The Role of Endoglin in the Resolution of Inflammation

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

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Abstract

Endoglin, a co-receptor of the TGF-β superfamily, is predominantly expressed in endothelial cells and in some myeloid cells and implicated as a potential modulator of immune responses. We previously demonstrated that Endoglin heterozygous (Eng⁺⁻) mice subjected to the dextran sulfate sodium colitis model developed persistent inflammation and epithelial ulceration, while Eng⁺⁺ mice recovered following the acute phase of disease. Our aim was to assess potential alterations in distribution and number of immune cells, expression of inflammatory mediators and mechanisms of oxidative burst in Eng⁺⁻ mice. While the number of overall T, B and myeloid cells was unaltered between the genotypes, changes in neutrophil regulating cytokines and angiogenesis mediating factors were observed in Eng⁺⁻ mice. In addition, downregulation of phagocyte oxidative burst enzymes point to potential defects in microbial clearance in Eng⁺⁻ mice. These findings suggest a role for endoglin in regulating immune and vascular functions during inflammation.
Dedication

To all of my family and friends,

for their love, faith and constant support
Acknowledgments

I would like to thank my supervisor, Dr. Michelle Letarte, for giving me the opportunity to work in her lab. Through her guidance, support and encouragement, I have learned so much more than I could have ever imagined. Dr. Letarte’s dedication will be a constant inspiration in my life. I would also like to extend my gratitude to my supervisory committee members, Dr. Dana Philpott and Dr. Shannon Dunn, for their helpful suggestions and advice as well as allowing me to learn from members of their lab.

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Last but not least, I am so thankful to my family and friends for their constant love and support.
Acknowledgment of contributions

Dr. Mirjana Jerkic

Performed the myeloperoxidase (MPO) counts, western blots for MPO, Nox2 and Smad2 as well as the H₂O₂ measurement assay. This data was used to generate Figures 3.9, 3.11 A and C, 3.12 and 3.13 A and B.

Dr. Christopher Waterhouse in Dr. Paul Kubes’ lab

Performed the MPO activity assay and confirmed the MPO counts results. This data was used to generate Figure 3.11 A and B.

Valentin Sotov

Performed the superoxide generation assay in isolated bone marrow cells, which is represented in Figure 3.13 C.

Niousha Ghamami, a summer student in our lab

Helped to generate the B cell flow cytometry data.
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<table>
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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>Alk</td>
<td>Activin receptor-like kinase</td>
</tr>
<tr>
<td>ALL</td>
<td>Acute lymphoblastic leukemia</td>
</tr>
<tr>
<td>AVM</td>
<td>Arteriovenous malformation</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>BMPER</td>
<td>BMP-binding endothelial cell precursor-derived regulator</td>
</tr>
<tr>
<td>CD</td>
<td>Crohn’s disease</td>
</tr>
<tr>
<td>CGD</td>
<td>Chronic granulomatous disease</td>
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<tr>
<td>DSS</td>
<td>Dextran sulfate sodium</td>
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<td>Dihydroethidium</td>
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<tr>
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<td>Eng^{+/+}</td>
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</tr>
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<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte colony-stimulating factor</td>
</tr>
<tr>
<td>GIPC</td>
<td>G Alpha Interacting Protein (GAIP)-interacting protein C terminus</td>
</tr>
<tr>
<td>Flk1</td>
<td>Fetal Liver Kinase 1 or VEGF receptor 2</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HHT</td>
<td>Hereditary hemorrhagic telangiectasia</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>UC</td>
<td>Ulcerative colitis</td>
</tr>
<tr>
<td>VCAM1</td>
<td>Vascular cell adhesion protein 1</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WB</td>
<td>Western blot</td>
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Chapter 1
Introduction

1 Introduction

1.1 Overview

Inflammation is a tightly regulated and complex physiological process that is required for clearance of pathogens (1). Inflammatory responses involve the cooperation of cells of both the innate and adaptive arms of the immune system as well as those residing at sites of inflammation, such as endothelial and epithelial cells (2). Resolution of inflammation after removal of aversive stimuli is required in order to prevent further damage to host tissues (3). Lack of resolution resulting in persistent inflammation has been implicated in multiple diseases, including arthritis, atherosclerosis and inflammatory bowel disease (IBD) (4). Identification of the cellular and molecular components of inflammation has been extensively pursued, however, much remains to be discovered in order to develop novel therapeutic strategies for chronic inflammatory diseases. In this study, we explored the role of a TGF-β superfamily co-receptor, endoglin, as a potential regulator of inflammatory responses involved in the resolution of inflammation. My thesis will focus on the role of endoglin in the immune system, and therefore an overview of the following topics will be presented first: (1) expression, structure and known functions of endoglin in the vascular and immune systems; (2) brief summary of the inflammatory response, TGF-β mediated regulation of inflammation and role of vascular endothelium and immune-driven angiogenesis in inflammation; (3) description of IBD and the *Endoglin* heterozygous (*Eng*+/−) mouse model of IBD.

1.2 Expression, structure and function of endoglin

1.2.1 Expression in cells and tissues

*A) Marker of the vascular endothelium*

Endoglin (CD105) was initially discovered as a surface marker of the human pre-B acute lymphoblastic leukemia (ALL) cell line, HOON, by generation of monoclonal antibodies to these leukemic cells (5). This study resulted in the production of the first monoclonal antibody (44G4)
specific for human endoglin. Interestingly, staining of tonsil and thymus histological sections with 44G4 demonstrated that endoglin expression was primarily on the cells that line the inner lumen of blood vessels, the endothelial cells. Examination of expression in multiple human organs, including lymph nodes and kidney revealed endoglin to be constitutively expressed on endothelial cells of all vessel types (i.e. arteries, veins, capillaries and high endothelial venules) (6). Using in situ hybridization and immunostaining, murine endoglin was also found to be primarily expressed on endothelial cells in all tested organs, including ovary, heart, placenta, spleen, intestine, stomach and thymus (7).

B) Present on subsets of hematopoietic cells

Endoglin is also expressed on leukemic cells within the peripheral blood and bone marrow of patients with either pre-B or myeloid leukemia (6). In early studies of human bone marrow, endoglin was found on some fetal pro-B and pre-B cells (CD34⁺CD19⁺) and on fetal and adult erythroid lineage cells (8, 9). Endoglin expression on murine long-term repopulating hematopoietic stem cells as well as other hematopoietic precursor cells has also been reported (10, 11). In terms of mature leukocytes, there is upregulation of surface endoglin on activated monocytes and macrophages in peripheral blood and in the red pulp of the spleen (12, 13). A summary of evidence demonstrating modulation of hematopoiesis and immune functions by endoglin is found in section 1.2.5.

C) Found on some mesenchymal cells and on syncytiotrophoblast

Initial studies of murine organs revealed that endoglin is also expressed on some stromal cells within the connective tissue of intestine, stomach, heart, skeletal muscle, uterus, ovary, testis and thymus (7). These cells were described as fibroblasts, fibroblast-like cells and undifferentiated mesenchymal stem cells, which are important for tissue regeneration and repair. In atherosclerosis patients, endoglin is upregulated in the vascular smooth muscle cells of vessels with plaques (14). Similarly, in a porcine model of induced arterial injury, increased endoglin expression was seen in endothelial cells, smooth muscle cells and myofibroblasts (15). At the 5-8 week gestation period, endoglin is expressed in human embryonic heart, specifically on the mesenchymal cells of the cushion tissue during valve formation and heart septation (16).

In addition, endoglin is found on the syncytiotrophoblast of human placenta, which is in direct contact with the maternal circulation and important for nutrient exchange and immune protection for the fetus (17). In preeclampsia, a condition that leads to maternal hypertension and
fetal distress during pregnancy, there is upregulation of endoglin in the syncytiotrophoblast (18). Rat and human mesangial cells, which are specialized cells of the kidney that surround the endothelium, also express endoglin (19, 20).

1.2.2 Elucidating the structure of endoglin led to its identification as a TGF-β co-receptor

Endoglin is a type I integral membrane glycoprotein that is primarily expressed as a disulfide-linked homodimer of 180 kDa (21). Human endoglin has a large extracellular domain (561 amino acids), a single transmembrane region (25 amino acids) and a cytoplasmic tail (47 amino acids). The primary amino acid sequences of murine and human endoglin share 72% identity, with the transmembrane and cytoplasmic regions being the most conserved (95%) (7). Although endoglin lacks domains with known enzymatic activity, the cytoplasmic tail is rich in serine and threonine residues regulated by phosphorylation. Two variants of membrane endoglin have been described: a long form (L-endoglin) which has all 47 amino acids in its cytoplasmic tail and a short form endoglin (S-endoglin), which only has a 13 amino acid tail (22). L-endoglin is by far the most predominant, however, increased S-endoglin expression is observed in senescent endothelial cells (23). Also, a 65 kDa soluble form of endoglin (sEng) is released at higher levels from the preeclamptic placenta into maternal circulation and serves as a diagnostic marker for this disease (18).

Although the crystal structure of endoglin remains to be resolved, a 3D reconstruction of the extracellular domain (25 Å resolution) was generated using single particle electron microscopy (24). Using software to predict the 3D structure of endoglin from its sequence, the extracellular domain was shown to consist of three distinct regions: the orphan domain at the N-terminus (a large domain with no significant homology to known proteins/domains) followed by two subdomains of the zona pellucida (ZP) family: ZP-N (at the N-terminal end) and ZP-C (at the C-terminal end).

The sequencing of betaglycan, a known transforming growth factor beta (TGF-β) type III receptor, revealed homology with endoglin and led to its identification as a component of the receptor complex that binds TGF-β1 and TGF-β3 isoforms (25). TGF-β is part of a superfamily of structurally related ligands (33 in mammals), which include TGF-β1, β2 and β3 isoforms,
activins and bone morphogenetic proteins (BMPs), all of which have distinct and pleiotropic functions in multiple cellular processes (26). More recently, the ability of the extracellular domain of endoglin to directly bind to various ligands of the superfamily was tested using surface plasmon resonance (27, 28). While endoglin does not bind directly to TGF-β, it can bind BMP9 and BMP10 on its own and with high affinity. The role of BMP9 in the immune system has not been investigated but it is known that TGF-β plays a major role in inflammation and its resolution. For this reason, my thesis is focused on how endoglin may modulate TGF-β mediated signaling in the context of an inflammatory model.

1.2.3 Role in vascular development and regulation

A) Mutations in endoglin lead to hereditary hemorrhagic telangiectasia

Mutations in the endoglin (ENG) gene lead to the vascular disorder, hereditary hemorrhagic telangiectasia type 1 (HHT1) (29). HHT is an inherited autosomal dominant disorder that occurs in 1/5000 to 1/8000 individuals (30). Mutations at other genetic loci have also been implicated in HHT. Mutations in the ACVRL1 (or ALK1) gene lead to HHT2 while mutations in SMAD4 are observed with HHT in association with juvenile polyposis (JPHT). Two other potential loci have been described: HHT3 (linked to chromosomal region 5q31) and HHT4 (linked to region 7p14). However 80-90% of patients carry mutations in ENG and ACVRL1 genes (31). All types of mutations have been documented in HHT patients (i.e. insertions, deletions, missense, nonsense, and splice) and the underlying cause of disease is haploinsufficiency, implying less functional protein being expressed (32).

Clinically, HHT patients exhibit frequent epistaxis (nosebleeds), gastrointestinal bleeding and in some cases, stroke and internal hemorrhages (30). These symptoms are attributed to the presence of abnormal vascular structures, known as arteriovenous malformations (AVMs), which represent direct connections between arteries in veins. Telangiectasia in mucosae and gut are small AVMs while large AVMs can occur in lungs, brain and liver; these structures are unstable and prone to hemorrhage (33). There are some genotype-phenotype differences in disease manifestations between HHT1 and HHT2 patients; pulmonary and cerebral AVMs are predominant in HHT1 whereas hepatic AVMs are more common in HHT2 (34). The observations that a deficiency in endoglin can lead to such abnormal structures imply that it is an important regulator of angiogenesis and of blood vessel stability and function.
B) Endoglin homozygous null mice have an embryonic lethal phenotype

In order to study the function(s) of endoglin, three independent groups (35-37) generated a homozygous null mouse (Eng<sup>−/−</sup>) model. In all cases, this phenotype is embryonically lethal and with death in utero at E10-10.5 due to substantial cardiovascular defects. Knockout embryos demonstrate growth retardation, internal hemorrhage, and defective heart valve formation. Endoglin was found on the cells that undergo endocardial-mesenchymal transformation, leading to cushion tissue formation resulting in valve development and heart septation, and is therefore an important regulator of early heart development (36).

Although vasculogenesis did occur in Eng<sup>−/−</sup> mice, there was evidence of impaired remodeling in extraembryonic (yolk sac) and embryonic vessels resulting in defective connection between extraembryonic and embryonic circulations. These defects included decreased vessel branching, disorganized enlarged and leaky vessels prone to hemorrhage. In one study, there was evidence of anemia in Eng<sup>−/−</sup> mice (37). Interestingly, impaired vascular smooth muscle cell formation around the vessels was also shown (35). In an inducible endothelial specific knockout model, loss of endoglin led to delayed formation and remodeling of vessels, development of AVMs, hemorrhage and evidence of impaired muscularization of vessels (38). These studies further emphasize endoglin as a key modulator of cardiovascular development.

C) Endoglin heterozygous mice are a model of HHT

Loss of a single ENG copy leads to HHT, and therefore the Endoglin heterozygous (Eng<sup>+/−</sup>) mouse model was next explored in order to study the mechanisms of HHT (36). Eng<sup>+/−</sup> mice show normal embryonic development and are viable. The initial Eng<sup>+/−</sup> mice were of 129/Ola background and developed multiple signs of HHT, including mucocutaneous telangiectasia, epistaxis, gastrointestinal bleeding and internal hemorrhage due to vessel abnormalities in intestine, liver, lungs and brain (39). However, since it was found that the 129/Ola strain is prone to developing abnormal lung and liver vasculature, these mice were subsequently backcrossed to the C57BL/6 strain (40). Early crosses showed a significant decrease in clinical signs of HHT: 7% in C57BL/6 mice compared to 36% in intermediate crosses and 72% in the 129 Ola/strain (41). These findings suggested that modifier genes are involved in regulating the function of endoglin.

In a study comparing the angiogenic response between Eng<sup>+/+</sup> and Eng<sup>+/−</sup> mice, mouse aortic endothelial cells (MAECs) derived from Eng<sup>+/−</sup> mice showed decreased proliferation, migration
and increased collagen synthesis (42). Using an in vitro capillary tube formation assay, Eng\(^{+/−}\) MAECs and human umbilical vein endothelial cells (HUVECs) derived from newborns with ENG mutations showed decreased tube formation, increased luminal area and wall thickness. Using in vivo assays, matrigel plugs containing growth factors and an ischemic injury model, decreased angiogenesis was observed in Eng\(^{+/−}\) mice. Although Eng\(^{+/−}\) mice on the C57BL/6 background do not show a prominent HHT phenotype, injury induced angiogenesis is impaired, suggesting that a second-hit is required to induce disease in a genetically predisposed host expressing a single ENG copy.

Vasomotor tone abnormalities were observed in Eng\(^{+/−}\) C57BL/6 mice, as revealed by impaired myogenic response leading to increased endothelial-dependent vasodilation (43). This impairment was shown to be associated with defective endothelial nitric oxide synthase (eNOS) leading to superoxide rather than NO production (44). The production of reactive oxygen species (ROS) was shown to be higher in several organs of Eng\(^{+/−}\) mice and is likely an important factor in disease initiation. These findings confirm a role for endoglin in regulation of eNOS and vasomotor function.

1.2.4 Modulation of TGF-β superfamily signaling pathways

A) Smad-dependent pathways

Members of the TGF-β superfamily of ligands are known to have distinct and pleiotropic effects on various cellular processes including proliferation, differentiation and apoptosis (45). TGF-β alone has been implicated as an important regulator of many physiological processes, including embryogenesis, organogenesis (i.e. cardiac and bone development), vasculogenesis, wound healing, angiogenesis, hematopoiesis and immune regulation (46). TGF-β superfamily ligands exert their function by binding to heteromeric receptor complexes at the cell surface composed of type I and type II serine/threonine kinase receptors (47). Despite a large number of ligands, there are only 5 known type II receptors and 7 type I receptors (activin-like kinases, ALKs) (26).

The canonical pathway transducing signals for the TGF-β superfamily of ligands is dependent on intracellular SMAD proteins (46). Briefly, the sequence of this signaling cascade is as follows: (1) ligand binding leads to transphosphorylation of the type I receptor by the type II receptor; (2) the type I receptor phosphorylates receptor regulated SMADS (R-SMAD, including
SMADs1, 2, 3, 5 and 8); (3) the R-SMADs form heteromeric complexes with the common-mediator SMAD (Co-SMAD4); (4) this complex translocates into the nucleus and along with other factors regulate transcription of specific genes. In addition, inhibitory SMADS (I-SMAD, 6 and 7) negatively regulate SMAD signaling by competing for Co-SMAD binding or causing degradation of R-SMADs (48).

