ABSTRACT

CD34, podocalyxin and endoglycan are cell surface sialomucins, which comprise the CD34-family of proteins. While many roles have been proposed for the CD34 family of molecules, little is known about their functions in disease.

In a phenylhydrazine-induced model of anemia, we demonstrated that podocalyxin is rapidly induced on early stress erythroid progenitors, from an early BFU-E stage, through to immature reticulocytes. Our data suggest that although podocalyxin is not required for recovery from anemia, it can be used as a simple marker of stress erythroid progenitors.

CD34 is expressed on mature inflammatory cells including mast cells, eosinophils and dendritic cell precursors. Interestingly, in an OVA-induced asthma model, \textit{Cd34}^{-/-} mice exhibited attenuated pathology and reduced immune cell infiltration into the alveoli and lung parenchyma. CD34 expression is present on lung and BAL eosinophils, and isolated \textit{Cd34}^{-/-} eosinophils exhibit a migration defect \textit{in vitro}. These findings suggest a key role for CD34 in asthma pathology.

Similarly, in a dextran sulfate sodium-induced colitis model, \textit{Cd34}^{-/-} mice exhibited significantly reduced pathology, associated with loss of CD34 expression on colon-infiltrating eosinophils. Eosinophil numbers were reduced in \textit{Cd34}^{-/-} tissues and induction of hypereosinophilia in \textit{Cd34}^{-/-} mice was sufficient to overcome the protection from DSS-induced disease. Thus, we have also demonstrated a key role for CD34 and eosinophil trafficking in ulcerative colitis.

Finally, in a B16 melanoma tumor model, \textit{Cd34}^{-/-} mice exhibit altered tumor growth
kinetics. At early stages, \( Cd34^{-/-} \) mice exhibited reduced tumor size, due to loss of CD34 expression on non-hematopoietic cells (likely vascular endothelial cells). Conversely, at later timepoints, \( Cd34^{-/-} \) mice exhibited increased tumor growth, due to loss of CD34 expression on hematopoietic cells. These findings suggest complex, opposing roles for CD34 in tumor growth due to effects on vascular integrity in early disease and immune cell-mediated tumor rejection later in the disease.

These findings suggest key roles for CD34-family proteins in a range of disease states. Podocalyxin expression is induced during embryonic and stress erythropoiesis and is an indicator of anemia. CD34 plays a key role in optimal immune cell migration during asthma, colitis and tumor growth.
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\( \Delta \text{dblGATA-1} \) Eosinophil-ablated mouse strain (deletion of two autoregulatory domains in the GATA-1 gene)

5-FU 5-fluorouracil

5-oxo-ETE 5-oxo-6,8,11,14-eicosatetraenoic acid

AHR Airway hyper-responsiveness

APC Allophycocyanine (fluorochrome)

AR Airway resistance

BAL Bronchoalveolar lavage

BFU-E Blast-forming unit - erythroid

BM Bone marrow

BMMCs Bone marrow-derived mast cells

BMP4 Bone morphogenic protein 4

CBA Cytokine bead array

CCR3 Eotaxin receptor

CD Crohn’s disease

CD34 \( Cd34 = \) gene, \( CD34 = \) protein

CFU-E Colony-forming unit - erythroid

CFU-Eos Colony-forming unit – eosinophil

CFU-GM Colony-forming unit – granulocyte / macrophage

CFU-Mast Colony-forming unit – mast cell

CFU-Meg/Mk Colony-forming unit - megakaryocyte

CFSE Carboxyfluorescein succinimidyl ester

CLP Common lymphoid progenitor

CMP Common myeloid progenitor

DAB 3,3’-Diaminobenzidine

DMBA 7, 12-dimethylbenz(a)anthracene

DMSO Dimethyl sulfoxide

DSS Dextran sulfate sodium

ECP Eosinophil cationic protein

EPO Eosinophil peroxidase

Epo Erythropoietin

Epo-R Erythropoietin (Epo) receptor
EpoR-H  Mutated erythropoietin receptor with intact Stat5 signalling
EpoR-HM Mutated erythropoietin receptor lacking Stat5 signalling
EPX  Eosinophil protein X (alternate name for eosinophil peroxidase)
ES cells  Embryonic stem cells
FACS  Fluorescence-activated cell sorting
FBS  Fetal bovine serum
FITC  Fluorescein isothiocyanate (fluorochrome)
GMP  Granulocyte – macrophage progenitor
H&E  Hematoxylin and eosin
HEV  High endothelial venule
hpf  High power field
HSC  Hematopoietic stem cell
IBD  Inflammatory bowel diseases
ICAM-1 Inter-cellular adhesion molecule (CD54) which binds LFA-1
i.p.  Intraperitoneal
i.v.  Intravenous
KLS  Sca-1⁺ c-kit⁺ Lineage marker⁻ cell subset
Ly5.1  CD45.1 isoform
Ly5.2  CD45.2 isoform
MBP  Major basic protein
MCh  Methacholine
MCP-1  Monocyte chemotactic protein-1 (CCL2)
MEP  Megakaryocyte – erythroid progenitor
MIP-1α  Macrophage inflammatory protein-1 (CCL3)
MMPs  Matrix metalloproteinases
NK cell  Natural killer cell
NKP  Natural killer cell progenitor
OVA  Ovalbumin
p.c.  Post-coitus
PCR  Polymerase chain reaction
PE  Phycoerythrine (fluorochrome)
PHIL  Eosinophil-ablated mouse strain (EPO promoter driving expression of diptheria toxin A)
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<tr>
<td>PHz</td>
<td>Phenylhydrazine</td>
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<tr>
<td>PMA</td>
<td>Phorbol myristate acetate</td>
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<td>Podxl</td>
<td>Podocalyxin</td>
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<tr>
<td>ProE</td>
<td>Proerythroblast</td>
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<tr>
<td>PSGL-1</td>
<td>P-selectin glycoprotein ligand-1</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
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<tr>
<td>s.c.</td>
<td>Subcutaneous</td>
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<tr>
<td>SCF</td>
<td>Stem cell factor</td>
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<tr>
<td>SD</td>
<td>Standard deviation of the mean</td>
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<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SHIP</td>
<td>SH2-containing inositol-5-phosphatase</td>
</tr>
<tr>
<td>SSC</td>
<td>Light side scatter (granularity)</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor alpha</td>
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<tr>
<td>TPA</td>
<td>12-O-tetradecanoylphorbol-13-acetate</td>
</tr>
<tr>
<td>Tpo</td>
<td>Thrombopoietin</td>
</tr>
<tr>
<td>UC</td>
<td>Ulcerative colitis</td>
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<td>VCAM-1</td>
<td>Vascular cell adhesion molecule-1 (CD106) which binds VLA-4</td>
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<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WBC</td>
<td>White blood cell</td>
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DEDICATION

To my parents….even though I haven’t said it often enough or clearly enough…thank you for everything and I love you.

And to my little brother, who fought long and hard and this February, just didn’t have the energy to keep fighting anymore. You will be sorely missed by all of us, I hope your pain is gone.
CO-AUTHORSHIP STATEMENT

Chapter 2. Steven Maltby and Michael R. Hughes designed and performed all research and wrote the paper. Lori Zbytnuik performed part of the research. Robert F. Paulson and Kelly M. McNagny designed research and edited the paper.

Chapter 3. Marie-Renee Blanchet designed research, performed research and wrote the paper. Steven Maltby helped with asthma disease timecourses (Figure 3.1 and 3.8), did all flow cytometry staining (Figure 3.5) and in vitro eosinophil migration assays (Figure 3.8) and helped write the paper. D. James Haddon performed some of the research and helped write the paper. Lori Zbytnuik and Helen Merkens performed part of the research. Kelly M. McNagny designed research and edited the paper.

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Chapter 5. Steven Maltby designed and performed all research and wrote the paper. Jennifer Baker helped with immunohistochemistry experiments. Kelly M. McNagny designed research and edited the paper.

Appendix A. Steven Maltby designed and performed all research and wrote the appendix. Michael R. Hughes, Justin Wong, and Lori Zbytnuik helped design and perform experiments. Kelly M. McNagny designed research and edited the manuscript.
CHAPTER 1. INTRODUCTION

1.1 CD34 AND THE CD34-RELATED FAMILY OF PROTEINS

CD34, podocalyxin and endoglycan are cell surface sialomucins, which comprise the CD34-family of proteins based on structural homology and a conserved genomic organization, as recently reviewed (Nielsen & McNagny, 2008; Furness & McNagny, 2006).

CD34 is best known for its expression on hematopoietic progenitors and has been extensively used, as a marker, to purify hematopoietic stem cells. CD34 binding to L-selectin in high endothelial venules (HEVs) led to suggestions that CD34 plays a critical pro-adhesive role during leukocyte migration in vivo (Baumheter et al., 1993). However, L-selectin binding requires a specific carbohydrate modification, which appears to be HEV-specific. More recently, CD34 expression has been demonstrated on immature and mature mast cells and eosinophils, where it acts as an anti-adhesive molecule, allowing efficient cell migration, and enhancing inflammatory responses.

Podocalyxin is expressed on kidney podocytes (from where it derives its name), particularly on specialized foot processes within the glomerulus (Kerjaschki et al., 1984). Podocalyxin expression is required for the formation of filtration slits between podocytes during development, which are critical for the filtration of urine and regulation of blood pressure (Doyonnas et al., 2001). More recent studies have
demonstrated that podocalyxin is expressed on hematopoietic progenitors and on erythroid progenitors during fetal embryogenesis and during recovery from anemia in adults, although its function on these cell types remains unclear.

Endoglycan, the most recently identified member of the CD34 family, is expressed predominantly on early hematopoietic progenitors and endothelial cells. One recent report demonstrated that endoglycan is expressed on mature human B cells, T cells and circulating monocytes, where it acts as a selectin ligand (Kerr et al., 2008).

The function of each CD34 family protein is largely dependent on post-translational modifications, which vary in different cell types. Several roles have been proposed for the CD34 family of molecules, including both pro- and anti-adhesive functions in cell migration, roles in cell proliferation and effects on cell morphology. However, little is known about both the intracellular and the extracellular ligands for each of these molecules or the molecular mechanism governing their functions.
1.1.1 Protein structure and cellular localization

CD34, podocalyxin and endoglycan are Type-I transmembrane proteins, present on the extracellular membrane. While the three proteins have relatively low primary sequence homology, they do share several conserved motifs, including a serine-, threonine-, proline-dense extracellular mucin domain, a globular domain, a single transmembrane domain, and a highly conserved cytoplasmic tail containing potential phosphorylation sites (Figure 1.1A). In addition to the primary amino acid structure, each molecule is extensively post-translationally modified by glycosylation and sialylation, greatly increasing the apparent mass of each molecule and altering protein function in different cell types (e.g. CD34 binding of L-selectin on HEVs). All three proteins are encoded by genes consisting of eight exons, with equivalent exons encoding equivalent protein domains in each protein (Figure 1.1B). Conserved structure and gene organization suggest that the three genes derived from a common ancestral gene, leading to the designation of the CD34 family of proteins (Y. Li et al., 2002; Nielsen et al., 2002; Nielsen & McNagny, 2008).

In addition to the similarities, the three proteins also have specific differences, which likely relate to differences in function. Differences include varying protein size (CD34 being the smallest and endoglycan the largest), an unpaired cysteine residue on endoglycan, which may allow homodimerization (Sassetti et al., 2000) and different C-terminal tail sequences (DTEL for CD34, DTHL for podocalyxin and endoglycan), which determine the intracellular binding partners of each molecule (Figure 1.1 modified from Nielsen & McNagny (2008).
Figure 1.1: CD34, podocalyxin and endoglycan protein structures, known intracellular binding partners and genomic organization.

(A) Schematic representations of the CD34 family protein structures, with structural domains and known intracellular ligands indicated (horizontal lines = O-glycosylation sites, arrow = sialylation sites, black circles = N-glycosylation sites). (B) Schematic representation of genomic organization for each gene, with each protein encoded by eight exons encoding corresponding protein domains (colour coding indicates the organization of coding regions for each structural domain). Figure adapted with permission from Nielsen & McNagny (2008).
CD34 (also known as CD34 antigen) was first identified by an antibody raised against the human KG-1a hematopoietic myeloid cell line in 1984 (Civin et al., 1984). This first study also identified expression on immature bone marrow cells, including progenitors *in vivo* (Civin et al., 1984). Since its discovery, CD34 has been extensively used as a marker of hematopoietic stem cells (HSCs) and progenitors, with an expanding list of cell types shown to express CD34, as discussed in the next section. CD34 is encoded on chromosome 1 in both humans and mice and the full-length protein consists of ~380 amino acids, depending on the species. An alternatively spliced shorter form has also been reported, which lacks the majority of the intracellular domain, although the functional significance and prevalence of this form remain unclear (Drew et al., 2005b; Nakamura et al., 1993). Following translation, the primary protein structure is extensively modified by N- and O-linked glycosylation and sialylation. Interestingly, this modification pattern varies across cell types, although little work has been done to characterize the location and extent of these modifications. One particular example is the presence of the sialyl Lewis X modification on CD34, which is involved in selectin binding, and is present on HEVs but not on other cell types (Baumheter et al., 1993). Structurally, CD34 consists of an extensive extracellular mucin domain and a cysteine globular domain and intracellularly, consists of a short linear cytoplasmic tail with several phosphorylation sites ending in a DTEL C-terminal sequence. The cytoplasmic tail is critical for interactions between CD34 and the molecule CrkL, which is currently the only known intracellular binding partner of CD34 (Felschow et al., 2001) as reviewed in Nielsen & McNagny (2008).
Podocalyxin (also known as PCLP, thrombomucin, gp135, GCTM2, TRA-1-60 or TRA-1-81) was first identified on renal glomerular epithelial cells from rat glomeruli in 1984 (Kerjaschki et al., 1984). The glomerulus stains extensively with cationic dyes, and analysis of lysates revealed a 140 kDa protein band that accounted for the majority of this staining, which was subsequently found to be podocalyxin (Kerjaschki et al., 1984). Podocalyxin is encoded on chromosome 6 in mice (chromosome 7 in humans), and has a primary protein sequence of ~500 amino acids. As with CD34, an alternatively spliced short form of podocalyxin has been reported (J. Li et al., 2001). Structurally, podocalyxin is very similar to CD34, with the main differences being an enlarged mucin domain, and a DTHL C-terminal sequence (Figure 1.1A). This difference in the cytoplasmic tail between CD34 and podocalyxin result in podocalyxin binding NHERF1 (Tan et al., 2006), NHERF2 (Y. Li et al., 2002; Orlando et al., 2001) and ezrin (Schmieder et al., 2004), rather than CrkL, and likely reflects differences in function between the two molecules. Importantly, the NHERF proteins and ezrin act as adaptor proteins, linking to the actin cytoskeleton, which may provide a mechanism through which podocalyxin alters cellular mobility and morphology.

Endoglycan (also known as PODXL2 or PCLP2) is the most recently identified member of the family, and was first characterized in 2000 based on sequence homology to CD34 and podocalyxin (Sassetti et al., 2000). Endoglycan mRNA and protein expression was detected in endothelial cells and hematopoietic progenitors (Sassetti et al., 2000). Endoglycan is encoded on chromosome 6 in mice
(chromosome 3 in humans), and has a primary sequence of ~600 amino acids, making endoglycan the largest protein in the family. While endoglycan also has the characteristic mucin domain shared by the family, it also has several unique features. The N-terminal region of endoglycan consists of a non-glycosylated domain containing glutamic acid-rich repeats (Sassetti et al., 2000). The extracellular globular domain of endoglycan also contains an unpaired cysteine residue (CD34 and podocalyxin have even numbers of cysteines), which is hypothesized to play a role in endoglycan homodimerization (Sassetti et al., 2000). The C-terminus of endoglycan has the same DTHL sequence as podocalyxin and binds to NHERF1 (Tan et al., 2006), although it remains unclear whether endoglycan can interact with NHERF2 and ezrin.
1.1.2 Expression patterns

On hematopoietic cell lineages, where I have focused most of my attention, CD34, podocalyxin and endoglycan are all expressed by hematopoietic stem cells and/or immature multipotent progenitors (Table 1.1) (McNagny et al., 1997; Sassetti et al., 2000; Young et al., 1995). Expression of all three molecules is then downregulated and largely absent on mature terminally-differentiated cell types, with several notable exceptions.

CD34 is present on committed circulating mast cell progenitors in both mouse and human, and remains expressed on mature tissue-resident mast cells in mice (Table 1.1 – blue) (Drew et al., 2002; Drew et al., 2005a). CD34 is also expressed at low levels on eosinophils in the marrow, spleen and peripheral tissues (Blanchet et al., 2007; Radinger et al., 2004) and on dendritic cell precursors, although it remains unclear whether expression persists on any mature dendritic cell populations (Table 1.1 – blue).
Table 1.1: Expression patterns of CD34, podocalyxin and endoglycan.

CD34, podocalyxin and endoglycan have common and unique distribution patterns within both hematopoietic and non-hematopoietic tissues. Cell types uniquely expressing only one family member, are highlighted (CD34 = blue, podocalyxin = orange, endoglycan = green). (+ = expressed, - = not expressed, -/+ = low or variable expression) Table contents updated and adapted with permission from Nielsen & McNagny (2008).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>CD34</th>
<th>Podocalyxin</th>
<th>Endoglycan</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hematopoietic</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stem cells</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Multipotent progenitors</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>T cells</td>
<td>-</td>
<td>-</td>
<td>+ (thymocytes)</td>
</tr>
<tr>
<td>B cells</td>
<td>-</td>
<td>-</td>
<td>+ (activated)</td>
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<tr>
<td>Dendritic cells</td>
<td>+ (precursor)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mast cells</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>+</td>
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</tr>
<tr>
<td>Macrophages</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Erythrocytes</td>
<td>-</td>
<td>+ (stress)</td>
<td>-</td>
</tr>
<tr>
<td>Platelets</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><strong>Non-hematopoietic</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muscle satellite cells</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hair follicle bulge cells</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Vascular endothelia</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Smooth muscle</td>
<td>-</td>
<td>-</td>
<td>+</td>
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<tr>
<td>Kidney podocytes</td>
<td>-</td>
<td>+</td>
<td>-/+</td>
</tr>
<tr>
<td>Neurons</td>
<td>-/+</td>
<td>+ (subset)</td>
<td>+ (30%)</td>
</tr>
<tr>
<td>Fibrocytes</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mesothelium</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
Podocalyxin is uniquely expressed on erythroid cells during fetal hematopoiesis, and quickly lost following birth (Doyonnas et al., 2005) (Table 1.1 – orange). However, under stress conditions, such as anemia, podocalyxin expression is induced on early BFU-E progenitors and persists through to enucleated immature reticulocytes in the periphery (Doyonnas et al., 2005; Maltby et al., 2009). In addition, podocalyxin is expressed on megakaryocytes and platelets in rats, and thrombocytes in chickens (Table 1.1 – orange) (McNagny et al., 1992; McNagny et al., 1997; Miettinen et al., 1999). Endoglycan is uniquely expressed on immature thymocytes and B cells (with increased expression following activation) (Table 1.1 – green) (Kerr et al., 2008).

On non-hematopoietic tissues all three proteins are expressed on vascular endothelia within blood vessels (Fina et al., 1990; Horvat et al., 1986; Sassetti et al., 2000; Young et al., 1995) and on neurons to varying degrees (Vitureira et al., 2005). CD34 is uniquely expressed on a number of progenitor/stem cell types including muscle satellite cells (Beauchamp et al., 2000) and hair follicle bulge stem cells (Trempus et al., 2003), and is also found on fibrocytes (Schmidt et al., 2003) (Table 1.1 – blue). Podocalyxin is uniquely expressed on podocytes within the glomerulus in the kidney (Kerjaschki et al., 1984) and mesothelia (Doyonnas et al., 2001; McNagny et al., 1997) (Table 1.1 – orange), while endoglycan is uniquely expressed on smooth muscle cells associated with vasculature (Table 1.1 – green) (Sassetti et al., 2000).
As the expression patterns make clear, each molecule has a unique and an overlapping expression pattern with the other two family members (Table 1.1/Figure1.2). Structural similarities between the molecules has led to suggestions that these molecules play similar functions, as will be discussed in the next sections. Thus, this overlapping expression raises the possibility that functional compensation may occur between molecules. On cell types expressing two or three of these proteins, such as hematopoietic stem cells, the loss of a single molecule (e.g. CD34) may not result in a significant phenotype, due to the action of another family member (e.g. podocalyxin). In an attempt to avoid this complication, we have mainly focused our attention on cell types that express only one of the CD34 family members.
**Figure 1.2: CD34, podocalyxin and endoglycan expression patterns on hematopoietic cell populations.**

Schematic of the hematopoietic lineage tree highlighting blood cell populations uniquely expressing one of CD34, podocalyxin or endoglycan, (CD34 = blue, podocalyxin = orange, endoglycan = green). Note: Multipotent precursors and HSCs are not highlighted as they express more than one family member. Expression patterns adapted from Nielsen & McNagny (2008).
1.2 PROPOSED FUNCTIONS OF THE CD34-RELATED PROTEINS

Despite the large number of studies using CD34 as a marker of hematopoietic stem cells, remarkably little is known about the precise functions of CD34 and its related molecules. Several potential functions have been proposed, including roles in promoting or blocking cell adhesion, disruption of tight junctions, progenitor cell proliferation and modulation of cell polarity and morphology, although these functions differ depending on the cell type analyzed (Figure 1.3).

In addition, the proposed functions for the CD34-related proteins have largely come from in vitro studies, and much of this work has yet to be explored in vivo. The functions of each protein will ultimately depend on the protein expression levels, overlapping expression of related molecules, expression of interacting partners within the same cell type and post-translational modification of the CD34-related molecules. Thus it remains unclear which function CD34, podocalyxin and endoglycan will have in each particular cell type across a range of different stimuli. To address these concerns, our studies have largely used knockout mouse models to determine the effects of loss of protein expression, particularly in disease models. It is important to note however that further work is required to link findings in vitro with the functional phenotypes observed in vivo, and thereby fully understand the mechanism of CD34, podocalyxin and endoglycan functions.
Figure 1.3: Proposed functions for the CD34-family of proteins in adhesion, anti-adhesion, differentiation, migration, proliferation and modulation of cell polarity.

Proposed functions for CD34, podocalyxin and endoglycan in vivo and in vitro include A) glycosylation-dependent pro-adhesion, particularly in HEVs and B) charge-dependent anti-adhesion on vasculature and hematopoietic cells in culture, C) blocking differentiation in multipotent progenitors, D) facilitation of migration both by disrupting cell junctions and improving leukocyte migration, E) enhancement of progenitor cell proliferation and F) modulation of cell polarity and morphology, particularly in the case of MCF-7 breast cancer cells. Figure modified and adapted with permission from Nielsen & McNagny (2008) and Nielsen & McNagny (2009).
A. Glycosylation-dependent pro-adhesion

B. Charge-dependent anti-adhesion

C. Inhibitor of differentiation

D. Disruption of tight junctions

E. Enhancer of progenitor cell proliferation

F. Modulation of cell polarity / morphology

Key:
- **L-Selectin**
- **CD34 or Podocalyxin**
- **HEV-specific glycosylation**
- **Integrins/integrin ligands**
- **Negative charge from sialic acid or sulfation**
- **Tight junctions**
- **Focal adhesions**
1.2.1 CD34 functions

As mentioned, CD34 is most commonly used as a marker of stem cell and progenitor populations. As such, the vast majority of CD34-associated literature has focused on comparisons of CD34\(^+\) and CD34\(^-\) populations or the use of CD34 to purify distinct cell populations. While this work is important and interesting, relatively little attention has focused on the actual function of CD34 or the effects of CD34 loss. The studies that have been performed suggest roles for CD34 in hematopoietic progenitor, mast cell and eosinophil migration and in maintenance of vascular integrity.

1.2.1.1 CD34 in maintenance of homeostasis and development

The best-characterized function of CD34 to date is the ability of endothelial CD34 expressed in HEVs to bind L-selectin on migrating leukocytes. Staining of peripheral lymph node HEVs with a recombinant L-selectin molecule revealed binding to carbohydrate modifications on four distinct glycoproteins (Baumheter et al., 1993; Fieger et al., 2003; Suzuki et al., 1996). Protein sequencing of one of these glycoproteins revealed it to be CD34 (Baumheter et al., 1993). Further, CD34 staining of HEV colocalized with bound L-selectin, demonstrating that CD34 is specifically modified in the HEV to bind L-selectin (Baumheter et al., 1993). An additional study demonstrated CD34 expression on vasculature in a range of organs and during disease (Baumhueter et al., 1994). However, while L-selectin binding to CD34 occurred in a subset of HEV vessels, L-selectin was not bound by CD34 in most tissues (Baumhueter et al., 1994). These studies demonstrate that CD34 acts
as a pro-adhesive molecule binding L-selectin within the HEV. However, the carbohydrate modification required for this interaction is limited to the HEV and CD34 binding to L-selectin is not found in other tissues.

As CD34 is expressed by both stem and progenitor cell populations, many expected that loss of CD34 expression, in a knockout model, would result in severe defects. Surprisingly, two separate CD34-knockout mouse strains resulted in viable animals with very minor phenotypes (Cheng et al., 1996; Suzuki et al., 1996). Suzuki et al. reported a minor defect in eosinophil recruitment to the lung in an asthma model in Cd34<sup>-/-</sup> mice (Suzuki et al., 1996). However, Cd34<sup>-/-</sup> mice did not exhibit any differences in L-selectin binding or leukocyte rolling in the HEV and exhibited normal hematopoiesis and recovery following 5-FU treatment (Suzuki et al., 1996). Cheng et al. reported a delay in erythroid and myeloid development from CD34-deficient ES cells, in an in vitro embryoid body assay (Cheng et al., 1996). In addition, isolated progenitor cells from their Cd34<sup>-/-</sup> mice revealed decreased colony formation and expansion in vitro (Cheng et al., 1996). However, in vivo, no defects were detected in baseline hematopoiesis or recovery from sublethal doses of irradiation (Cheng et al., 1996). Taken together, both Cd34<sup>-/-</sup> mouse strains reveal a very minor phenotype, with potential roles for CD34 on eosinophil migration and progenitor expansion.
To determine a potential function for CD34 on hematopoietic stem cells, our group compared the bone marrow reconstitution efficiency of Cd34⁻/⁻ bone marrow cells, to wildtype controls. An initial study demonstrated an impaired ability of CD34-deficient stem cells to reconstitute the bone marrow in a transplantation model, suggesting that CD34 plays a role in homing and migration of hematopoietic precursors (Drew et al., 2005b). To further clarify this role, a subsequent study explored the effects of irradiation on this phenotype. Fetal liver cells derived from wildtype Ly5.1 or Cd34⁻/⁻ (Ly5.2) embryos were mixed 1:1 and injected into either lethally irradiated or non-irradiated W/W' animals (Nielsen & McNagny, 2007). Twelve weeks after injection, recipient animals were sacrificed to assess the relative bone marrow reconstitution levels of wildtype versus Cd34⁻/⁻ cells (Nielsen & McNagny, 2007). Assessment of Ly5.1 versus Ly5.2 expression on the c-kit⁺ portion of the bone marrow (as W/W' mice lack c-kit expression) revealed decreased contribution by Cd34⁻/⁻ bone marrow in non-irradiated recipients, but surprisingly, no difference in the irradiated recipient animals (Nielsen & McNagny, 2007). The authors suggested that CD34 may play an anti-adhesive role on stem cells in vivo and be required for efficient stem cell migration in the non-irradiated reconstitution model (Nielsen & McNagny, 2007). However, in lethally-irradiated recipients, irradiation damages the vasculature, inducing an increase in vascular permeability. Thus, in the lethally-irradiated host, Cd34⁻/⁻ stem cells exhibit no deficiencies reaching the appropriate bone marrow niche, as the vascular tissue has been disrupted (Nielsen & McNagny, 2007).
1.2.1.2 CD34 in inflammatory conditions

While $Cd34^{-/-}$ mice exhibit no major obvious defects, further studies have increased our understanding of CD34 functions under conditions of stress and disease, when homeostasis is perturbed.

While CD34 expression is best known on hematopoietic stem cells, an initial screen characterizing CD34 expression patterns revealed high expression levels on mature bone marrow-derived mast cells (BMMCs) in vitro and primary peritoneal mast cells in vivo in mice (Drew et al., 2002). Intriguingly, BMMCs grown from $Cd34^{-/-}$ animals demonstrated an increased propensity to aggregate in vitro, suggesting that CD34 has as an anti-adhesive function on mast cells (Drew et al., 2005b). In vivo, mast cell repopulation of the peritoneal cavity was impaired in $Cd34^{-/-}$ animals, in a water ablation model, particularly when the closely related sialomucin, CD43, was also deleted (Drew et al., 2005b), suggesting a role for CD34 in optimal mast cell homing and migration.

Further characterization of CD34 expression on mature hematopoietic cells also revealed expression on eosinophils, particularly in severe asthmatics (Radinger et al., 2004). As mast cells and eosinophil play key roles in the pathogenesis of asthma, we analyzed the effect on CD34-deficiency on asthma severity (Chapter 3) (Blanchet et al., 2007). Our findings demonstrate that, as with mast cells, CD34 expression is required for optimal migration of eosinophils in an in vitro transwell
assay (Blanchet et al., 2007). In addition, \textit{Cd34}\textsuperscript{−/−} mice exhibit attenuated asthma pathology, with reductions in inflammatory tissue infiltration and airway hyperresponsiveness, associated with decreases in lung mast cell and eosinophil accumulation (Blanchet et al., 2007).

Continuing from these studies, we also assessed the pathology of \textit{Cd34}\textsuperscript{−/−} mice in a dextran sulfate sodium (DSS)-induced model of ulcerative colitis (Chapter 4). Colon-infiltrating eosinophils are present in patients with ulcerative colitis (Makiyama \textit{et al.}, 1995), although there has been some controversy about the importance of eosinophils in disease pathology. Our findings demonstrate that, similar to our asthma findings, \textit{Cd34}\textsuperscript{−/−} mice are protected from DSS-induced colitis, with decreased colon pathology and decreased inflammatory cytokine production. Following DSS-induction, CD34 expression was highest on eosinophils within the inflamed colon and eosinophilia alone (in IL5\textsuperscript{Tg} animals) was sufficient to induce baseline colon inflammation, suggesting that CD34 and eosinophils are both important for ulcerative colitis disease pathology.

