-2C).
Figure 2-1 *ESE-1* is expressed in human neutrophils and undergoes rapid downregulation *ex vivo*.

(A-C). ESE-1 protein is expressed in the nucleus and undergoes rapid protein degradation in human neutrophils from normal healthy individuals. Neutrophils isolated from the peripheral blood of each donor were incubated *ex vivo* in culture media for 0, 4, 8, and 21 hours, and at each time point were nuclear fractionated and analyzed for the expression of ESE-1 protein by Western blot. Lactose dehydrogenase (LDH) and Small Ubiquitin-like Modifier (SUMO) proteins were used as cytoplasmic and nuclear markers, respectively. Human lung adenocarcinoma cell line (A549) was used as a positive control (+) for ESE-1 protein. (D). RT-qPCR analysis of the normal healthy neutrophils for the expression of ESE-1 mRNA over the same time course of *ex vivo* incubation. The number of individuals and raw Ct values (mean±SD) of ESE-1 and GAPDH per time point are shown.
**Figure 2-2** ESE-1 is modestly upregulated in sepsis patient neutrophils but its level does not correlate with degree of apoptosis.

(A). ESE-1 is modestly upregulated in human septic neutrophils. The expression of ESE-1 transcript was compared in 10 healthy donors (HD; n=10) and 20 septic patients (SP; n=20) by RT-qPCR. The raw Ct values (median±SD) for each group are shown for ESE-1 and TATA-binding protein (TBP). GAPDH was found to be an unsuitable internal reference when directly comparing healthy with septic neutrophils, because GAPDH itself was significantly elevated in sepsis likely due to increased anaerobic metabolism. Statistical analysis was performed by two-tailed Student’s t-test with Welch’s correction assuming non-equal variance. (B). Western blot showing the expression of ESE-1 in eight sepsis patients (SP; n=8) and two healthy donors (HD; n=2). Healthy donor neutrophils were treated with or without LPS (1 μg/mL) for 4 hours to compare with the septic neutrophils. ‘-’= no LPS; ‘+’=added LPS. A549 cell line was used as a positive control for the ESE-1 protein. (C). Relative expression of ESE-1 at transcriptional level plotted against percentage of apoptosis in each of the 20 septic patients.
Figure 2-3 Ectopically expressed ESE-1 undergoes rapidly downregulation in nucleofected HL-60 cells.

(A). Flow cytometry gating for EGFP+ cells in nucleofected HL-60 with pEGFP-C3-empty or pEGFP-C3-ESE-1. Note the rapid reduction of EGFP+ cells within 24 hours of nucleofection in the pEGFP-ESE-1-nucleofected cells compared to the pEGFP-C3 control vector. (B). Western blot showing the expression of nucleofected EGFP-ESE-1 and EGFP alone in HL-60 cells at 5 and 10 hours. The pEGFP-C3 vector contains a CMV promoter which increases the gene expression with LPS treatment. Representative data from three independent experiments.
**ESE-1 is upregulated during HL-60 granulocytic differentiation**

As an independent confirmation of the expression of *ESE-1* in human neutrophils, we found that *ESE-1* transcription was induced during all-trans retinoic acid (ATRA)-mediated HL-60 differentiation, a human promyelocytic cell line often used as an *in vitro* model of human neutrophils (Breitman et al., 1980; Ozeki and Shively, 2008) (Figure 2-4). ATRA is a well-known mediator of HL-60 differentiation into neutrophil-like cells (Breitman et al., 1980; Congleton et al., 2011; Jian et al., 2011; Otake et al., 2005; Ozeki and Shively, 2008), which include inhibited cell growth (Breitman et al., 1980; Ozeki and Shively, 2008) (Figure 2-4A), increased expression of mature human neutrophil extracellular marker CD66 (Elghetany, 2002; Ozeki and Shively, 2008) (Figure 2-4B), and spontaneous apoptosis (Jian et al., 2011; Martin et al., 1990; Otake et al., 2005; Ozeki and Shively, 2008) (Figure 2-4C,D). While undifferentiated HL-60 cells had almost no basal expression of ESE-1 (Ct>30), treatment of early passage HL-60 cells with different concentrations of all-trans retinoic acid (ATRA) for 6 days resulted in a transcriptional upregulation of ESE-1 (Figure 2-4E, F). This correlated with the degree of granulocytic maturation by CD66 staining (Figure 2-4B) and with an increase in spontaneous apoptosis (Figure 2-4C) and blunted cell growth (Figure 2-4A) characteristic of mature neutrophils. However, the level of *ESE-1* even in HL-60 cells differentiated with 5 μM ATRA was not comparable to that of primary neutrophils and the ESE-1 protein was not as readily detectable by Western blot, making it difficult to use the cell line to study ESE-1 protein function.
Figure 2-4 *ESE-1* is transcriptionally upregulated during ATRA-mediated HL-60 granulocytic differentiation.

(A). HL-60 undergoes growth arrest during ATRA-mediated granulocytic differentiation. Ethanol (EtOH) at 0.1% was used as the vehicle control (=0 µM ATRA), and error bar shows the standard deviation (SD). The growth curve is a presentative of four independent experiments. (B). HL-60 granulocytic differentiation is accompanied by dose-dependent increase in the expression of human neutrophil marker CD66 with increasing dose of ATRA. The percentage of ATRA-treated HL-60 cells was quantified by flow cytometry on Day 6 of ATRA treatment. (C). Flow cytometric analysis of ATRA-differentiated HL-60 cells on Day 6 for the proportion of apoptotic cells.
Representative figures of CD66 single-stain and Annexin V/7-AAD double-stain flow cytometric analysis of the HL-60 cells on Day 6 of ATRA treatment. (E). *ESE-1* is transcriptionally upregulated during HL-60 granulocytic differentiation. *ESE-1* mRNA expression in HL-60 cells differentiated with varying concentrations of ATRA (Day 6 and over Days 3-5 with 5 μM) was determined by RT-qPCR. Graph showing mean±SD from three and two independent experiments, respectively. (F). Table showing raw numbers of % of CD66, apoptosis, and Ct values of ESE-1 and GAPDH from RT-qPCR (mean±SD) for graphs A-E. All graphs show mean±SD from three independent experiments, and statistical analysis was by one-way ANOVA with Bonferroni’s post-test on all pairs. *P<0.05, **P<0.01, ***P<0.001.

**Elf3-/- mice show no difference in phenotype in in vivo model of endotoxemia**

To determine whether *Elf3* deletion had any impact on sepsis pathogenesis in vivo given the expression of *ESE-1* in human neutrophils, we intraperitoneally injected the *Elf3-/-* and WT littermates with 5 mg/kg LPS, as a model of non-lethal endotoxemia. Equal volume of sterile PBS was injected as the procedural control. 5 mg/kg LPS was sufficient to cause egression of the bone marrow reservoir as indicated by a significant decrease in the percentage of the mature neutrophil (CD11b<sup>hi</sup>Gr-1<sup>hi</sup>) population (Ueda et al., 2005) (Figure 2-5B), as well as rapid extravasation of the circulating neutrophils (Figure 2-5F). However, there were no gross differences in the physiological parameters such as body temperature (Figure 2-5H) or body weight (Figure 2-5I) at this particular dose and there was no difference in neutrophil count or activation (Figure 2-5D, E) between WT and *Elf3-/-* animals nor in the degree of neutrophil apoptosis during the resolution of inflammation at 24 hours post-LPS administration (Figure 2-5G). While less IL-5, IL-9, and M-CSF were observed in the peripheral blood of *Elf3-/-* in the early phase of acute endotoxemia (Figure 2-6), these may have been due to other immune cell types such as eosinophils and T-cells.
Figure 2-5 ESE-1/Elf3 is not expressed in murine neutrophils and has no effect on neutrophil function in animal model of non-fatal endotoxemia.

(A). Table showing RT-qPCR results in raw Ct values of Elf3 (mean±SD) using three different primer sets in FACS-sorted mouse bone marrow neutrophils (CD11b<sup>high</sup>Gr-1<sup>high</sup>, purity >99%) and lungs of the wild-type (WT) C57BL/6 mice, from eight (n=8) and six (n=6) animals, respectively. (B). Elf3 gene deletion has no effect on neutrophil maturation in the Elf3-/-(KO) mice. Six (n=6)

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<th>Ct(Elf3 - primer 1)</th>
<th>Ct(Elf3 - primer 2)</th>
<th>Ct(Elf3 - primer 3)</th>
<th>Ct(GAPDH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophil (n=8)</td>
<td>32.68±0.39</td>
<td>30.24±0.07</td>
<td>32.99±0.82</td>
<td>14.17±0.73</td>
</tr>
<tr>
<td>Lung (n=6)</td>
<td>20.84±0.68</td>
<td>20.23±0.59</td>
<td>20.27±0.67</td>
<td>15.87±0.32</td>
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</tbody>
</table>
and nine (n=9) animals were intraperitoneally injected with 200 µL PBS or 5 mg/kg of LPS (O111:B4) respectively in each of WT and KO, and analyzed at 24 hours post-LPS administration. (C). A representative flow cytometry analysis showing the gating strategy for CD11b^{high}Gr-1^{high}, CD11b^{low}Gr-1^{high}, CD11b^{int}Gr-1^{int}, total CD11b^{*}Gr-1^{*}, and the counting beads (top far right). (D-E). *Elf3* deletion has no effect on neutrophil re-compensation (by immature cell marker c-Kit) or activation (by CD16/32) following LPS intraperitoneal injection (24 hours). (F). Changes in the total number of peripheral neutrophils over the 24 hour period of LPS injection. n=3-8 per each timepoint. Statistical analysis by two-way ANOVA; ns=not significant. (G). Proportion of apoptotic neutrophils (Annexin V+) in the peripheral blood at 24 hours post LPS-injection in WT and KO mice (n=8, n=7 respectively). Statistical analysis by two-tailed Student’s t-test. (H). Changes in body temperature in WT and KO (n=5 each) over 8 hours upon 10 mg/kg LPS injection. Same volume of PBS were injected in control WT mice (n=2). (I). Changes in body weight in WT and KO (n=12) with PBS or LPS (5 mg/kg) intraperitoneal injection at 0 and 24 hours.
Figure 2-6 *Elf3/-* mice have a reduced amount of circulating IL-5, IL-9, and M-CSF during acute endotoxemia.

Cytokine analysis of WT and KO plasma (n=7) at 2 hour of LPS (10 mg/kg) injection. Statistical analysis by Student’s t-test. Whole blood was collected by cardiac puncture into EDTA-coated tubes and centrifuged at 2,000 g for 15 min 4 °C to collect the plasma. Cytokines were quantified by Quantibody Mouse Cytokine Array from RayBiotech Inc, Norcross, GA following manufacturer’s instructions.
**Murine neutrophils do not express Elf3**

To verify the effectiveness of the animal model given these only slight differences, we sorted mature bone marrow neutrophils (CD11b<sup>high</sup>Gr-1<sup>high</sup>) from C57BL/6 WT mice by fluorescence-activated cell sorting (FACS), and analyzed the cells for the expression of *Elf3* by qPCR using three different primer sets. We chose to isolate the cells from the bone marrow, as it contains a large reservoir of neutrophils that are functionally comparable to those from the periphery in mice (Boxio et al., 2004). Mouse blood contains relatively little circulating neutrophils (10-25% of total peripheral white blood cells) in contrast to humans, whose blood leukocytes mainly consists of neutrophils (50-70%) (Mestas and Hughes, 2004). Based on the previously identified epithelial-restricted expression pattern of Elf3, mouse lungs were included as a positive control (Figure 2-5A). We observed minimal *Elf3* expression in the mouse neutrophils by all of three qPCR primer pairs (Ct>30), while there was a robust expression of Elf3 in the mouse lungs (Ct of 20). (Figure 2-5A). Therefore, this lack of *Elf3* expression in mature murine neutrophils may account for the absence of discernible phenotype in the *Elf3*<sup>-/-</sup> mice.
2.5 Discussion

Increasing evidence has shown a role of ESE-1 in inflammation and immunity (Oliver et al., 2012). However, the incidence or function of ESE-1 in neutrophils has never been explored, despite previous studies which examined the expression of ESE-1 in peripheral blood lymphocyte and in cell lines of T-cells (Jurkat) and macrophages (DU-528 and U937) (Oettgen et al., 1997; Tymms et al., 1997) as well as a study showing involvement of Ets factors in the regulation of neutrophil elastase and proteinase 3 gene expression (Nuchprayoon et al., 1999). We thus investigated the expression of ESE-1 in peripheral neutrophils of 10 healthy donors and, to address the possibility of ESE-1 being only present during inflammation, in 20 septic patients (Figure 2-2A). To our surprise, by qPCR and Western blot using A549 human lung adenocarcinoma cell line as a positive control, ESE-1 was detectable at both RNA (Ct of around 27) and protein level, and it was moderately upregulated in septic patient neutrophils. Furthermore, consistent with previous findings of ESE-1 as a nuclear transcription factor, we found ESE-1 residing mostly in the nucleus with negligible amounts in the cytoplasm (Figure 2-1A-C).

Studying the function of ESE-1 in human neutrophils proved to be challenging, however, due to the instability of ESE-1 protein in neutrophils in culture. As early as 2 hours (data not shown), the expression of ESE-1 plummeted at the mRNA level, and continued to decline over time (Figure 2-1D), which was accompanied by a delayed yet still rapid degradation of the ESE-1 protein (Figure 2-1A-C). This may indicate possible presence of a yet unidentified serum factor that is required for ESE-1 induction and preservation in vivo. Intriguingly, LPS treatment was not enough to rescue the rapidly dissipating ESE-1 (Figure 2-2B, 2-3B), and we observed a large variability of ESE-1 expression in septic patient neutrophils (Figure 2-2A). More patients and better patient stratification will be needed to implicate a potential role of ESE-1 in human neutrophils during sepsis. However, while this may largely be reflective of the disease heterogeneity such as varying causes of disease onset and different state of progression at the time of blood collection, it is also possible that in some of the patients ESE-1 is post-translationally modified without changes in its absolute protein amount. In fact, ESE-1 has previously been shown to be stabilized by serine phosphorylation that could alter its subcellular localization and protein half-life in breast cancer (Manavathi et al., 2007), and NetPhos2.0 server predicts 15 potential serine phosphorylation sites on ESE-1 protein with P-score of >0.95. Thus whether ESE-1 is post-translationally modified in
sepsis is subjected to further investigation. Additionally, we also found that GAPDH was unsuitable as an internal reference for the RT-qPCR when directly comparing healthy with septic neutrophils, because GAPDH itself was significantly elevated in sepsis likely due to increased anaerobic metabolism. We found that TATA-binding protein (TBP) was a better alternative for normalization (Figure 2-2A).

Neutrophils have a short life-span which makes them difficult to study in vitro (El Kebir and Filep, 2013). Because mouse models have been used extensively to identify the function of Ets factors in hematopoiesis and immune cells, we used Elf3 knockout (KO) mice to gain additional insight into the role of ESE-1/Elf3 in neutrophil differentiation and function. To determine whether Elf3 deletion had any impact on neutrophil biology during inflammation, we intraperitoneally injected the Elf3 KO and WT littermates with 5 mg/kg LPS as a model of non-fatal endotoxemia, and examined neutrophil maturation, activation, and egression from the bone marrow. Based on our finding of increased expression of ESE-1 in differentiating HL-60 cell line, we also compared the WT and Elf3 KO bone marrow for any possible defect in granulopoiesis, by the proportion of CD11b\textsuperscript{high}Gr-1\textsuperscript{high}/CD11b\textsuperscript{low}Gr-1\textsuperscript{high}/CD11b\textsuperscript{med}Gr-1\textsuperscript{med} with or without LPS stimulation as described previously (Ueda et al., 2005). The LPS was sufficient to cause egression of the bone marrow reservoir as indicated by a significant decrease in the percentage of the mature neutrophil (CD11b\textsuperscript{high}Gr-1\textsuperscript{high}) population (Figure 2-5B, C), as well as rapid extravasation of the circulating neutrophils (Figure 2-5F). However, there was no difference in neutrophil count between WT and KO animals, nor in the degree of neutrophil apoptosis during the resolution of inflammation at 24 hours post-LPS administration (Figure 2-5G), consistent with the lack of Elf3 expression in murine neutrophils (Figure 2-5A).