Since TGF-β can act on endothelial cells in both pro- and anti-angiogenic fashions, the role of endoglin in modulating TGF-β signals has been studied (49). Endoglin is thought to modulate TGF-β signaling by interacting with the ligand binding receptor (TβRII in the case of TGF-β) through its extracellular and cytoplasmic domains (50-52). In most cell types, TGF-β acts through ALK5 and TβRII, however in endothelial cells, it also acts through ALK1 and TβRII (Figure 1.1) (53-56). Signaling through ALK5 leads to SMAD 2/3 activation resulting in transcription of genes involved in vessel maturation such as PAI-1 (plasminogen activator inhibitor 1). Signaling via ALK1, induces phosphorylation of SMAD 1/5/8, which then upregulates expression of pro-angiogenic genes such as ID1 (inhibitors of differentiation and DNA binding 1) (49).

The regulation of endothelial cell proliferation has been examined in multiple studies and conflicting views have emerged. In one study, overexpression of endoglin in cells led to increased TGF-β dependent proliferative responses that were dependent on ALK1 and TβRII (57). In contrast, another study found that endothelial cells lacking endoglin proliferate faster and have enhanced TGF-β mediated anti-proliferative responses (58). There is also evidence that ALK1 signaling is dependent on ALK5 activation (59, 60). These findings show a complex role or endoglin in modulating Smad-dependent pathways.
Figure 1.1 Endoglin modulates distinct TGF-β signaling pathways in endothelial cells

TGF-β1 binds to TβRII, which recruits and phosphorylates type I receptors ALK5 and ALK1. Endoglin binds to TβRII and can modulate both pathways. Activation of ALK5 leads to signaling via SMAD 2/3, interaction with SMAD 4 and translocation into the nucleus to modulate transcription of genes, such as PAI-1. ALK1 signaling leads to activation of SMAD 1/5/8 pathway, which also involves SMAD 4, resulting in transcription of genes, such as Id-1.
B) enOS and other binding partners of endoglin

As mentioned previously, eNOS is responsible for NO production by endothelial cells. Proper activation of eNOS occurs when there is coupling of oxygen to L-arginine metabolism (61). Our laboratory demonstrated previously that endoglin binds to both eNOS and the chaperone Hsp90 and that these interactions are essential for maintaining eNOS in a coupled state (44). In cells deficient in endoglin, these interactions are impaired leading to an uncoupling of the enzyme and generation of superoxide rather than NO. In addition lower eNOS levels are present in \textit{Eng}^{+/−} mice (62).

Direct interaction of the endoglin cytoplasmic domain with scaffolding proteins, such as zyxin, β-arrestin2 and GIPC (GAIP-interacting protein C terminus) has also been documented (63-65). Although interaction with β-arrestin2 does not affect TGF-β mediated Smad1/5/8 or Smad2/3 phosphorylation, β-arrestin2 does negatively regulate Erk1/2 activation (MAP kinase pathway) and subsequently cell migration (64). GIPC can modulate TGF-β mediated Smad1/5/8 phosphorylation and also negatively regulate endothelial cell motility (65). Thus endoglin is critical to maintaining endothelial cell function integrity via direct binding to several cytoplasmic proteins.

1.2.5 Role in the immune system

A) Hematopoiesis

Endoglin (CD105) was first discovered in cells of hematopoietic origin and its potential role in hematopoiesis has been investigated. Within the Flk1\textsuperscript{+} (VEGF receptor II) compartment of cells, which identifies early hematopoietic cells, the CD105\textsuperscript{+} cells were a transient subset with the most hematopoietic potential (66). In vitro differentiation assays using embryonic stem (ES) cells demonstrated that while \textit{Eng}^{+/+} and \textit{Eng}^{+/−} ES cells appear normal, \textit{Eng}^{−/−} ES cells have reduced number of CD45\textsuperscript{+} leukocytes due to decreased myelopoiesis and erythropoiesis. Lymphopoiesis was not defective in \textit{Eng}^{−/−} ES cells.

Endothelial and hematopoietic stem cells are derived from a common but transient precursor called the hemangioblast. The CD105\textsuperscript{+}Flk1\textsuperscript{+} population was found to have the strongest ability to form these hemangioblasts (67). \textit{Eng}^{−/−} (but not \textit{Eng}^{+/−}) ES cells have decreased capacity to generate hemangioblasts and hematopoietic precursors. In addition, \textit{Eng}^{−/−} ES cells show decreased expression of mesoderm and hematopoietic related genes (i.e. PDGFR, Gata2, Gata3,
Gata4, Mesp1, Tal1 and Lmo2), and lower levels of Smad1 and phosphorylated-Smad1 (68). Interestingly, introduction of a constitutively active form of Alk1 in Eng$^{+/−}$ ES cells caused increased hemangioblast differentiation whereas constitutively active Alk5 caused a further decrease. Overexpression of endoglin in ES cells led to increased hemangioblast production with more CD105$^{+}$Flk1$^{+}$ precursors and increased erythropoiesis (69). As the first appearance of hematopoiesis occurs at E7.0-7.5, the role of endoglin in early specification of hematopoietic lineages was further explored in isolated cells from E7.5 embryos (70). CD105$^{+}$Flk1$^{+}$ cells from embryos show early hematopoietic and endothelial markers (i.e. VE-cadherin and CD31, CD41, c-Kit) whereas CD105$^{+}$Flk1$^{−}$ cells harbor more markers of erythroid precursors (CD71). Altogether, these findings suggest a crucial role for endoglin as a regulator of early hematopoiesis, specifically myelopoiesis and erythropoiesis, and of endothelial progenitors by potentially modulating TGF-β effects on hematopoiesis.

In isolated adult murine bone marrow cells, knockdown of endoglin leads to some impairment of TGF-β mediated suppression of proliferation (71). Utilizing bone marrow reconstitution assays, knockdown or overexpression of endoglin in donor bone marrow did not alter overall T, B or myeloid cell reconstitution in recipient mice; however, some changes in erythrocyte precursor numbers were observed. Finally, a study to determine markers of early megakaryocyte precursors, found that within the mouse bone marrow hematopoietic progenitor population (Lin$^{−}$Sca1$^{−}$Kit$^{+}$): $\text{CD150}^{+}\text{CD9}^{lo}\text{CD105}^{hi}$ cells had mostly erythroid potential, the $\text{CD150}^{+}\text{CD9}^{hi}\text{CD105}^{lo}$ contained mainly megakaryocyte progenitors and the $\text{CD150}^{+}\text{CD9}^{lo}\text{CD105}^{lo}$ identified bipotential erythroid megakaryocyte precursors (72). These findings indicate that while endoglin is an important regulator of early hematopoiesis, it may be primarily involved in erythropoiesis and potentially in the development of megakaryocytes in adult bone marrow.

**B) Immune responses**

In terms of function of endoglin in immune responses, an experimental coronary heart ligation-induced myocardial infarction model showed increased heart pathology associated with decreased microvessels in Eng$^{+/−}$ mice compared to Eng$^{+/+}$ mice (73). Since blood mononuclear cells (MNCs), which include endothelial progenitors, circulating endothelial cells and bone marrow-derived monocytic cells, migrate to the site of injury to aid in new vessel growth, the recruitment of HHT1 patient derived and control MNCs was assessed. Treatment of Eng$^{+/−}$ mice
with control MNCs improved the heart pathology whereas treatment of Eng⁺/⁻ mice with MNCs derived from patients with HHT1 showed impaired neoangiogenesis and decreased recruitment to the infarct site. As a follow-up to this study, the total number of CD14⁺ monocytes in HHT1 peripheral blood was not altered relative to controls but the total number of CD34⁺ cells was higher in HHT1 patients (74). SDF1-α (CXCL12), a chemoattractant that binds to CXCR4, is important for chemotaxis of MNCs (75). Although the expression of CXCR4 was elevated in HHT1 MNCs, the expression of a negative regulator of CXCL12-CXCR4 interaction, CD26, was also upregulated on HHT1 MNCs (76). The motility of HHT1 MNCS towards CXCL12 was reduced; however, inhibition of CD26 with Diprotin-A improved migration of these cells. In addition, pre-treatment of HHT1 MNCs with Diprotin-A was able to improve the recruitment of cells in the same myocardial infarction model. Given that endoglin may be important in myelopoiesis and is expressed by activated monocytes, these findings constitute the first evidence of a function for endoglin in regulating an immune response.

1.3 Inflammation and its resolution

1.3.1 Cellular and molecular components of inflammation

A) Acute inflammation

The primary purpose of the inflammatory response is to reinstate homeostasis by protecting the host from spread of infectious pathogens and ensuring proper restoration of tissue structure and function (1). Studies of the mechanisms that govern inflammation have identified this response as complex, specific and involving a diverse array of cellular and molecular mediators (2-4, 77, 78). In most cases, there are 4 major components in inflammation: (1) inducers (i.e. bacterial pathogen-associated molecular patterns, virulence factors and allergens), (2) sensors (i.e. innate pathogen-recognition receptors such as TLRs and NLRs), (3) mediators (i.e. chemokines/cytokines and organ-specific factors), (4) effectors (i.e. leukocytes and endothelial cells) (78). The type of inducers, sensors and mediators can vary and as a result elicit a number of effectors; for instance, viral infections typically induce type I interferon Th1 responses whereas parasites skew toward a Th2 response (IL-4, IL-5 and IL-13). As effector molecules that eliminate pathogens can be toxic to host cells, inflammation should ideally be short-lived (acute) and resolved as soon as infectious agents are removed (4).
In general, the sequence of acute inflammation, usually triggered by infection or injury, is as follows: (1) activation of innate receptors on local cells (i.e. resident macrophages, mast cells, epithelial cells, iNKT cells), (2) production of cytokines/chemokines, pro-inflammatory lipid mediators (i.e. leukotrienes and prostaglandins) and vasoactive compounds, (3) activation of endothelial cells leading to vasodilation, increased permeability to allow plasma components to site (antibodies and complement proteins) and extravasation of leukocytes (primarily neutrophils, monocytes and NK cells), (4) production of effector molecules (i.e. ROS) and (5) resolution of inflammation after clearance of pathogenic organisms in order to inactivate immune cells and induce repair of tissue (i.e. inhibition by TGF-β and lipid-based resolving factors such as resolvins and protectins) (2, 77).

B) Chronic inflammation

If the acute response fails to remove the pathogen, there is recruitment of additional leukocytes, including monocytes and lymphocytes, resulting in development of persistent inflammation (3). While chronic inflammation can result from improper clearance of infections, other triggers have been implicated, most notably autoimmune responses and allergens. Many chronic inflammatory diseases, such as IBD and multiple sclerosis, involve both genetic and environmental factors leading to dysregulated and sustained immune responses (4). Although the types of immune cells involved can vary depending on the disorder (i.e. Th1, Th2 and Th17), hallmarks of chronic inflammation also include the presence of granulomas (i.e. aggregation of macrophages), development of tertiary lymphoid tissue and co-existence of acute phase and chronic phase cells, implying continual re-initiation of inflammation (77). Upregulation of pro-inflammatory factors (i.e. IL-6, TNF-α) and downregulation of anti-inflammatory factors (i.e. IL-10) have also been observed in the development of chronic inflammation. Exacerbation of these responses contributes to continual damage and altered structure of the involved tissue resulting in disruption of normal functions.

1.3.2 Role of TGF-β in resolution of inflammation

TGF-β is a key regulator of immune responses that has been implicated in many chronic inflammatory disorders (79). Knockout of both copies of Tgfb1 (β1 isoform) leads to a multi-organ autoimmune response (80). As TGF-β has been identified as potent inhibitor of immune activation in most leukocyte subsets, it is considered a key regulator of inflammation resolution (81). The role of TGF-β in dampening T cell responses has been extensively studied and a
number of mechanisms have been identified: (1) inhibition of naïve T cell proliferation (i.e. through downregulation of IL-2), (2) inhibition of Th1 and Th2 differentiation and subsequent cytokine production, (3) inhibition of CD8+ CTL differentiation and proliferation, (4) promotion of T-regulatory (T-reg) cell development and (5) regulation of Th17 differentiation (82).

Similarly, in B cells, TGF-β can inhibit activation and proliferation of progenitor and mature B cells, promote IgA class switching, attenuate B cell response to low-affinity antigens and regulate B cell tolerance (83).

TGF-β is a potent chemoattractant and activator of monocytes by increasing adhesion molecule expression and cytokine production; however, once monocytes differentiate into macrophages, TGF-β inhibits their cytokine and ROS production (79). In granulocytes, TGF-β can also mediate recruitment to sites of inflammation and regulate the function of these cells (84, 85). TGF-β is also important in the wound healing process of inflammation as it stimulates fibrosis by recruiting and activating fibroblasts (production of extracellular matrix components collagen and fibronectin) and promoting epithelial cell proliferation (86-88). Interestingly, while non-immune cells produce TGF-β, many leukocytes secrete TGF-β upon activation in order to initiate resolution and healing processes (83).

1.3.3 Activation of endothelial cells and immune-driven angiogenesis

A) Leukocyte adhesion cascade

Endothelial cells have an active and prominent role in inflammatory responses (89). During inflammation, local innate cells release cytokines (i.e. IL-1β and TNF-α) and vasoactive compounds that induce activation of endothelial cells by increasing permeability, upregulating adhesion molecule expression (i.e. VCAM1 and MADCAM1) and secretion of chemokines (i.e. CXCL8, CCL2, CXCL1) (90). Endothelial cells immobilize these chemokines on their surface via glycosaminoglycans to prevent dispersion (91). As the type of infectious agents can vary and as a result require different inflammatory mediators, the type of chemokines and adhesion molecules upregulated on the endothelium determines the specific leukocyte subsets that are recruited (92). In addition, endothelial cells can also be directly activated by bacterial molecules through expression of innate receptors such as TLRs and NLRs (93).

Once endothelial cells are activated, a specific sequence of events termed the leukocyte adhesion cascade is triggered: capture/rolling of leukocytes on the endothelium, firm adhesion
and transmigration/extravasation (90). For each step, specific classes of adhesion molecules are required: (1) rolling is largely mediated by selectins (L-selectins on most leukocytes and P- and E-selectins on endothelial cells), but leukocyte integrins ($\alpha_4\beta_1$) and endothelial Ig-superfamily adhesion molecules (VCAM1) are also involved, (2) leukocyte arrest is triggered by chemokine binding to leukocytes, resulting in higher affinity binding of their integrins to endothelial cells (i.e. LFA1 to ICAM1 and $\alpha_4\beta_7$ integrin to MADCAM1) and (3) transmigration/extravasation of leukocytes through the endothelium via either paracellular route which involves redistribution of junctional molecules such as ICAM1 and JAM-A, or transcellular route which requires formation of vesiculo-vacuolar organelles and is dependent on $Ca^{2+}$ and SNARE-complexes (94, 95).

B) Immune-driven angiogenesis

Besides the role of endothelium in leukocyte activation, it has long been known that there is an important co-dependence between inflammation and angiogenesis (growth of new blood vessels from pre-existing vessels) (96). Angiogenesis is a highly coordinated event involving several cell types and tightly regulated by a careful balance of pro- and anti-angiogenic (angiostatic) factors that occurs during physiological processes such as wound healing and the menstrual cycle (97). Abnormal expansion of the microvasculature under inflammatory conditions, termed pathological angiogenesis, has been identified as a contributing factor to the exacerbation of inflammation as it provides inflammatory cells more access to the nutrients and oxygen required to sustain inflammation (98). This pathological angiogenesis has been observed in chronic inflammatory diseases, including IBD (99). Angiogenesis in chronic inflammation can be attributed to production of pro-angiogenic factors (i.e. VEGFA, PDGF, bFGF) by immune cells, such as neutrophils and macrophages, in a specialized process that is called immune-drive angiogenesis. In addition, a number of pro-inflammatory cytokines, including IL-1$\beta$ and TNF-$\alpha$, have demonstrated pro-angiogenic effects. Figure 1.2 illustrates the several factors that are produced by infiltrating leukocytes that mediate immune-driven angiogenesis.
Chronic gut inflammation is marked by increased immune cell infiltration in the lamina propria, releasing cytokines and growth factors providing an endothelium-activating and pro-angiogenic environment. Macrophages (MΦ), neutrophils (light purple), other antigen-presenting cells (APC) and T cells (red) are emerging as key sources of angiogenic factors and angiogenesis-modulating cytokines. In addition, gut epithelium (row of cells), and endothelium (lining the lumen of the vessel below), secrete additional mediators. ROS/NO production also facilitates the release of angiogenic factors. *Adapted from Chidlow et al, Am J Physiol Gastrointest Liver Physiol 293:G5-G18, 2007 (99).*
1.3.4 Inflammatory bowel disease and the DSS experimental model

A) Inflammatory bowel disease

IBD is a chronic inflammatory disorder that can affect the entire mucosal tissue of the gastrointestinal tract (GI) with two major clinically defined forms: Crohn’s disease (CD) and ulcerative colitis (UC) (100). CD can affect any point on the GI tract, with the terminal ileum being the most common, patchy and segmental in comparison to UC, which mainly affects the rectum. Symptoms of IBD include chronically relapsing flares, diarrhea, abdominal pain and rectal bleeding. Although there is high concordance in monozygotic twins in CD compared to UC, IBD is considered to be a complex multifactorial disorder that is mediated by the interaction of several factors including genetic susceptibility, intestinal microbiota, other environmental factors, dysregulated immune responses and more recently pathological angiogenesis (101, 102). As a result, IBD is thought to be an inappropriate immune response to microbiota in genetically susceptible hosts.