Following from these previous studies, we also assessed the susceptibility of \textit{Cd34}\textsuperscript{−/−} mice to the KBxN-serum induced model of auto-immune arthritis (Blanchet \textit{et al.}, 2009). In the arthritis model, mast cells are critical for disease induction, thus we expected that \textit{Cd34}\textsuperscript{−/−} mice would exhibit attenuated disease as a result of decreased mast cell accumulation in the joint tissue. Surprisingly, \textit{Cd34}\textsuperscript{−/−} mice actually had
increased disease susceptibility, due to a previously unidentified vascular defect (Blanchet et al., 2009). \(Cd34^{-/-}\) mice had increased vascular permeability following induction with either auto-immune serum or treatment with TNF\(\alpha\) alone, in an independent series of experiments (Blanchet et al., 2009). These findings demonstrate that, in addition to the role of CD34 in eosinophil and mast cell homing, CD34 expression on vascular endothelia plays a role in the maintenance of vessel integrity during inflammation.
1.2.1.3 CD34 in tumor development and cancer

In developing tumors, CD34 expression is used as a marker of tumor vasculature (Chen et al., 2009; Murakami et al., 2009) and CD34+ staining is used to characterize vascular patterns within tumor tissues (Tardio, 2008). One study of oral squamous cell carcinoma, demonstrated that the CD34 staining patterns may be used to predict metastasis (Kademani et al., 2009). A “penetrating” staining pattern of CD34 expression on tumor biopsies was significantly associated with regional metastasis (Kademani et al., 2009). Importantly however, and similar to the evaluation of CD34 expression on stem cells, these studies use CD34 expression merely as a marker of vasculature and have not explored a potential function for CD34 on tumor vasculature.

In a colorectal polyp tumor model, we demonstrated the importance of CD34 on infiltrating mast cells in tumor growth (Gounaris et al., 2007). In mast cell-deficient animals, polyp formation was decreased (Gounaris et al., 2007). This decrease in growth could be reversed by reconstitution with wildtype, but not Cd34+/−, bone marrow-derived mast cells (Gounaris et al., 2007). These findings demonstrate that mast cells in tumor sites alter tumor growth, and further, CD34 expression on mast cells plays a key role in mast cell migration to sites of tumor initiation.

In the lone study examining a functional role for CD34 in tumor cells, Trempus et al demonstrated reduced tumor growth following treatment of Cd34+/− animals with
7,12-dimethylbenz(a)anthracene (DMBA) and 12-O-tetradecanoylphorbol-13-acetate (TPA) (Trempus et al., 2007). At higher doses of DMBA treatment, Cd34\(^{-/-}\) mice could be induced to produce tumors, although at lower efficiency than wildtype controls (Trempus et al., 2007). Further study, suggested a decreased capacity of hair follicle bulge stem cells (which normally express CD34) in Cd34\(^{-/-}\) mice to activate and switch to a proliferative state following TPA exposure (Trempus et al., 2007). These findings clearly demonstrated a role for CD34 in follicle stem cell proliferation, which results in decreased tumor growth following DMBA and TPA treatment in Cd34\(^{-/-}\) animals (Trempus et al., 2007).

These studies have indicated that CD34 expression is present within developing tumors, both on tumor vasculature and on infiltrating mast cell populations. In addition, in a DMBA-induced tumor model, loss of CD34 expression results in decreased tumor growth, likely due to a direct effect on hair follicle bulge stem cells. In Chapter 5, I further explore a role for CD34 on non-tumor cells during tumorigenesis, by monitoring B16 melanoma tumor growth in Cd34\(^{-/-}\) animals. Our findings demonstrate that loss of CD34 results in decreased tumor growth at early timepoints, due to impaired vessel function. However, at later timepoints, tumor growth is increased in Cd34\(^{-/-}\) mice, due to impaired immune cell infiltration of tumors, resulting in decreased tumor rejection and clearance.
1.2.2 Podocalyxin functions

While relatively little is understood about CD34 function, even less is known about the role of podocalyxin in vivo. As discussed below, Podxl<sup>−/−</sup> mice die perinatally due to a defect in kidney development (Doyonnas et al., 2001), making it difficult to study the effects of podocalyxin-deficiency in adult animals. Using the Ly5.1/5.2 bone marrow reconstitution model, we have been able to confirm podocalyxin expression patterns and determine the effects of podocalyxin-deficiency on adult hematopoiesis. In addition, assessment of tissues from a range of different cancers has demonstrated that podocalyxin expression occurs in many tumor types and may play a role in promoting tumor migration and metastasis (Casey et al., 2006; Nielsen et al., 2007; Schopperle et al., 2003; Somasiri et al., 2004; Nielsen & McNagny, 2009).

1.2.2.1 Podocalyxin in development

Unlike the Cd34<sup>−/−</sup> mouse strains, deletion of the Podxl gene resulted in a severe phenotype and perinatal lethality (Doyonnas et al., 2001). Podxl<sup>−/−</sup> embryos displayed gut herniation (omphalocele) and frequent edema (Doyonnas et al., 2001). The resulting pups die within 24 hours of birth due to a defect in kidney development, resulting in impaired urine production (Doyonnas et al., 2001). Within the kidneys, podocytes in the glomerulus extend foot processes around blood vessels, forming spaces through which the urine is filtered. In wildtype animals, podocalyxin is normally expressed along these foot processes, within the space between neighboring processes. In Podxl<sup>−/−</sup> animals, foot process extension was impaired leading to a failure to form gaps between podocytes (Doyonnas et al.,
2001). Our group hypothesized that this defect results in the increased blood pressure and edema observed in Podxl<sup>-/-</sup> embryos, leading to death shortly after birth. These findings suggest that similar to CD34, podocalyxin has an anti-adhesive function, on kidney podocytes, creating the separation between cells required for proper glomerulus function.

In addition to defects in kidney development, a recent publication demonstrates a key role for podocalyxin in aortic lumen formation during embryogenesis (Strilic et al., 2009). During early vessel formation, endothelial cells form junctions and both CD34 and podocalyxin were found to be targeted to the junctional interface between cells where nascent lumens form (Strilic et al., 2009). As development progresses, this interface expands to ultimately form the vessel lumen in the embryonic aorta (Strilic et al., 2009). The colocalization of CD34 and podocalyxin to the endothelial cell interface prior to lumen formation was also seen in neovascularization near tumor sites in adult animals (Strilic et al., 2009). Most interestingly, Podxl<sup>-/-</sup> embryos exhibited a delay in embryonic lumen formation, demonstrating that podocalyxin expression at the endothelial interface plays a functional role in creating the vessel lumen (Strilic et al., 2009).
1.2.2.2 Podocalyxin in hematopoiesis

On hematopoietic cells, characterization of podocalyxin expression in embryos revealed early expression in the embryonic yolk sac in both mice and chicks (McNagny et al., 1997), progressing to the fetal liver and ultimately to the bone marrow and spleen at birth, mirroring the localization of hematopoietic stem cells and progenitors (Doyonnas et al., 2005). Further, isolated podocalyxin-positive progenitor cells were able to fully reconstitute myeloid and lymphoid lineages when injected into lethally-irradiated hosts, demonstrating that podocalyxin is expressed by a hematopoietic stem cell population (Doyonnas et al., 2005). In addition, high levels of podocalyxin expression were found on fetal erythroid cells, with levels declining following birth, suggesting that podocalyxin may play a role in embryonic erythropoiesis (Doyonnas et al., 2005).

A recent study also showed that high-dose erythropoieitin (Epo) administration was sufficient to induce podocalyxin expression on erythroblasts both in vitro and in vivo (Sathyanarayana et al., 2007). Podocalyxin expression was induced following Stat5-dependent signaling through the Epo receptor (Epo-R). Using a mouse strain lacking Stat5 signaling from the Epo-R, they also demonstrated abnormal reticulocyte retention in the marrow (Sathyanarayana et al., 2007). The authors suggested this effect is due to an inability of erythroid progenitors to upregulate podocalyxin surface expression in these mice, and that podocalyxin is required as an anti-adhesive molecule for optimal reticulocyte migration (Sathyanarayana et al., 2007).
Continuing on from these studies, in Chapter 2 (Maltby et al., 2009), we further characterized the expression pattern of podocalyxin during adult stress erythropoiesis. We demonstrate that podocalyxin is expressed on early erythroid cells from the BFU-E progenitor stage through to immature reticulocytes in the periphery (Maltby et al., 2009). However, our findings demonstrate that loss of podocalyxin, in Podxl⁻/⁻-reconstituted animals, had no effects on recovery from chemically-induced anemia (Maltby et al., 2009). Thus, we propose the use of podocalyxin as a simple marker to isolate early stress erythroid progenitors from tissues, but the functional importance of podocalyxin expression on erythroid cells remains to be clarified.
1.2.2.3 Podocalyxin in cancer

In addition to roles in embryonic development and stress erythropoiesis, several functions have been identified for podocalyxin on developing tumor cells and during cancer metastasis. A number of studies have identified podocalyxin expression in testicular, breast, prostate, and pancreatic cancers, as well as embryonic carcinomas and leukemia (Casey et al., 2006; Heukamp et al., 2006; Kelley et al., 2005; Ney et al., 2007; Schopperle et al., 2003; Schopperle & DeWolf, 2007; Sizemore et al., 2007; Somasiri et al., 2004).

One study, assessing the importance of podocalyxin expression during breast cancer progression, shows that podocalyxin is expressed and also plays a key role in tumor progression and metastasis (Somasiri et al., 2004). Analysis of a tissue microarray of breast cancer samples indicated podocalyxin expression on a minor subset of tumors (Somasiri et al., 2004). Linking this data with clinical outcome data demonstrated that high podocalyxin expression was correlated with an increase in metastasis and poor clinical outcomes (Somasiri et al., 2004). In addition, podocalyxin overexpression in the MCF-7 breast cancer cell line resulted in disruption of cell-cell junctions and decreased cell adhesion in vitro (Somasiri et al., 2004). In prostate cancer, increased tumor aggressiveness risk was also linked to a chromosomal region containing the Podxl gene, suggesting that podocalyxin may play a role in tumor progression (Casey et al., 2006). These findings suggest that in addition to podocalyxin expression on a variety of tumors, podocalyxin decreases tumor cell adhesion, resulting in increased tumor invasiveness and metastasis.
A follow-up study using podocalyxin-overexpressing MCF-7 cells provides further insight into the mechanism of podocalyxin function on breast cancer cells (Nielsen et al., 2007). Confocal and electron microscopy imaging of MCF-7 cells demonstrated that increased podocalyxin expression results in microvilli formation on the apical cell surface (Nielsen et al., 2007). In addition, localization of podocalyxin to the apical cell surface resulted in recruitment of the intracellular ligand NHERF-1 (Nielsen et al., 2007). Microvilli formation required expression of the extracellular portion of podocalyxin, but not the intracellular tail (Nielsen et al., 2007). However, association of podocalyxin with NHERF-1 was abolished when the intracellular tail of podocalyxin was absent (Nielsen et al., 2007). These findings suggest that the upregulation of podocalyxin on breast cancer cells is sufficient to alter cell morphology, which may explain the increased tumor invasiveness in vivo. In addition, podocalyxin expression alters NHERF-1 localization and as NHERF-1 plays a role as an adaptor protein, altered localization may have multiple downstream effects on signalling and cell behavior.

While these studies have clearly identified podocalyxin expression in a number of cancers, there has been relatively little study of the direct effect of podocalyxin function on tumor progression. Structural homology between podocalyxin and CD34 suggests that podocalyxin may play similar anti-adhesive role to CD34. On podocalyxin-expressing tumor cells in vivo, this may explain the increased tumor aggressiveness, resulting in increased metastasis and poor clinical outcomes. However, much of podocalyxin function is modulated by the specific glycosylation
patterns and no studies have analyzed whether podocalyxin glycosylation is modified on tumor cells. Thus, it remains unclear whether the increased microvilli formation and mobility observed in vitro relates to the function of podocalyxin in vivo and whether this function is similar in other cancer types.
1.2.3 Endoglycan functions

The least is known about endoglycan function, although due to shared structure and binding partners with podocalyxin, endoglycan is thought to have similar functions \textit{in vivo}. An initial study of endoglycan function demonstrated that endoglycan can bind to L-selectin, as do CD34 and podocalyxin (under certain circumstances), with subtle differences in the mechanism of binding (Fieger \textit{et al.}, 2003).

A recent study expanded on this observation by demonstrating the presence of endoglycan on human B cells, T cells and circulating monocyte progenitors (Kerr \textit{et al.}, 2008). On B cells, endoglycan was constitutively expressed and further upregulated following phorbol myristate acetate (PMA) activation, concurrent with a down regulation of the P-selectin glycoprotein ligand-1 (PSGL-1) (Kerr \textit{et al.}, 2008). Endoglycan isolated from these cells interacted with an antibody recognizing the sialyl Lewis X acid residue, indicating that endoglycan is appropriately modified on B cells to interact with selectins (similar to CD34 on HEVs) (Kerr \textit{et al.}, 2008). Using purified endoglycan from B cells, the group also demonstrated interactions with P- and E-selectin, and overexpression of endoglycan on a B cell line resulted in increased adhesion to a P-selectin substrate (Kerr \textit{et al.}, 2008). However, it remains unclear whether the appropriate sialyl Lewis X modification is present on endoglycan on all cell types, or if this interaction is unique to certain cell subsets, as is the case with CD34.
1.3 AIMS OF THE STUDY / RATIONALE

My work has focused on CD34 and podocalyxin function on hematopoietic cell subsets during disease conditions, specifically on a role in cell migration. In my studies, I have aimed to better characterize 1) the expression patterns of CD34 and podocalyxin and 2) the effects of losing gene expression, in knockout models at baseline and during disease. As CD34, podocalyxin and endoglycan have unique and overlapping expression patterns, I have focused on diseases involving cell types uniquely expressing only one of these molecules, in order to reduce the likelihood of functional compensation (highlighted in Table 1.1 and Figure 1.2). My findings have improved our understanding of CD34 and podocalyxin functions in vivo and also increased our understanding of these disease processes in general.

In a phenylhydrazine (PHz)-induced anemia model (Chapter 2) (Maltby et al., 2009), we demonstrated that podocalyxin expression is induced on stress erythroid populations from the BFU-E stage through to immature reticulocytes, following treatment with PHz or Epo (Maltby et al., 2009). We hypothesized that this expression may be required for erythroid cell proliferation and release under conditions of stress, but podocalyxin-deficiency had no effect on the kinetics of anemia recovery (Maltby et al., 2009). We were able to clearly demonstrate podocalyxin expression on erythroid progenitor populations within the bone marrow and spleen and thus proposed podocalyxin as a simple marker to sort stress erythroid progenitors (Maltby et al., 2009). These findings are further supplemented by data not presented in the original manuscript (Appendix A), demonstrating that
podocalyxin-deficient stress erythroid cells exhibit normal cell survival and osmofragility, normal BFU-E forming capacity and podocalyxin expression is present in the peripheral blood under conditions of chronic anemia (in SHIP−/− mice).

In subsequent chapters I focused on the function of CD34 in disease, particularly on mast cells and eosinophils (as well as vascular endothelia, in a melanoma model). In an OVA-induced asthma model (Chapter 3) (Blanchet et al., 2007), we focused on the role CD34 plays on mast cell and eosinophil migration. We hypothesized that CD34 is required for efficient migration of mast cells and eosinophils into the lung during disease, and that Cd34−/− animals would exhibit decreased disease symptoms. We were able to show that decreased disease did occur in these animals, with decreases in both mast cell and eosinophil infiltration (Blanchet et al., 2007). In addition, we showed a cell-intrinsic defect in eosinophil migration using isolated Cd34−/− eosinophils in an in vitro cell migration assay (Blanchet et al., 2007).

Continuing from the asthma model, I assessed the role of CD34 in a DSS-induced model of ulcerative colitis (Chapter 4). Eosinophil migration and degranulation are integral to the development of ulcerative colitis, and similar to our findings in the asthma model, we demonstrated that Cd34−/− mice were less susceptible to the development of colitis. The predominant colon-infiltrating cell type expressing CD34 in wildtype mice was eosinophils, and eosinophilia alone, in IL5Tg mice, was
sufficient to induce low levels of colon shortening (a primary readout in mouse models of colitis). The importance of CD34 expression on eosinophils in this model was also supported by findings that loss of CD34 expression on hematopoietic cells alone was sufficient to attenuate disease symptoms.

In a B16 melanoma tumor model, I assessed the function of CD34 on tumor growth (Chapter 5). As mast cells and eosinophils are both suggested to alter tumor growth kinetics, I hypothesized that \( Cd34^{-/-} \) mice would have a reduced incidence of immune cells infiltration into tumors resulting in decreased tumor growth. Somewhat surprisingly, I found a significant role for CD34 on non-hematopoietic lineages, resulting in decreased tumor growth at early timepoints, suggesting an important role for CD34 in vascular endothelial cell function. At later timepoints, CD34 loss on hematopoietic cells resulted in increased tumor growth, with concurrent reduction in mast cell tumor infiltration.
1.4 REFERENCES


adenocarcinomas from adenocarcinomas of the biliary and gastrointestinal tracts. *Human Pathology, 38*(2), 359-364.


pluripotent stem cell markers are expressed on podocalyxin in embryonal carcinoma. *Stem Cells, 25*(3), 723-730.


CHAPTER 2. PODOCALYXIN SELECTIVELY MARKS ERYTHROID-COMMITTED PROGENITORS DURING ANEMIC STRESS BUT IS DISPENSABLE FOR EFFICIENT RECOVERY

2.1 INTRODUCTION

Erythropoiesis is a tightly regulated process that ensures red cell homeostasis under steady state conditions while maintaining a reserve of progenitors for rapid recovery during anemic challenge. Although homeostatic and stress-induced erythropoiesis require the erythroid survival factor erythropoietin (Epo), the intracellular molecular targets of Epo-receptor (Epo-R) signaling, and the context with which these signals are interpreted, differ (reviewed by Socolovsky (2007)). In general, optimal recovery following acute anemic stress depends on hypoxia-induced upregulation of Epo (Ebert & Bunn, 1999), together with other stress-induced erythropoiesis modulators such as stem cell factor (SCF) (Broudy et al., 1996; Perry et al., 2007), bone morphogenic protein 4 (BMP4) (Lenox et al., 2005; Perry et al., 2007) and glucocorticoids (Bauer et al., 1999). In mice, stress erythropoiesis occurs in the fetal liver and adult spleen and is distinct from bone marrow steady state erythropoiesis. In particular, stress erythropoiesis utilizes a specialized population of progenitor cells that have the potential to rapidly produce large numbers of new erythrocytes (Lenox et al., 2005). BMP4 expression is induced in the mouse spleen in response to tissue hypoxia and works to promote the differentiation of stress blast-forming unit erythroid (BFU-E). SCF promotes the

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expansion of stress BFU-E and hypoxia synergizes with the other two signals to maximize the response (Perry et al., 2007). Together with Epo, these signals coordinate the rapid proliferation and differentiation of stress erythroid progenitors (Perry et al., 2007).

Podocalyxin, initially described as a surface marker of kidney podocytes (Dekan et al., 1991; Kerjaschki et al., 1984), is a member of the CD34 family of sialomucins (reviewed by Furness & McNagny (2006)) expressed on vascular endothelia (Horvat et al., 1986; Kershaw et al., 1995), hemangioblasts (Hara et al., 1999), mesothelial cells (Doyonnas et al., 2001), early hematopoietic precursors (Doyonnas et al., 2005; Kerosuo et al., 2004; McNagny et al., 1997), and some epithelial tumors associated with poor prognosis (Casey et al., 2006; Sizemore et al., 2007; Somasiri et al., 2004). CD34 family members share some common structural features including a heavily glycosylated and sialated extracellular domain, a single-pass transmembrane region and a C-terminal intracellular domain that is conserved between vertebrate species (Furness & McNagny, 2006). Although functions for the CD34 family have been defined in other tissue types, their role in hematopoietic cells remains largely elusive. We have shown that CD34 can act as an anti-adhesive on hematopoietic cells (Drew et al., 2005) with functional consequences in models of hematopoietic-mediated disease (Blanchet et al., 2007; Gounaris et al., 2007) and that podocalyxin appears to share a similar anti-adhesive function, at least in non-hematopoietic cells, such as kidney podocytes (Doyonnas et al., 2001) and metastatic human breast cancer cells (Somasiri et al., 2004).
During mouse embryonic development, podocalyxin-positive cells are detected in fetal liver and yolk sac as early as embryonic day 10.5. This expression continues throughout embryonic development, with decreasing levels of podocalyxin-positive hematopoietic cells detected towards birth. Interestingly, at birth up to half of nucleated hematopoietic cells in the spleen, bone marrow and peripheral blood undergo a transient period of increased podocalyxin expression that tapers off by one to two weeks of age (Doyonnas et al., 2005). Notably, peak levels of podocalyxin expression in these tissues correlate to periods of hypoxic stress during early development and around the time of birth (Porayette & Paulson, 2008) - a potential clue in ultimately understanding podocalyxin’s function. Podocalyxin expression is retained on a subset of c-Kit$^+$ Sca-1$^+$ Lin$^-$ (KLS) hematopoietic progenitors in the bone marrow of adult mice (Doyonnas et al., 2005). This subset of KLS cells have superior long-term hematopoietic reconstituting potential compared to the podocalyxin-negative KLS subset isolated from the same bone marrow preparation (Doyonnas et al., 2005). With respect to more lineage-restricted hematopoietic cells, podocalyxin expression is rapidly induced on adult definitive erythroid cells in mouse spleen and bone marrow following phenylhydrazine (PHz)-induced anemia (Doyonnas et al., 2005) and following treatment with high doses of Epo (Sathyanarayana et al., 2007).
Recently, Sathyanarayana et al. (2007) showed high-dose Epo administration was sufficient to induce podocalyxin expression on staged erythroblasts in vitro and on bone marrow erythroblasts in vivo. In that study, podocalyxin expression was, in part, associated with EpoR-mediated activation of the Jak2/Stat5 pathway. Epo induction of podocalyxin expression was not observed in a transgenic mouse strain expressing an EpoR with an intracellular-domain truncation (EpoR-HM), which lacks a tyrosine residue critical for Stat5 signal transduction but retains the Jak2-activating domain (Sathyanarayana et al., 2007; Zang et al., 2001). In addition, Sathyanarayana et al. demonstrated that the EpoR-HM mutant mice exhibit abnormal reticulocyte retention in the marrow following high-dose Epo treatment, speculating that this effect may be due, at least in part, to the inability of these mice to upregulate podocalyxin surface expression. In contrast, the EpoR-H mutant mouse, which has a mutation truncating the same C-terminal 108 amino acids, but retains the Stat5-binding tyrosine residue (Zang et al., 2001) does not display this reticulocyte-retention phenotype and can upregulate podocalyxin surface expression on erythroid cells in response to Epo. Accordingly, the authors postulated a key role for podocalyxin in the release of erythroid cells from the bone marrow and spleen into the periphery during erythropoietic expansion induced by Epo administration.

In this study, we asked whether podocalyxin expression modulates efficient recovery from hemolytic anemia or the appearance of reticulocytes in the periphery following high-dose Epo treatment. Since podocalyxin-deficient (Podxl−/−) mice die perinatally (Doyonnas et al., 2001) we used lethally irradiated mice reconstituted
with fetal liver cells derived from day 15.5 Podxl+/ or Podxl+/+ embryos for these studies. The chimeric mice we generated have wild-type podocalyxin expression in all peripheral tissues, but lack functional podocalyxin expression in hematopoietic cells. Our results suggest that podocalyxin is completely dispensable for recovery from phenylhydrazine (PHz)-induced and 5-fluorouracil (5-FU)-induced anemia. Furthermore, ablation of podocalyxin does not result in accumulation of marrow or splenic erythroblasts or reticulocytes during anemic stress or following high-dose Epo administration. In addition, our data suggest that podocalyxin is abundantly expressed by stress-induced BFU-E and CFU-E progenitors in the spleen and marrow. The expression of podocalyxin on these pre-blast erythroid progenitors suggests that this CD34-family member may be an effector in response to not only Epo, but also other hypoxia-induced factors. Finally, we provide evidence that podocalyxin is a highly specific marker for the isolation and study of erythroid progenitors in mouse models of anemia.
2.2 MATERIALS AND METHODS

2.2.1 Hematopoietic-reconstituted (chimeric) mice

All mice were maintained in specific pathogen-free facility at the Biomedical Research Centre (UBC) and experimental protocols were conducted in accordance with approved and ethical treatment standards of the University of British Columbia. Recipient Ly5.1 C57Bl/6 mice and splenectomized Ly5.1 C57Bl/6 mice (6-8 weeks old) (Jackson Laboratory, Bar Harbor, Maine) were lethally irradiated (11 Gy) and injected (intravenously) with 2x10^6 fetal liver cells derived from congenic (Ly5.2) Podxl^-/- or Podxl^+/- day 15.5 post coital (p.c.) embryos (tissue samples from each embryo were genotyped by PCR) (Doyonnas et al., 2005). To determine reconstitution levels 10 weeks post-transplant, peripheral blood was sampled from the saphenous vein and labeled with antibodies against Ly5.1 and Ly5.2 (BD Pharmingen, Mississauga, ON), following red cell lysis. Full reconstitution was defined as >95% donor-derived (Ly5.2) CD45^+ cells contributing to myeloid and lymphoid cells in the periphery. Resting peripheral blood parameters were examined in reconstituted mice using a Hemavet 950 automated blood analyzer, calibrated for mouse samples (Drew Scientific Inc., Oxford, CT, USA).

2.2.2 Administration of Epo, PHz and 5-FU

Reconstituted mice were injected (intraperitoneally) with 200 U/mouse Epo (Eprex, Janssen-Ortho Inc., Toronto, ON), 60 mg/kg PHz prepared in RPMI (Gibco-Invitrogen, Burlington, ON), 120 mg/kg of 5-FU (Sigma-Aldrich, Oakville, ON) or vehicle control at the intervals described in the text. Peripheral blood hematocrit
levels were determined manually and reticulocytes were assayed with thiazole orange (Sigma-Aldrich, Oakville, ON). Reticulocyte indices were calculated by normalizing reticulocyte percentages to a hematocrit of 0.45 (Riley et al., 2001; Riley et al., 2002).

### 2.2.3 Antibodies and flow cytometry

Erythroid population subsets from bone marrow and spleen were determined by flow cytometry using a PE-conjugated rat anti-mouse Ter119 (BD PharMingen, Mississauga, ON), FITC-conjugated rat anti-mouse CD71 (Cedarlane Laboratories, Burlington, ON) and forward scatter as described by Liu et al. (Liu et al., 2006). Podocalyxin surface expression was detected using rat anti-mouse podocalyxin (MAB1556, R&D Systems, Minneapolis, MN, USA) followed by biotin-conjugated goat anti-rat IgG (Cedarlane Laboratories) and avidin-APC (BD Pharmingen, Mississauga, ON). DRAQ5 (Biostatus Ltd., Shepshed, UK) nucleic acid staining was performed following the manufacturer’s specifications. Data was collected on a BD FACSCalibur (BD Biosciences, Mountain View, CA, USA) and analyzed with FlowJo software (Treestar, Inc., Ashland, OR, USA).

### 2.2.4 Cell sorting and colony assays

Bone marrow and spleen cells were sorted based on lineage marker immunolabeling and podocalyxin expression as described previously (Doyonnas et al., 2005). Briefly, single-cell suspensions of marrow and spleen tissues isolated from mice 72 hours following PHz induction were labelled with PE-conjugated
antibodies against B220 (clone RA3-6B2), CD3 (clone 17A2), Gr-1, Ter119 (clone Ly76) and Mac-1 (clone M1/70) (all from BD Pharmingen) and podocalyxin (mentioned previously). Cell populations were sorted on a FACSDiva cell sorter (BD Biosciences) using the gates as shown in the figures. The collected populations were enumerated manually by nucleated cell counts (3% acetic acid in water) on a hemocytometer or by flow cytometry and then plated at several concentrations in semi-solid culture to assay for BFU-E and CFU-GM colonies (MethoCult® GF M3434, Stem Cell Technologies Inc. (STI), Vancouver, BC) or for late BFU-E and CFU-E colonies (MethoCult® M3334; STI, Vancouver, BC). BFU-E and CFU-GM colonies were scored on Day 7 to 10 and late BFU-E and CFU-E were scored on Day 2 to 3 using standard methods. Megakaryocyte progenitors (CFU-MK) were assayed by plating sorted cells from marrow or spleen derived from PHz-treated mice (as previously mentioned) in serum-free media containing IL-6, IL-3 and thrombopoietin (Tpo) (Megacult®-C, STI) onto chambered slides (Nunc, Rochester, NY). Slides were fixed and dehydrated at Day 8 and then stained for acetylcholinesterase-positive colonies (Jackson, 1973). The fold enrichment of BFU-E and CFU-E were estimated by calculating a ratio of bursts or colonies obtained from sorted populations compared to counts obtained from plating non-fractionated spleen or bone marrow (i.e., presort starting material).
2.3 RESULTS

2.3.1 Deletion of podocalyxin does not alter blood lineage homeostasis

Because $Podxl^{/-}$ mice die perinatally, irradiated hematopoietic chimeras were used to assay podocalyxin function during stress-induced erythropoiesis in adult mice. We first analyzed peripheral blood parameters of mice fully reconstituted with $Podxl^{/-}$ and $Podxl^{+/+}$ embryonic fetal liver. $Podxl^{+/+}$ and $Podxl^{/-}$ hematopoietic-reconstituted mice display similar resting hematocrit, total red blood cell, platelet and white blood cell counts (Table 2.1). Both groups of reconstituted mice exhibit significantly higher levels of white blood cells compared to non-irradiated C57Bl/6 controls. These data demonstrate that, at rest, loss of podocalyxin does not affect peripheral blood homeostasis, although irradiation and reconstitution has an effect on resting WBC levels.
Table 2.1: Peripheral blood parameters of lethally irradiated recipient mice reconstituted with *Podxl*+/+ or *Podxl*−/− fetal liver.

