STAT3-DEFICIENCY IN BONE MARROW HEMATOPOIETIC STEM CELLS RESULTS IN DYSFUNCTIONAL MACROPHAGES AND CORRELATES WITH CROHN'S DISEASE-LIKE PATHOGENESIS

A Dissertation in

Anatomy

by

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Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

May 2011
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ABSTRACT

The most well-known subsets of inflammatory bowel disease (IBD) are ulcerative colitis (UC) and Crohn’s Disease (CD). Currently, the etiology of CD is unknown but it is thought to result from excessive T helper (Th)1-cell mediated inflammation. There is no gender or age bias, but CD is most often detected between 15-30 years of age. Macrophages are tissue specific monocytes and are the first line of defense in innate immunity; once activated they produce interleukin (IL)-12, IL23 and tumor necrosis factor-alpha (TNFα), proinflammatory cytokines, which induce a Th1-cell mediated immune response. Th1-cells can synthesize several proinflammatory cytokines including IL6, TNFα and interferon-gamma (IFNγ). Signal transducers and activators of transcription (STAT) proteins play an important role in mediating intestinal cytokine signaling and it is unknown if the loss of these proteins initiate severe downstream modifications that consequently result in CD.

When compensating for a deficiency in STAT3, STAT1 levels increase, which leads to an increase in IFNγ signaling, which promotes Th1-cell mediated inflammation. IL10, which is mediated through STAT3 signaling, is an anti-inflammatory cytokine that is secreted from activated macrophages. IL10 inhibits excessive macrophage activation which suppresses the macrophage induced synthesis of TNFα, and thus is responsible for down-regulating Th1-cell mediated
immune responses. This suggests that STAT3 mediates mucosal immune tolerance during an innate immune response.

Breeding a bone marrow (B)-TIE2 promoter driven Cre recombinase gene-targeted mouse with a STAT3 lox-P (F/F) mouse created a conditional (specific to bone marrow hematopoietic stem cells) STAT3 knock-out. This tissue specific STAT3 deficient mouse is suitable for in vivo studies because it exhibits a spontaneous Crohn’s-like pathogenesis in the small and large intestines, also characterized by an over production of pro-inflammatory cytokines and excessive macrophage infiltrations. In addition to these morphological changes, one functional change demonstrated a loss of NADPH oxidase activity, which is an important response induced by the innate immune system to kill phagocytosed materials (Welte et al., 2003). The overall goal of this project was to determine the role of STAT1 and STAT3 signaling in the development of inflammatory bowel disease. My working hypothesis was that defective innate immunity in Crohn’s disease may correlate with a loss in STAT3 activity. My specific hypothesis was that STAT3-deficiency in bone marrow hematopoietic stem cells results in dysfunctional macrophages and correlates with Crohn’s Disease-like pathogenesis.

For these studies gene-targeted mice were generated and expanded. Homozygous C57BL/6 mice with two floxed STAT3-SH2 domains and heterozygous Tie2Cre+ mice were backcrossed to the BALB/c background. To generate and expand the bone marrow specific STAT3-deficient mouse model homozygous BALB/c STAT3FF mice were bred with BALB/cTie2Cre+ mice. Wild-type littermates were used as controls to standardize the weaning and milking process for a more accurate comparison. STAT1-deficient
(STAT1<sup>+/−</sup>) mice were also used as a control in this study. The initial disruption of the STAT1 allele was generated by Durbin, et al., in 1996.

To identify the effects of wild type, STAT3- and STAT1- deficiency on monocyte distribution, the first experiment was designed to identify the specific population of monocyte-derived cells associated with CD-like phenotypes in the wild type, STAT1-, and STAT3-, deficient mouse. These studies determined that there were significant increases in myeloid-derived cells, including granulocytes and monocytes, as well as a significant decrease in lymphocytes circulating in the peripheral blood. There was severe myeloid hyperplasia in bone marrow specific STAT3-deficient animals and an increase in CD44<sup>low</sup>CD11b<sup>+</sup> cells, both F4/80<sup>+</sup> and F4/80<sup>−</sup>.

The second experiment was designed to determine the effects of wild type, STAT3- and STAT1- deficiency on macrophage function. For this experiment, resident peritoneal macrophages were isolated and purified for analysis. These data determined that early stage macrophages, at four weeks, were significantly dysfunctional in specific and non-specific phagocytosis along with a significant reduction in ROS and NADPH production.

To determine the effects of wild type, STAT3- and STAT1- deficiency on macrophage mediated cytokine synthesis and signaling a third experiment was designed to analyze the cytokine profiles of the system (serum) and the local environment (cultured macrophages). These data identified that macrophages isolated and cultured from bone marrow specific STAT3-deficient mice secrete
normal levels of IL3 and IFNγ, but exhibit a significant reduction in the production
of GM-CSF, IL1β, IL6 and TNFα.

In conclusion, this study determined that STAT3 signaling is required for
normal macrophage functions and that abnormal macrophages critical for the
pathogenesis of a Crohn's-like disease in these mice models. It was demonstrated
that the role of STAT3 activity in monocyte development and function has a
regulatory function, for differentiating numbers and phagocytic functional ability.
This study also established that the macrophage mediated cytokine synthesis is
abnormal and is involved in this Crohn's-like pathogenesis. This project uses a
spontaneous mouse model of CD that clinically resembles Crohn’s disease in human
patients. These data support the known theory that abnormal innate immunity is
essential in Crohn’s disease. Therefore, these data suggest that STAT3 proteins and
macrophages should be targeted in future therapies and clinical trials.
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<td>ANOVA</td>
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<td>dulbecco's modified eagle medium</td>
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<td>DVM</td>
<td>Doctor of Veterinary Medicine</td>
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<td>et al</td>
<td>and others</td>
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<td>μg</td>
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<td>S.E.M.</td>
<td>standard error of the mean</td>
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<td>STAT</td>
<td>signal transducers and activators of transcription</td>
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<td>T&lt;sub&gt;a&lt;/sub&gt;</td>
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<tr>
<td>TNBS</td>
<td>trinitrobenzen sulfonyl acid</td>
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<td>TNF</td>
<td>tumor necrosis factor</td>
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<td>w/v</td>
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ACKNOWLEDGEMENTS

I would like to thank Dr. Samuel Shao-Min Zhang, my thesis advisor and a great mentor. There were several times throughout this research when I really wanted to give up, and I cannot thank you enough for pushing me through it. Your words of encouragement and helpful advice were paramount to my success in the program. Thank you for providing a listening ear and a collaborative spirit; I could not have asked for a better mentor.

Thank you to my committee members: Dr. Colin Barnstable, Dr. Lisa Poritz and Dr. Patricia McLaughlin. You were tough on me, but I appreciate you pushing me to succeed. Thank you for your suggestions and for your time commitment to my education; I appreciated all of your feedback.

A special thank you is necessary for Lucy Lou and Christopher Siefring, my lab mates, for all their encouragement, wisdom and laughter. You made research much better by suffering through it with me. You will always be my lab family, and I miss you now that we’ve all moved on. Also a special thank you to Chen Yang for continuing my work, I wish you the best of luck with it.

Lastly, I would like to thank my family, friends and sorority sisters. Their prayers and continual words of encouragement have seen me through many frustrating situations. Without their love and support I wouldn’t have made it this far within my academic career.
CHAPTER ONE

INTRODUCTION
1.1. Current Problems in Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a common designation for a disease that causes abnormal swelling and chronic, relapsing inflammation within the digestive tract (Mudter et al., 2005). Identified and described in the late 1800s (Thorlaken, 1924, Cullinan, 1938), IBD pathogenesis has posed an intriguing mystery to many researchers. The most well-known subsets of IBD are Crohn’s disease and ulcerative colitis, although there are many variations and unclassified, indeterminate, IBD’s. Ulcerative colitis (UC) primarily affects the mucosal layer of the rectum and extends proximally into the large intestines, forming ulcers and continuous superficial mucosal inflammation (Cullinan, 1938; Podolsky, 2002). Crohn’s disease (CD) is transmural, affecting not only the mucosa but also the underlying layers of the intestinal wall, with granulomas, discontinuous ulcerations and stricture formation between the bowel and skin or other surrounding organs (Podolsky, 2002; Welte et al., 2003). The primary differences between UC and CD are location and treatment (Xavier and Podolsky, 2007).

In 1932, Dr. Burrill Crohn and his colleagues, Dr.’s Ginzbur and Oppenheimer, described “Regional ileitis”, which was inflammation seen within the small intestine and colon, now referred to as Crohn’s disease (Crohn et al., 1932; Bockus and Lee, 1935; Aufses, 2001; Batra et al., 2009). CD can affect the entire gastrointestinal (GI) system, from mouth to anus, but is primarily located within the iliocecal region, small intestines and some colon. Surgical interventions, removal of disease infected bowel and strictures, are not curative in CD which contrasts the success seen in UC.
Although there is no gender or age bias with CD, it can be difficult to diagnose and there are ethnic risks; persons of Jewish heritage have the highest risk for CD while African Americans have the lowest (Lakatos, 2006; Veluswamy et al., 2010). Within the United States 150 out of 100,000 people (0.15%) are affected by CD (Quaglietta et al., 2007). Most often, CD is detected in individuals between the ages of 20 and 30, but some children can experience severe symptoms and side effects related to Crohn's, such as stunted growth and delayed sexual development, which aids in early detection (Griffiths et al., 1993; Hildebrand et al., 1994; Pfefferkorn et al., 2009; Lee et al., 2010).

Crohn's disease (CD) is thought to result from an abnormal over responsiveness to normal commensal gut flora and resultant from multiple genetic risk factors (Neuman, 2007; Strober et al., 2007). Patients have episodes of severe inflammation that commonly affect the terminal ileum, but any part of the GI tract can be involved. The disease is characterized by chronic inflammation of the mucosa and submucosa of the intestine that includes the prominent development of granulomatous lesions (Shanahan, 2002; Podolsky, 2002; Welte et al., 2003; Marks and Segal, 2008). Chronic transmural inflammation of the intestines coincides with discontinuous ulcerations and stricture formations between the bowel and skin or other surrounding organs, this immunopathogenesis is unique to CD (Marks and Segal, 2008). Genetic analysis of patients with Crohn's disease and their families has identified disease susceptibility genes that are expressed in monocytes, and other
myeloid derived cells of the small intestine (Ogura et al., 2001; Cho 2007; Quaglietta et al., 2007; Xavier and Podolsky, 2007).

A genetic cause of the pathogenesis of Crohn’s disease has been suggested because of hereditary factors. A greater concordance of disease seen within monozygotic twins than in dizygotic twins (Hugot et al., 2001; Cho 2007) prompted genome-wide linkage studies within familial samples, which successfully identified several susceptibility loci (Tysk, et al., 1988; Ahmad et al., 2004; Cho 2007). One CD susceptibility gene identified was the nucleotide-binding oligomerization domain (NOD) 2. It is the general sensor of most, if not all, bacteria and is a critical component of pathogen recognition within the innate immune system (Uematsu et al., 2010). Mutations and uncommon polymorphic variants of NOD2 proteins are strongly associated with the presence of CD, with around 30% of patients carrying a loss-of function mutation in NOD2 (Hugot et al., 2001; Ogura et al., 2001). The genetic variation and mutations of the NOD2 gene are associated with the development of mucosal inflammation and increased susceptibility to microbial infections (Quaglietta et al., 2007).

NOD2 serves as an intracellular receptor for the muramyl dipeptide derived from bacterial peptidoglycan, and its stimulation leads to activation of transcription factors, and the induction of genes encoding pro-inflammatory cytokines (Girardin et al., 2003; Inohara et al., 2003; Quaglietta et al., 2007). This proinflammatory response is believed to be important for the clearance of gut bacteria whose presence would otherwise lead to sustained chronic inflammation. It has also been
demonstrated that NOD2 has a protective effect in activated macrophages because it induces the production of the anti-inflammatory cytokine IL10 and other proinflammatory mediators, such as nuclear factor-κB (NFκB) (Abbott et al., 2004; Netea et al., 2004; Quaglietta et al. 2007). NOD2 is not the only genetic variant that is associated with CD, and all susceptibility loci that have been found demonstrate a critical link between CD and innate immunity, including autophagy and phagocytosis (Xavier and Podolsky, 2007).

Another theory for Crohn’s disease is that exposure to external microorganisms are producing an inappropriate immune response. Intestinal epithelial cells function to provide a barrier against excessive exposure to microflora and mucosal antigens, which aids in downregulating the immune response (Strober et al., 2002). It is believed that the disruption critical components, like tight junctions, of the mucosal barrier can lead to increased intestinal permeability (Poritz et al., 2007). Increased intestinal permeability allowing penetration of toxic substances and macromolecules enhances the exposure to microorganisms, which contributes to the exacerbated immune.

Mouse models are important for understanding the proinflammatory and anti-inflammatory mechanisms that are interacting in IBD (Strober et al., 2007). These mouse models are critical in biomedical research because of transgenic and gene targeting technology that will allow genetic manipulation. Animal models were developed to study the in vivo affects of intestinal inflammation; the goal was
to analyze and assess the pathogenesis of mucosal inflammation and its correlation with human IBD (Strober et al., 2002).

Dextran sulfate sodium (DSS) causes a disruption of the epithelial layers of the mucosa within the intestine which alters mucosal barrier function and leads to colitis (Okayasu et al., 1990; Cooper et al., 1993; Kullmann et al., 2001; Poritz et al., 2007). Any change in the barrier function leads to the activation of mucosal phagocytes, by microfloral substances, which then stimulates the release of proinflammatory cytokines and subsequent inflammation (Strober et al., 2002).

Trinitrobenzene sulfonic acid (TNBS) is a haptenating agent that elicits a T-helper (Th)-1 cell mediated response (Neurath et al., 1995; Strober et al., 2002). This model was important to determine the initial events in the development of mucosal inflammation (Yamada et al., 1992). It has also allowed for the analysis of specific antigen and cellular immune response relationships. TBNS colitis has been shown to be closely associated with tumor necrosis factor (TNF)α, which has lead researchers to believe that TNFα may be necessary for the initiation and persistence of the Th1 response in this model of colitis.

Physical agent-induced colitis is important to prove the critical role of mucosal microflora in mucosal inflammation development and the associated epithelial barrier function. Permeability to macromolecules may be the initial step before macrophage activation. Never-the-less macrophages are influenced by microorganisms to secrete immune mediators that can lead to inflammation. Newer models where developed to aid in understanding the proinflammatory and anti-
inflammatory mechanism and to develop efficacious treatment options in humans (Strober et al., 2002).

Transgenic mice and knock outs are newer models used to study intestinal inflammation. These models fail to transcribe an important cytokine, receptor or protein that will cause systemic modifications that can be examined and compared to a wild type control (Strober et al., 2002). Models with targeted genetic deletions have been examined to understand the influence of the genetic defect.

These models include mice with NOD2/CARD15 (caspase recruitment domain family, member 15) mutations (Maeda et al., 2005; Wehkamp et al., 2005) that were created to mimic the genetic defect identified in human CD. Bone marrow derived macrophages were isolated and examined after stimulation of NOD ligand muramyl dipeptide (MDP). These macrophages produced elevated levels of IL-1β and had an enhanced sensitivity to MDP, relative to the wild type control. Meada and colleagues suggest that the macrophages of human patients with NOD2 mutations have an increased responsiveness to bacteria which results in high levels of secreted proinflammatory cytokines (Maeda et al., 2005).

Severe combined immunodeficiency (SCID)-Transfer model initially demonstrated that the inflammation was primarily caused by a Th1-mediated T cell response (Powrie et al., 1994; Leach et al., 1996). SCID models are also useful to study the regulatory cells in mucosal inflammation (Strober et al., 2002). Other models of mucosal inflammation include: interleukin (IL)-10 deficiency colitis (Kuhn et al., 1993), transforming growth factor (TGF)-β deficient mice (Shull et al.,
1992), C₃H/HeJ Bir mice (Cong et al., 1998). These data provide more evidence from clinical and experimental studies that reveal the abnormalities of the innate immune system existing during CD pathogenesis.

1.2. Abnormal Immunity in Crohn’s Disease

Mucosal immune responses must maintain a sensitive balance between immune tolerance, preventing inflammatory reactions to a myriad of innocuous microorganisms, and the innate immune responses at the mucosal surfaces. It is believed that Crohn’s disease is a multifactorial disease, produced by a combination of genetic susceptibility, exacerbated responses to the external microorganisms and a subsequent inappropriate inflammatory response. When normal immune mechanisms are inappropriately directed against harmless antigens or genetically mutated to produce inappropriate inflammatory responses there is a resultant dysregulation of adaptive and innate immune responses.

Adaptive immunity within CD is mediated by the activation of T cells. T cells act as both regulatory and effector cells in mucosal inflammation (Strober et al., 2002). T cell-mediated responses require the induced synthesis of effector molecules, including proinflammatory and anti-inflammatory cytokines, which are produced by innate immune cells. Dysregulated T cell-mediated responses are characteristic of CD and mucosal inflammation (Mudter, et al., 2005).

CD4+ effector T cells act mainly through the production of cytokines and are divided into T-helper (Th) cell subsets that release different but overlapping
cytokine profiles. Th1 cells secrete TNFα, IL-6 and interferon (IFN)-γ, which is the main macrophage-activating cytokine (Paul and Seder, 1994; Rugtveit et al., 1995). Th17 cells secrete IL-17, IL-6, TNFα and some chemokines, all of which act to recruit acute inflammatory cells, neutrophils, to sites of infection early in the adaptive immune response (Luger et al., 2008). Th2 cells secrete IL-4, IL-5, and have a CD40 ligand on their surface, all of which activate B cells (Paul and Seder, 1994). In addition to these proinflammatory cytokines Th-cell subsets also secrete IL-10 which inhibits macrophage activation (Burrows et al., 2001). During the earliest stages of activation CD4+ T cells produce IL-2, which is important for proliferation and only very small amounts of IL-4 and IFN-γ (Paliard et al., 1988).

Systemic Th1-mediated responses can clear persistent pathogens and produce inflammatory responses that can damage the surrounding mucosa. Therefore they are triggered as secondary responders by the surveying innate immunity. TNF-α may assist in killing infected macrophages but can also cause intestinal damage. A chronic disease, like Crohn's disease, is marked with an overproduction of proinflammatory cytokines, including TNFα, by Th1 and Th17-cells (Mudter et al., 2005). Therefore some treatment methods use anti-TNFα for CD therapy.

Regulatory mechanisms, from macrophages, within the gut restrict immune responsiveness from aggressive T cells; if these regulatory mechanisms, i.e. IL-10 signaling, were compromised strong inflammatory T-cell responses can commence. In CD, this dysregulated response leads to a Th1-cell mediated response,
overproduction of IFN-γ and TNF-α, which is driven by secreted IL-12 or IL-23 (Fuss
et al., 2006; Ooi et al., 2009). In current theories of pathogenesis, the Th1 mediated
proinflammatory response is the most accepted model of Crohn’s disease.