In terms of implicated genes, many genome-wide association studies of human IBD have identified multiple genetic loci that contribute to the disease. These genes can regulate a vast array of responses, including barrier function, innate and adaptive immune pathways (i.e. NOD2, IL-23, IL-6, IL-17, IL-12, IL-10), ROS generation, autophagy, ER stress and metabolic pathways (101, 103, 104). A multitude of animal models have been developed in order to understand disease mechanisms, including genetic (i.e Il10 KO mice) or chemically induced models as seen with the dextran sulfate sodium (DSS) experimental colitis (105). These models have implicated multiple cellular and molecular mediators of IBD (i.e. neutrophils, monocytes/macrophages, Th1 cells, Th2 cells, Th17 cells, T-regs, IL-6, IL-12p40, IL-23 and TGF-β) (106).

B) The DSS model of IBD

The DSS mouse model was first developed by Okayasu et al. (1990) (107). The addition of DSS to the drinking water results in disruption of the gut epithelium leading to a robust inflammatory response accompanied by weight loss, diarrhea, blood in the stools and mortality. Acute and chronic colitis have been observed in the DSS model by varying the concentration, duration and frequency of DSS administration (108). Acute colitis usually is induced by administration of 2-5% of DSS for 4-9 days, whereas chronic disease can be induced by continual low dose or cyclical administration. The colonic structure of DSS-treated mice is drastically altered, with shortening of length, epithelial damage and ulceration, increased
polymorphonuclear cells in lamina propria and submucosa, cryptitis and crypt abscesses (109). The DSS model has been utilized in various genetically modified mice (i.e. TLR KO mice) to understand IBD mechanisms as well as to test novel therapeutic agents (110).

As athymic, SCID and SCID/NK-depleted mice are still susceptible to DSS-treatment, this experimental model is considered largely mediated by an innate immune response (111). While BALB/c mice show an attenuated colitis response, the C57BL/6 background is considered a robust responder to DSS-induced colitis after a single challenge with increased neutrophils, T and B cells in the colon of colitic mice (112, 113). In a study comparing single challenge DSS regiment (acute) and multiple challenge DSS regiment (chronic), Th1-Th17 cytokines (IL-6, IL-17, TNF-α and CXCL1) were predominant serum factors as opposed to Th2 cytokines (IL-6, IFNγ, IL-4 and IL-10) in the chronic disease (114). In addition, DSS-treatment of mice overexpressing a potent pro-angiogenic factor, vascular endothelial growth factor A (VEGF-A), results in exacerbated colitis with pathological angiogenesis, and so the DSS model can be utilized to study inflammatory and angiogenic responses in IBD (115).

1.3.5 DSS-treated Eng+/− mouse model

In order to study the potential contribution of endoglin to inflammatory responses, Eng+/− and control Eng+/+ mice were challenged with DSS (116). Mice were given a 5-day course of 3% DSS and were monitored up to 26 days for weight loss, diarrhea and fecal blood. Weight loss began around day 5, with days 7-9 representing the peak of disease in both groups of mice; however, Eng+/− mice demonstrated a slower rate of recovery than control mice. Similarly, diarrhea and fecal blood levels were most pronounced at day 7 in both groups, however, by days 18-26, Eng+/− mice presented worse clinical symptoms of colitis than their counterparts. Histological examination of the colon revealed the distal colon to be the most affected in both genotypes followed by the cecum and then the transverse colon. At day 9, leukocyte infiltration and massive crypt damage was maximal in both groups, however, by days 18-26 there was resolution of inflammation in Eng+/+ mice and persistent disease in Eng+/− mice (Figure 1.3). Formation of lymphoid follicles at epithelial abscesses, where direct contact with antigen occurs, was also noted in both groups of colitic mice. In addition, Eng+/− mice presented signs of pathological angiogenesis at days 18-26 due to increased vessel numbers and elevated VEGF-A protein and mRNA levels.
Figure 1.3 Persistent inflammation and pathological angiogenesis in the distal colon of DSS-challenged Eng^{+/−} mice
Images taken at day 26 of the DSS colitis course in both Eng^{+/+} and Eng^{+/−} mice illustrate massive infiltration by leukocytes (black arrows) and incomplete crypt regeneration in the Eng^{+/−} mice whereas Eng^{+/+} mice show minimal signs of inflammation. There is increased thickness of the mucosal, submucosal, muscular and particularly serosal layers where vessels (v) are numerous and often dilated. Both images are at the same magnification (Bar = 100 mm).
1.4 Inflammation and its resolution

1.4.1 Rationale

Endoglin is a key modulator of angiogenesis and endothelial function via either potentiation or inhibition of TGF-β superfamily responses. While there are some indications of endoglin modulation of immune responses, its specific role and the mechanisms involved remain to be resolved. Leukocytes and endothelial cells are important and play interdependent roles in inflammation. Therefore it is necessary to establish if endoglin regulates both endothelial and immune cell mediated responses. The DSS model of IBD was chosen to study the function of endoglin in inflammation as it has been shown to engage both immune and vascular systems. Indeed, DSS-treated $Eng^{+/−}$ mice present signs of persistent disease and pathological angiogenesis suggesting a regulatory role for endoglin in inflammation.

1.4.2 Hypothesis

The development of persistent inflammation in DSS-treated $Eng^{+/−}$ mice is due to underlying immune and angiogenic defects.

1.4.3 Specific Objectives

(I) To analyze the number and distribution of subsets of leukocytes by flow cytometry under basal and inflammatory conditions in the gut lamina propria, spleen and bone marrow of $Eng^{+/−}$ congeneric versus control mice.

(II) To measure mRNA and protein levels of angiogenic/inflammatory factors in the distal colon of DSS-treated $Eng^{+/−}$ and control littermates.

(III) To test for functional defects in potentially altered immune subsets in $Eng^{+/−}$ mice.
Chapter 2
Materials and Methods

2 Materials and Methods

2.1 Mice

Congenic 14–15-week-old \textit{Eng}^{+/−} mice on the C57BL/6 background and control \textit{Eng}^{++} littermates were used. \textit{Eng}^{+/−} mice were first generated by homologous recombination using embryonic stem cells of 129/Ola origin, and then backcrossed to C57BL/6 (36, 41). Mice were housed in a specific pathogen-free facility. All protocols were approved by the Animal Care Committee at the Toronto Centre for Phenogenomics, in accordance with the Canadian Council on Animal Care. The genotype of each mouse was assessed at weaning time by β-galactosidase staining of an ear punch and subsequently confirmed by polymerase chain reaction (PCR) using tail DNA (39).

2.2 DSS-induced colitis

As previously established (116), mice drank acidified water supplemented with 3% DSS (DSS, m.w. 36,000–50,000; MP Biomedicals) for 5 days and were then returned to acidified water. Mice were examined daily for up to 22 days for body weight, water intake, activity, fur appearance, and diarrhea as described before (116). Mice were sacrificed at days 0 (prior to DSS) 7-9 and 18-22. At time of sacrifice, mice were anesthetized with ketamine (100 mg/kg intraperitoneally, i.p.) and xylazine (10 mg/kg i.p.), perfused with 10 mL of phosphate-buffered saline (PBS) through the left ventricle and the colon was removed, cut longitudinally and feces were gently scraped. For cell isolation, colons were immediately placed on ice in PBS (with 2% fetal bovine serum, FBS) and for RNA and protein expression, the distal portion of the colon was placed in biopsy cassettes and frozen in liquid nitrogen. Spleens, femurs and tibiae were also harvested for leukocyte isolation.
2.3 Isolation of colonic lamina propria, splenocytes and bone marrow leukocytes

2.3.1 Lamina propria

The isolation protocol for colonic lamina propria cells was adapted from previously established protocols (117, 118). For each experiment, the isolated colons were pooled, 4 colons/genotype at day 0 and 3 colons/genotype for colitic mice. Each colon was cut into small pieces and briefly washed in media [Roswell Park Memorial Institute medium (RMPI) 1640 supplemented with 10% FBS, sodium pyruvate, 20mM HEPES, non-essential amino acids, penicillin-streptomycin and β-mercaptoethanol]. Epithelial cells were removed by incubating the tissue in PBS containing 1% FBS, 5mM EDTA, 1mM DTT and penicillin-streptomycin with vigorous shaking at 37°C for 10 minutes. This step was repeated twice more and at the end of each incubation step, the tissue was strained on a 100 µm mesh and added to fresh solution. All tissue was washed again in media to remove residual EDTA and DTT and was digested in media (without β-mercaptoethanol), supplemented with 5mM Ca^{2+}, 2mg/mL of collagenase D (Roche Diagnostics) and 20µg/mL of DNase I (Roche Diagnostics), with shaking at 37°C for 60 minutes. The digestion solution containing the colonic tissue was first strained through a 100 µm mesh and the filtrate was spun at 500 x g for 5 minutes at 4°C. The pellet was resuspended in media and then passed through 70 µm and 40 µm strainers to generate a single cell suspension. The number of living lamina propria cells were counted using trypan blue (Life technologies). The isolated cells were spun and resuspended in PBS with 2% FBS, 0.1% sodium azide and 10mM HEPES (SB buffer) and kept on ice.

2.3.2 Spleen

The spleen was placed on a 100 mm petri dish in SB buffer and mashed using a syringe plunger. The filtrate was passed through a 70 µm strainer, spun at 500 x g for 5 minutes at 4°C. Red blood cells (RBC) were lysed using RBC lysis buffer (eBioscience) for 5 minutes at room temperature and the reaction was stopped by adding PBS. The cells were spun, resuspended in SB buffer, passed through a 40 µm strainer and counted.
2.3.3 Bone marrow

The femurs and tibias from individual mice were flushed with alpha-Minimum Essential Medium into a petri dish using a 10 mL syringe with a 25 5/8-gauge needle. The marrow aggregates were dissociated by filtering through a 20-gauge needle and a 70 µm strainer and centrifuged at 500 x g for 5 min at 4°C. After RBC lysis, a single cell suspension in SB buffer was prepared and the live cells were counted.

2.4 Multi-color flow cytometry analysis

The single cell suspensions were seeded at 10^6 cells/well in a conical-bottom plate, spun at 500 x g for 5 min at 4°C and stained using the LIVE/DEAD fixable violet dead cell stain kit (Life technologies). The Fc receptors were then blocked using anti-mouse CD16/CD32 (eBioscience) at a concentration of 1 µg/10^6 cells and the cells were stained with various combinations of fluorochrome-conjugated antibodies against surface antigens of mouse B, T and myeloid cells. There were 4 different staining panels, with PE-Texas Red anti-CD45 (Life technologies) present in all panels: (1) B cell panel (Alexa Fluor 700 anti-CD19, Allophycocyanin anti-CD5, Alexa Fluor 488 anti-CD1d, eBioscience; Allophycocyanin-Cyanine 7 anti-IgM, Biolegend) (119), (2) total T cell panel (Allophycocyanin anti-CD3, eBioscience; Fluorescein isothiocyanate anti-CD4, Phycoerythrin anti-CD8, BD Biosciences) (113), (3) T-regulatory cells (Mouse regulatory T cell staining kit #1 containing Fluorescein isothiocyanate anti-CD4, Allophycocyanin anti-CD25, Phycoerythrin anti-Foxp3, eBioscience) and (4) myeloid cells (Peridinin Chlorophyll Protein Complex Cyanine 5.5 anti-CD11b, Phycoerythrin - Cyanine 7 anti-F480, eBioscience; Alexa Fluor 700 anti-Ly6C, Fluorescein isothiocyanate anti-Ly6G, BD Biosciences) (120-122).

Fluorescence-minus-one controls were utilized to determine gate positions during analysis and for compensation controls, the anti-rat/hamster Ig κ Compensation Particles Set (BD Biosciences) was used. The samples were next fixed in 2% paraformaldehyde for 10 minutes for analysis on the LSRII flow cytometer (BD Biosciences). All data was analyzed using the FlowJo software (Tree Star Inc.) and subset distribution (expressed as a percentage of total CD45^+ leukocytes) was determined. An example of myeloid cell analysis in the spleen is shown in Figure 2.1. Using these percentages and the live cell counts per mouse obtained after isolation, the number of cells/mouse in each of these subsets was calculated.
Figure 2.1 Flow cytometry analysis of myeloid subsets in the spleen of a DSS day 22 Eng+/+ mouse

The living cells are first gated using the LIVE/DEAD fixable violet stain to eliminate dead cells. Within the live compartment, the CD45+ leukocytes are gated followed by identification of CD11b+ myeloid cells. The myeloid compartment is then subdivided into F480+ and F480− cells.

While some cells within F480+ cells were Ly6G+, which likely belong to an undifferentiated subset, the rest of these F480+ cells are considered to be macrophages. In the F480− compartment, monocytes (Ly6C+Ly6G−) and neutrophils (Ly6C+/Ly6G+) were found.
2.5 Differential blood counts

50-60 µL of blood was collected from the saphenous vein of each mouse for hematological analysis using 200 µL EDTA-coated capillary tubes (Drummond Scientific). The blood was transferred into a 0.5 mL tube and gently mixed to prevent coagulation. The differential count was determined using a Multispecies Hematology Analyzer, the Hemavat 950FS Hematology Analyzer (Drew Scientific Group), to measure the following parameters: RBC count, hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, platelet/thrombocyte count and white blood cells (neutrophils, lymphocytes, monocytes, basophils and eosinophils).

2.6 RNA isolation and PCR array

In order to assess for differential mRNA expression profiles between Eng^{+/+} and Eng^{+/−} mice, total RNA was extracted from ~25-30 mg of distal colonic tissue using TRIzol reagent (Life technologies) according to the manufacturer’s protocol. The RNA was further cleaned up using the Qiagen RNeasy Mini Kit with an on-column DNase I treatment (Qiagen). The quality of the extracted RNA was assessed by the Agilent 2100 bioanalyzer (Agilent Technologies) and samples with RNA integrity numbers between 9-10 were used for expression analysis.

The RT^2 Profiler PCR Arrays (Qiagen) enable simultaneous analysis of mRNA expression of multiple genes using a Real-time PCR based method. Two different arrays, the mouse angiogenesis array (Cat. No. PAMM-024A) and the TGF-β/BMP signaling pathway array (Cat. No. PAMM-035A), were used to profile mRNA expression levels of genes within the distal colons of days 18-21 mice. Each array contained 84 different pathway-related genes as well as 5 housekeeping genes: (i.e. GAPDH), a genomic DNA contamination control, reverse transcription controls and positive PCR controls. Briefly, the RNA samples were first converted to first strand cDNA using the RT^2 First Strand Kit and then mixed with the RT^2 qPCR Master Mix, which enables SYBR Green real-time PCR detection. As one plate represents a single sample, the mixture is added into all wells of the plate, with each well containing pre-dispensed gene specific primer sets. The plates were run on the Standard 7500 Real Time PCR system from Applied Biosystems and the results are expressed as an Eng^{+/−}/Eng^{+/+} fold-change.
2.7 Protein extraction from colonic and bone marrow tissues

Total proteins from the distal colonic tissue were extracted by homogenization of frozen tissue (~25-30 mg) in a lysis solution (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 1 mM EDTA, 1% Triton X-100) supplemented with complete protease inhibitors (Fermentas). The homogenates were rotated at 4°C for 30 minutes and centrifuged at 15,800 x g for 20 minutes and the supernatants collected. The protein concentration was measured using the Bio-Rad protein assay (Bio-Rad Laboratories). The protein extracts of isolated bone marrow cells, as described in section 1.3.3, were obtained by using the CelLytic mammalian tissue cell lysis reagent (Sigma-Aldrich) supplemented with protease inhibitors according to the protocol provided. Protein concentrations were obtained as described for colonic tissue.

2.8 Cytokine/Chemokine profiles

The protein levels of 14 cytokines/chemokines (IL-12p40, IL-1β, IL-6, IL-10, CXCL1, CCL11, IL-17, IL-4, IFNγ, TNF-α, GM-CSF, M-CSF, IL-12p70 & G-CSF) were measured in distal colon using a Milliplex multi-analyte profiling magnetic bead based immunoassay kit (specifically the Mouse Cytokine/Chemokine Panel I, EMD Millipore). Using distinctly color-coded microspheres/beads, each coated with a specific capture antibody (i.e. one bead type for each cytokine), this assay allows quantitative measurement of multiple analytes within a single sample. Prior to addition of the bead mixture, the protein extracts from frozen distal colonic tissue were first diluted 8x to decrease interference by detergent. After incubating the lysates with the beads for 16-18 hours with shaking, biotinylated detection antibody are added and mixed for 60 minutes followed by addition of a Streptavidin-Phycoerythrin conjugate, the reporter molecule. Using the Luminex 200 instrument (Luminex Corporation), the beads first pass through a laser that excites the internal dyes to identify the specific microsphere (i.e. IL-6) and then through a second laser that excites PE to quantify the amount of the factor. The results are presented as picograms (pg) of analyte per milligram (mg) of tissue.
2.9 Myeloperoxidase assays

2.9.1 MPO positive cells

Distal colon samples were frozen using OCT and sectioned longitudinally. In order to determine the number of MPO\(^+\) cells, the sections were stained with 0.05% Diaminobenzidine with 1.5% hydrogen peroxide (\(\text{H}_2\text{O}_2\)) for 30 minutes and then counterstained with Mayer’s hematoxylin. Slides were later analyzed using Olympus BX50 microscope, Leica DFC 300F camera, and “OpenLab” software, quantifying 10 fields per slide at 400 x magnifications.