<table>
<thead>
<tr>
<th>Genotype of donor</th>
<th>Hematocrit</th>
<th>RBC (x10⁶/mL)</th>
<th>WBC (x10³/mL)</th>
<th>Platelet (x10³/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Ly5.2) fetal liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Podxl</em>+/+ (n=8)</td>
<td>44 ± 2</td>
<td>10.0 ± 0.6</td>
<td>23 ± 3</td>
<td>1050 ± 184</td>
</tr>
<tr>
<td><em>Podxl</em>−/− (n=10)</td>
<td>44 ± 1</td>
<td>10.1 ± 0.2</td>
<td>21 ± 4</td>
<td>913 ± 314</td>
</tr>
<tr>
<td>Ly 5.1 (n=4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(non-reconstituted, non-irradiated control)</td>
<td>48 ± 1</td>
<td>10.2 ± 0.3</td>
<td>12 ± 5*</td>
<td>880 ± 81</td>
</tr>
</tbody>
</table>

All recipients are female, engraftment at ten weeks >95% Ly 5.2. Data shown as mean ± standard deviation. * represents p<0.05; significantly different compared to reconstituted chimeras.
2.3.2 Podocalyxin is a highly specific-marker of early erythroid progenitor and erythroblast populations during anemic stress response

To determine if podocalyxin is expressed by erythroid progenitors at pre-erythroblast stages of development we injected mice with PHz and sorted lineage-negative (Lin−) populations based on the presence or absence of surface-expressed podocalyxin (Figure 2.1). The bone marrow and spleen Lin−podocalyxin+ populations derived from PHz-treated anemic mice are highly enriched for BFU-E and CFU-E but contain very low to no CFU-GM or CFU-MK potential (Figure 2.1). Conversely, the podocalyxin-negative fraction consists predominantly of CFU-GM and CFU-MK (Figure 2.1) and lacks erythroid potential. Using this cell-surface labelling scheme, at 72 hours post-PHz treatment, we were able to enrich for late BFU-E (Day 3) and CFU-E by approximately 100-fold from bone marrow and 10-fold from spleen. Furthermore, we enriched early BFU-E (Day 10) by approximately 20 to 50-fold (compared to non-fractionated tissues) from bone marrow and spleen.
Figure 2.1: Podocalyxin is a specific marker of stress-induced blast-forming unit erythroid (BFU-E) and colony-forming unit erythroid (CFU-E).

Immunofluorescence profile (left panels) used to sort lineage–negative (Lin–) (B220/CD3/Mac1/Ter119/Gr–1), podocalyxin+ or podocalyxin– cells from (A) bone marrow and (B) spleen for plating and colony assays (right panels) 72 hours following phenylhydrazine induction. Frequency of BFU–E, CFU–E, colony–forming unit granulocyte macrophage (CFU–GM) and colony–forming megakaryocytes (CFU–Mk) are shown. Colony assays were performed in triplicate or quadruplicate plates and the results shown are representative of two separate experiments.
In contrast, only a subset of lineage-negative bone marrow cells harvested from non-stressed mice is podocalyxin+, with much lower levels of podocalyxin expression (approximately 10-fold). Both the podocalyxin-positive and podocalyxin-negative populations of lineage-negative non-stressed bone marrow contain BFU-E, CFU-E, CFU-GM and CFU-spleen potential (data not shown). Thus, the presence or absence of podocalyxin expression does not offer an advantage in enrichment of erythroid-progenitors in mice during homeostasis. Therefore, podocalyxin is a highly specific marker of early erythroid progenitors in both bone marrow and spleen during anemic recovery, but not under normal, unstressed conditions.

2.3.3 Podocalyxin-deficient hematopoietic chimeras do not accumulate erythroblasts in bone marrow or spleen

Podocalyxin has been postulated to play a role in reticulocyte release from hematopoietic reservoirs following high-dose Epo administration (Doyonnas et al., 2005; Zang et al., 2001). If true, we would expect fewer or delayed appearance of circulating reticulocytes in Podxl+/−-chimeric mice following high-dose administration of Epo or induction of anemia. Accordingly, this delayed-release would be accompanied by accumulation of late erythroblasts and reticulocytes in bone marrow and spleen.

To test this hypothesis, we compared Epo-induced erythroblast expansion in bone marrow and spleen of Podxl+/+ and Podxl−/− hematopoietic-reconstituted mice. Podocalyxin is highly expressed on proerythroblast (CD71+Ter119low) and
CD71^Ter119^ cells in the bone marrow and spleen and peripheral blood reticulocytes 72 hours after administration of high-dose Epo (Figure 2.2). Compared to Podxl^+/+-reconstituted mice, Epo-treated Podxl^+-reconstituted mice display a slightly lower proportion of CD71^Ter119^low (proerythroblast stage) (Socolovsky et al., 2001) and CD71^Ter119^high blasts in bone marrow but comparable proportions of these populations in the spleen (Figure 2.2). At this time point, DRAQ5 staining of cells demonstrated that a significant proportion (approximately 35%) of CD71^Ter119^ cells were DRAQ5-negative (i.e., enucleated), while nearly all of the CD71^Ter119^ population consisted of DRAQ5-negative erythrocytes). Under conditions of homeostatic red cell turnover, the CD71^Ter119^high population contains developing orthochromatic and reticulocyte populations (Socolovsky et al., 2001). However, since the CD71^Ter119^high population does not express podocalyxin following Epo induction, it is likely that this population does not contain newly produced erythrocytes (Figure 2.2). These data, along with the podocalyxin flow-cytometry profiles suggests that, following Epo induction, podocalyxin-positive reticulocytes exit the spleen and marrow at the CD71^Ter119^ stage (i.e., as CD71^+ “stress reticulocytes”) and retain podocalyxin expression in the periphery through to the mature reticulocyte stage. Importantly, the proportion of this population (CD71^Ter119^DRAQ5^) is similar in bone marrow and spleen of Podxl^-/- and Podxl^+/+ chimeras. Thus, we do not observe accumulation of these erythrocyte precursors at their sites of development (Figure 2.2).
Figure 2.2: Podocalyxin-deficient erythroblasts and reticulocytes do not aberrantly accumulate at their sites of development.

(A) Comparison of podocalyxin expression levels in proerythroblasts (ProE, CD71^Ter119^{low}), CD71^Ter119^+, CD71^Ter119^+ populations (left panel) and blood reticulocytes (right panel) 72 hours after a single injection of erythropoietin (Epo). Mean fluorescence intensity (MFI) was determined by subtracting nonspecific background signal detected in Podxl^-/^- blasts of the same stage. (B) In vivo erythroblast population profile 72 hours after Epo treatment in the marrow and spleen of Podxl^+/+ (solid bars) and Podxl^-/- (open bars) chimeras. (n = 6, * represents p < 0.05, Error bars = SEM). (C) A representative CD71/Ter119 profile (left panel) of spleen erythroblasts 72 hours following high–dose Epo–administration together with the podocalyxin expression and nuclear content (DRAQ5^+) of the Ter119^+ populations (right panel). Immunolabeling of surface–expressed podocalyxin is represented by black line and the isotype control by the grey–filled line. (D) Based on the analysis scheme presented in (C), the nuclear content of CD71^Ter119^+ erythroblasts following 72 hours Epo treatment is shown. The fraction of DRAQ5–negative blasts (i.e., enucleated) in the CD71^Ter119^+ population from bone marrow and spleen were assayed for several individual Podxl^+/+ (solid bars) and Podxl^-/- (open bars) Epo–treated chimeras. (n = 3; Error bars = SEM).
2.3.4 Deletion of podocalyxin does not alter release of reticulocytes into the peripheral blood

In order to assess reticulocyte release kinetics in Podxl<sup>-/-</sup> chimeric mice in more detail, we next monitored the appearance of peripheral reticulocytes at various times following high-dose Epo treatment. During succeeding days following Epo induction, mice exhibit an increase in red cell hematocrit and reticulocyte index (Figure 2.3B). Both the amplitude and kinetics of these measured blood parameters are comparable in Podxl<sup>+/+</sup> and Podxl<sup>-/-</sup> chimeras, providing further support that Epo-induced podocalyxin expression is not required for reticulocyte release into the periphery. In addition, we found that the number of Epo-induced circulating immature and mature reticulocytes in Podxl<sup>-/-</sup> and Podxl<sup>+/+</sup> chimeras is comparable at 72 hours following Epo-administration (Figure 2.3A). We conclude that Podxl<sup>-/-</sup> erythroid progenitors expand normally and CD71<sup>+</sup> “stress reticulocytes” are released and mature normally in response to high-dose Epo treatment.
Figure 2.3: Kinetics of reticulocyte release in Podxl\(^{+/-}\) and Podxl\(^{-/-}\)–chimeric mice.

(A) Peripheral blood reticulocyte index, normalized to a hematocrit of 0.45. (n = 9; Error bars = SEM). Pooled data were not statistically significant as assessed by two–way ANOVA. (B) Reticulocyte indices following a single dose injection of erythropoietin (Epo) delivered intraperitoneally (200 U/mouse). Injections were staggered to allow assessment of all mice at a common endpoint. Podxl\(^{+/-}\) [(A) solid bars or (B) solid lines, closed squares] and Podxl\(^{-/-}\) (A) open bars or (B) dotted lines, open squares. (n = 3 per time point; Error bars = SEM).
2.3.5 Podocalyxin is dispensable for recovery from chemically-induced anemia

We next assessed the ability of Podxl<sup>−/−</sup> and Podxl<sup>+/+</sup> chimeras to recover from PHz-induced anemia. Both Podxl<sup>−/−</sup> and Podxl<sup>+/+</sup> chimeras exhibited the typical drop and recovery in hematocrit associated with chemically-induced anemia. As with Epo-treatment, we did not detect significant differences in the PHz sensitivity or time of recovery from anemia in Podxl<sup>−/−</sup>-reconstituted mice. The peripheral blood reticulocyte index (Figure 2.4A, right panel) and hematocrit (Figure 2.4A, left panel) were similar in the kinetics of recovery in Podxl<sup>−/−</sup>-reconstituted mice compared to Podxl<sup>+/+</sup> chimeras.

Because we could not detect a difference in the recovery response of podocalyxin-deficient mice, we reasoned that delaying the anemia recovery response may allow us to unmask a more subtle recovery defect. The mouse spleen is the primary site of erythropoiesis in response to hypoxic stress and optimal recovery from PHz-induced anemia is heavily dependent on the BMP4 dependent stress erythropoiesis pathway mediated in the mouse spleen or liver (Lenox et al., 2005). For this reason, splenectomized mice display delayed recovery kinetics from PHz-induced anemia. We therefore generated splenectomized Podxl<sup>−/−</sup> and Podxl<sup>+/+</sup> chimeric mice in an effort to uncover a role for podocalyxin in anemia recovery that would require the mobilization of erythroid progenitors to nonsplenic extramedullary sites, the expansion of bone marrow blasts, and, the release of reticulocytes to the periphery.
Figure 2.4: Podocalyxin is dispensable for efficient anemia recovery.

(A) Hematocrit (left panel) and reticulocyte index (right panel) of chimeric mice following phenylhydrazine (PHz) treatment (two consecutive injections at the times indicated). Hematocrit recovery plots of (B) splenectomized chimeric mice following PHz–induction at the time points indicated and (C) chimeric mice following single injection of 5–fluorouracil (5–FU) at day 0. Podxl+/− (solid lines, closed squares) and Podxl−/− (dotted line, open squares) in all plots. (n = 5; Error bars = SD).
Despite the loss of the primary site of anemia recovery in mice, we could not detect a difference in the kinetics of hematocrit recovery of splenectomized $Podxl^{-/-}$ and $Podxl^{+/+}$ chimeras following PHz-induced anemia (Figure 2.4B).

Treatment of mice with 5-FU causes the death of actively proliferating hematopoietic cells and pancytopenia (as cycling blood progenitors die), followed by a recovery phase as quiescent hematopoietic cells are induced to expand to recover hematopoietic tissue. In a 5-FU hematopoietic recovery experiment, $Podxl^{-/-}$-reconstituted chimeras did not exhibit defects in hematocrit recovery (Figure 2.4C). Importantly, although 5-FU treatment induces pancytopenia, cells expressing podocalyxin during 5-FU recovery shared an erythroblast surface antigen phenotype (not shown). We conclude that podocalyxin expression is not essential for appropriate erythroid expansion, differentiation or reticulocyte release in response to chemically-induced anemia.
2.4 DISCUSSION

While surface expression of podocalyxin is absent on erythroblasts and reticulocytes under homeostatic conditions, it is rapidly induced in response to administration of high-dose Epo. Newly generated reticulocytes are released from spleen and marrow as CD71\textsuperscript{+}Ter119\textsuperscript{+}podocalyxin\textsuperscript{+} “stress reticulocytes” under these conditions of suprabasal erythroblast expansion. Therefore, like CD71, the presence of podocalyxin on peripheral blood reticulocytes marks nascent red cells at times of high erythropoietic rate. Mice transplanted with either Podxl\textsuperscript{-/-} or Podxl\textsuperscript{+/+} hematopoietic cells exhibit normal peripheral blood parameters and normal kinetics of recovery from anemia.

In this study we have identified expression of podocalyxin on BFU-E, late BFU-E and CFU-E stages of development. During recovery from anemic stress (induced by either PHz or 5-FU), and concurrent with an increased erythropoietic rate, podocalyxin is expressed on the surface of these erythroid progenitor stages and downstream erythroblasts. Because podocalyxin is expressed on both splenic and marrow BFU-E and CFU-E, its expression must not be strictly limited to BMP4-responsive stress progenitors (Lenox et al., 2005; Perry et al., 2007). Importantly, the lineage-negative podocalyxin-positive population in PHz-induced spleen and bone marrow contain little or no CFU-GM or CFU-MK potential. Although podocalyxin expression has been reported on platelets in rats (Miettinen et al., 1999) and chicken thrombocytes (McNagny et al., 1997), we have not detected surface-expressed podocalyxin on resting peripheral blood platelets in mice (not shown).
With respect to the recent Sathyanarayana et al. study (Sathyanarayana et al., 2007); our findings suggest that the notable accumulation of enucleated erythroblasts (CD71^+Ter119^+DRAQ5^-) observed in the bone marrow of EpoR-HM transgenic mice cannot be attributed to their inability to induce podocalyxin expression. Instead, our work suggests that podocalyxin expression on erythroid progenitors is dispensable for efficient Epo-induced expansion of erythroblasts and reticulocyte release into the periphery. Although we cannot absolutely rule out a subtle role for podocalyxin in the release of stress-induced reticulocytes from hematopoietic tissues, our experiments argue against assigning a critical role for podocalyxin in this process. Instead, it is likely that other mediators downstream of a Jak2/Stat5 axis are responsible for the accumulation of erythroblasts in the marrow of EpoR-HM transgenic mice with deficient Stat5 activation.

An important caveat to these findings is the fact that endothelial cells comprising the vasculature of the erythroid developmental niche in bone marrow and spleen are likely capable of expressing podocalyxin (Horvat et al., 1986; Kershaw et al., 1995). However, other than the special case of L-selectin mediated adhesion to high endothelial venules (which constitute less than 1% of total endothelial vasculature) (Baumheter et al., 1993; Fieger et al., 2003; Sarangapani et al., 2004; Sassetti et al., 1998), a possible role for podocalyxin (and other CD34-family members) in vascular integrity or heterotypic interaction with hematopoietic cells has not been elucidated. Since mice transplanted with Podxl^- hematopoietic cells retain a Podxl^+/+ niche, we cannot rule out a role for endothelial-expressed podocalyxin in anemic recovery and,
to date, the expression of podocalyxin on vascular endothelial in response to hypoxic stress has not been studied. The resolution of this issue awaits the generation of mice with a conditional podocalyxin allele, permitting tissue specific deletion on vascular endothelial cells (in progress).

During basal erythropoiesis the expansion of erythroblasts is controlled, in part, by Fas/FasL mediated apoptosis among differentiating erythroblasts interacting with one another (Liu et al., 2006) or macrophages in erythroblastic islands. The signaling mechanisms that are responsible for this suppression are not known, nor are the mechanisms that determine the spectrum of Fas/FasL expression levels in developing blasts that allow only some to survive (Socolovsky, 2007). One of the unique targets of high-dose Epo or stress-induced erythroblasts expansion is the suppression of Fas and FasL surface expression (Liu et al., 2006). Induced surface expression of podocalyxin under the same conditions that suppress Fas/FasL expression suggest that these features of stress-induced erythroblasts may be linked through common mechanisms. Since a pro- or anti-adhesive role for podocalyxin may be expected to influence the efficiency of Fas/FasL mediated apoptosis in erythroblast populations, we assayed Annexin-V on stress-induced or Epo-stimulated erythroblasts in vivo but did not observe a difference in podocalyxin-deficient blasts (Appendix A). In addition, erythroblastic islands appeared to form normally in Podxl<sup>−/−</sup> fetal liver (Hughes and McNagny, unpublished observations).
Although it is clear that podocalyxin expression can be induced directly via Epo-induced pathways in erythroblasts (Sathyanarayana et al., 2007), it is not clear if this response is a unique feature of EpoR mediated signaling pathways or a property of erythroid-committed progenitors. Although the BFU-E compartment is not particularly Epo-sensitive during basal erythropoiesis, in response to anemia recovery, podocalyxin surface expression is induced in this progenitor compartment. One possibility is that induction of this expression could be mediated by Epo-induced pathways in BFU-E that have become highly Epo-responsive due to stress activated sensitization or, perhaps, podocalyxin surface expression can be induced by other erythropoietic-inducing humoral factors or hypoxia-sensing mechanisms. In either case, increased expression of podocalyxin in these progenitors is a key demonstration of the participation of BFU-E and CFU-E in response to hypoxic stress in both the marrow and spleen.

Sorting based on podocalyxin in cells lacking common lineage markers greatly enriched early progenitor populations that preferentially developed along the erythroid lineage, under stress conditions. Thus, podocalyxin could serve as a valuable research tool for enrichment of immature erythroid progenitor populations in anemic mice for future studies. We propose the use of podocalyxin as a marker for a simple sorting strategy (in mice) to study BFU-E and CFU-E populations induced by hypoxic stress. Finally, since most in vitro studies of BFU-E and CFU-E rely on high concentrations of Epo, these culture conditions likely induce podocalyxin expression even in progenitors that have not been exposed to anemic stress. Thus, it is likely
that studies examining erythroid colony formation or the interaction of erythroid progenitors with extracellular matrices, other erythroblasts or non-erythroid cells are conducted under conditions that induce podocalyxin surface expression.
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2.6 REFERENCES


CHAPTER 3. CD34 FACILITATES THE DEVELOPMENT OF ALLERGIC
ASTHMA

3.1 INTRODUCTION

Asthma is a pulmonary inflammatory disease characterized in humans by airway inflammation, airway hyper-responsiveness (AHR) and tissue remodeling (Hogg, 1997). In mice, induction of asthma leads to a Th2-polarized cytokine response, development of antigen-specific IgEs, a dramatic infiltration of inflammatory cells (eosinophils, lymphocytes and neutrophils) into the lung tissue and alveolar space, and increased airway resistance (AR) (Blanchet et al., 2005). The importance of individual hematopoietic cell types such as eosinophils and mast cells in asthma pathogenesis is still debated. Although some studies using mast cell and/or eosinophil-deficient mice have shown that these cell types are required for AHR and airway inflammation (Kobayashi et al., 2000; Lee et al., 2004), alternative reports (using a different strain of eosinophil-deficient mice on a different genetic background) suggest development of AHR and airway inflammation is eosinophil-independent but that these cells play a role in collagen deposition and remodeling (Humbles et al., 2004). It is known that many of the inflammatory factors released by eosinophils and mast cells in the lung, such as IL-5, TNFα, monocyte chemotactic protein-1 (MCP-1/CCL2) and IL-6 (Conti & DiGioacchino, 2001; Doganci et al., 2005; Hogg, 1997; Mukhopadhyay et al., 2006) play an important role in perpetuating lung inflammation. In addition, degranulation by eosinophils and mast cells provokes the

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release of bronchoconstrictive agents such as leukotrienes and bradykinins, which play a key role in development of AHR (Kim et al., 2006; Terawaki et al., 2005). In summary, although mast cells, eosinophils and their products are known to have potent immunomodulatory functions, their precise role in allergic asthma remains controversial.

Many pathways are involved in the recruitment of mast cells and eosinophils to the site of inflammation. Human eosinophils in vitro respond to a wide variety of chemotactic agents including eotaxin, RANTES and 5-oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE) (Ferland et al., 2001; Ferland et al., 2004). Migration of human eosinophils through basement membrane components is dependent on intracellular calcium flux (Liu et al., 1999), release of matrix metalloproteinases (MMPs) (Okada et al., 1997), and activation of the plasminogen/plasmin pathway (Ferland et al., 2001). Migration of murine eosinophils from the circulation to the alveolar space is known to require adhesion via P-selectin glycoprotein ligand-1 (PSGL-1)/P-selectin interactions (Larbi et al., 2003) and \( \alpha 4\beta 1 \) integrin/vascular cell adhesion molecule-1 (VCAM-1) interactions (Jia et al., 1999). Although the importance of mast cell recruitment to the lung versus local proliferation in situ has been debated, recent experiments suggest that mast cells are recruited to lung via \( \alpha 4\beta 1 \) or \( \alpha 4\beta 7 \) integrin and VCAM-1-dependent adhesion (Abonia et al., 2006).
CD34 is a cell surface sialomucin originally identified as a marker of hematopoietic stem cells (HSC), early hematopoietic progenitors and vascular endothelia (Delia et al., 1993) and was more recently identified on mast cells and eosinophils (Drew et al., 2005; Radinger et al., 2004). In fact, it was shown that in a mouse model of asthma, 50% of the recruited eosinophils were CD34+ (Radinger et al., 2004). Surprisingly, despite the wide attention CD34 has garnered as a marker of hematopoietic precursors (>14,000 citations), little is known of its function on these cells. On high endothelial venules (HEVs), a specialized type of lymph node endothelium, CD34 is modified with a rare glycan structure, sialyl Lewis-X, which allows it to function as an adhesive ligand for L-selectin (Fieger et al., 2003). However, the essential modifications to make CD34 a ligand for selectins have not been observed on CD34 expressed by the vast majority of vascular endothelial cells or hematopoietic cells, and therefore its function on most cell types has remained obscure.

Attempts to clarify the role of CD34 on hematopoietic precursors through two independent gene deletion studies have yielded cryptic and inconsistent phenotypes. One line of Cd34−/− mice exhibited essentially normal hematopoietic cell development and function in vivo, but a modest reduction in colony number and size from early hematopoietic precursors, suggesting a possible defect in proliferation or differentiation (Cheng et al., 1996). A second line of Cd34−/− mice showed no defects in hematopoietic cell development but a cryptic defect in eosinophil migration to the
lung in response to allergens, possibly the result of CD34 loss as a selectin ligand from lung endothelium (although this was never clearly resolved) (Suzuki et al., 1996).

By examining cultured mast cells from \textit{Cd34}^{-/-} mice, we recently found that loss of CD34 leads to a clear enhancement of mast cell adhesion and aggregation that can be fully reversed by ectopic re-expression of the protein (Drew, Merzaban \textit{et al}., 2005). These data suggest that CD34 serves to reduce adhesion, potentially enhancing the mobility of cells. Here we have exploited these observations to examine the relevance of CD34 expression in mast cells and eosinophil-dependent allergic responses. We find that CD34-deficiency leads to highly attenuated allergic inflammation and airway restriction \textit{in vivo} and impaired eosinophil trafficking through extracellular matrix \textit{in vitro}. The results suggest an essential role for this anti-adhesin in normal mast cell and eosinophil function \textit{in vivo} and in asthma pathogenesis.
3.2 MATERIALS AND METHODS

3.2.1 Mice

Mice were maintained in specific pathogen-free conditions at The Biomedical Research Centre and the local animal care committee approved all procedures. Six to eight week-old females C57Bl/6 (wt) and Cd34<sup>−/−</sup> (kindly provided by Dr T.W. Mak (Suzuki et al., 1996) and bred at The Biomedical Research Centre) were used throughout, unless otherwise stated. Cd34<sup>−/−</sup> mice have been backcrossed more than 5 generations onto a C57Bl/6 background and show no evidence of rejection of Cd34<sup>−/−</sup> bone marrow in transplantation or parabiosis experiments with C57Bl/6 mice.

3.2.2 Induction of asthma and assessment of alveolar inflammation

Asthma was induced in wt and Cd34<sup>−/−</sup> mice as previously described (Blanchet et al., 2005) with minor modifications. Briefly, mice were sensitized intraperitoneally with 0.2% chicken ovalbumin (OVA) mixed with Al(OH)<sub>3</sub> (both from Sigma, St Louis, MO) on days 1 and 8. Mice were subsequently intranasally challenged over several days, as shown in Figure 3.1A, with 50 µl 2% OVA. On day 28, the mice were anaesthetized with 200 mg/kg ketamine/10 mg/kg xylazine and blood was collected by cardiac puncture for analysis of blood eosinophil frequency. Bronchoalveolar lavages (BAL) were performed by three subsequent infusions and aspirations of 1.0 ml sterile PBS. Total BAL cells were counted and differential counts obtained from cytospin preparations stained with modified May-Grunwald Giemsa stain (HemaStain Set; Fisher Scientific, Kalamazoo, MI). Analysis of total and differential
BAL counts was also repeated with Cd34−/− mice and wt littermates.

A section of the left lung was fixed in 10% formalin for histological studies. The remainder of each lung was digested in 20 U/ml collagenase IV (Sigma) for 30 minutes and total inflammatory cells were used for the OVA recall assay and eosinophil sorting. In a separate experiment, lung hematopoietic cells were separated by gradient Percoll centrifugation, counted and used for eosinophil frequency analysis.

For eosinophil morphology, CCR3+CD3−B220− cells were sorted from total BAL cells, cytospun onto microscope slides and stained with modified May-Grunwald Geimsa stain. All images were captured on a Zeiss Axioplan2 microscope (Zeiss, Toronto, ON) using 40X/0.75 dry or 100X/1.30 oil objectives. Images were captured using a Qimaging Retiga EX CCD camera (Minneapolis, MN) and Openlab 4.0.4 software.

3.2.3 Histology

Formalin-fixed (overnight) lung tissue was embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E) for evaluation of lung inflammation or toluidine blue for evaluation of mast cell infiltration. Histological scores were obtained in a blinded fashion from H&E stained slides. A relative score from 0 to 5
was attributed (0 = no sign of disease, 5 = profound inflammation) for each of the following: perivascular, peribronchial and parenchymal infiltration and epithelial damage for a potential maximum score of 20, by scanning the entire lung section. Mast cell infiltration was evaluated by counting numbers of mast cells in each section from the toluidine blue stained slides.

3.2.4 OVA recall assay and cytokine production

Total lung cells were obtained from OVA-sensitized and challenged wt and Cd34⁻/⁻ mice. Cells (500 000) were plated in RPMI media with 10% fetal bovine serum and 1% penicillin/streptomycin and stimulated with increasing doses of OVA. Supernatants were collected after overnight culture (24 hours) and cytokine production was evaluated using a cytometric bead essay (Mouse Inflammatory Cytokine Kit; BD Biosciences, San Diego, CA) according to the manufacturer's instructions.

3.2.5 Assessment of airway hyperresponsiveness

On day 29, wt and Cd34⁻/⁻ mice were anaesthetized with a 200 µl intraperitoneal injection of a 10 mg/ml ketamine/1 mg/ml xylazine solution, tracheotomized and intubated with an 18 G catheter. Airway resistance (AR) was measured with a Flexivent apparatus (SCIREQ, Montreal, QC, Canada). Respiratory frequency was set at 160 breaths/min with a tidal volume of 0.2 ml and a positive end-point expiratory pressure of 2-4 ml H₂O was applied. Increasing concentrations of
metacholine (Mch; 0 to 125 mg/kg) were administered via the jugular vein. AR was recorded every 15 seconds by a snapshot measure. Baseline AR was reached prior to administering subsequent doses of MCh. The percentage increase in AR was calculated for each MCh dose.

3.2.6 Flow cytometric analysis of BM progenitors, BAL, blood and tissue eosinophils

Cells were blocked with 10% mouse serum prior to staining. The following antibodies were used: biotinylated rat anti-mouse B220 (clone RA-6B2), CD3 (clone 2C11), CD34 (BD Pharmingen, San Diego, CA), CD4, and Gr-1 (clone Ly-6G) followed by streptavidin-APC (BD Pharmingen); rat anti-mouse CCR3-PE (R&D Systems, Minneapolis, MN); Sca-1-APC (eBioSciences); anti-mouse CD45.2-PerCP, IL5R-alpha-PE (clone T21.2), Mac-1-PE, and Ter119-PE (all from BD Pharmingen). CCR3+/B220−/CD3− BAL cells were sorted using a FACSaria (BD Biosciences, Mountain View, CA). Sorted cells were cytospun and Giemsa-stained to verify eosinophil purity. Alternately, BAL cells were stained for CCR3 and CD34 to assess eosinophil surface marker expression. The frequency of CD45+/CCR3+/B220−/CD3− cells (eosinophils) in blood and collagenase-treated individual lungs was analyzed. For analysis of bone marrow (BM) progenitors, BM was flushed from femurs of OVA-induced asthmatic mice with 10 ml PBS-0.1% BSA and populations of B220+, CD3+, CD4+, Gr-1+, Sca-1+, IL5Ralpha+, Mac-1+, and Ter119+ cells were assessed. BAL cell CD34 staining, blood, lung tissue, and BM data were collected on a BD FACSCalibur (BD Biosciences). Data were analyzed with FlowJo software (Treestar, Ashland,
3.2.7 Eosinophil migration assay

Sorted BAL CCR3⁺ eosinophils were placed in the upper chamber of 24-well Biocoat Matrigel Invasion Chambers (BD Biosciences). Eotaxin (30 ng/ml) (synthesized at the Biomedical Research Centre) was added in the lower chamber as a chemoattractant. Chambers were incubated at 37°C + 5% CO₂ for 18h. At the end of the incubation period, cells in both the upper and lower chambers were removed by aspiration and 10,000 microbeads were added to each sample to allow rapid counting by FACS. Briefly, for each sample, beads were gated and samples collected until 5000 total beads had been counted. The number of cell events were then determined by gating and this number was multiplied by 4, to determine total cell number in the original sample. For both wt and Cd34⁻/⁻ cells, the migration index was calculated as follows: the number of cells in the lower chamber of the Matrigel Invasion Chamber was divided by the total number of cells in both lower and upper chamber and multiplied by 100. That migration percentage was then divided by the percentage of migration in a control chamber lacking Matrigel or eotaxin (spontaneous migration), and multiplied by 100.