Innate immunity induces the first line of defense, both effector and
regulatory cells, due to the presence of macrophages and other antigen presenting
cells (APCs) within intestinal tissues. Since the immune system is highly reactive it
must be tightly controlled through a balance between responsiveness (protective
immunity) and non-responsiveness (homeostasis) called immune tolerance. Immune tolerance is mediated by innate immune cells. A genetic failure to regulate
inflammation and an associated failure to control mucosal immunity may give rise
to CD (Strober et al., 2002).

The integrity of the intestinal immunity depends on the functional activity of
macrophages and their ability to orchestrate an appropriate immune response. They are the initiators of mucosal immunity and are critical factors in the regulation
of inflammation. Local intestinal macrophages may be responsible for mucosal
homeostasis. Activated macrophages primarily function as effector cells, with some
regulatory activity. Takeda and colleagues have demonstrated that macrophages
from mice with myeloid cell-specific signal transducer and activator of transcription
(STAT)3 deficiency are highly activated and induce excessive IL-12, TNFα, IL-6 and
IL-1β but cannot produce important regulatory cytokines like IL-10 (Takeda et al.,
1999). Additionally, STAT3 has been implicated in the development of Crohn’s
disease-like pathogenesis due to defects within the innate immunity, specifically
myeloid derived cells (Welte et al., 2003). Thus, it has been proposed that defects in innate immunity and in the regulation of inflammation act synergistically to promote the pathology of CD.

1.3. STAT Signaling Plays a Role in Crohn’s Disease

Signal transducers and activators of transcription (STAT) proteins respond to cytokine signaling, phosphorylate, dimerize and translocate into the nucleus from their latent cytoplasmic location to induce gene transcription of additional cytokines and chemokines (Darnell, 1997; Mudter and Neurath 2003). To date, there are seven characterized STAT proteins in mammalian cells: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6. This seven member protein family was discovered for their involvement in specific signaling from IFN receptors (Dale et al., 1989; Fu et al., 1992; Darnell et al., 1994), recent studies with STAT knockout mice have elucidated the functional role of these proteins in disease (Akira, 1999).

STAT activity depends on the interaction of the Src Homology (SH)2 domain with a phosphotyrosine residue (Fu et al., 1992; Stahl et al., 1995). There is a high degree of specificity when it comes to SH2-phosphopeptide binding and STAT dimerization, therefore when STAT1 and STAT3 form a heterodimer they also create a unique DNA-binding complex (Kisseleva et al., 2002). Cytokines, growth factors, and other signaling molecules are responsible for activating STAT proteins (Schlessinger and Ullrich, 1992; Larner et al., 1993; Darnell, 1997).
Once activated STATs translocate into the nucleus to transcribe new genes therefore contributing to the production of the proinflammatory and anti-inflammatory molecules within an immune response. (Darnell, 1997; Akira, 1999; Levy and Darnell 2002) Thus, if STAT activation is lost the resultant phenotype is due to the inability of the immune system to function effectively (Leonard and O'Shea, 1998). STAT activation is a critical component in the signal transduction cascade for cytokine signaling, which is also a very complex and interactive entity within the immune system (Darnell, 1997; Akira 1999; O’Shea et al., 2002; Levy and Darnell et al., 2002). Therefore it is extremely important to understand the effects of a specific loss of STAT activation and the pathogenesis that confers.

Cytokines generally function in the same way: (1) Cytokine binds to its cell surface receptor, (2) Receptor dimerization and activation of janus kinase (JAK) tyrosine, (3) phosphorylation of tyrosine residues creating docking sites for STATs, (4) JAKs phosphorylate STATs, which then dimerize and translocate into the nucleus to activate gene transcription (Darnell, 1997; Leonard and O’Shea, 1998; Akira 1999; O’Shea et al., 2002; Levy and Darnell et al., 2002). Gene transcription will include the generation, production and secretion or expression of new cytokines and chemokines that will affect the immune system either in a proinflammatory, anti-inflammatory or an immunoregulatory way (Darnell, 1997; Akira, 1999; Levy and Darnell 2002).

The hematopoietin and interferon receptors all signal through the JAK-STAT pathway and activate different combinations of STATs with different effects (Levy et
al., 1988; Dale et al., 1989; Fu et al., 1992; Darnell et al., 1994; Fu, 2006). Therefore
STATs also play a critical role in hematopoiesis and the development of the immune
system (Mudter and Neurath, 2003). Of these functions by STAT signaling, regulating cytokine signaling pathways and generating functional immunological responding cells might play a critical role in the pathogenesis of inflammatory bowel disease (Takeda et al., 1999; Akira 2000; Welte et al., 2003).

STAT3 activation is important for the regulation of intestinal homeostasis. At
the mucosal surface of the gut STAT3 activation restores the balance between CD4+
effector T cell responses and immune tolerance, preventing inflammatory reactions
to a multitude of antigens. It has been determined that the intestines of STAT3
deficient mice were transmurally inflamed and thickened by the cellular infiltration
of myeloid lineage cells (Welte et al., 2003). STAT3 activates inflammatory T cells in
the presence of IL-6 (Atreya et al., 2000). Intestinal T cells in CD present with an
increase in STAT3 activation, which was attributed to an induction of antiapoptotic
signals allowing the accumulation of activated T cells (Atrya et al., 2000; Lovato et
al., 2003)

STAT3 also plays a pivotal role in mediating anti-inflammatory cytokines,
such as IL-10 and TGF-β which can explain the extreme intestinal inflammation
observed in STAT3 deficient mice. STAT1, on the other hand, promotes
inflammation and contributes to the development of adaptive immune T cells. It is
believed that STAT proteins evolved to aid in developing the immune system and to
mediate intestinal cytokine signaling (Mudter et al., 2005). Furthermore, it has been
suggested that an imbalance of these two proteins lead to the formation of CD, expressing overproliferative macrophage infiltrates and excessive Th1-cell mediated inflammation. Although it has been shown that these proteins are important, it is unknown if the resultant disease pathogenesis occurs through several independent signaling pathways, both myelopoietic and lymphopoietic, or if the loss of one signaling pathway, IL-10/STAT3 in macrophages, is the cause of severe downstream consequences.

Animals with STAT3 deficiency in hematopoietic stem cells have been generated in this lab (Welte et al. 2003). The pathology of this bone marrow specific STAT3-deficient (Btie2Cre*STAT3FF) mouse model, similar to human CD patients, is as follows: (1) transmural developmental variations in the small intestine (especially the ileum) and cecum, (2) increased inflammatory changes (transmural inflammation), (3) intestinal and peritoneal fusions, (4) loss of mucosal texture (mucosal erosion), (5) discontinuous ulcerations, (6) myeloid lineage infiltrations (including neutrophils and macrophages), (7) granuloma-like structures, (8) increase bowel wall thickness, (9) crypt abscesses, filled with necrotic myeloid cells, (10) aggregated epitheloid cells, (11) edema in the submucosa, and (12) serosal thickening.

STAT1 is essential for IFNγ signaling (Durbin et al., 1996; Meraz et al., 1996; Leonard and O'Shea, 1998). An important contribution that IFNγ/STAT1 signaling makes to mucosal immunity is the ability to activate T-bet, a transcription factor, in mucosal T cells, Neurath et al., in 2002 demonstrated that elevated expression of T-bet is enough to enhance Th-1 cell-mediated colitis (Neurath et al. 2002). This
supports the theory that STAT1 is important in developing and perpetuating the Th1 response upon activation by IFN (Meraz et al., 1996; Ramana et al., 2002).

One phenomenon that has been observed within this lab is the recovering affects that STAT1 deletion has on STAT3 deficiency (Zhang, unpublished data). Through the generation of an additional STAT1 deletion in the STAT3-deficient mouse it has been shown that there are no gross morphological disorders, as well as no CD-like pathology which suggests that an imbalance of STAT1/STAT3 signaling is detrimental to the gut. An imbalance in STAT1 and STAT3 can lead to disproportionate production of IL-12 and IL-10 from macrophages, thus contributing to the immunopathogenesis seen in colitis models (Kamada et al., 2005).

Hyperproduction of IL-12 and IL-23, as seen within CD, is a result of a disrupted IL-10/STAT3 pathway within the macrophage. IL-10 inhibits the expression of costimulatory molecules (B7-1/B7-2) and MHC class II proteins on the cell surface of monocytes and macrophages which are necessary for T cell recognition and activation. Additionally, IL-10 limits the monocytic production of proinflammatory cytokines and chemokines, prevents dendritic cells from trafficking to the lymph node, as antigen presenting cells (APCs), and thus prevents Th1-cell differentiation and recruitment. Therefore IL-10 indirectly regulates the function of T helper cells and can directly regulate the Th1 and Th2 responses through by T cell activation (Couper et al., 2007).
1.4. Hypothesis and Specific Goals of This Study

My working hypothesis is that defective innate immunity in Crohn’s disease may correlate with a loss in STAT3 activity. My specific hypothesis is that STAT3-deficiency in bone marrow hematopoietic stem cells results in dysfunctional macrophages and correlates with Crohn’s Disease-like pathogenesis.

Preliminary studies show that bone marrow specific STAT3 deficiency can lead to a spontaneous mouse model with Crohn’s disease like pathogenesis. The loss of STAT3 allows for an unbalanced STAT1 activation, and this STAT1 elevation allows for IFNγ signaling to function, unopposed. The built in regulatory pathway (IL-10-STAT3) has been interrupted. The depletion of STAT1 from germline DNA leaves STAT3 active within the system and based on preliminary studies does not contribute to an inflammatory bowel disease, although these mice are more susceptible to any other form of infection, including norovirus. Most interestingly, when STAT1 and STAT3 are deleted concurrently, the preliminary studies demonstrate a recovery from the diseased phenotype. There is no significant inflammation within the intestines and the overall gross morphology has also recovered.

The defects in the immune responses that contribute to the altered cytokine productions and Crohn's-like phenotype is due to a defect in the innate immunity, specifically within macrophage function and ability regulate the adaptive immunity. Therefore, macrophages are the initiators of disease and due to dysregulated
function and development resulting in an inability to maintain the integrity of intestinal immunity. The following experiments were designed to test the hypothesis.

**Specific Aim 1, Determine the effects of wild type, STAT3-, and STAT1-deficiency on monocyte distribution.** The aim of this experiment was to identify which specific population of monocyte-derived cells is associated with CD-like phenotypes in the wild type, STAT3-, and STAT1-deficient mouse. Peripheral blood smears and bone marrow smears were made to examine the erythroid:myeloid ratio, and to examine the differentiation ability of monocytic precursors. Fluorescence activated cell sorting (FACS) was implemented to analyze the distribution of myeloid-derived cells within the spleen, thymus, mesenteric lymph node and bone marrow.

**Specific Aim 2, Determine the effects of wild type, STAT3-, and STAT1-deficiency on monocyte function.** The aim of this experiment was to examine the functional macrophage changes that occur in the wild type, STAT3-, and STAT1-deficient mouse. Resident peritoneal macrophages were isolated, purified and analyzed using cell culture techniques and functional assays. Fc-OxyBURST® analysis, non-specific fluid phase endocytosis, and reduction assays were performed to quantify the phagocytic capability of the macrophages. Fluorescence activated cell sorting (FACS) was implemented to analyze the purity of isolated cells, the analysis of specific and non-specific phagocytosis.
Specific Aim 3, Determine the consequences of wild type, STAT3-, and STAT1-deficiency on cytokine production and signaling. This experiment was to examine the expression levels of the secreted cytokines and determine the cytokine profile in the wild type, STAT3-, and STAT1-deficient mouse. Samples were taken of serum and cell culture supernatant. The cytokine levels were calculated using the Bioplex® system with a multiplex array (Bio-Rad).
CHAPTER TWO

GENERATION AND CHARACTERIZATION OF GENE-TARGETED MICE IN BALB/C BACKGROUND
2.1. Introduction

A germline deletion of STAT3 is embryonically lethal demonstrating that STAT3 is important during early embryogenesis; STAT3 activation has been detected during development and postimplantation (Duncan et al., 1997; Takeda et al., 1997; Akira, 1999). Therefore to assess the functional role of STAT3 in adult tissues conditional gene knockouts using Cre-loxP recombination technology has to be used (Akira, 2000; Takeda and Akira, 2001; Welte et al., 2003).

First found in P1 bacteriophages, Cre-lox recombination has been a useful mechanism for gene manipulation (Sternberg and Hamilton, 1981; Sauer and Henderson, 1988; Nagy, 2000). Generation of STAT3 Cre-loxP mice with a targeted deletion in the hematopoietic stem cells was used as described previously (Welte et al., 2003). Briefly, exons 18-20 encode the Src homology 2 (SH2) domain of STAT3 and are responsible for the protein's function (Fu et al., 1992; Shuai et al., 1994; Akira, 1999). First, floxed-STAT3 mice were generated by flanking the SH2 domain with two loxP (locus of x over P1) sites inserted in introns 17 and 20 (F allele). These recognition signals target the critical tyrosine residues that are important for STAT3 function. Second, transgenic technology must be employed to introduce the Cre gene to the mouse genome; in this case the Cre expression was controlled by a tissue-specific tyrosine kinase immunoglobulin-like and EGF (epidermal growth factor)-like domain (Tie)2 gene promoter (Welte et al., 2003). Deletion through DNA recombination is then catalyzed by the Cre (cyclization recombination) protein (C allele).
Hematopoietic stem cells and endothelial progenitor cells are targeted by the Tie2 promoter gene because they share the same antigenic determinants (Koni et al., 2001). These progenitor cells are both derived from a common multipotent cell precursor called a hemangioblast. Hemangioblasts are derived from extraembryonic mesoderm and then differentiate to form the blood islands in the yolk sac of early embryos; this is the definition of hematopoiesis (Choi et al., 1998; Huber et al., 2004). Under the control of a Tie2 promoter the Cre expression is directed to affect all circulating cells, with hematopoietic precursors, and endothelial cells that contribute to the vessels themselves (Schlaeger et al., 1997).

Generation of a homozygous STAT3-deficient mouse was a result of breeding a bone marrow (B)-Tie2 promoter driven Cre recombinase gene targeted mouse with a STAT3 lox-P (F/F) mouse. This created a conditional (specific to hematopoietic stem cells) STAT3 knock-out in a C57BL/6/129 background. The animal model contains a homozygous allelic deletion of the SH2 domain (STAT3-\text{loxP}^{F/F}) and a heterozygous C allele (Cre^{+/\cdot} gene). This mouse is good for in vivo studies because it exhibits a spontaneous Crohn’s-like pathogenesis (Welte et al., 2003).

The pathology of the bone marrow specific STAT3-deficient (Btie2Cre^{\star}\text{STAT3}^{FF}) mouse model, similar to human CD patients, is as follows: (1) transmural developmental variations in the small intestine (especially the ileum) and cecum, (2) increased inflammatory changes (transmural inflammation), (3) intestinal and peritoneal fusions, (4) loss of mucosal texture (mucosal erosion), (5)
discontinuous ulcerations, (6) myeloid lineage infiltrations (including neutrophils and macrophages), (7) granuloma-like structures, (8) increase bowel wall thickness, (9) crypt abscesses, filled with necrotic myeloid cells, (10) aggregated epitheloid cells, (11) edema in the submucosa, and (12) serosal thickening. Btie2Cre*STAT3FF mice rarely survive past 8 weeks; most are dead within 4-6 postnatal weeks (Welte et al., 2003).

For these experiments all efforts were put forth to optimize the breeding and expansion of these animals in a pure C57BL/6 background. However, nothing seemed to work in reproducing enough amounts of homozygous animals described previously with a C57BL/6/129 mixed background (Welte et al., 2003). STAT1 deficient animal (Durbin et al., 1996) in C57BL/6 background showed a similar situation with very low productive rates. Therefore it was important to transfer the transgenic mutations to a new genetic background. BALB/c was chosen because the genetic background is more sensitive to disease than C57BL/6 mice (Chiodini and Buergelt, 1993; Pal-Ghosh et al., 2008). BALB/c strains were generated by backcrossing the C57BL/6 strains with pure BALB/c mice for at least six generations to produce a significantly pure background.

2.2. Experimental Procedures

2.2.1. Animals

BALB/c mice, 6 to 8 weeks old, were purchased from the Jackson Labs for initial backcrossing. All animals were housed in pathogen-free conditions in the
barrier wing in the Animal Research Facilities operated by Division of Animal Care of The Pennsylvania State University, College of Medicine, Hershey, PA. The veterinary staff routinely monitors the health of the animals housed in the facilities. Veterinary services were available 24 hours a day, in case of emergency. The transgenics are housed in a 12hr lights on and 12hr lights off facility. All experimental procedures were established in accordance with the institutional animal care and use committee (IUCAC) guidelines and approved protocols from the Animal Care Committees.

2.2.2. Scheme to Transfer Gene-Targeted Animals to BALB/c Background

To generate BALB/c bone marrow specific STAT3-deficient mice the C57BL/6 mice were back-crossed to the pure BALB/c background for at least six generations. The probability of C57BL/6 mice was 0.016%. To maintain the integrity of the inbred strain, sibling mating and parent-offspring mating schemes were employed. Care was taken to avoid creating subtypes during breeding schemes and transfer of mutations (Jackson Laboratory Resource Manual). Parent-offspring mating was also important to prevent genetic drift.

During the transfer, several generations were maintained on the C57BL/6 background, the BALB/c:C57BL/6 mixed background and the BALB/c background. Once the phenotype, and corresponding genotype, was confirmed the mixed backgrounds were euthanized. The same strategies were employed to generate BALB/c STAT1-deficient mice.
2.2.3. Breeding and Expansion

Homozygous C57BL/6 mice with two floxed STAT3-SH2 domains and heterozygous Tie2Cre+ mice were backcrossed to the BALB/c background. To generate and expand the Btie2Cre+STAT3FF mice homozygous BALB/c STAT3FF mice were bred with BALB/cTie2Cre+ mice. At weaning age the mice were phenotypically indistinguishable so all offspring were genotyped. Wild-type controls were considered to be without Cre expression, they were either homozygous floxed STAT3FF mice or heterozygous floxed STAT3F+ mice. Using wild-type littermates as controls standardizes the weaning and milking process and accounts for a more accurate comparison.

STAT1-deficient (STAT1−/−) mice were also used as a control in this study. STAT1 was functionally inactivated by the deletion of a protein coding region that correlated with a loss of nearly four complete exons that encoded the DNA-binding domain in the genomic DNA (Durbin et al., 1996). STAT1−/− were first generated from heterozygous mutant breeding as an inbred strain expanded from sibling matings and from parent-offspring matings. A breeding scheme was created to allow homozygous mutant breeding once viable and fertile homozygotes were generated. Following the breeding strategies suggested by the Jackson Laboratory Resource Manual, the integrity of the homozygous mutant breeding was maintained by verifying the genotype of the breeders every second generation.
2.2.4. Genotyping

The “cre--loxP” breeding scheme produces a litter of wild-type, heterozygous and homozygous mutants that are phenotypically indistinguishable at weaning age. The genotype was determined by genomic polymerase chain reaction (PCR), which has been described previously (Welte et al., 2003). STAT1 primers were designed and made in this lab, based off data described previously (Durbin et al., 1996; Zhang, unpublished data). DNA samples were collected by ear clips in mice >10 days old and then digested in a lysis buffer with fresh proteinaseK to isolate the DNA (Pearson and Stirling, 2003). ProteinaseK digests native proteins and inactivates endogenous RNases and DNases. Samples were incubated for a minimum of 3 hours at ~60°C, and then boiled for 5 minutes to deactivate the proteinaseK (Roche). Once the DNA has been isolated the PCR cocktail can be mixed (Table 1).