2.9.2 MPO enzymatic activity

The activity of MPO in distal colon was measured using previously established protocols (123). Briefly, frozen tissue was homogenized in hexadecyltrimethylammonium bromide buffer to release MPO from the tissue. The homogenates were centrifuged at 19,000 x \(g\) at 4°C for 15 minutes and the supernatant added to PBS containing a known amount of \(\text{H}_2\text{O}_2\) and a hydrogen donor, o-dianisidine dihydrochloride. The rate of change in absorbance at 450 nm over a 90 second period was determined and one unit of MPO activity was defined as that degrading one micromole of hydrogen peroxide per minute at 25°C. The values are expressed as units (U) of MPO activity per mg of tissue.

2.10 Western blots

Distal colon and bone marrow lysates, as prepared in section 2.7, were incubated in Laemmli buffer at 95°C for 5 minutes and electrophoresed on 4%–12% gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis gels (Life Technologies). The proteins were then electrotransferred and the Polyvinylidene fluoride membranes blocked with 5% milk in TBS-T (20mM Tris, pH 7.6, 137 mM NaCl, 0.1% Tween-20) for 1 hour at 25°C. The blots were incubated overnight at 4°C with the following antibodies: MPO (mouse IgG\(_{2B}\), 1:1000; R&D Systems, McKinley Place, MN), Nox2 (gp91phox/Nox2, mouse IgG1, 1:1000; BD Biosciences, Mississauga, ON) or \(\beta\)-actin (mouse IgG1, 1:10,000; Sigma-Aldrich, Oakville, ON). After washing with TBS-T for 30 minutes, blots were incubated for 60 minutes with appropriate secondary antibodies (IgGs conjugated with horseradish peroxidise, 1:10,000). The enhanced chemiluminescence reagent (Perkin Elmer, Shelton, CT) was used for detection. Band intensities were quantified and expressed relative to \(\beta\)-actin.
2.11 Superoxide and H₂O₂ assays

2.11.1 H₂O₂ assay in colonic samples

H₂O₂ levels were measured using Amplex Red assay (Molecular Probes) in accordance to manufacturer’s instructions and as previously described (124). Distal colon and cecum were carefully dissected from control or DSS-treated mice, flushed with PBS and homogenized in 25 mM HEPES buffer, pH 7.4, plus 1 mM EDTA and protease inhibitors. After centrifugation at 6000 x g for 5 minutes at 4°C, the supernatants were incubated in a microtiter plate at 37°C for 1 hour with Amplex red, horseradish peroxidase and L-Arginine (NOS substrate, 1 mM, Sigma-Aldrich, Oakville, ON). The effects of 100 µM Apocynin (NADPH oxidase inhibitor, Sigma-Aldrich) on the reaction were tested. Fluorescence was quantified using 544 nm excitation and 590 nm emission and levels normalized for protein content.

2.11.2 Superoxide production by isolated bone marrow neutrophils

Bone marrow neutrophils were isolated using a previously established protocol (125). As described before, after flushing the bone marrow of femurs and tibias (pooled bones from 3 mice/genotype) with alpha-Minimum Essential Medium and disaggregation with a 20-gauge needle, the cell suspension was centrifuged at 1000 x g for 5 minutes at 4°C and the pellet was resuspended in 3 mL of α-MEM. The cells were layered onto a discontinuous Percoll gradient (Sigma-Aldrich) of 82%/65%/55% and spun at 700 x g for 30 min at 4°C. Mature neutrophils were collected at the 82%/65% interface. The neutrophils were incubated with 5 µM Dihydroethidium (DHE, Life Technologies) for 15 minutes, which upon reaction with superoxide anions forms ethidium and intercalates with DNA (126), with or without phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) stimulation (30 minutes post DHE incubation). The number of fluorescent nuclei is quantified using the CY3 fluorescence channel of the NIKON Cellomics ArrayScan® VTI HCS Reader (Thermo Scientific). The results are represented as a fold-change over the number of fluorescent cells without PMA stimulation.

2.12 Statistical analysis

Parametric data was assessed using the Student’s t-test or one-way ANOVA followed by multiple comparisons using Tukey’s post hoc testing. Non-parametric data was analyzed using the Mann-Whitney test or the Kruskal-Wallis Test with pairwise comparisons. For the colonic
H$_2$O$_2$ measurement results, the paired t-test was utilized. Statistical significance was accepted at $P < 0.05$. All statistical analysis, with the exception of the PCR array data, was performed using the IBM Statistical Package for the Social Sciences software. The PCR array data was analyzed using an Excel-based PCR array data analysis template provided by Qiagen. Data are presented as means ± SEM, with the exception of the PCR array data and the DHE assay results, which are expressed as fold-changes.
3 Results

3.1 *Eng*<sup>+/−</sup> mice exhibit delayed recovery when challenged with the DSS model of IBD

Previously, we demonstrated that *Eng*<sup>++</sup> mice subjected to the DSS colitis model develop persistent clinical and histopathological signs of disease, while *Eng*<sup>+/−</sup> mice recover (116). In all DSS-induced colitis experiments conducted in this study, *Eng*<sup>+/−</sup> and *Eng*<sup>+/+</sup> mice received 3% DSS in drinking water for 5 days and were then returned to normal water. They were monitored daily for clinical signs of disease, including diarrhea, blood in the stools and weight changes, to determine if the animals followed the previously observed course of disease. Figure 3.1 illustrates the change in body weight observed during my experiments. Weight loss began at day 4 and at the same rate in both *Eng*<sup>+/−</sup> and *Eng*<sup>+/+</sup> mice until day 9, with days 7-9 representing the peak of disease. At day 10, both groups of mice began regaining body weight, however, *Eng*<sup>+/−</sup> mice demonstrated a delay in recovery rate, which was significantly different from that of *Eng*<sup>+/+</sup> during days 13-17.

3.2 *Eng*<sup>+/+</sup> and *Eng*<sup>+/−</sup> mice show increased gut leukocyte numbers during colitis

Previously reported characterization by confocal microscopy of leukocytes in the colon of DSS treated mice followed until day 33, revealed increased numbers of B, T and myeloid subsets after colitis induction (113). In order to study potential alterations in immune subsets in colitic *Eng*<sup>+/−</sup> mice, the distribution (expressed as percentage of CD45<sup>+</sup> leukocytes) of total T, B and myeloid cells in isolated colonic lamina propria cells was assessed by flow cytometry prior to DSS administration (day 0) and during colitis (days 7-9 and 18-22).

The distribution of total B (CD19<sup>+</sup>) lymphocytes, a major gut immune subset estimated at 26-35% of leukocytes, was unchanged after colitis induction and between genotypes (Table 3.1). However, at days 18-22 of colitis, there was a significant increase in the total number of B cells (0.4-0.5 x 10<sup>6</sup> cells to 2.3-2.5 x 10<sup>6</sup> cells) in both *Eng*<sup>+/+</sup> and *Eng*<sup>+/−</sup> mice (Table 3.2). We also
used additional B cell markers to differentiate between B1 and B2 cells (119). Under basal and inflammatory conditions, all CD19+ cells were CD5−, indicating that these cells were likely of B2 origin. While the majority of CD19+ cells were IgM+ in both Eng+/+ and Eng−/− mice, there was some increase in IgM+ cells with colitis, 70-84% (days 0 and 7-9) to 90% (days 18-22) of the CD19+ cells in both groups, suggesting increased activation with colitis. The CD1d marker, a MHC class I related molecule known to present lipid antigens (127), was found to be expressed on 26-35% of the CD19+IgM+ cells with no changes with colitis or genotype.

The percentage of T lymphocytes (CD3+ cells) was maintained at ~13-16% before and during colitis and for both genotypes (Table 3.1). The total number of T cells increased by days 18-22 of colitis in both groups of mice (Table 3.2). The majority of T cells in the gut under basal and inflammatory conditions were CD4+ (44-61% of CD3+ cells), while the remaining were mostly CD8+ T cells (17-27%). The distribution of CD4+FoxP3+ T-reg cells was also assessed in both groups at day 12 and estimated at 2-4% of leukocytes with no difference between Eng+/+ and Eng−/− mice.

Myeloid cells (CD11b+) were present in the colon under non-inflammatory conditions and at the same frequency (~19-22%) in Eng+/+ and Eng−/− mice (Table 3.1). At days 7-9 of colitis, the overall myeloid distribution was significantly expanded in Eng+/+ mice and showed a trend in Eng−/− mice. By days 18-22, the myeloid proportion in Eng+/+ mice declined but remained somewhat elevated in Eng−/− mice (P=0.077 between days 0 and 18-22). In terms of total cells present, both groups of mice showed higher numbers of myeloid cells at days 18-22 of colitis compared to day 0 (Table 3.2).

We confirmed that there is an increase in the number of B, T and myeloid cells with colitis with no differences between the genotypes. Although there were no significant alterations in overall distribution of T and B subsets, there was a trend for a higher percentage of myeloid cells in Eng−/− mice at days 18-22. As DSS-induced colitis is thought to be primarily an innate mediated model (111), the subsets within the CD11b+ compartment were further studied.
Figure 3.1 *Eng*<sup>+</sup>/<sup>-</sup> mice exhibit lower weight recovery than *Eng*<sup>+</sup>/<sup>+</sup> mice during days 13-17 of DSS-induced colitis

*Eng*<sup>+</sup>/<sup>+</sup> and *Eng*<sup>+</sup>/<sup>-</sup> mice were given a 5-day course (bar) of 3% DSS in their drinking water followed by return to normal water. All mice were weighed daily and their weight calculated as a percentage of the starting weight. Results represent mean ± SEM (N=94-95 mice for each genotype). *P<0.05 vs. *Eng*<sup>+</sup>/<sup>+</sup> mice at the corresponding time point, from days 13-17.
Table 3.1 Distribution of colonic leukocytes during DSS-induced colitis in \textit{Eng}^{+/+} and \textit{Eng}^{+/-} mice

<table>
<thead>
<tr>
<th>Experimental day(s)</th>
<th>\textit{Eng}^{+/+}</th>
<th>\textit{Eng}^{+/-}</th>
<th>\textit{Eng}^{+/+}</th>
<th>\textit{Eng}^{+/-}</th>
<th>\textit{Eng}^{+/+}</th>
<th>\textit{Eng}^{+/-}</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>31 ± 5</td>
<td>35 ± 5</td>
<td>14 ± 1</td>
<td>13 ± 1</td>
<td>22 ± 3</td>
<td>19 ± 2</td>
</tr>
<tr>
<td>7-9</td>
<td>33 ± 1</td>
<td>35 ± 8</td>
<td>14 ± 1</td>
<td>14 ± 1</td>
<td>37 ± 1*</td>
<td>32 ± 8</td>
</tr>
<tr>
<td>18-22</td>
<td>33 ± 2</td>
<td>26 ± 4</td>
<td>16 ± 1</td>
<td>16 ± 1</td>
<td>23 ± 1</td>
<td>30 ± 5</td>
</tr>
</tbody>
</table>

Results represent mean ± SEM (For both genotypes, N=3 experiments for day 0, 5 for days 7-9 and 4 for days 18-22). *P<0.05 vs. corresponding day 0.

Table 3.2 The total number of all major colonic leukocytes is increased at days 18-22 of colitis

<table>
<thead>
<tr>
<th>Experiment day(s)</th>
<th>\textit{Eng}^{+/+}</th>
<th>\textit{Eng}^{+/-}</th>
<th>\textit{Eng}^{+/+}</th>
<th>\textit{Eng}^{+/-}</th>
<th>\textit{Eng}^{+/+}</th>
<th>\textit{Eng}^{+/-}</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.4 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>0.2 ± 0.01</td>
<td>0.2 ± 0.01</td>
<td>0.3 ± 0.02</td>
<td>0.3 ± 0.04</td>
</tr>
<tr>
<td>7-9</td>
<td>0.9 ± 0.1</td>
<td>1.6 ± 0.4</td>
<td>0.5 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>1.2 ± 0.2</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>18-22</td>
<td>2.5 ± 0.3*</td>
<td>2.3 ± 0.4*</td>
<td>1.2 ± 0.2*</td>
<td>1.5 ± 0.2*</td>
<td>1.8 ± 0.2*</td>
<td>2.9 ± 0.8*</td>
</tr>
</tbody>
</table>

Results represent mean ± SEM (For both genotypes, N=3 experiments for day 0, 5 for days 7-9 and 4 for days 18-22). *P<0.05 vs. corresponding day 0.
3.3 Neutrophils and monocytes are the major contributors to the expansion of the gut myeloid compartment during colitis

The CD11b\(^+\) myeloid cells were further characterized using additional markers to identify macrophages (CD11b\(^{+}\)F480\(^{-}\)Ly6C\(^{+/C-}\)Ly6G\(^{-}\)), monocytes (CD11b\(^{+}\)F480\(^{-}\)Ly6C\(^{+}\)Ly6G\(^{-}\) cells) and neutrophils (CD11b\(^{+}\)F480\(^{-}\)Ly6C\(^{+/C-}\)Ly6G\(^{+}\) cells) (120, 122). Prior to DSS induction, the myeloid cells within the lamina propria of Eng\(^{+/+}\) and Eng\(^{+/−}\) mice were predominantly CD11b\(^{+}\)F480\(^{-}\) and likely macrophages. Following colitis, myeloid cells were mostly CD11b\(^{+}\)F480\(^{-}\) (monocytes and granulocytes) as shown by representative flow cytometry profiles (Figure 3.2A).

The percentage of CD11b\(^{+}\)F480\(^{-}\) cells, which mainly encompasses total macrophages, was unchanged at the peak of disease (days 7-9) compared to day 0, but was significantly reduced (19-22% at day 0 to 7-11% at days 18-22) by days 18-22 in both Eng\(^{+/+}\) and Eng\(^{+/−}\) mice (Figure 3.2B). The total number of macrophages per mouse increased by colitis days 18-22 in Eng\(^{+/−}\) mice but not in Eng\(^{+/+}\) mice (Figure 3.2C). While there were no F480\(^{-}\)Ly6G\(^{+}\) cells found in the lamina propria at any time point, colitis induction lead to an increase in both groups of mice in the distribution (from 0.1% at day 0 to 35-50% of CD11b\(^{+}\)F480\(^{-}\) cells during colitis) and number of F480\(^{-}\)Ly6C\(^{+}\) cells, which likely represent activated macrophages (128).

The major myeloid subset seen in colitis was composed of CD11b\(^{+}\)F480\(^{-}\) cells, and therefore the neutrophil and monocyte distribution was next analyzed. As shown in the representative images of CD11b\(^{+}\)F480\(^{-}\) cells from days 8 and 18 in figure 3.3A, neutrophils (Ly6C\(^{+/C-}\)Ly6G\(^{-}\)) and monocytes (Ly6C\(^{+}\)Ly6G\(^{-}\)) were the two major gut myeloid cell types that infiltrated the colon of Eng\(^{+/+}\) and Eng\(^{+/−}\) mice throughout colitis. Neutrophils were not present prior to colitis and were maintained at 8-11% of total leukocytes at days 7-9 and 18-22 with no difference between genotypes (Figure 3.3B). In terms of total numbers, a trend for higher neutrophil numbers was observed in Eng\(^{+/−}\) mice at days 18-22 (Figure 3.3C). As observed with neutrophils, monocytes infiltrated the colon after DSS administration; however, there was a trend towards lower monocyte distribution (9% vs 5% of total leukocytes) in Eng\(^{+/−}\) mice versus Eng\(^{+/+}\) mice during colitis days 7-9 (\(P=0.076\)) but numbers were similar at days 18-22 (Figure 3.3D). The total number of monocytes was similar in both groups of mice at days 7-9 with no further change at days 18-22 (Figure 3.3E).
Figure 3.2 Decreased percentage of colonic CD11b⁺F480⁺ cells with DSS-induced colitis in *Eng*⁺/⁺ and *Eng*⁺/- mice but higher number of macrophages observed in colitic *Eng*⁺/- mice

A) Representative flow cytometry contour plots of colonic lamina propria cells, isolated at day 0 and day 22 of a DSS-induced colitis experiment, show the distribution of CD11b⁺F480⁺ and CD11b⁺F480⁻ cells. All plots were first gated for CD11b⁺ cells (not shown) and the percentage of CD45⁺ leukocytes is indicated. B) The distribution of CD11b⁺F480⁺ cells decreases in both groups by days 18-22. C) The number of CD11b⁺F480⁺ cells increases in *Eng*⁺/- mice by days 18-22. Results represent mean ± SEM (For both genotypes, N=3 experiments for day 0, 5 for days 7-9 and 4 for days 18-22). *P<0.05 vs. corresponding day 0.
Figure 3.3 Neutrophils and monocytes infiltrate the colon during DSS-induced colitis
A) Representative flow cytometry contour plots of colonic lamina propria cells isolated from both groups of mice at days 8 and 18 of the colitis course. Cells were initially gated for CD11b\(^+\)F480\(^-\) cells (not shown) and analyzed for neutrophils (Ly6C\(^+\)/Ly6G\(^+\)) and monocytes (Ly6C\(^+\)Ly6G\(^-\)). B) and C) Histograms revealing the emergence of neutrophils with colitis and their similar distribution in Eng\(^{+/+}\) and Eng\(^{+/−}\) mice, but a trend for a higher number in Eng\(^{+/−}\) mice. D) and E) Histograms showing that DSS induces the appearance of monocytes in the lamina propria with no significant difference between the heterozygotes and littermate controls. Results represent mean ± SEM (For both genotypes, N=3 experiments for day 0, 5 for days 7-9 and 4 for days 18-22).
DSS colitis led to a decrease in the distribution of macrophages and increased proportion of monocytes and neutrophils in the gut of both groups of mice. While higher numbers of macrophages were found in colitic Eng\(^+/−\) mice, there were no differences in the number of infiltrating neutrophils and monocytes. In order to assess if Eng\(^+/−\) mice show alterations in systemic immune responses, the splenic leukocyte subsets were next analyzed at all time points.