3.2.8 Bone marrow reconstitutions

BM was isolated from both wt (Ly 5.2) and Cd34⁻/⁻ (Ly 5.2) mice. Cells (10⁷) were injected intravenously into C57Bl/6-Ly 5.1 lethally-irradiated recipients and 12 weeks
were allowed for complete BM reconstitution. Reconstitution was evaluated by Ly 5.2 vs. Ly 5.1 expression on peripheral blood and mice were considered reconstituted when more than 80% of hematopoietic cells (in Ly 5.1 recipients) originated from the Ly 5.2 donors. Reconstituted mice were then sensitized and challenged with OVA and inflammatory parameters evaluated as previously described.

3.2.9 Statistics

For analysis of total cells, hematopoietic cell subtypes in BAL and AHR, statistical analyses were performed using an ANOVA table followed by a Fisher post hoc test. For analysis of histology, cytokine release, haematopoietic progenitors, blood and lung eosinophil frequency and eosinophil migration, unpaired t-tests were used.
3.3 RESULTS

3.3.1 Allergic airway inflammation is attenuated in Cd34^{-/-} mice

To examine the role of CD34 expression in mast cell and eosinophil responses, wt and Cd34^{-/-} mice were sensitized with OVA-alum twice intraperitoneally and subsequently challenged intranasally with OVA (Figure 3.1A). As shown in Figure 3.1B, OVA sensitization and challenge in wt mice caused an inflammatory cell infiltration in the BAL when compared to control mice (2.94 ± 0.42 x 10^6 cells / ml in OVA-sensitized mice compared to 0.05 ± 0.01 x 10^6 cells / ml in naïve mice). Interestingly, Cd34^{-/-} mice exhibited an attenuated response, with less than half the number of infiltrating cells in the BAL (1.16 ± 0.32 x 10^6 cells / ml, Figure 3.1B) compared to challenged wt animals. To clarify which cell types were present in the infiltrates, differential counts were performed (Figure 3.1C). In wt mice the majority of infiltrating cells (>50%) were eosinophils followed by equal frequencies (approximately 20%) of neutrophils, lymphocytes and macrophages. Interestingly, Cd34^{-/-} mice showed a similar profile of inflammatory cell infiltration with a clear reduction in overall cell numbers, suggesting a general decrease in the degree of inflammation (Figure 3.1C). Similar results were obtained in Cd34^{-/-} and wt littermate comparisons (data not shown).
Figure 3.1: Protocol for OVA sensitization and BAL analysis.

(A) Schematic of the sensitization and challenges protocol used to induce asthma in wt and Cd34⁻/⁻ mice. (B) Total cell counts in BAL from naive and challenged (OVA) asthmatic wt and Cd34⁻/⁻ mice (n=6; error bars=SEM). (C) Differential count results from naive and challenged (OVA) wt and Cd34⁻/⁻ mice (n=6; Error bars = SEM).
Attenuation of inflammatory responses in \( Cd34^{-/-} \) mice was further confirmed by histological analyses. H&E- and toluidine blue-stained tissues were blindly evaluated and inflammation scores were awarded (0 = no sign of disease, 5 = profound inflammation) for the following: perivascular infiltration, peribronchial infiltration, epithelium damage and parenchymal infiltration for a potential total score of 20. As shown in Figure 3.2A, OVA sensitization and challenge induced potent perivascular, peribronchial and parenchymal inflammatory cell infiltration. Scoring of histological inflammation (Figure 3.2B) revealed a significantly milder tissue inflammation in \( Cd34^{-/-} \) mice compared to wt (score = 10.3 ± 0.9 in wt vs. 6.6 ± 1.2 in \( cd34^{-/-} \), \( p = 0.03, n = 6 \)). The main differences between the two groups were observed in peribronchial and parenchymal infiltration, which were lower in \( Cd34^{-/-} \) mice. One can note the obvious differences in lung tissue inflammation when challenged wt and \( Cd34^{-/-} \) lung sections are compared (Figure 3.2A). Although \( Cd34^{-/-} \) mice still showed signs of inflammatory cell infiltration around bronchi and vessels, infiltration in wt mice was more severe and extended deeper to the parenchyma.
Figure 3.2: H&E-stained lung sections from naive and OVA-challenged asthmatic mice.

Lung sections were fixed in 10% formalin 24 hours after the last OVA challenge. (A) naive wt mouse, naive Cd34−/− mouse, OVA-challenged wt mouse, and OVA-challenged Cd34−/− mouse. (B) Mean total histologic scores for peribronchial, perivascular, and parenchymal infiltration and epithelium damage. A score of 1 to 5 (0=no sign of disease, 5=profound inflammation) for each parameter was blindly attributed after analysis of 5 fields from each sample. (n=6; Error bars = SEM for each group).
3.3.2 Asthmatic Cd34⁻/⁻ mice do not develop airway hyperresponsiveness (AHR) in response to OVA challenges

In addition to inflammatory cell infiltration, a second major hallmark of allergic asthma is the development of AHR. We assessed airway resistance (AR; a measure of AHR) in naive and OVA-sensitized mice through a metacholine (MCh) challenge approach. As shown in Figure 3.3, naive wt and Cd34⁻/⁻ mice showed similar responses to MCh prior to OVA sensitization. As expected, OVA-asthmatic wt mice displayed significantly increased AR in response to MCh. Strikingly, OVA-sensitized Cd34⁻/⁻ mice exhibited no increase in AR (Figure 3.3), even at the highest doses of MCh tested on this strain (500 µg/kg; data not shown). Cd34⁻/⁻ mice demonstrated a significantly decreased response to MCh compared to wt starting at the 3.9 µg/kg dose and throughout all higher dosages for the rest of the challenge (p < 0.05 for every dose, n = 6). Surprisingly, the MCh response in Cd34⁻/⁻ OVA-challenged mice was significantly lower than in Cd34⁻/⁻ naive mice starting at the 15.6 µg/kg dose (p < 0.05 for 15.6 µg/kg dose and following doses; n = 6). Thus, Cd34⁻/⁻ mice show a complete failure in developing this second phenotypic hallmark of asthma and were indeed less responsive than naïve mice.
Figure 3.3: Airway resistance (AR) in naive and OVA-challenged mice.

Naive and OVA-challenged mice were administrated increasing doses of MCh (intravenously) and the increase in airway resistance from baseline (R [% increase]) was measured for each MCh dose. (* represents p < .05 of OVA-challenged mice compared to respective naive controls; n=6 for each dose; Error bars = SEM.)
3.3.3 Normal antigen-specific recall responses in OVA-challenged Cd34^-/- mice.

To test whether the attenuated allergic responses in Cd34^-/- mice was due to an inability to effectively present antigen or produce the appropriate cytokines, total lung cells from wt and Cd34^-/- mice were isolated and stimulated ex vivo with OVA to evaluate antigen-specific cytokine responses. Results from 3 independent cytokine production experiments are compiled and presented in Figure 3.4. No statistically significant difference was found between wt and Cd34^-/- cells in their ability to release TNF, IL-6, MCP-1 (CCL2) or IL-5 (on a per cell basis) in response to OVA sensitization. We conclude that there are no major defects in antigen presentation or cytokine production in Cd34^-/- mice.
Figure 3.4: Cytokine recall assay of cells isolated from asthmatic mice.

Isolated total lung tissue cells were cultured for 24 hours with the indicated doses of OVA and cytokine release was measured. (A) TNF, (B) IL-6, (C) MCP-1 (CCL2), and (D) IL-5 release in response to OVA-stimulation using equivalent numbers of isolated lung tissue inflammatory cells. There was no statistically significant difference in OVA-specific cytokine release between wt and Cd34−/− mice (n=3 independent experiments).
3.3.4 Normal frequency of hematopoietic precursors, increased eosinophil frequency in blood and decreased numbers of lung eosinophils in OVA challenged asthmatic Cd34\(^{-/-}\) mice

To elucidate which steps in eosinophil recruitment to the lung were affected by loss of CD34, we first examined the profiles of hematopoietic precursor cells in the BM of \(wt\) and \(Cd34^{-/-}\) mice following allergen challenge. No statistically significant differences were observed in the profiles of eosinophil progenitors, granulocytes, macrophages, B lineage, T lineage or erythroid lineage cells in either naive animals (not shown) or allergic animals (Figure 3.5A I and II). This suggests that differences in the asthmatic response are not a result of deficiencies in progenitor cell development or differentiation in the BM. We also tested the frequency of blood and lung tissue eosinophils (CD45\(^{+}\)/CCR3\(^{+}\) cells) and noted a significant increase in blood CD45\(^{+}\)/CCR3\(^{+}\) cells in \(Cd34^{-/-}\) / OVA-challenged mice (6.55 % ± 0.44) compared to \(wt\) (4.53 % ± 0.55; \(p = 0.02, n = 6\)) (Figure 3.5B). This was complemented by a decrease in total CD45\(^{+}\)/CCR3\(^{+}\) cells/ lung in OVA-challenged \(Cd34^{-/-}\) mice (2.43 x10\(^{6}\) ± 0.22 eosinophils / lung) compared to \(wt\) (4.54 x10\(^{6}\) ± 0.53 eosinophils / lung; \(p = 0.01, n = 4\)) (Figure 3.5C).
Figure 3.5: Bone marrow progenitor populations, frequency of blood eosinophils, and lung tissue eosinophil numbers in wildtype and Cd34−/− mice.

(A) Frequency of bone marrow hematopoietic progenitor markers in OVA-challenged mice. (Ai) B220+ indicates B lymphocytes; c-kit+, immature progenitors; Gr1+, granulocytes; Mac-1+, monocytes; and Ter-119+, erythroid cells; (Aii) Lin−Sca−IL5R+ indicates eosinophil progenitors; and CD3+, T lymphocytes. All cells were analyzed by FACS. (B) Frequency of blood CD45+/CCR3+ cells (eosinophils) in naive and OVA-challenged wt and Cd34−/− mice. (C) Frequency of CD45+/CCR3+ cells in hematopoietic (CD45+) cell preparations from individual lungs of naive and OVA-challenged wt and Cd34−/− mice was analyzed by FACS. Total hematopoietic cells/lung counts were obtained and used to determine total eosinophils/lung. (n=4-6; * represents p< 0.05; Error bars = SEM)
3.3.5 Defective mast cell trafficking and eosinophil migration in Cd34⁻/⁻ mice

The failure of Cd34⁻/⁻ mice to develop a robust allergic response despite normal production of inflammatory cells in BM and response to antigen in cytokine recall assays suggests that other defects underlie the attenuated inflammatory response in these animals. Previously, we have shown that, on mast cells, CD34 can act as a potent inhibitor of cell adhesion and that mice lacking CD34 exhibit defects in mast cell trafficking. Since AHR has previously been reported to be defective in mice lacking mast cells, we analyzed the number of mast cells in wt and Cd34⁻/⁻ lungs before and after OVA challenge. Although wt and Cd34⁻/⁻ mice showed similar numbers of lung mast cells prior to challenge (as presented in Figure 3.6), wt mice exhibited a potent recruitment of mast cells to the lungs after OVA challenge (18.1 ± 1.5 mast cells / section versus 3.3 ± 0.6 mast cells/section in naives; p < 0.0001, n = 6), while Cd34⁻/⁻ mice exhibited a clear impairment in the recruitment of mast cells after OVA challenge (6.3 ± 1.5 mast cells/section; p = 0.0002 compared to wt ; n = 6). We conclude that impaired mast cell recruitment is likely one of the causes for the reduced AHR in Cd34⁻/⁻ mice.
Figure 3.6: Mast-cell lung infiltration in response to OVA challenge.

The number of mast cells/lung section was obtained from toluidine blue–stained lung sections of naive and challenged mice (n=6; Error bars = SEM for each group).
Additionally, studies have suggested that the trafficking of eosinophils to the lung during an allergic response can occur in the absence of mast cells (Kobayashi et al., 2000). Thus, although the lack of AHR in Cd34⁻/⁻ mice is likely, in part, due to impaired mast-cell recruitment, the failure of eosinophils to infiltrate the lung may be due to a separate defect. Interestingly, recent reports have suggested that eosinophils can also express CD34 (Radinger et al., 2004). To confirm this observation and to test whether CD34 loss has a role in eosinophil trafficking, we compared BAL eosinophils from wt and Cd34⁻/⁻ mice for expression of CD34 and for their ability to migrate in vitro. To identify eosinophils, BAL fluid and dissociated lung tissue cells were co-stained for CD34 and the eotaxin receptor (CCR3), a well-known marker of eosinophils that is required for their efficient homing to the lung during allergic inflammation (Hylkema et al., 2002). Figure 3.7A,B shows identical levels of CCR3 expression by wt and Cd34⁻/⁻ BAL eosinophils, potentially suggesting no inherent defect in the ability of these cells to respond to eotaxin. Differential analysis of Giemsa-stained cytospin preparations from CCR3⁺/B220⁻/CD3⁻ sorted cells (Figure 3.7C) revealed 98% eosinophil purity, and no obvious visible difference in cell morphology between purified wt and Cd34⁻/⁻ eosinophils. Wt CCR3⁺ cells exhibited a low but reproducible level of staining with CD34 antibody, while CCR3⁺ cells from Cd34⁻/⁻ mice showed no such staining, confirming the presence of this molecule on eosinophils (Figure 3.7D,E). CD34 staining levels on Cd34⁻/⁻ eosinophils were equivalent to unstained controls from both wt and Cd34⁻/⁻ cells (data not shown).
Figure 3.7: FACS profiles of CCR3 and CD34 expression on BAL and parenchymal cells from asthmatic mice, and migration assay results.

(A,B) FACS profiles showing granularity (as measured by side light scatter [SSC]) versus CCR3 fluorescence staining on purified cells. (C) Giemsa staining of cytospin preparations from CCR3+ sorted cells. Eosinophils (CCR3+CD3−B220−) were sorted from total BAL from OVA-challenged asthmatic mice. (D,E) Single-color histograms showing CD34 staining intensity on SSChi CCR3+ gated populations from panels A and B. Gray-filled histogram represents staining intensity from Cd34−/− cells, while the black line represents staining of wt cells. (F) Matrigel migration assay of wt and Cd34−/− sorted eosinophils. The migration index (in response to 30 nM eotaxin) was compared between wt (normalized to 100% migration) and Cd34−/− mice (n=4 independent experiments using pooled BALs cells from 4-6 mice for each experiment; Error bars = SEM).
Interestingly, CCR3\(^{+}\) cells in the BAL fluid showed higher levels of CD34 than CCR3\(^{+}\) cells in the underlying lung tissue, suggesting a possible correlation between CD34\(^{+}\) cells and cell invasiveness. To test this notion, CCR3\(^{+}\)/B220\(^{-}\)/CD3\(^{-}\) sorted cells from BAL of wt and Cd34\(^{-/-}\) mice were compared in a side-by-side chemotaxis assay for their ability to migrate through Matrigel in response to eotaxin. Cd34\(^{-/-}\) eosinophils exhibited 44.9 ± 8.6\% lower ability to migrate through Matrigel when compared to wt cells (Figure 3.7F; n = 4; p = 0.002). Since differential selectin binding by Cd34\(^{-/-}\) and WT eosinophils is also a potential explanation for poorer eosinophil trafficking in the lung, we used FACS to determine the ability of wt and Cd34\(^{-/-}\) sorted BAL eosinophils to bind L-, P- and E-selectin. Binding was identical between the 2 populations (data not shown). We therefore conclude that Cd34\(^{-/-}\) eosinophils have an intrinsic defect in their ability to migrate through matrix in vitro.

3.3.6 Phenotype of Cd34\(^{-/-}\) mice is due to lack of CD34 expression on hematopoietic cells

Although all these data are consistent with an intrinsic defect in the ability of Cd34\(^{-/-}\) eosinophils and mast cells to chemotax, CD34 is also expressed by most vascular endothelial cells, including those in the lung. Thus, the possibility remained that some defects in the allergic homing of Cd34\(^{-/-}\) cells were due to loss of CD34 from the vascular endothelia. To address this issue, we generated hematopoietic chimeras by reconstituting lethally-irradiated Ly5.1 C57BL/6 mice with wt or Cd34\(^{-/-}\)
(Ly5.2) BM. In each case, peripheral blood cells were analyzed 12 weeks post-transplant to ensure that more than 80% of the white blood cells of these chimeras were derived from donor BM. These mice were then sensitized and challenged with OVA as in previous protocols. Total and differential cell counts in the BAL are shown in Figure 3.8. As expected, mice reconstituted with wt BM showed an increase in total BAL inflammatory cells in response to OVA compared to naive controls. Interestingly, the number of inflammatory cells was again approximately 50% lower in mice that were reconstituted with Cd34⁻/⁻ BM. Moreover, differential count analysis shows that the differential profile of each hematopoietic population is similar between the 2 groups, but the number of eosinophils and lymphocytes was significantly lower in Cd34⁻/⁻ reconstituted mice compared to wt reconstituted mice. As these results match results in non-reconstituted animals, we conclude that the allergic phenotypes observed in Cd34⁻/⁻ mice is due to a loss of CD34 expression in cells of hematopoietic origin (i.e. eosinophils and mast cells) rather than a loss of this molecule from the nonhematopoietic microenvironment.
Figure 3.8: BAL analysis of wildtype and Cd34<sup>−/−</sup> bone marrow–reconstituted mice.

(A) Total cells in the BAL of wt bone marrow–reconstituted mice (wt in Ly5.1) or Cd34<sup>−/−</sup> bone marrow–reconstituted mice (Cd34<sup>−/−</sup> in Ly5.1) following OVA sensitization and challenge. (B) Differential counts of BAL cells from wt bone marrow–reconstituted mice (wt in Ly5.1) or Cd34<sup>−/−</sup> bone marrow–reconstituted mice (Cd34<sup>−/−</sup> in Ly5.1). (* represents p < .05 when wt are compared with Cd34<sup>−/−</sup>; n=6; Error bars = SEM).
3.4 DISCUSSION

Although CD34 is best known as a marker of HSCs and early hematopoietic precursors, recent studies have shown that it is also expressed by eosinophils and mast cells (Drew et al., 2005; Radinger et al., 2004). Here we have addressed its role on these mature haematopoietic cells using a well-defined, eosinophil- and mast cell-dependent asthma model. 

\( Cd34^{-/-} \) mice exhibited a complete absence of AHR and a striking attenuation in the degree of inflammatory cell infiltration, two major hallmarks of allergic asthma. Since: (1) these effects are known to be eosinophil- and mast cell-dependent; (2) these cells types are rapidly recruited to the lung in response to allergic challenge; and (3) both cell types have recently been shown to express CD34, we focused our attention on these cells in the mutant mice. We noted a clear defect in the recruitment of both cell types in \( Cd34^{-/-} \) mice.

Our eosinophil result suggests that lack of CD34 expression impairs the capacity of hematopoietic cells to cross from the circulation into the lung tissue and then to leave the tissue towards the alveolar space. Each of these steps involves movement through the basement membrane and extracellular matrix components. This migration process can be mimicked, by using Matrigel-coated transwell migration chambers, which consist of matrix proteins. Since eosinophils that lack expression of CD34 showed an impaired capacity to migrate through Matrigel and \( Cd34^{-/-} \) OVA-challenged mice had higher frequency of blood eosinophils and lower tissue eosinophils, it is likely that CD34 is required for optimal transmigration from the circulation into the lung tissue and alveolar space, and that suboptimal migration
underlies the reduced number of eosinophils in the BAL of \( Cd34^{--} \) mice.

How does CD34 loss impair allergic responses? We observed no defects in the ability of \( Cd34^{--} \) mice to generate hematopoietic subsets in the BM, nor did we observe any defects in the ability of lung infiltrating hematopoietic cells to produce inflammatory cytokines in response to specific antigen challenge. Thus, \( Cd34^{--} \) mice appear to be fully competent to mount an appropriate immune response. The best evidence to date suggests CD34 serves a proadhesive or an antiadhesive function depending on its cellular context. On HEVs in lymph nodes, CD34 has previously been shown to act as a glycosylation-dependent ligand for L-selectin on naive lymphocytes and to facilitate their trafficking into the lymph nodes (Delia et al., 1993). Although CD34 is normally expressed on lung endothelium, several experiments would argue against a pro-adhesive function for CD34 on these cells as the cause of defects in CD34-deficient mice. Firstly, CD34 binding to L-selectin is known to be highly dependent on HEV-specific glycosylation of CD34 and the appropriate modifications are not present on CD34 expressed by lung endothelia or eosinophils. Secondly, we observed defective migration of \( Cd34^{--} \) eosinophils in \textit{in vitro} chemotaxis assays in the absence of vascular endothelial cells or L-selectin, suggesting an intrinsic hematopoietic defect rather than a vascular defect. Finally, and most importantly, all observed defects in allergic responses in \( Cd34^{--} \) mice could be recapitulated in chimeric \textit{wt} mice that had been transplanted with \( Cd34^{--} \) BM. Since these mice have \textit{wt} endothelium and non-hematopoietic cells, the data would argue that the failure to develop allergic asthma is due to intrinsic defects in the
Particularly relevant to the present study, we have previously shown that CD34 is a marker of mast cells and their precursors and that loss of the CD34 leads to a clear increase in their adhesiveness (Drew et al., 2005). We have also shown that enhanced adhesion can be reversed by the ectopic re-expression of CD34 in mast cells. Finally, we have shown that loss of CD34 in mice results in no difference in kinetics of mast-cell proliferation or survival but striking defects in HSC and mast cell trafficking, presumably due to enhanced adhesion (Drew, Merzaban et al., 2005; Nielsen & McNagny, 2007). Recent studies, using a similar model as the one described here, have suggested that the rapid increase in the frequency of lung mast cells during allergic challenge is the result of VCAM-1 and \( \alpha_4\beta_1 \) integrin-dependent mast cell recruitment, rather then local mast cell proliferation in situ (Abonia et al., 2006). Thus, the decreased mast cell migration into the lungs of allergic \( Cd34^- \) mice likely reflects impaired trafficking of these cells.

Previous reports have suggested that approximately 50% of eosinophils recruited to the lung during allergic lung inflammation express CD34 (Radinger et al., 2004). We have re-examined this issue by comparing \( Cd34^- \) and wt eosinophils. Our data suggests that CD34 is expressed at low levels by all eosinophils, but the highest level by those that have migrated to the BAL. We have also shown that \( Cd34^- \) eosinophils have a clear defect in the ability to migrate in chemotaxis assays in vitro.
and to infiltrate lungs *in vivo*. It is unlikely that this was due to differences in cell maturation, shape or chemokine signalling, as CCR3 expression was equivalent in both strains of mice and analysis of Giemsa-stained eosinophil preparations suggests no obvious difference in shape or maturation status between *Cd34<sup>−/−</sup>* and *wt* eosinophils, but could be explained by differences in cell flexibility or impaired intracellular signalling in *Cd34<sup>−/−</sup>* cells. Moreover, the absence of L-selectin or vascular endothelial cells to act as an adhesive substrate in *in vitro* assays, again, would argue against a proadhesive role for CD34 on eosinophils and would instead suggest that the defect in migration is the result of enhanced adhesiveness of cells lacking this highly-charged sialomucin.

Using mast-cell deficient (Kobayashi *et al.*, 2000) or eosinophil deficient (Lee *et al.*, 2004) mice, other groups have shown that development of full AHR in mouse models of asthma is dependent on the presence of mast cells and eosinophils. Therefore, the lack of AHR in *Cd34<sup>−/−</sup>* mice could be due to the decreased recruitment of both these cell populations to the lung. On the other hand, although the recruitment of eosinophils to the lung is reduced 2-3 fold in *Cd34<sup>−/−</sup>* mice, it is noteworthy that it is not completely eliminated and is still substantially higher than that of naive mice while AHR is completely abrogated, which could argue against a direct link between eosinophil trafficking and development of AHR. This interpretation would be supported by previous reports showing that an independent strain of eosinophil-deficient mice (Δdbl GATA mice) develop AHR in the complete absence of eosinophils (Humbles *et al.*, 2004), and by several studies in human that
failed to show a correlation between eosinophil infiltration and AHR (Wills-Karp &
Karp, 2004). Finally, the fact that \( Cd34^{-/-} \) OVA-challenged mice exhibit even less
AHR than \( Cd34^{-/-} \) naive mice would again argue against a direct link between
eosinophil trafficking to the lungs and AHR. One of many possible explanations for
the complete lack of AHR in \( Cd34^{+/+} \) mice could be that CD34 is required for airway
smooth muscle contraction or for airway smooth muscle cell progenitor recruitment.
In this regard, it is intriguing that smooth muscle cells have been reported to express
CD34 in humans (Schmidt et al., 2003). Further studies of smooth muscle progenitor
recruitment in a more chronic model of asthma may shed light on this possibility.

Mast cells and eosinophils are associated with a wide variety of pathological
inflammatory responses including allergy, arthritis, multiple sclerosis-like syndromes
and lung fibrosis. Our data suggest that loss of CD34 from mast cells and
eosinophils could lead to attenuation of much of the inflammatory damage
associated with these relatively common and severe diseases and that it may be an
ideal target molecule for therapy. The fact that \( Cd34^{+/+} \) mice show no major defects
in normal development would suggest that therapies based on transiently impairing
its expression should be well-tolerated. Further experiments will be required to
evaluate the importance of CD34 in these additional diseases.
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CHAPTER 4. CD34 IS REQUIRED FOR INFILTRATION OF EOSINOPHILS INTO THE COLON AND PATHOLOGY ASSOCIATED WITH DSS-INDUCED ULCERATIVE COLITIS

4.1 INTRODUCTION

The two major forms of inflammatory bowel disease (IBD), Crohn’s disease (CD) and ulcerative colitis (UC), are multi-factorial intestinal disorders, which have become increasingly prevalent in Western society. Clinical symptoms of these disorders include weight loss, diarrhea, bleeding, fever and shortening of the colon (Hendrickson et al., 2002). Typical histological findings from colon tissue from UC patients reveal the infiltration of leukocytes into the gut mucosa, goblet cell depletion, crypt abscesses and distortion of mucosal glands (Hendrickson et al., 2002). Despite the increasing prevalence of these disorders, our understanding of how they originate and develop is still quite poor.

The immune cells resident in the intestines are continuously exposed to both endogenous and exogenous antigens and play a key role in regulating food tolerance and defense against gut pathogens. Dysregulation of this immune response results in mucosal inflammation and tissue damage, leading to the onset of disease (Torres & Rios, 2008). One key cell type in the diseased colon is eosinophils, and several lines of evidence suggest a role for eosinophils in the

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pathology of ulcerative colitis, in both humans and in animal models. In UC and CD patients, colon eosinophil numbers are significantly elevated compared to healthy controls (Bischof et al., 1996; Carvalho et al., 2003; Makiyama et al., 1995). Eosinophils also respond to a number of cytokines associated with IBD, including eotaxin, which is increased in the serum of IBD patients (Chen et al., 2001), IL-5 and TNFα, both of which are present in perfusion fluids isolated from patients with ulcerative colitis (Lampinen et al., 2001). Additionally, levels of the eosinophil granule proteins eosinophil peroxidase (EPO) / eosinophil protein X (EPX), major basic protein (MBP) and eosinophil cationic protein (ECP) in biopsy specimens from UC patients correlate with disease severity (Carlson et al., 1999; Sangfelt et al., 2001). While the potential role of eosinophils in IBD development is not currently clear, tissue eosinophilia is well accepted in other disease conditions, such as eosinophilic gastroenteritis and esophagitis (Bischof et al., 1999; Powell et al., 2010).

In mice, oral administration of dextran sulfate sodium (DSS) provokes intestinal inflammation with similar features to human UC and serves as a valuable model of colitis. While increasing interest has focused on the role of infiltrating immune cells on disease pathology, much of the focus has highlighted roles for neutrophils. However, several studies have also indicated a critical role for eosinophils in colitis pathology. Eotaxins act as chemoattractants for eosinophil migration, such that eotaxin-1−/−, eotaxin-2−/− and eotaxin-1/2−/− mice develop ameliorated forms of colitis, after DSS treatment (Ahrens et al., 2008; Forbes et al., 2004). Further, decreased
disease severity is associated with decreased eosinophil infiltration into the colon in eotaxin-1\(^{-/-}\) animals (Forbes et al., 2004), demonstrating the importance of eosinophil migration in disease pathology. The adhesion molecule ICAM-1 is also expressed by circulating eosinophils and plays a key role in eosinophil migration into the large intestine following hapten-induction of colitis (Forbes et al., 2006). Furthermore, a key role for eosinophil degranulation, particularly release of EPO and ECP, has been shown in DSS-induced ulcerative colitis. EPO\(^{-/-}\) mice, or mice treated with the EPO-inhibitor resorcinol, developed attenuated DSS-induced colitis (Forbes et al., 2004) and antibody blockade of ECP in a rat DSS model results in reduced disease pathology (Shichijo et al., 2005). Finally, total ablation of eosinophils in both the PHIL and the \(\Delta\)dblGATA-1 mouse models results in decreased disease severity and gut pathology in two independent studies (Ahrens et al., 2008; Vieira et al., 2009).

Despite findings of a critical role for eosinophils in colitis pathology, somewhat surprisingly, deletion of IL-5 has no effect on disease severity (Kopf et al., 1996). IL-5 promotes eosinophil development and expansion and while IL-5\(^{-/-}\) mice have normal peripheral eosinophil numbers, they are unable to develop eosinophilia (Kopf et al., 1996). Several groups have demonstrated that IL-5-deficient mice exhibit normal DSS colitis induction, and demonstrate that IL-5 is not required for the development of ulcerative colitis (Forbes et al., 2004; Stevceva et al., 2000). Taken together, these findings suggest that while eosinophil migration and degranulation are critical for disease pathology, IL-5-mediated eosinophil expansion and survival is not required.
CD34 is a transmembrane sialomucin and although it has been widely used as a hematopoietic stem cell marker (HSCs) for almost 30 years and more recently for other stem cell/progenitor types, such as muscle satellite cells (Beauchamp et al., 2000), adipogenic precursors (Joe et al., 2010) and epidermal precursors (Trempus et al., 2003), its function remains unclear. In high endothelial venules (HEVs), where CD34 is uniquely glycosylated to bind L-selectin, CD34 promotes cell adhesion in the recruitment of naïve lymphocytes to secondary lymphoid organs (Baumheter et al., 1993; Baumhueter et al., 1994). However, HEV cells appear to be the only cell type able to modify CD34 for L-selectin interaction, thus L-selectin binding is not thought to be a general function for CD34 on other cell types (Nielsen & McNagny, 2008).