Primers were designed at a length of 25-30 bases, with 40-50% G+C, to flank the region of interest (Table 2). Care was taken to ensure that the 3’ ends weren’t complementary to avoid the possible formation of primer-dimers. A general PCR program (Table 3) was used on a MyCycler™ Thermal Cycler (BioRad). To optimize the PCR protocol the melting temperatures (T_m) and annealing temperatures (T_a) of the primers were calculated and matched to be as close as possible. Gradient PCRs were also setup and performed to determine the best annealing temperature for the primer pairs. Simplified calculations were as followed:

\[ T_m = 2[A+T] + 4[G+C] \]

\[ T_a = T_m - 5^\circ \]
**Table 1: GoTaq® - Green Master Mix 2x (PROMEGA)**

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<th>Component</th>
<th>Volume</th>
<th>Final Conc.</th>
</tr>
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<td>1X</td>
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<tr>
<td>PROMEGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward Primer</td>
<td>0.25-2.5ul</td>
<td>0.1-1.0uM</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>0.25-2.5ul</td>
<td>0.1-1.0uM</td>
</tr>
<tr>
<td>DNA</td>
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<td>&lt;250ng</td>
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**Table 2: Sequences of Primers for Genomic PCR**

<table>
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<th>Gene</th>
<th>Forward Primer sequence 5'-3'</th>
<th>Reverse Primer sequence 5'-3'</th>
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<tr>
<td>STAT1</td>
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<td>CTGATCCAGGCAGGCCTTGG</td>
</tr>
<tr>
<td>Cre</td>
<td>CGATGCAACGATATGGAG</td>
<td>CGCATACAGTCGAACACGC</td>
</tr>
<tr>
<td>STAT3*</td>
<td>ATTTGGAACCTGGGACCAAGTGG</td>
<td>ACATGTTACTTACAGGGTGTGTG</td>
</tr>
<tr>
<td>*(l-17F, E-17B)</td>
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<td></td>
</tr>
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**Table 3: PCR Program for Genotyping**

<table>
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<th>Time</th>
<th>Repeats</th>
<th>Descriptions</th>
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<td>3mins</td>
<td>initial denature</td>
</tr>
<tr>
<td>Cycle 2</td>
<td>95°C</td>
<td>30sec</td>
<td>Denature</td>
</tr>
<tr>
<td></td>
<td>58°C</td>
<td>1min</td>
<td>Anneal</td>
</tr>
<tr>
<td></td>
<td>72°C</td>
<td>1min</td>
<td>(Repeat x40)</td>
</tr>
<tr>
<td>Cycle 3</td>
<td>72°C</td>
<td>4min</td>
<td>final extension</td>
</tr>
<tr>
<td>Cycle 4</td>
<td>4°C</td>
<td>∞</td>
<td>refrigeration (hold)</td>
</tr>
</tbody>
</table>
Figure 1: Genomic PCR: Generation of bone marrow specific STAT3 deletion. Verification by genomic PCR (A) PCR product is approximately 510bp for loxP sites (F allele), (B) deleted allele structure (C) PCR product is approximately 300bp for Cre (C allele). (D) Genotyping of STAT3-F allele, lanes 2, 4 and 6. (E) Genotyping Tie2-Cre, lanes 3, 5, and 7. (Welte et al., 2003)

Figure 2: Endpoints and Collection: Animals were weaned and genotyped at three weeks, 21-28 days old. Time points for sample collection was between 4-5 weeks (28-35 post-natal days) and between 7-8 weeks (42-49 post-natal days)
To see the PCR products, 1-2% agarose solutions were mixed with ethidium bromide and molded into gels with 8-20 wells. The DNA was added and run at 90-120mV depending on the gel size, for approximately 60mins. Product size was determined by a 100bp DNA ladder (Figure 1). Pictures were taken under UV light with BIORAD® Chemidoc system.

2.2.5. Survival and Morbidity

Mice were observed for any clinical signs of distress. Mice with reduced body mass, hunched posture and hypoactivity were supplied with wet food on the bottom of the cage to promote food intake and to prevent malnutrition. The mice were collected, as shown in Figure 2, between 4 to 5 weeks (max post natal day was 35, PN35) and also between 7 to 8 weeks (max post natal day was 49, PN49). Morbidity and mortality was recorded for a survival study and those with high morbidity rankings were closely monitored for further signs of distress and euthanized prior to endpoints if necessary.

2.3. Results

2.3.1. Generation of STAT1- and STAT3-Deficient Mice in BALB/c Background

In this study BALB/c mice with STAT3-deficiency were generated from breeding Tie2Cre+ mice with homozygous STAT3FF mice. The transgenic C57BL/6 lines were backcrossed to the BALB/c background and after six generations were
Figure 3: Transfer of the Mutations to BALB/c Backgrounds. To generate Btie2Cre−STAT3FF mice, the mutant strains had to be backcrossed to BALB/c for six generations. A breeding scheme was designed using sibling and parentoffspring matings to ensure that the purity of the BALB/c background was maintained. Litters were generated at the expected Mendelian ratios.
considered pure, with a 1/64 (0.016%) chance of C57BL/6 interference. Litters appeared normal at birth and were generated at the expected Mendelian ratio.

Backcrossing C57BL/6 STAT1\(^{-/-}\) mice to the BALB/c inbred strain used a parent-offspring and sibling mating schemes for six generations (Figure 3). Heterozygous mating produced a normal frequency of STAT1\(^{+/}\), STAT1\(^{-/-}\) and STAT1\(^{+/+}\). Once mutants were generated homozygous breeding was used.

### 2.3.2. Increase of Breeding Rates in BALB/c Background

General comparison of the two strains is shown in Table 4. Briefly, both strains are inbred, and are weaned at approximately 4 weeks. C57BL/6 mice are black, docile and generate smaller litters, when compared to BALB/c. BALB/c mice are white, generate larger litters and males are more aggressive. Originally C57BL/6 STAT1\(^{-/-}\) pups had a neonatal survival rate of 0%, until fostering was implemented. Briefly, the STAT1\(^{-/-}\) C57BL/6 pups were removed from their birth cage and introduced to nesting and bedding materials of the foster moms, so that they could carry her scent (Jackson Laboratory Resource Manual). Foster mothers were STAT1\(^{-/-}\) BALB/c to allow differentiation of the fosterlings by color. C57BL/6 STAT1\(^{-/-}\) mice had short reproductive cycles and produced sporadic small litters (≤5 pups) it was rare for moms to nest pups. Meanwhile, BALB/c STAT1\(^{-/-}\) mice had larger litters (≥9 pups), were very fertile with long reproductive life spans and pups met sexual maturity much younger than C57BL/6 (Table 5).
The Cre-loxP strains were more stable on the BALB/c background; reproductive cycles, fertility and breeding rates were all significantly increased. BALB/c litters with Cre expression were much larger (≥9 pups) while C57BL/6 litters would yield ≤5 pups (Table 6).

Table 4: Comparison of Genetic Strains

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>BALB/cJ</th>
<th>C57BL/6J</th>
</tr>
</thead>
<tbody>
<tr>
<td>Color</td>
<td>inbred</td>
<td>inbred</td>
</tr>
<tr>
<td>Weaning Age (wks)</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Mean litter size</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Wean:Born ratio</td>
<td>1</td>
<td>0.8</td>
</tr>
<tr>
<td>temper</td>
<td>Aggressive</td>
<td>Docile</td>
</tr>
</tbody>
</table>

Table 5: Strain Comparison of STAT1-Deficient Mice

<table>
<thead>
<tr>
<th>STAT1^{−/−}</th>
<th>C57BL/6</th>
<th>BALB/c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breeding</td>
<td>Normal Frequencies; Failure to thrive</td>
<td>Normal Frequencies</td>
</tr>
<tr>
<td>Litter Size</td>
<td>≤5</td>
<td>≥9</td>
</tr>
</tbody>
</table>

Table 6: Strain Comparison of STAT3-Deficient Mice

<table>
<thead>
<tr>
<th>Btie2Cre^{−/−}STAT3FF</th>
<th>C57BL/6</th>
<th>Balb/c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breeding</td>
<td>Longer than Normal Frequencies</td>
<td>Normal Frequencies</td>
</tr>
<tr>
<td>Litter Size</td>
<td>≤4</td>
<td>≥9</td>
</tr>
<tr>
<td>Morbidity</td>
<td>Appears ~3wks</td>
<td>Appears ~7.5wks</td>
</tr>
<tr>
<td>Survival</td>
<td>4-5wks</td>
<td>8-9wks</td>
</tr>
<tr>
<td>Homozygous Ratio</td>
<td>↓ 5%</td>
<td>~20-30%</td>
</tr>
</tbody>
</table>
Figure 4: Gross Morphology of Btie2Cre•STAT3FF Mouse: Btie2Cre•STAT3FF mice (A) were phenotypically distinguishable from their wild-type littermates (B) at eight weeks.
Figure 5: Survival and Morbidity Curve: C57BL/6 and BALB/c mice were observed for signs of clinical distress and survival. C57BL/6 mice (yellow) appeared malnourished and didn’t survive past 5 weeks. BABL/c mice (green) presented with disease >5 weeks and didn’t survive past 9 weeks.
2.3.3. Increase of Life Span in BALB/c STAT3-deficient Animals

The C57BL/6 Btie2Cre*STAT3FF mice were smaller than their littermates, appeared hunched and hypoactive but mice never survived past five weeks. The diseased phenotype was significantly distinguishable in BALB/c Btie2Cre*STAT3FF mice in comparison to their wild type littermate controls at 7 to 8 weeks of age (Figure 4 and 5). These mice appeared healthy at weaning age, but within four weeks lost body mass, became lethargic and hypoactive, had hunched postures, greasy and stained fur, appeared pale in limbs, tail and snout and they isolated themselves from their littermates.

2.4. Discussion

Bone marrow specific STAT3-deletion leads to a spontaneous Crohn’s disease-like pathogenesis in C57BL/6 mice; these mice are an important tool to analyze the mechanisms of Crohn’s disease because of the close pathological resemblance (Welte et al., 2003). In these studies most of the C57BL/6 STAT3-deficient mice died within four weeks. To characterize the functional loss and subsequent diseased phenotype in bone marrow specific STAT3-deficient mice, it was important to prolong the survival.

In this study mice with STAT3-deficiency were generated from breeding Tie2Cre+ mice with homozygous STAT3FF mice. These strains were backcrossed to BALB/c for at least six generations and the progeny genotype was determined by genomic PCR. These mice had shown signs of clinical distress; they were lethargic, listless and hypoactive, with hunched posture, porphoryn staining of the eyes and
greasy fur, their limbs, tail and snout were pale and they isolated themselves. This phenotype was unique to the BALB/c strains and wasn't seen in the C57BL/6 mice.

The different phenotypic presentation supports a genetic component of CD. The pathogenesis of Crohn’s-like disease in the C57BL/6 mouse as previously described was generated on a mixed 129:C57BL/6 background (Welte et al., 2003). This could possibly account for the phenotypic differences that conferred on each genetic background in this study. Interestingly, differences in intestinal inflammation have also been noted in chemical-induced mucosal inflammation studies (Strober et al., 2002). Unlike the five week survival of the C57BL/6 model, the bone marrow specific STAT3-deficient BALB/c mice survived to eight weeks, but rarely survived past nine.

Along with the increased survival rates, BALB/c STAT3-deficient mice were a better model because breeding rates, fertility and reproductive life spans were also significantly increased. During the course of this study only five homozygous STAT3-deficient C57BL/6 mice were generated. The litters were also smaller and infrequent on the C57BL/6 background. Therefore, BALB/c mice produced a better model to study the functional effects of STAT3-deficiency in hematopoietic stem cells.

This phenomenon was also demonstrated with STAT1-deficiency. There were no breeding problems or interferences with neonatal survival on the BALB/c background. Meanwhile, C57BL/6 STAT1(-/-) females were poor mothers; they would not nest their pups. Therefore litter fostering was necessary to maintain the
C57BL/6 STAT1\(^{-/-}\) mouse line. Foster mothers were normally BALB/c STAT1\(^{-/-}\) mice to allow for easy division of the fosterlings.

Homozygous sibling mating for BALB/c STAT1\(^{-/-}\) expansion produced the expected frequencies. They were comparable in size to wild-type pups, if not bigger and had no apparent dysfunction. Behavior and activity levels were normal, along with food and water intake. Once weaned these mice appeared to reach sexual maturity quickly, they were very fertile and were able to maintain a long reproductive life. The males did appear to be more aggressive and often had to be separated, and housed in single cages, which was unique to the STAT1\(^{-/-}\) strains.

It has been demonstrated that STAT1\(^{-/-}\) mice are prone to infection (Durbin et al., 1996; Meraz et al., 1996). Most C57BL/6 STAT1\(^{-/-}\) mice were prone to ulcerative dermatitis and abscesses that presented within the flank and under the shoulder blades. In the BALB/c STAT1\(^{-/-}\) mice there was seldom, if any, cases of ulcerative dermatitis and no abscesses.

Few Btie2Cre\(^+\)STAT3FF mice were collected on the C57BL/6 background in this study. There were no phenotypically significant changes yet these mice died before five weeks of age. The BALB/c Btie2Cre\(^+\)STAT3FF mice demonstrated a severe phenotype that made it possible to phenotypically distinguish between the disease homozygotes and the wild type littermate controls at 7 to 8 weeks of age.

Overall it was important to prolong the survival times and increase the survival rates of the spontaneous Crohn’s disease-like pathogenesis in a mouse model to identify the functional effects of STAT3-deficiency in hematopoietic stem
cells. The mice generated in this study demonstrate that a similar disease phenomenon occurs on the BALB/c background due to a bone marrow specific deletion of STAT3. Meanwhile, the generation and expansion of these BALB/c transgenes also provided a more stable mutant line with a significant increase in offspring.
CHAPTER THREE

ALTERATION OF MYLOPOIESIS IN STAT3-DEFICIENT ANIMALS
3.1. Introduction

STAT3 deletion is embryonically lethal, therefore to study STAT3-deficiency in adult tissues Cre-loxP recombination techniques need to be employed (Duncan et al., 1997; Takeda et al., 1997; Akira, 1999; Welte et al., 2003). In 2003, Welte et al. generated a bone marrow specific STAT3-deficient mouse model that resulted in a Crohn’s disease-like pathogenesis (Welte et al., 2003). This STAT3-deficient model demonstrated an infiltration of myeloid-derived cells in the gastrointestinal system and an expansion of myeloid derived cells in the bone marrow (Welte et al., 2003). In order to understand the changes that occur due to this STAT3-deficiency within hematopoietic stem cells it is important to characterize the general phenotype of the mouse model.

Clinically human patients with Crohn’s disease can suffer from malnutrition and poor body development (Bresson and Schmitz, 1992; Stokes, 1992; Kelly, 1999). It was important to identify if a similar phenomenon occurs within the spontaneous mouse model. Furthermore, analysis of the cellular distribution in peripheral blood and bone marrow and the myeloid cell populations were also important to characterize the diseased phenotype described in bone marrow specific STAT3-deficient mouse.

The immunological pathogenesis of CD is poorly understood; various mouse models were generated to address this problem but had concluded that CD is a Th1 cell mediated response (Shanahan, 2002; Podolsky, 2002; Strober et al., 2002; Ostanin et al., 2009). This observation prompted several studies on T cell dysfunction in CD (Parronchi et al., 1997; Kosiewicz et al., 2001; Strober et al.,
2003). However, recent studies have concluded that there must be another critical component involved in mucosal immunity that is initiating the early inflammation (Welte et al., 2003; Marks and Segal, 2008; Smith et al., 2009; Marks et al., 2010). Meanwhile, a reduction in absolute numbers of T cells has been reported to be a result of STAT3 deletion in bone marrow cells (Lee et al., 2002). Some CD patients receiving allogeneic hematopoietic stem cell transplantation (HSCT) have been cured, indicating a critical immunological component of the disease (Casanova and Abel, 2009). Therefore it is important to characterize the cellular components of these STAT3-deficient mice so that an accurate comparison of dysfunction and mucosal immunity can be investigated.

In order to characterize the mechanisms by which STAT3-deficiency initiates a CD-like pathogenesis, it is necessary to evaluate the functional role of STAT3 in cellular development and distribution. The aim of these studies was to analyze the cellular distribution in peripheral blood and bone marrow and to characterize the predominant myeloid-derived cell populations.

### 3.2. Experimental Procedures

#### 3.2.1. Animals

Bone marrow, hematopoietic stem cell, specific STAT3-deficient mice (Btie2Cre*STAT3FF) on a BALB/c background were used for this study. Littermate wild-type animals, heterozygous or homozygous with no Cre expression, were used as controls. STAT1 knockout mice (STAT1<sup>+/−</sup>) on BALB/c background were also used as a control. At each time point a minimum of 10 animals were used per group
(60 total animals). All animals were housed in pathogen-free conditions in the barrier wing in the Animal Research Facilities of The Pennsylvania State University, College of Medicine, Hershey, PA. All experimental procedures were established in accordance with the Institutional Animal Care and Use Committee (IUCAC) guidelines and approved protocols from the Animal Care Committees.

3.2.2. Genotyping

To verify the specific STAT3 or STAT1 deletion genomic PCR was implemented using primer sets that determine the presence of Cre expression (Cre primers), recognized the loxP sites (F primers) and identified the STAT1 deletion (STAT1 primers) (as described in chapter 2).

3.2.3. Antibodies

The following antibodies were used to stain the extracellular surface immune markers: CD44 – FITC Clone IM7 (eBiosciences) which is a phagocytosis receptor expressed by bone marrow myeloid cells and lymphocytes (Wittig et al., 1999; Vachon et al., 2007); CD11b (Mac1) – Cy5 Clone M1/70 (eBiosciences) which is expressed by granulocytes; F4/80 – PE clone BM8 (eBiosciences) which is expressed by macrophages and is referred to as a pan macrophage marker.
3.2.4. Physical Measurements of Animals

Animals were euthanized with CO$_2$ asphyxiation. After euthanasia mice were measured (cm) from snout to rump. Body weights (g) were recorded for each animal to calculate organ to body ratio. Animals were monitored and unique observations were recorded to identify the appearance and behavior of the transgenes.