3.4 Reduced B and T lymphocytes and increased myeloid cells in spleen of colitic Eng\(^+/+\) and Eng\(^+/−\) mice

In both groups of mice, the percentage of total CD19\(^+\) B cells was unaltered between days 0 and 7-9 (~43-50%), but by days 18-22, there was a significant reduction in total B cells to ~ 16-22% of CD45\(^+\) leukocytes (Table 3.3). CD19\(^+\) cells were predominantly CD5 IgM\(^+\)CD1d\(^−\) B2 cells and unaltered between Eng\(^+/+\) and Eng\(^+/−\) mice. CD19\(^+\)CD5\(^+\) B1 cells were found at a low frequency (0.5% of leukocytes) in both groups of mice and unchanged after DSS colitis. In comparison to the gut, the spleen contained large numbers of B cells that were unchanged between days 0 and 7-9 in all mice. During days 20-22 of colitis, Eng\(^+/−\) mice had lower numbers of B cells than their days 0 and 7-9 counterparts, and Eng\(^+/+\) mice at days 20-22 (Table 3.4).

The splenic distribution of CD3\(^+\) T cells in Eng\(^+/+\) and Eng\(^+/−\) mice was ~30-32% at days 0 and 7-9; however, by days 20-22 of colitis, there was a contraction of total T cells to ~ 10-13% (Table 3.3). At all observed time points and in both genotypes, CD4\(^+\) and CD8\(^+\) T cell subsets each accounted for ~50% of CD3\(^+\) cells (data not shown). T-regulatory cells were also assessed at day 12 and found at a frequency between 2-3% in both groups of mice. As observed for the distribution, neither group of mice demonstrated a change in T cell numbers between days 0 and 7-9 (Table 3.4). During days 20-22 of colitis, Eng\(^+/−\) mice exhibited a significant decrease in total T cell number compared to day 0 while Eng\(^+/+\) mice showed a slight decrease that did not reach significance.

Prior to DSS-induction and at days 7-9, CD11b\(^+\) myeloid cells represented the minority of spleen leukocytes (~5-9%); this was followed by an extensive increase to ~42-47% in Eng\(^+/+\) and Eng\(^+/−\) groups by days 20-22 (Table 3.3). The total number of myeloid cells was also substantially increased in all mice at that time point (Table 3.4). Thus there is a major increase in the proportion and number of myeloid cells in the spleen of mice, at 20-22 days of the colitis course with no genotype difference.
Table 3.3 *Eng*⁺/+ and *Eng*⁺/- mice show a decrease in percent splenic B and T cells and an increase in myeloid distribution by days 20-22 of DSS colitis

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Experiment day(s)</th>
<th>B cells</th>
<th>T cells</th>
<th>Myeloid cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Eng</em>⁺/+</td>
<td><em>Eng</em>⁺/-</td>
<td><em>Eng</em>⁺/+</td>
<td><em>Eng</em>⁺/-</td>
</tr>
<tr>
<td>Spleen</td>
<td>0</td>
<td>43 ± 1</td>
<td>44 ± 1</td>
<td>32 ± 2</td>
</tr>
<tr>
<td></td>
<td>7-9</td>
<td>50 ± 4</td>
<td>46 ± 3</td>
<td>30 ± 2</td>
</tr>
<tr>
<td></td>
<td>20-22</td>
<td>22 ± 3*#</td>
<td>16 ± 3*#</td>
<td>13 ± 2*#</td>
</tr>
</tbody>
</table>

Results represent mean ± SEM (For both genotypes, N=7 mice for day 0, 8 for days 7-9 and 11-13 for days 20-22). *P*<0.05 vs. corresponding day 0, *P*<0.05 vs. corresponding days 7-9.

Table 3.4 Extensive increase in splenic myeloid cell number at days 20-22 of colitis

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Experiment day(s)</th>
<th>B cells</th>
<th>T cells</th>
<th>Myeloid cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Eng</em>⁺/+</td>
<td><em>Eng</em>⁺/-</td>
<td><em>Eng</em>⁺/+</td>
<td><em>Eng</em>⁺/-</td>
</tr>
<tr>
<td>Spleen</td>
<td>0</td>
<td>42 ± 5</td>
<td>35 ± 2</td>
<td>30 ± 3</td>
</tr>
<tr>
<td></td>
<td>7-9</td>
<td>39 ± 8</td>
<td>30 ± 2</td>
<td>24 ± 5</td>
</tr>
<tr>
<td></td>
<td>20-22</td>
<td>32 ± 3</td>
<td>20 ± 2*#†</td>
<td>19 ± 2</td>
</tr>
</tbody>
</table>

Results represent mean ± SEM (For both genotypes, N=7 mice for day 0, 8 for days 7-9 and 11-13 for days 20-22). *P*<0.05 vs. corresponding day 0, *P*<0.05 vs. corresponding days 7-9, †P<0.05 vs corresponding *Eng*⁺/+ mice.
We confirmed previous findings of decreased splenic B and T cells with DSS colitis and no change in these subsets between the genotypes (113). As expected, the overall myeloid compartment in the spleen greatly expanded during colitis, however, with no difference between \( \text{Eng}^{+/+} \) and \( \text{Eng}^{+/}\) mice. Due to this extensive increase, the subsets within the CD11b\(^+\) population were next analyzed to test for differences in genotype.

### 3.5 Increased macrophages, neutrophils and monocytes in the spleen of DSS-treated \( \text{Eng}^{+/+} \) and \( \text{Eng}^{+/}\) mice

The immune subsets within the splenic CD11b\(^+\) myeloid compartment were next analyzed using the F480, Ly6C and Ly6G markers to differentiate between macrophages, neutrophils and monocytes (as shown in the colon). The representative flow contour plots in figure 3.4A show that in \( \text{Eng}^{+/+} \) and \( \text{Eng}^{+/}\) mice, both CD11b\(^+\)F480\(^+\) and CD11b\(^+\)F480\(^-\) cells were present at low frequencies at day 0 and subsequently increased by day 20 of the colitis course, with CD11b\(^+\)F480\(^-\) cells being the predominant population at this time point. In contrast to the colonic lamina propria, low levels of neutrophils (CD11b\(^+\)F480\(^-\)Ly6C\(^+\)/Ly6G\(^+\) cells) and monocytes (CD11b\(^+\)F480\(^-\)Ly6C\(^+\)Ly6G\(^-\) cells) were found in the spleen at day 0 followed by extensive expansion of both subsets by day 20 of colitis in all groups (Figure 3.4B).

For all mice, the distribution of CD11b\(^+\)F480\(^+\) cells was initially found to be \(~1-2\%\) of total leukocytes and increased to \(~5\%\) by days 20-22 (Figure 3.5A). At days 0 and 7-9, F480\(^+\)Ly6C\(^-\) cells were found at a higher frequency (70-80\% of CD11b\(^+\)F480\(^+\) cells) than F480\(^-\)Ly6C\(^+\) cells (15-30\%), however, the distribution of F480\(^-\)Ly6C\(^+\) cells increased (40-50\%) at days 20-22 of colitis. Some F480\(^-\)Ly6G\(^+\) cells (7-9\% of CD11b\(^+\)F480\(^+\) cells), likely to be an undifferentiated subset, were found in the spleen by days 20-22, also with no difference between the groups. The total number of F480\(^-\) cells also increased in all groups of mice at this time point (Figure 3.5B). An extensive increase in neutrophil proportion and number (Figure 3.5C & D) was observed in isolated splenocytes of all mice at days 20-22. Similarly, F480\(^-\)Ly6C\(^+\) monocytic cells had the highest frequency and number at days 20-22, with no difference between the genotypes (Figure 3.5E & F). Overall, \( \text{Eng}^{+/+} \) and \( \text{Eng}^{+/}\) mice demonstrated a similar increase in the distribution and number of macrophages, neutrophils and monocytes in the spleen during DSS-induced colitis, suggesting that the persistent disease in \( \text{Eng}^{+/}\) mice is not attributed to an alteration in the number of leukocytes in that organ.
Figure 3.4 Expansion of myeloid subsets in the spleen of colitic mice
A) Representative flow cytometry contour plots of splenocytes isolated at days 0 and 20 of a DSS-induction experiment. Cells were initially gated for CD11b (not shown). The gates reveal the distribution of CD11b$^+$F480$^+$ and CD11b$^+$F480$^-$ cells prior to DSS-induction and the subsequent increase of both populations with colitis. B) The CD11b$^+$F480$^-$ population can be divided into two major subsets: neutrophils (Ly6C$^-$Ly6G$^+$ cells) and monocytes (Ly6C$^+$Ly6G$^-$ cells). Both cell types are found at low frequencies in the spleen at day 0, but are increased substantially by day 20 in both Eng$^{+/+}$ and Eng$^{+/−}$ mice.
Figure 3.5 Increased proportion and total number of the major splenic myeloid subsets at days 20-22 of colitis
A) The distribution and B) number of spleen macrophages were unchanged by days 7-9 after DSS induction but were significantly expanded by days 20-22 in both groups of mice. C) The proportion of neutrophils and D) their total number were also significantly increased by days 20-22. E) Monocyte distribution and F) number were also much increased by days 20-22. Results represent mean ± SEM (For both genotypes, N=7 mice for day 0, 8 for days 7-9 and 11-13 for days 20-22). *P<0.05 vs. corresponding day 0, #P<0.05 vs. corresponding days 7-9.
3.6 Higher distribution and number of bone marrow myeloid cells at days 20-22 is predominantly due to more neutrophils

As endoglin is expressed on some hematopoietic stem cells and implicated in myelopoiesis (66), the myeloid cells of the bone marrow were also characterized by flow cytometry before and during colitis. The bone marrow under basal conditions contained a high percentage (~63-66%) and number of myeloid cells in Eng\textsuperscript{+/+} and Eng\textsuperscript{+/−} mice (Table 3.5). Following DSS treatment, an increase in the proportion (~83-84%) and number of myeloid cells was observed at days 20-22, with no difference between Eng\textsuperscript{+/+} and Eng\textsuperscript{+/−} mice. For both genotypes, CD11b\textsuperscript{−}F480\textsuperscript{−} cells were found at a higher frequency at day 0 than day 22, while CD11b\textsuperscript{−}F480\textsuperscript{−} cells increased with colitis, as shown in the representative flow cytometry plots in figure 3.6A. The percentage of CD11b\textsuperscript{+}F480\textsuperscript{+} cells was not changed from day 0 to days 7-9 but was decreased by day 22 in both groups (Figure 3.7A). The total number of CD11b\textsuperscript{+}F480\textsuperscript{+} cells was also unaltered at days 7-9 but declined by days 20-22 in Eng\textsuperscript{+/−} mice (Figure 3.7B).

Prior to induction of colitis, F480\textsuperscript{+}Ly6C\textsuperscript{−}Ly6G\textsuperscript{+} undifferentiated cells were found in similar proportions to the F480\textsuperscript{−}Ly6C\textsuperscript{+}Ly6G\textsuperscript{−} monocytic cells; by day 22, only the undifferentiated subset declined in all mice (Figure 3.6B). The decline in percent and number of undifferentiated cells was identified as early as days 7-9 and maintained during colitis days 20-22 (Figure 3.7C & D). The frequency of F480\textsuperscript{+}Ly6C\textsuperscript{+}Ly6G\textsuperscript{−} monocytic cells was higher in Eng\textsuperscript{+/+} and Eng\textsuperscript{+/−} mice at days 7-9 and decreased to basal levels in Eng\textsuperscript{+/+} mice and even lower levels in Eng\textsuperscript{+/−} mice by days 20-22 (Figure 3.7E). In terms of number of cells, there was a significant increase in Eng\textsuperscript{+/−} mice and trend in Eng\textsuperscript{+/+} mice at days 7-9 with a decline to basal numbers in Eng\textsuperscript{+/−} mice at days 20-22 (Figure 3.7F).
### Table 3.5 Increased bone marrow myeloid cell distribution and number at days 20-22 of colitis in both Eng$^{+/+}$ and Eng$^{+/-}$ mice

<table>
<thead>
<tr>
<th>Experiment day(s)</th>
<th>$Eng^{+/+}$</th>
<th>$Eng^{+/-}$</th>
<th>$Eng^{+/+}$</th>
<th>$Eng^{+/-}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>63 ± 2</td>
<td>66 ± 1</td>
<td>18 ± 3</td>
<td>16 ± 2</td>
</tr>
<tr>
<td>7-9</td>
<td>67 ± 2</td>
<td>64 ± 2</td>
<td>18 ± 3</td>
<td>16 ± 2</td>
</tr>
<tr>
<td>20-22</td>
<td>83 ± 1</td>
<td>84 ± 1</td>
<td>41 ± 4</td>
<td>38 ± 6</td>
</tr>
</tbody>
</table>

Results represent mean ± SEM (For both genotypes, N=6 mice for day 0, 9 for days 7-9 and 11-13 for days 20-22). *$P$<0.05 vs. corresponding day 0, #$P$<0.05 vs. corresponding days 7-9.
Figure 3.6 The distribution of major bone marrow myeloid subsets is similar in Eng^{+/+} and Eng^{+-} mice

A) Flow cytometry contour plots were first gated for CD11b^{+} cells (not shown) and F480 expression examined at days 0 and 22 of the DSS induced colitis. The CD11b^{+}F480^{-} cells constitute the majority of cells and mostly represent neutrophils, which appear increased by day 22. B) The CD11b^{+}F480^{+} population was analyzed further and consists of macrophages (Ly6C^{+}Ly6G^{-}) and an undifferentiated myeloid subset (Ly6C^{-}Ly6G^{+}).
Figure 3.7 DSS colitis induction leads to a decline in undifferentiated myeloid cells and to a transient increase in macrophages in the bone marrow
A) The proportion of total CD11b\(^+\)F480\(^+\) cells was reduced by days 20-22 of colitis in both groups of mice. B) A significant decrease in the total number of CD11b\(^+\)F480\(^+\) cells was observed in Eng\(^{+/—}\) mice but not in Eng\(^{+/+}\) at days 20-22. C) The distribution and D) number of CD11b\(^+\)F480\(^+\)Ly6C\(^+\)/Ly6G\(^+\) undifferentiated cells were decreased during colitis, at the two time points tested. E) Macrophages exhibited an increase in percentage by days 7-9, and were subsequently reduced. F) The total number of macrophages was also transiently increased by days 7-9. Results represent mean ± SEM (For both genotypes, N=6 mice for day 0, 9 for days 7-9 and 11-13 for days 20-22). *P<0.05 vs corresponding day 0, #P<0.05 vs corresponding days 7-9.
Figure 3.8 Colitis mediated changes in the distribution and number of bone marrow neutrophils and monocytes are similar in Eng^{+/+} and Eng^{+-} mice

A) Flow cytometry contour plots were first gated for CD11b^{+} F480^{-} cells (not shown) and neutrophil and monocyte gates set based on Ly6G and Ly6C expression. A representative profile of the days 0, 7 and 22 is shown for both Eng^{+/+} and Eng^{+-} mice. B) The distribution of neutrophils was decreased by days 7-9 and expanded by days 20-22 in both groups of mice. C) The total number of neutrophils also showed an increase at days 20-22. D) The monocyte proportion and E) number were increased at days 7-9 and 20-22 of the colitis experiments. Results represent mean ± SEM (For both genotypes, N=6 mice for day 0, 9 for days 7-9 and 11-13 for days 20-22). *P<0.05 vs. corresponding day 0, #P<0.05 vs. corresponding days 7-9.
Neutrophils and monocytes were also found in the CD11b^+ F480^- fraction at all tested time points (Figure 3.8A). Under basal conditions, bone marrow neutrophils were found in high proportion and number (Figure 3.8B & C). After DSS-induction, the frequency of neutrophils declined by days 7-9 in all mice, indicating exit from the bone marrow, followed by an extensive increase by days 20-22 indicating re-population. Although the total number of neutrophils did not decrease at days 7-9, expansion by days 20-22 was demonstrated in both groups (Figure 3.8C). Monocytic cell distribution increased during DSS days 7-9 and although there was a decline by days 20-22, higher than basal levels were still observed at this stage of disease (Figure 3.8D). Higher number of monocytes was also observed with colitis in both genotypes (Figure 3.8E).