Murine models have been used extensively to identify therapeutic targets for human burns, trauma, and sepsis. Sepsis, in particular, is a deadly disease that affects more than 18 million people per year worldwide with 30-50% chance of death (Slade et al., 2003), causing a significant burden of morbidity and mortality at an enormous economic cost. Yet despite extensive research and almost 150 Phase II and III clinical trials, there has been no success in the development of effective targeted therapy for sepsis (Marshall, 2014). This may partially, or even primarily, be attributed to the failure of in vivo models to accurately replicate the human conditions, concerns of which have recently been raised by many scientists (Raven, 2012). While obvious deficiencies, such as the use of young, healthy, and inbred mice that are far from the human condition which involve mostly
aged individuals with comorbidities, may be corrected relatively easily, it is time to re-think how we approach the experimental design and interpretation of results from animal studies. It is critical to first recognize the problem and better understand its sources.

Much of the information on Ets factors in immune function has been derived from genetically altered rodent models, which were based for future human studies. A targeted analysis of Ets factors in human bone marrow, for example, only included a few selected genes such as PU.1 and Elf1 that were identified from mouse models (Bjerregaard et al., 2003). We thus may be missing out on potentially a number of other genes that have important functions in human neutrophils, yet which remain unidentified based on the conclusions made from animal studies. It is already known that human and mouse neutrophils differ quite significantly. Murine neutrophils lack defensins that are present in humans (Mestas and Hughes, 2004), and human and mouse neutrophil proteinase 3 and L-selectins have different substrate specificities (Hajjar et al., 2010; Zollner et al., 1997). There are also major differences in the intracellular signaling mechanisms, such as the PI3K pathway, which is critical in primed respiratory burst in humans but not in mice (Condliffe et al., 2005). Additionally, there is still a lack of side-by-side comparison of human and mouse neutrophil transcriptome in various disease models, but where it is available, the transcriptomic association was found to be weak (Gentile et al., 2014). A recent study by Seok et al (Seok et al., 2013) systematically compared the genomics of peripheral leukocytes in human disease with mouse models of burn, trauma, and sepsis and demonstrated poor correlation in the ortholog genes, especially in the models of endotoxemia. However, it is important to note that the substantial difference in the composition of peripheral leukocytes between human and mouse may have resulted in seemingly larger differences, owing to the fact that peripheral leukocytes are dominated by neutrophils in humans but by B lymphocytes in mice (Mestas and Hughes, 2004).

To address these challenges, there have been recent progresses in the generation of humanized animal models which produce functional human neutrophils (Coughlan et al., 2012). Unfortunately, although this is an impressive advancement, the percentage of human neutrophils is still very low, and it remains to be tested how closely they mirror human neutrophils in a disease setting. Generation of humanized animal models to study human neutrophils in vivo have been largely limited by poor reactivity of murine cytokines with human myeloid cells. Reconstitution of myeloid cells still continues to be a challenge, though attempts using viral expression, transgenic animal models and knock-in models to express human myelogenic factors such as CSF-1(Chen et
al., 2009), IL-3, and GM-CSF (Billerbeck et al., 2011) have shown modest improvements in myeloid engraftment. More research is certainly required to address these issues. Meanwhile, alternative strategies to study ESE-1 function in human neutrophils may include the use of human induced-pluripotent stem (iPS) cells (Morishima et al., 2011) to manipulate ESE-1 gene expression during its differentiation into mature neutrophils. Analysis of more sepsis patient neutrophils with better disease stratification will also help to implicate a role of ESE-1 in human neutrophils during sepsis.

Diseases of the immune system are often systemic and difficult to treat. There thus has been an arduous effort to understand the biology of the key players, such as neutrophils, in forms of genomic, transcriptomic, and proteomic analyses. In vivo models have also been an indispensable part of this venture, albeit with severe limitations. However, as part of this ongoing struggle in basic and clinical research to find immune-modulating therapies, we would like to call for a heightened awareness on the danger of generalizations and assumptions from previous discoveries. Here we show that what was earlier thought as an epithelium-specific protein is constitutively expressed in human neutrophils, which opens up a plenitude of new research questions on the role of ESE-1 and other ‘epithelial-specific’ Ets factors in different facets of neutrophil function. Given the major difference in the effect of ESE-1 and Elf3 on immune cells of different species, different approaches will be required to study ESE-1 in human neutrophils, information of which may help to uncover mechanisms of neutrophil function in human inflammatory diseases that have been overlooked for decades.
Chapter 3

3  ESE-1 acts as a negative regulator of cyclooxygenase-2 in human rheumatoid arthritis synovial fibroblasts

A modified version of this chapter has been submitted to Cell and Bioscience: Chan Mi Lee, Sahil Gupta, Jiafeng Wang, Elizabth M. Johnson, Leslie Crofford, John C. Marshall, Mohit Kapoor, Jim Hu. Epithelium-specific Ets transcription factor-1 acts as a negative regulator of cyclooxygenase-2 in human rheumatoid arthritis synovial fibroblasts. Cell and Bioscience, 2016. Accepted.
3.1 Abstract

Introduction: Rheumatoid arthritis (RA) is characterized by excessive synovial inflammation. Cyclooxygenase-2 (COX-2) is an enzyme that catalyzes the conversion of arachidonic acid (AA) into prostaglandins. Epithelium-specific Ets transcription factor-1 (ESE-1) was previously demonstrated to upregulate COX-2 in co-operation with nuclear factor kappa B (NFκB) in macrophages and chondrocytes. However, the role of ESE-1 in RA pathology has remained unclear. In this study, we aimed to elucidate the relationship between ESE-1 and COX-2 in RA synovial fibroblasts (RASFs) using a knockdown approach to determine how ESE-1 contributes to RA pathogenesis.

Method: Endogenous levels of ESE-1 and COX-2 in human RASFs were analyzed by RT-qPCR and Western blot, and ESE-1 was knocked down using helper-dependent adenovirus (HD-Ad). Intracellular localization of ESE-1 was examined by nuclear fractionation and PGE2 was quantified using competitive ELISA. To confirm the ESE-1-COX-2 relationship in other cellular systems, COX-2 was also measured in SW982 synovial sarcoma cell line and ESE-1 knockout (KO) murine macrophages.

Results: ESE-1 and COX-2 were induced by IL-1β in RASFs that corresponded with an increase in PGE2. Knockdown of ESE-1 led to a significant upregulation of COX-2 at later phase of IL-1β stimulation. ESE-1 was localized in the nucleus, occupying disparate cellular compartments to NFκB when COX-2 was increased. Knockdown of ESE-1 also transcriptionally upregulated COX-2 in SW982 and ESE-1 KO murine macrophages, suggesting that ESE-1 may be involved in the resolution of inflammation.

Conclusion: ESE-1 acts as a negative regulator of COX-2 in human RASFs and its effect on COX-2 is NFκB-independent.

3.2 Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by the progressive destruction of the joints due to excessive inflammation in the synovium, which can lead to deformities and loss of joint function in severe cases. Inflammation in the RA synovial tissue is perpetrated by the production of inflammatory cytokines and secreted mediators from infiltrating
immune cells and activated synovial fibroblasts (McInnes and Schett, 2007). Prostaglandins (PGs) are key mediators responsible for RA symptoms of pain and swelling (Fattahi and Mirshafiey, 2012). Synthesis of PG requires conversion of arachidonic acid released from cell membranes to prostaglandin H$_2$ (PGH$_2$), the critical step of which is catalyzed by cyclooxygenase-2 (COX-2), also known as the PGH$_2$ synthase. PGH$_2$ is further metabolized to bioactive forms such as PGE$_2$, prostacyclin, prostaglandin D$_2$, and prostaglandin F$_2\alpha$, by their respective synthases in different cell types (Fattahi and Mirshafiey, 2012; Ricciotti and FitzGerald, 2011). COX-2 is highly expressed in the RA synovial lining due to the persistent presence of proinflammatory cytokines such as IL-1β, TNF-α, and IL-6, and is a key biosynthetic enzyme regulating PG production in the synovium (Crofford et al., 1994; Siegle et al., 1998). PGE$_2$ is the major PG that is generated by chondrocytes and synovial fibroblasts (Martel-Pelletier et al., 2003), and clinical responses to non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to correlate with reduced levels of PGE$_2$ in the synovial fluid (Flower, 2003; Seppala et al., 1990). COX-2 inhibitors such as celecoxib effectively control arthritis symptoms (Flower, 2003).

COX-2 gene activation is complex and employs numerous regulatory factors specific to different stimuli, as exemplified by the COX-2 promoter which contains two NFκB motifs, two activator protein 1 (AP-1) sites and two cAMP-response elements (CREs) among others (Appleby et al., 1994). Several Ets factors have also been shown to regulate COX-2 expression, including Ets-1 (Teruyama et al., 2001), Pea3 (Howe et al., 2001) and PU.1 (Joo et al., 2004), and Elk1 (Zhang et al., 2013) in different tissue contexts. Ets family of transcription factors are characterized by the highly conserved E26 transformation-specific (Ets) DNA binding domain, which recognizes GGAA/T core consensus sequence within the promoter and enhancer regions of target genes (Oliver et al., 2012). Unlike most Ets factors which are expressed in hematopoietic cells, however, a subgroup of Ets proteins called epithelium-specific Ets factors (ESE’s) has epithelium-restricted expression pattern under basal conditions. Interestingly, ESE-1, the prototype of ESE subfamily, is highly sensitive to inflammatory stimulation (Brown et al., 2004), where it was found to be expressed in the human RA synovial tissue (Grall et al., 2003). It was also transcriptionally upregulated by proinflammatory stimuli such as IL-1β, TNF-α, or LPS in the resident cell types including synovial fibroblasts, chondrocytes, osteoblasts, and macrophages, typically displaying a peak expression between 2-6 hours and dissipation by 24 hours in most cells (Grall et al., 2003). ESE-1, or Elf3 in mice, was similarly found to transactivate COX-2 promoter in murine
macrophages and human chondrocytes in cooperation with NFκB (Grall et al., 2005), suggesting its critical role in RA pathogenesis. However, initial analyses had revealed ESE-1 to be predominantly expressed in the cytoplasm of the cells (Grall et al., 2003), leaving discrepancies in how it might function as a transcription factor in situ. Additionally, the prolonged expression of ESE-1 mRNA in RASFs beyond 24 hours of IL-1β stimulation unlike in other cell types has left the relationship between ESE-1 and COX-2 in RASFs elusive. As RASFs and synovial macrophages are prominent cell types present in the terminal layer of the hyperplastic synovial tissue which secrete inflammatory cytokines and matrix-degrading enzymes (Bartok and Firestein, 2010; Neumann et al., 2010), elucidation of the role of ESE-1 in COX-2 regulation is important to gain better understanding of the molecular events that occur in RA synovial tissues.

Previous studies primarily focused on investigating functional significance of ESE-1 by ESE-1 overexpression, where ESE-1 cDNA was transfected into cell lines along with luciferase constructs to investigate the transactivation of ESE-1 on its target genes. However, ectopic gene expression can lead to supraphysiological expression of the gene of interest, as well as cell toxicity from the transfection procedure itself. Also, overexpression by transfection may not accurately reflect the temporal behaviour of a protein, and may thus lead to artificial interaction or co-localization of proteins that do not normally co-exist under physiological conditions. ESE-1 overexpression could also have accompanied co-induction of its other target genes, giving rise to confounding results. Therefore, we sought to elucidate the relationship between ESE-1 and COX-2 in human RASFs using a knockdown approach with helper-dependent adenoviral (HD-Ad) vector, which has all of the viral genes removed to render it much less immunogenic than conventional adenoviruses (Brunetti-Pierri and Ng, 2010; Vetrini and Ng, 2010), and in Elf3 knockout mouse bone marrow-derived macrophages (BMDMs) to avoid side effects from transfection- or transduction-mediated gene manipulation. In this study, we show for the first time that ESE-1 negatively regulates COX-2 in human RASFs.
3.3 Materials and Methods

Reagents

DMEM, RPMI-1640, fetal bovine serum (FBS) and L-glutamine were purchased from Gibco Life Technologies Ltd., Burlington, Ontario, Canada. Penicillin/streptomycin, phosphate buffered saline (PBS) were from Wisent, St. Bruno, Quebec, Canada. Human recombinant IL-1β was product of R&D Systems (Minneapolis, MN, USA), and LPS endotoxin (Escherichia coli, serotype O128:B12) and DEAE-Dextran hydrochloride of Sigma (Oakville, Ontario, Canada), while murine IL-4 was from Peprotech, Quebec, Canada. Antibodies used in this study were: COX-2 rabbit polyclonal antibody from Thermo Fisher Scientific (Burlington, Ontario, Canada), and COX-2 (C-20) goat polyclonal, NFκB p65 (C-20), p50 (H-119) and Lamin A (H-102) rabbit polyclonal antibodies from Santa Cruz (Dallas, TX, USA). ESE-1 rabbit monoclonal antibody was produced in our laboratory in collaboration with Epitomics, Burlingame, CA, USA (Lee et al., 2015). Hsp90 rabbit polyclonal and β-actin mouse monoclonal antibodies were purchased from Cell Signaling Technology (Whitby, Ontario, Canada).

Preparation of RASFs

Synovial tissues were obtained at the time of joint replacement surgery from patients with RA who fulfilled the revised American Rheumatism Association criteria for this disease (Seppala et al., 1990). Experiments were carried out according to a protocol that was approved by the Institutional Review Board in Vanderbilt University, Nashville, TN, and patient informed consent was obtained. RASF were prepared as previously described (Appleby et al., 1994). Briefly, minced synovial tissues were digested overnight with 1 mg/ml collagenase (Type I, Sigma, St. Louis, MO) in DMEM in a humidified 5% CO2 incubator at 37°C and the isolated cells were cultured in 175 cm² culture flasks in DMEM supplemented with 20% FBS, L-glutamine (2 mM), penicillin (100 units/ml) and streptomycin (100 μg/ml). At greater than 95% confluency, the adherent RSF were passaged by digestion with 0.05% trypsin/EDTA and used for cell culture experiments.

Cell Culture

Cell lines SW982 and A549 were obtained from American Type Culture Collection, Rockville, MD, and were cultured in DMEM supplemented with 10% FBS and 100 IU/mL penicillin, and
100 μg/mL streptomycin in 5% CO₂ at 37°C. Cells were starved in serum-deprived medium containing 0.5% FBS, in which transduction and cytokine stimulation were also performed.

**Infection of cells with helper-dependent adenovirus (HD-Ad)**

*ESE-I* gene was knocked down in human synovial fibroblasts and SW982 cells using shRNA helper-dependent adenoviral vector expressing two shRNAs prepared as previously described (Wu et al., 2008; Yu et al., 2015), with added modifications from (Fasbender et al., 1997). Briefly, cells were seeded at 100,000 cells per well in growth medium on 6-well plates a day prior to transduction, and 5,000 virus DNA particles per cell equivalent of 100 MOI were complexed with 520.5 ng DEAE-Dextran by incubation for 30 minutes at room temperature in 0.5% FBS DMEM. The DEAE-virus mixture was added to cells by replacing the growth medium. C4HSU empty vector was used as control. The cells were incubated for 2 hours in a 5% CO₂ at 37°C, after which 20% FBS DMEM was added to achieve a final concentration of 10% FBS. The cells were then incubated for additional 48 hours, and the medium was removed and replaced with 0.5% FBS DMEM for 24 hour starvation before being stimulated with 10 ng/mL IL-1β.