3.2.5. Organ and Tissue Collections

Mesenteric and pancreatic tissue was removed from the stomach before it was excised between the esophageal sphincter and the pyloric sphincter. The small intestines were removed from the duodenopyloric junction to the ileocecal junction. The mesenteric fat was removed and the intestinal fecal contents were gently washed out with a phosphate buffered saline – neutral buffered formalin (PBS-NBF) mixture. The cecum was cleared of all mesenteric fat and lymph nodes and excised between the ileocecal junction and the extreme proximal end of the colon. The colon was excised from the extreme proximal end to the anal canal and the mesenteric fat and lymph nodes were removed.

The lumen of the tubed structures was washed with a PBS-NBF mixture to prevent the degeneration of the mucosal surfaces and to clear the fecal contents. The cecum and stomach were injected with a PBS-NBF mixture to prevent the degeneration of the mucosal folds and then immersed in 10% NBF.
3.2.6. Blood Collection and Smear

After euthanasia, terminal cardiac punctures were performed to collect peripheral blood samples. 27G needles were used with a 1CC syringe to collect approximately 0.7ml of blood per animal.

A drop (~1ul) of blood was placed directly on a glass slide and spread over its surface with the edge of another slide. The blood was allowed to air dry at room temperature and then fixed with methanol for 1min. The staining was performed with Wright Giemsa, a polychromatic stain (Table 7). Briefly, red blood cells stain pink, granules stain lilac to purple while nuclei stain a deep blue. Once dry the slides were cover slipped with permount. Then the smears were then examined under light microscopy with a high-power lens and oil immersion and differential counts were calculated.

3.2.7. Bone Morrow Collections and Smear

Bone marrow aspirates were collected, prepared and smeared over a glass slide for analysis. Once dry, the smear was fixed with methanol (1min) and stained with Wright Giemsa stain (drop method), wash with diH2O and coverslipped. These samples were scored for myeloid hyperplasia (≤4). Cellular progenitors, distribution of cells and the erythroid to myeloid ratio were also analyzed. All scoring and additional analysis was done in a blind fashion by a DVM in the comparative medicine department (The Pennsylvania State University, College of Medicine).
3.2.8. Myeloid Cell Collections

Important immunologic tissues were harvested immediately after animal euthanasia to investigate the distribution of immune cells. The thymus, midsection of the spleen, a mesenteric lymph node and bone marrow aspirates were placed in a fresh Eppendorf tube with 1mL of a PBS:FACS buffer mix and stored on ice until preparation for flow cytometry.

Single-cell suspensions were made from tissue fragments. Briefly, tissues were gently mashed and filtered through a 100um (for tissues) or 40um (for bone marrow) nytex nylon mesh, with 1 to 2 mL of FACS buffer (eBiosciences). The chilled single cell suspensions were then aliquoted into 11X75mm polypropylene tubes (USA Scientific) until FACs analysis.

3.2.9. Histology

Fresh tissues were collected for histological examination and immersed in 10% neutral buffered formalin (NBF; Sigma), which is composed of 4% w/v formaldehyde with phosphate buffers. Once the fixation was complete the tissues were washed with 1XPBS, transferred to 70% EtOH and stored at 4°C until processed into paraffin (Table 8). Briefly, samples were dehydrated through a series of graded alcohols and then immersed in paraffin wax.

Once in paraffin the tissue samples were melted by submersion in a hot paraffin bath (65°C) until embedding. Block molds were prepared and warmed on a
Table 7: Wright Giemsa Polychromatic Stain.

<table>
<thead>
<tr>
<th>Granules</th>
<th>Color</th>
<th>Targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basophilic</td>
<td>deep blue</td>
<td>Nucleic DNA; Cytosolic RNA</td>
</tr>
<tr>
<td>Azurophilic</td>
<td>purple</td>
<td>Leukocytic lysosomes - granules</td>
</tr>
<tr>
<td>eosinophilic (acidophilic)</td>
<td>pink</td>
<td>Erythrocytic cytoplasm - hemoglobin</td>
</tr>
<tr>
<td>Neutrophilic</td>
<td>lilac</td>
<td>Cytoplasmic granules - neutrophils</td>
</tr>
</tbody>
</table>

Table 8: Paraffin Section Processing Procedures

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>70% EtOH</td>
<td>1hr</td>
<td></td>
</tr>
<tr>
<td>80% EtOH</td>
<td>1hr</td>
<td></td>
</tr>
<tr>
<td>90% EtOH</td>
<td>1hr</td>
<td></td>
</tr>
<tr>
<td>100% EtOH</td>
<td>1hr</td>
<td></td>
</tr>
<tr>
<td>100% EtOH</td>
<td>1hr</td>
<td></td>
</tr>
<tr>
<td>Xylene</td>
<td>1hr</td>
<td></td>
</tr>
<tr>
<td>Paraffin 65°C</td>
<td>30min</td>
<td></td>
</tr>
<tr>
<td>Paraffin 65°C</td>
<td>30min (with vacuum)</td>
<td></td>
</tr>
</tbody>
</table>
hot plate, while hot paraffin was poured into the mold. The tissues were positioned in the block mold for the most effective sectioning techniques (e.g. open longitudinal samples of the intestines are positioned with the cut surface down). Once the tissue was in the desired orientation the mold was placed on a cooling plate, while the cassette back was placed over the mold for an identifying lid. The block mold was cooled for a minimum of 30mins until the tissue block could be removed and stored.

Before sectioning the tissue blocks they were placed tissue side down in an ice bath for a minimum of 10 minutes while the microtome was being prepared. The tissue block was inserted into the microtome and trimmed at 20um sections. Once the desired sectioning location has been reached the block was sectioned at 5um, a ribbon of at least four sections was removed from the microtome blade and floated on top of a water bath. The sections were then floated on top of a pre-labeled, clean, glass slide.

3.2.10. FACS (Flow Cytometry) Analysis

Single-cell suspensions were prepared from the tissues collected and extracellular immune markers were targeted for staining. The cells were stained for 30mins on ice (~4°C) and incubated in the dark with different antibodies as shown in 3.2.3. The cells were then washed with 2mL FACs buffer and centrifuged at 500xg for 5 mins. The supernatants were decanted and the cells were resuspended in the residual buffer in addition to 200uL of additional FACs buffer. Samples were analyzed on a BD LSRII (Becton Dickinson, San Jose, CA, USA), with a minimum of
100,000 live events collected according to the manufacturers protocol. All recorded cell files were analyzed with FlowJo software (Treestar.com), only granulocytic cells were analyzed, which have a higher forward scatter (FSC) than red blood cells.

3.2.11. Statistical Analysis

Variables with number values (i.e. weight change) were compared using one-way ANOVA with a Newman-Keuls post-test for multiple comparisons (GraphPad Prism, San Diego, CA). To compare surface marker expression, a two way ANOVA, followed by Bonferroni t-test was performed. When necessary an unpaired student t test was used to calculate two-tailed p values between the groups (GraphPad Prism, San Diego, CA). Statistical significance was considered achieved when p<0.05 (*), p<0.01 (**) or p<0.001 (***)

3.3. Results

3.3.1. Differences in Physical Examinations

Btie2Cre*STAT3FF mice were hypoactive, lethargic and sometimes appeared listless. They isolated themselves from their littermates and appeared unkempt with porphyrin staining around the eyes, greasy fur, hunched posture and soiled anogenital regions. Mice also appeared pale in both upper and hind limbs and on the snout and tail. STAT1(−/−) mice were comparable to wild-type controls.
Figure 6: Body Weights: (A) At 4 weeks, BALB/cBtie2Cre·STAT3FF mice are significantly smaller than wild-type (WT) *** (p<0.001) and STAT1(−/−) mice ### (p<0.001). STAT1(−/−) are also significantly larger than the wild-type * (p<0.05). (B) At 8 weeks, BALB/cBtie2Cre·STAT3FF mice are significantly smaller than wild-type ^^^ (p<0.001) and STAT1(−/−) mice ^^^^^ (p<0.001). STAT1(−/−) are also significantly larger than the wild-type ^ (p<0.05) at this time. Bars are mean ± SEM. (A. n=14(WT), n=11(Btie2Cre·STAT3FF), n=17(STAT1(−/−)); B. n=16(WT), n=13(Btie2Cre·STAT3FF), n=17(STAT1(−/−)).
Figure 7: Body Lengths: (A) At 4 weeks, BALB/cBtie2Cre·STAT3FF mice are significantly shorter than wild-type (WT) *** (p<0.001) and STAT1(−/−) mice ### (p<0.001). (B) At 8 weeks, BALB/cBtie2Cre·STAT3FF mice are significantly shorter than wild-type ^^^ (p<0.001) and STAT1(−/−) mice %%% (p<0.001). Bars are mean ± SEM. (A. n = 14(WT), n = 11(Btie2Cre·STAT3FF), n = 17(STAT1(−/−)); B. n=16(WT), n=13(Btie2Cre·STAT3FF), n=17(STAT1(−/−))).
When comparing gross body weights and lengths the Btie2Cre*STAT3FF mice were significantly smaller (p<0.001) than the wild-type and the STAT1\textsuperscript{(-/-)} control mice at 4 and 8 weeks. STAT1\textsuperscript{(-/-)} weighed significantly (p<0.05) more than the wild-type control animals at 4 and 8 weeks, but there was no significant difference in body lengths (Figure 6 and 7). There was also a significant increase in STAT1\textsuperscript{(-/-)} body weight in comparison to wild-type at 4 and 8 weeks. When comparing body length there was no significance between the STAT1\textsuperscript{(-/-)} and wild-type controls. This indicates that the lost of STAT3 in hematopoietic stem cells contributes to reduced body weight and size, similar to a CD-like phenotype.

### 3.3.2. Differences in Upper GI Development

The gastrointestinal system was weighed and compared to the body weight as a percentage ratio (Figure 8). At 4-5 weeks the intestine of the Btie2Cre*STAT3FF mice were significantly increased in comparison to wild-type (p<0.01), this could be indicative of inflammation. There was also a significant reduction in the stomach (p<0.05) and cecum (p<0.01) when compared to wild-type. At the same time point the STAT1\textsuperscript{(-/-)} mice had a significant reduction in stomach weight (p<0.05) and colon (p<0.05) a significant reduction in the small intestines (p<0.001) in comparison to the wild-type and bone marrow specific STAT3-deficient mice. At 7-8 weeks this phenotype in STAT1\textsuperscript{(-/-)} mice was recovered in the stomach and small intestine, but the colon was significantly reduced compared to wild-type (p<0.01) and STAT3-deficient (p<0.001) mice.
Figure 8: Organ:Body Weight Histogram; (A.) At 4 weeks there is a significant increase in the bone marrow specific STAT3 deficient mouse small intestines **(p<0.01), and a significant reduction in the stomach *(p<0.05) and cecum ***(p<0.01) in comparison with wild-type. Additionally STAT1(*/-) mice demonstrate a significant reduction in stomach *(p<0.05), small intestine ***(p<0.001) and colon *(p<0.05) in comparison to wild-type. While there is a significant reduction in the stomach *(p<0.05) and small intestine ***(p<0.01) in comparison to Btie2Cre*STAT3FF mice. (B.) At 8 weeks Btie2Cre*STAT3FF mice demonstrated a significant increase in small intestine in comparison to wild-type ****(p<0.001) and STAT1(*/-) ###*(p<0.001). Btie2Cre*STAT3FF also show an increase in the colon ***(p<0.001) and a reduction in the cecum *(p<0.05) when compared to STAT1(*/-). Additionally, when compared to wild-type the Btie2Cre*STAT3FF mice show a reduction in the cecum ***(p<0.01) and the STAT1(*/-) were reduced in the colon ***(p<0.01). Bars are mean ± SEM. (A. n = 14(WT), n = 11(Btie2Cre*STAT3FF), n = 17(STAT1(*/-)); B. n=16(WT), n=13(Btie2Cre*STAT3FF), n=17(STAT1(*/-)).
At this time point the Btie2Cre*STAT3FF mice still presented with significant increases within the small intestine in comparison to the wild-type (p<0.001) and STAT1\(^{-/-}\) (p<0.001) control. Btie2Cre*STAT3FF also show a significant reduction in the cecum when compared to the wild-type (p<0.01) and STAT1\(^{-/-}\) (p<0.05) mice. These data demonstrates a trending increase in the small intestines and colon, which could be the first signs of inflammation or edema.

### 3.3.3. Transmural Inflammation in STAT3-Deficient Animals Compared with Wild-type Animals

Bone marrow specific STAT3-deficiency results in a Crohn's disease-like pathogenesis. In addition to the increase in intestinal weight in comparison to the body weight, these STAT3-deficient mice show significant transmural inflammation and increase in bowel wall thickness compared to wild-type mice (Figure 9). Within the cecum there was a significant increase in cellular infiltration and presence of ulceration of the mucosal surfaces. There was also a loss of normal intestinal cellular architecture and crypt development in the colon of the Btie2Cre*STAT3FF mouse in comparison to the wild-type control. Significant changes were present in the serosa, smooth muscle, submucosa and mucosa of STAT3-deficient mice in comparison to the wild-type control.
Figure 9: H&E Staining: Bone marrow specific STAT3-deficiency results in Crohn's disease like pathogenesis. There was cellular infiltration and ulceration within the cecum (X4,A; X20,B) Transmural inflammation, abnormal crypt development and wall thickening was present within the colon in comparison with the wild-type (C&D X10, hematoxylin and eosin).
3.3.4. Increase of Circulating Granulocytes and Decrease of Circulating Lymphocytes

Differential counts of blood smears were performed (Figure 10). The ratio of white blood cells (WBCs) in wild-type samples were similar to the normal values previously described (Nemzek et al., 2001). The percentages of WBCs in STAT3-deficient mice were: lymphocytes ~40%, monocytes ~22%, neutrophils ~22%, eosinophils ~14% and basophils ~2% (Figure 11). Monocytes were significantly increased in Btie2Cre*STAT3FF (p<0.05) in comparison to wild-type and STAT1-deficient mice. Eosinophils are significantly increased in Btie2Cre*STAT3FF mice when compared to wild-type (p<0.05) and STAT1-deficient (p<0.01) mice. There was a significant reduction in the number of lymphocytes in the Btie2Cre*STAT3FF, in comparison to wild-type (p<0.05) and STAT1-deficient (p<0.05) mice (Figure 12). These results indicate that there is an increase in myeloid-derived cells present within the peripheral blood.

3.3.5. Severe Myeloid Hyperplasia in Bone Morrow of STAT3-Deficient Animals

Bone marrow smears were performed to examine the distribution of myeloid progenitors. The bone marrow was scored in a double blind fashion, the cells were graded on a scale of 4 for myeloid hyperplasia, following the scoring criteria for previously described (Long et al., 1986), and assessed for the cellular distribution (Figures 13). In comparison to the wild-type and STAT1(−/−) control mice there was a significant increase in myeloid hyperplasia (p<0.001) in Btie2Cre*STAT3FF mice.
Figure 10: Peripheral Blood Smear, Mouse, Wright Giemsa Stain. 40x. Low magnification blood smears under light microscopy. (A.) Wild-type smear; normal ratio of myeloid:lymphoid distribution of WBC’s as described in Nemzek et al., 2001. (B.) Btie2C+STAT3FF smear; increased ratio of myeloid:lymphoid distribution. Marked increase of monocytes and eosinophils. (C.) STAT1(+/−) smear; normal distribution retained.
Figure 11: Distribution of White Blood Cells. Histogram of Peripheral Blood Distribution, wild-type similar to normal values as described in Nemzek et al., 2001. Marked elevation in monocytes, neutrophils and eosinophils in Btie2Cre*STAT3FF and STAT1 deficient mice, the ratio of myeloid:lymphoid distribution is altered. (n = 5 (WT), n = 5 (Btie2Cre*STAT3FF), n = 5 (STAT1^{-/-})).
Figure 12: Distribution of Peripheral Blood Cells. Differential counts of blood smears were performed and analyzed with GraphPad Prizm software. (A) Circulating monocytes in bone marrow specific STAT3-deficient mice and STAT1-deficient mice are significantly increased (p<0.05) in comparison to wild-type. (B) Circulating lymphocytes are reduced significantly in bone marrow specific STAT3-deficient mice when compared to wild-type (p<0.01) and STAT1-deficient (p<0.05) mice. (C) Circulating neutrophils in bone marrow specific STAT3-deficient mice and STAT1-deficient mice are significantly increased (p<0.05) in comparison to wild-type. (D) Circulating eosinophils are significantly increased in bone marrow specific STAT3-deficient mice when compared to wild-type (p<0.01) and STAT1-deficient (p<0.01) mice. Bars are mean ± SEM. (n = 5 (WT), n = 5 (Btie2Cre+STAT3FF), n = 5 (STAT1−/−))
Figure 13: Bone Marrow Smears, Wright Giemsa Stain. 100x. A. Wild-type bone marrow smear; mild myeloid hyperplasia, normal cellular distribution and normal myeloid:erythroid ratio. B. Btie2Cre·STAT3FF bone marrow smear; marked myeloid hyperplasia, predominant neutrophils, unbalanced myeloid:erythroid ratio, conspicuous eosinophils and megakaryocytes. C. STAT1(-/-) bone marrow smear; mild myeloid hyperplasia, balanced myeloid:erythroid ratio and normal differentiation with scattered eosinophils.
Figure 14: Scoring of Myeloid Hyperplasia. The presence of myeloid hyperplasia is reflected by the scoring criteria previously described (Long et al., 1986). Conducted as a blind study the Btie2Cre+STAT3FF slides were scored for severe myeloid hyperplasia when compared to wild-type (p<0.01) and STAT1 (p<0.01) mice. Bars are mean ± SEM. (n = 5 (WT), n = 7 (Btie2Cre+STAT3FF), n = 5 (STAT1(-/-))).

<table>
<thead>
<tr>
<th>Wild type</th>
<th>Mild myeloid hyperplasia, normal cellular differentiation, erythroid and megakaryocytes are within normal limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Btie2Cre+STAT3FF</td>
<td>Marked myeloid hyperplasia, predominant neutrophils and conspicuous eosinophils and megakaryoblasts</td>
</tr>
<tr>
<td>STAT1(-/-)</td>
<td>Mild myeloid hyperplasia, myeloid:erythroid ratio is balanced, normal differentiation in both, scattered eosinophils</td>
</tr>
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</table>
In these Btie2Cre*STAT3FF mice, neutrophils were predominant, eosinophil precursors, as well as megakaryoblasts, were frequent and conspicuous.

Within STAT1<sup>-/-</sup> mice the myeloid:erythroid ratio was more appropriately balanced and there was also normal differentiation in both series. Eosinophils were scattered but present and megakaryocytes were infrequent, and there was no apparent myeloid hyperplasia present in STAT1<sup>-/-</sup> mice. Meanwhile wild-type mice presented with mild hyperplasia and normal cellular differentiation, both erythroid and megakaryocyte lineages were within normal limits and the erythroid to myeloid ratio was normal (Table 9). Together, these data suggest that there is an increased myeloid population in the Btie2Cre*STAT3FF mice.