In both groups of mice, DSS treatment leads to a transient increase in bone marrow macrophages, exit of neutrophils followed by significant expansion and higher monocytic cells. While there were colitis-dependent changes in all tested bone marrow myeloid subsets, Eng^+/− mice showed no impairment in their ability to generate and release these cells.

3.7 Differential peripheral blood leukocyte count shows less circulating lymphocytes and more neutrophils with colitis

The blood cell differential count was next performed in Eng^+/+ and Eng^+/− mice to test for potential defects in recruitment of immune subsets. The number of erythrocytes was within the normal range at all time points (Table 3.6) (129). During colitis, both groups of mice demonstrated a decrease in the total number of lymphocytes and a small increase in the monocyte population in both groups of mice; however these changes were within the normal range. In terms of neutrophils, they were increased by days 18-21 reaching significance in the Eng^+/− mice and a trend in Eng^+/+ mice, however, these changes were minimal within the physiological range (1.9–11.5 x 10^9 cells/L) in both groups. Although this method was an indirect way to assess leukocyte recruitment, no major changes were observed between Eng^+/+ and Eng^+/− mice, suggesting similar ability to recruit cells during inflammatory conditions.
Table 3.6 Changes in peripheral blood leukocyte differential count with colitis

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Day 0</th>
<th>Day 7</th>
<th>Days 18-21</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\text{Eng}^{+/+}$</td>
<td>$\text{Eng}^{+/-}$</td>
<td>$\text{Eng}^{+/-}$</td>
</tr>
<tr>
<td>Erythrocytes</td>
<td>11606 ± 284</td>
<td>11642 ± 110</td>
<td>10045 ± 726</td>
</tr>
<tr>
<td>White blood cells</td>
<td>11.5 ± 0.9</td>
<td>13.1 ± 1.2</td>
<td>7.9 ± 1.3</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>9.5 ± 0.8</td>
<td>11.2 ± 1.1</td>
<td>5.5 ± 0.9</td>
</tr>
<tr>
<td>Monocytes</td>
<td>0.3 ± 0.05</td>
<td>0.3 ± 0.04</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>1.6 ± 0.4</td>
<td>1.5 ± 0.3</td>
<td>1.7 ± 0.3</td>
</tr>
</tbody>
</table>

Results represent mean ± SEM (For both genotypes, N=5 mice for day 0, 6-7 for days 7-9 and 18-22). *$P<0.05$ vs. corresponding day 0, #$P<0.05$ vs. corresponding days 7-9.
3.8 Suppressed mRNA expression of key angiogenic and TGF-\(\beta\)/BMP related genes and lower Smad2 levels in \(Eng^{+/+}\) mice.

As the persistent inflammation observed in \(Eng^{+/+}\) mice was not associated with major defects in leukocyte subset distribution or numbers, the levels of the factors that regulate angiogenesis and inflammation were next measured in the distal colons of colitic mice. Given that \(Eng^{+/+}\) mice develop pathological angiogenesis and have defective TGF-\(\beta\)-mediated signaling, dysregulated expression of angiogenesis and TGF-\(\beta\) related genes might contribute to increased inflammation in \(Eng^{+/+}\) mice. In order to characterize differential gene expression between \(Eng^{+/+}\) and \(Eng^{+/+}\) mice during colitis, we measured mRNA expression profiles of multiple angiogenic and TGF-\(\beta\)/BMP pathway-related genes, at days 18-21 of the colitis course. All results are expressed as a ratio of the values obtained for \(Eng^{+/+}\) relative to those of \(Eng^{+/+}\) mice (Table 3.7).

The first array representing genes that regulate angiogenesis, demonstrated a decrease in expression of key genes involved in angiogenesis and inflammation. Decreased expression of a potent anti-angiogenic factor, thrombospondin-1 (Thbs1) (130), was found in \(Eng^{+/+}\) mice, suggesting that downregulation of an angiostatic gene is associated with increased angiogenesis in our model. The reduction in Thbs1 mRNA was confirmed at the protein level by Western blot analysis (data not shown). There was also a slight downregulation of placental growth factor, which is a member of the VEGF family of pro-angiogenic factors (131). Surprisingly, there was a decrease in the mRNA levels of pro-inflammatory cytokines/chemokines CXCL1, Eotaxin (CCL11), IL-1\(\beta\) and especially IL-6, which are also known to have pro-angiogenic effects (99, 132, 133). The decrease in endoglin mRNA expression observed serves as a positive control for the array, as \(Eng^{+/+}\) mice express a single copy of the gene and should express half levels of corresponding mRNA.

The second array, which included genes related to TGF-\(\beta\)/BMP pathways, showed decreased expression of all significantly altered genes, for CD79a, a membrane protein that is part of the B cell receptor complex, and showed increased expression suggesting higher B cell activation in the colon of \(Eng^{+/+}\) mice (134). The lower mRNA levels of endoglin and IL-6 were confirmed in this array. Decreased levels of additional angiogenesis regulating genes, BMP endothelial cell precursor-derived regulator (BMPer) and TGF-\(\beta\)-induced (Tgfbi), were observed (135, 136).
Follistatin, which is known to regulate inflammation-induced tissue repair (137, 138), was also downregulated in colitic \textit{Eng}^{+/−} mice. Interestingly, higher levels of total and phosphorylated SMAD2 were found in both groups of colitic mice at days 18-21 mice, indicating an increase in total SMAD2 level causing a proportional increase in phosphorylated SMAD2 and TGF-β signaling with colitis. However, \textit{Eng}^{+/−} mice showed lower levels of SMAD2 (total and phosphorylated) than \textit{Eng}^{+/+} mice, implying decreased TGF-β signaling through the Alk5 pathway in the distal colon of these mice (Figure 3.9). SMAD1 levels (total and phosphorylated) were not different with colitis or between the genotypes, indicating that the Alk1 signaling pathway is likely not involved in the colitis response (data not shown). TGF-β has been implicated in modulating expression of genes such as IL-6 and Tgfbi, and therefore the downregulation of these factors in the distal colon of \textit{Eng}^{+/−} mice may be attributed to defective TGF-β mediated signaling (139).

Pathological angiogenesis in colitic \textit{Eng}^{+/−} mice is associated with dysregulation of genes that modulate angiogenesis and tissue repair, some of which are regulated by TGF-β/BMP pathways. Although histological analysis indicated persistent inflammation in the colons \textit{Eng}^{+/−} mice, there were decreased mRNA levels of several pro-inflammatory cytokines/chemokines. These findings prompted us to measure protein levels of key cytokines/chemokines to test for differences in cytokine production by \textit{Eng}^{+/−} mice.
Table 3.7 Downregulated angiogenesis and TGF-β/BMP pathway related genes in Eng\textsuperscript{+/−} vs. Eng\textsuperscript{+/+} mice at days 18-21 of colitis

<table>
<thead>
<tr>
<th>Angiogenesis Related Genes</th>
<th>Fold-change</th>
<th>T-test P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endoglin</td>
<td>-2.03</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td>CXCL1</td>
<td>-3.60</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td>IL-6</td>
<td>-15.14</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td>Eotaxin (CCL11)</td>
<td>-1.95</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td>Pgf</td>
<td>-1.69</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td>Thbs1</td>
<td>-2.48</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td>IL-1β</td>
<td>-3.82</td>
<td>$P = 0.05$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TGF-β/BMP Related Genes</th>
<th>Fold-change</th>
<th>T-test P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endoglin</td>
<td>-2.36</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td>BMPER</td>
<td>-2.22</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td>CD79A</td>
<td>2.28</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td>Follistatin</td>
<td>-4.11</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td>Tgfbi</td>
<td>-1.78</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td>IL-6</td>
<td>-11.2</td>
<td>$P &lt; 0.05$</td>
</tr>
</tbody>
</table>

Pgf (Placental growth factor), Thbs1 (Thrombospondin 1), Tgfbi (Transforming growth factor, beta induced). N=4 mice for Eng\textsuperscript{+/+} group and N=5 for the Eng\textsuperscript{+/−} group.
Figure 3.9 Lower total and phosphorylated Smad2 expression in distal colons of days 18-21 Eng<sup>+/−</sup> mice

Total Smad2 and P-Smad2 levels increase with colitis in both genotypes, but to a lesser extent in Eng<sup>+/−</sup> mice. Results represent mean ± SEM (N=3 mice for day 0 and 9-12 for days 18-21). *P<0.05 vs. corresponding day 0 total Smad2, †P<0.05 vs. corresponding day 0 P-Smad2, ‡P<0.05 vs. corresponding total Smad2 Eng<sup>+/−</sup> mice, ‡†P<0.05 vs. corresponding P-Smad2 Eng<sup>+/−</sup> mice. These experiments were performed by Dr. Mirjana Jerkic.
3.9 Higher levels of neutrophil regulating cytokines/chemokines in days 7-9 $Eng^{+/-}$ mice

The distal colonic protein levels of several cytokines and chemokines that were identified by the PCR array and additional factors implicated in the DSS model were measured in both groups of mice and at all time points (112, 114). The 14 cytokines/chemokines factors that were measured include: CXCL1, G-CSF, IL-6, CCL11, IL-1β, IL-10, TNF-α, IFNγ, IL-17, M-CSF, IL-12p40, IL-12p70 and IL-4. Table 3.8 lists all the results while Figure 3.10 highlights the most significant changes. The neutrophil chemoattractant (140), CXCL1, was elevated in all mice at days 7-9, with $Eng^{+/-}$ mice exhibiting a higher concentration than $Eng^{+/+}$ mice, followed by return to basal levels by days 18-21 in both groups (Figure 3.10A). Similarly, G-CSF increased at days 7-9, with a trend for a higher amount in $Eng^{+/-}$ mice ($P=0.074$) and subsequently decreased in both groups of mice by colitis days 18-21 (Figure 3.10B). IL-6 (Figure 3.10C) and CCL11 (Figure 3.10D) levels were elevated at peak of disease and back to basal levels at days 20-22 with no difference between the genotypes. IL-1β and IL-10 levels also increased only during days 7-9 in all mice but to a lesser extent than the aforementioned factors (Figure 3.10E & F). For TNF-α, the overall expression was minimal, with increased levels observed in both groups of mice at days 7-9.

Overall, the levels of neutrophil regulating factors, CXCL1 and G-CSF, were found to be greatly increased in day 7-9 $Eng^{+/-}$ mice, however, by days 18-21 these factors decreased to basal levels in both groups. Higher levels of the pro-inflammatory factor IL-6 and the eosinophil chemoattractant, CCL11, were also only found at day 7-9 in colitic mice, but with no difference between the genotypes. Although there were high protein levels of CXCL1, CCL11, IL-1β and IL-6 at days 7-9, the decreased mRNA levels of these cytokines/chemokines at days 18-21 could be attributed to a negative feedback loop and possibly dysregulated TGF-β responses.
Table 3.8 Expression of Cytokines and Chemokines in the distal colon during DSS-induced colitis in Eng$^{+/+}$ and Eng$^{+/−}$ mice.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Day 0</th>
<th>Days 7-9</th>
<th>Days 18-21</th>
<th>Day 0</th>
<th>Days 7-9</th>
<th>Days 18-21</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCL1</td>
<td>10.9 ± 0.4</td>
<td>699 ± 193*</td>
<td>122 ± 74</td>
<td>11.6 ± 2.3</td>
<td>1614 ± 261*†</td>
<td>138 ± 75</td>
</tr>
<tr>
<td>G-CSF</td>
<td>1.3 ± 0.3</td>
<td>2940 ± 967*</td>
<td>91 ± 57</td>
<td>1.2 ± 0.3</td>
<td>5740 ± 1209*‡</td>
<td>100 ± 49</td>
</tr>
<tr>
<td>IL-6</td>
<td>2.3 ± 0.2</td>
<td>1330 ± 526*</td>
<td>102 ± 67</td>
<td>2.6 ± 0.2</td>
<td>2609 ± 735*</td>
<td>220 ± 142</td>
</tr>
<tr>
<td>CCL11</td>
<td>45 ± 4</td>
<td>479 ± 75*</td>
<td>122 ± 23</td>
<td>58 ± 9</td>
<td>638 ± 45*</td>
<td>119 ± 20</td>
</tr>
<tr>
<td>II-1β</td>
<td>13 ± 2</td>
<td>60 ± 16*</td>
<td>73 ± 53</td>
<td>14 ± 1</td>
<td>51 ± 13*</td>
<td>42 ± 20</td>
</tr>
<tr>
<td>IL-10</td>
<td>9.2 ± 2.0</td>
<td>36 ± 11*</td>
<td>24 ± 4</td>
<td>8.0 ± 2.3</td>
<td>24 ± 3*</td>
<td>18 ± 4</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1.9 ± 0.7</td>
<td>10.6 ± 2.7*</td>
<td>3.9 ± 1.7</td>
<td>1.8 ± 0.9</td>
<td>5.8 ± 0.8*</td>
<td>3.0 ± 0.9</td>
</tr>
<tr>
<td>IFNγ</td>
<td>3.4 ± 0.6</td>
<td>17.7 ± 9.4</td>
<td>10.1 ± 4.2</td>
<td>2.7 ± 0.1</td>
<td>4.8 ± 1.6</td>
<td>9.2 ± 4.3</td>
</tr>
<tr>
<td>IL-17</td>
<td>1.1 ± 0.3</td>
<td>6.2 ± 2.4</td>
<td>5.2 ± 2.7</td>
<td>1.0 ± 0.2</td>
<td>2.0 ± 0.6</td>
<td>5.3 ± 2.1</td>
</tr>
<tr>
<td>M-CSF</td>
<td>1.5 ± 0.6</td>
<td>8.7 ± 3.8</td>
<td>1.4 ± 0.4</td>
<td>1.5 ± 0.7</td>
<td>25.1 ± 16.5</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>8.0 ± 1.5</td>
<td>7.3 ± 2.2</td>
<td>8.3 ± 2.8</td>
<td>8.6 ± 3.5</td>
<td>8.3 ± 2.5</td>
<td>3.7 ± 1.8</td>
</tr>
<tr>
<td>IL-12p40</td>
<td>6.1 ± 1.5</td>
<td>3.9 ± 1.0</td>
<td>3.6 ± 0.8</td>
<td>6.2 ± 1.4</td>
<td>1.8 ± 0.7</td>
<td>2.0 ± 0.6</td>
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<td>IL-12p70</td>
<td>0.8 ± 0.4</td>
<td>1.0 ± 0.3</td>
<td>0.4 ± 0.2</td>
<td>1.2 ± 1.0</td>
<td>1.0 ± 0.4</td>
<td>0.4 ± 0.4</td>
</tr>
<tr>
<td>IL-4</td>
<td>1.4 ± 0.1</td>
<td>1.7 ± 0.1</td>
<td>1.4 ± 0.1</td>
<td>1.6 ± 0.1</td>
<td>1.4 ± 0.1</td>
<td>1.3 ± 0.1</td>
</tr>
</tbody>
</table>

Results represent mean ± SEM (For both genotypes, N=4 mice for day 0, N=8 8 for days 7-9 and days 20-22). *P<0.05 vs. corresponding day 0, †P<0.05 vs. corresponding Eng$^{+/+}$, ‡P=0.074 vs. corresponding Eng$^{+/+}$. 
Figure 3.10 Elevated levels of neutrophil regulating factors in Eng\(^{+/-}\) mice during days 7-9 of DSS colitis

Cytokine and chemokine levels were measured in distal colon extracts of control and colitic mice and are expressed in pg/mg of tissue protein. A) CXCL1, B) G-CSF, C) IL-6, D) CCL11, E) IL-1β and F) IL-10 levels were all increased by days 7-9 of colitis in both groups of mice, relative to control samples. In addition, higher levels of CXCL1 (A) and G-CSF (B) were observed in samples from Eng\(^{+/-}\) than Eng\(^{+/+}\) mice at days 7-9. Results represent mean ± SEM (For both genotypes, N=4 mice for day 0, 8 for 7-9 and 20-22). *P<0.05 vs. corresponding day 0, †P<0.05 vs. corresponding Eng\(^{+/+}\), ‡P=0.074 vs. corresponding Eng\(^{+/+}\).
3.10 Decreased colonic myeloperoxidase expression and activity in Eng\textsuperscript{+/−} mice compared to Eng\textsuperscript{+/+} mice at days 18-21

As higher levels of neutrophil activating cytokines were found in Eng\textsuperscript{+/−} mice, the levels and activity of a key granulocyte derived effector molecule, the enzyme myeloperoxidase (MPO), was next tested (141). Higher activity of the MPO enzyme, which is important for ROS-mediated bacterial clearance, has been reported in DSS models of IBD (114). Here we used 3 different assays to measure MPO expression and activity. The number of MPO\textsuperscript{+} cells detected on tissue sections was higher in distal colons of days 9-12 Eng\textsuperscript{+/−} mice, with a trend for higher numbers in Eng\textsuperscript{+/+} mice (P=0.072) (Figure 3.11A). Although Eng\textsuperscript{+/+} mice maintained higher MPO\textsuperscript{+} cells at day 19, Eng\textsuperscript{+/−} mice demonstrated a trend towards lower counts (P=0.062) compared to their Eng\textsuperscript{+/+} counterparts. Increased MPO enzymatic activity was observed in both groups of mice at colitis days 9-12 relative to day 0; MPO activity remained elevated in Eng\textsuperscript{+/+} mice at day 19, but was significantly reduced in Eng\textsuperscript{+/−} mice (Figure 3.11B). Western blot analysis confirmed that MPO levels were increased in both groups at days 7-9; however, at days 18-21, lower MPO levels were found in Eng\textsuperscript{+/−} versus Eng\textsuperscript{+/+} mice (Figure 3.11C). Thus although there were no differences in the number of neutrophils in the gut between the genotypes, our data show lower MPO expression and activity in colitic Eng\textsuperscript{+/−} mice, suggesting potential defects in neutrophil function and potentially bacterial clearance.