In addition to expression on progenitor cell types, CD34 is also expressed on differentiated leukocytes, including mast cells (Drew et al., 2002), dendritic cell precursors and eosinophils (Blanchet et al., 2007; Radinger et al., 2004). Our studies with Cd34−/− mice demonstrate that CD34 plays a key role in anti-adhesion on these mature cell types. CD34-deficient mast cells exhibit dramatically enhanced homotypic cell aggregation in vitro and delayed tissue homing in vivo in a water ablation model, particularly when the related sialomucin, CD43, is also deleted (Drew et al., 2005). Likewise, mast cell and eosinophil accumulation in the lung in an OVA-induced asthma model is significantly decreased in Cd34−/− mice and Cd34+/−
eosinophils exhibit impaired migration in an *in vitro* Matrigel transwell assay (Blanchet *et al.*, 2007). Further, we have observed that the reduced accumulation of mast cells and eosinophils in *Cd34*<sup>-/-</sup> tissues correlates with reduced disease symptoms and that CD34 loss specifically on hematopoietic cells is sufficient to block disease (Blanchet *et al.*, 2007). Finally, HSCs derived from CD34-deficient mice show significantly decreased bone marrow repopulation in competitive reconstitution assays (Nielsen & McNagny, 2007), demonstrating that on hematopoietic precursor cells, CD34 is critical for optimal cell migration.

Based on these previous findings, we hypothesized that CD34 plays a similar role in facilitating eosinophil migration in other inflammatory disease models. As ulcerative colitis, in a DSS-induced mouse model, appears to be dependent on eosinophil migration, we tested whether CD34-deficiency leads to attenuated symptoms in DSS induction of experimental colitis. In this study, we show that *Cd34*<sup>-/-</sup> mice exhibit a significantly milder colitis than *Cd34*<sup>+/+</sup> mice and that the primary inflammatory population expressing CD34 within the inflamed colon is eosinophils. Further, using hematopoietic chimeras, we demonstrate that CD34 expression on hematopoietic cells correlates with the attenuated disease phenotype. Interestingly, our studies show that peripheral eosinophil levels decrease with the onset of disease pathology and that eosinophilia alone, in the absence of DSS treatment (in IL5<sup>Tg</sup> animals), is sufficient to induce low levels of colon shortening. Finally, we demonstrate that eosinophilia in *Cd34*<sup>-/-</sup> mice is sufficient to overcome protection from disease. This study further supports the model that eosinophils, and
particularly eosinophil migration, are required for the development of ulcerative colitis pathology. It also furthers our understanding of CD34 expression and function in disease and suggests CD34 is a potential therapeutic target to inhibit eosinophil migration in disease.
4.2 MATERIALS AND METHODS

4.2.1 Mice

We used Cd34\(^{-/-}\) (kindly provided by Dr. T. W. Mak (Suzuki et al., 1996)) and IL-5 transgenic (IL5\(^{Tg}\); NJ. 1638 CD3\(^{IL-5^+}\)) mice expressing IL-5 under the control of the CD3 promoter (kindly provided by Dr. J. Lee (Lee et al., 1997)) with C57Bl/6 controls throughout our experiments. Sex- and age-matched six-to-ten week-old mice were maintained in specific pathogen-free conditions at The Biomedical Research Centre and all procedures were approved by the local animal care committee.

4.2.2 Bone marrow reconstitutions

To assess the role of CD34 on hematopoietic versus non-hematopoietic populations, chimeras were created using the Ly5.1/5.2 reconstitution model. Briefly, bone marrow was isolated from either donor wildtype (Ly 5.2) and Cd34\(^{-/-}\) (Ly 5.2) or Ly5.1 mice and transplanted into recipient Ly5.1 or wildtype and Cd34\(^{-/-}\) mice respectively, as specified in the text. Marrow cells (~\(10^6\)) were injected intravenously into lethally-irradiated (11 Gy) recipient animals and the animals were rested for 10-12 weeks to allow for complete bone marrow reconstitution. Reconstitution levels were evaluated by staining for Ly 5.2 and Ly 5.1 expression in peripheral blood and mice were considered fully reconstituted when hematopoietic cells were > 80% donor-derived.
4.2.3 Induction and assessment of DSS ulcerative colitis

Experimental ulcerative colitis was induced in mice as previously described (Okayasu et al., 1990). Briefly, drinking water was supplemented with 3.5% w/v dextran sulfate sodium (DSS 36,000 – 50,000 kDa; MP Biomedicals LLC, Solon, OH) for 7 days. On day 7, mice were switched to regular drinking water.

To assess disease severity and progression, mice were monitored daily for body mass, diarrhea and rectal bleeding by visual inspection. Diarrhea and bleeding were each given a score of 0 or 1 based on the presence of symptoms and added together to give a daily diarrhea/bleeding score (0-2). Body weight was measured daily and expressed as percentage body mass by normalization to the initial body mass at day 0. In some experiments, mice were bled via the saphenous vein to assess peripheral blood eosinophil levels on days 0 and 7.

On day 8, animals were sacrificed and tissues collected. The intestine was excised and colon length measured. Excised colon tissues were gently rinsed in sterile PBS and processed for flow cytometry, cytokine assessment or histology as outlined below.
4.2.4 Colon-infiltrating cell isolation

Colon tissues were cut into pieces and incubated in digestion solution (200 U/ml collagenase IV (Sigma-Aldrich, St. Louis, MO) in PBS) for 60 min at 35°C. Digested tissues were then forced through a 70 µm nylon strainer, rinsed in PBS and collected. Cells were spun down, resuspended in 3 ml of 30% Percoll solution and centrifuged for 15 min at 1300 rpm to remove contaminating tissues and fat. The resulting cell pellet was again washed in sterile PBS and plated for flow cytometry staining.

4.2.5 Flow cytometry staining of peripheral blood and bone marrow eosinophils and colon-infiltrating immune cells

Peripheral blood was obtained from the saphenous vein into EDTA-coated tubes and bone marrow was prepared by flushing the extracted femurs. Red cells were removed using hypotonic lysis buffer, and unlysed cells were washed and used for further FACS analysis. Cells were first blocked in 10% mouse serum and stained for CD45 and CCR3 expression (using PE-conjugated anti-CCR3 (R&D Systems, Minneapolis, MN) and FITC-conjugated anti-CD45.2). Cells isolated from colon tissues were stained with biotinylated anti-CD34 (BD Pharmingen, San Diego, CA) followed by streptavidin-APC (BD Pharmingen), in addition to antibodies against CD45 and CCR3. In reconstituted animals, antibodies recognizing CD45.1 and CD45.2 (conjugated to Alexa 488 and PerCP, respectively) were used to identify donor versus recipient-derived hematopoietic cells within the colon tissue.
To assess cell frequencies and CD34 expression, data was collected on a BD FACSCalibur (BD Biosciences, Mountain View, CA) and analyzed with FlowJo software (Treestar, Ashland, OR). Total white blood cell counts were determined manually using a hemacytometer. To confirm the identity of CD34-expressing cells, CD45<sup>+</sup>CCR3<sup>+</sup> cells were sorted from single cell suspensions isolated from lamina propria cells using a FACSVantage cell sorter (BD Biosciences, Mountain View, CA). Sorted cells were cytopspun onto microscope slides and stained using a Hema 3 staining kit (Fisher Diagnostics, Middletown, VA) to verify the presence of eosinophils.

**4.2.6 Histology & immunohistochemistry**

Colon tissue samples were rinsed in PBS, fixed in 10% neutral-buffered formalin overnight and embedded for sections. Tissue sections were stained with H&E for general morphology, toluidine blue to assess mast cell accumulation or processed for immunohistochemistry.

Scoring of disease pathology in H&E-stained sections was performed as previously described (Cooper et al., 1993). Crypt damage was assessed by visual assessment at 100X magnification and grading based on severity (0-4), with 0 representing normal baseline morphology, 1 = ~1/3 crypt loss with mild inflammation,
2 = ~2/3 crypt loss with moderate inflammation, 3 = total crypt loss with intact epithelial layer and severe inflammation and 4 = total crypt loss with extensive loss of surface epithelium, ulceration and massive tissue inflammation along the distal colon. In addition, total numbers of lymphoid aggregates were assessed at 200X magnification to confirm the presence of lymphocyte by cell morphology along each colon length by visual assessment.

To visualize tissue eosinophils, colon sections were immunostained with antibodies recognizing mouse major basic protein (MBP) purchased from Dr. Jamie Lee (Mayo Clinic, Scottsdale, AZ, USA). Control slides were stained using a non-specific rat IgG1 isotype control (Cedarlane Labs Ltd, Burlington, ON). Primary antibodies were then stained using HRP-conjugated rabbit anti-rat secondary antibodies (Dako Canada Inc., Mississauga, ON), visualized using DAB reagent (Vector Laboratories Inc., Burlingame, CA) and counterstained with 0.1% methyl green. Eosinophils in three random high power fields (200X) were counted from each tissue and averaged.

All slides were analyzed on a Zeiss Axioplan2 microscope (Toronto, ON) and images were captured using a Qimaging Retiga EX CCD camera (Minneapolis, MN) and Openlab 4.0.4 software (PerkinElmer, Waltham, MA).
4.2.7 Cytokine bead arrays

Mouse colon and cecum tissues were dissected and washed in PBS to remove feces. Tissue was homogenized in 500µl PBS with protease inhibitor cocktail (Calbiochem, Gibbstown, NJ) using a TissueLyser II (Qiagen, Mississauga, ON). Homogenates were spun down and the supernatant was collected. Cytokine concentrations of MIP-1α, MCP-1, IL-6 and TNF-α were measured using the BD Flex CBA kit, according to manufacturer’s specifications. Samples were collected on a BD FACSCalibur and analyzed using BD FCAPArray software. Cytokine levels were normalized to total protein content, as determined using a BCA Protein Assay kit (Thermo Scientific, Rockford, IL).

4.2.8 Statistics

Unpaired student’s t tests were used for statistical analysis of numbers of lymphoid aggregates, pathology scores, colon length, cytokine assessment and comparison of individual time-points on disease timelines. To compare disease timelines, two-way ANOVA analysis was performed.
4.3 RESULTS

4.3.1 Ulcerative colitis pathology is attenuated in Cd34\(^{-/-}\) mice

Following administration of 3.5 % DSS in drinking water, as expected, wildtype animals exhibited a gradual decrease in total body mass over an 8-day timeline (Figure 4.1A). Along this timeline, mice also exhibited rectal bleeding and diarrhea from day 4 onwards, with a steady increase in symptom severity (Figure 4.1B). In Cd34\(^{-/-}\) animals, some weight loss and diarrhea occurred, although weight loss and diarrhea/bleeding scores were significantly lower, compared to the wildtype controls (Figure 4.1A/B). On day 8, mice were sacrificed and colon tissues were processed to assess disease severity. At the endpoint, colon lengths in both wildtype and Cd34\(^{-/-}\) DSS-induced animals were significantly decreased, compared to untreated animals (Figure 4.1C). Further, colon lengths in wildtype animals were significantly shorter than Cd34\(^{-/-}\) colons at sacrifice, indicating greater disease severity in wildtype animals (Figure 4.1C).
Figure 4.1: Symptoms of DSS-induced ulcerative colitis are attenuated in Cd34⁻/⁻ mice.

Mice were monitored for 8 days following administration of 3.5% DSS in drinking water as described in Materials & Methods. A) Daily body mass measurements, normalized to a starting body mass of 100%. B) Daily disease symptom scores for the presence of diarrhea and/or bleeding (0-2). C) Final colon length measurements following sacrifice on day 8. (Data is representative of 5 separate experiments; DSS-treated animals n=6, Untreated controls n=2; * represents p < 0.05, ** represents p < 0.01; Error bars = SEM).
Histological analysis of colon samples from DSS-induced animals provides further evidence of decreased disease severity in \( Cd34^{-/-} \) animals. In untreated control animals, no differences were seen in colon morphology between wildtype and \( Cd34^{-/-} \) animals (Figure 4.2C/D). Normal crypt structure and muscle layer thickness, as well as an absence of cellular infiltrates were seen in both genotypes at baseline. As expected, following DSS treatment, wildtype colon sections exhibited extensive cellular infiltration, edema, muscle thickening, loss of both crypt morphology and surface epithelia structure, lymphoid aggregates (Figure 4.2B) and extensive ulceration by day 8, particularly in the distal colon (Figure 4.2E/G). Multiple ulceration sites were also present along the length of the colon (Figure 4.2E). In contrast, \( Cd34^{-/-} \) colon tissues had relatively little crypt loss and surface epithelia remained intact in the distal colon, although low levels of edema, cellular infiltrate and tissue damage were present (Figure 4.2F/H). Further, very few lymphoid aggregates were present in \( Cd34^{-/-} \) animals (Figure 4.2B), and these tended to be smaller in size than in wildtype animals and restricted to the lamina propria. Clinical scoring of colon pathology confirms a statistically significant reduction in disease pathology scores (Figure 4.2A), with reduced disease severity in \( Cd34^{-/-} \) animals following DSS treatment, compared to wildtype controls. These findings suggest that CD34 plays a role in DSS-induced ulcerative colitis, and that loss of CD34 expression protects against severe disease symptoms.
Figure 4.2: Reduced disease pathology and ulceration in Cd34⁻/⁻ colons.

On day 8, following DSS treatment, colon tissues were fixed overnight in 10% formalin, paraffin-embedded for longitudinal sections, H&E stained and analyzed by microscopy. A) Clinical pathology scores from Cd34⁺/⁺ and Cd34⁻/⁻ colon sections (0 = no sign of disease, 4 = severe disease as outlined in Methods). B) Number of lymphoid aggregates along the length of the distal colon. Representative images of distal colon morphology from control C) Cd34⁺/⁺ and D) Cd34⁻/⁻ animals and DSS-treated E/G) Cd34⁺/⁺ and F/H) Cd34⁻/⁻ animals captured at 100X magnification. (n=5 for each genotype; * represents p < 0.05, ** represents p < 0.01; Error bars = SEM).
Additionally, assessment of inflammatory cytokine levels in colon tissues, isolated from DSS-treated animals, revealed significant induction of IL-6, MCP-1, TNFα and MIP-1α in wildtype animals. Induction above baseline was also seen in Cd34−/− animals, but again, these levels were reduced compared to wildtype animals (Figure 4.3). Other cytokines (IL-2, IL-3, IL-4, IL-5, IL-9, IL-10, IL-13 and IFNγ) were also assayed, but were below detectable levels in all samples, although IL-5 was detectable in IL5Tg colon tissues at baseline, as expected (data not shown). Taken together, these findings demonstrate that Cd34−/− animals exhibit a decreased susceptibility to DSS-induced ulcerative colitis, with decreased disease symptoms, decreased disease pathology and decreased local inflammatory cytokine production.
Figure 4.3: Reduced inflammatory cytokine production in Cd34⁻/⁻ colons.

On day 8, following DSS treatment, colon and cecum tissues were isolated and dissociated in PBS. Cytokine concentrations in the collected supernatants were measured by cytometric bead array (CBA). Resulting cytokine values were normalized to total protein content and presented as ug of cytokine / mg of total protein. Levels of A) TNFα, B) MCP-1, C) MIP-1α and D) IL-6 are shown. (Data are representative of two separate experiments, n=4 per genotype, * represents p < 0.05, ** represents p < 0.01; Error bars = SEM).
4.3.2 CD34 is primarily expressed by tissue eosinophils and Cd34\(-/-\) mice exhibit reduced eosinophil infiltration following DSS exposure

To characterize CD34 expression patterns on colon-infiltrating cells, we next performed flow cytometric analysis on isolated colon cells from DSS-treated animals. As eosinophils have been shown to play a key role in DSS-induced colitis, and we previously showed a role for CD34 in eosinophil migration, we focused on eosinophils in colon infiltrate. Colon tissue isolates were processed and stained for CD34, CD45 and CCR3 expression and assessed by flow cytometry. Following DSS administration, CD45\(^+\) cells represented a large proportion of total colon cells (Figure 4.4A), but were largely absent at baseline in healthy animals (data not shown). Among the CD45\(^+\) cell population, \(~30\%\) also expressed CCR3 (Figure 4.4A) and sorting of CD45\(^+\)CCR3\(^+\) cells demonstrates that this population consists of eosinophils (Figure 4.4C). Only low cell numbers were successfully sorted from digested colon tissues, but all identifiable cells had eosinophil morphology.

Staining for CD34 expression revealed a significant proportion of the total CD45\(^+\) cells in DSS-induced colon tissue express CD34 in wildtype colon (Figure 4.4B – black line). Staining of CD45\(^+\) cells isolated from Cd34\(^{-/-}\) colon reveals no CD34-positive staining, confirming that the staining seen in wildtype DSS-treated colons is specific for CD34 (Figure 4.4B – grey-shaded). The CD34\(^+\) staining observed on the total CD45\(^+\) population was further enriched by gating on CCR3\(^+\)SSC\(^{hi}\) eosinophils (Figure 4.4D – black line). Again, staining from Cd34\(^{-/-}\) colon tissues revealed no CD34-positive staining on CD45\(^+\)CCR3\(^+\) cells (Figure 4.4D – grey-shaded). All
CD34$^+$ cells from isolated colon tissue were CD45$^+$SSC$^{hi}$ and ~80% also fell within a CCR3$^{hi}$ gate. The remaining CD34 expression seemed to be found on CCR3$^{lo}$ cells. These findings demonstrate that eosinophils are the primary cell type expressing CD34 in DSS-induced colon tissue of wildtype animals.
Figure 4.4: CD34 is expressed by CD45^CCR3^ eosinophils in inflamed colon tissue.

On day 8 following DSS treatment, colon tissues were excised, collagenase digested and processed for flow cytometry. A) A representative flow cytometry profile of CD45/CCR3 staining on colon isolates, indicating the gating used to distinguish total CD45^+ cells and CD45^+/CCR3^+ cells (numbers = % of cells in the CD45^CCR3^+ or CD45^+ gates). Histograms of CD34 expression on B) total CD45^+ cells and D) CD45^+/CCR3^+ cells. Black lines represent staining on Cd34^+/+ cell isolates, grey-filled histogram represent staining intensity from Cd34^-/- cell isolates. C) Cytospin images of sorted CD45^+/CCR3^+ cells from colon, demonstrating eosinophil morphology. (Plots are representative of 3 separate experiments).
Colon tissues were also immuno-stained using antibodies recognizing the eosinophil-specific protein MBP, to confirm the presence of eosinophils within DSS-treated colon tissues. MBP-staining clearly revealed the presence of tissue-infiltrating eosinophils within both the submucosal and mucosal layers (Figure 4.5A – brown staining), compared to isotype control-stained tissues (Figure 4.5B). At baseline, low levels of eosinophils were present in both wildtype and Cd34⁻/⁻ colon tissues, mainly localized within the intestinal crypts (data not shown). Following DSS treatment, eosinophils were present throughout the submucosal and mucosal layers at greatly increased numbers. Eosinophil numbers were quantified by counting three random high power fields for each sample. Quantification revealed a much greater number of infiltrating eosinophils in wildtype animals than in Cd34⁻/⁻ animals, with roughly twice as many eosinophils present in wildtype animals (205.5 +/- 11.7 vs 120.7 +/- 13.2 total eosinophils in Cd34⁻/⁻) (Figure 4.5C). These findings demonstrate that CD34 expression is induced on eosinophils in DSS colitis, and that loss of CD34 expression results in dramatically decreased eosinophil infiltration of the colon.
**Figure 4.5: DSS induces colon recruitment of peripheral blood eosinophils, which is attenuated in Cd34⁻/⁻ mice.**

Formalin-fixed, paraffin-embedded colon tissues were sectioned and prepared for immunohistochemistry by staining with an antibody recognizing MBP or with isotype control (rat IgG1), visualized using an HRP-coupled secondary antibody and DAB developing reagent and analyzed by microscopy. Representative images of A) MBP (brown staining) and B) control isotype staining on DSS-treated colon tissues. C) Average submucosal, mucosal and total eosinophil numbers per high power field (hpf) (200X). Three random fields were selected for each sample and averaged. DSS-treated animals were bled through the saphenous vein on day 7 and sacrificed to obtain bone marrow cells, and assessed by flow cytometry for eosinophil numbers (CD45⁺/CCR3⁺). D) Bone marrow eosinophil levels, CCR3⁺ of total CD45⁺ cells. Peripheral blood eosinophil levels, represented as E) proportion CCR3⁺ of CD45⁺ cells, or F) total eosinophil numbers per mL of blood, calculated based on relative eosinophil numbers (% CCR3⁺ of total CD45⁺) and total white blood cell counts, in Cd34⁺/⁺ and Cd34⁻/⁻ animals before and after disease (n=5 for DSS-treated, 2-3 for controls for histology; n=2-5 for peripheral blood and bone marrow counts; * represents p < 0.05, ** represents p < 0.01, *** represents p < 0.001; Error bars = SEM).
4.3.3 DSS-induced ulcerative colitis results in decreased circulating eosinophil numbers

As our data suggests that eosinophils are the key cell type expressing CD34 during colitis, we also characterized the frequency of eosinophils in the bone marrow and peripheral blood throughout the course of disease. At steady state, eosinophils (CCR3+/CD45+) represent ~3% of the total hematopoietic cells (CD45+) in the bone marrow (Figure 4.5D) and 1-2% of total circulating leukocytes (Figure 4.5E), in both wildtype and Cd34⁻/⁻ animals. Following DSS induction, the levels of eosinophils increased slightly in the bone marrow to ~4.5% (Figure 4.5D). Interestingly, and somewhat unexpectedly, by day 7 following DSS induction, the levels of peripheral blood eosinophils decreased from baseline levels to <1% of total leukocytes (Figure 4.5E). When normalized to total white blood cell counts, this reveals a decrease in total eosinophil numbers in the periphery (Figure 4.5F), as eosinophils are recruited into the colon, in agreement with recent findings (Vieira et al., 2009). Further, as Cd34⁻/⁻ mice exhibited a slight reduction in both percentage eosinophils (Figure 4.5E) and total white blood cell counts (5.27 +/- 0.76 X 10⁶ cells/mL vs. 6.88 +/- 0.47 X 10⁶ cells/mL in wildtype) at steady state, they also exhibited significantly fewer total peripheral blood eosinophils, compared to wildtype animals (Figure 4.5F), suggesting Cd34⁻/⁻ have a lower baseline pool of eosinophils, despite similar eosinophil production in the bone marrow.

The reduction in eosinophil numbers following DSS induction was unexpected, as other models of disease associated with eosinophils, such as asthma, are often
associated with blood eosinophilia, as a result of rapid bone marrow precursor expansion. This may reflect the speed of colitis onset in the DSS model, which may be too rapid for a large induction of bone marrow eosinophil precursor expansion. Regardless, this finding demonstrates that peripheral blood eosinophilia is clearly not required for pathology in ulcerative colitis and may provide further insight into the mechanism of colitis induction.

4.3.4 Attenuation of DSS-induced ulcerative colitis is due to loss of CD34 on bone marrow populations, and not the microenvironment

In addition to its expression on hematopoietic precursors, mast cells and eosinophils, CD34 is also expressed on non-hematopoietic cell lineages (such as vascular endothelia). We therefore used bone marrow chimeras to assess the relative importance of CD34 expression on hematopoietic versus non-hematopoietic cells, in terms of effects on disease severity. First, wildtype (Ly5.1) mice were lethally irradiated and reconstituted with either wildtype or Cd34⁻/⁻ bone marrow (Ly5.2), to assess the role of hematopoietic CD34 expression on disease outcomes. Following DSS administration, both wildtype and Cd34⁻/⁻-reconstituted animals exhibited similar levels of weight loss, potentially reflecting the increased age of reconstituted animals or irradiation-induced damage to the mucosal epithelia (Figure 4.6A). Significantly, wildtype bone marrow-reconstituted animals demonstrated an increase in diarrhea/bleeding scores, more severe shortening of the colon and increased colon pathology at the day 8 endpoint, compared to Cd34⁻/-reconstituted animals (Figure 4.6C/E/F). These findings suggest that loss of CD34 expression
exclusively on bone marrow-derived cells is sufficient to protect from DSS-induced colitis.

In a parallel series of experiments, chimeric animals were generated to assess the relative importance of CD34 expression on non-hematopoietic cells. For these experiments, wildtype or Cd34⁻/⁻ recipients (Ly5.2) were irradiated and reconstituted with wildtype (Ly5.1) bone marrow. Following DSS treatment, no significant difference was seen in weight loss, diarrhea/bleeding scores, colon shortening or pathology between wildtype or Cd34⁻/⁻ recipients (Figure 4.6B/D/G/H). These findings suggest that CD34 expression on hematopoietic cells is more important for the progression of DSS-induced ulcerative colitis, and CD34 expression on non-hematopoietic cells has a minimal role in disease progression.
Figure 4.6: CD34 expression on hematopoietic, but not non-hematopoietic, cell populations, is critical for disease severity.

Chimeric animals were generated by lethal irradiation of recipient animals followed by injection with donor bone marrow cells. Mice were allowed 10-12 weeks to recover and assessed for donor reconstitution based on Ly5.1/5.2 staining in peripheral blood, then administered 3.5% DSS for 7 days. Daily body mass measurements, normalized to a starting body mass of 100%, daily disease symptom scores, final colon length measurements and tissue pathology scores were measured for all mice. A/C/E/F) Data for Ly5.1 recipient animals reconstituted with either Cd34+/+ or Cd34-/- bone marrow to assess hematopoietic contributions. B/D/G/H) Data for Cd34+/+ or Cd34-/- recipients reconstituted with Ly5.1 bone marrow to assess non-hematopoietic contributions. (Data is representative of 2 experiments with each chimera set; DSS-treated animals mass, disease scores and pathology scores n=5; colon length n=10; Untreated controls n=2-3; * represents p < 0.05, ** represents p < 0.01; Error bars = SEM).
4.3.5 Hypereosinophilia results in increased CD34 expression on colon eosinophils and colon shortening, in the absence of DSS exposure

To provide further support for the role of eosinophils in colon inflammation and disease we also assessed IL5\textsuperscript{Tg} mice, which exhibit profound eosinophilia, for intestinal pathology. The IL5\textsuperscript{Tg} mice express IL5 under the control of the CD3 promoter, and are hypereosinophilic with $\sim60\%$ eosinophils in the blood, bone marrow and spleen at steady state (Lee et al., 1997). Without DSS treatment, colon length in IL5\textsuperscript{Tg} animals was significantly reduced, on both the wildtype and $Cd34^{-/-}$ backgrounds, compared to non-IL5\textsuperscript{Tg} controls (Figure 4.7A). In the tissues of these mice at steady state, CD34 expression was undetectable on CD45\textsuperscript{+} cells in the peripheral blood and only marginally expressed in the bone marrow and spleen of $Cd34^{+/+}$IL5\textsuperscript{Tg} mice (Figure 4.7B – black lines), when compared to staining in $Cd34^{-/-}$ IL5\textsuperscript{Tg} tissues (Figure 4.7B – grey shaded). This marginal CD34\textsuperscript{+} staining was enriched further on CD45\textsuperscript{+}CCR3\textsuperscript{+}SSC\textsuperscript{hi} cells, similar to our observations in DSS-treated colon (Figure 4.7C).