**RNA isolation and real-time quantitative PCR (RT-qPCR)**

Total RNA was isolated using GE Illustra RNAspin Mini Kit (GE Healthcare Life Sciences, Baie-D’Urfe, Quebec) as per manufacturer’s instructions. For real-time quantitative PCR, after spectrophotometry quantification, 1 μg of RNA was reversed transcribed in a final volume of 20 μL using Superscript VILO Mastermix with Superscript III (Invitrogen, Carlsbad, CA) and the resulting cDNA template (10 ng) was used for qPCR reaction using Power SYBR Green PCR Master Mix from Life Technologies (Burlington, Ontario, Canada). ViiA™7 Real-Time 384-well PCR System from Life Technologies was used for the amplification and analysis. For relative ΔΔCt quantification, qPCR signals were normalized using GAPDH and fold changes were calculated according to Livak and Schmittgen (Livak and Schmittgen, 2001). The primer sequences used for human and mouse samples are provided below:
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<th>Primer Sequence</th>
<th>Source/Reference</th>
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<td>ESE-1 5'-'GGCGTCTTTCAAGTCTCGG-3' (Forward) 5'-CTCCCCGTGTTGAGGAGCT-3' (Reverse)</td>
<td>(Lee et al., 2015)</td>
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<td>RELA 5'-'ACTTCCCCCTGCTCTCGATG-3' (Forward) 5'-CAACCCCTCTTCCTCATTC-3' (Reverse)</td>
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<td>Fizz1 5'-'TCCCCAGTGATACCTGATGAGA-3' (Forward) 5'-CCACAGCCGAGACGTCCGATAC-3' (Reverse)</td>
<td>(Maresz et al., 2008)</td>
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<td>Ym1 5'-'GGCCACATTTATCTTGGGAGAATCTG-3' (Forward) 5'-CCACTGGATCATCTCAGATGC-3' (Reverse)</td>
<td>(Maresz et al., 2008)</td>
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<tr>
<td>GAPDH 5'-'GAAGGTGAAGGTCGGAGTC-3' (Forward) 5'-GAAGATGGTGATGGGATTTC-3' (Reverse)</td>
<td>(Lee et al., 2015)</td>
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**Cytoplasmic nuclear fractionation and Western blot**

Nuclear and cytoplasmic extracts were prepared from human RASFs by nuclear/cytoplasmic separation as previously described (Suzuki et al., 2010). In summary, cells grown in 10 cm dishes were washed twice with ice-cold PBS, and pelleted cells were resuspended in 900 µL of hypotonic buffer containing 0.1% NP-40 in PBS containing protease inhibitors (Roche; Mississauga, Ontario, Canada) and triturated five times, after which they were immediately centrifuged at 500 x g at 4 °C. The supernatant was collected and designated as the cytoplasmic extract, while the pellet was washed once with 1 mL of 0.1% NP-40 PBS buffer, re-centrifuged, and lysed in 180 µL 6xSDS...
sample buffer (2% (w/v) SDS, 58.3 mM Tris-HCl (pH 6.8), 6% (v/v) glycerol, 5% (v/v) 2-β-mercaptoethanol, 0.02% (w/v) bromophenol blue) and was designated as the nuclear extract. The nuclear extract was sonicated at Level 2 on Misonix 3000 sonicator for 5-10 seconds. Lysates were separated by electrophoresis on 10% SDS-PAGE gel, and transferred to nitrocellulose membrane (Amersham; GE Healthcare, Mississauga, Ontario, Canada). Membranes were blocked with 5% (w/v) nonfat milk in TBST (50mM Tris-Cl pH 7.5, 150 mM NaCl, 0.05% Tween-20) for 1 hour at room temperature and probed for ESE-1 (1:3,000), COX-2 (1:500), Hsp90 (1:1,000), β-actin (1:4,000), or Lamin A (1:500) overnight at 4 °C. Protein signals were detected with HRP-conjugated secondary antibodies at a dilution of 1:4,000 using ECL Western blotting detection system (Amersham Pharmacia Biotech, Baie-D’Urfe, Quebec, Canada).

**Prostaglandin E2 (PGE2) quantification**

PGE2 was quantified using a competitive binding ELISA kit (R&D Systems) according to the manufacturer’s protocol. Cell medium was centrifuged to remove particulates and the supernatant was diluted 3-fold before the assay. The plate was read with plate reader VersaMaxPLUS ROM v1.21 with SoftMax Pro v5.3b12 software at the absorbance of 450 nm with wavelength correction at 540 nm to correct for the optical imperfections in the plate. The concentration of PGE2 was calculated against a standard curve ranging from 0 to 2,500 pg/mL.

**ESE-1/Elf3 knockout mice and bone marrow-derived macrophage culture**

*Elf3/-* mice on a C57BL/6 background were housed in pathogen-free condition at Toronto Centre for Phenogenomics (TCP), Toronto, Canada, and all procedures were approved by the Toronto Centre for Phenogenomics Animal Care Committee (Animal Use Protocol #0062). Bone marrow was flushed from femur and tibia of *Elf3/-* mice and wild-type littermates into single cell suspension and cultured in 20% L-929 conditioned media containing for 7 days as previously described (Lee and Hu, 2013). Wild-type littermates were used as controls. The purity of bone marrow-derived macrophages was measured by flow cytometry with CD11b and F4/80 following methods from (Teruyama et al., 2001). 4 x 10^5 of mature BMDMs were subjected to 100 ng/mL LPS or 10 ng/mL IL-4 for 18 hours to drive M1 and M2 polarization, respectively.
Statistical analysis

Statistical analysis was performed by two-tailed Student's t-test with Welch’s corrections for unequal variances where appropriate, or by one-way paired ANOVA for multiple comparisons with Tukey’s post-test or Bonferroni’s post-test for selected pairs using GraphPad Prism 5.03 (GraphPad Software, Inc., La Jolla, CA). P-values of less than 0.05 were considered statistically significant.

3.4 Results

ESE-1 and COX-2 are induced by IL-1β in human RASFs

ESE-1 was previously shown to be rapidly upregulated by proinflammatory stimuli in human RASFs and maintained up to 24 hours (Brown et al., 2004; Grall et al., 2003). To investigate its relationship to COX-2 expression, we stimulated primary RASFs with 10 ng/mL human IL-1β and quantified the amount of ESE-1 and COX-2 mRNA by RT-qPCR (Figure 3-1A) and protein levels by Western blot (Figure 3-1C). We observed that ESE-1 mRNA expression peaked around 6 hours and was reduced slightly at 24 hours. Transcriptional levels of COX-2, on the other hand, showed gradual increase until 6 hours and downregulation at 24 hours post IL-1β stimulation. Protein levels of ESE-1 varied among RA patients, indicating heterogeneity of the patient population and potentially the presence of other factors which may modulate ESE-1 expression (Figure 3-2A). COX-2 protein, however, accumulated over time in most patients as previously shown (Crofford et al., 1997) (Figure 3-2B), which correlated with increase in PGE₂ concentration (Figure 3-1B).
Figure 3-1 ESE-1 and COX-2 are induced by IL-1β in human RASFs

(A). Changes in ESE-1 and COX-2 transcriptional levels in human patient RASFs (n=5) during IL-1β (10 ng/mL) stimulation by quantitative RT-PCR normalized to GAPDH. (B). Quantification of prostaglandin E2 (PGE2) in the culture media of human RASFs (n=6) stimulated with 10 ng/mL of IL-1β for the designated time points. (C). Representative Western blot showing changes in ESE-1 and COX-2 at protein level, using Hsp90 as a loading control. Bars show mean±SEM, *P<0.05, **P<0.01, ***P<0.001
Figure 3-2 ESE-1 and COX-2 protein expression in four patient RASFs.

(A) Western blot of ESE-1, COX-2, and Hsp90 of four patient RASFs treated with 10 ng/mL IL-1β over a 24 hour time course. (B) Densitometry analysis of ESE-1 and COX-2 bands normalized to Hsp90.
RASFs can be effectively transduced by helper-dependent adenovirus (HD-Ad)

Helper-dependent adenovirus (HD-Ad) provide an attractive alternative means of gene delivery to non-viral vectors or other virus types, by its high carrying capacity of 37 kb and low immunogenicity from having all its viral coding sequences removed (Brunetti-Pierri and Ng, 2010; Vetrini and Ng, 2010). HD-Ads have been successfully produced and used in our laboratory as a potential tool for cystic fibrosis (CF) gene therapy (Cao et al., 2013), as well as a research tool to knockdown ESE-1 in a number of studies (Wu et al., 2008; Yu et al., 2015). However, similar to other adenoviruses, HD-Ad requires specific receptors to mediate viral attachment and gene transfer, notably the coxsackie virus and adenovirus receptor (CAR), which the fibroblasts are known to be lacking (Hidaka et al., 1999).

As expected, transduction with virus alone in human RASFs proved ineffective irrespective of viral dose (Figure 3-3A), despite yielding close to 100% transduction in A549 lung adenocarcinoma cell line (data not shown). Non-covalent complexing of recombinant adenovirus with cationic molecules, however, has been demonstrated to significantly increase viral attachment and thus the efficiency of gene transfer by neutralizing the net negative surface charge on virus particles and the cell membrane (Fasbender et al., 1997; Kushwah et al., 2007). In an attempt to optimize viral infection in human RASFs, therefore, we complexed HD-Ad with DEAE-Dextran, which resulted in 100% cells being transduced at 100 MOI, with the expression lasting for more than 96 hours. We were able to achieve up to 90% knockdown of ESE-1 with HD-Ad carrying shESE-1 construct using this infection protocol (Figure 3-3B). Although it was inevitable that the virus led to some degree of inflammation and ESE-1 activation, by 72 hours from the initial exposure to virus particles, the background PGE2 and COX-2 expression were comparable to basal levels prior to IL-1β stimulation. There was also minimal cell toxicity, if any, conferring a significant advantage over transfection. This indicates that with optimization with charge-neutralizing polymers such as DEAE-Dextran, HD-Ad can be an effective gene delivery tool for hard-to-transduce cell types such as immune cells and fibroblasts, and to study immune-responsive or cell survival genes that can be affected by transfection.
Figure 3-3 Knockdown of ESE-1 leads to increased COX-2 and PGE2 production in RASF.

(A). Representative picture of human RASFs successfully transduced with helper-dependent adenovirus containing EGFP construct (HD-Ad-EGFP) with and without DEAE-Dextran. (B). Knockdown of ESE-1 leads to the transcriptional upregulation of COX-2 following 24 hours of IL-1β stimulation (n=6). Scale bar shows 100 μm. (C). Transcriptional upregulation of COX-2 is accompanied by increased production of PGE2 (n=6) (D). Representative Western blot showing changes in ESE-1 and COX-2 proteins in RASFs transduced with control (C4HSU) or shESE-1 HD-Ad. Bars show the mean±SEM, *P<0.05, **P<0.01, ***P<0.001, by one-way ANOVA Bonferroni’s post-test.
Figure 3-4 Changes in COX-2 with ESE-1 knockdown in human patient RASFs.

(A) Western blot showing changes in COX-2 protein level following ESE-1 knockdown by HD-Ad-shESE-1 in four different patient RASFs. (B) Time point analysis of ESE-1 and COX-2 transcriptional levels (n=5) during IL-1β stimulation after C4HSU or shESE-1 HD-Ad viral transduction. ns=not significant, *P<0.05, ***P<0.001. (C) qPCR analysis of ESE-1 knockdown in human RASFs (n=6) for MMP-1 and MMP-13, and (D) RelA, and NFκB1. ns=not significant.
Knockdown of ESE-1 leads to upregulation of COX-2 and increased PGE₂ production.

In all patient RASF studied, knockdown of ESE-1 led to a significant upregulation of COX-2 at both RNA (Figure 3-3B) and protein (Figure 3-3D) levels. This correlated with increased concentration of PGE₂ in the cell media (Figure 3-3C), indicating functional significance of ESE-1 on COX-2 activity. Interestingly however, ESE-1 knockdown had no effect on COX-2 basal level of expression or early phase of induction, and the effect of ESE-1 knockdown on COX was only visible at 24 hour time point (Figure 3-4B) when the initial IL-1β-induced inflammation was largely resolved. Similarly, while adenovirus binding to cultured synoviocytes is known to trigger COX-2 expression through the MAPK pathway, this subsides by 24 hours (Crofford et al., 2005) and in our study, transduced cells were stimulated with IL-1β 72 hours after initial exposure to the virus when both COX-2 and PGE₂ were comparable to the basal levels, thus minimizing confounding results from the inflammatory reaction to the viral vector. Additionally, knockdown of ESE-1 had no effect on metalloproteinase activity in RASFs, as shown by insignificant changes in MMP-1 or -13 mRNA expression (Figure 3-4C), indicating that ESE-1 may be a specific effector for resolving inflammatory responses.

ESE-1 is localized in the nucleus

ESE-1 was previously detected in the cytoplasm by immunostaining of RA patient tissue sections (Grall et al., 2003). However, cytoplasmic/nuclear fractionation of activated human RASFs in in vitro revealed nuclear expression of ESE-1 (Figure 3-5A,B), which was also consistent following shESE-1 HD-Ad viral transduction, where nuclear decrease in ESE-1 led to cytoplasmic increase in COX-2 (Figure 3-5D). Furthermore, despite findings of ESE-1 cooperating with NFκB to transactivate target genes such as iNOS in endothelial cells (Rudders et al., 2001) and COX-2 in macrophages (Grall et al., 2005), NFκB was localized in the cytoplasm by 24 hours post IL-1β stimulation in RASFs (Figure 3-5C,D). This was consistent with the previous finding where NFκB activated by IL-1β in RASFs resolved and returned to normal levels by 4 hours of IL-1β stimulation (Crofford et al., 1997). Therefore while NFκB may be responsible for the transcriptional upregulation of ESE-1 (Wu et al., 2008), it seems unlikely that NFκB is involved at the 24 hour time point when ESE-1 knockdown enhances COX-2 expression. Nuclear localization of ESE-1 is in alignment with its known function as a transcription factor, and it may regulate other genes implicated in COX-2 regulation.
Figure 3-5 ESE-1 is expressed in the nucleus of RASFs.

(A). ESE-1 protein level increases with IL-1β stimulation (n=4), shown by Western blot on whole cell lysates (WCLs) of stimulated or unstimulated RASFs. #1-#4 denotes patients #50, 58, 77, and 22, and A549 lysate was included as positive control for ESE-1 protein. (B). ESE-1 protein is exclusively expressed in the nucleus, with Hsp90 and Lamin A as cytoplasmic and nuclear markers, respectively. Western blot of nuclear fractionated RASFs stimulated with 10 ng/mL IL-1β for 24 hours. (C). ESE-1 and COX-2 are minimally present in RASFs without IL-1β stimulation. (D). Knockdown of ESE-1 by HD-Ad-shESE-1 leads to increase in COX-2 expression in the cytoplasm in IL-1β treatment in RASFs compared to C4HSU control vector. Western blot showing results from two different patient RASFs.
SW982 cell line shows different pattern of ESE-1 expression from human primary RASFs

The transcriptional expression pattern of ESE-1 in response to IL-1β has been studied in numerous non-epithelial cell lines, including human chondrocytes (T/C28a2, C28/I2, and C20A4), osteoblasts (LB-12), monocytes (THP-1), gliomas (U-138 MG and U-373 MG), and endothelial cells (HUVECs), where ESE-1 was shown to be one of the few Ets factors that were specifically responsive to IL-1β-mediated activation, with typical induction pattern of peak expression between 2-6 hours and dissipation by 24 hours in most cell types (Brown et al., 2004; Grall et al., 2003; Grall et al., 2005; Rudders et al., 2001).

Studies have shown that SW982 synovial sarcoma cell line is representative of human primary synovial fibroblasts (Chang et al., 2014; Yamazaki et al., 2003). However, the expression pattern of ESE-1 in SW982, where ESE-1 peaked at 2 hours and underwent drastic downregulation at 24 hours, and protein expression peaking at 6 hours and subsequently undergoing degradation, was different from that of RASFs (Figure 3-6A,B), indicating that it is not a good representative cell line for our purposes. The knockdown of ESE-1 still had a visible effect on COX-2 upregulation at only 24 hours (Figure 3-6C), when ESE-1 protein was minimally present. p65 also dissipated by 24 hours of IL-1β, which made the performance of ChIP very difficult (data not shown). Therefore, it is possible that ESE-1 plays an indirect role or has other unknown function in COX-2 expression, such as mRNA stability, not just acting as a transcription factor, and the function of ESE-1 as a transcript, for example as a competing endogenous RNA, has never been explored.
Figure 3-6 ESE-1 downregulation leads to increased COX-2 expression in SW982 cell line.