3.11 Defective colonic DSS-induced H\textsubscript{2}O\textsubscript{2} production in Eng\textsuperscript{+/−} mice is accompanied by lower Nox-2 levels

As Eng\textsuperscript{+/−} mice show impaired regulation of ROS production, we further analyzed ROS production in the colon of colitic mice by measuring levels of a phagocyte NADPH oxidase, Nox-2, and of H\textsubscript{2}O\textsubscript{2}, a downstream byproduct of increased ROS (142). Colonic Nox-2 expression was increased in all mice at days 7-9 of colitis and persisted in the Eng\textsuperscript{+/+} mice till days 18-21 (Figure 3.12A). However, Nox-2 expression was significantly reduced in colitic Eng\textsuperscript{+/−} versus Eng\textsuperscript{+/+} mice. H\textsubscript{2}O\textsubscript{2} levels were elevated with DSS colitis in Eng\textsuperscript{+/+} mice, comparing days 20-21 to day 0. Interestingly, Eng\textsuperscript{+/−} mice showed an increase in the basal level of H\textsubscript{2}O\textsubscript{2} levels, which was no further increased by colitis (Figure 3.12B). The H\textsubscript{2}O\textsubscript{2} production was dependent on NADPH oxidase activity, as was decreased by the inhibitor apocynin. Thus the decrease in Nox-2 with colitis and the lack of a further increase in H\textsubscript{2}O\textsubscript{2} levels (despite the increase in basal H\textsubscript{2}O\textsubscript{2} levels) in Eng\textsuperscript{+/−} mice suggest impaired ROS mediated bacterial clearance.
Figure 3.11 Decreased myeloperoxidase expression and activity in colonic samples of days 18-21 Eng^{+/-} mice
A) The number of MPO^{+} cells present on colonic sections were increased at days 9-12 of the colitis course in both groups of mice. At day 19, the number of MPO^{+} cells was still elevated in Eng^{+/+} mice but was much reduced in Eng^{+/-} mice. B) Similar findings were observed when colonic tissue samples were tested for MPO enzymatic activity. C) MPO expression was also assessed by Western blot analysis of colonic sample extracts, and results confirmed increased MPO levels in days 7-9 in both groups and decreased MPO levels by days 18-21 Eng^{+/-} mice. Results represent mean ± SEM (For counts: N=6 mice at day 0, 7 at days 9-12 and 5-7 at day 19; for MPO counts: N=7-8 mice at day 0, 8 at days 9-12 and 6-7 at day 19; For MPO WB: N=6 mice at day 0, 4-5 at days 7-9 and 4 at day 19). *P<0.05 vs. corresponding day 0, #P<0.05 vs. corresponding days 9-12, †P<0.05 vs. corresponding Eng^{+/+}, ‡P=0.062 vs. corresponding Eng^{+/+}. These experiments were performed by Dr. Mirjana Jerkic and Dr. Chris Waterhouse.
Figure 3.12 Lower Nox-2 expression and impaired ROS production in colonic samples of days 18-21 Eng+/− mice

A) Distal colon Nox-2 levels, measured by WB shows increase in protein level at colitis days 7-9 in both groups, but only by days 18-21 in Eng+/− mice. *P<0.05 vs. corresponding day 0, #P<0.05 vs. corresponding days 7-9, †P<0.05 vs. corresponding Eng+/+ mice. B) H₂O₂ levels were higher by days 20-21 than at day 0 in Eng+/+ mice; they were also higher in Eng+/− under basal conditions and no further increased by colitis. Apocynin, an NADPH oxidase inhibitor, inhibits the H₂O₂ production in all groups. *P<0.05 vs. corresponding basal day 0, #P<0.05 vs. corresponding basal, †P<0.05 vs. corresponding basal day 0 Eng+/+. Results represent mean ± SEM (For Nox-2 WB: N=5 mice for day 0, 3 for days 7-9 and N=18 for days 18-21; for H₂O₂ assay, N=6-14 mice for day 0 and 4-7 mice for days 20-21). These experiments were performed by Dr. Mirjana Jerkic.
3.12 Normal MPO, Nox-2 and ROS production in \( \text{Eng}^{+/–} \) bone marrow

The alterations in MPO, Nox-2 and ROS production in the colon of colitic \( \text{Eng}^{+/–} \) mice suggested either an intrinsic neutrophil defect or the response of neutrophils to the inflamed colonic environment during colitis progression in \( \text{Eng}^{+/–} \) mice. The bone marrow expression levels of MPO and Nox-2 were next analyzed to test if the differences in the colon were intrinsic to myeloid cells. MPO expression increased at days 7-9 of colitis in both genotypes, however, by days 18-21 MPO levels returned to basal levels in all groups (Figure 3.13A). Nox-2 expression increased in bone marrow of \( \text{Eng}^{+/+} \) mice by days 18-21 and a trend for higher levels was observed in \( \text{Eng}^{+/–} \) mice (Figure 3.13B). Although MPO and Nox-2 expression increased with colitis, normal activation of bone marrow phagocytes was observed in both \( \text{Eng}^{+/+} \) and \( \text{Eng}^{+/–} \) mice. In addition, at days 0, 12 and 25, neutrophils derived from both genotypes generated the same level of superoxide when stimulated with PMA (Figure 3.13C). As a result, the capacity to generate ROS is not defective in \( \text{Eng}^{+/–} \) bone marrow, suggesting that intrinsic neutrophil defects do not contribute to colitis progression in \( \text{Eng}^{+/–} \) mice.
**Figure 3.13 Bone marrow Nox-2 and MPO levels and ROS production by isolated bone marrow neutrophils are not different between Eng$^{+/+}$ and Eng$^{+/−}$ mice**

A) MPO expression increased in bone marrow of days 7-9 colitic mice and declined to basal levels by days 18-21 in both groups of mice. B) Total bone marrow Nox-2, measured by WB, increased at days 18-21 in Eng$^{+/+}$ mice with a trend for higher levels in Eng$^{+/−}$ mice. *P<0.05 vs. corresponding day 0, #P<0.05 vs. corresponding days 9-12. C) Superoxide (O$_2^−$) production, measured by DHE assay, was tested in isolated neutrophils from days 0, 12 and 25; it was increased in all time points and in both genotypes with PMA stimulation. *P<0.05 vs. corresponding unstimulated group. For WB experiments, results represent mean ± SEM (For Nox-2 WB: N=3 mice for day 0, 3-4 for days 7-9 and 4-5 for days 18-21; For MPO WB: N=3 mice for day 0, 3-4 for days 7-9 and 4-5 for days 18-21). For DHE assay, results are plotted as fold change relative to the unstimulated Eng$^{+/+}$ control for each time point (N=8 samples for days 0 and 12 and 4 for day 25). These experiments were performed by Dr. Mirjana Jerkic and Valentin Sotov.
Chapter 4
Discussion

4 Discussion

Our studies indicate that the recovery from DSS-induced colitis is delayed in Eng\(^{+/−}\) mice compared to wild-type mice. This difference was not attributed to major changes in the number and distribution of infiltrating or resident colonic leukocytes. Furthermore, no major changes were observed in spleen or bone marrow immune subsets between colitic Eng\(^{+/−}\) and wild type mice. However, dysregulation of factors that control angiogenesis, inflammation, TGF-β/BMP signaling and neutrophil activation was observed in Eng\(^{+/−}\) mice. Furthermore, downregulation of enzymes responsible for the oxidative burst of neutrophils and macrophages was observed and may contribute to impaired bacterial clearance and subsequent resolution of inflammation.

4.1 Confirmation of slower recovery in DSS-challenged Eng\(^{+/−}\) mice

The DSS model of colitis is a very useful tool in understanding the cellular and molecular mechanisms disrupted in IBD pathogenesis. Typically, acute responses are studied immediately after administration of a single cycle of 2-5% DSS for 4-9 days whereas for chronic colitis, multiple cycles of DSS are utilized (114). When given a single challenge of DSS, the C57BL/6 strain is a stronger responder than the BALB/c strain, as these mice develop acute colitis with worse clinical symptoms (i.e. weight loss and diarrhea) and according to some reports, sustained colonic disease (112). In our previous study, challenging Eng\(^{+/+}\) and Eng\(^{+/−}\) mice with 3% DSS for 5 days resulted in a similar and pronounced disease course in both groups, reaching a peak at days 7-9, followed by delayed recovery from clinical symptoms observed till days 19-26 in Eng\(^{+/−}\) mice compared to their Eng\(^{+/+}\) littermates (116). In this study, we confirmed a similar colitis progression in both groups of mice until days 7-9 and a slower rate of recovery in Eng\(^{+/−}\) mice, most noticeable between days 13-17; however, the difference between colitic Eng\(^{+/+}\) and Eng\(^{+/−}\) mice was not as pronounced as previously reported. The severity of DSS induced colitis is dependent on several factors including the particular batch of DSS, dosage, duration, manufacturer and resident microflora composition (108). The fact that the mice used in this study were housed in a cleaner facility from the previous experiments may contribute to the dampened
disease response in $Eng^{+/−}$ mice. However, given that this difference in recovery rate is maintained, we continued with the characterization of the immune response in both groups prior to induction of colitis, at the peak of inflammation (days 7-9) and during disease progression or recovery (days 18-22).

4.2 Regulation of T and B cell numbers during colitis is not defective in $Eng^{+/−}$ mice

The colonic lamina propria normally contains a large reservoir of immune cells, including lymphocytes and myeloid cells, which are important for maintaining gut homeostasis (117). In non-inflammatory conditions, T and B cells together represent the majority of immune cells in the colon. The administration of DSS results in the destruction of the protective mucus and epithelial layers of the gut, enabling bacterial translocation and resulting in increased lamina propria lymphocyte numbers (107). The increase in colonic B cell numbers during DSS induced colitis was not impaired in $Eng^{+/−}$ mice; however, elevated expression of mRNA for CD79A (Ig-alpha), a member of the B cell receptor complex (BCR) important for BCR expression and function, suggests increased B cell activation in $Eng^{+/−}$ mice (134). This may be explained by increased antigen exposure in $Eng^{+/−}$ mice, due to decreased gut epithelial regeneration and development of abscesses as reported previously (116).

In addition to expansion of B cell numbers, increased colonic CD3$^+$ T cell numbers has been reported at day 25 in study in which mice were given a 3% DSS course for 6 days (113). While there were higher numbers of total T cells, we did not observe differences in subset distribution (T helper cells, cytotoxic T lymphocytes and T-regulatory cells) between the groups. In terms of T helper cell subsets, there are reports of increased IL-17 and IFNγ in colon as well as higher Th1, Th17 and Th2 cytokines in plasma of DSS-treated mice (112, 114). In contrast, our findings demonstrated colonic IL-4, IFNγ and IL-17 levels were at the limit of detection and not elevated with colitis in either group. Analysis of cytokine production in isolated lamina propria cells may have been a more sensitive approach to study these factors. In addition, $Eng^{+/+}$ and $Eng^{+/−}$ mice demonstrated a similar decrease in spleen B and T cells, confirming a previous study of C57BL/6 mice, and unaltered peripheral blood lymphocytes counts (113, 129). Overall, the DSS-induced changes in colonic and systemic B and T lymphocyte numbers during inflammation appear unaltered in $Eng^{+/−}$ mice.
4.3 The potential contribution of macrophages and/or eosinophils to persistent disease in Eng<sup>+/−</sup> mice

Myeloid cells are crucial regulators of the DSS model of inflammation, and monocytes/macrophages and granulocytes have been shown to play central roles in mediating innate and adaptive responses during colitis (111, 143). Under basal conditions, the gut mucosa is the organ that contains the largest macrophage population that is continually replenished by IL-8 and TGF-β mediated recruitment of circulating monocytes (144). Although these macrophages phagocytose and digest microorganisms to prevent translocation, they normally do not promote pro-inflammatory response (i.e. IL-6 and IL-1β secretion) (145). During gut inflammation, such as infection-induced, pro-inflammatory cytokines/chemokines (i.e. CCL2, type I interferon) as well as TLR ligands are involved in the recruitment of additional Ly6C<sup>+</sup> blood monocytes to the gut to aid in bacterial clearance (122). Overall, the slower recovery in Eng<sup>+/−</sup> mice was not associated with defective recruitment and development in the bone marrow of these circulating monocytes (CD11b<sup>+</sup>F480<sup>−</sup>Ly6C<sup>+</sup> cells) during colitis.

The CD11b<sup>+</sup>F480<sup>+</sup> subset, usually representing macrophages, was the predominant immune subset in the colon of Eng<sup>+/−</sup> and wild type mice, under basal conditions, suggesting that maintenance of this population in the gut is normal in Eng<sup>+/−</sup> mice. In contrast to similar studies using DSS-treated C57BL/6 mice (113, 146), we did not observe an increase in the number and distribution of CD11b<sup>+</sup>F480<sup>+</sup> cells in the colon during the acute phase (days 7-9). While there was a decline in distribution of this colonic subset by days 18-22 in both groups of mice due to infiltration by monocytes and granulocytes, an increase in the total number of CD11b<sup>+</sup>F480<sup>+</sup> cells was demonstrated only in Eng<sup>+/−</sup> mice. In the spleen, we were able to confirm the increase in CD11b<sup>+</sup>F480<sup>+</sup> cells by days 20-22, which was similar in both genotypes. In addition, although endoglin has been shown to be involved in myeloid differentiation, examination of similar markers in the bone marrow demonstrated no intrinsic impairment in the development of these cells. Interestingly, as much as one third of colonic F480<sup>+</sup> cells have been previously identified as eosinophils, characterized by flow cytometry as MHCII<sup>lo</sup>SSC<sup>hi</sup> as opposed to MHCII<sup>hi</sup>SSC<sup>lo</sup> macrophages (121). While analysis of histologic sections of the colon at days 18-22 confirmed this population to be predominantly macrophages, there are a number of studies that demonstrated an important role of eosinophils in the DSS model and IBD and may be implicated at days 7-9 (128, 146, 147).
Eosinophils, members of the granulocyte family, are normally found as resident cells in many organs (i.e. GI tract, thymus, spleen and ovaries), however, increased numbers have been implicated in the pathogenesis of many inflammatory diseases including asthma and IBD (148). Functionally, eosinophils are most notably recognized for their ability to clear parasitic infections through many effector molecules (i.e. ROS); however, their involvement in regulation of multiple immune pathways through production of various cytokines (i.e IL-4, IL-12, IFNγ, IL-1β, IL-6, IL-10, TGF-β) has been suggested (149). In IBD patients, elevated levels of secreted eosinophil lipid and granular proteins (i.e. major basic protein, eosinophil peroxidase, eosinophil cationic protein and eosinophil-derived neurotoxin) have been shown to correlate with disease severity (150, 151). Although we did not observe a difference in colonic CCL11 levels, a potent chemoattractant for eosinophils (133), between Eng^{+/−} and Eng^{+/−} mice, eosinophil recruitment and activity have been associated with worse disease in DSS-treated mice (147). Indeed, elevated levels of CCL11 (eotaxin-1) have been shown in IBD patients (146). More recently, it was found that infiltration of CD11b^{−/−}F480^{−/−}Ly6C^{hi} inflammatory monocytes/macrophages, expressing markers of M1 and M2 macrophage subsets (i.e Arginase 1, IL-10, CCL4, IL-1β, IL-6, CXCL2), in the gut of colitic mice is involved in recruitment of eosinophils after DSS induction (128). While there was a similar number of these inflammatory monocytes/macrophages in both Eng^{+/+} and Eng^{+/−} mice, the delayed recovery in Eng^{+/−} mice could be attributed to increased eosinophil effector molecules and/or a defect in regulation of this subset by inflammatory monocytes/macrophages.