In IL5\textsuperscript{Tg} animals, significant CD45\textsuperscript{+} inflammatory cell infiltrates were present in the colon at steady state, compared to non-IL5\textsuperscript{Tg} control animals, where very few cells were seen (data not shown). Intriguingly, the highest CD34 expression, in the absence of DSS treatment, was seen on colon-infiltrating cells, and was again enriched on CD45\textsuperscript{+}CCR3\textsuperscript{+}SSC\textsuperscript{hi} eosinophils isolated from the colon (Figure 4.7C). These data suggest that eosinophilia alone, in the absence of an external inflammatory stimulus can induce mild colon pathology, with shortening of the colon
and immune cell infiltrate, which is associated with the influx of CD34$^+$ eosinophils into the colon. Further, these findings suggest that while eosinophilia clearly is not required for the development of ulcerative colitis per se, it may contribute to disease severity and baseline inflammation in the absence of induced colitis.
**Figure 4.7: IL5-induced eosinophilia, leads to colon shortening, with CD34 expression on colon infiltrating eosinophils.**

Colon tissues from unchallenged, non-DSS treated, IL5\textsuperscript{Tg} and Cd34\textsuperscript{+/−}/IL5\textsuperscript{Tg} mice at baseline were excised, measured and processed for flow cytometry. Blood, bone marrow and spleen tissues were also processed for flow cytometry as described in methods. A) Baseline colon length of Cd34\textsuperscript{+/−} and Cd34\textsuperscript{−/−} animals with or without the IL5 transgene. Histogram profiles of CD34 expression on B) total CD45\textsuperscript{+} cells and C) CD45\textsuperscript{+}/CCR3\textsuperscript{+}SSC\textsuperscript{hi} cells in peripheral blood, marrow, spleen and colon isolates. For all histograms, black lines represent staining on cells from Cd34\textsuperscript{+/−}/IL5\textsuperscript{Tg} tissues and grey-filled histograms represent background staining of cells from Cd34\textsuperscript{−/−}/IL5\textsuperscript{Tg} tissues. (Histograms are representative of 3 separate experiments; n=9-12 for colon length; ** represents p < 0.01; Error bars = SEM).
A

Colon Length (mm)

<table>
<thead>
<tr>
<th>Cd34+/+</th>
<th>Cd34+/- IL5Tg</th>
<th>Cd34+/+</th>
<th>Cd34+/- IL5Tg</th>
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</table>

B. CD45+ Total

Blood | Marrow | Spleen | Colon

CD34

C. CD45+ / CCR3+ / SSChi

Blood | Marrow | Spleen | Colon

CD34
4.3.6 Eosinophilia restores DSS-induced colitis susceptibility in Cd34\(^{-/-}\) mice on an IL5\(^{Tg}\) background

Since our findings in Cd34\(^{-/-}\) mice suggest that impaired eosinophil migration is sufficient to reduce disease, we also assessed whether we could override this phenotype under conditions of IL-5-induced eosinophilia. Cd34\(^{+/+}\)IL5\(^{Tg}\) and Cd34\(^{-/-}\)IL5\(^{Tg}\) mice were treated with DSS, and assessed for disease induction over 8 days. Contrary to our findings on a non-IL5\(^{Tg}\) background, on the IL5\(^{Tg}\) background, mice exhibited similar weight loss and diarrhea-bleeding symptoms regardless of Cd34 genotype (Figure 4.8A/B). In fact, Cd34\(^{-/-}\)IL5\(^{Tg}\) animals exhibited slightly more colon shortening at the day 8 time point than Cd34\(^{+/+}\)IL5\(^{Tg}\) controls (Figure 4.8C) and histological assessment revealed no difference in clinical score (Figure 4.8D). Quantification of colon eosinophil accumulation following DSS exposure revealed a greatly increased number of eosinophils in IL5\(^{Tg}\) mice (Figure 4.8E), in excess of numbers seen in wildtype non-IL5\(^{Tg}\) animals (Figure 4.5C). Further, eosinophil numbers were equally elevated in Cd34\(^{-/-}\)IL5\(^{Tg}\) animals, following DSS exposure (Figure 4.8E). These findings demonstrate that hypereosinophilia, induced by increased IL-5 production, is sufficient to overcome the decreased eosinophil migration and decreased susceptibility to colitis observed in Cd34\(^{-/-}\) animals and provide further support for the hypothesis that eosinophils play a role in pathogenesis.
**Figure 4.8: Eosinophilia is sufficient to restore full susceptibility to DSS-induced colitis in Cd34\(^+\)/- mice.**

*Cd34\(^+/\) and Cd34\(^-/\) mouse strains were crossed onto the IL5\(^Tg\) background to induce eosinophilia. Mice were then treated with 3.5% DSS for 7 days and monitored daily for A) body mass and B) diarrhea and bleeding. On the day 8 endpoint, C) total colon length was measured. Colon tissues were fixed, embedded and stained by H&E and assessed for D) clinical pathology as discussed previously and E) average submucosal, mucosal and total eosinophil numbers were counted per high power field (hpf) (200X). Three random fields were selected for each sample and averaged. (n=4, * represents p < 0.05, Error bars = SEM).*
4.4 DISCUSSION

Mice treated with DSS develop an acute ulcerative colitis that closely resembles human colitis and serves as a valuable model of disease. Since there is 1) controversial evidence suggesting an essential role for eosinophils in ulcerative colitis and 2) data showing that CD34 is required for efficient eosinophil trafficking, we investigated the effect of CD34 loss on disease outcomes. Following DSS treatment, we found a significant attenuation of disease symptoms in Cd34⁻/⁻ mice. Cd34⁻/⁻ mice exhibit less severe weight loss, reduced colon shortening and lower overall diarrhea/rectal bleeding. In addition, histological analysis revealed decreased eosinophil infiltration, decreased clinical pathology, reduced ulceration, reduced numbers of lymphoid aggregates and reduced inflammatory cytokine production in Cd34⁻/⁻ colon tissues. Furthermore, we found that eosinophils entering the colon express high levels of CD34 and are the predominant CD34-expressing hematopoietic cell type in the colon following disease onset.

Our previous findings have demonstrated key roles for CD34 in optimal cell migration of hematopoietic cells, particularly mast cells and eosinophils. On mast cells, CD34 expression prevented cell-cell aggregation in vitro (Drew et al., 2005). In an asthma model, Cd34⁻/⁻ mice exhibit decreased disease pathology due to CD34 loss on hematopoietic cells, accompanied by a reduction in both mast cell and eosinophil recruitment to the lung. In addition, Cd34⁻/⁻ eosinophils exhibit impaired migration in an in vitro assay (Blanchet et al., 2007), suggesting that loss of CD34 expression, alone, is sufficient to impair eosinophil migration. In this study, we
propose a similar role for CD34 on eosinophils in the DSS-induction model of ulcerative colitis, whereby CD34 expression is required for efficient eosinophil infiltration of the colon, which in turn leads to tissue damage and pathology.

Our findings demonstrate that CD34 is prominently expressed on CD45\(^+\)CCR3\(^+\)SSC\(^{hi}\) eosinophils within the colon tissues following DSS-treatment. The presence of eosinophils within inflamed colon tissues was confirmed by MBP-staining of colon sections. Low levels of eosinophils were present at baseline, with a dramatic increase in tissue eosinophils at the day 8-endpoint. Similar to our findings in the asthma model, \(Cd34^+/\) mice exhibited drastically reduced numbers of eosinophils within the colon, compared to wildtype controls. CD34 is also expressed by mast cells, although toluidine blue staining revealed very low levels of mast cells present in tissues both before and after DSS-treatment (~1-2 cells / section; data not shown). Thus, it is unlikely that CD34 expression on mast cells accounts for the differences in disease pathology seen in \(Cd34^+/\) mice. These findings support the hypothesis that CD34 is required for optimal infiltration of eosinophils into the colon, following DSS-treatment, and that eosinophil infiltration, in turn, is required for the development of severe colitis pathology.

Assessment of cytokine levels in colon tissue isolates also revealed reduced inflammation in \(Cd34^+/\) mice. Following DSS-induction, levels of the pro-inflammatory cytokines TNF\(\alpha\) and IL-6 were increased in wildtype mice, as
previously published (Alex et al., 2009; Dieleman et al., 1994), and found at lower levels in Cd34⁻/⁻ animals. TNFα and IL-6 are released by resident macrophages, following DSS-induced increases in mucosal permeability (Kitajima et al., 1999) and subsequent exposure to commensal gut flora. In fact, blocking TNFα, using anti-TNFα antibodies *in vivo*, results in decreased disease pathology in the chronic DSS colitis mouse model (Kojouharoff et al., 1997), and has had success in patients with Crohn’s disease (van Dullemen et al., 1995). Interestingly, we also detected high levels of the chemokines MIP-1α and MCP-1 in the colon of wildtype, compared to Cd34⁻/⁻ mice, although these chemokines do not appear to be released into the serum during acute DSS-induced colitis, according to the literature (Alex et al., 2009). Interestingly, MIP-1α administration is sufficient to exacerbate inflammatory bowel disease in a TNBS-induced disease model (Pender et al., 2005). It remains unclear, in our model, whether any of these cytokines are the cause of the reduced eosinophil accumulation and disease severity observed in Cd34⁻/⁻ mice, or merely an effect. Our previous findings demonstrated a cell-intrinsic defect in Cd34⁻/⁻ eosinophil migration *in vitro* (Blanchet et al., 2007), but the reduced local cytokine levels in Cd34⁻/⁻ mice may exacerbate this defect. Alternatively, the reduced levels of inflammatory cytokines in colon tissues of Cd34⁻/⁻ mice may be a direct result of the decreased eosinophil accumulation, resulting in reduced local tissue damage and inflammation.
To further clarify the role CD34 plays in ulcerative colitis, we used bone marrow chimeras to determine the relative importance of CD34 on hematopoietic versus non-hematopoietic tissues. These findings reveal a critical role for CD34 on hematopoietic cells, as wildtype (Ly5.1) recipient animals reconstituted with $Cd34^{-/-}$ bone marrow exhibited attenuated disease severity similar to that seen in $Cd34$-null animals. These findings suggest that CD34 expression on non-hematopoietic lineages (ie endothelial cells) has a minimal effect on disease pathology and further support the conclusion that CD34 expression on eosinophils is critical for disease progression.

By crossing $Cd34^{-/-}$ animals onto the IL$5^{Tg}$ background, to induce hypereosinophilia, we were able to restore susceptibility to DSS-induced colitis. Following DSS treatment, $Cd34^{-/-}IL5^{Tg}$ mice had similar symptoms and pathology and slightly increased colon shortening, when compared to $Cd34^{+/+}IL5^{Tg}$ control animals. Further, tissue eosinophil counts in IL$5^{Tg}$ animals (~300 cells/hpf) exceeded eosinophil numbers in wildtype mice after DSS treatment (~200 cell/hpf), regardless of CD34 expression. Thus, chronic eosinophilia alone is sufficient to overcome the reduced eosinophil accumulation and restore disease susceptibility in $Cd34^{-/-}$ animals.
Intriguingly, following DSS-treatment, we observed decreased eosinophil numbers in the peripheral blood of wildtype mice, despite a slight increase in bone marrow production, likely due to recruitment of eosinophils from the periphery to the inflamed tissue, which is in agreement with recent findings (Vieira et al., 2009). This finding is quite different from observations in models such as asthma, and was somewhat surprising. In models of allergic disease, eosinophil numbers commonly increase and disease pathology is associated with peripheral blood eosinophilia. An absence of peripheral blood eosinophil expansion in DSS-induced colitis may reflect the acute nature of disease in this model. In DSS-induced colitis, the inflammatory insult leads to an acute recruitment of eosinophils into the colon within 7 days. In contrast, ovalbumin-induced asthma requires ~1 month of priming and challenges to induce disease. Extended timecourses may provide sufficient time for the expansion of bone marrow eosinophils and subsequent peripheral blood eosinophilia. This same phenomenon may also explain the observation that IL-5−/− mice exhibit normal disease pathology in colitis (Forbes et al., 2004; Stevceva et al., 2000). IL-5 is a critical regulator of eosinophil expansion and survival, and as such, normal colitis susceptibility in IL-5−/− mice has cast doubt on the role of eosinophil in pathology of this disease. Our findings suggest that eosinophil expansion, which is important in allergy, is dispensable for normal disease progression in colitis and instead, factors regulating eosinophil recruitment such as eotaxin-1/2 (Ahrens et al., 2008; Forbes et al., 2004), ICAM-1 (Forbes et al., 2006) or CD34 are more important. This is a very important observation when considering the development of novel therapies for the treatment of ulcerative colitis and suggests that targeting eosinophil recruitment,
rather than eosinophil expansion, will be more therapeutic.

IL-5\(^{Tg}\) mice exhibit significant chronic eosinophilia with > 60% eosinophils (as a proportion of total white blood cells) in peripheral blood, bone marrow and spleen. We observed a small, but significant decrease, in colon length at steady state in both IL5\(^{Tg}\) and Cd34\(^{-/-}\)IL5\(^{Tg}\) animals, compared to controls. In addition, prior to disease induction, IL5\(^{Tg}\) colon tissues exhibited significant inflammatory cell infiltrates, including CCR3\(^{+}\) eosinophils. Initial reports on IL5\(^{Tg}\) mice revealed that \(~70\%\) of these mice spontaneously die by 12 months of age (Lee et al., 1997). Interestingly, surviving animals display severe inflammatory pathologies including skin lesions and lower bowel inflammation (Lee et al., 1997). Our findings suggest that even at younger ages (6-12 weeks) IL5\(^{Tg}\) mice have chronic, low-level gut inflammation, with eosinophil infiltration. Further, in the absence of DSS treatment, CD34 expression is present at low levels in the bone marrow and spleen, and more significantly, is increased on colon-infiltrating eosinophils. These findings demonstrate that chronic exposure to IL5 and eosinophilia are sufficient to induce low-level colon inflammation, which may progress to more severe disease and explain the pathology in aged IL5\(^{Tg}\) animals.

Eosinophils have been implicated in a variety of inflammatory diseases and our data demonstrate that the loss of CD34 results in attenuation of ulcerative colitis, with decreased pathology, likely due to decreased eosinophil recruitment. As Cd34\(^{+/-}\)
mice exhibit no major defects in development or in maintaining homeostasis, these findings suggest that the CD34 molecule, itself, may be a valuable therapeutic target for the treatment of inflammatory diseases, including ulcerative colitis, by inhibiting immune cell infiltration into the tissue.
4.5 ACKNOWLEDGEMENTS

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CHAPTER 5. OPPOSING ROLES FOR CD34 IN B16 MELANOMA TUMOR GROWTH ALTER EARLY STAGE VASCULATURE AND LATE STAGE IMMUNE CELL INFILTRATION

5.1 INTRODUCTION

Cancer initiation begins from mutational changes within a normal cell population, resulting in unrestricted cell proliferation and tumor development. Continued tumor growth results from complex interactions between tumor cells and the surrounding host microenvironment. Ultimately, tumor size is governed by both the growth and proliferation of tumor cells, by cues from the local microenvironment and interactions with immune cells. For tumor progression beyond 1 mm$^3$, angiogenesis and integration into the local blood supply is required (Folkman et al., 1989; Hanahan & Folkman, 1996; Ribatti et al., 2004). Subsequent growth is affected by remodeling of the surrounding tissues and extracellular matrix, the supply of nutrients and survival factors for proliferating tumor cells and evasion of host immune responses.

Extensive study has focused on the initial mutations involved in carcinogenesis, with great advances in our understanding of the roles of oncogenes in tumor progression. These studies have given us insight into the steps involved in initiating tumor growth and how mutations cooperate in tumor progression. While these studies provide insight into tumor initiation, a growing body of literature recognizes

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1 A version of this chapter will be submitted for publication. Steven Maltby and Kelly M. McNagny (2010) Opposing roles for CD34 in B16 melanoma tumor growth alter early stage vasculature and late stage immune cell infiltration.
the role and importance of the surrounding microenvironment in altering tumor growth. In this study, we focused on CD34 function in a B16 melanoma model, and characterized the effects on tumor growth resulting from CD34 loss on both hematopoietic and non-hematopoietic cell populations.

The cell surface sialomucin CD34 is best known for its expression on hematopoietic stem cells and also on immature hematopoietic cells, vascular endothelia (Delia et al., 1993), eosinophils (Blanchet et al., 2007; Radinger et al., 2004) and mast cells (Drew et al., 2005). CD34 is most frequently used as a cell surface marker to identify progenitor cells and surprisingly little is known about its function in vivo. One exception is the role of CD34 as an L-selectin ligand following a very specific glycan modification in the high endothelial venules (HEVs) of lymph nodes, which creates a sialyl Lewis-X structure (Baumheter et al., 1993). However, this specific post-translational modification is limited to the HEV, while on other cell types CD34 does not act as a ligand for L-selectin. In fact, total loss of CD34 expression in two separate Cd34−/− transgenic animal models resulted in very minimal phenotypes, with a possible defect in progenitor cell proliferation or differentiation in one case (Cheng et al., 1996) and a mild eosinophil migration defect in the other (Suzuki et al., 1996).

Our previous findings have demonstrated a role for CD34 on mast cells and eosinophils as an anti-adhesive molecule. Mast cells derived from Cd34−/− bone
marrow have an increased level of homotypic adhesion, compared to \(Cd34^{+/+}\) control cells (Drew, Merzaban et al., 2005). \(In vivo\), \(Cd34^{-/-}\) mice demonstrate reduced mast cell seeding of the peritoneal cavity following water ablation (in the absence of the sialomucin \(Cd43\)) and reduced lung infiltration during asthma (Blanchet et al., 2007; Drew, Merzaban et al., 2005). Similarly, \(Cd34^{-/-}\) animals exhibit reduced eosinophil recruitment to the lung during asthma, despite a corresponding increase in peripheral blood levels suggesting a reduced migration potential for \(Cd34^{-/-}\) eosinophils (Blanchet et al., 2007). In support of these findings, isolated CD34 deficient eosinophils from asthmatic lungs demonstrate reduced migration \(in\ vita\o\) using transwell migration assays (Blanchet et al., 2007).

In the context of endothelial cells, recent evidence suggests a potent role the CD34-related molecule podocalyxin in vessel lumen formation. Podocalyxin and CD34 share a high level of structural homology and an overlapping expression pattern on vascular endothelia. A recent study of vascular development demonstrated that CD34 and podocalyxin colocalize to sites of aortic lumen formation in the embryonic aorta and in vessels near developing tumors (Strilic et al., 2009). In addition, \(Podxl^{+/+}\) embryos exhibited a delay in lumen formation, demonstrating that expression of podocalyxin on vascular endothelia is required for proper vessel formation (Strilic et al., 2009). Furthermore, a recent study by our group suggested increased vascular leakage in \(Cd34^{-/-}\) animals in an arthritis model, suggesting a role for CD34 on vascular endothelia in maintaining vascular integrity during disease states (Blanchet et al., 2009). Taken together, these studies suggest
a key role for CD34 and related molecules in the development and maintenance of vascular integrity during development and disease.

In studies of tumor development, CD34 expression is often used as a marker of tumor vasculature (Chen et al., 2009; Murakami et al., 2009) and CD34⁺ staining is used to characterize vascular patterns within tumor tissues (Tardio, 2008). In one study of oral squamous cell carcinoma, CD34 staining patterns were used to predict metastasis (Kademani et al., 2009). A “penetrating” staining pattern of CD34 expression on tumor biopsies is significantly associated with regional metastasis (Kademani et al., 2009), although it remains unclear whether CD34 plays a direct role in clinical outcome.

In the lone study examining a functional role for CD34 in tumors, Trempus et al. demonstrated reduced tumor induction in Cd34⁻/⁻ animals following treatment with 7,12-dimethylbenz(a)anthracene (DMBA) and 12-O-tetradecanoylphorbol-13-acetate (TPA) (Trempus et al., 2007). At high doses of DMBA treatment, Cd34⁻/⁻ mice could be induced to produce tumors, although at lower efficiency than wildtype controls (Trempus et al., 2007). Further study suggested a decreased capacity of hair follicle bulge stem cells (which normally express CD34) in Cd34⁻/⁻ mice to activate and switch to a proliferative state following TPA exposure (Trempus et al., 2007). These findings clearly demonstrated a role for CD34 in tumor growth following DMBA and TPA treatment, as a result of altered follicle stem cell proliferation in Cd34⁻/⁻ animals.
(Trempus et al., 2007). This study provided important insight into CD34 function in follicle bulge stem cells during tumor initiation.

As mentioned previously, CD34 expression is not limited to the tumor vasculature. Both mast cells and eosinophils express CD34 and are frequently found at developing tumor sites. As reviewed in (Maltby et al., 2009; Murdoch et al., 2008), a range of hematopoietic cells infiltrate tumor sites and interact with tumor cells and the surrounding microenvironment. Mounting evidence suggests that these infiltrating cell populations directly and indirectly affect tumor cell survival and alter tumor growth. Local mast cells are present in a variety of tumor types, and alter tumor angiogenesis, localized tissue remodeling and the host immune response against developing tumors (Maltby et al., 2009). Blocking mast cell function, with interventions such as cromolyn administration or implanting tumors into mast-cell deficient mice have demonstrated a key role for mast cells in pancreatic tumors (Soucek et al., 2007). In a colorectal polyp tumor model, we demonstrated the importance of CD34 on infiltrating mast cells in tumor growth (Gounaris et al., 2007). In mast cell-deficient animals, polyp formation was decreased (Gounaris et al., 2007). This decrease in growth could be reversed by reconstitution with wildtype, but not Cd34−/−, bone marrow-derived mast cells (Gounaris et al., 2007). Eosinophils also infiltrate into necrotic regions of tumors, with a proposed function in tumor rejection, by damaging proliferating tumor cells (Cormier et al., 2006). Since our group has previously shown an important role for CD34 in both eosinophil and mast cell trafficking in models of disease we speculated that Cd34−/− animals would have
reduced immune cell infiltration into tumor sites. Alternatively, since CD34 family members can also regulate vascular lumen formation and integrity, CD34 could also play a “non-hematopoietic” role in tumor progression.

Despite the presence of CD34 within tumors on both vasculature and infiltrating immune cells, our study is the first to address a potential role for CD34 expression within the surrounding microenvironment during tumor growth. To address the potential function of CD34 on both hematopoietic and non-hematopoietic cell subsets we monitored B16 melanoma growth at several timepoints in wildtype and Cd34−/− mice, as well as bone marrow-reconstituted chimeras. Here we report the complex role CD34 plays within the developing tumor. Our results demonstrate that at early stages, tumor growth is impaired in Cd34−/− animals, due to loss of CD34 specifically on non-hematopoietic subsets. In addition, fewer lung metastases were found in Cd34−/− mice using an intravenous model. In contrast, at late timepoints tumor growth is increased in Cd34−/− animals, specifically when CD34 expression is lost on hematopoietic cells, likely reflecting reduced tumor rejection by infiltrating immune cells. In addition, we found reduced mast cell infiltration at all stages of tumor growth in Cd34−/− animals.

This study highlights the complex role CD34 plays on endothelia within the tumor microenvironment and hematopoietic cells surrounding tumor sites, and the diverse interactions between hematopoietic and non-hematopoietic cells governing tumor
growth. This study also highlights the importance of characterizing tumor development across a range of tumor stages, as CD34 loss creates opposing effects on tumor growth at different endpoints.
5.2 MATERIALS AND METHODS

5.2.1 Mice

\(Cd34^{-/-}\) (kindly provided by Dr. T. W. Mak. (Suzuki et al., 1996)) and IL5 transgenic (IL5\(^{Tg}\); NJ. 1638 CD3\(^{IL-5^+}\)) mice expressing IL-5 under the control of the CD3 promoter (kindly provided by Dr. J. Lee (Lee et al., 1997)) were used with C57Bl/6 controls throughout our experiments. Six-to-eight week-old age- and gender-matched mice were maintained in specific pathogen-free conditions at The Biomedical Research Centre and all procedures were approved by the local animal care committee.

5.2.2 Bone marrow reconstitutions

To assess the role of CD34 on hematopoietic versus non-hematopoietic populations, chimeras were created using the Ly5.1/5.2 reconstitution model. Briefly, bone marrow was isolated from either donor wt (Ly 5.2) and \(Cd34^{-/-}\) (Ly 5.2) or Ly5.1 mice and transplanted into recipient Ly5.1 or wt and \(Cd34^{-/-}\) mice respectively, as specified for each experiment in the text. Cells (~\(10^6\)) were injected intravenously into lethally-irradiated (11 Gy) recipient animals and then the animals were rested for 10-12 weeks to allow for complete bone marrow reconstitution. Reconstitution levels were evaluated by staining for Ly 5.2 and Ly 5.1 expression on peripheral blood cells; mice were considered reconstituted when hematopoietic cells were found to be > 80% donor-derived.
5.2.3 B16F1 melanoma tumor induction

The B16F1-OVA melanoma cell line was kindly provided by Dr. C. Parrish (Mattes et al., 2003). The cell line was maintained in RPMI media (Gibco) supplemented with 10% FBS, penicillin, streptomycin and L-glutamine. Adherent cells were trypsinized and collected by centrifugation. To induce subcutaneous tumor growth, 5 X 10^5 cells were injected over the shoulder, and the tumors allowed to grow for 14 to 19 days as indicated in the text. Tumor growth was assessed by measuring tumor volume using manual calipers (calculated as L X W^2 / 2) and tumor mass at the endpoint. Lung metastases were induced by injection of 3 X 10^5 cells intravenously into the tail vein and the mice were sacrificed 12 days later to assess local tumor numbers on the lung exterior. Excised solid tumors were fixed overnight in 10% formalin, paraffin-embedded and prepared for histology by H&E and toluidine blue staining. For immunohistochemistry studies, tumors were excised and frozen on an aluminum block at -20C, then embedded in cutting medium (OCT, Tissue-TEK) for histology.

5.2.4 Histology

To assess mast cell infiltration into tumors, toluidine blue staining was performed on fixed tumor sections. Mast cells were quantified in healthy tissues immediately surrounding tumors and within developing tumors. Cell counts were normalized to tumor circumference and tumor area respectively, calculated from the measured diameter of each tumor section. Slides were analyzed on a Zeiss Axioplan2 microscope (Toronto, ON) at 200X magnification and images were captured using a
Qimaging Retiga EX CCD camera (Minneapolis, MN) and Openlab 4.0.4 software.

Immunohistochemistry was performed as previously described (Baker et al., 2008; Kyle et al., 2007). Five minutes prior to euthanization, mice were administered an intravenous injection of the fluorescent dye DiOC\(_7\)(3) (carbocyanine, Molecular Probes), 0.6 mg/mL, dissolved in 75% (v/v) DMSO: 25% sterile water. Snap-frozen tumor sections 2-3 mm from the tumor edge were sectioned, dried and imaged for carbocyanine fluorescence, then fixed in 50% (v/v) acetone/methanol for 10 min at room temperature. Vasculature was visualized using an anti-mouse CD31 antibody (MEC 13.3, BD PharMingen) and CD34 expression was evaluated using an anti-mouse CD34 antibody (RAM34, BD PharMingen). Tissues were imaged using a robotic fluorescence microscope (Ziess Imager Z1), a cooled, monochrome CCD camera (Retiga 4000R, Qimaging), a motorized slide loader and x-y stage (Ludl Electronic Products) and customized NIH-ImageJ software. Images were then inverted, overlaid and cropped, with staining artefacts removed by visual inspection. Carbocyanine intensity was assessed by sorting pixels based on relative distance from the nearest CD31-positive vessel, reported in 1.5 um increments and subtracting background staining intensity. Vessel density was assessed by measuring the relative distance of each pixel from CD31\(^+\) vessels and vascular perfusion was assessed by colocalization of carbocyanine fluorescence with CD31 staining using image analysis software for the entire tumor section.
5.2.5 Statistics

For the analysis of differences in tumor mass, volume and histology data, unpaired student t-tests were used. Where indicated, ANOVA analyses were performed to determine differences in pooled data sets.
5.3 RESULTS

5.3.1 Cd34<sup>-/-</sup> animals exhibit reduced B16 melanoma growth at day 14

To determine the impact of CD34 expression within the microenvironment, 5 X 10<sup>5</sup> B16-F1-OVA melanoma cells were injected subcutaneously into Cd34<sup>-/-</sup> animals and wildtype Cd34<sup>+/+</sup> C57Bl/6 controls. Animals were sacrificed 14 days later, and the tumors were excised, weighed and processed for histology. In Cd34<sup>-/-</sup> animals, tumor growth was significantly reduced, with final tumor masses less than half that of tumors excised from wildtype controls (Figure 5.1A). Similar results were obtained when tumor cells were injected intravenously in a melanoma metastasis model. Animals were injected with 3 X 10<sup>5</sup> cells i.v. and sacrificed 12 days later to assess lung tumor numbers by visual inspection of the lung surface. In this model, Cd34<sup>-/-</sup> also yielded fewer final lung metastases, compared to wildtype controls (Figure 5.1B), demonstrating reduced tumor growth and initiation in the absence of CD34 expression.

To determine where CD34 was expressed in the resulting B16 tumors, and confirm the absence of CD34 expression in tumors from Cd34<sup>-/-</sup> animals, tumors were processed and stained using anti-CD34 antibodies. Staining demonstrates clear vascular localization for CD34 and an overlapping staining pattern with the common vascular marker CD31 (Figure 5.1C), as previously reported (Chen et al., 2009). As expected, CD34 staining was absent in tumors excised from Cd34<sup>-/-</sup> mice, although CD31 staining patterns appear largely unaffected (Figure 5.1C). In addition, carbocyanine (a small molecular weight fluorescent dye) was infused into mice 5
minutes before sacrifice to illuminate functionally perfused vasculature. Interestingly, carbocyanine fluorescence was more intense proximal to functional vasculature in \( Cd34^- \) tumors (Figure 5.1C), which will be further discussed later. These staining patterns confirm CD34 expression on tumor vasculature within B16-F1 tumors in wildtype mice, and suggest a slight increase in vascular permeability in the \( Cd34^- \) animals.
Figure 5.1: Melanoma growth is reduced in Cd34<sup>−/−</sup> animals at day 14 with CD34 expression on tumor vasculature.

A) Average final tumor masses 14 days following subcutaneous injection of 5X10<sup>5</sup> B16 melanoma cells. (Data pooled from three experiments, Cd34<sup>+/+</sup> n=12; Cd34<sup>−/−</sup> n=15) B) Average number of lung tumor metastases following sacrifice 12 days after intravenous injection of 3 X 10<sup>5</sup> B16 melanoma cells, counted on the lung surface. (Cd34<sup>+/+</sup> n=5, Cd34<sup>−/−</sup> n=6, * represents p < 0.05; Error bars = SEM). C) Representative photomicrographs from Cd34<sup>+/+</sup> and Cd34<sup>−/−</sup> tumor sections stained for CD34 and CD31 expression or carbocyanine fluorescence, as indicated (CD31 false-colored red, CD34 blue, carbocyanine green).
A

[Graph showing tumor mass (g) for Cd34+/+ and Cd34-/-]

B

[Graph showing lung metastases (n) for Cd34+/+ and Cd34-/-]

C

[Images showing CD31, CD34, and Carbo staining for Cd34+/+ and Cd34-/-]
5.3.2 Reduced tumor growth at early timepoints is due to lack of CD34 expression on non-hematopoietic cells

As CD34 expression is reported on a wide range of cell types, including vascular endothelia and hematopoietic cells (eosinophils and mast cells), we generated bone marrow-reconstituted chimeras to assess the lineages responsible for the altered phenotype in early tumor growth. To assess the effect of CD34 loss on non-hematopoietic lineages, wildtype and Cd34\(-/-\) animals were irradiated and reconstituted with unsorted bone marrow from wildtype Ly5.1 congenic animals. Following a ten-week recovery, peripheral blood from the chimeric animals was assessed for expression of Ly5.1 and Ly5.2 (C57Bl/6) to confirm effective replacement of all hematopoietic subsets with donor cells. Resulting chimeras retained wildtype CD34 expression on all hematopoietic lineages, but lacked CD34 on non-hematopoietic tissues, including vascular endothelia, in Cd34\(-/-\) animals. Subcutaneous injection of these animals with B16 melanoma cells, again, resulted in reduced tumor growth in wildtype bone marrow-reconstituted Cd34\(-/-\) animals, compared to wildtype bone marrow-reconstituted Cd34\(+/-\) animals, as determined 14 days following injection (Figure 5.2A/B). The reductions in tumor growth were less severe than the reductions seen in non-reconstituted Cd34\(-/-\) animals, but final tumor volumes were statistically significantly reduced (Figure 5.2B). These findings demonstrate that CD34 expression by non-hematopoietic cells is integral for optimal tumor growth seen at this early endpoint in wildtype mice.
To assess the importance of CD34 expression on hematopoietic lineages at the day 14-endpoint, reciprocally transplanted chimeras were generated. In this case, wildtype Ly5.1 animals were reconstituted with either Cd34+/+ or Cd34−/− bone marrow. Following the 10-week recovery period, chimerism was confirmed and the animals were injected subcutaneously with melanoma cells. In these chimeras, no differences were seen in tumor growth at day 14 post-injection (Figure 5.2C), suggesting that while CD34 expression is required on non-hematopoietic cells for optimal tumor growth, its expression on hematopoietic cells had no effect at the early, day 14, endpoint.
Figure 5.2: Reduced tumor growth at day 14 correlates with a lack of CD34 expression on non-hematopoietic cells.