(A). Analysis of ESE-1, COX-2, RelA and NFκB1 transcriptional levels by RT-qPCR in SW982 cells during IL-1β (10 ng/mL) stimulation. (B). Representative Western blot of SW982 stimulated with IL-1β (10 ng/mL) over the 24 hour time period. (C). Time course analysis of SW982 cells transduced with C4HSU or shESE-1 HD-Ad vectors and stimulated with IL-1β over 24 hours, started 72 hours post-transduction. *P<0.05, **P<0.01, ***P<0.001, by one-way ANOVA Bonferroni’s post-test, data shown are representative of two independent experiments with n=3 each.
**ESE-1/Elf3 knockout (KO) macrophages also show increased COX-2 expression**

Macrophages are phagocytic cells which play a crucial role in the first line of defense against pathogens or environmental toxins, and provide the link between innate and adaptive immune response (Biswas et al., 2008; Mege et al., 2011; Murray and Wynn, 2011b). They express a wide range of Toll-like receptors (TLRs) and pattern-recognition receptors to detect endogenous danger signals and display an incredible plasticity and their functions can be significantly and specifically altered by surrounding cytokines (Mosser and Edwards, 2008). Two discrete classes of macrophage have been recognized, namely M1, which is proinflammatory or classically activated, and M2, which is alternatively activated and takes on more immune-regulatory role (Biswas and Mantovani, 2010; Gordon and Martinez, 2010; Martinez et al., 2006; Pena et al., 2011). The bacterial lipopolysaccharide (LPS) and the Th1 cytokine interferon-γ (IFN-γ) induce M1 polarization, while Th2 cytokines such as IL-4, -13 and -10 lead to M2 phenotype. Classically activated M1 macrophages have increased production of pro-inflammatory cytokines such as TNF-α and IL-12, IL-23, nitric oxide (NO), and reactive oxygen species (ROS) and have increased antigen presentation and microbicidal activity, while M2 macrophages typically produce anti-inflammatory cytokines such as IL-10, IL-1 receptor antagonist (IL-1ra) and promote tissue remodeling and repair (Gordon and Martinez, 2010; Gordon and Taylor, 2005; Mantovani et al., 2005; Sica and Mantovani, 2012).

ESE-1 was previously shown to modulate COX-2 in RAW264.7 murine macrophage cell line (Grall et al., 2005). Therefore, to address the difference in cell type, we also examined COX-2 levels in ESE-1/Elf3 knockout (KO) bone marrow-derived macrophages (BMDMs). Use of Elf3 KO BMDMs circumvented having to expose cells to additional inflammation from transfection-or transduction-mediated gene manipulation. *In vitro* differentiated BMDMs by CD11b and F4/80 staining were almost 100% pure (Figure 3-7A). When subjected to LPS, however, Elf3 KO BMDMs showed increased transcriptional level of COX-2 (Figure 3-7B). Because COX-2 is one of the hallmark genes of M1, or pro-inflammatory macrophage phenotype (Mantovani et al., 2013; Martinez et al., 2006), we also checked other genes related to M1- versus M2-polariation and observed that Elf3 KO BMDMs were slightly skewed towards M1 (Figure 3-7C,D). This indicates that ESE-1 can have anti-inflammatory role in macrophages which may be subjected to further analysis.
Figure 3-7 ESE-1 knockout (KO) bone marrow derived macrophages (BMDMs) show increased COX-2 expression and an increased propensity towards M1 phenotype. (A). Expression of macrophage maturation markers CD11b and F4/80 in BMDMs derived from WT or Elf3 KO C57BL/6 mice. Bone marrow cells isolated from the WT or Elf3 mice were differentiated ex vivo in 20% L-929-conditioned media and analyzed by flow cytometry. The graph shows n=12 for WT and KO mice. (B). ESE-1 knockout BMDMs show increased COX-2 expression following LPS (100 ng/mL) treatment. BMDMs were plated at 4 x 10^5/well on 6-well plates and stimulated with 100 ng/mL LPS or 10 ng/mL IL-4 for 18 hours, after which they were lysed for RNA isolation and qPCR analysis (n=6). (C). shows expression of genes related to M1 polarization and (D). M2-related genes in WT and KO BMDMs (n=6) treated with 100 ng/mL LPS (“M1”) and 10 ng/mL IL-4 (“M2”) for 18 hours prior to analysis. Statistical analysis by one-way ANOVA with Bonferroni’s post-test for selected pairs, ns=not significant, *P<0.05, **P<0.01.
3.5 Discussion

It is undisputable that understanding the pathogenesis of RA is critical for its prevention and treatment. However, persistent inflammation arises not only from persistent elicitation but also from incomplete resolution, and in pursuit of finding causative mechanisms, primary focus on effectors of pro-inflammatory response may have left some effectors playing dual or complex roles unrecognized from experimental approaches chosen to demonstrate one relationship but not the other. Our study illustrates one such example with an Ets transcription factor, ESE-1. ESE-1 was previously shown to co-operate with NFκB and positively regulate COX-2 by binding to the Ets-binding site on the COX-2 promoter (Grall et al., 2005). However, by gene knockdown approach, we made an opposite observation in human RASFs, where knockdown of ESE-1 led to an upregulation of COX-2, which correlated with increased levels of PGE2. The use of HD-Ad-mediated knockdown is advantageous over previously employed conventional transfections, given its higher efficacy of gene delivery and long-term expression, as well as much lower cellular toxicity and immunogenicity.

It is important to note that previous findings on ESE-1 have been based on overexpression studies, but with insufficient consideration on the effect of the transfection procedure itself. Ectopic gene expression can lead to supraphysiological expression of the gene of interest, as well as cell toxicity from the transfection. Also, overexpression by transfection may not accurately reflect the temporal behavior of a protein, and may thus lead to artificial interaction or co-localization of proteins that normally do not co-exist under physiological conditions. For example, in RASFs, NFκB is resolved within four hours following IL-1β stimulation (Crofford et al., 1997), yet the effect of ESE-1 knockdown was only evident at 24 hour time point, when the initial inflammation induced by IL-1β had mostly resolved and ESE-1 and NFκB were disparately localized in the nucleus and cytoplasm, respectively. Also, knockdown of ESE-1 had no effect on COX-2 induction in RASF or SW982, indicating that ESE-1 may not play a direct role in regulating COX-2 transcription as previously thought. Rather, given that PGE2 has been shown to prolong COX-2 mRNA half-life through the p38 MAPK pathway, ESE-1 may be functioning more as a downstream effector of PGE2 signaling than IL-1β at the 24 hour time point. In fact, transcriptional activation of COX-2 in NIH 3T3 fibroblasts by PGE2 was found to require C/EBP and CRE-1 sites but not NFκB (Bagga
et al., 2003), suggesting that ESE-1 may be interacting with protein partners other than NFκB or assuming other functions at later time points.

COX-2 regulation is complex, and occurs at both transcriptional and non-transcriptional levels. The exact transcription factor complexes that are recruited at the COX-2 promoter site vary by cell type and stimulation (Kang et al., 2007). Sequence analysis of the 5’-flanking region of the human COX-2 gene has identified several potential transcriptional regulatory elements, including two nuclear factor kappa B (NF-κB) sites, an SP1 site, a CAAT enhancer binding protein (C/EBP), nuclear factor for interleukin-6 expression (NF-IL6) motif, two AP-2 sites, an E-box, and a TATA-box, as well as a peroxisome proliferator response element (PPRE), two cyclic AMP response elements (CRE), and a sterol response element (SRE) (Tanabe and Tohnai, 2002). Additionally, COX-2 can be regulated post-transcriptionally by its mRNA stability with the involvement of molecules such as HuR, microRNA 101a and 199a and alternative polyadenylation (Harper and Tyson-Capper, 2008) and by long-noncoding RNAs (Krawczyk and Emerson, 2014), as well as at its protein level by N-glycosylation at Asn-594 and by substrate-dependent suicide inactivation (Mbonye et al., 2008). Therefore, elucidation of the exact role of ESE-1 in COX-2 regulation warrants a separate study.

However, one possible mechanism by which ESE-1 exerts its repressor function on COX-2 transcription may be through ESE-3, another closely related Ets factor and a direct target gene of ESE-1. Unlike ESE-1 which typically peaks at 2 hours following cytokine stimulation, ESE-3 was found to peak around 24 hours in human airway epithelial cells (Wu et al., 2008). While Wu et al. did not consider the possibility of ESE-3 acting as a reciprocal repressor of ESE-1, their data indicates that overexpression of ESE-3 was in fact more effective in inhibiting ESE-1 transcription than ESE-1 itself (Wu et al., 2008). Similarly, p38 MAPK plays a crucial role in prolonging COX-2 mRNA stability by PGE2 (Dean et al., 1999; Lasa et al., 2000), and ESE-3 is known to act as a downstream repressor of p38 MAPK pathway under certain conditions (Tugores et al., 2001). Therefore, it is possible that ESE-3 acts as a reciprocal repressor of ESE-1 at later time point when IL-1β is degraded, and that this feedback loop is defective in RASFs. Given that ESE-3 is also upregulated during stress-induced senescence in human fibroblasts (Fujikawa et al., 2007), dysregulation in the ESE-1-ESE-3-MAPK regulatory loop may be involved in sustaining the non-senescent phenotype of RASFs.
Lastly, although knockdown of ESE-1 also resulted in upregulation of COX-2 in SW982 cells, the pattern of ESE-1 expression induced by IL-1β was very different, indicating SW982 is not a truly representative model to study the role ESE-1 in human RASFs in vitro. Because most of the ESE-1 targets have been identified in immortalized cell lines, this points to the need to develop better cell model systems that more closely mimic RASFs in situ, as well as experimental tools which minimally interfere with immune-responsive function of the target proteins. Nonetheless, our findings reveal new insights into the role of ESE-1 in rheumatoid arthritis, as it is the first time to demonstrate that ESE-1 may assume an anti-inflammatory role under physiological conditions to prevent excessive tissue damage during an inflammatory response, by negatively regulating COX-2 in human RASFs.
4 The effect of ESE-1 deletion in other immune cells: macrophages and B cells
4.1 Abstract

Introduction: E26 transformation-specific (Ets) protein family of transcription factors are closely involved hematopoiesis. Epithelial-specific Ets transcription factor-1 (ESE-1) or Elf3 in mice, is a member of the Ets family which is known to be constitutively expressed in epithelial cells and to play an important role in epithelial tissue differentiation. However, ESE-1 can be induced by proinflammatory cytokines in non-epithelial such as endothelial cells and fibroblasts, and Elf3-dendritic cells have shown to be hypermaturated. As previous studies have shown that macrophage differentiation can be influenced by different cytokines and growth conditions, we investigated whether Elf3 deletion had any effect on myelopoiesis in Elf3-mice.

Methods and Results: We first analyzed the overall proportion of different immune cell lineages in the bone marrow by single stain flow cytometry, and analyzed the expression of Elf3 during macrophage colony stimulating factor (M-CSF)-driven ex vivo monocytic differentiation of fluorescence-activated cell (FACS)-sorted myeloid progenitors by reverse transcription quantitative PCR (RT-qPCR), as well as surface markers of maturated macrophages by multi-color flow cytometry. We observed no significant alterations in Elf3-macrophages in maturation state or activation. However, Elf3-macrophages showed slightly diminished ability to produce Th1-polarizing proinflammatory cytokine IL-12 in response to LPS, as determined by ELISA. Interestingly, Elf3-mice had reduced number of circulating B lymphocytes, but the source could not be pinpointed to any evident changes in the bone marrow stroma, growth factor expressions, B cell apoptosis, or final B cell maturation in the spleen.

Conclusions: Elf3 does not play a significant role in myelopoiesis. However, it may play a previously unidentified role in B cell development, where Elf3 mice have reduced number of circulating B cells but without gross defects in the spleen or in the bone marrow.

4.2 Introduction

Hematopoietic stem cells (HSCs) are rare cells (1 in 10^4-10^5 in the adult mouse bone marrow) which can self-renew and give rise to multiple cell lineages of the hematopoietic system. In a hierarchical differentiation steps into committed progenitors, HSCs undergo gradual loss of the self-renewal potential while acquiring distinct features of the terminally differentiated mature cells (Yeung et al, 2009). There are two major sites of hematopoiesis in adults: the bone marrow and
the spleen. During embryonic development, HSCs originally reside in the liver and the placenta, and migrate into the bone marrow and spleen around embryonic day 17 (E17.5) (Christensen et al., 2004; Gekas et al., 2005; Ottersbach and Dzierzak, 2005). Of note, spleen is an active site of hematopoiesis especially during pathological conditions such as myeloproliferative diseases or cancer (Cesta et al., 2006; Morita et al., 2011). Although the frequency of CD34−KLS (c-Kit++Lin−Sca-1++) HSCs is much lower in the spleen in the steady-state, the spleen-derived cells retain similar colony-forming activity as well as repopulating and self-renewal capacity as those from the bone marrow (Morita et al., 2011).

The formation of myeloid cells, called myelopoiesis, involves a sequential development steps through which hematopoietic stem cells (HSCs) undergo lineage commitment to common myeloid progenitors (CMPs), granulocyte-macrophage progenitors (GMPs), and eventually to monocytes or neutrophils (Hume, 2006). Stepwise differentiation into mature myeloid cells require specific transcriptional regulators which promote certain lineage acquisitions but oppose other development pathways. Key factors include PU.1, an Ets transcription factor whose expression is critical for the generation of CMP from stem cells and maintained at high levels for myeloid lineages but progressively downregulated in erythroid and T-cell differentiation (Anderson et al., 2002; Arinobu et al., 2007; Chen et al., 1995; DeKoter and Singh, 2000; DeKoter et al., 1998; Henkel et al., 1996; Leddin et al., 2011; Olson et al., 1995; Rosenbauer et al., 2006); CCAAT/enhancer binding proteins especially C/EBPα (Dahl et al., 2003; Hohaus et al., 1995; Porse et al., 2005; Zhang et al., 1996) and C/EBPε (Yamanaka et al., 1997) which are essential in the generation of GMP and neutrophilic differentiation; and interferon-regulatory factor (IRF8) (Holtschke et al., 1996; Tamura et al., 2000) and growth-factor independent 1 (Gfi-1) (Hock et al., 2003; Hock and Orkin, 2006; Karsunky et al., 2002) in determining monocytic/macrophage versus granulocytic fates from GMP, respectively (Rosenbauer and Tenen, 2007).

Ets family of transcription factors are characterized by the conserved Ets DNA binding domain. Members of the Ets protein family are closely involved in hematopoiesis and immune cell regulation (Gallant and Gilkeson, 2006). Major examples include PU.1, which is a critical determinant of myeloid fate of the common myeloid progenitors (CMPs) (Anderson et al., 1998) and important in the normal differentiation of B cell and T cell populations (McKercher et al., 1996; Scott et al., 1994), Ets1/2, a downstream regulator of monocyte colony-stimulating factor receptor (M-CSFR) signaling, which drives macrophage differentiation (Klappacher et al., 2002),
Spi-B in B cell formation and function (Garrett-Sinha et al., 1999; Hu et al., 2001; Su et al., 1997; Su et al., 1996), and Fli-1 in megakaryocyte differentiation and HSC maintenance (Hart et al., 2000; Kruse et al., 2009). More importantly, Ets factors are known to act together in a concerted manner in some lineages, with transient expression of one factor followed by another (Anderson et al., 1999).