4.4  Leukocyte recruitment and infiltration is normal in Eng^{+/−} mice

During acute inflammation, neutrophils are one of the earliest responders to inflammatory cues and fulfill a multitude of antimicrobial effector functions (3). In addition to being the site of neutrophil development, the mouse bone marrow also functions as the main reservoir for neutrophils (152). We found that the percentage and number of bone marrow neutrophils were the same in wild type and Eng^{+/−} mice under basal conditions, indicating that there was no intrinsic defect in the generation of neutrophils in these mice. During inflammation, such as with DSS colitis, cytokines/chemokines that activate and promote recruitment of neutrophils from the bone marrow into circulation are secreted from the site of inflammation (108). These factors include: (1) G-CSF, which is important for neutrophil survival, proliferation and release, (2)
CXCL1 and (3) CXCL2, both of which are homologs to human IL-8 and are potent chemoattractants for neutrophils (153).

TGF-β has also been identified as a strong chemoattractant for neutrophils (84, 85). The decline in bone marrow neutrophils and increase in peripheral blood neutrophils was similar in both groups indicating normal recruitment to the site of inflammation in Eng+/− mice. In addition, the DSS-induced expansion of bone marrow neutrophils at days 18-21 (154) was unaltered between Eng+/+ and Eng+/− mice. We can therefore conclude that the recovery of neutrophils in the bone marrow was not defective in Eng+/− mice. In agreement with the observation that the colonic tissue of Eng+/− mice produced a higher level of CXCL1 and G-CSF at days 7-9 of colitis, there was a trend for higher numbers of neutrophils in Eng+/− mice. Furthermore, the previously observed increased vascular permeability and vessel number in Eng+/− mice may also account for the increase in colonic macrophages and neutrophils during DSS-colitis. In summary, the development and recruitment of neutrophils was not found to be impaired in Eng+/− mice as illustrated in the model shown in Figure 4.1.
Figure 4.1 Summary model of leukocyte recruitment in DSS-treated $Eng^{+/-}$ mice

DSS-induced colitis led to an increase in colonic factors, such as CXCL1 and G-CSF, which promote recruitment and proliferation of myeloid cells from the bone marrow. Although the number of peripheral blood neutrophils (yellow) and monocytes (blue) was normal in $Eng^{+/-}$ mice, increased transmigration of neutrophils through the vessels and higher macrophage numbers (green) were observed in the colon of colitic $Eng^{+/-}$ mice. The DSS model also leads to systemic bacterial translocation. We did not observe impairment in the ability of myeloid cells to migrate to the spleen in $Eng^{+/-}$ mice. Adapted from Chidlow et al, Am J Physiol Gastrointest Liver Physiol 293:G5-G18, 2007 (99).
4.5 Dysregulation of factors controlling angiogenesis and inflammation in colitic Eng\textsuperscript{+/-} mice

At days 19-26, we found that the sustained inflammation in Eng\textsuperscript{+/-} mice was accompanied by pathological angiogenesis in the gut and increased levels of the pro-angiogenic factor, VEGF-A (116). As there were minimal changes in leukocyte numbers in Eng\textsuperscript{+/-} mice during DSS colitis, the colonic expression profile of factors regulating angiogenesis, inflammation and TGF-\(\beta\)/BMP pathways was assessed at days 18-21. All the genes that were significantly altered in Eng\textsuperscript{+/-} mice, with the exception of CD79A, were downregulated, including: thrombospordin-1 (Thsb1), placental growth factor (Pgf), TGF-\(\beta\)-induced (Tgfbi), BMP binding endothelial regulator (BMPER), follistatin, IL-6, IL-1\(\beta\) and CCL11. Although many of these molecules exert pro-angiogenic effects on the vascular endothelium, some are negative regulators of angiogenesis, suggesting that an imbalance in angio- and angiostatic factors may contribute to disease progression in Eng\textsuperscript{+/-} mice. For instance, while there were lower mRNA levels of the pro-angiogenic VEGF family member, Pgf (131), there was downregulation of an important angiostatic factor, Thbs1 (130). In the multi-challenge DSS model of chronic colitis, Thbs1 knockout mice demonstrated an increase in vessel number in the colon and dysplasia (155). The decrease in Thbs1 mRNA in colitis was confirmed by Western blot in our studies, suggesting that Thbs1 may indeed be normally implicated in down-regulating inflammation-driven angiogenesis.

Interestingly, many TGF-\(\beta\)/BMP regulating pathway members were expressed at lower mRNA levels in the colons of Eng\textsuperscript{+/-} mice. Furthermore, decreased total Smad2 levels and a proportional decrease in phosphorylated Smad2 leading to decreased TGF-\(\beta\) signaling was observed in colitic Eng\textsuperscript{+/-} mice. The decrease in Tgfbi expression, a secreted protein important for modulating cell adhesion to the extracellular matrix (ECM) and upregulated by TGF-\(\beta\) stimulation (136, 156), also supports the notion of impaired TGF-\(\beta\) signaling in colitic Eng\textsuperscript{+/-} mice. BMPER, which was first identified in endothelial precursor cells, is a secreted protein found in the ECM that functions as an extracellular regulator of BMP signaling through interaction with BMP 2, 4 and 6 (157). In vitro studies found that the action of BMPER is dose-dependent, as lower levels enhance HUVEC sprouting and migration through activation of BMP4 signaling, while higher levels of BMPER inhibit this pro-angiogenic effect (135). Bmper\textsuperscript{+/-} mice subjected to the oxygen-induced retinopathy model, demonstrated faster hypoxia-
induced retinal revascularization (158). In addition, BMPER has been found to regulate inflammation-induced activation of endothelium, such as expression of the adhesion molecules ICAM1 and VCAM1 (159). In an atherosclerosis mouse model, Bmper\textsuperscript{+/−} mice demonstrated enhanced plaque formation, increased macrophages and expression of the aforementioned adhesion molecules in these lesions (160). Furthermore, knockdown of BMPER in zebrafish leads to impaired hematopoiesis and vascular network formation (161). Follistatin, a single chain monomeric glycoprotein expressed as a soluble protein or associated with cell surface proteoglycans, is known to regulate activin, a TGF-β superfamily ligand (137). In the acute DSS and trinitrobenzene sulfonic acid colitis models, administration of exogenous follistatin enabled some restoration of epithelial barrier function and of colonic architecture (138, 162).

While decreased Smad2 levels and reduced expression of Tgfbi imply attenuation of TGF-β signaling in colitic Eng\textsuperscript{+/−} mice, the down-regulation of BMPER and follistatin suggests alterations in BMP and activin mediated angiogenesis, repair and inflammatory responses. Endoglin is known to modulate responses not only to TGF-β1 and -β3 but also to several BMPs and to activin (51). These results suggest that endoglin alters these factors that play an important role in inflammation-driven angiogenesis.

In contrast to expected results (112), down-regulation of mRNA levels for the pro-inflammatory and pro-angiogenic cytokines/chemokines CCL11, CXCL1, IL-1β and IL-6 was observed in days 18-21 colitic Eng\textsuperscript{+/−} mice. While the protein levels of these mediators were initially increased to similar levels in both Eng\textsuperscript{+/−} and wild type mice at days 7-9 of the DSS course, these factors were restored to basal levels by days 18-21. This decrease in transcript levels may represent a strong negative feedback loop, with IL-6 demonstrating the most dramatic down-regulation. IL-6 can be produced by various cells types (i.e. lymphocytes, monocytes, fibroblasts, keratinocytes and endothelial cells) and is known to regulate a wide range of biological activities. These include: promotion of acute phase protein production (i.e. C-reactive protein), activation of adhesion molecule expression on endothelial cells (i.e. ICAM1 and VCAM1), production of chemokines in many cell types (i.e. CCL2 and CCL8), activation of antibody production in B cells and stimulation of neutrophil production in the bone marrow (163, 164). Elevated levels of IL-6 have been found in patients with IBD and in many IBD models (165, 166). Although TGF-β can interfere with IL-6 induced STAT1 and STAT3 activation in intestinal epithelial cells in a Smad2 dependent fashion (167), there is some evidence that TGF-β
can also stimulate IL-6 expression using Smad2/3 signaling (139). In addition to TGF-β regulation, BMP6 has also been shown to promote macrophage expression of IL-6 and IL-1β (168, 169). At days 18-21, the combination of impaired epithelial repair and the down-regulation of pro-inflammatory cytokine IL-6 in Eng-/- mice, which may be attributed to dysregulation of TGF-β/BMP mediated signaling, could cause impaired immune responses to invading microbes and as a result, altered recovery. Figure 4.2 briefly summarizes the findings of the altered TGF-β/BMP signaling pathways in colitic Eng-/- mice.
Figure 4.2 Dysregulated TGF-β/BMP signaling pathways contribute to persistent disease in Eng<sup>+/−</sup> mice
Endoglin modulates TGF-β superfamily pathways. In the current study, we observed a decrease in SMAD 2/3 levels, suggesting reduced response to TGF-β. Many of the factors altered in Eng<sup>+/−</sup> colitic mice, as demonstrated by the PCR array data are downstream of this pathway. Interestingly, altered expression of factors, including Thbs1 and IL-6, have been attributed to dysregulation of the SMAD 1/5/8 pathway. Overall, the decreased expression of these factors may contribute to the impaired epithelial and tissue repair, pathological angiogenesis and potentially dampening of immune responses associated with persistent disease in Eng<sup>+/−</sup> mice.
4.6 Decreased MPO and Nox2 levels in Eng\textsuperscript{+/−} mice may lead to improper bacterial clearance

Although there were indications for higher colonic neutrophil numbers in Eng\textsuperscript{+/−} mice, lower levels of the neutrophil ROS regulating molecules, MPO and Nox2, were found in colitic Eng\textsuperscript{+/−} mice. Upon recruitment and activation, neutrophils employ a number of strategies against invading microorganisms, including: engulfment/digestion of bacteria, production of ROS, degranulation to release antimicrobial molecules into the extracellular space (i.e. elastases and defensins) and release of neutrophil extracellular traps (NETs), which are dense strands of DNA and proteins that capture bacteria (140, 170). Neutrophils can also mediate activation of leukocytes through secretion of cytokines and chemokines, such as CCL2 and IL-1β (171). As a decrease in ROS regulating enzymes was observed in the colon of colitic Eng\textsuperscript{+/−} mice, we will now focus on the implications of reduced ROS production on bacterial clearance.

During an inflammatory response, phagocytes (i.e. macrophages and neutrophils) are activated and produce large amounts of ROS, which are potent molecules that can efficiently kill microbes; however, if this production is not tightly controlled, damage to host tissue through reaction with lipids, proteins and DNA can occur (141). The phagocyte NADPH oxidase is essential for efficient microbial killing by producing superoxide ions that are rapidly dismutated into H\textsubscript{2}O\textsubscript{2}. NADPH oxidase is a complex of proteins, including: gp91\textsuperscript{phox} (Nox2, catalytic subunit), p22\textsuperscript{phox}, p67\textsuperscript{phox}, p47\textsuperscript{phox} and small GTP-binding protein (Rac) (142). In resting state, the p47\textsuperscript{phox} subunit SH3 domain is bound to its auto-inhibitory region; however, upon activation (i.e. phagocytosis and PMA stimulation) the phosphorylation of p47\textsuperscript{phox} allows binding to p22\textsuperscript{phox} and Rac translocation (172).

Impairment in phagocyte respiratory burst resulting in inefficient bacterial and fungal clearance can also be detrimental to the host. In patients with chronic granulomatous disease (CGD), where 65% have Nox2 mutations, defective ROS production increases susceptibility to infection and in some cases, causes IBD-like symptoms (173). In IBD patients with active disease, defective recruitment and production of superoxide in neutrophils may contribute to disease pathogenesis (174, 175). In contrast, DSS-treated gp91\textsuperscript{phox} −/− mice demonstrated decreased ulceration and neutrophil infiltration, and lower II-6 and II-10 levels indicating ROS involvement in IBD (176). Initially, Eng\textsuperscript{+/−} and wild type colitic mice show the same increase in
Nox2; however as colitis progress, Nox2 goes down in Eng$^{+/+}$ mice while maintained in wild type mice. Eng$^{+/+}$ mice showed elevated basal levels of H$_2$O$_2$ in the distal colon, in agreement with an eNOS-dependent rise in superoxide production reported in lungs of these mice (Toporsian 2010). However, no further elevation of ROS was induced by DSS colitis in Eng$^{+/+}$ mice, whereas an increase in ROS was seen in colitic Eng$^{+/+}$ mice. This suggests that Eng$^{+/+}$ mice were less able to increase ROS production in response to an inflammatory stimulus and consequently may have lower anti-microbial activity. We could rule out an intrinsic bone marrow neutrophil defect, as Nox2 levels and ROS production in PMA stimulated neutrophils were not different in Eng$^{+/+}$ compared to wild type mice.

MPO is another neutrophil enzyme that synthesizes highly reactive ROS (177). MPO is concentrated in the azurophilic (primary) granules of neutrophils. It is also present in some monocytes, where it declines after differentiation into tissue macrophages (178). As observed in other DSS colitis studies (114), higher colonic MPO activity and levels were observed in colitic mice; however like Nox2, MPO remained higher in Eng$^{+/+}$ mice throughout colitis whereas it decreased with time in the colitic Eng$^{+/+}$ mice. The key substrate for MPO is H$_2$O$_2$, which binds to halides to form hypochlorous acid (141). There is some evidence of dependence of MPO on Nox2 activity, as NADPH oxidase is responsible for superoxide and subsequently H$_2$O$_2$ production; furthermore, some CGD patients exhibit reduced MPO activity (142). Treatment of human CGD neutrophils with PEGylated d-amino acid, which increases H$_2$O$_2$ levels, increased MPO activity and improved bacterial killing (179). Interestingly, in a renal ischemia-reperfusion model, lower MPO levels were observed in Eng$^{+/+}$ mice; however, in contrast to our findings, this was due to decreased infiltration (180). While the relationship, if any, between the expression of Nox2 and MPO levels and endoglin remains to be determined, lower Nox2 activity may further reduce MPO activity by decreasing substrate availability.

Although the role of endoglin in regulating Nox2 and MPO is unclear, deficiencies in these oxidative burst regulators leads to defective formation of NETs and subsequently impairment in microbial clearance (181, 182). More recently, the signaling lymphocyte-activation molecule family (SLAMF, 1-9) of receptors was described on the surface of many hematopoietic cells. These molecules were found to play multiple roles in immune processes, including co-stimulation, T cell cytokine production, NK and CD8 T cell mediated toxicity and phagocyte functions (183). In Slamf$^{+/+}$ mice, impaired macrophage clearance of gram-negative bacteria due
to decreased Nox2 activity and delayed phagosome maturation was observed (184). In contrast, macrophage expression of Slamf8 was found to negatively regulate Nox2 through inhibition of p47phox phosphorylation (185). In neutrophils, loss of Slamf6 resulted in increased IL-6, IL-12, TNF-α and lower ROS production (186). As endoglin has been shown to regulate monocyte activity, dysregulated expression of the Slam family may contribute to alterations in the oxidative burst enzymes. Figure 4.3 summarizes the current findings of impaired phagocyte oxidative burst activity in Eng+/- mice.
Figure 4.3 Impairment of neutrophil mediated bacterial clearance in $Eng^{+/−}$ mice with persistent inflammation

Decreased myeloperoxidase levels and activity in colon despite a trend for a higher neutrophil number suggests defective neutrophil activity in $Eng^{+/−}$ mice. In addition, lower levels of the phagocyte NADPH oxidase component, Nox2, and a lower than expected rise in ROS production further point to decreased antimicrobial activity in colitic $Eng^{+/−}$ mice. In addition to direct effects of ROS, the lower levels of MPO and Nox2 could lead to defective NET formation. Interestingly, potential alterations in members of the SLAM family of receptors that regulate phagocyte ROS activity may contribute to lower ROS production in $Eng^{+/−}$ mice. Adapted from Nathan C, Nature Reviews Immunology 6(3):173-82, 2006 (187).
4.7 Concluding remarks

While endoglin has been shown to regulate myelopoiesis and some monocyte functions, the recruitment, expansion and development of leukocytes was not found to be severely impaired in DSS-treated Eng\(^{+/−}\) mice. Defects in resolution of inflammation in Eng\(^{+/−}\) mice were accompanied by alterations in factors that modulate angiogenesis and inflammation, several belonging to the TGF-β/BMP superfamily. In addition, we also report a novel role of endoglin in regulating phagocyte ROS production during inflammatory responses. There is still much that remains to be studied in order to understand the specific mechanisms whereby endoglin mediates recovery from inflammation. As TGF-β has been identified as an important regulator of multiple pathways during inflammation, our study indicates a role for endoglin in mediating at least some of these processes, including immune, vascular and tissue repair responses.

4.8 Future Directions

In order to further explore the role of endoglin in regulating neutrophil and macrophage responses, the systemic levels of MPO and Nox2 will be analyzed in the spleen and potentially the mesenteric lymph nodes of colitic Eng\(^{+/+}\) and Eng\(^{+/−}\) mice. The expression levels of SLAMs (1,6,8) known to regulate phagocyte ROS production in distal colons of Eng\(^{+/+}\) and Eng\(^{+/−}\) mice will also be assessed. If phagocyte function is defective, we postulate that persistent inflammation will be associated with less bacterial clearance. The 16S bacterial rRNA levels will be measured in distal colonic tissue of colitic mice. In addition, NET formation assays will be performed on sections of colonic tissues during colitis to determine if there is impairment in the ability of neutrophils to trap bacteria in Eng\(^{+/−}\) mice.
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