A) Average final tumor mass and B) volume at day 14 in Cd34+/+ or Cd34−/− animals reconstituted with wildtype Ly5.1 bone marrow. (Data pooled from two experiments, Cd34+/+ n=13, Cd34−/− n=14) C) Average final tumor mass at day 14 in reciprocal reconstitutions of wildtype Ly5.1 animals reconstituted with Cd34+/+ or Cd34−/− bone marrow (n=9, * represents p < 0.05; Error bar = SEM).
5.3.3 Reduced tumor growth in Cd34⁻/⁻ mice is associated with increased dye extravasation at early stages

To assess vascular integrity in tumor tissues, we injected fluorescent carbocyanine dye intravenously 5 minutes prior to sacrifice, at the day 14 endpoint. Measurement of carbocyanine fluorescence proximal to tumor vasculature at this timepoint revealed a statistically significant increase in fluorescence intensity in Cd34⁻/⁻ tumors, compared to wildtype controls (Figure 5.3A). Increased fluorescence in Cd34⁻/⁻ tumors persisted proximal to tumor vasculature, with levels decreasing with distance away from the vessel (Figure 5.3B).

Tumor tissues were also immunostained for CD31 expression and revealed slightly lower, although not statistically significant, average distance of each point within the tumor from the nearest CD31⁺ vessel (Figure 5.3C). Lower average distance corresponds to a slightly higher vessel density in the Cd34⁻/⁻ tumors at the day 14 endpoint. Colocalization of carbocyanine fluorescence and CD31 expression was used to assess the percentage of perfused, functional vessels in each tumor. Colocalization revealed a higher proportion of perfused vessels in Cd34⁻/⁻ tumor tissues, compared to Cd34⁺/+ (Figure 5.3D). These findings demonstrate an increase in carbocyanine dye extravasation in tumor lacking CD34 expression and increased vascular perfusion within tumor blood vessels.
Figure 5.3: Increased carbocyanine dye extravasation and vessel perfusion in tumors from \textit{Cd34}\textsuperscript{-/-} animals.

On day 14, carbocyanine was injected five minutes prior to sacrifice. In tumor sections, fluorescence intensity was measured A) proximal to CD31\textsuperscript{+} vessels (at distance 0 µm) and B) as a function of distance from the nearest blood vessel (CD31\textsuperscript{+}) in day 14 tumors. C) Following immunohistochemical staining for CD31 expression, the average distance of each point within the tumor from the nearest CD31\textsuperscript{+} staining pixel was calculated, using image analysis software. D) Proportion of perfused vessels, corresponding to the percentage of CD31\textsuperscript{+} pixels colocalized with carbocyanine staining. For carbocyanine data, one outlier per genotype was removed prior to analysis, with very low fluorescence intensity, likely due to improper injection. (n=5 per genotype for carbocyanine stains, n=6 for average distance and vessel perfusion, * represents p< 0.05; ** represents p<0.01; Error bars = SEM).
5.3.4 Paradoxically, Cd34\(^{-/}\) animals exhibit increased tumor growth at later timepoints, as a result of CD34 loss on hematopoietic cells

As tumor growth was significantly reduced 14 days following injection, possibly due to a vascular defect, we hypothesized that this difference would increase with further tumor development. To address this hypothesis, Cd34\(^{-/}\) animals were again injected with 5 X 10\(^5\) B16 cells subcutaneously and tumors were allowed to grow for 19 days. Between the day 14 and day 19 sacrifice timepoints, tumors masses increased from ~0.4 grams to >1.0 gram on average. Surprisingly, we observed that with additional time, tumors in Cd34\(^{-/}\) animals approached or even surpassed final tumor size in Cd34\(^{+/}\) animals (Figure 5.4A). Because there is no statistically significant difference at this timepoint between final tumor masses of tumors from Cd34\(^{+/}\) and Cd34\(^{-/}\) animals, these findings suggest a dramatically enhanced tumor growth between days 14 and 19 in Cd34\(^{-/}\) animals.

To determine the relative importance of CD34 expression on hematopoietic versus non-hematopoietic cells for the size difference at this later timepoint, we again grew tumors in bone marrow-chimeric animals. Using wildtype Ly5.1 animals reconstituted with either Cd34\(^{+/}\) or Cd34\(^{-/}\) bone marrow, we found a statistically significant increase in final tumor mass at the later day 19 timepoint in Cd34\(^{-/}\)-reconstituted animals (Figure 5.4B). These findings suggest that CD34 expression on hematopoietic cells is critical for the growth increase observed at later timepoints, with CD34 loss resulting in an increase in tumor growth.
Figure 5.4: Melanoma growth in $Cd34^{+/+}$ animals surpasses growth in $Cd34^{+/+}$ animals by day 19, in association with loss of CD34 expression on hematopoietic cells.

A) Average tumor masses following sacrifice at day 19 in $ Cd34^{+/+}$ and $ Cd34^{-/-} $ animals. (Pooled from three experiments, $ Cd34^{+/+} $ n=15, $ Cd34^{-/-} $ n=16) B) Average final tumor mass at day 19 endpoint in wildtype Ly5.1 animals reconstituted with either $ Cd34^{+/+} $ or $ Cd34^{-/-} $ bone marrow. (Pooled from two experiments, $ Cd34^{+/+} $ recon n=11, $ Cd34^{-/-} $ recon n=13). (* represents p < 0.05; Error bars = SEM).
5.3.5 \textit{Cd34}^{−/−} mast cells infiltrate developing tumors with decreased frequency

As mentioned, mast cells have been proposed to play various roles in the tumor microenvironment, with suggested functions in both promotion and suppression of tumor growth (Maltby \textit{et al.}, 2009). Our lab has shown CD34 to be a molecule critical for efficient mast cell homing and migration (Blanchet \textit{et al.}, 2007; Drew, Merzaban \textit{et al.}, 2005). Therefore, we stained tumor samples for the presence of mast cells using toluidine blue staining, to characterize and quantify mast cell accumulation within developing tumors.

Toluidine blue staining revealed distinct mast cell accumulation around the periphery of developing tumors at both day 14 and day 19 endpoints, consistent with previous reports. The majority of mast cells were positioned in healthy tissues directly bordering tumors (Figure 5.5A), with a small but significant group of mast cells penetrating into the edges of developing tumors (Figure 5.5B). Tumor-infiltrating mast cells were most commonly seen on the periphery of tumors, with no mast cells seen towards the centre of tumor masses.

Mast cell numbers were quantified within stained tumor sections, and enumerated based on their presence at the tumor periphery or within the tumor mass. At the early (day 14) timepoint, there was a trend towards reduced peripheral and infiltrating mast cell numbers in tumors from \textit{Cd34}^{−/−} animals, compared to \textit{Cd34}^{+/+} controls (Figure 5.5C). Due to high variability between samples, this difference was
not statistically significant. However, at the later timepoint (day 19), there were significantly fewer mast cells observed within the tumor in the Cd34⁻/⁻ animals (Figure 5.5D).

We also assessed mast cell numbers in tumors from chimeric animals at each timepoint where tumor size differences were observed, to determine whether mast cell accumulation correlated with altered tumor growth. At day 14, in chimeras lacking CD34 on non-hematopoietic tissues, no difference in mast cell infiltration was seen, with similar numbers at both tumor periphery and within tumor masses (Figure 5.5E). Conversely, at day 19, in chimeras lacking CD34 expression on hematopoietic cells, there were a reduced number of mast cells present within tumor tissue (Figure 5.5F). These findings demonstrate reduced mast cell infiltration into tumors at all timepoints, specifically when CD34 expression was absent on hematopoietic lineages, while mast cell infiltration and accumulation were unaffected in animals with wildtype CD34 expression on hematopoietic cells.
**Figure 5.5: Mast cell infiltration into tumors is reduced when cells lack CD34 expression.**

Images of toluidine blue-stained tumor sections reveal mast cell presence in A) peripheral tissues surrounding tumors and B) infiltrating into tumor tissues (arrows indicate mast cells, dotted line designates tumor boundary). Mast cell counts in tumors from $Cd34^{+/+}$ and $Cd34^{-/-}$ animals at C) day 14 (pooled from three experiments; $Cd34^{+/+}$ n=13, $Cd34^{-/-}$ n=16) and D) day 19 (pooled from three experiments; n=13). Counts in tumors from E) day 14 in $Cd34^{+/+}$ or $Cd34^{-/-}$ animals reconstituted with wildtype Ly5.1 marrow (pooled from three experiments, $Cd34^{+/+}$ recon n=13, $Cd34^{-/-}$ recon n=14) and F) day 19 in wildtype Ly5.1 animals reconstituted with either $Cd34^{+/+}$ or $Cd34^{-/-}$ bone marrow (pooled from three experiments, $Cd34^{+/+}$ recon n=11, $Cd34^{-/-}$ recon n=13). (* represents p < 0.05; Error bars = SEM).
5.3.6 Eosinophilia in IL5<sup>Tg</sup> animals is associated with decreased tumor growth

Eosinophils have been suggested to play a role in the clearance of melanoma tumors and we hypothesize that the increased growth at later timepoints in Cd34<sup>-/-</sup> mice may be due to impaired eosinophil infiltration. We also assessed the effect of increased eosinophil infiltration, by injecting B16 melanoma cells into IL5<sup>Tg</sup> animals, which exhibit profound blood eosinophilia (Lee <i>et al.</i>, 1997). IL5<sup>Tg</sup> animals were sacrificed at day 15 following subcutaneous injection of B16 melanoma cells and exhibited significantly reduced tumor growth compared to littermate controls (Figure 5.6A).

In the resulting tumors, mast cell infiltration was also assessed to determine the effect of eosinophilia on mast cell infiltration. In tumor sections from IL5<sup>Tg</sup> animals, there was no significant difference in mast cell infiltration or mast cell accumulation at the tumor periphery, compared to littermate controls (Figure 5.6B). These findings demonstrate that increased eosinophil numbers result in a decrease in tumor growth, without affecting mast cell tumor infiltration.
Figure 5.6: Tumor growth is reduced in IL5<sup>Tg</sup> eosinophilia mice.

A) The average final tumor mass at day 15 of tumors from IL5<sup>Tg</sup> eosinophilic mice and wildtype animals and B) mast cell counts from toluidine blue-stained tissues. (n=5, * represents p < 0.05; Error bars = SEM).
5.4 DISCUSSION

CD34 expression in the tumor microenvironment has been extensively reported in human and mouse cancers. In human tumor samples, CD34 expression patterns are often used to diagnose and distinguish tumor types (Tardio, 2008). Notably for our study, CD34 expression was generally absent in melanoma tumor samples (Tardio, 2008), and indeed we found no significant CD34 staining on tumor cells from B16-F1-OVA tumors (Figure 5.1C). Across a broad range of tumor types, CD34 is commonly expressed on tumor vasculature and also on spindle cells in a variety of skin lesions, as reviewed in (Tardio, 2008). Within these lesions, CD34 expression has been used a diagnostic marker, particularly in dermatofibrosarcoma protuberans, epithelioid sarcoma and pleomorphic hyalinizing angiectactic tumors, although it remains unclear whether CD34 has a functional role in these cancers (Tardio, 2008). CD34 staining in tumor biopsies has also been proposed as a potential prognostic indicator for patient survival, as CD34 staining correlates with tumor angiogenesis (Quon et al., 2009) and angiogenesis studies frequently use CD34 as a marker for tumor vasculature, somewhat interchangeably with CD31 (Chen et al., 2009; Murakami et al., 2009). One study has also reported a role for CD34 on tumor-forming cells during cancer progression, finding that Cd34−/− mice have fewer tumors following DMBA/TPA treatment (Trempus et al., 2007). Despite these findings, little work has focused on a functional role for CD34 on tumor vasculature or the surrounding microenvironment.
In our study, we observed dual functional roles for CD34 on non-tumor cells in B16 melanoma progression. In Cd34−/− animals, a lack of CD34 on non-hematopoietic cells affected the tumor vasculature at early timepoints, and was associated with decreased tumor size at day 14. Reduced tumor numbers were also observed in Cd34−/− mice in a 12-day lung metastasis model. At later timepoint (day 19), tumor size is increased following CD34 loss on hematopoietic cells. Throughout tumor development, we also demonstrate a reduction in intratumor stained mast cells ifollowing loss of CD34 expression on hematopoietic lineages.

At early timepoints, impaired tumor growth in Cd34−/− animals is due to loss on a non-hematopoietic cell population, most likely vascular endothelial cells. Our previous work has demonstrated a potential role for CD34 on endotherialia in maintenance of vessel integrity in inflammatory disease (Blanchet et al., 2009). Cd34−/− mice exhibit a striking increase in vascular leakage in an arthritis model, as well as increased leakage following treatment with TNFα (Blanchet et al., 2009). Likewise, inactivation of the related molecule podocalyxin, has been shown to delay the formation of the vascular aortic lumen during embryogenesis (Strilic et al., 2009). Further, podocalyxin and CD34 were shown to localize at the site of lumen formation during early vessel growth, in a Lewis Lung carcinoma tumor model (Strilic et al., 2009). Thus, there is a precedent to suggest a role for this family of molecules in angiogenesis and vasculogenesis.
The proposed impairment in $Cd34^{-/-}$ vessel function may be sufficient to explain the reduced tumor growth early in tumor development we observe in our study. However, at day 14, there were no significant differences in vessel density (Figure 5.3C), tumor necrosis, tumor cell proliferation or tissue hypoxia between $Cd34^{+/+}$ and $Cd34^{-/-}$ tumors (data not shown), suggesting that the impairment may occur early in tumor growth and may be corrected by day 14, or that the defect has a more subtle effect on vessel function. In support of this second hypothesis is our finding of increased carbocyanine extravasation proximal to tumor vessels in $Cd34^{-/-}$ mice (Figure 5.1C, Figure 5.3A/B). This increase could be explained by impaired vessel integrity in $Cd34^{-/-}$ tumors, altered blood flow or extravascular dye perfusion. Interestingly, this slight increase in carbocyanine extravasation was also observed in a Lewis Lung carcinoma tumor assay, although there was no difference in final tumor mass in that study (data not shown). In addition, we observed slightly higher levels of vessel perfusion in $Cd34^{-/-}$ tumors (Figure 5.3D), which may be a result of the increased dye extravasation or may reflect a difference in tumor angiogenesis. These findings support a role for CD34 in maintenance of vessel integrity and fit with our previous findings of increased vascular leakage in inflammatory conditions (Blanchet et al., 2009).

Interestingly, the reduction in tumor growth at day 14 in reconstituted $Cd34^{-/-}$ animals was less severe than the reduction seen in non-reconstituted $Cd34^{-/-}$ animals. This suggests that while CD34 expression on non-hematopoietic cells is required for the tumor growth difference seen at this early endpoint, CD34
expression on hematopoietic cells may play an additional role. In fact, in the non-reconstituted \( Cd34^{-/-} \) mice we observed a slight reduction in mast cell infiltration of developing tumors, which is not seen in tumors grown in \( Cd34^{+/+} \) mice reconstituted with \( Cd34^{+/+} \) bone marrow (Figure 5.5C/E). Mast cells have been suggested to play a role promoting early tumor angiogenesis by releasing factors such as VEGF and in tissue remodeling to provide space for tumor expansion (Maltby et al., 2009). We speculate that while CD34 expression on the endothelia is the most important factor for early tumor vessel integrity, mast cell infiltration may also contribute to angiogenesis. Thus, in addition to altered vessel integrity due to loss of CD34 on endothelial cells, reduced mast cell migration in animals with \( Cd34^{-/-} \) hematopoietic cells could result in a synergistic reduction in angiogenesis and impaired vessel function.

In addition to mast cells, CD34 is also expressed by eosinophils, which have been proposed to play a role in tumor rejection (Mattes et al., 2003). Our previous studies have demonstrated a key role for CD34 in optimal eosinophil migration (Blanchet et al., 2007). We speculate that increased tumor growth at late timepoints in animals with \( Cd34^{-/-} \) hematopoietic cells may be due to impaired eosinophil migration, which in turn results in impaired tumor clearance. To further explore a potential role for eosinophils, we also assessed the effect of increased eosinophil numbers by inducing tumor growth in IL5\(^{Tg}\) animals. Eosinophilia in IL5\(^{Tg}\) has been reported to result in reduced tumor growth in a sarcoma model (Simson et al., 2007), and we found a significant reduction in melanoma tumor growth in IL5\(^{Tg}\) mice (Figure 5.6A).
This experiment also addressed a potential concern that the observed reduction in mast cell infiltration in Cd34⁻/⁻ animals could be an artefact of decreased tumor size, rather than due to loss of CD34. As the small tumors from IL5Tg mice exhibit no decrease in mast cell infiltrates, it provides further support that the mast cell migration defect observed in tumors from Cd34⁻/⁻ mice is a specific effect of CD34 loss on mast cells. It should also be noted that in addition to the pro-tumorigenic roles for mast cells in angiogenesis, many studies have indicated a role for mast cells in tumor clearance, via mechanisms similar to those ascribed to eosinophils. Thus, the decreased mast cell infiltration observed in Cd34⁻/⁻ tumors at day 19 may contribute to a reduced tumor clearance, which would result in the observed increased tumor size at this timepoint.

In conclusion, our study demonstrates that endothelial CD34 expression plays a key role early in tumor development and is required on non-hematopoietic cells to establish proper vasculature. In Cd34⁻/⁻ animals at all timepoints, tumor-infiltrating mast cell numbers were reduced, and at later timepoints tumor growth surpassed that of Cd34⁺/+ controls. The differences at later timepoints can be attributed to CD34 loss on hematopoietic cells and likely reflects a reduced capacity of Cd34⁻/⁻ cells to induce immune tumor rejection. This study presents novel insights into CD34 function on vasculature and expands our understanding of a role for CD34 in hematopoietic cell migration during tumor development.
5.5 ACKNOWLEDGEMENTS

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5.6 REFERENCES


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CHAPTER 6. CONCLUSION

My research has focused on the functional roles of CD34 and podocalyxin, particularly relating to hematopoietic cell migration in disease pathogenesis. Both Cd34<sup>-/-</sup> mice and wildtype mice reconstituted with Podxl<sup>-/-</sup> bone marrow appear normal at steady state, with no apparent defects in maintaining homeostasis (Cheng et al., 1996; Doyonnas et al., 2005; Suzuki et al., 1996). However, we have demonstrated altered phenotypes under disease conditions, when homeostasis is perturbed. These findings have improved our understanding of CD34 and podocalyxin functions in vivo, and also improved our understanding of basic disease processes in general.

6.1 CHAPTER FINDINGS

In Chapter 2 (Maltby et al., 2009), we demonstrated that expression of podocalyxin occurs on stressed erythroid cells, from an early BFU-E stage through to peripheral blood immature reticulocytes. These findings support the hypothesis that during anemia, erythroid cells express podocalyxin and exit the spleen (and bone marrow) at a CD71<sup>+</sup>Ter119<sup>+</sup> stage, rather than the CD71<sup>-</sup>Ter119<sup>+</sup> stage, as had been commonly thought (Socolovsky, 2007). However, loss of podocalyxin expression had no obvious effects on erythroid release and recovery from anemia, in contrast to proposed roles in the literature (Sathyanarayana et al., 2007). Data presented in Appendix A supports these findings, demonstrating that loss of podocalyxin on erythroid cells has no effects on BFU-E potential, Annexin V staining or erythroid osmofragility. Nevertheless, our work also revealed podocalyxin to be a simple
marker for the enrichment of stress erythroid progenitors. In addition, podocalyxin is expressed during conditions of chronic anemia, as seen in SHIP−/− mice. Taken together, these findings demonstrate that podocalyxin is expressed during stress erythropoiesis, but is not required for recovery from anemia, or to produce functional erythroid cells.

In Chapter 3 (Blanchet et al., 2007), we demonstrated reduced asthma severity in Cd34−/− mice, in an OVA-alum challenge model. The attenuated disease phenotype correlated with reduced mast cell and eosinophil infiltration into the lung and bronchoalveolar space following allergen challenge. Further, reconstituted animals lacking CD34 expression exclusively on hematopoietic cells, also exhibited attenuated disease, demonstrating a critical role for CD34 on hematopoietic cells in asthma pathology. Further study revealed CD34 expression at low levels on lung and BAL eosinophils, and a cell-intrinsic migration defect in eosinophils isolated from Cd34−/− animals, as compared to Cd34+/+. These findings demonstrate a role for CD34 in optimal eosinophil migration, likely through an anti-adhesive role, as we’ve previously reported for mast cells (Drew et al., 2005).

In Chapter 4, in a chemically-induced model of ulcerative colitis, we demonstrated a key role for CD34 in gut inflammation. Cd34−/− mice exhibited significantly reduced disease pathology, as quantified by weight loss, diarrhea and bleeding, colon shortening and tissue histology. As seen in the asthma model, reduced disease
pathology was associated with loss of CD34 on hematopoietic cells specifically. Further, our results demonstrated CD34 expression on colon-infiltrating eosinophils, with significantly reduced eosinophil numbers in Cd34^{-/-} tissues following disease induction. Under conditions of hypereosinophilia (IL5^{Tg} model), colon tissues exhibited chronic low-level inflammation, assessed by colon length, and CD34 expression on colon eosinophils. In addition, hypereosinophilia was sufficient to overcome the disease protection identified in Cd34^{-/-} mice. These findings demonstrate a role for CD34 in the progression of DSS-induced colitis, suggesting that CD34 expression on eosinophils is necessary for full disease and that it may be a valid therapeutic target for the treatment of IBD. In fact, we are currently working with a humanized mouse model (expressing human CD34 and lacking mouse CD34 expression) to test the feasibility of a CD34 targeted approach, for the treatment of disease.

Finally, in Chapter 5, we demonstrated altered tumor growth kinetics in Cd34^{-/-} mice, with both a non-hematopoietic and a hematopoietic component. At early stages, Cd34^{-/-} mice exhibited reduced tumor size, and reduced tumor numbers in a metastasis model. CD34 expression was present on tumor vasculature, and loss of CD34 expression on non-hematopoietic cells was sufficient to reduce tumor size at an early day 14 endpoint. In addition, we observed increased dye extravasation in Cd34^{-/-} tumors, suggesting CD34 plays a role in the maintenance of vessel integrity, supporting previous data for a role in vessel integrity (Blanchet et al., 2009; Strilic et al., 2009). At later timepoints, Cd34^{-/-} mice exhibited increased or equal tumor size,
as a result of Cd34 ablation on hematopoietic cells, compared to Cd34+/+ mice. At all timepoints, CD34 expression on hematopoietic cells was associated with an increase in mast cell accumulation within tumors. Our findings suggest that CD34 plays dual roles during tumor growth, with a non-hematopoietic pro-tumorigenic role in maintenance of vessel function and a haematopoietic anti-tumorigenic role at later timepoints (Figure 6.1).
Figure 6.1: CD34 expression regulates vascular integrity and immune cell accumulation during tumor growth

CD34 is expressed on vascular endothelia within the developing tumor, and loss of CD34 expression results in decreased tumor growth at early timepoints, associated with impaired vessel function and increased dye extravasation. Loss of CD34 expression on hematopoietic lineages results in decreased mast cell tumor infiltration and an increase in tumor mass at later timepoints, likely due to decreased immune cell-mediated tumor rejection.
6.2 GENERAL CONCLUSIONS

A key step in understanding the role of any given protein is the characterization of that protein’s expression pattern. Within my thesis work, we broadened the characterization of podocalyxin, demonstrating rapid induction on early erythroid cells during stress responses on early erythroid progenitors (BFU-E), which persists on immature reticulocytes in the periphery (Chapter 2) (Maltby et al., 2009). Further, our studies demonstrated expression of, and a functional role for, CD34 in eosinophil migration within both the lung (Chapter 3) (Blanchet et al., 2007) and the inflamed colon (Chapter 4) and a role for CD34 in maintenance of vessel integrity, on non-hematopoietic cells, in a tumor model (Chapter 5).

While these studies have focused specifically on CD34 and podocalyxin, our studies have also more broadly improved our understanding of basic disease processes. Key differences exist between “normal” basal erythropoiesis and “stress” erythropoiesis. Stress erythropoiesis derives from a unique subset of progenitors, which are BMP4-responsive (Lenox et al., 2005) and regulated by Fas/FasL interactions (Liu et al., 2006). These progenitors share features with embryonic erythroid cells and interestingly, podocalyxin expression is prominent on embryonic erythroid cells (Doyonnas et al., 2005). One speculation we have made is that expression on stress erythroid cells may reflect the immature nature of these progenitors.
Inadvertently, our studies of podocalyxin expression during stress erythropoiesis led to the observation that podocalyxin is expressed on CD71+Ter119+ cells, surprisingly absent on CD71-Ter119+ cells in the spleen and marrow, but still present on immature reticulocytes in the periphery. In the literature, erythroid development is proposed to pass through these three subsets in sequence (Socolovsky, 2007), which would require a rapid downregulation and subsequent upregulation of podocalyxin on immature erythroid cells. The more likely explanation for these findings is that stress erythroid cells maintain podocalyxin expression and actually exit the spleen and marrow at the CD71+Ter119+ stage, at an earlier stage than previously thought.

In the asthma model, we identified a remarkable degree of protection from disease pathology following ablation of CD34, specifically on hematopoietic lineages. Interestingly, while CD34 was only expressed by eosinophils and mast cells within this model, Cd34-/- animals exhibited greatly reduced macrophage, lymphocyte and neutrophil lung infiltration (Blanchet et al., 2007). This finding may reflect a role for eosinophils and mast cells in the recruitment of macrophage and lymphocyte populations. Alternatively, degranulation by infiltrating eosinophils and mast cells leads to tissue damage and may result in increased immune cell recruitment in response to inflammation. In an ulcerative colitis model, we identified a similar degree of protection in Cd34-/- animals. Again, this protection was conveyed by loss of CD34 on hematopoietic cells, likely due to impaired eosinophil migration. These findings suggest that novel therapies targeting mast cell and eosinophil migration,
potentially by interfering with CD34 function, or the ability of these cell types to recruit other immune cells, may be sufficient to reduce asthma and colitis symptoms.

Our findings in the DSS-induced ulcerative colitis model also demonstrate that colitis occurs in the absence of blood eosinophilia, a result that is quite different from the asthma model. This difference may be due to differences in the length of the colitis and asthma timelines. Asthma induction occurs over several weeks and involves an initial intraperitoneal sensitization stage, following by sequential intranasal challenge. The length of this treatment allows for the induction of bone marrow eosinophil precursor expansion and increased blood eosinophil numbers. However, the DSS colitis model only requires 8 days, with overt disease pathology starting at day 4. This comparatively short timeline, compared to asthma, likely does not leave time for significant eosinophil expansion in the bone marrow, and there are no observed increases in peripheral eosinophil levels. This distinction between diseases is important, as many therapies have targeted eosinophil proliferation as a means to interfere with eosinophil function, and these approaches will likely not have effects on ulcerative colitis. Our data suggests that while eosinophils are critical for disease progression, eosinophil expansion is not required in the DSS-induced model and that therapies targeting eosinophil migration are more likely to be successful. It will be interesting to see if decreased peripheral eosinophil numbers are observed in patients with chronic ulcerative colitis pathology, which would presumably allow for the expansion of marrow eosinophil production. Conversely, we found that eosinophilia alone (in IL5\textsuperscript{Tg} mice) results in eosinophil accumulation in the colon.
tissues and increased susceptibility to colitis, in the absence of overt pathology at steady state. The early low-level inflammation likely explains the age-related gut pathology seen in ~50% of aged IL5\textsuperscript{Tg} animals previously reported (Lee et al., 1997). Importantly, this observation may have important implications for the treatment and observation of patients exhibiting profound, chronic eosinophilia in clinics and suggest that chronic eosinophilia alone could increase disease susceptibility in this subset of patients.

The tumor studies in Chapter 5 emphasize the importance of analyzing a range of endpoints, and highlight the complex signals that affect tumor growth \textit{in vivo}. Importantly, all of our studies used “wildtype” B16-F1-OVA melanoma cells, and thus all of the observed differences in tumor growth were due to the presence or loss of CD34 expression on the tumor microenvironment alone. Most of the cancer studies to date have focused on specific changes within tumorigenic cells with emphasis on the importance of initial mutations in tumor development. However, increasing attention has focused on the role of non-tumor cells in cancer development and demonstrated the importance of a range of cells within the tumor microenvironment. In fact, mast cells and eosinophils, which both express CD34 have been shown to have potent pro- and anti-tumorigenic effects as I reviewed in (Maltby \textit{et al.}, 2009) and shown by (Mattes \textit{et al.}, 2003; Simson \textit{et al.}, 2007). In our study, injected melanoma cells are the same for all models, so any differences observed are a direct result of loss of CD34 expression within the tumor microenvironment. Our study demonstrates that both non-hematopoietic and hematopoietic cell lineages
alter tumor growth, with opposing effects following ablation of the single molecule, CD34. These findings highlight the balance of pro-tumorigenic and anti-tumorigenic functions within the tumor microenvironment, which can shift at different stages of tumor progression, and reveal the complex and often contradictory roles a molecule can play in developing tumors.