Elf3, the murine homolog of ESE-1 in humans, is a member of the ETS family of transcription factors which is constitutively expressed in epithelial cells (Oliver et al., 2012) but which is highly induced during inflammation in almost all cell types (Brown et al., 2004; Grall et al., 2003; Grall et al., 2005; Rudders et al., 2001; Wu et al., 2008). However, despite numerous studies implicating Elf3 in tissue differentiation and induction by proinflammatory cytokines, as well as regulation of important genes involved in myeloid cell function such as cyclooxygenase-2 (COX2) (Grall et al., 2005), lysozyme (LYS) (Lei et al., 2007), inducible nitric-oxide synthase (iNOS/NOS-2) (Rudders et al., 2001), and angiopoietin-1 (Ang-1) (Brown et al., 2004), there has been no previous study on the role of Elf3 in hematopoiesis. Additionally, macrophage differentiation is marked by distinct stages of early phase Ras-dependent cell proliferation and terminal phase METS-mediated growth suppression (Guidez et al., 1998; Klappacher et al., 2002). Dual role of Elf3 in differentiation has been recognized in the skin, where it suppresses the expression of basal keratin 4 (K4) that is important in mediating early proliferation, while concurrently activating the small proline-rich protein 2A (SPRR2A) promoter associated with late differentiation (Brembeck et al., 2000). Thus, similar dual-role scenario of Elf3 in cell proliferation and differentiation pathways may also apply to macrophages in response to differentiating cytokines. Given these previously demonstrated roles of Elf3 in tissue differentiation and inflammatory response, I hypothesized that deletion of Elf3 will have an impact on myelopoiesis and aimed to investigate the role of Elf3 in macrophage differentiation and function.
4.3 Materials and Methods

Reagents

DMEM, RPMI-1640, fetal bovine serum (FBS), and L-GluataMAX™ were purchased from Gibco Life Technologies Ltd., Burlington, Ontario. Penicillin/streptomycin, phosphate buffered saline (PBS), and Hank’s balanced salt solution (HBSS) were from Wisent, St. Bruno, Quebec, and lipopolysaccharide (LPS) endotoxin (Escherichia coli, serotype O128:B12) and ABTS Liquid Substrate Solution for ELISA assays were purchased from Sigma, Oakville, Ontario. Antibodies used for flow cytometry were as follows: anti-mouse APC-Cy7 B220 (CD45R) (RA3-6B2), PE-Cy7 CD127 (IL-7R) (A7R34), APC CD43 (S11), BV421 CD24 (M1/69), APC-Cy7 Sca-1 (Ly6A/E) (D7), APC PDGFRα (CD140a) (APA5) from BioLegend, San Diego, CA; anti-mouse FITC IgM (II/41), PE CD11b (M1/70), APC Ly-6G/Gr-1 (RB6-8C5), PE Ly-6C (HK1.4), PE CD3e (145-2C11), FITC CD34 (RAM34), PE Ly-6A/E (Sca-1) (D7), APC CD117 (c-Kit) (2B8), PE F4/80 (BM8), PE-Cy7 CD16/32 (93), PE CD14 (Sa2-8), FITC CD11c (N418), PE MHC class II (M5/114.15.2), PE CD80 (B7-1) (16-10A1), Alexa Fluor 488 c-Fms (M-CSFR, CD115) (AFS98), APC CD86 (B7-2) (GL1), FITC CD31 (PECAM-1) (390), PE PDGFRβ (CD140b) (APB5) and anti-mouse CD16/32 purified antibody from eBioscience, San Diego, CA; anti-mouse BV510 IgD (11-26c.2a) and BV605 CD21 (7G6) from BD Horizon, and FITC Ly-6G/Gr-1 (1A8) and PerCP-Cy5.5 CD11b (M1/70) from BD Pharamingen™, BD Biosciences. Appropriate isotype control antibodies from respective companies were used for negative staining. 7-AAD for dead cell exclusion and CompBead Plus Negative Control (BSA) and BD CompBead Plus Anti-Rat Ig, κ beads were obtained from BD Biosciences, Mississauga, Ontario. Mouse Hematopoietic Progenitor Cell Enrichment Kit was from STEMCELL Technologies, Vancouver, BC.

ESE-1/Elf3 knockout mice and whole blood analysis

Elf3−/− mice on C57BL/6 background were maintained in pathogen-free conditions at the Toronto Centre for Phenogenomics (TCP), and wild-type littermates were used as controls. All experiments were reviewed and approved by the TCP Animal Care Committee (ACC) for the humane use of animals (Animal Use Protocol #0062). For the analysis of peripheral white blood cells, whole blood was collected by cardiac puncture under isoflurane anesthesia with EDTA-coated syringes, and aliquots were run on the Hemavet 950FS Hematology Analyzer at the Centre for Modeling
Human Disease (CMHD), TCP, Toronto. For the preparation of whole blood for flow cytometry analysis, 500 µL of whole blood was lysed with 5 mL of red blood cell (RBC) Lysis Buffer (155 mM NH₄Cl 10 mM KHCO₃, 1 mM EDTA) and incubated at room temperature for 5 minutes. Cells were then centrifuged and washed twice with cold staining medium (2% FBS 1 x HBSS, 10 mM HEPES pH 7.2) and counted on a hemocytometer with trypan blue cell exclusion. Cells were resuspended at 10⁷/mL for antibody staining.

**Preparation of single cell suspension of mouse bone marrow cells and differentiation of bone marrow-derived macrophages (BMDM)**

Bone marrow was flushed from femur and tibia of 9-12 week-old C57BL/6 mice with 27G needle with RPMI-1640 media supplemented with 10% FBS and made into single cell suspensions by passing through the 70µm nylon filter. Red blood cells were lysed with ammonium chloride lysis solution (155 mM NH₄Cl 10 mM KHCO₃, 1 mM EDTA) and washed with cold staining medium (2% FBS 1 x HBSS, 10 mM HEPES pH 7.2). The single cell suspension was then either stained for flow cytometry as below, or plated at 4 x 10⁵ cells per 10 cm non-tissue culture-treated petri dishes in 10 mL of 20% L-929-conditioned media (20% L-cell conditioned media, 10% FBS, 2 mM-glutaMAX, 10 IU/mL penicillin and 10 µg/mL streptomycin in 1x RPMI), and designated as Day 0. On Day 3, additional 5 mL of the growth media were added per petri dish. On Day 6-8, cells were harvested by EDTA-mediated detachment with 5 mM EDTA in sterile PBS. For stimulation with LPS, 4 x 10⁵ of mature BMDMs seeded on 6-well plates were starved overnight and subjected to 100 ng/mL LPS or cytokines as indicated for 18 hours before analysis as previously described (Lee and Hu, 2013).

**Isolation of single cell suspension from mouse spleen**

For spleen cell analysis, mice were euthanized by CO₂ or cervical dislocation, and the spleen was collected by cutting through the skin just below the ribcage on the left uppermost side. Isolated spleen was placed on a 70 µm nylon mesh cell strainer immersed in 5 mL of complete medium (1xRPMI-1640 containing 10% FBS 2 mM L-glutamine, 100 U/mL penicillin/streptomycin, 50 µM 2β-mercaptoethanol) at room temperature, cut into three to six pieces, and crushed with plunger from a 1 mL syringe until only the white fibrous tissue was left. Using fresh chilled medium and a Pasteur pipet, the petri dish and the cell strainer were rinsed to ensure that all cells have been recovered. The cells were pelleted by centrifugating at 400xg for 5 minutes at 4 °C, treated with
1mL/spleen of RBC lysis buffer at room temperature, washed and resuspended in flow staining buffer.

**Flow cytometry and Fluorescence-Activated Cell Sorting (FACS)**

Bone marrow or spleen cells in single cell suspension were stained at 1 x 10^7 cells/mL concentration in staining buffer (2% FBS 1xHBSS 10 mM HEPES pH 7.2) with conjugated flow antibodies for 30 min at 4 °C in the dark, washed twice with the staining buffer, and fixed with 2% formaldehyde and stained with Fixable Violet (Invitrogen, Burlington, Ontario) where appropriate, or stained with 7-AAD for dead cell exclusion before running on flow cytometer. Stained cells were acquired on LSRII flow cytometer (BD, Franklin Lakes, NJ), and minimum 20,000 of live events were collected and analyzed using Flowjo Data Analysis Software (Flowjo LLC, Ashland, OR). The gates for analysis were set using Fluorescence Minus One (FMO) gating controls.

For FACS of common myeloid progenitors, non-progenitor cells were first removed from the prepared bone marrow cells by immunomagnetic negative selection using EasySep™ Mouse Hematopoietic Progenitor Cell Isolation Kit with RoboSep™ (STEMCELL Technologies, Vancouver, BC). This removed terminally differentiated cells by using antibodies recognizing the lineage antigens such as CD3, CD11b, CD19, CD45R, Gr-1 and Ter119 conjugated to magnetic beads. The enriched progenitor cells were stained with CD34, Sca-1, c-Kit, and CD16/32 and sorted on FACSARia II (BD Bioscience, San Jose, CA) for common myeloid progenitors (CMPs; CD34^+ Sca-1^− c-Kit^− CD16/32^lo^) and granulocyte myeloid progenitors (GMPs; CD34^+ Sca-1^− c-Kit^− CD16/32^hi^). The sorting purity was approximately 95% on average. The sorted CMPs were plated on 12-well plates in 1xRPMI-1640 medium containing 10% FBS, 2 mM glutamine and 100 ng/mL murine M-CSF (Peprotech, Quebec, Canada) and collected on indicated days for qPCR analysis.

**RNA Isolation and real-time quantitative PCR (RT-qPCR)**

Total RNA from mouse bone marrow cells and bone marrow-derived macrophages were isolated using GE Illustra RNASpin Mini Kit (GE Healthcare Life Sciences, Baie-D’Urfe, Quebec) according to the manufacturer’s instructions. For quantitative real-time RT-PCR, total RNA (1 µg) was reversed transcribed using random hexamers and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) and the resulting cDNA template (20 ng) was used for qPCR reaction
using Power SYBR Green PCR Master Mix from Life Technologies, Burlington, Ontario. ABI Prism 7700, from Applied Biosystems, Foster City, CA was used for the amplification and analysis. For relative ΔΔCt quantification, qPCR signals were normalized to GAPDH. The primer sequences used in this study are outlined below:

<table>
<thead>
<tr>
<th>B-cell growth factors</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCL12</td>
<td>5’- TGCATCAGTGACGGTAAACCA -3’ (Forward) 5’- TTTCTCCAGCCGTGCAACAATC -3’ (Reverse)</td>
</tr>
<tr>
<td>FLT3L</td>
<td>5’- GCCTGGAGCCCAAATTCTCTC -3’ (Forward) 5’- GCTGAAGTAACGTCAGGTGTC -3’ (Reverse)</td>
</tr>
<tr>
<td>IL-7</td>
<td>5’- TTCTCCACTGATCTTGGTCTT -3’ (Forward) 5’- AGCAGCTTTTTGTGATCATCAC -3’ (Reverse)</td>
</tr>
<tr>
<td>BAFF</td>
<td>5’- TTCCATGGGCTTCTCAGCCTT -3’ (Forward) 5’- GGAATTGTTGGGCAGGTGTTT -3’ (Reverse)</td>
</tr>
<tr>
<td>SCF</td>
<td>5’- CGGGAATCTCTGTGCTGATAA -3’ (Forward) 5’- GGCTCTTCGGAGATTCTTT -3’ (Reverse)</td>
</tr>
<tr>
<td>CCL5</td>
<td>5’- GCTGCTTTGCCATACCTCCTCC -3’ (Forward) 5’- TCGAGTGACAAACAGGTGAT -3’ (Reverse)</td>
</tr>
<tr>
<td>RANKL</td>
<td>5’- CAGCAGCATGCTCTGTCTGTGA -3’ (Forward) 5’- CTGGGATTTTCATGGAGTCTCA -3’ (Reverse)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Other Ets factors</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>mEhf</td>
<td>5’- CCAGCCAACAACCTCCTTCTAA -3’ (Forward) 5’- GCCATTCCTCACAGGATCTATG -3’ (Reverse)</td>
</tr>
<tr>
<td>mElf5</td>
<td>5’- GAGCATCAGACAGCCTGTA -3’ (Forward) 5’- GGTGAAGTGACAGGAGATTCT -3’ (Reverse)</td>
</tr>
<tr>
<td>mPea3</td>
<td>5’- ACCTCAGTCACTTCAGAGAC -3’ (Forward) 5’- TTTCTGACTCTGTGGTGG -3’ (Reverse)</td>
</tr>
<tr>
<td>mEts1</td>
<td>5’- CTCCGAGACGAAAGAATAATG -3’ (Forward) 5’- ACTCATTCACAGCACAATCAC -3’ (Reverse)</td>
</tr>
<tr>
<td>mEr81</td>
<td>5’- GTCTGTTCAGTCAAGAGC -3’ (Forward) 5’- CTGGGATGGCTGAGGTGGTGT -3’ (Reverse)</td>
</tr>
<tr>
<td>mElf3</td>
<td>5’- GAGATGGTCTTCTGACTATAAG -3’ (Forward) 5’- GTGGGATGGATGTCTCGGAT -3’ (Reverse)</td>
</tr>
</tbody>
</table>

**Cytokine measurement by ELISA**

Secreted murine IL-12, TNF-α, IL-6, IL-1β, and IL-10 in culture media from stimulated BMDM cells were quantified using sandwich Avidin-HRP-ABTS ELISA kits from Peprotech, Quebec, Canada following the manufacturer’s protocols. Samples for analysis were prepared by centrifuging culture medium at maximum speed for one minute and diluting at 1:3 to 1:10 with Diluent Buffer (0.05% Tween-20, 0.1% BSA in PBS). The standard curve was prepared by serial
dilution of the provided standard from 0.01 µg/mL to 0 in Diluent Buffer and incubating with the pre-coated capture antibody as the samples. Following incubation of the samples and standards with the capture antibody and biotinylated detection antibody, avidin-HRP conjugate followed by ABTS Liquid Substrate were added before detection on Molecular Devices Spectra Max 190 with SoftMax Pro Software 5 at 405 nm with wavelength correction at 650 nm. Cytokine concentrations were calculated using the standard curve generated from the serially diluted standard against its absorbance.

**Determination of Nitrite Concentration**

The production of nitric oxide (NO) by activated BMDM was quantified using Griess Reagent Kit for Nitrite Determination (Molecular Probes, Burlington, Ontario) according to the manufacturer’s instructions. Briefly, N-(1-naphthyl)ethylenediamine (Component A) and sulfanilic acid (Component B) was mixed at equal volumes to form the Griess Reagent and used immediately. In a microplate, 20 uL of Griess Reagent was mixed with 150 uL of culture supernatant (nitrite-containing sample) and 130 uL of deionized water was added and incubated at room temperature for 3 minutes. Samples for analysis were prepared by collecting supernatant from centrifuged cell culture medium at appropriate time point and experimental conditions. For the calibration curve, the nitrite standard solution (Component C) was diluted to concentrations ranging between 1-100 uM by serial dilution and mixed with Griess Reagent and deionized water as the experimental samples. The photometric reference sample was prepared by mixing 20 uL of Griess Reagent with 280 uL of deionized water. The microplate was read at 548 nm against the reference sample, and nitrite concentrations were calculated using a standard curve of nitrite concentration against absorbance.
4.4 Results

Elf3/− bone marrow contains higher proportion of CD11b⁺ and Gr-1⁺ cells and less CD19⁺ cells under steady state

To investigate whether ESE-1/Elf3 had any role in the development of immune cells, we first checked the distribution of different immune cell lineages in the wild-type and Elf3/− mouse bone marrow by single-stain flow cytometry of different lineage markers such as CD11b, Gr-1, CD11c, CD14 and F4/80, CD19, and CD3, for myeloid cells, B cells, and T cells, respectively. We opted to first perform single staining flow cytometry, as multi-color staining can significantly decrease resolution due to extensive spectral overlap and corresponding amount of compensation that is required for accurate analysis (Herzenberg et al., 2006; Yeung and So, 2009), which can mask minor differences in cell populations that may have been captured without compensation. Interestingly, we found a modestly increased proportion of CD11b⁺ and Gr-1⁺ cells, and decreased frequency CD19⁺ cells compared to the wild-type littermate under resting conditions (Figure 4.1). As CD11b and Gr-1 are expressed on developing myeloid cells (Francke et al., 2011) and CD19 is expressed on both maturating and mature B-cells (Nagasawa, 2006), this finding led us to first question whether Elf3 deficiency led to any defect in myelopoiesis, and whether the decrease in CD19⁺ cells was due to relative quantification, as we did not observe any difference in the total number of bone marrow cells between gender- and age-matched WT and Elf3/− mice (data not shown).
Elf3 is minimally expressed during monocyctic differentiation and Elf3-/- mice have no defect in myelopoiesis

Given the lack of any gross defect in neutrophil development or function in Elf3-/- mice (Chapter 2), we decided to focus on the effect of Elf3 deletion on monocyctic differentiation. We first analyzed whether there was any inherent difference in the prevalence of myeloid progenitor cells that was responsible for the higher numbers of CD11b+ and Gr-1+ cells in the bone marrow, by quantifying precursors by flow cytometry using cell surface markers known to be associated with common myeloid progenitor (CMP) and granulocyte myeloid progenitor (GMP) cells. However, we observed no significant defect in the frequency of CMP or GMP in the Elf3-/- bone marrow (
Figure 4-2). Macrophage colony-stimulating factor (M-CSF) is the key inducer of macrophage proliferation and differentiation (Barreda et al., 2004), and progenitor cells from the bone marrow can be isolated and induced to undergo macrophage differentiation in vitro. Therefore, we next examined the expression of Elf3 over the time course of ex vivo differentiation of fluorescence-activated cell (FACS)-sorted myeloid progenitors (by the expression of CD34+Sca-1-c-Kit+ cell surface markers (Yeung and So, 2009) from mouse bone marrow with 100 ng/mL murine M-CSF for 7 days. We analyzed changes in Elf3 transcriptional level by RT-qPCR over a time-course of BMDM development and observed no significant expression throughout the CMP monocytic differentiation. Additionally, we compared the maturation state of Elf3/- BMDM to that of wild-type littermate controls by flow cytometric profiling of known surface markers associated with macrophage differentiation, such as CD11b, F4/80, c-Fms (M-CSFR), Ly-6C and Ly-6G (Murray and Wynn, 2011a). Consistent with the lack of evident expression of Elf3 during monocytic differentiation of sorted myeloid progenitors with M-CSF, we did not observe any difference in macrophage maturation in Elf3/- BMDMs (Figure 4-3).