3.3.6. Specific Myeloid Population Is Associated with Gastrointestinal Phenotypes in STAT3 Deficient Animals

Myeloid derived cells share a common progenitor within the bone marrow, this means that monocytes, macrophages and granulocytes all respond to the same cell surface markers, such as F4/80 and CD11b. CD44 is supposed to identify those cells that express a phagocytic receptor (Wang <i>et al.</i>, 2002; Vachon <i>et al.</i>, 2006).

To identify the CD44<sup>high</sup>CD11b<sup>+</sup>F4/80<sup>+</sup> cells, total single cell suspensions from select tissues were stained with FITC-CD44, Cy5-CD11b and PE-F4/80 (eBioscience). They were gated for CD44<sup>high</sup> cells and subjected to FACS analysis. Percentages of CD11b<sup>-</sup>F4/80<sup>+</sup>, CD11b<sup>+</sup>F4/80<sup>+</sup>, and CD11b<sup>+</sup>F4/80<sup>-</sup> cells were
Figure 15: FACs Analysis and Controls. The phenotypes of myelomonocytic populations were characterized by staining with Cy5-CD11b, PE-F4/80 and FITC-CD44. Single cell-suspensions were created from hematopoietic tissues, spleen, bone marrow, mesenteric lymph nodes and thymus. Representative cell populations analyzed on LSRII (Becton Dickinson, San Jose, CA, USA) and Flowjo (TreeStar.com) from (A.) wild-type; (B.) Btie2Cre\(^{-}\)STAT3FF and (C.) STAT1\(^{-/-}\) Control staining represented as a histogram overview to compensate for fluorescence expression. (D.) PE-F4/80 compensation (red); (E.) Cy5-CD11b compensation (blue); (F.) FITC-CD44 compensation (green).
Figure 16: Detection of Monocyte-Derived Markers: To identify the specific myelomonocytic cell populations, total single cell suspensions from select tissues were stained with FITC-CD44, Cy5-CD11b and PE-F4/80 (eBioscience). They were gated for CD44	extsuperscript{hi} or CD44	extsuperscript{lo} cells and subjected to FACS analysis. Percentages of CD11b	extsuperscript{hi}F4/80	extsuperscript{hi}, CD11b	extsuperscript{hi}F4/80	extsuperscript{lo}, and CD11b	extsuperscript{lo}F4/80	extsuperscript{lo} cells were recorded. In the bone marrow, there is a significant increase of CD44	extsuperscript{lo}CD11b	extsuperscript{hi}F4/80	extsuperscript{hi} **(p<0.01), in comparison to wild-type, and CD44	extsuperscript{lo}CD11b	extsuperscript{hi}F4/80	extsuperscript{lo} *(p<0.05) cells in Btie2Cre*STAT3FF in comparison to wild-type and #(p<0.05) STAT1(-/-) mice. In the spleen, there is a significant increase of CD44	extsuperscript{lo}CD11b	extsuperscript{hi}F4/80	extsuperscript{lo} *(p<0.05), in comparison to STAT1(-/-), and CD44	extsuperscript{lo}CD11b	extsuperscript{lo}F4/80	extsuperscript{lo} *(p<0.05) cells in Btie2Cre*STAT3FF in comparison to wild-type and **(p<0.01) STAT1(-/-) mice. Also, there is an increase in CD44	extsuperscript{hi}CD11b	extsuperscript{hi}F4/80	extsuperscript{lo} cells in Btie2Cre*STAT3FF in comparison to *(p<0.05) wild-type and *(p<0.01) STAT1(-/-) mice. CD44	extsuperscript{hi}CD11b	extsuperscript{hi}F4/80	extsuperscript{lo} cells in STAT1(-/-) are significantly reduced in comparison to *(p<0.05) wild-type. There is a significant decrease of CD44	extsuperscript{hi}CD11b	extsuperscript{lo}F4/80	extsuperscript{lo} cells **(p<0.01) in the thymus of Btie2Cre*STAT3FF mice, and a significant increase of CD44	extsuperscript{hi}CD11b	extsuperscript{hi}F4/80	extsuperscript{lo} **(p<0.01) and CD44	extsuperscript{hi}CD11b	extsuperscript{hi}F4/80	extsuperscript{lo} ***(p<0.001) cells in the thymus of STAT1(-/-) in comparison to wild-type. Also, Btie2Cre*STAT3FF mice have a significant increase of CD44	extsuperscript{hi}CD11b	extsuperscript{hi}F4/80	extsuperscript{lo} cells compared to wild-type *(p<0.05) and STAT1(-/-) ***(p<0.01) controls. Bars are mean ± SEM. (n = 3 (WT), n = 3 (Btie2Cre*STAT3FF), n = 3 (STAT1(-/-))).
recorded (Figures 15 and 16). There is a significant decrease of CD44$^{\text{high}}$CD11b$^{-}$F4/80$^{+}$ cells (p<0.01) in the thymus of bone marrow specific STAT3-deficient mice, and a significant increase of CD44$^{\text{high}}$CD11b$^{+}$F4/80$^{+}$ (p<0.01) and CD44$^{\text{high}}$CD11b$^{+}$F4/80$^{+}$ (p<0.001) cells in the thymus of STAT1. There is also a significant increase in CD44$^{\text{high}}$CD11b$^{+}$F4/80$^{-}$ cells (p<0.05) in the spleen of the STAT3-deficient mouse when compared to the wild-type and STAT1$^{(-/-)}$ controls. The STAT1$^{(-/-)}$ had a significant decrease in splenic CD44$^{\text{high}}$CD11b$^{-}$F4/80$^{-}$ cells (p<0.05) in comparison to the wild-type controls.

Next the cells were gated for CD44$^{\text{low}}$ cells and subjected to FACS analysis. Percentages of CD11b$^{-}$F4/80$^{+}$, CD11b$^{+}$F4/80$^{+}$, and CD11b$^{+}$F4/80$^{-}$ cells were recorded. When compared to the wild-type control there is a significant increase of CD44$^{\text{low}}$CD11b$^{+}$F4/80$^{+}$ cells (p<0.01) and CD44$^{\text{low}}$CD11b$^{+}$F4/80$^{-}$ cells (p<0.05) in the bone marrow of Btie2Cre$^{+}$STAT3FF mice. Also, there is a significant increase of CD44$^{\text{low}}$CD11b$^{+}$F4/80$^{+}$ cells (p<0.05) and CD44$^{\text{low}}$CD11b$^{+}$F4/80$^{-}$ cells (p<0.01) in the spleen in the Btie2Cre$^{+}$STAT3FF when compared to the STAT1$^{(-/-)}$ control. Meanwhile, CD44$^{\text{low}}$CD11b$^{+}$F4/80$^{-}$ cells in STAT1$^{(-/-)}$ mice were significantly reduced (p<0.05) in the bone marrow when compared to the wild-type controls. These results indicate that there is a specific increase in the CD44$^{\text{low}}$CD11b$^{+}$ cell population in BtieCre$^{+}$STAT3FF mice.

3.4. Discussion

In 2003, Welte et al., determined that the intestines of STAT3 deficient mice (Btie2Cre$^{+}$STAT3FF) were transmurally inflamed and thickened by the cellular
infiltration of myeloid lineage cells (Welte et al., 2003). The data in this study shows that at four weeks Btie2Cre\*STAT3FF mice have a significant increase in small intestine mass in comparison to body weight; there is also a trending increase in the colon mass. There was also a statistically significant reduction in body mass and length of bone marrow specific STAT3-deficient mice. This reduction suggests that there are severe developmental defects as a result of STAT3 deficiency.

Btie2Cre\*STAT3FF mice presented with severe myeloid hyperplasia and a predomination of neutrophils within the bone marrow. Although STAT3 may not be required for neutrophilic differentiation, it’s important for regulating neutrophilic numbers (Takeda et al., 1999; Lee et al., 2002). STAT3 is also a very important cell cycle control protein that is required for the induction of a G1 cycle arrest in granulopoiesis (Coffer et al., 2000; Takeda and Akira, 2000). It is equally as important in growth-factor induced myeloid differentiation through GM-CSF.

Hematopoietic derived myeloid cells were also upregulated in the peripheral blood of Btie2Cre\*STAT3FF mouse. There was a significant increase in the number of macrophages and eosinophils. Rothenberg et al., 2001 demonstrated that eosinophil accumulation was associated with weight loss and an increased production of IL-5 signaling (Rothenberg et al., 2001). These data also support the theory that the dysregulation of innate immune responses correlates with the pathogenesis of CD (Welte et al., 2003).

The loss of STAT3 phenotypically appears as myeloid hyperplasia within the bone marrow and an increase of myeloid derived cells within the peripheral blood.
There was also a significant reduction in the number of circulating T cells in the peripheral blood of the Btie2Cre*STAT3FF mice. The reduction in circulating T cells could be attributed to a loss in IL-2 induced proliferation because of defective STAT3 signaling (Akashi, et al., 1998). Defective regulatory cells also correlates with a loss in T cell development and thymic dysfunction (Strober et al., 2002).

Conversely, bone marrow STAT3-deficient mice demonstrated a significant increase in CD44^{low}CD11b^{+} cell populations, F4/80^{+} and F4/80^{-}, in bone marrow and spleen. CD44 is a transmembrane adhesion molecule that triggers phagocytic ingestion by macrophages and regulates inflammation (Vachon et al., 2006). A CD44 dysfunction may indicate defective macrophage function (Wang et al., 2002). Splenic granulocytes have also been demonstrated to be elevated in the STAT3-deficient mice (Kortylewski et al., 2005). Therefore the significant increase in CD44^{low}CD11b^{+} cell populations in the Btie2Cre*STAT3FF mice are indicative of dysfunctional myeloid-derived cells. Although not specifically identified within Crohn’s-disease like pathogenesis, the transgenic Btie2Cre*STAT3FF mouse does present with significant reductions of CD44^{high}CD11b^{-}F4/80^{+} cells which could indicate thymic dysfunction.

Overall the Btie2Cre*STAT3FF mice demonstrate a significant reduction in body mass and length, with a significant increase in intestinal mass. These mice also show a significant increase in circulating myeloid-derived cells in the peripheral blood, and a significant increase in myeloid hyperplasia within the bone marrow. Important immunologic tissues, like the bone marrow and spleen, also demonstrate
a significant increase in myeloid-derived cell populations, with little to no expression of the phagocytic receptor, CD44. Therefore, bone marrow specific STAT3-deficiency results in significant alterations in myelopoiesis.
CHAPTER FOUR

EARLY STAGE MACROPHAGE DYSFUNCTIONS IN STAT3-DEFICIENT, BUT NOT STAT1-DEFICIENT AND WILD-TYPE ANIMALS
4.1. Introduction

Macrophage precursors, monocytes, are not ascribed any phagocytic abilities when circulating within the peripheral blood (Andus et al., 1991; Rugtveit et al., 1995). They migrate through the periphery in response to any necrotic material, pathogens and inflammation (Rugtveit et al., 1994). Once the circulating monocytes reach their target they migrate into the tissues to differentiate into macrophages (Mahida et al., 1989; Geissmann et al., 2003; Geissmann et al., 2008). The differentiated macrophages phagocytose cell and tissue debris, bacteria and dead neutrophils (Djaldetti et al., 2002; Bilitewski, 2008). If this phagocytic activity is compromised normal immune responses cannot occur leading to tissue damage and subsequently more inflammation (Xavier and Podolsky, 2007; Casanova and Abel, 2009).

Macrophages have the ability to act as sentinels or first responders and survey the intestinal environment, as the first line of defenders (Xavier and Podolsky, 2007). Macrophages become the main cell within the inflammatory site once the short-lived neutrophils are spent (Bilitewski, 2008). The mucosal immune system has to sample the environment to differentiate between the harmful and harmless microorganisms; therefore there are specific and non-specific methods for recognition (Ezekowitz, 1990; Czop and Kay, 1991).

Macrophages are phagocytic and antigen presenting cells. All phagocytic cells have the ability to recognize and engulf foreign particles through pattern recognition receptors on the cell-surface and kill the pathogen through the
production of reactive oxygen species (ROS) (Djaldetti et al., 2002; Bilitewski, 2008). Phagocytosis is a critical response to a myriad of biologic events such as tissue remodeling and pathogen clearance (Djaldetti, et al., 2002). The ability to phagocytose harmful material and monitor the intestinal contents is crucial to activate innate responses and critical to the functional integrity of the mucosal immunity (Xavier and Podolsky, 2007). There are a variety of events responsible for phagocytosis including fluid phase endocytosis (pinocytosis), recognition and binding of particles by cell surface receptors (i.e. FcyR, complement receptors, TLR) and a cytosolic architectural change to engulf the offending particles (Djaldetti et al., 2002). A phagosome is formed once the pseudopodia fuse within the cytosol of the phagocytic cell, maturation includes the formation of a phagolysosome. The destruction of the ingested materials occurs through an acid-base balance, hydrolysis and radical attack due to the production of ROS (Dahlgren and Karlsson; 1999; Fang, 2004; Biliteski, 2008). These events are necessary for the normal function of the innate immune system, any failure within these macrophages can significantly influence the pathogenesis of disease.

In 1930, Cappell demonstrated that all cells, from small lymphocytes to large macrophages, are present within the peritoneal fluids (Cappell, 1930; Harveit, 1964). He described the peritoneal population as “fully developed large macrophages” (Cappell, 1930). Peritoneal macrophages have been widely used to obtain much of the current knowledge of macrophage biology since Cohn and colleagues in the 1960s lead the way (Steinman and Moberg, 1994; Ghosn et al.,
Macrophages are actively phagocytic in body fluids (Djaldetti et al., 2002; Ghosn et al., 2010) and easily accessible for experimental purposes. Therefore these experiments utilized peritoneal macrophages to study the functional effects of STAT3-deficiency in hematopoietic stem cells. These studies investigate specific phagocytosis and ROS production, non-specific phagocytosis for immune surveillance as well as nicotinamide adenine dinucleotide phosphate (NADPH-) oxidase production from redox reactions.

4.2. Experimental Procedures

4.2.1. Animals and Genotyping

Bone marrow hematopoietic stem cell specific STAT3 knockout mice (Btie2Cre*STAT3FF) on a BALB/c background were used for this study. Littermate wild-type animals, heterozygous or homozygous with no Cre expression, were used as controls. STAT1 knockout mice (STAT1^{+/−}) on BALB/c background were also used as a control. All animals were housed in pathogen-free conditions in the barrier wing in the Animal Research Facilities of The Pennsylvania State University, College of Medicine, Hershey, PA. All experimental procedures were established in accordance with the Institutional Animal Care and Use Committee (IUCAC) guidelines and approved protocols from the Animal Care Committees.

To verify the specific STAT3 or STAT1 deletion genomic PCR was implemented using primer sets that determine the presence of Cre expression (Cre
primers), recognized the loxP sites (F primers) and identified the STAT1 deletion (STAT1 primers) (as described in Chapter 2).

4.2.2. Antibodies

The following antibodies were used to stain the extracellular surface immune markers: CD11b(Mac1) – Cy5 Clone M1/70 (eBiosciences) which is expressed by granulocytes; F4/80 – PE clone BM8 (eBiosciences) which is expressed by macrophages and is referred to as a pan macrophage marker.

4.2.3. Macrophage Collection and Purification

Resident peritoneal macrophages where collected from mouse peritoneal exudates, following a modified version of Edelson et al., 1975 with Davies and Gordon protocol (Edelson et al., 1975; Davies and Gordon, 2004). Briefly, once euthanized with CO₂ asphyxiation the mice were sterilized with 70% EtOH. The thoracic skin was cut right above the sternum and, with the animals arms pinned down, the skin was gently ripped from the sternum, peritoneum and pelvis. Care was taken not to rip any abdominal vessels to prevent spills of blood into the peritoneal cavity. The peritoneum was sterilized again with 70% EtOH and injected at the distal end with ~10mL of cold, sterile PBS with a 22G needle, bevel down. After a 10second and gentle shaking the peritoneal exudate was collected from the proximal end in a clean, sterile 5mL syringe with a 18G needle, bevel up, and stored
in a 15mL tube held on ice until use. Macrophages for functional assays were spun down and washed with chilled sterile 1xPBS and used immediately.

The cells were plated at 1x10^6 cell/ml, cultured in polystyrene dishes in 37°C incubators with 5% CO_2 and allowed to adhere for 24hrs. Once adherent the cells were washed twice with sterile 1XPBS and grown in macrophage culture medium GIBCO® RPMI 1640 (GIBCO, Life Technologies) media supplemented with 2mM glutamine, 20mM HEPES, 10% heat-inactivated FCS, 50IU penicillin, 50ug streptomycin (MP Biomedicals, 200x Penicillin-Streptomycin), gentamicin and amphotericin B (Cascade Biologics, Invitrogen) for 72hrs.

4.2.4. Cell Imaging

Peritoneal exudates macrophages (PEM)s were examined by an Olympus IX 70 microscope with the DIC filter and SPOT™ imaging software (SPOT Imaging Solutions, Diagnostic Instruments, Inc., Sterling Heights, MI, USA.)

4.2.5. FcOxyBURST® Assay (Analysis of FcγR-Mediated Phagocytosis)

For this assay the manufacturer’s protocol was followed, but briefly, fresh PEMs were isolated and resuspended in glucose-PBS in preparation for the assay. Kreb’s Ringer’s PBS (KRP buffer: 1.0mM Ca^{2+}, 1.5mM Mg^{2+} and 5.5mM glucose) was prewarmed to 37°C and used to respuspend the PEMs 30mins prior to flow analysis. Baseline measurements were taken and then the immune complexes were added. Fluorescence related to oxidation the FcOxyBURST® immune complexes was
assayed by flow cytometry using a FACSCalibur every 30sec for 2mins. The cell suspension was then incubated for 30mins and reread.

4.2.6. FITC-Dextran Assay (Fluid Phase Endocytosis-Macropinocytosis)

Pooled peritoneal macrophages were incubated with fluorescein isothiocyanate (FITC)-Dextran (M.W. 40kDa; Sigma) for 30 minutes at 37°C or at 4°C. The cells were then washed with PBS three times and prepared for analysis on the FACSCalibur (Becton Dickinson, San Jose, CA, USA).

4.2.7. Nitroblue Tetrazolium (NBT) Reduction Assay (ROS Generation)

PEMs were collected and spun down for 10mins at 1500rpm at 4°C. The supernatant was aspirated and then the pellet was resuspended in 20% NBT/DMEM solution (Suh, et al., 2006). The cells were then incubated at 37°C for 1 hour. The reaction was then stopped with 3ml ice cold PBS. The cells were spun at 1500rpm for 10mins at 4°C. The pellet was aspirated and smeared on a clean glass slide. Once dry, the slides were fixed with methanol for 1min, rinsed with diH2O and counterstained with Safranin-O for 1.5mins. Excess stain was rinsed and the slides were observed under light microscopy.