Integrating our findings, I propose the following general model of CD34 function at inflammatory sites (Figure 6.2). Following initiation of an inflammatory response (allergen exposure, local tissue damage, etc.), eosinophil and mast cell infiltration occurs in response to the production or release of molecules such as IL-5, eotaxin and SCF. Cell migration from neighbouring tissues and peripheral blood, and subsequent migration within the tissue is mediated, in part, by CD34 expression on these cell types. Ablation of CD34 expression results in a cell-intrinsic decrease in mast cell and eosinophil migration (Drew et al., 2005; Blanchet et al., 2007). Within inflamed tissues, eosinophils and mast cells release a variety of mediator molecules, including histamine (which increases local vascular permeability), proteases and peroxidases (which damage local tissues) and various pro-inflammatory molecules (which promote further immune cell recruitment). Thus, reduced inflammatory cell accumulation in the tissues of \( Cd34^{-/} \) mice results in a decrease in both immune cell recruitment and the tissue damage associated with disease pathology. Intriguingly, recent unpublished findings in our lab demonstrate reduced chemokine receptor surface expression following loss of podocalyxin expression. Thus, in addition to a role in anti-adhesion, CD34 types proteins may play a similar role in chemokine
receptor stabilization to optimize cell migration. Somewhat paradoxically, CD34 also modulates vascular integrity, and loss of CD34 results in increased dye extravasation and local edema following exposure to inflammatory mediators (Chapter 5) (Blanchet et al., 2009). One model to explain the increased vessel leakage is the hypothesis that CD34 is required for establishment of proper cell polarity in endothelial cells. Expression of podocalyxin in MCF-7 cells helped establish cell polarity, and overexpression expanded the apical surface, eventually leading to reduced cell adhesion onto plates (Nielsen et al., 2007). Loss of CD34 and podocalyxin expression in the developing embryonic aorta also leads to a delay in vessel lumen formation (Strilic et al., 2009). Thus, one hypothesis is that CD34 and podocalyxin play a role in the early establishment of apical/basal polarity within developing vessels. If CD34 or podocalyxin is absent, this process is delayed and ultimately leads to impaired endothelial polarity and disorganized vascular structure, leading to decreased vessel integrity and increased vascular leakage. The observed decreased vessel integrity and decreased immune cell infiltration in Cd34−/− mice are somewhat counterintuitive, and how this occurs remains unclear.

The alternative hypothesis has been raised that the attenuated disease phenotypes observed in Cd34−/− mice may reflect impaired T cell or dendritic cell function, resulting in generalized immunosuppression. CD34 is expressed within the HEV with a particular sialyl-Lewis-X modification that binds L-selectin and plays a role in naïve T cell homing (Baumheter et al., 1993). The absence of this binding in Cd34−/− mice could, in principle, lead to altered T cell homing and impaired T cell
function. In each of our disease models, we have demonstrated that *Cd34* /− bone marrow-reconstituted mice, which lack CD34 expression in HEVs, exhibit attenuated disease. This finding suggests that loss of CD34 expression in the HEV is not critical for differences in disease severity, but T cell functionality in *Cd34* /− mice has not been thoroughly characterized. CD34 is also expressed on subsets of dendritic cells (Blanchet, Bennett & McNagny, unpublished observations), which are important for the development of adaptive immune responses. Any impairment in dendritic cell function in *Cd34* /− mice could result in reduced adaptive immune responses and explain the attenuated disease phenotype. We have not explicitly tested dendritic cell function in an asthma model, although our findings suggest that the adaptive immune response is fully functional in *Cd34* /− mice. We demonstrated that wildtype and *Cd34* /− mice produce equal levels of cytokines in recall assays (Figure 3.4) and *Cd34* /− asthmatic mice actually exhibit increased numbers of blood eosinophils following allergen challenge (Figure 3.5). However, the loss of CD34 expression did result in impaired dendritic cell migration and dampened immune responses in a mouse model of hypersensitivity pneumonitis (Blanchet, Bennett & McNagny, unpublished observations), so the importance of CD34 on dendritic cells in an asthma model should be explored in the future.

Our findings have broadened our understanding of roles for CD34 and podocalyxin in disease models, but a disconnect still remains between *in vitro* data and the *in vivo* cell functions attributed to each of the CD34 family of molecules. For example, *in vitro* studies have demonstrated intracellular association between podocalyxin and
NHERF1, NHERF2 and ezrin, which in turn associate with the actin cytoskeleton. Linking with the actin cytoskeleton likely plays a key role in the formation of microvilli observed in podocalyxin-overexpressing MCF-7 cells (Nielsen et al., 2007), although this phenotype was not NHERF-dependent and the direct mechanism is unknown. Even further, while we clearly show a cell-intrinsic effect of CD34 loss on eosinophil migration, we can only speculate on how CD34 performs this function mechanistically. For this reason, extensive further work is needed in characterizing CD34 modifications in response to stimuli and the intracellular ligand it binds, particularly under conditions of disease and a further characterization of both extracellular and intracellular interacting partners in different cell types.
Figure 6.2: CD34 mediates eosinophil and mast cell accumulation, and vascular integrity under inflammatory conditions

At inflammatory sites, CD34 is expressed on vascular endothelial cells and infiltrating mast cells and eosinophils. Loss of CD34 results in decreased vessel integrity and an increase in vascular permeability under inflammatory conditions. Further, loss of CD34 on infiltrating immune cells results in decreased eosinophil infiltration from peripheral blood and decrease mast cell recruitment from local tissues. Reduced eosinophil and mast cell numbers results in a decrease in local degranulation, which affects vascular permeability (via histamine release) and the local tissue damage and further immune cell recruitment associated with local pathology. (Black arrow weights correspond to differences in effect size for each genotype)
Wildtype conditions

Cd34⁻/⁻ conditions

Key

- Vascular Endothelia
- Macrophage
- Mast cell
- Eosinophil
- Lymphocyte
- Neutrophil
- CD34
- Decreased
6.3 FUTURE DIRECTIONS

One key advance in the study of CD34, podocalyxin and endoglycan functions will be the development of new conditional knockout animals. These tools will allow us to specifically delete each molecule within different cell subsets in isolation, and allow the further dissection of our observed phenotypes. To study podocalyxin function during stress erythropoiesis, a conditional Podxl-knockout mouse model will be extremely useful. In mice, the highest expression levels of podocalyxin are observed perinatally (Doyonnas et al., 2005). At this stage in development, hematopoietic cells are engaged in a rapid burst in proliferation and migration, from the fetal liver to seed the bone marrow. Our hypothesis is that podocalyxin may play a role in optimal cell migration, so any defect in Podxl−/− animals would most likely be observed at this timepoint. In fact, podocalyxin expression during adult stress erythropoiesis may reflect the “embryonic” nature of stress erythroid cells, and podocalyxin expression may be more important during fetal or perinatal erythropoiesis. Unfortunately, to date we have been unable to analyze these early time windows, as Podxl−/− mice die rapidly after birth, and bone marrow chimera studies only allow us to explore mature adult hematopoiesis. Using conditional knockout animals, specifically ablating podocalyxin expression only on hematopoietic or erythroid lineages would allow us to characterize any defects at this timepoint or during early development, when a phenotype is most likely to arise. In the asthma, colitis and tumor models, specific deletion of Cd34 on individual hematopoietic lineages would also provide further insights into the relative roles of mast cells versus eosinophils and the importance of each cell type in disease.
progression in vivo. More specifically, eosinophil-specific ablation of CD34 could demonstrate whether the decreased migration observed in Cd34−/− eosinophils is due to a cell-intrinsic or an environmental defect in Cd34−/− mice.

One interesting observation from our tumor studies is that loss of CD34 expression on non-hematopoietic cells, most likely vascular endothelia, results in impaired vessel integrity. This finding fits with the recent observation that vascular leakage is increased in Cd34−/− mice following arthritis induction or stimulation with TNF (Blanchet et al., 2009). Interestingly, a role for CD34 in vessel lumen formation has also been suggested, based on similarities between CD34 and podocalyxin (Strilic et al., 2009). While CD34 and podocalyxin expression on vasculature is well documented, these findings could open a new area of study into the function of these molecules during both angiogenesis and the maintenance of steady-state vessel integrity. Again, this is an area of the CD34 and podocalyxin literature where the mechanisms are very poorly understood. Studies in a tumor model have been somewhat hampered by the necessity of using lethally-irradiated mice to explore non-hematopoietic effects, and irradiation is known to damage and alter vessel function. Further studies analyzing angiogenesis and vessel function in vitro, such as aortic ring angiogenesis assays, or in vivo, using implanted angioreactors, could provide valuable insights into CD34 function on vasculature.
In addition to animal models and new transgenics, further work is needed at the cellular and sub-cellular level, to clarify the mechanisms of CD34 and podocalyxin functions. While we have identified disease phenotypes and defects in cell-based assays, the cellular and molecular mechanism of these differences has eluded us. One line of future experiments is a basic functional comparison of isolated $Cd34^{+/+}$ and $Cd34^{-/-}$ eosinophils, comparing activation states, degranulation and migration. While our findings demonstrate a defect in eosinophil migration, we could not confirm whether this was a direct effect on anti-adhesion following CD34 loss, or a difference in activation state of purified eosinophils. Comparison of signalling events in stimulated $Cd34^{+/+}$ and $Cd34^{-/-}$ eosinophils could also provide information about potential roles for CD34 in signal transduction. Further, screening for potential extracellular and intracellular binding proteins that interact with CD34 should be performed, as it would provide a starting point to understand how these molecules work. It is unlikely that CD34 and podocalyxin act alone, and the identification of interacting partners would provide valuable insights into how these molecules exert their effects. In addition, while we have identified expression of CD34 and podocalyxin on many new cell types, we have not compared the glycosylation patterns of each molecule on any of these cell subsets. Glycosylation-based differences in protein function have already been identified for CD34 on HEVs (Baumheter et al., 1993), but whether these differences are present on other cell types has been unexplored.
6.4 REFERENCES


APPENDIX A. SUPPLEMENTARY DATA ON PODOCALYXIN EXPRESSION AND FUNCTION DURING STRESS ERYTHROPOIESIS

A.1 BACKGROUND

In addition to the experiments described in Chapter 2, we performed a number of assays to characterize potential differences between Podxl+/+ and Podxl−/− erythroid cells. Our assessments demonstrated similar BFU-E capacity, red cell structural integrity and homing for Podxl−/− cells, compared to wildtype, and these data were not included in the final Experimental Hematology manuscript. This appendix contains a summary of these experiments, demonstrating that the loss of podocalyxin has no major effects on adult murine erythroid development or erythroid structure.

A.2 MATERIAL AND METHODS

A.2.1 Mice

As outlined in Chapter 2, all mice were maintained in specific pathogen-free facility at the Biomedical Research Centre (UBC) and experimental protocols were conducted in accordance with approved and ethical treatment standards of the University of British Columbia. Recipient Ly5.1 C57Bl/6 mice (6-8 weeks old) (Jackson Laboratory, Bar Harbor, Maine) were lethally irradiated (11 Gy) and injected (intravenously) with 2x10^6 fetal liver cells derived from congenic (Ly5.2) Podxl−/− or Podxl+/+ day 15.5 post coital (p.c.) embryos (tissue samples from each embryo were genotyped by PCR) (Doyonnas et al., 2005). To determine reconstitution levels 10
weeks post transplant, peripheral blood was sampled by saphenous vein and labeled with antibodies against Ly5.1 and Ly5.2 (BD Pharmingen, Mississauga, ON), following red cell lysis. Full reconstitution was defined as >95% donor-derived (Ly5.2) CD45+ cells contributing to myeloid and lymphoid cells in the periphery.

SH2-containing inositol-5-phosphatase knockout (SHIP−/−) and wildtype (SHIP+/+) (6-8 weeks old) congenic littermates (Helgason et al., 1998) were generously provided by Dr. Gerry Krystal (BC Cancer Agency, Vancouver). Genotyping was performed as previously described (Helgason et al., 1998).

A.2.2 Antibodies and flow cytometry

Podocalyxin expression on erythroid subset populations in bone and spleen were determined by flow cytometry as already described in section 2.2.3 of this manuscript. Annexin V staining was performed according to manufacturers specifications (Molecular Probes, Eugene, OR).

A.2.3 Fetal liver erythroid colony assessment

Fetal liver samples were collected from congenic Podxl−/− and Podxl+/+ embryos at d15.5 p.c. and dissociated by passing them through a 19G needle. Cells were plated at several concentrations in semi-solid culture (M3434, Stem Cell
Technologies Inc, Vancouver, BC) with recombinant mouse stem cell factor (SCF) (R&D Systems Inc, Minneapolis, MN) and several concentrations of erythropoietin as described in the text and assayed for BFU-E 7 days after plating using standard methods.

A.2.4 Osmofragility assay

Assessment of erythrocyte osmofragility was performed as previously described (Mukherjee et al., 2007). Briefly, peripheral bleed samples from reconstituted animals were suspended in PBS and diluted to 1% hematocrit. Samples were then mixed with various concentrations of NaCl solution ranging from 0.10 to 0.85% (w/v). Following incubation, supernatants were analyzed spectrophotometrically at 540 nm and readings were normalized to 100% and 0% lysis based on measurements of 0.10% and 0.85% NaCl conditions respectively.

A.2.5 Short-term homing assay

Reconstituted animals were injected on 5 consecutive days with Epo (i.p.) 20 U/mouse/day. Animals were then sacrificed and following a red cell lysis, spleen cells were stained with biotinylated antibodies recognizing CD3, B220, Mac1 and Gr1. Stained cells were then incubated with streptavidin beads and depleted using magnetic depletion according to manufacturers specifications (AutoMACS, Miltenyi Biotec Inc, Auburn, CA). The remaining cells were then labelled using CFSE or
Tracker Orange (Molecular Probes, Burlington, ON) according to manufacturers specifications, counted and mixed in equal numbers. Cells were injected intravenously (i.v.) into C57Bl/6 recipients, which were sacrificed 18 hours later. Spleen and bone marrow were then isolated and analyzed by flow cytometry to assess numbers of migrating donor cells.

A.2.6 Administration of phenylhydrazine

SHIP\(^{+/+}\) and SHIP\(^{-/-}\) mice were injected (i.p.) twice with 60 mg/kg PHz prepared in RPMI (Gibco-Invitrogen, Burlington, ON) 24 hours apart. Animals were sacrificed three days later, and tissues were stained for B220 and podocalyxin expression.

A.2.7 Statistics

An unpaired student’s \( t \) test was used for statistical analysis to compare differences between wildtype and \( Podxl^{-/-} \) cells in all assays.
A.3 RESULTS

A.3.1 Podocalyxin is expressed on immature erythroid populations during stress, but loss has no effect on erythrocyte development or apoptosis

To better understand the role of podocalyxin in stress erythropoiesis, we analyzed erythroid progenitor subsets during stress erythropoiesis to determine where podocalyxin expression occurs, as described in Chapter 2. I have included further figures in this appendix chapter to show the gating strategy used to determine podocalyxin expression on immature subsets. Following Epo induction (at 72 hours), podocalyxin expression was induced in the bone marrow, spleen and peripheral blood. Highest levels of podocalyxin were seen on the ProE (CD71+Ter119lo) and CD71+Ter119+ subsets in marrow and spleen (Figure A.1B/D). Further, expression was highest on the EryA basophilic populations (Ter119+CD71+FSChi), with decreasing levels on EryB late basophilic and polychromatic populations (Ter119+CD71+FSClo) (Figure A.1B/D), as described by Liu et al (Liu et al., 2006). Expression was then absent on the CD71+Ter119+ population, which contains the more mature orthochromatic erythroblasts and reticulocytes (Liu et al., 2006). As demonstrated in Chapter 2, loss of podocalyxin had no effect on total numbers or proportions of each of these subsets, when compared to cells from wildtype animals. The apparent podocalyxin staining on CD71−Ter119− cells was found to be due to background staining of B220+B cells, and was found non-specifically on the same population in Podxl−/− (data not shown).
Figure A.1: Podocalyxin expression on immature erythroid subsets within the bone marrow and spleen.

Wildtype mice were injected with a single dose of Epo (200U / mouse) and sacrificed 72 hours later. Bone marrow and spleen cells were isolated and stained for CD71, Ter119 and podocalyxin staining. A) Bone marrow and C) spleen gates for erythroid subsets, based on levels of CD71 and Ter119 staining and FSC. B) Bone marrow and D) spleen podocalyxin staining levels on the indicated subsets (histogram colors correspond to the color of the gating labels). Data is representative of 3 experiments.
At baseline and following Epo induction, we then analyzed annexin V staining of erythroid subsets to identify potential subtle differences in cell death or apoptosis, in the absence of podocalyxin. Flow cytometry revealed low levels of annexin V staining in marrow and spleen across all cell subsets at baseline, with no differences between $Podxl^{-/-}$ or $Podxl^{+/+}$ chimeras (Figure A.2A). Gating on specific erythroid subsets within the baseline marrow and spleen demonstrated low levels of annexin V staining particularly on CD71$^{+}$Ter119$^{+}$ (Figure A.2D) and higher levels on CD71$^{+}$Ter119$^{+}$ (Figure A.2C). However, no difference was seen in staining intensity between $Podxl^{+/+}$ and $Podxl^{-/-}$ tissues. These findings demonstrate that while podocalyxin expression is induced early in erythroid development, loss of podocalyxin has no major effect on erythroid differentiation or cell turnover at baseline in the spleen and bone marrow.
Figure A.2: Annexin V staining of erythroid subsets at baseline in marrow and spleen.

*Podxl*+/+ and *Podxl*−/−-reconstituted chimeras at baseline were sacrificed and unlysed bone marrow and spleen tissues were processed for flow cytometry. Annexin V staining levels on A) total ungated, B) total Ter119+, C) CD71+Ter119+ and D) CD71−Ter119+ gated cell populations. (grey-filled represents staining levels on *Podxl*−/− cells, black line represents staining on *Podxl*+/+ cells)
A.3.2 Podxl−/− fetal liver exhibits normal BFU-E capacity

As we have previously shown high levels of podocalyxin in developing fetal liver (Doyonnas et al., 2005), we assessed Podxl−/− embryos to determine if podocalyxin plays a critical role in embryonic erythropoiesis. Assessment of BFU-E colony formation capacity in Podxl−/− and Podxl+/− d15.5 (p.c.) embryos revealed low levels of BFU-E growth at low doses of Epo (10mU) with SCF (Figure A.3). Culturing fetal liver cells at higher Epo levels (500mU and 3000mU) with SCF yielded increased BFU-E growth. However, at all levels of Epo tested, cells isolated from Podxl−/− fetal liver yielded similar BFU-E colony numbers to cells from Podxl+/+ fetal liver (Figure A.3). These findings suggest that while podocalyxin is expressed at high levels during fetal erythropoiesis, its expression is not required for erythroid blast formation in vitro.
Figure A.3: Normal levels of BFU-E colonies are found in the Podxl/⁻ fetal liver.

Fetal liver cells from d15.5 p.c. embryos were plated in methylcellulose media containing Epo and varying doses of Epo with SCF. Resulting BFU-E colonies were determined by microscopy at day 7 of culture. (n=2 replicate plates from one experiment, error bars = SEM)
A.3.3 Circulating peripheral erythrocytes in Podxl\textsuperscript{−/−} animals exhibit normal osmofragility

Following identification of podocalyxin expression on immature erythroid cells, we hypothesized that loss of podocalyxin expression during development may result in impaired mature erythrocytes. One hypothesis was that loss of podocalyxin expression during development might result in altered structure in mature cells. To determine whether loss of podocalyxin expression affects structural integrity of mature erythrocytes, we performed an osmofragility assay. In an osmofragility assay, differences in structural integrity will appear as a change in which salt concentration is able to lyse the mature red cells. Circulating erythrocytes from Podxl\textsuperscript{+/+} and Podxl\textsuperscript{−/−} animals both lysed between concentrations of 0.5 and 0.55% NaCl, with equal lysis across the full range of NaCl solution tested (Figure A.4). These results demonstrate that the loss of podocalyxin expression during erythroid development does not result in any major alterations in mature erythrocyte structural integrity \textit{in vivo}. 
Figure A.4: Normal osmofragility of mature erythrocytes isolated from Podxl<sup>−/−</sup> animals.

Peripheral blood from reconstituted animals was diluted in PBS to 1% hematocrit and plated in NaCl solutions, ranging from 0.10 – 0.85% and analyzed spectrophotometrically to determine osmofragility of mature erythrocytes. (n=5 for each genotype, Error bars = SEM)
A.3.4 Loss of podocalyxin has no effect on homing of developing erythroid progenitors

Podocalyxin has been suggested by us and others (Doyonnas et al., 2005; Sathyanarayana et al., 2007) to play a role in cell-cell adhesion and homing. We hypothesized that podocalyxin expression may occur during stress erythropoiesis as a means of anti-adhesion, allowing release of immature erythroid progenitors to the periphery. Podxl⁻/⁻ erythroblasts would remain trapped in the spleen and bone marrow, whereas Podxl⁺/⁺ would release into the periphery during times of stress. To directly assess differences in cell migration/homing for Podxl⁻/⁻ cells, we isolated erythroid progenitors and tracked their movement following injection into recipient animals. Following Epo induction, we enriched an erythroid fraction from the spleen by depleting non-erythroid populations expressing the lineage markers CD3, B220, Mac-1 and Gr-1. In recipient animals, labelled spleen cells accumulated at low levels in the marrow (~0.1% of total marrow) and spleen (~0.5-1% of total spleen). Dye swap experiments demonstrated no impact of the cell labels on cell accumulation in these tissues (Figure A.5). No significant difference was found between accumulation of Podxl⁻/⁻ and Podxl⁺/⁺ cells in these tissues, although there was some variability between experiments. This suggests that in our enriched erythroid population, podocalyxin does not play a role in homing of cells to the marrow and spleen, or retention of cells in these organs.
Figure A.5: Homing of erythroid-enriched cell populations in recipient animals.

Spleen cells from Epo-induced animals were depleted using antibodies against CD3, B220, Mac-1 and Gr-1. Cells were then labelled with CFSE or Tracker Orange and injected into recipient animals intravenously. Recipient animals were sacrificed 18 hours later, and fluorescent donor cell levels were assessed in marrow and spleen by flow cytometry. A) and B) represent two experiments, with dyes swapped for each genotype, to control for potential dye effects on tissue accumulation. (n=2 or 3 per genotype, error bars = SEM)
A.3.5 SHIP\(^{-/-}\) mice express podocalyxin constitutively under conditions of chronic anemia

The loss of SH2-containing inositol-5-phosphatase (SHIP) expression results in chronic anemia. We analysed SHIP\(^{-/-}\) animals to determine whether podocalyxin expression is unique to acute anemic stress or is expressed during a wide range of anemic conditions. Flow cytometry of SHIP\(^{-/-}\) animals reveals that podocalyxin is constitutively expressed in both the spleen and the marrow under basal conditions, when compared to non-anemic SHIP\(^{+/+}\) littermates (Figure A.6 – black line). Following PHz induction resulted in a further induction of podocalyxin expression in both SHIP\(^{-/-}\) and SHIP\(^{+/+}\) animals, in both tissues (Figure A.6 – red line). These findings also demonstrate that SHIP function is not required for podocalyxin induction following PHz injection. Thus, podocalyxin expression occurs during acute stress erythropoiesis (induced by Epo or PHz), but also during conditions of chronic anemia, and does not require functional SHIP expression.
Figure A.6: Podocalyxin is constitutively expression in chronically anemic SHIP−/− mice.

Wildtype and SHIP−/− PHz-injected and uninjected mice were sacrificed. Marrow and spleen cells were processed and stained for B220 and podocalyxin expression. Gating on B220− cells, representative histograms of podocalyxin staining on SHIP+/+ A) bone marrow and B) spleen and SHIP−/− C) bone marrow and D) spleen. (grey-filled represents unstained background fluorescence, black line represents uninjected mice, red line represent PHz-injected mice 72 hours after injection)
A.4 DISCUSSION

Our study characterizing podocalyxin expression and suggesting podocalyxin as a marker to sort early stress erythroid progenitors is present in Chapter 2 of this thesis and published in Experimental Hematology. The intention of this appendix was to expand on this dataset and present the experimental findings that were not included in the final manuscript. Our finding of podocalyxin specifically on cells of the erythroid lineage in fetal liver and stress erythropoiesis suggests that this molecule has a function on this cell type. However, as presented here, we were unable to find any major functional difference between Podxl−/− or Podxl+/+ reconstituted animals, in terms of erythroid populations and differentiation, cell death and structural integrity.

One major limitation to these studies is the requirement to use bone marrow-reconstituted animals due to neonatal lethality in Podxl−/− animals. While reconstituted chimera studies have the advantage of allowing us to focus specifically on the function of podocalyxin on hematopoietic cells, there is the inherent limitation that we cannot identify potential effects on erythroid development caused by podocalyxin loss on non-hematopoietic cell types. In addition, reconstitution requires lethal irradiation, followed by ten weeks of recovery, meaning that we were unable to compare the effects of podocalyxin loss on erythropoiesis early in life, when rapid expansion of the erythroid lineage occurs and more subtle defects may be apparent. To address some of these issues, and explore much more subtle potential differences caused by podocalyxin loss, lab members have generated and
are characterizing transgenic animals allowing conditional deletion of the podocalyxin gene locus in specific cell lineages using the Cre/Lox system. These animals will allow us to analyze at much earlier timepoints and without requiring irradiation.

Our findings that podocalyxin expression occurs in SHIP−/− animals during conditions of chronic anemia suggests that podocalyxin induction is not unique to the acute stress erythropoiesis model. Assessing podocalyxin induction/expression in further chronic anemia models may shed insight to whether podocalyxin expression is as a general feature of chronic anemia. Further, subjecting Podxl−/− animals to conditions of chronic anemia may reveal subtle defects in erythropoiesis, which are not obvious under acute conditions. In addition, translation of these findings into humans could serve as a valuable tool for characterizing and diagnosing anemia. Assessment of podocalyxin on peripheral blood reticulocytes could be used clinically as an indicator of anemia or as a read-out of abnormal erythropoiesis in patients.
A.5 REFERENCES


APPENDIX B. UBC RESEARCH ETHICS BOARD CERTIFICATES OF APPROVAL

THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE

Application Number: A07-0399
Investigator or Course Director: Kelly McNagny
Department: Biomedical Research Centre (BRC)
Animals:

- Mice IL-5 40
- Mice C57Bl/6 40
- Mice PHIL 40
- Mice CD34 KO 40

Start Date: January 1, 2006
Approval Date: March 26, 2010

Funding Sources:

Funding Agency: Allergy, Genes and Environment Network (AllerGen) - Networks of Centres of Excellence (NCE)
Funding Title: Role of mast cells and eosinophils in allergic inflammation and fibrosis in the lung

Funding Agency: Canadian Institutes of Health Research (CIHR)
Funding Title: CD34 in inflammatory cell migration and function

Funding Agency: Michael Smith Foundation for Health Research
Funding Title: Cell commitment and differentiation in murine erythropoiesis and eosinophilopoiesis models

Unfunded title: N/A
The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

Office of Research Services and Administration
102, 6190 Agronomy Road, Vancouver, BC V6T 1Z3
Phone: 604-827-5111 Fax: 604-822-5093
ANIMAL CARE CERTIFICATE

Application Number: A06-1485

Investigator or Course Director: Kelly McNagny

Department: Biomedical Research Centre (BRC)

Animals:

- Mice PHIL 50
- Mice Fpx-cre 80
- Mice IL5Tg 30

Start Date: January 1, 2009

Approval Date: November 10, 2009

Funding Sources:

- Funding Agency: Michael Smith Foundation for Health Research
  Funding Title: Cell commitment and differentiation in murine erythropoiesis and eosinophilopoiesis models

- Funding Agency: Canadian Institutes of Health Research (CIHR)
  Funding Title: CD34 inflammatory cell migration and function

- Funding Agency: Allergy, Crops and Environment Network (AllerCon) Networks of Centres of Excellence (NCE)
  Funding Title: Role of mast cells and eosinophils in allergic inflammation and fibrosis in the lung

Unfunded title: N/A

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

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Office of Research Services and Administration
102, 6190 Agronomy Road, Vancouver, BC V6T 1Z3
Phone: 604-827-5111 Fax: 604-822-5093
ANIMAL CARE CERTIFICATE

Application Number: A06-1483

Investigator or Course Director: Kelly McNagny

Department: Biomedical Research Centre (BRC)

Animals:

- Mice C57Bl/6-CD34 120
- Mice Balbc 296
- Mice PHIL 120
- Mice C57Bl/6 280
- Mice WBB5F-1 J-Kit w/ Kit w-v 120

Start Date: July 21, 2003

Approval Date: November 16, 2009

Funding Sources:

Funding Agency: Allergy, Genes and Environment Network (AiGenNet) - Networks of Centres of Excellence (NCE)
Funding Title: CanGoFar - Programme B diagnostics and therapeutics

Funding Agency: Canadian Institutes of Health Research (CIHR)
Funding Title: CD34 in inflammatory cell migration and function

Funding Agency: Stem Cell Network (SCN) - Networks of Centres of Excellence (NCE)
Funding Title: Cell therapy for muscular disease

Funding Agency: Canadian Institutes of Health Research (CIHR)
Funding Title: Role of mast cells and eosinophils in allergic inflammation and fibrosis of the lung

Funding Agency: Michael Smith Foundation for Health Research
Funding Title: CD34 in inflammatory cell migration and function
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Unfunded title: N/A

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

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A copy of this certificate must be displayed in your animal facility.

Office of Research Services and Administration
102, 6190 Agronomy Road, Vancouver, BC V6T 1Z3
Phone: 604-827-5111 Fax: 604-822-5093
ANIMAL CARE CERTIFICATE
BREEDING PROGRAMS

Application Number: A09-0222

Investigator or Course Director: Kelly McNagny

Department: Biomedical Research Centre (BRC)

Animals:

- Mice Enslg 300
- Mice WBB6F-1 Jkit W/kit w-v 200
- Mice CD34KO 600
- Mice NHERF-KO 50
- Mice LY 5.1 200
- Mice Inpp5D 400
- Mice Il5-Ig 140
- Mice PHIL 180
- Mice Podo KO 200
- Mice Dali 2-40
- Mice GFP 50
- Mice C57Bl/6 600
- Mice Ank1 260

Approval Date: September 30, 2009

Funding Sources:

Funding Agency: Canadian Institutes of Health Research (CIHR)
Funding Title: CD34 in inflammatory cell migration and function

Funding Agency: Canadian Institutes of Health Research (CIHR)
Funding Title: The role of podocalyxin in hematopoietic and vascular function
The Animal Care Committee has examined and approved the use of animals for the above breeding program.

This certificate is valid for one year from the above approval date provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.