However, we also questioned whether the differentiation conditions from progenitor cells had an effect on the later macrophage function. Indeed, previous publications indicated that GM-CSF-maturated macrophages from bone marrow precursors display M1 phenotype with preferential production of TNF-α, IL-6, IL-12p70 and IL-23, while M-CSF induces M2 phenotype. The M2 phenotype was characterized by the enhanced production of IL-10 and CCL2 (Fleetwood et al, 2007; Bailey et al, 2011), which in part was due to differential dependence to type I interferon signaling where M-CSF culture led to increased expression of basal IFN-β and the activation of type I IFN genes such as Stat1, Stat2, Irf7, Ccl12 and Cxcl10 (Fleetwood et al, 2009). Therefore, to address this possibility, we also differentiated the bone marrow cells from WT C57BL/6 mice in either 100 ng/mL murine M-CSF or 20 ng/mL GM-CSF for 8 days and treated them with M1 (LPS, IFN-γ) or M2 (IL-4, IL-10, IL-13)-polarizing cytokines. Disappointingly, none of GM-CSF or M-CSF-treated cells gave perceivable Elf3 signal (Ct values 34~39; data not shown).
Figure 4-2 ESE-1 mRNA expression is undetectable during monocytic differentiation.

(A). Frequency of CMP (CD34⁺Sca-1⁻Kit⁺CD16/32⁺) and GMP (CD34⁺Sca-1⁻c-Kit⁺CD16/32⁻) in the bone marrow of WT (n=6) and Elf3⁻/⁻ mice (n=7). (B). Common myeloid progenitor (CMP) and granulocyte-macrophage progenitor (GMP) cells (CD34⁺Sca-1⁻c-Kit⁺) were FACS-sorted from WT bone marrow by and cultured in 100 ng/mL murine M-CSF. Cells were lysed at indicated time points and total RNA was isolated for qPCR analysis for the level of Elf3 transcript. Day of sorting was indicated as ‘Day 0’. Lin⁻Sca-1⁺c-Kit⁺ cells (‘LSK’ HSC = Hematopoietic Stem Cell) were sorted and lysed immediately after sorting. 3LL Lewis Lung Carcinoma cell line was used as Elf3 positive control. (C). Table showing the raw qPCR Ct values and the number of animals included per data point.
Figure 4-3 Elf3 deletion has no effect on macrophage maturation.

(A-D). Bone marrow cells isolated from the WT or Elf3 were differentiated ex vivo in 20% L-929-conditioned media as a source of M-CSF (=CSF-1) and analyzed for various markers of macrophage maturation. The graph shows n=12 for WT and n=11 for KO from four independent experiments. F4/80 is associated with mature macrophages and c-Fms (=M-CSFR) is involved in macrophage differentiation and proliferation (Klappacher et al, 2002). Ly-6C is a monocytic marker and Ly-6G is a granulocytic marker. CD11b+Ly-6C–Ly-6G– is considered a mature macrophage phenotype (Francke et al, 2011). Statistical analysis by two-tailed Student’s t test.
*Elf3* deletion has no effect on macrophage activation but attenuates IL-12 production

Although *Elf3* induction could not be detected in during BMDM differentiation, previous findings have indicated that *Elf3* is induced by LPS in RAW264.7 murine macrophage cell line though absent in resting cells (Grall et al., 2005). Additionally, *Elf3* abrogation in dendritic cells (DCs), which share the common progenitor with macrophages, has been shown to display impaired ability to differentiate naïve T cells into Th1 or Th17 lineage due to reduced IL-12 and IL-6 production, respectively (Kushwah et al., 2011). Therefore, we postulated that *Elf3* will be induced during macrophage activation despite the lack of expression during macrophage differentiation. To test whether *Elf3* expression can be induced by proinflammatory cytokines or stimuli, therefore, we attempted to replicate the previous study by Grall *et al.* by subjecting RAW264.7 to 100 ng/mL LPS treatment at 400,000 or 1,000,000 cells/well densities on 6-well plates. Untreated Lewis Lung Carcinoma (3LL) cell line (at 0 hour) was included as a positive control for *Elf3* expression.

To our surprise, however, we did not observe any significant induction of *Elf3* by LPS in RAW264.7 cells or by various proinflammatory cytokines in mature BMDM cells (Figure 4-4). Consistent with the lack of *Elf3* expression during macrophage activation, there was no visible defect in the BMDM activation as shown by no difference in MHC class II, CD80, and CD86 surface expression upon stimulation with LPS (Figure 4-5). Still, we questioned whether *Elf3* deletion in macrophages play a similar role in DCs, especially in their ability to produce pro-inflammatory cytokines. We thus measured their cytokine production by stimulating mature WT and *Elf3*-/- BMDMs with LPS and quantifying the levels of secreted cytokines such as TNF-α, IL-1β, IL-6, IL-12, and IL-10 as well as nitric oxide (NO) by ELISA and Griess reagent assay, respectively, and observed modest reduction in IL-12 production by *Elf3*-/- macrophages, with similar trends for IL-6 and IL-1β (Figure 4-6). Therefore, it seems that *Elf3* plays some role in macrophage function but to minimal extent, given its expression levels below detection.
Figure 4-4 *Elf3* is minimally expressed during LPS-induced macrophage activation in RAW264.7 and during other cytokine stimulation in murine bone marrow-derived macrophages (BMDMs).

(A). Murine macrophage cell line RAW264.7 were plated at either 4 x 10^5/well or 1 x 10^6/well density on 6-well plates and were stimulated with 100 ng/mL LPS for different durations. Cells were lysed and subjected to qPCR analysis. Lewis Lung Carcinoma cell line (3LL) was used as a positive control for mElf3 expression, where Ct values ranged from 22-27. The data is a representative of four independent experiments. Similar results were obtained with RAW264.7 cells treated with a mix of 10 ng/mL TNF-α and 10ng/mL IL-1β, as well as in BMDM cells from WT animals, shown in raw Ct values in (B).
Figure 4-5 *Elf3* deletion has no effect on macrophage activation.

(A). Representative flow cytometry plots for the percentage of cells expressing activation markers induced by LPS. (B). Table showing mean±SD values of percentage of WT and KO BMDM cells positive for indicated markers with and without LPS treatment. Representative (n=3) of two independent experiments. There was no statistically significant difference between WT and KO BMDMs. BMDMs were generated by *in vitro* differentiation of bone marrow cells in 20% L-cell medium, and on Day 7 were incubated in the growth medium with or without 100 ng/mL LPS for 18 hours.
**Figure 4-6** *Elf3* deletion leads to decreased production of IL-12 in activated macrophages.

The amount of inflammatory cytokines and nitric oxide released from WT and *Elf3*-/-(KO) BMDMs were quantified by ELISA from culture medium with or without LPS (100 ng/mL) stimulation. Statistical analysis by one-way ANOVA with Bonferroni’s post-test. n=3, representative of two independent experiments.

*Elf3*-/-(KO) have reduced number of circulating B cells

To examine whether the altered distribution of immune cells types in the bone marrow was reflected by the cell population in the peripheral blood, we inspected the immune cell numbers in the peripheral blood by Hematology Analyzer. We found that *Elf3*-/-(KO) mice had a reduced peripheral white blood cell (WBC) count compared to their WT littermates, which was largely due to reduced number of lymphocytes (**Figure 4-7A,B**). To identify which of the circulating lymphocyte population was reduced, we stained whole peripheral leukocytes with CD3 and CD45R/B220 antibodies for flow cytometry, and observed that this reduction was mostly due to decreased number of circulating B-cells (CD3^+^B220^+^) (**Figure 4-7C**).

B cell development occurs primarily in the bone marrow, where immature B cells undergo negative selection against autoantigens to express a functional B-cell receptor (BCR) (Hartley et al., 1991;
Nemazee and Burki, 1989; Norvell et al., 1995). The surviving cells, which account for 10% of immature B cells in mice, express surface IgM and IgD and are released into the circulation to transit into the spleen (Forster and Rajewsky, 1990), where they further maturation into follicular mature (FM) B cells or marginal zone (MZ) B-cells (Allman et al., 1992). Follicular B cells are also known to recirculate between secondary lymphoid organs, including the spleen, lymph nodes, bone marrow, and Peyer’s patches (Okada and Cyster, 2006; Perez-Andres et al., 2010). In mice, distinct cell surface markers are used to distinguish immature (CD24\textsuperscript{hi}) B cells, such as transitional B cells, from mature (CD24\textsuperscript{lo}) B cells, such as FM and MZ B cells (Loder et al., 1999). Additional cell surface markers have been used to subdivide murine transitional immature B cells (CD24\textsuperscript{hi}) into two distinct populations: transitional type 1 (T1; CD24\textsuperscript{hi}, CD21\textsuperscript{lo}, CD23\textsuperscript{lo}, IgM\textsuperscript{hi}, IgD\textsuperscript{lo}) and transitional T2 (CD24\textsuperscript{hi}, CD21\textsuperscript{hi}, CD23\textsuperscript{hi}, IgM\textsuperscript{hi}, IgD\textsuperscript{hi}) (Loder et al., 1999). Therefore, we performed further analysis of the circulating B cells by B220, IgM, CD24, and CD21 to identify which population was affected, and found that B cells of “transitional” phenotype expressing high levels of CD21 and CD24 were reduced in $Elf3^{-/-}$ mice (Figure 4-7D), which indicate that there is reduced number of immature B cells transitioning from the bone marrow to the spleen.
Figure 4-7 Elf3-/- mice have reduced number of circulating B cells in the peripheral blood.

(A). Number of different white blood cell (WBC) types in the whole blood of WT and Elf3 KO mice. Values showing mean±SD, statistical analysis by Student’s t-test. *P<0.05, **P<0.01.(B). Graph of table A. (C). Elf3-/- mice have a reduced number of circulating B-cells, identified as CD3+B220+ cells by flow cytometry. Statistical analysis by Student’s t-test, ***P<0.001. (D). Elf3-/- mice have reduced proportion of circulating immature B-cells (B220low/CD24high) that have a transitional phenotype (E), showing flow cytometry analysis of B220 and Annexin V+ stained whole blood leukocytes from n=6 animals. Statistical analysis by Student’s t-test, **P<0.01.
Elf3-/- mice have no defect in B-cell development or the bone marrow microenvironment

Because there was no significant defect in the natural apoptosis of Elf3-/- B cells in circulation (Figure 4-7E), we went to examine the two major sites of B cell development, the bone marrow and spleen, to determine the source of reduced number of B-cells, particularly that of CD19+ cells in the bone marrow and immature transitional B cells in the periphery. B-cell precursors arise from the common lymphoid progenitor [CLP; Lin Kit<Sca-1>^lo IL-7R^+(Izon et al., 2001; Kondo et al., 1997)] cells, and are positive for the B-cell-lineage marker B220. Developing B cells can be divided into four subsets according to their cell surface markers, including B220, Kit, CD19, FLT3, CD24, CD43, and IgM (Nagasawa, 2006). The four subsets are referred to as fractions A (pre-pro B cells; B220^+CD19^-CD24^-CD43^-IgM^-), B/C (pro-B cells; B220^+CD19^+CD24^-CD43^-IgM^-), and D (pre-B cells; B220^+CD19^+CD24^-CD43^-IgM^-) (Hardy and Hayakawa, 2001; Hardy et al., 2007; Nagasawa, 2006), where the final immature B cells (B220^+CD19^-CD24^-CD43^-IgM^-IgD^-) arise from the fraction D cells and subsequently exit the bone marrow to reach the spleen. However, multi-color flow cytometric analysis did not reveal major changes in B cell precursor fractions (Figure 4-8A), though there was a slight reduction in the number of mature (IgM^-IgD^-) B-cells which may account for the reduced percentage of CD19+ cells in the Elf3-/- bone marrow. Similarly, we observed no difference in the overall B cell population in the Elf3-/- spleen (Figure 4-8B) as previously reported (Kushwah et al., 2011) and this may indicate that the slight delay in B cell development in the bone marrow is later recovered in the spleen.

Adherent bone marrow stromal cells form specific cellular niches that are indispensable for early B-cell development, by providing the necessary secretory factors and cell adhesion (Acosta-Rodríguez et al., 2007; Lebien and Tedder, 2008). The generation and maintenance of committed B cell precursors such as pre-pro-B cells, for example, require CXC chemokine ligand 12 (CXCL12)-abundant reticular (CAR) cells and interleukin-7 (IL-7) secreted by the stromal cells is absolutely required for proliferation and differentiation of pro-B cells (Egawa et al., 2001; Hunte et al., 1996; Namen et al., 1988; Von Freeden-Jeffry et al., 1995). CXCL12 and FLT3L are essential for the development of pre-pro B cells, where knockout mice show severe lack of very early B cell precursors (Egawa et al., 2001; Sitnicka et al., 2003; Sitnicka et al., 2002). Interestingly, FLT3L also synergizes with IL-7 (Hunte et al., 1996; Sitnicka et al., 2003), which is known to induce pro-B cell proliferation (Peschon et al., 1994; Von Freeden-Jeffry et al., 1995), and is
required for B cell precursor survival (Opferman et al., 2003). SCF, which is a ligand for the class III receptor tyrosine kinase Kit, is required for B cell development in adults from the pro-B cell stage (Driessen et al., 2003), whereas RANKL is involved in the development of pre-B and immature B cells (Yasuda et al., 1998). Moreover, B-cell activation factor member of the tumor necrosis factor (TNF) family (BAFF) and the related factor a proliferation-inducing ligand (APRIL) play an integral role in the B cell growth and survival of murine B cells and in eliciting T-independent IgA responses in the periphery (Castigli et al., 2004; Castigli et al., 2005; Mackay et al., 2003). Therefore, to examine whether this was due to an alteration in the bone marrow microenvironment, we analyzed the mRNA levels of various cytokines and growth factors known to play a role in B-cell development and hematopoiesis, such as CXCL12, FLT3L, IL-7, and SCF, as well as the composition of bone marrow stromal cells, including mesenchymal stem cells (MSC) (Sca-1^+PDGFRα^+), CAR cells (Sca-1^+PDGFRβ^+), and endothelial cells (Sca-1^+CD31^+) (Cheung et al., 2014; Ehninger and Trumpp, 2011), by flow cytometry. However, we observed no major alterations in the stromal cell population (Figure 4-9A) or in the bone marrow expression of hematopoietic growth factors (Figure 4-9B), suggesting an alteration in the B-cells (CD19^+) themselves.
Figure 4-8 *Elf3*-/- mice have no major defect in B-cell development.