4.2.8. FACS (Flow Cytometry) Analysis

The peritoneal exudate macrophages (PEMs) were trypsinized, washed and subsequently analyzed by flow cytometry for characterization. Typically ~10^6 cells
were stained with 1:100 dilution of the working solution of PE-F4/80 and Cy5-CD11b for 30mins in the dark. FACS analysis was performed on the FACSCalibur (Becton Dickinson, San Jose, CA, USA).

4.2.9. Statistical Analysis

Unpaired student $t$ test was used to calculate two-tailed P values between the groups (GraphPad Prism, San Diego, CA). Statistical significance is considered achieved when $p<0.05$ (*), $p<0.01$ (**) or $p<0.001$ (***)

4.3. Results

4.3.1. Evaluation of Purified Peritoneal Macrophages

Isolated and purified PEMs were analyzed on the FACSCalibur (Becton Dickinson, San Jose, CA, USA) the cell population, according to forward scatter (FSC) and side scatter (SSC), was granulocytic (Figure 17). Using the LSRII (Becton Dickinson, San Jose, CA, USA) to distinguish the cell suspension with PE-conjugated F480 (pan-macrophage marker) and CY5-conjugated CD11b (cell surface marker) to distinguish that the adherent cells contained 90-95% of double positive cells (F4/80+CD11b+) cells (Figure 18). This indicates that this method of isolation and purification yields 90-95% of myeloid derived cells.

4.3.2. Reduction in FcγR-Mediated Phagocytosis

FcγR-mediated phagocytosis was measured by the internalization of immune complexes prior to an oxidative burst in the phagosomal vacuole of freshly collected
Figure 17: Isolation and Purification of Peritoneal Exudate Macrophages; Peritoneal exudates macrophages (PEM cells) were harvested by peritoneal exudate. (A & B.) FACS analysis was performed on FACScalibur and FSC and SSC determined the cells were granulocytic. PEM cells were stained with 1:100 dilution of PE-F4/80 and Cy5-CD11b. (C.) Single stained control cells with PE-F4/80 confirmed that 89.6% of cells were F4/80+ cells. (D.) Single stained control cells with Cy5-CD11b confirmed that 93.9% of cells were F4/80+ cells. (E.) Immunostained sample cells confirmed that purity by adherence produced 93.7% cells with F4/80+CD11b+ expression, similar to macrophage expression. (F.) Phase contrast picture of adherent cells.
Figure 18: Adherent Peritoneal Exudate Macrophages. One characteristic of macrophages that distinguish them from other cells within the myeloid lineage is their ability to adhere to plastic polystyrene plates. (A) Phase contrast picture of Wild-type PEMs. (B) Phase contrast picture of Btie2Cre*STAT3FF cells. (C) Phase contrast picture of STAT1<sup>−/−</sup>. There were no gross morphological differences between the three transgenes.
PEMs, following the manufactures protocol. The immune complexes were probes with the FcOxyBURST® assay from Molecular Probes (Invitrogen). The reagent consists of insoluble BSA-anti-BSA immune complexes that are covalently labeled with dichlorodihydrofluorescein (H₂DCF). Once ingested the oxidation of the H₂DCF to DCF in the phagosomal vesicle produces a green fluorescence that can be monitored by flow cytometry.

\[
H_2DCF + O_2 \rightarrow O_{-2} + O_{-2} + 2H^+ + DCF \text{ (fluorescence)}
\]

This test was important to measure specific phagocytosis which recognizes the Fc portion of immunoglobins. Fc-receptors are on the surface of phagocytic cells to recognize the antibodies on the surface of a pathogen. This specific binding facilitates phagocytosis of neutralized microorganisms, induces killing, uptake, stimulation and activates a respiratory burst (Suchard et al., 1997; Fitzer-Attas et al., 2000; Bilitewski, 2008). Phagocytic activation is initiated by γ-chain signaling, it is important for the initiation of an inflammatory response and can cause tissue damage if not regulated. Fc-receptor macrophages are one of the most important cells in the innate response because of the bacterial recognition and subsequent ingestion to destroy the pathogen.

Fc-receptor mediated phagocytosis and generation of oxidative bursts were significantly reduced in the STAT3-deficient mice in comparison to wild-type (p<0.01) and STAT1\(^{(-/-)}\) (p<0.001) mice at four weeks of age (Figure 19A). This phenomenon was recovered at eight weeks of age, both STAT3-deficient and STAT1\(^{(-/-)}\) mice normalized to the wild-type control (Figure 19B). Indicating that
Figure 19: FcOxyBURST® Assay. Specific phagocytosis by FcyR-mediated recognition was measured at 4 weeks and at 8 weeks. (A) At 4 weeks Btie2Cre-STAT3FF macrophages are significantly reduced in comparison with wild-type **(p<0.01) at the initial uptake, 60 seconds and 30 min incubation. At every time point the STAT1(-/-) deficient macrophages were significantly increased ###(p<0.001) in comparison to bone marrow specific STAT3-deficient and *** (p<0.001) wild-type mice. (B) There is no significance between the transgenes, the functional activity has been recovered within normal limits, in comparison to wild-type control. Bars are mean ± SEM. (A. n = 7 (WT), n = 5 (Btie2Cre-STAT3FF), n = 7 (STAT1(-/-)) B. (n = 9 (WT), n = 9 (Btie2Cre-STAT3FF), n = 7 (STAT1(-/-))).
early stage macrophages are dysfunctional, and this dysfunction may correlate with immature cells.

4.3.3. Reduction of Macropinocytosis in Early Stage

Fluid phase endocytosis, non-specific phagocytosis, determines the cells ability to macropinocytose. The FITC-dextran molecule that was used had a molecular weight (M.W.) of 40kDa (FDx40), this molecule was determined to be ingested by macropinocytosis by Araki et al., in 1996. The ability to macropinocytose includes the ability for the cell margin to extend actin rich “ruffles” (phagocytic “arms”) to produce an intracellular vesicle called a pinosome. This process is stimulated by GM-CSF, and creates macropinosomes >0.2um in diameter. FACs analysis allowed the determination of fluorescence from single cells, allowing for a more accurate demonstration of phagocytic function (Bilitewski, 2008).

At 4 weeks the bone marrow specific STAT3 deficient mouse has a significantly reduced ability to macropinocytose materials in comparison to the wild-type (p<0.05) and the STAT1 deficient (p<0.05) mouse (Figure 20A). This loss was recovered at 8 weeks of age to be within the normal limits of endocytosis (Figure 20B). These results support the theory of immature early stage macrophages.
Figure 20: Macropinocytosis of FDx40. Non-specific phagocytosis was measured by the ability to ingest FITC-dextran (M.W. 40kDa) (FDx40) molecules. (A.) At 4 weeks cultured macrophages from bone marrow specific STAT3-deficient mice have a significant reduction of the ability to phagocytose FITC-dextran *(p<0.05)* in comparison with wild-type and # (p<0.05) STAT1-deficient controls. (B.) This functional loss was recovered at eight weeks of age. Bars are mean ± SEM. (A. n = 7 (WT), n = 5 (Btie2Cre·STAT3FF), n = 7 (STAT1<sup>+/−</sup>) B. (n = 9 (WT), n = 9 (Btie2Cre·STAT3FF), n = 7 (STAT1<sup>+/−</sup>)).
Figure 21: Nitroblue Tetrazolium Reduction Assay. The ability to reduce tetrazolium to a blue formazan measures the ability to produce NADPH and ROS. This assay demonstrates that bone marrow specific STAT3-deficient mice have a significant reduction in redox capabilities compared to wild-type controls *(p<0.05) and STAT1(−/−) controls ##(p<0.01). Bars are mean ± SEM. (n = 7 (WT), n = 5 (Btie2Cre−STAT3FF), n = 7 (STAT1(−/−))).
4.3.4. ROS Production Are Reduced

This assay measures the metabolic activity of phagocytes through a non-specific response to nitroblue tetrazolium (NBT), its focus is on the ability to produce oxygen radicals (O$_2^-$ and OH$^-$) and the reduction of tetrazolium dye to an insoluble formazan (Thannickal and Fanburg, 2000). It determines the redox capabilities of the phagocytic cell based on oxidative reductive reactions (Baehner et al., 1976; Thannickal and Fanburg, 2000; Pompeia et al., 2003, Suh et al., 2006).

The phagocytic activity of Btie2Cre$^*$STAT3FF macrophages is significantly lower (p<0.05) than the wild-type mice (Figure 21). There is also a significant reduction (p<0.01) in Btie2Cre$^*$STAT3FF macrophages phagocytic activity than the STAT1$^{$-/-$}$ deficient mice. The ability to produce reactive oxygen species ROS was also measured in the Fc-receptor mediated assay, due to the respiratory bursts responsible for producing fluorescence. These data demonstrated that at 4 weeks, the production of ROS from Btie2Cre$^*$STAT3FF macrophages were significantly reduced in comparison to wild-type (p<0.01) and STAT1$^{$-/-$}$ (p<0.001). This dysfunction was recovered at 8 weeks. Therefore the production of NADPH and ROS are significantly reduced in bone marrow specific STAT3-deficient mice. Thus, an excess number of these dysfunctional cells can contribute to the mucosal damage that is seen in intestinal inflammation.
4.4. Discussion

Macrophages are critical cells within innate immunity. Their ability to phagocytose materials and sample the surrounding environment allows the host to distinguish between harmful and harmless microorganisms and is extremely important to maintain the integrity of intestinal immunity (Rugtveit et al., 1995; Kortylewski et al., 2005). The presentation of antigenic material to the adaptive immune system is critical to a normal response of immunity, without it there is a dysregulation of immune function that can subsequently lead to tissue damage.

Phagocytosis is a critical mechanism of macrophage function, it is the ability of the cell to engulf foreign materials or microorganisms that might cause harm to the system (Bilitewski, 2008). It has been suggested that macrophages in Crohn’s disease are the key initiators and culprits of pathogenesis (Smith et al., 2009; Casanova and Abel, 2009). Currently it is unknown if the functional loss in macrophages is through inadequate pattern recognition signals, specific phagocytosis, through dysregulated pseudopodia that engulf macromolecules, non-specific phagocytosis, or through other dysfunctional mechanisms (Klippel and Bilitewski, 2007; Marks and Segal, 2008; Marks et al., 2009; Smith et al., 2009). The aim of these studies was to the measure the phagocytosis of fluorescence labeled particles and immune complexes through specific and non-specific binding. Coupling these assays with FACs analysis allows the determination of fluorescence from single cells (Bilitewski, 2008).

The antibody-pathogen immune complex, specific recognition, through Fc-receptor mediated phagocytosis demonstrated a significant reduction in specific
phagocytosis within Btie2Cre\textsuperscript{+}STAT3FF mice compared to wild-type and STAT1-deficient controls. This phenomenon occurred at early stages of development, coinciding with weaning ages, around four weeks old. The loss of specific phagocytic activity was recovered at eight weeks of age. Non-specific phagocytosis of surrounding macromolecules was also significantly reduced at four weeks of age, but yet recovered at eight weeks, in Btie2Cre\textsuperscript{+}STAT3FF mice, in comparison with wild-type and STAT1\textsuperscript{(-/-)} controls.

Functional properties of macrophages rely not only on their ability to phagocytose materials but also the production of reactive oxygen species (ROS) through respiratory bursts. The cytokine, IFN\textgamma has been attributed to stimulating and priming the macrophages to induce the release of ROS (Rugtveit et al., 1995). The over production of ROS can lead to tissue damage and contribute to the intestinal erosion seen within CD. The production of ROS was also measured in the Fc-receptor (FcR)-mediated assay. These data demonstrated that at four weeks, the Btie2Cre\textsuperscript{+}STAT3FF mouse was unable to produce a normal response from the macrophages. Interestingly this dysfunction was recovered at eight weeks, during the presence of disease. This assay is limited in that the macrophages could be FcR specific, highly capable of ingesting materials, but could have an inability to produce ROS during the induction of the respiratory burst which produces measureable fluorescence. Therefore it is unknown if the macrophages are truly dysfunctional in FcR-mediated phagocytosis or just in the production of ROS. Either way, the macrophages are dysfunctional in some capacity.
Bone marrow specific STAT3-deficient mice also demonstrated a failure to reduce NBT, which indicates that there is a loss of oxidative reduction reactions in the macrophages. The redox reaction is a result of NADPH activities, and the generation of ROS, therefore the failure to reduce NBT correlates with a failure of the normal redox reactions (Baehner et al., 1976; Pompeia et al., 2003).

Specific phagocytosis is reduced in Btie2Cre*STAT3FF macrophages at four weeks in comparison to wild-type and STAT1(-/-) macrophages, but this phenotypic loss is recovered at eight weeks of age. Additionally, non-specific phagocytosis is also reduced at four weeks and recovered at eight weeks. There is a possibility that at four weeks this reduced activity can be attributed to immature macrophages. Therefore at eight weeks when the activity has come within normal limits the long-lived macrophages have either matured or significantly increased in sensitivity to counterbalance the loss in activity.

Therefore because specific and non-specific phagocytic activity, along with the ability of the macrophages to produce reactive oxygen species (ROS) occurs these data present evidence that the macrophages are dysfunctional. Based on prior data (chapter 3) macrophages are also in excess, therefore, elevations in dysfunctional macrophage numbers may lead to the dysregulation of the innate immune system. In accordance with Welte and colleagues, 2003, this can contribute to the proposed pseudoactivated innate immune system (Welte et al., 2003).
CHAPTER FIVE

ANALYSIS OF CYTOKINE PRODUCTION IN STAT3-, STAT1-
DEFICIENT, AND WILD-TYPE MACROPHAGES
5.1. Introduction

Once activated macrophages have the ability to produce a dynamic cytokine response including an array of proinflammatory, antiinflammatory and immunoregulatory cytokines and chemokines (Santor et al., 1994; Kanai et al., 2001). Current theories have implicated T lymphocytes as being the culprits of Crohn’s disease, due to the Th1 cytokine profile expressed in disease but this theory remains unproven (Shanahan, 2002; Podolsky, 2002; Xavier and Podolsky, 2007; Smith et al., 2009). In 2003, Welte and collaborators demonstrated a connection between the innate immunity, specifically myeloid-derived cells and STAT3-deficiency on the pathogenesis of Crohn’s-like disease (Welte et al., 2003). In addition, recent genome-wide association studies (GWAS) have also redirected IBD research to investigate the implications of the innate immunity and their associated cytokine responses (Barrett et al., 2008; Smith et al., 2009).

Cytokines are small signaling molecules that allow cells within the immune system to communicate and respond to specific stimuli (Rogler and Andus, 1998). The biological functions of cytokines depend on mediated gene activation or repression of signaling by downstream modulators (Larner et al., 1993). Signal transducers and activators of transcription (STAT) proteins that respond to cytokine signaling, phosphorylate, dimerize and translocate into the nucleus from their latent cytoplasmic location to induce gene transcription of additional cytokines and chemokines (Darnell, 1997; Leonard and O'Shea, 1998). Regulating cytokine signaling pathways plays a critical role in the pathogenesis of inflammatory bowel disease (MacDonald, 1999; Hugot et al., 2001; Smith et al., 2009).
Determination of the cytokine profile before disease onset is of extreme importance to allow a true evaluation of the immune reactivity and complications of the disease. Macrophage function, phenotype and activation are regulated by the production of cytokines and transcription of STAT proteins (Hu, et al., 2007). The cytokine, IFNγ, is one of the most potent endogenous activators of macrophage function (Hu, et al., 2007), it has been attributed to stimulating and priming macrophages for antigen presentation and to induce the release of ROS. Additionally, GM-CSF was also thought to alter the production of ROS through the maturation and increased viability of macrophages, but it has now been shown that GM-CSF is not needed (Coleman et al., 1988; Rugtveit et al., 1995). However, GM-CSF is important the generation of pseudopodia for non-specific phagocytosis.

Phosphorylated STATs dimerize and translocate into the macrophage nucleus to activate gene transcription. Gene transcription will include the generation, production and secretion or expression of new cytokines and chemokines that will affect the immune system either in a proinflammatory, anti-inflammatory or an immunoregulatory way. The aim of these studies was to investigate the expression levels of the secreted cytokines and determine the cytokine profile for the bone marrow specific STAT3-deficient mice in comparison to wild-type and STAT1(−/−) controls. Samples were taken from the serum, for a systemic profile, and from cultured macrophages for a local profile.
5.2. Experimental Procedures

5.2.1. Animals and Genotyping

Bone marrow hematopoietic stem cell specific STAT3 knockout mice (Btie2Cre*STAT3FF) on a BALB/c background were used for this study. Littermate wild-type animals, heterozygous or homozygous with no Cre expression, were used as controls. STAT1 knockout mice (STAT1⁻/⁻) on BALB/c background were also used as a control. All animals were housed in pathogen-free conditions in the barrier wing in the Animal Research Facilities of The Pennsylvania State University, College of Medicine, Hershey, PA. All experimental procedures were established in accordance with the Institutional Animal Care and Use Committee (IUCAC) guidelines and approved protocols from the Animal Care Committees.

To verify the specific STAT3 or STAT1 deletion genomic PCR was implemented using primer sets that determine the presence of Cre expression (Cre primers), recognized the loxP sites (F primers) and identified the STAT1 deletion (STAT1 primers) (See Chapter 2).

5.2.2. Serum Collections

Terminal cardiac blood punctures yield approximately ~700ul of blood per animal. Once collected the blood is allowed to clot for 20-30 minutes at room temperature (RT). Since samples are collected and compiled for use at a later date, the fresh samples are spun down at 1,000x g for 10mins and collect the supernatant. Supernatant is stored in -80°C until use. Finally, when preparing samples for
analysis the samples are defrosted, spun down and diluted (if necessary) with serum standard buffer.

5.2.3. Macrophage Culture

PEMs were isolated and purified through cell culture in GIBCO® RPMI 1640 (GIBCO, Life Technologies) media supplemented with 2mM glutamine, 20mM HEPES, 10% heat-inactivated fetal calf serum (FCS), 50IU penicillin, 50ug streptomycin (MP Biomedicals, 200x Penicillin-Streptomycin), gentamicin and amphotericin B (Cascade Biologics, Invitrogen) (macrophage culture medium; MCM; Weigmann et al., 2007) until adherent. After 24hrs, the cells were washed twice in 1XPBS to remove non-macrophages. The cells were allowed to grow and mature for 72hrs and the supernatant was collected. Debris was removed by centrifugation and only the supernatant was tested.

5.2.4. Bio-plex® Bio-Rad Cytokine Assay

The Bio-Plex® Bio-Rad cytokine Assay is a multiplex bead-based sandwich immunoassay that allows the sampling and measurement of secreted cytokines. These beads are able to bind with high affinity to the secreted product and aids in quantification of small concentrations. Bio-plex® Bio-Rad cytokine assay utilizes the Luminex system to analyze 25 cytokines and chemokines simultaneously using a Procarta® cytokine assay kit. The Procarta® cytokine assay kit was developed as a multiplex immunoassay based on Luminex xMAP® technology and it quantitatively
measures cytokine levels in unknown samples of serum, plasma or tissue culture supernatant. The assay kit allows the user to detect cytokine profiles in a 96-well format and for this experiment the manufacturer’s protocol was followed (Figure 22). The cytokine expression profile of the serum and macrophage supernatants was measured using this BioPlex® BioRad Luminex system. Serum standards were diluted in assay buffer and standards for supernatants were diluted in MCM. Standards were prepared in a serial dilution for calibrating the standard curve. Through this process and the Luminex system we are able to definitively conclude the cytokine profile of the samples collected.