(A). Number of B-cells and their progenitors in the WT and *Elf3*-/- bone marrow by flow cytometry. n=7 per group. (B). Total percentage of B-cells (CD3-B220+) and maturing B-cells (B220+IgM+) in the spleen. n=6 per group. Further stratification by CD21 and CD24 also revealed no difference. (C). 6-color flow cytometry gating strategy for (A). Statistical analysis by two-tailed Student’s t-test of WT and KO values revealed no statistically significant pair (unmarked).
Figure 4-9 *Elf3*-/-(KO) bone marrow has minimal changes in the stromal cell composition or the gene expression of hematopoietic growth factors.

(A). Proportion of mesenchymal stromal cells (MSC; Sca-1+PDGFRα+), CXCL-12-abundant reticular cells (CAR; Sca-1-PDGFRβ+), and endothelial cells (EC; Sca-1+CD31+) in WT and *Elf3*-/-(KO) mice determined by flow cytometry. n=5 per group. Statistical analysis by two-tailed Student’s t-test of WT and KO values revealed no statistically significant pair (unmarked). (B). RT-qPCR of various hematopoietic growth factors in the bone marrow of untreated WT and *Elf3*-/-(KO) mice. n=15 per group, mean±SD. Statistical analysis by Student's t test, and with Welch's correction for unequal variance for BAFF.
Figure 4-10 Expression of other Ets factors in WT and Elf3-/- BMDMs.

Mature BMDMs were treated with (+) or without (-) LPS (100 ng/mL) for 18 hours and harvested for RT-qPCR analysis to check the expression of other Ets factors. n=3, one time experiment.

4.5 Discussion

Given the epithelium-specific nature of ESE-1, many studies have focused on the role of ESE-1 in epithelial development (Oliver et al., 2012). However, ESE-1 expression can also be elicited by cytokines in non-epithelial cells (Brown et al., 2004; Grall et al., 2003; Rudders et al., 2001; Wu et al., 2008) and regulate the function of myeloid cells in both innate and adaptive immune system, such as macrophages (Grall et al., 2005) and DCs (Kushwah et al., 2011). Lineage determination in the bone marrow requires tightly regulated interaction between the developing cell and the bone marrow stroma (Rosenbauer and Tenen, 2007; Rosmarin et al., 2005), and the differentiation and maturation of myeloid cells are modulated by the action of hemapoietic or myelopoietic cytokines that activate specific signaling molecules. JAK/STAT, Raf/MEK/ERK, and PI3K-Akt pathways are some of the key intracellular signaling pathways involved in myeloid differentiation (Miranda and Johnson, 2007). In addition, recent findings suggest that macrophage function is influenced by their development conditions, where exposure to M-CSF or GM-CSF can lead to increased
propensity to be M2 or M1-polarized (Fleetwood et al., 2009; Fleetwood et al., 2007), respectively, and that cell density during differentiation (Lee and Hu, 2013) or activation (Jacobs and Ignarro, 2003) can also affect their function. We therefore questioned whether ESE-1 could act as a downstream transcription factor of these pathways in immune cells, and investigated the effect of ESE-1/Elf3 deletion in bone marrow cell homeostasis and macrophage maturation in vivo.

Initial assessment of immune cell lineages in the bone marrow revealed that Elf3-/− bone marrow had a modest shift towards the myeloid cell lineages (CD11b+, Gr-1+), with respective reduction in B cells (CD19+). Further analysis of the bone marrow Elf3 deletion showed no effect on the frequency of common myeloid progenitor (CMP) or granulocyte-macrophage progenitors (GMP), and Elf3 expression similarly could not be detected during ex vivo monocytic differentiation of fluorescence-activated cell (FACS)-sorted myeloid progenitors (by the expression of CD34+Sca-1−c-Kit+ cell surface markers (Yeung and So, 2009) from mouse bone marrow treated with 100 ng/mL murine macrophage colony-stimulating factor (M-CSF). Consistent with this lack of Elf3 expression, bone marrow-derived macrophages (BMDMs) from wild-type (WT) and Elf3-knockout (KO) mice flow revealed no significant alterations in the expression of monocytic/macrophage cell surface markers of, indicating that Elf3 does not play a role during monocytic differentiation.

However, Gr-1 includes two epitopes, Ly-6C and Ly-6G, which has been used to classify myeloid-derived suppressor cell (MDSC) populations in addition to nuclear morphology and mechanisms of immunosuppression involved in tumor immune evasion. Mononuclear MDSCs, which are CD11b+Ly-6G−Ly-6Chigh, for example, have been designated as ‘monocytic’ (Youn and Gabrilovich, 2010), and produce high NO but low amounts of ROS from increased STAT1 and iNOS expression. Also, in support of their immature nature, monocytic subsets have been shown to be able to differentiate to macrophage or dendritic cells in vitro and in vivo (Kusmartsev and Gabrilovich, 2003; Kusmartsev and Gabrilovich, 2005). On the other hand, ‘granulocytic’ population, which are neutrophil-like with multi-lobed nuclei, express CD11b+Ly-6G+Ly-6Chigh and produce high levels of ROS yet low levels of NO, which is thought to be due to upregulated STAT3 and NAPDH activity (Rosenburg and Sinha, 2009; Gabrilovich and Nagaraj, 2009; Condamine and Gabrilovich, 2010). Therefore, the presence of more CD11b+ and Gr-1+ cells in Elf3-/− bone marrow may still indicate increased tendency of Elf3-/− mice to shift to increased myelopoiesis in disease states, and may have a particular relevance to tumor immune response, as
tumor cells are known to secrete growth factors such as G-CSF, GM-CSF, VEGF and IL-6 to drive myelopoiesis and encourage bone marrow exit of immature CD11b+Gr-1+ myeloid cells (Gordon and Taylor, 2005). In addition, while Gr-1 is a well-known marker of murine neutrophils (Eash et al., 2010), it is also expressed on precursors of monocytes and macrophages, and higher frequency of Gr-1+ cells may indicate increased presence of less differentiated, immature cells (Francke et al., 2011; Huang et al., 2006). Interestingly, there were also reduced number of circulating peripheral monocytes in Elf3/- mice (Figure 4-7A,B), which may suggest decreased monocyte release or increased monocyte apoptosis in the periphery. However, the difference in the absolute number was quite low to warrant an in-depth study.

Another possibility behind higher frequency of CD11+ and Gr-1+ cells may be that Elf3/- stromal cells secrete less M-CSF under physiological conditions, even though Elf3/- myeloid progenitors have full potential to mature when subjected to excessive M-CSF in ex vivo cultures. A possible approach to assess alterations in the Elf3/- bone marrow stroma may be to co-culture sorted WT CMP population with either WT or KO BM-isolated stromal cells (Anjos-Afonso and Bonnet, 2008) and observing cell proliferation of the myeloid progenitor cells by anti-BrdU staining with subsequent flow cytometric analysis, as well as quantifying secreted M-CSF in the co-culture media. In addition, while we focused on macrophages, CD11b is also expressed in natural killer (NK) cells (Biron and Brossay, 2001; Korbel et al., 2004), and preliminary data suggested that there may be slightly lower incidence of NK cells (by NKp46 staining) (data not shown) which may be subjected to more analysis. Furthermore, unlike a previous publication which showed the induction of Elf3 with LPS treatment in RAW 264.7 cells with Western blot (Grall et al., 2005), we did not observe such induction at the mRNA level when we performed the qPCR under same treatment conditions, and similar results were obtained when stimulated with a combination of TNF-α and IL-1β or when BMDMs were used. Careful review of results from Grall et al., however, indicated few shortcomings in the study, such as missing loading control for Western blot and ambiguous supply source of Elf3 antibody. Consistent with the lack of Elf3 expression during macrophage activation, there was no alteration in the Elf3/- BMDM activation as shown by no difference in MHC class II, CD80, and CD86 surface expression upon stimulation with LPS.

We discovered a lower percentage of CD19+ cells in the Elf3/- bone marrow as well as B220+CD24hi circulating B cells in the peripheral blood as a serendipity. Because negatively
selected B cells expressing functional BCR emerge from the bone marrow and travel to the spleen to further differentiate into follicular- or marginal zone B cells, we looked into the bone marrow and the spleen which are the two major sites of B cell development, to identify the source of reduced number of B-cells. However, we were unable to find major defect in either location. The reduced percentage of CD19+ cells in the bone marrow may be a result of slightly reduced number of naïve immature (IgM+IgD+) B-cells, which may have also been responsible for the modest reduction in the number of transitional (T1 and T2) phase B cells in the circulation. Unfortunately, the number of (IgM+IgD+) B-cells did not result in a statistically significant finding to make a solid conclusion, but including more mice may show a clearer difference. Additionally, mature splenic follicular B cells can also recirculate to the bone marrow where they are positioned in perisinusoidal niche, and respond in a T cell-independent manner to blood-borne pathogens (Cariappa et al., 2007; Cariappa et al., 2005) and therefore may have partially contributed to the reduced number of CD19+IgD+ cells in the bone marrow. Additionally, while we did not observe any significant alterations in the overall B cells, Annexin-V and CD24 co-staining may have revealed specific increase in apoptosis in immature transitional B cells. We also did not find any discernible alterations in the stromal cell population (Figure 4-9A) or in the bone marrow expression of B cell function-related growth factors (Figure 4-9B).

Overall, however, these findings indicate that Elf3 may have a role in B cell development which was not previously recognized. Furthermore, a single knockout of Elf3 out of almost 30 Ets factors may not show a dramatic effect due to compensation from other Ets, which may complexity to the analysis. In fact, preliminary assessment showed that Elf3 KO BMDMs tended to have higher levels of Peas3 and Elf5 under basal conditions (Figure 4-10). Therefore, it could be that though there is no gross defect, the type of intracellular signal pathways activated differ or that other Ets factors are upregulated to compensate for the loss of function during myelopoiesis or B lymphopoiesis. Investigating whether there is any alteration in myelopoiesis in Elf3/-Elf5/- or Elf3/-Pea3/- double knockout mice may be the possible next step to determine the role of compensation among Ets factor, and Elf3/- mice may show less severe phenotype in disease models that involve B cells, such as systemic lupus erythematosus (Sang et al., 2012; Seavey et al., 2011), given the underlying lower B cell production or survival.
Chapter 5

5 Summary and Future Directions
5.1 Summary

Ets factors are involved in various cellular processes including cell differentiation, development, regeneration, and survival (Hollenhorst et al., 2011). ESE-1 is a member of a unique subfamily of Ets factors which have been shown to be constitutively expressed in epithelial cells (reviewed in Oliver et al., 2012). However, despite its notion as being primarily expressed in epithelial tissues, increasing evidence has suggested close involvement of ESE-1 in inflammation. ESE-1 is highly upregulated in response to various proinflammatory stimuli such as IL-1β, TNF-α and LPS in cells of non-epithelial origin, including smooth muscle cells, endothelial cells, and fibroblasts. In addition, ESE-1 has been shown to be a downstream target of NFκB signaling and regulate a number of genes implicated in inflammation, such as NOS2, COX-2, LYS, Trl11, and IL-6. ESE-1 has mostly been demonstrated to play a role in epithelial tissue differentiation and transformation. We had also previously demonstrated that the expression of ESE-1 is highly induced during inflammation (Oliver et al., 2012; Wu et al., 2008), and that it is a regulator of dendritic cell (DC)-driven T cell differentiation (Kushwah et al., 2011). Therefore, we attempted to further study the role of ESE-1 in human inflammatory diseases such as sepsis and rheumatoid arthritis, as well as hematopoietic development of immune cells in Elf3-/- mice.

Septic shock is a serious medical condition with mortality rate as high as 50% (Artero et al., 2012; Stearns-Kurosawa et al., 2013). However, inadequate knowledge of molecular mechanisms of septic pathogenesis has limited the development of effective therapies, where attempts to neutralize inflammatory cytokines using antibodies (Schulte et al., 2013) as well as many other therapeutic strategies (Rice and Bernard, 2005) have been unsuccessful (Schulte et al., 2013; Seam and Suffredini, 2007; Seeley et al., 2012). Thus, better understanding of the disease progression and identification of novel molecular targets are crucial for the development of more effective treatment. Initial analysis has shown lack of ESE-1 mRNA expression in human peripheral blood (PBL) (Oettgen et al., 1997; Tymms et al., 1997). However, given its rapid induction in non-epithelial cells in response to proinflammatory cytokines, we hypothesized that ESE-1 will be highly upregulated in human neutrophils during severe inflammation in sepsis. Instead, we surprisingly found that ESE-1 is constitutively expressed at both RNA and protein levels in human neutrophils, but that it rapidly disappears upon primary cell isolation and culture, which may explain why it has eluded detection in earlier studies. Unfortunately, attempts to further study the
implication of ESE-1 expression in human neutrophils in an in vivo endotoxemia model was hindered by the lack of expression of ESE-1/Elf3 in murine neutrophils, which may be an added evidence of inter-species difference and cautions against assuming findings across species.

As another model of human inflammatory disease, we studied the expression and function of ESE-1 in human rheumatoid arthritis synovial fibroblasts (RASFs) derived from patients suffering from rheumatoid arthritis (RA). RASFs show proliferative and invasive phenotype which play a central role in the pathogenesis of RA. ESE-1 was previously shown to be induced in RASFs by IL-1β (Grall et al., 2003; Brown et al., 2004) and to positively regulate COX-2 expression in cooperation with NFκB in macrophages (Grall et al., 2005). However, the functional significance of ESE-1 overexpression in RA synovial tissues has been unknown. We found, for the first time using HD-Ad-mediated knockdown of ESE-1, that ESE-1 negatively regulates COX-2 in human RASFs and that this regulation occurs at a later phase of the inflammatory response. The negative regulation of COX-2 by ESE-1 was also functionally relevant by direct changes in levels of PGE2, the final product of COX-2 enzymatic activity. This indicates that ESE-1 may be involved in the resolution of inflammation unlike previously thought, and this discrepancy may be attributed to confounding responses to transfection procedures in earlier studies. Additionally, ESE-1 was found to be localized in the nucleus rather than the cytoplasm, consistent with its known function as a transcription factor. It also did not co-localize in the same cellular compartment as NFκB when exerting its effect, indicating that ESE-1 regulation of COX-2 expression may occur through a NFκB-independent, currently unknown mechanism.

Given that ESE-1 is known to be involved in many tissue differentiation and that it can be expressed in immune cells in response to cytokines, we also studied its role in myelopoiesis using Elf3-/- mice. Interestingly, we found no major alteration in CMP or GMP frequency or the stromal cell composition in the Elf3-/- bone marrow, but modestly higher proportion of CD11b+ and Gr-1+ cells and a respective reduction in the percentage of CD19+ B cells. However, Elf3 deletion had no effect on the differentiation of bone marrow-derived macrophages (BMDM), which was consistent with the fact that Elf3 was not induced during macrophage differentiation from myeloid progenitors. There was also no evident induction of Elf3 during macrophage activation in response to LPS, but Elf3-/- BMDMs demonstrated diminished ability to secrete IL-12, indicating that Elf3 can play a minor role in macrophage function consistent with the previous finding where Elf3-/-
DCs could not drive Th1 T cell differentiation due decreased IL-12 production (Kushwah et al., 2011). Moreover, we observed less circulating B cells in the peripheral blood of Elf3−/− mice, which may have arisen from delayed or reduced IgM+IgD+ cells in the bone marrow that usually migrate to the spleen for further development. These are called ‘transitional’ immature B-cells, and also express high levels of CD24 and CD21 to distinguish them from re-circulating mature splenic follicular B cells. However, as there was no gross defect in the overall B cell population in the spleen, it may be that Elf3−/− B cells eventually recover any delay or alterations during its growth in the bone marrow later in their splenic development. Overall, Elf3 seems to play a rather minor role in macrophage function or B cell development, but this may be due to functional compensation from other Ets factors, and may become more evident when subjected to an inflammatory insult under a diseased state.