5.3.5. Cytokine Multiplex Bead Analytes

Twenty-five cytokines and chemokines were analyzed simultaneously using the Bio-plex® system. The bead analytes are antigen-specific antibodies that are able to bind the antigen with high affinity, even if the antigen concentration is low. A separately labeled antibody (detection antibody) is then added to recognize a different epitope on the first antibody which is then used to detect the bound antigen. Finally the antibody-bead-antigen-detection antibody complex is labeled with streptavidin-PE to be read on the Bio-Plex® Luminex reader (Figure 22).

5.3.6. Calibration and Standard Curves

Quantitation of the bead analytes are completed through standard curves and calibrations. This is important because the Bio-plex assays are multiplex bead-
Figure 22: Bioplex Schematic: Procarta® Cytokine Assay Kit: To test 25 cytokines from an unknown sample the manufacturers protocol was followed,
based sandwich immunoassays, which use the same principle of a sandwich ELISA. A standard curve is constructed to determine the amount of the unknown antigen by using a labeled reference antigen, a standard, which is added at varying amounts. Standard reference samples are prepared and tested along with the unknown samples. Comparison with the standard curve allows the concentration of the antigen in the unknown samples to be calculated. The standard reference samples are derived from a series of known analyte concentrations to construct the signal intensity vs. concentration plot for the standard curve.

5.3.7. Computational Data Analysis

The standard reference samples and the curve-fitting is a critical component when determining the accuracy of the Bio-Plex ® assay results. The mathematical model most used for sandwich immunoassays is the logistic regression model (Baud 1993) which is a non-linear regression. In brief the x-axis represents the log of the concentration (pg/ml) and the y-axis is the response or the relative median fluorescence intensity (MFI). The data collected was analyzed with two logistic equations, four-parameter (4PL) or five-parameter (5PL) to determine the best results. The statistical measure applied to assess the “goodness of fit” is the back calculation of standards or the standard recovery (Nix and Wild 2001).

\[
\text{Observed concentration/expected concentration} \times 100
\]
Using the standard recovery method allows us to calculate the relative error in the sample calculations. This program is automatically installed in the Bio-Plex ® Manager Software.

5.3.8. Statistical Analysis

Unpaired student $t$ tests were used to calculate two-tailed $p$ values between the groups (GraphPad Prism, San Diego, CA). Statistical significance is considered achieved when $p<0.05$ (*), $p<0.01$ (**) or $p<0.001$ (***)

5.3. Results

5.3.1. Normal level of hematopoietic stem cell cytokine IL-3

In the presence of IL-3 macrophages generate and expand from hematopoietic stem cell progenitors (Stanley and Jubinsky, 1984). Therefore to understand the stimulation of macrophage differentiation and expansion it was important to compare the levels of IL-3 expression from Btie2Cre·STAT3FF macrophages and serum to wild-type and STAT1(-/-) mice. When comparing the amount of IL-3 produced by bone-marrow specific STAT3-deficient mice to the wild-type and STAT1-deficient controls, there was no significant difference detected regardless of sample (Figure 23). Therefore these data indicate that Btie2Cre·STAT3FF macrophage progenitors are stimulated and induced to differentiate normally. However, according to previous data there is a significant increase in myeloid hyperplasia in these mice indicating that the regulation of this process is impaired.
**Figure 23: Normal IL-3 Secretion:** There were no significant differences in IL-3 production and secretion in cultured macrophage supernatant (A) or serum (B) when comparing bone marrow specific STAT3-deficient mice to wild-type or STAT1(−/−) controls. Bars are mean ± SEM. (A. n = 12 (WT), n = 7 (Btie2Cre·STAT3FF), n = 9 (STAT1(−/−)); B. n = 10 (WT), n = 5 (Btie2Cre·STAT3FF), n = 10 (STAT1(−/−)))
5.3.2. Decrease of GM-CSF secretion in STAT3-deficient Macrophages

Macrophage proliferation and differentiation is strongly influenced and regulated by the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) (Thorens et al., 1987; Stanley and Jubinsky, 1984). To understand the regulation of proliferation and differentiation signals it was important to examine the levels of GM-CSF among the groups. There was a significant reduction (p<0.01) of GM-CSF production and secretion in Btie2Cre+STAT3FF mice in comparison to wild-type and STAT1(-/-) controls (Figure 24). These data indicate that there is a significant reduction in the regulation of hematopoietic differentiation, which could contribute to the myeloid hyperplasia demonstrated in bone marrow specific STAT3-deficient mice.

5.3.3. Decrease of MCP-3 secretion in STAT3-deficient Macrophages

Monocyte chemoattractant protein (MCP)-3 regulates macrophage function and works with CCR2 to regulate the migration of monocytes from the bone marrow to the blood (Tsou et al., 2007). To understand the regulation monocyte homeostasis it was important to examine the levels of MCP-3 among the groups. In cultured cells, there was a significant reduction (p<0.01) of MCP-3 production and secretion in Btie2Cre+STAT3FF mice in comparison to wild-type and (p<0.05) STAT1(-/-) controls (Figure 24). STAT1(-/-) mice also show a significant reduction (p<0.01) in comparison to wild-type. In the system there was also a significant decrease of MCP-3 in STAT3-deficient mice when compared to (p<0.05) wild-type
Figure 24 GM-CSF and MCP-3. Cytokine production measured from the supernatant of macrophages after 5 days of culture or in the serum collected from cardiac puncture. There is a significant reduction *** (p<0.01) of GM-CSF production and secretion in Btie2Cre+STAT3FF macrophages when compared to wild-type control. There was no significant difference between Btie2Cre+STAT3FF or STAT1(−/−) controls. GM-CSF was normal in the serum. In cultured cells, there was a significant reduction of MCP-3 in Btie2Cre+STAT3FF mice compared to **(p<0.01) wild-type and *(p<0.05) STAT1(−/−) controls. STAT1(−/−) mice also show a significant reduction **(p<0.01) compared to wild-type. In the system there was also a significant decrease of MCP-3 in STAT3-deficient mice when compared to *(p<0.05) wild-type and **(p<0.05) STAT1(−/−) mice. Bars are mean ± SEM. (n = 12 (WT), n = 7 (Btie2Cre+STAT3FF), n = 9 (STAT1(−/−))).
5.3.4. Decrease of IL-1 and IL-6 in STAT3-deficient Macrophages

To understand the acute phase response in bone marrow specific STAT3-deficient macrophages it was important to examine the levels of secreted IL-6 and IL-1β in comparison to the wild-type and STAT1 controls. In comparison with the wild-type, levels of IL-6 and IL-1β are significantly reduced, (p<0.001) and (p<0.01) respectively, in BtieCre*STAT3FF mice, which show that the acute phase response in macrophages is significantly impaired. Additionally, a significant reduction (p<0.001) in IL-6 and (p<0.05) in IL-1β is demonstrated when comparing STAT1-deficient mice to wild-type mice (Figure 25). IL-6 is a potent mediator that stimulates lymphocyte proliferation and differentiation in response to a pathogenic insult (Marks et al., 2006). However, IL-6 has been described as a pleiotropic cytokine based on its ability to activate STAT1 and STAT3, as well as activating proinflammatory and anti-inflammatory pathways (Reindl et al., 2006; Hu et al., 2007). These results indicate that the production of these acute phase response cytokines from macrophages in bone marrow specific STAT3-deficient and STAT1-deficient mice are significantly impaired or being inhibited.
Figure 25: Acute Phase Response Cytokines: Macrophages were cultured for 5 days and supernatant was tested for IL-6 and IL-1β, secreted acute phase response cytokines. There was a significant reduction *** (p<0.001) in IL-6 secretion in Btie2Cre-STAT3FF macrophages, and *** (p<0.001) STAT1(-/-) compared to wild-type. There is also a significant reduction * (p<0.05) in the secretion of IL-1β in Btie2Cre-STAT3FF and * (p<0.05) STAT1(-/-) macrophages. Bars are mean ± SEM. (n = 12 (WT), n = 7 (Btie2Cre-STAT3FF), n = 9 (STAT1(-/-)))
5.3.5. Increase of IL-5 and IL-9 in STAT3-deficiency

IL-5 is a potent eosinophil differentiation and activation factor. Human Crohn’s disease patients present with increased eosinophil numbers, viability and CD44 activity (Lampinen et al., 2008). To understand if this phenomenon also occurs in the bone marrow specific STAT3-deficiency macrophages it was important to examine the levels of IL-5 secreted in comparison to the wild-type and STAT1-deficient controls. There was a significant increase in the systemic expression of IL-5 in Btie2Cre*STAT3FF mice in comparison with (p<0.01) wild-type and (p<0.01) STAT1(−/−) control mice (Figure 26). Additionally there was a local increase in IL-5 from the macrophages of STAT3-deficient mice in comparison with the (p<0.05) STAT1(−/−) control mice. These data indicate that there is a significant increase in eosinophil activity. Thus, this data suggests that the elevation of IL-5 may also be related to eosinophilia and tissue damage.

In the presence of IL-9 there is an increase in mucus production in the gut and eosinophils expand from hematopoietic stem cell progenitors (Noelle and Nowak, 2010). IL-9 has also be considered a regulatory cytokine in the GI due to the monocyte deactivation, inhibition of respiratory burst activity and down regulation of TNFα (Pilette et al., 2002). Therefore to understand the deactivation of macrophages and the upregulation of eosinophil numbers it was important to compare the levels of IL-9 expression from Btie2Cre*STAT3FF macrophages and serum to wild-type and STAT1(−/−) mice. There was a significant increase when comparing the amount of systemic IL-9 produced by bone-marrow specific STAT3-
Figure 26: IL-5 and IL-9 Levels: Macrophages were cultured for 5 days and supernatant was tested for IL-5 and IL-9, serum samples were also tested for systemic levels. There was a local increase in IL-5 from the macrophages of STAT3-deficient mice in comparison with the *(p<0.05) STAT1(/−) control mice. Additionally there was a significant increase in the systemic expression of IL-5 in Btie2Cre+STAT3FF mice in comparison with **(p<0.01) wild-type and ##(p<0.01) STAT1(/−) control mice. There was a significant increase in the IL-9 expression in macrophages of STAT3-deficient mice in comparison to wild-type **(p<0.01) and STAT1(/−) *p<0.05) mice. Additionally there was a significant increase in systemic IL-9 expression in STAT3-deficient mice to the wild-type (p<0.01) and STAT1-deficient (p<0.01) controls. Bars are mean ± SEM. (n = 12 (WT), n = 7 (Btie2Cre+STAT3FF), n = 9 (STAT1(/−)))
deficient mice to the wild-type (p<0.01) and STAT1-deficient (p<0.01) controls. (Figure 26). Additionally there was also a significant increase in the IL-9 expression in macrophages of STAT3-deficient mice in comparison to wild-type (p<0.01) and STAT1(−/−) (p<0.05) mice. Therefore these data indicate that Btie2Cre*STAT3FF macrophage progenitors are stimulated and induced to differentiate normally. However, according to previous data there is a significant increase in myeloid hyperplasia in these mice indicating that the regulation of this process is impaired.

5.3.6. Decrease of TNFα in STAT3-deficient Macrophages

Macrophages receive an apoptotic signal from TNFα that is important for destroying the phagocytosed materials from the site of infection or inflammation. Human Crohn’s disease patients have presented with decrease levels of TNFα secretion from their monocyte-derived macrophages (Smith et al., 2009). To understand if this phenomenon also occurs in the bone marrow specific STAT3-deficiency macrophages it was important to examine the levels of TNFα secreted in comparison to the wild-type and STAT1-deficient controls. There was a significant reduction (p<0.05) in the production of TNFα in Btie2Cre*STAT3FF mice and STAT1(−/−) mice in comparison to wild-type controls (Figure 27). These data indicate that there is a significant reduction in the ability to “kill” phagocytosed materials within the macrophages. Thus, this data suggests that the elevation of systemic TNFα that has been demonstrated in Crohn’s disease may be the result of other activated cells, such as dendritic cells or NK cells. Meanwhile the inability to kill
Figure 27: TNFα Production: Macrophages were cultured for 5 days, and supernatant was analyzed for TNFα secretion. There was a significant reduction *(p<0.05)* in the production and secretion of TNFα in both STAT3- and * (p<0.05) STAT1-deficient mice when compared to wild-type controls. Bars are mean ± SEM. (n = 12 (WT), n = 7 (Btie2Cre*STAT3FF), n = 9 (STAT1+/−)).
phagocytosed materials could result in over activation of the immune cell, in response to a persistent antigenic microorganism.

5.3.7. Normal levels of IFNγ in STAT3-deficient Macrophages

IFNγ, which signals through STAT1, is primarily responsible for priming and activating functional capacities of macrophages including the enzymatic production of ROS, the ability to present antigenic materials and coordinate antibacterial immunity, synthesize new pro-inflammatory cytokines, such as TNFα and IL6, and the synthesis of other IFNγ responsive genes, i.e. FcγRI antibody receptor for specific phagocytosis (Rosenberger and Finlay, 2003; Hu et al., 2007). Therefore it was important to understand the levels of IFNγ secretion from Btie2Cre*STAT3FF macrophages in comparison with the wild-type and STAT1-deficient controls. There was no significant difference detected between the bone marrow STAT3-deficient mice and the wild-type or STAT1(-/-) control mice (Figure 28). These data indicate that the macrophages are still being primed by autocrine or paracrine activity, yet the production of IL-6 and TNFα is significantly reduced (previous data). Additionally, prior data demonstrated a significant reduction in the specific phagocytic activity in these macrophages (chapter 4). Together, these data suggest that there is a dysfunction in the transcription of cytokines after macrophage activation.
Figure 28: IFNγ Levels: IFNγ is important for priming macrophages and inducing the production of additional proinflammatory cytokines, such as IL-6 and TNFα. Macrophages were cultured for 5 days and the supernatant was analyzed for the production of IFNγ. There are no significant differences in the production and secretion of IFNγ, when comparing the STAT3-deficient, STAT1-deficient and wild-type mice. Bars are mean ± SEM. (n = 12 (WT), n = 7 (Btie2Cre⁺STAT3FF), n = 9 (STAT1⁻⁻))
5.3.8. Increase of IL-12p40 and IL-12p70 in STAT3-deficiency

IL-12 is a Th1 cell-mediated proinflammatory cytokine. Therefore it was important to understand the levels of IL-12 secretion from Btie2Cre+STAT3FF mice in comparison with the wild-type and STAT1-deficient controls so that the disease cytokine profile could be identified. In comparison to wild-type control mice there was a local decrease in IL-12p40 from the macrophages of STAT3-deficient mice (p<0.05) and STAT1(−/−) (p<0.01) mice. Additionally there was a significant decrease in the local expression of IL-12p70 in comparison to wild-type in Btie2Cre+STAT3FF mice (p<0.05) and (p<0.01) STAT1(−/−) mice. However, there was a significant increase in the IL-12p40 expression in of STAT3-deficient mice in comparison to (p<0.05) STAT1(−/−) mice while STAT1(−/−) mice were significantly reduced in comparison to wild-type (p<0.05) mice. Additionally there was a significant increase in systemic IL-12p70 expression in STAT3-deficient mice in comparison to the wild-type (p<0.05) and STAT1-deficient (p<0.05) controls (Figure 29). Together, these data suggest that this disease phenotype is a Th1 cell-mediated response.
Figure 29: IL-12(p40) and IL-12(p70) Levels: Macrophages were cultured for 5 days and supernatant was tested for IL-12, serum samples were also tested for systemic levels. In comparison to wild-type control mice there was a local decrease in IL-12p40 from the macrophages of STAT3-deficient mice *(p<0.05) and STAT1(−/−) **(p<0.01) mice. Additionally there was a significant decrease in the local expression of IL-12p70 in comparison to wild-type in Btie2Cre*STAT3FF mice *(p<0.05) and *(p<0.01) STAT1(−/−) mice. However, there was a significant increase in the IL-12p40 expression in of STAT3-deficient mice in comparison to *(p<0.05) STAT1(−/−) while STAT1(−/−) mice were significantly reduced in comparison to wild-type *(p<0.05) mice. Additionally there was a significant increase in systemic IL-12p70 expression in STAT3-deficient mice in comparison to the wild-type *(p<0.05) and STAT1-deficient *(p<0.05) controls. Bars are mean ± SEM. (n = 12 (WT), n = 7 (Btie2Cre*STAT3FF), n = 9 (STAT1−/−))
5.4. Discussion

These studies have demonstrated that there are defective responses from macrophages collected from bone marrow specific STAT3-deficient mice, indicating that there is a defective innate immunity in this model of Crohn's disease. The macrophages tested were cultured for 5 days; therefore it is important to note that the secreted levels of cytokines could not have been suppressed by any humoral factors. It’s been shown that the number of neutrophils circulating was normal in comparison with the wild-type and STAT1 deficient models (see Chapter 3). Therefore, it is possible that the development and responsiveness of other progeny within the myeloid lineage is unaffected, meaning that the secretory level changes of cytokines are localized to the macrophages, thus indicating that these cells are dysfunctional within the innate immunity.

It is possible that local weak inflammatory responses can provoke a systemic proinflammatory state similar to what is seen in human Crohn’s disease. It was demonstrated that the bone marrow STAT3-deficient mouse produced normal levels of IL-3 for hematopoietic differentiation, but the regulating factors, GM-CSF, that define the generated macrophages were significantly decreased. It is possible that the monocytes and macrophages that are working within the gastrointestinal system are immature, and dysfunctional.

GM-CSF is important for the macrophage to initiate macropinocytosis and to stimulate the ruffling or projection of phagocytic arms to ingest surrounding materials. Myeloid derived cells share a common progenitor within the bone
marrow, this means that monocytes, macrophages and granulocytes all respond to the same restricted growth factors, such as granulocyte-macrophage colony-stimulating factor (GM-CSF). GM-CSF is important for the regulation of macrophage function and also for the stimulation of macropinocytosis through the actin rich ruffles needed to ingest particles. The cytokine in control of regulating the differentiation of macrophages from their hematopoietic precursors is also GM-CSF.

The ability to release acute response cytokines (IL-6 and IL-1β) are significantly reduced and possibly contributing to the chronic, slow developing intestinal inflammation that is seen within these bone marrow deficient STAT3 mice. IL-6 is also a potent mediator of lymphocyte proliferation and differentiation that is secreted by macrophages. This cytokine also induces the secretion of acute-phase proteins from the liver.