5.2 Future Directions

Based on the current findings, there are a number of possible future studies which may help determine the exact role of ESE-1 in cells involved in inflammation and the pathogenesis of human inflammatory diseases:

5.2.1 Correlate the neutrophil expression of ESE-1 with morbidity and survival of sepsis patients

Determination of diagnostic or therapeutic value of any potential candidate molecule (Faix, 2011; Faix, 2013; Pierrakos and Vincent, 2010; Walley, 2013) requires a careful analysis of its expression levels at various disease states and time points, as well as the ease of detection. Although we were not able to come to any sort of conclusion or find any correlation between the expression of ESE-1 in human neutrophils from sepsis patients with disease severity or mortality due to limitations in time, resources and the availability of septic patient cells, accumulating more data points on the expression of ESE-1 in septic neutrophils may provide valuable insights into the potential usefulness of ESE-1 expression as a prognostic, or even diagnostic, factor. Increased patient cohort will enable sub-categorization by the nature of disease pathogenesis and patient survival, which will help better identify where and when ESE-1 is highly upregulated in sepsis patient neutrophils. This may be more true if used in combination with existing biomarkers of
sepsis, such as procalcitonin (PCT) and C-reactive protein (CRP) (Faix, 2013; Pierrakos and Vincent, 2010; Walley, 2013).

Additionally, given its previously observed rapid and transient nature of induction upon inflammatory stimulation (Brown et al., 2004; Grall et al., 2005; Rudders et al., 2001; Wu et al., 2008), ESE-1 expression may be dynamically regulated in neutrophils during the course of sepsis with varying degrees of RNA and protein stability. The dynamic expression of ESE-1 during human sepsis may be demonstrated by incubating healthy human neutrophils in septic patient sera for 0, 2, 8, 12, 24hrs as a model of sepsis onset and measuring the level of ESE-1 mRNA and protein. From our preliminary observation on the rapid degradation of both EGFP-ESE-1 mRNA and protein in nucleofected HL-60 cells, it is possible that ESE-1 protein undergoes rapid degradation under basal conditions which is blocked in some patients with sepsis. Whether ESE-1 is protected from protein degradation by serine phosphorylation may be tested by immunoprecipitating serine-phosphorylated proteins using pan phospho-serine antibody and blotting with ESE-1 antibody. Better understanding the individual condition and health status at the time of analysis will also help to indicate any influential factors on ESE-1 expression and potential mechanisms of protein stabilization.

5.2.2 Investigate whether ESE-1 regulates neutrophil differentiation and its function in humans

As sepsis is essentially a human disease, no extensive animal studies can provide better relevant information than the actual human primary neutrophils, as animal models do not faithfully represent the human conditions. Unfortunately however, neutrophils, including the human acute promyelocytic leukemia cell line HL-60, are notoriously difficult to transflect (Basiouni et al., 2012; Esendagli et al., 2009), which limits performing many of the traditional functional studies. Use of lentiviral transduction in mature human neutrophils is also discouraged due to ineffective transduction and failure to express functional proteins (Geering et al., 2011b). Additionally, optimized nucleofection using the Amexa nucleofector still proved quite cytotoxic, as it causes cell membrane disruption by electric shock under a serum-stripped environment.

HL-60 cell line has traditionally been used as a model of neutrophil differentiation (Collins et al., 1978; Newburger et al., 1979). However, they do not produce fully mature neutrophils and lack
secondary and tertiary granules (Collins et al., 1977). In this case, therefore, use of neutrophil differentiation system based on human induced pluripotent stem cells (iPSCs) to study the role of ESE-1 in granulocytopenesis may provide greater, and more reliable insights into its function. A number of recent studies have reported patient- and disease-specific iPSCs (Agarwal et al., 2010; Park et al., 2008; Raya et al., 2009) as well as generation of fully mature and functional neutrophils from human dermal fibroblast-derived iPSCs (Brault et al., 2014; Choi et al., 2009; Flynn et al., 2015; Morishima et al., 2011; Sweeney et al., 2014). Characterization of step-wise maturation and a range of neutrophil functions by key surface markers such as CD45, CD11b, CD33, and CD16, presence of secondary and tertiary granules (80% and 60% positive, respectively) and myeloperoxidase activity (>90% positive), expression of myeloid transcription factors, chemotactic response to fMLP, phagocytosis and phagosome-dependent reactive oxygen production indicated that neutrophils differentiated from iPSCs were normal and fully mature (Morishima et al., 2011; Sweeney et al., 2014). The differentiation protocol has been further optimized to improve purity (50-70% from 30-40%) and shorter differentiation period (25 days from 27-30), using patient-derived somatic cells from chronic granulomatous disease (CGD) patients (Brault et al., 2014).

More importantly, clustered regularly interspaced short palindromic repeat (CRISPR) loci with CRISPR-associated (Cas) genes (CRISPR/Cas) system has emerged as a new and efficient tool for gene-editing that was adapted from immune defense mechanism in bacteria and archaea against bacteriophages (Cho et al., 2013; Cong et al., 2013; Jinek et al., 2013; Mali et al., 2013) and has been successfully employed in human iPS cells (Hou et al., 2013) and for phenotypic correction of mutations in myeloid cell lineages in genetic hematological disorders (Flynn et al., 2015; Mukherjee et al., 2011). The CRISPR/Cas-mediated gene correction in iPS cells is more efficient and safe using lentiviral vectors (Mukherjee et al., 2011), as the introduced wild-type copy of the gene is under correct endogenous control and there is no residual foreign exogenous DNA to cause insertional mutagenesis. Additionally, CRISPR/Cas technique avoids aberrant silencing of the transgene due random gene insertion or lack of cell-type specificity that are associated with viral insertions (Brendel et al., 2013; Chiriaco et al., 2014; Santilli et al., 2010). Therefore, while manipulation of ESE-1 gene expression is challenging in neutrophils by conventional means, recent advances in stem cell and gene editing technologies to introduce or delete ESE-1 will greatly enable future studies on the role of ESE-1 during neutrophil development and how its upregulation
during neutrophilic differentiation is regulated.

5.2.3 Investigate whether ESE-1 regulates neutrophil apoptosis and its mechanisms

ESE-1 expression was downregulated over the course of spontaneous apoptosis in healthy donor neutrophils, and upregulated in sepsis. Additionally, ESE-1 was upregulated during HL-60 granulocytic differentiation, with its expression being the highest when the HL-60 started to undergo spontaneous apoptosis (Breitman et al., 1980; Martin et al., 1990), suggesting that it may have a function in regulation of neutrophil apoptosis. Understanding the molecular mechanisms of neutrophil apoptosis is important not only for the advancement of our knowledge on sepsis, but also for the development of better therapeutics. As ESE-1 is usually confined in the nucleus and acts as a transcriptional factor (Oliver et al., 2012), investigating whether it is involved in regulating neutrophil apoptosis by regulating the transcriptional changes of the bcl-2 family of apoptosis regulators may be of value.

Extrinsic and intrinsic stimuli of neutrophil apoptosis signal through distinct molecular pathways (Geering and Simon, 2011; Luo and Loison, 2008). Therefore, to identify whether ESE-1 is a downstream target of a specific pathway or a converging point for all apoptotic mediators, ESE-1 expression at both mRNA and protein levels may be assessed in freshly isolated human neutrophils from blood of healthy donors and septic patients subjected (5 x 10^6 per treatment) to various inducer of neutrophil apoptosis as shown by a number of ex vivo studies, such as FasL (3 ng/mL) for 4-12 hours (Croker et al., 2011), TNF-α (25 ng/mL) for up to 8 hours (Cross et al., 2008; Geering et al., 2011a), R-roscovitine (20 μM) for 6-24 (Leitch et al., 2010) hours, or LY294002 (10 μM) for 72 hours along with their appropriate vehicle controls. The successful induction of apoptosis can be confirmed by Western blot of cleaved caspase-3 or degraded Mcl-1 as appropriate or by flow cytometry of cleaved caspase-3 or Annexin V. Specific enhancement of ESE-1 expression by a given apoptotic inducer can implicate ESE-1 involvement in the particular apoptotic pathway.

Neutrophils constitutively express both the pro-apoptotic (Bax, Bad, Bak, Bid and Bik) and anti-apoptotic (Mcl-1, A1, Bcl-xL) proteins of the bcl-2 family, as well as increased expression of apoptosome components Apaf-1 and procaspase-9 (Fox et al., 2010; Luo and Loison, 2008; Witko-Sarsat et al., 2011), whose balance is crucial in the normal maintenance of neutrophil apoptosis.
iPSC-differentiated neutrophils with knockdown or overexpression of ESE-1 may be treated with a specific apoptosis inducer that upregulates ESE-1 as identified from above, and changes in the transcription of bcl family of proteins may be determined by performing an Real-Time (RT$^2$) Profiler PCR Array available from SABioscience (Qiagen). Identifying common gene signatures regulated by ESE-1 in iPS-derived neutrophils incubated with 20 μM R-roscovitine or 10 μM LY294002 for 8 hours in culture may also provide insights into direct gene targets of ESE-1 and potential mechanism of regulation. Results from the RT array can be confirmed by Western blots for changes at the protein level, and this approach may give a global overview into the possible gene targets of ESE-1 during neutrophil apoptosis.

5.2.4 Investigate mechanisms of COX-2 regulation by ESE-1 in RASFs

As mentioned in Chapter 3, one possible mechanism by which ESE-1 exerts its repressor function on COX-2 transcription may be through ESE-3, another closely related Ets factor and a direct target gene of ESE-1 (Kas et al., 2000; Wu et al., 2008), which is known to act as a downstream repressor of the p38 MAPK pathway under certain conditions (Tugores et al., 2001). It is now clear from accumulating evidence that COX-2 gene is regulated both transcriptionally and post-transcriptionally following IL-1β stimulation. In human synovial fibroblasts (SFs), in particular, the COX-2 final product PGE$_2$ has been implicated in prolonging COX-2 mRNA half-life in a positive autocrine feedback loop by activating the p38 mitogen-activated protein kinase (MAPK) pathway, where PGE$_2$ also upregulated p38 MAPK via the prostaglandin EP4 receptor (Faour et al., 2001). In addition, PGE$_2$ alone was able to reverse the inhibitory effect of COX-2 inhibitor NS-398, while SB202190, a p38 inhibitor, suppressed IL-1β-induced COX mRNA protein synthesis by 80% (Faour et al., 2001). Similarly, in the presence of actinomycin D in human SFs, COX-2 mRNA declined within 2 hours following 3-4 hours of IL-β stimulation, but could last for 16 hours in the presence of PGE$_2$ (Faour et al., 2001).

Proinflammatory cytokines, such as IL-1β, that induce COX-2 gene expression also often result in the phosphorylation p38 by the dual-specificity kinase MAPK kinase 6 (M KK6) (Cuenda et al., 1997; Han et al., 1996; Raingeaud et al., 1996) activated by membrane proximal small GTPases of the Rho family (Ichijo, 1999; Tibbles and Woodgett, 1999), which in turn phosphorylates several downstream kinases such as MAPK-activated protein kinase 2 (MAPKAPK-2) and -3
(Freshney et al., 1994; McLaughlin et al., 1996; Rouse et al., 1994). The involvement of the MAPK pathway in regulating COX-2 mRNA stability has been demonstrated by a number of studies, where specific p38 inhibitors impede accumulation of COX-2 transcript (Guan et al., 1997; Guan et al., 1998a; Guan et al., 1998b; Lapointe and Isenović, 1999; Lasa et al., 2000; Matsuura et al., 1999; Pouliot et al., 1997; Reiser et al., 1998; Ridley et al., 1998; Ridley et al., 1997). In IL-1β-stimulated HeLa cells or human monocytes stimulated with bacterial LPS, in particular, inhibition of p38 leads to a rapid and specific destabilization of COX-2 mRNA, but without affecting COX-2 transcription (Dean et al., 1999; Ehrnsperger et al., 1997; Ridley et al., 1998).

Regulation of mRNA stability is mediated by sequences found in the 3’ untranslated region (3’ UTR). There are two major types of COX-2 transcripts of differing length, 4.6 and 2.8 kb, respectively, that arise from alternative polyadenylation sites (Newton et al., 1997; Ristimäki et al., 1996). The most abundant 4.6 kb transcript has a 2,515 nucleotide-long 3’ UTR containing 22 copies of AUUUA-rich element (ARE), which is subject to regulation by RNA binding proteins and microRNAs (Caput et al., 1986; Chen and Shyu, 1994; Chen and Shyu, 1995; Jing et al., 2005; Shaw and Kamen, 1986; Zhang et al., 2002). The strength and duration of COX-2 mRNA expression following IL-1β was found to be post-transcriptionally regulated by p38/MAPKAPK-2/hsp 27 cascade in HeLa cells, which involved the binding of AU-rich-element/poly(U) binding factor I to a short 123-nucleotide-long regulatory fragment on the COX-2 mRNA 3’-UTR (Lasa et al., 2000).

Therefore, given that knockdown of ESE-1 only led to an increased transcriptional level of COX-2 at 24 hours following IL-1β in RASFs and when the concentration of PGE₂ was at the highest level, it is very possible that ESE-1 functions in response to PGE₂. To test this, the same experiments may be performed in the presence of pure PGE₂ instead of IL-1β, to see whether COX-2 upregulation with ESE-1 knockdown occurs at an earlier time point. To investigate whether ESE-1 exerts its function by inducing ESE-3, the expression level of ESE-3 may also be measured by RT-qPCR and Western blot in correlation with that of ESE-1, and HD-Ad which express shRNA-ESE-3 may be designed and used in similar knockdown experiments in human RASFs, to determine whether ESE-3 is the critical player in the negative regulatory effect on COX-2 observed in ESE-1 knockdown. Additionally, to demonstrate that ESE-3 works downstream of MAPK p38 pathway, specific MAPK inhibitors may be used to observe changes in ESE-3
expression level, while a rescue model with ESE-3 overexpression in the presence of a p38 inhibitor may reveal that ESE-3 is a crucial effector of the MAPK pathway. Analysis of expression and interaction of RNA binding protein such as HuR (Dixon et al., 2001), CUGBP2 (Mukhopadhyay et al., 2003), Apobec-1 (Anant et al., 2004), AUFI/hnRNP D (Lasa et al., 2000), tristetraprolin (TTP) (Fenger-Gron et al., 2005; Phillips et al., 2004; Young et al., 2009), and T-cell intracellular antigen 1 (TIA-1) (Dixon et al., 2003) with regions of COX-2 mRNA 3’-UTR following ESE-1 or ESE-3 knockdown may also provide additional insights into the exact mechanism by which ESE-1 regulates COX-2 expression.

5.3 Concluding Remarks

Epithelium-specific Ets transcription factor-1 (ESE-1), as its name implies, has been extensively studied in the context of epithelial cell differentiation and transformation. However, increasing evidence has shown that despite its absence in non-epithelial cells such as fibroblasts, endothelial cells, and smooth muscle cells, ESE-1 is a key Ets factor which is rapidly induced and highly expressed in response to proinflammatory cytokines such as IL-1β and TNF-α. A number of immune function-related gene targets of ESE-1 had also been identified, suggesting that ESE-1 is an important regulator of immune response. In an attempt to further delve into the function of ESE-1 in human inflammatory diseases such as sepsis and rheumatoid arthritis, therefore, we used primary cells derived from human patients, as well as molecular techniques that have been better optimized, to verify and advance the pre-existing knowledge on ESE-1. Because final cell function is closely related to its development, we also used Elf3-/- mice to investigate the effect of Elf3 deletion in the myeloid cell lineages, with surprising discoveries. This thesis work is the first to demonstrate the constitutive expression of ESE-1 in human neutrophils, the negative regulation of COX-2 by ESE-1 in human RASFs, and the involvement of Elf3 in maintaining normal circulating B cell in mice. These findings summon more studies to better define the exact role and mechanism of ESE-1 in mediating disease pathogenesis, which has important health and therapeutic implications. As a special member of the Ets family of transcription factors, I believe that ESE-1 will continue to evolve beyond its notion of being epithelium-specific, to be recognized as an important Ets factor with multiple functions in human physiology and the immune system.
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