The phenotype and activation of macrophages is being affected and altered in the STAT3 deficient mice. Although IFNγ is a potent and endogenous activator of macrophages, the macrophages themselves are dysfunctional. Macrophages are potent producers of cytokines that are critical for the induction of a T cell response, such as IL-12 and IL-23. The loss of STAT3 in hematopoietic stem cells demonstrates a systemic increase of IL-12(p40) and IL-12(p70). However, the local macrophage environment shows a reduction of IL-12 in STAT3- and STAT1-deficient mice. This indicates that the disease model is a Th1 cell-mediated response, but macrophages are not primarily associated with this increase of IL-12 in spontaneous CD-like pathogenesis in STAT3-deficient model.
IFNγ is within normal limits in the Btie2Cre*STAT3FF macrophages, which have activated STAT1 signaling. This cytokine is responsible for activating the enzymatic production of reactive oxygen species and it primes the macrophages to increase the ability to destroy pathogens. Additionally, it enhances macrophages ability to coordinate antibacterial immunity and synthesize IL-12 and TNFα and other IFNγ responsive genes like FcγRI antibody receptor for phagocytosis. (Rosenberger and Finlay 2003). Low concentrations of IFNγ create a positive feedback loop and increased levels of STAT1 activation enhance responses. IFNγ triggers the synthesis of TNFα in macrophages, but the Btie2Cre*STAT3FF macrophages present with a reduced secretion of TNFα, along with macrophages from CD patients (Smith et al., 2009). This phenomenon may be explained by the theories: (1) immature macrophages are activated that cannot synthesize TNFα, or transport it out of the cell, (2) transcription of the signal is impaired through dysfunctional IFN receptors (α and β chain) or there is a dysfunction downstream of the STAT activation. Therefore the primed and active macrophages lose the ability to regulate the intestinal immunity effectively.

Meanwhile, the possibly immature and dysfunctional macrophages are being activated and primed by IFNγ signaling and then cannot effectively initiate the production and secretion of TNFα for the ability to apoptose. Phagocytic cells are important to remove debris and pathogenic materials from the site of infection or insult. Macrophages receive an apoptotic signal from TNFα that is important for destroying these materials. If these materials aren't removed through apoptosis
secondary secretion of proinflammatory cytokines could drive the development and initiation of chronic inflammation.

These data confirm what other researchers have concluded that macrophages in CD secrete abnormally low levels of TNFα. Although this might seem contrary to the elevations of systemic TNFα that is seen, this might be contributed to the excessive number of macrophages that are circulating within the system. Therefore, it is possible that these poorly differentiated, and dysfunctional macrophages have lost the ability to perform programmed death and are immortal.

Based on prior data, it appears that excessive dysfunctional macrophages are being primed and activated but they cannot produce and secrete the right cytokine profile. Macrophages in CD do not secrete the normal quantities of proinflammatory cytokines (Smith et al., 2009). Therefore it can be argued that the weak immune response of these macrophages leave the system prone to an enhanced and increased response to a secondary immune responders, i.e. Th1 and Th17 cells that are stimulated to “save the day”.
CHAPTER SIX

CONCLUDING DISCUSSION
6.1. Abnormal Macrophage Functions Are Essential for CD Like Phenotype

Over 30 years ago, Segal and Loewi suggested that there was some deficiency in the acute inflammatory response and defective neutrophil recruitment in Crohn's Disease (Segal and Loewi, 1976). However, Crohn's research, up until recently, had been focused on T cell activation disorders because of the Th1-mediated cytokine profile of the disease (Xavier and Podolsky, 2007). In 2003, Welte and colleagues demonstrated that STAT3-deletion was crucial to the innate immunity and this deficiency lead to a Crohn's disease-like pathogenesis (Welte et al., 2003).

Myeloid-derived innate immune cells specifically correlate with Crohn's disease (CD) due to a loss of STAT3 activity and consequently result in defective innate immunity. It has already been demonstrated that macrophages, rather than T cells, are important in the initiation of CD (Zhou et al., 2009). In this study, four week old mice with bone marrow specific STAT3-deficiency demonstrated local and systemic abnormalities in innate immune cell accumulation and immune responsiveness. Additionally, the functional ability of early stage macrophages to phagocytose materials was shown to be significantly reduced in the four week old STAT3-deficient model.

Interestingly, the functionality of these macrophages appears to recover within normal limits at eight weeks of age, in the midst of a full inflammatory infection of the intestines. This indicates that an early dysfunction and dysregulation of the innate system is to blame for a highly reactive secondary, adaptive, immune response that is maintaining intestinal inflammation. At this
time, mice are no longer reliant on the innate immunity to destroy pathogenic insult but to turn off the secondary, adaptive, inflammatory responses.

Data shown here indicates that there is an increased number of macrophages and myeloid derived granulocytic cells that are circulating in the blood, differentiating in the bone marrow, and infiltrating in the tissues. Therefore it is important to note that other myeloid lineage cells may be contributing to the CD-like phenotype. However, the increase in these myeloid-derived cells has not increased the effectiveness of the immune system, thus these cells might also be dysfunctional or deemed to be immature.

There is a possibility that other myeloid lineage cells, such as dendritic cells, can contribute to the CD-like phenotype that is seen within the STAT-3 deficient model. This study was limited to the exploration of macrophages and should be continued to other specialized cells within the innate immune system. Therefore, these further studies can illuminate the true cell that is critical for the pathogenesis of CD. However, what we can conclude from this research is that the innate immunity is dysfunctional in the early stages of this CD-like phenotype and can be attributed to the pathogenesis of this disease.

The current theory that Crohn’s disease is associated with a failure to regulate inflammation induced by commensal gut bacteria and subsequently resulting from T cell-mediated inflammation is not entirely true (Korzenik, 2007). T cell responses develop slowly and require the induced synthesis of effector molecules, initially produced and secreted by the innate immune cells (Medzhitov and Janeway, 1997). Therefore it is important to note that even though T cells may be major mediators in maintaining the disease phenotype they are not the primary
initiators. Defective STAT3-deficient T cells have no enhanced responsiveness within intestinal immunity (Takeda et al., 1998; Welte et al., 2003), but as my data has shown the loss of STAT3 activity within the innate immunity, specifically in macrophages, contributes to an intestinal inflammation similar to Crohn’s disease.

STAT activation and signaling is necessary for polarizing the secondary T cell response either toward a Th1-mediated response, primarily producing IFNγ, or a Th2-mediated response, primarily producing IL-4 (Mudter and Neurath, 2003). STAT1 activation, which is elevated in monocytes and neutrophils within human colitis, has been shown promote Th1 cell mediated colitis due to the transcription factor T-bet (Schreiber et al., 2002; Neurath et al., 2002). Concurrently, in mucosal inflammation T cells express an increased activation of STAT3, contributing to anti-apoptotic signals and accumulation of cells in the mucosa (Atreya et al., 2000; Lovato et al., 2003); my data demonstrates that there was a significant reduction in the number of lymphocytes that were circulating in the periphery suggesting that the loss of STAT3 promoted an apoptotic fate for the T cells. Alternatively, there T cells could have failed to form. These data provide evidence that the loss of STAT activity within T cells is not detrimental to the function of mucosal immunity, but it is the increased expression of STAT1, and the loss of STAT3 expression in myeloid-derived cells, namely macrophages, that is crucial to inflammation.

B-cell activation is associated with Th2 mediated inflammation. Th2 cells secrete IL-4, IL-5, IL-9 and IL-13, as well as having CD40 ligand on their surface (all of which activate B cells) and IL-10 which inhibits macrophage activation. CD40 is
involved in the activation of B cells and macrophages; the binding of CD40 on B cells promotes growth and isotype switching, whereas on macrophages CD40 induces them to secrete TNF-α and become receptive to much lower concentrations of IFN-γ. In 1997, Mizoguchi and colleagues investigated B cells in intestinal inflammation of T cell receptor (TCR)-α chain deficient mice and discovered a protective, rather than pathologic, role (Mizoguchi et al., 1997; Strober et al., 2002).

Within this study, I investigated CD19STAT3FF mice, which exhibit a STAT3 deficiency in the B cells, and there were no gross abnormalities to report (data not shown). These data support the conclusion that B lymphocytes are not involved in intestinal inflammation; the key mediators are CD4+ Th1 cells (Davidson et al., 1996; Reindl et al., 2007).

6.2. STAT3 Signaling Is Required for Normal Macrophage Functions

In 2005, Musso and colleagues demonstrated that STAT3 activation was restricted to inflamed areas and detected in macrophages and T cells, but not neutrophils (Musso et al., 2005). The loss of STAT3 activity in macrophages inhibits the production of a critical regulatory cytokine, IL-10 (Takeda et al., 1999). This study also presents data that demonstrates that the loss of STAT3 activity results in dysfunctional macrophages during the early stages of mucosal immunity.

Both STAT1 and STAT3 have been implicated as mediators of inflammatory bowel disease (Schreiber et al., 2002; Welte et al., 2003; Musso et al., 2005; Fu, 2006). STAT1 is an important mediator of IFNγ signaling, as well as Th1 cell differentiation and macrophage priming (Durbin et al., 1996; Meraz et al., 1996;
Leonard and O'Shea, 1998). The loss of STAT3 activity in macrophages mimics an elevated activation of STAT1 in macrophages, due to unopposed signaling, which leads to chronic inflammation, similar to Crohn’s disease (Schreiber et al., 2002). In these studies, STAT1-deficient mice demonstrated enhanced functional capabilities in early stage macrophages, indicating that the active STAT3 protein is essential to this myeloid-derived cell.

At the early stages of immunity macrophages from STAT1-deficient mice had a significantly increased functional capacity for phagocytosis and ROS production. This normalized at eight weeks, when the STAT3-deficient mice phenotypically presented with a CD-like disease. This occurrence can show the dichotomous nature of STAT1 and STAT3, identifying that the loss of STAT3 presents with a reduction of macrophage function, while the loss of STAT1 (the unopposed activity of STAT3) can lead to a significantly increased function within early macrophages. Although these two phenomena normalize at 8 weeks (the onset of disease) it is most likely that this change in early development results in CD-like pathogenesis.

STAT3 is a very important cell cycle control protein that is required for the induction of a G1 cycle arrest in granulopoiesis and it is equally as important in growth-factor induced myeloid differentiation through GM-CSF (Miranda and Johnson, 2007). In 1999, Takeda and colleagues demonstrated that mice with a conditional STAT3-deficiency in myeloid-derived cells, specifically macrophages and neutrophils, exhibit an impaired anti-inflammatory response (Takeda et al., 1999). Those data suggest that STAT3 plays an important role in the function of mature myeloid cells (Takeda et al., 1999; Miranda and Johnson, 2007). Although STAT3
may not be required for neutrophilic differentiation, it’s important for regulating neutrophilic numbers (Lee et al., 2002, Miranda and Johnson, 2007). Therefore the myeloid hyperplasia demonstrated within the bone marrow and the increase of myeloid derived cells within the peripheral blood of the STAT3-deficient mouse model used in this study can be primarily attributed to the loss of STAT3 activity.

The STAT3-deficiency in bone marrow hematopoietic stem cells presented with significantly increased myeloid hyperplasia and a predominant number of neutrophils in bone marrow in comparison to wild type and STAT1-deficient controls. Additionally, eosinophil precursors were frequent and conspicuous along with megakaryoblasts. These data demonstrate that there was a marked increased in myeloid derived cells present within the bone marrow.

Within STAT1-deficient mice the myeloid to erythroid ratio was more appropriately balanced, with normal differentiation in both series. Eosinophils were scattered but present and megakaryocytes were infrequent. There was no apparent myeloid hyperplasia present. Meanwhile wild-type mice presented with mild myeloid hyperplasia and normal cellular differentiation, both erythroid and megakaryocyte lineages were within normal limits. Therefore, from these data, it can be said that STAT3 is important for regulated myeloid differentiation and production within the bone marrow, and subsequent circulation within the peripheral blood. Dysregulation of this capability contributes to a Crohn’s like phenotype.
Bone marrow specific STAT3-deficiency produced macrophages that had a significant reduction in non-specific and specific phagocytosis, which is the primary function of the macrophages (Henson et al., 2001; Savill et al., 2002). Both specific and non-specific functions were restored at the time of disease, therefore there it is a possibility that this can be attributed to immature early stage macrophages, and the functional recovery is due to the maturation, or that the actual number of immature macrophages have increased due to infiltrating myeloid-derived cells. Based on data collected in this study, macrophages precursors, myelomonocytic cells, are in excess. Therefore it can be concluded that an elevation in dysfunctional macrophage numbers leads to dysregulation of the innate immune system and correlates with Crohn’s disease-like pathogenesis.

Functional properties of macrophages rely not only on their ability to phagocytose materials but also the production of reactive oxygen species (ROS) through respiratory bursts (Rugtveit et al., 1995). The cytokine, IFNγ has been attributed to stimulating and priming the macrophages to induce the release of ROS, meanwhile the over production of ROS can lead to tissue damage and contribute to the intestinal erosion seen within CD. GM-CSF was also thought to alter the production of ROS through the maturation and increased viability of the cells, but it has been shown that GM-CSF is not needed (Rugtveit et al., 1995; Coleman et al., 1988). This study demonstrated that at four weeks, the bone marrow STAT3-deficient mouse was unable to produce a normal ROS response. Interestingly this dysfunction was recovered at eight weeks, during the presence of disease. STAT3-deficient mice also demonstrated a failure to reduce nitroblue tetrazolium (NBT),
which indicates that there is a loss of oxidative reductive reactions within the macrophages. Redox reactions are a result of NADPH activities and the generation of ROS, therefore the failure to reduce NBT correlates with a failure of the normal redox reactions (Baehner et al., 1976; Pompeia et al., 2003).

These data demonstrated that at four weeks, the bone marrow specific STAT3-deficient mouse was unable to produce a normal “killing” response. But, during the presence of disease, this dysfunction was recovered. These data suggest that these macrophages are functionally immature at four weeks, and a persistence of pathogenic insult, and macrophage activation, can cause an excessive secondary immune response leading to tissue damage and chronic inflammation, as seen in CD.

6.3. Dysfuntional Macrophages Alters Local Cytokine Profiles

IL-3 and GM-CSF are synthesized and released to act on bone marrow hematopoietic stem cells to stimulate the production of macrophages and granulocytes, both of which are important nonspecific effector cells in innate and adaptive immunity (Mahida, 2000). When comparing the amount of IL-3 produced, bone-marrow specific STAT3-deficient mice are comparable to the wild-type and STAT1-deficient mice. This suggests that the stimulation of myeloid progenitors is normal, but based on bone marrow studies it has been demonstrated the myeloid to erythroid ratio is abnormal in STAT3-deficient mice, with a significantly increased production of myeloid progenitors.

Myeloid derived cells share a common progenitor within the bone marrow, this means that monocytes, macrophages and granulocytes all respond to the same
restricted growth factors, such as granulocyte-macrophage colony-stimulating factor (GM-CSF). GM-CSF is important for the regulation of macrophage function and also for the stimulation of macropinocytosis through the actin rich ruffles needed to ingest particles. The cytokine in control of regulating the differentiation of macrophages from their hematopoietic precursors is also GM-CSF. Bone marrow specific STAT3-deficiency produced macrophages with a significantly reduced production of GM-CSF which may correlate to the functional loss in the macrophage activity.

Dysregulated cytokine responsiveness in myeloid cells can trigger mucosal inflammation like Crohn's disease (Reindl et al., 2007). In the most acute immune responses it is likely that both innate and adaptive immune cells will be involved in an effective response and the decision to differentiate into the needed cells is made early. One important determinant of the differentiation pathway is the mix of cytokines produced by cells of the innate immune system.

IL-6 is also a potent mediator of lymphocyte proliferation and differentiation that is secreted by macrophages. This cytokine also induces the secretion of acute-phase proteins from the liver. The ability to release acute response cytokines (IL-6 and IL-1β) are significantly reduced and possibly contributing to the chronic, slow developing intestinal inflammation that is seen within bone marrow deficient STAT3 mice. Upon activation by signals released from microorganisms or from infected tissues, macrophages are stimulated to release an array of cytokines and chemokines to attract neutrophils, eosinophils and other effector cells from the adaptive immunity to respond to the offense. Macrophages not only function on the
external environment but they also have the ability to stimulate themselves to better respond to the insult at hand. The inability to produce an acute phase response contributes to a chronic state of inflammation.

These studies demonstrated an increased production of IL-5 which is a classical Th2 cytokine. This cytokine elevation may be the contributing factor to the significant elevation in eosinophils that have been previously described circulating within the peripheral blood as well as the hematopoietic derived myeloid cells that were upregulated in the STAT3-deficient mouse. Rothernburg et al., 2001 demonstrated that eosinophil accumulation was associated with weight loss and an increased production of IL-5 signaling. These data also support the theory that the regulation of innate, regulatory and inflammatory immune responses are important to the pathogenesis of CD.

Phagocytic cells are important to remove debris and pathogenic materials from the site of infection or insult (Maderna and Godson, 2003). Macrophages receive an apoptotic signal from TNFα that is important for destroying these materials. If these materials aren’t removed through apoptosis secondary secretion of proinflammatory cytokines could drive the development and initiation of chronic inflammation. These data confirm what other researchers have concluded that macrophages in CD secrete abnormally low levels of TNFα (Smith et al., 2009). Although contrary to the elevations of systemic TNFα that is seen, this might contribute to the excessive number of macrophages that are circulating within the system.
IFNγ is comparable to normal in the bone marrow specific STAT3-deficient mouse. This cytokine is responsible for activating the enzymatic production of ROS within macrophages and primes the macrophages to increase the ability to destroy pathogens (Rugtveit et al., 1995). It enhances macrophages ability to coordinate antibacterial immunity and synthesize IL-12 and TNFα. Other IFNγ responsive genes are FcγRI antibody receptor for phagocytosis (Rosenberger and Finlay, 2003). Low concentrations of IFNγ create a positive feedback loop and increase levels of STAT1 activation which enhances responses including Th1 cell mediated responses.

In these studies bone marrow specific STAT3-deficient mice demonstrate a reduced secretion of TNFα, although IFNγ secretion is comparable to normal. Possible explanations for this phenomenon are: (1) immature macrophages are activated that cannot synthesize TNFα, or transport it out of the cell (Smith et al., 2009), (2) transcription of the signal is impaired through dysfunctional IFN receptors (α and β chain) or (3) there is a dysfunction downstream of the STAT activation. Regardless of the cause, these dysfunctional macrophages in STAT3 deficient mice are producing defective local cytokine profiles that correlate with CD-like pathogenesis.

6.4. Innate Immunity Plays Important Roles in CD Phenotypes

Systemic Th1-mediated responses can clear persistent pathogens but can also produce an inflammatory response that can damage the surrounding mucosa; therefore they are triggered as secondary responders by the surveying innate immunity. Regulatory mechanisms from the innate immunity, specifically
macrophages, restrict immune responsiveness from aggressive T cells; if these regulatory mechanisms, IL-10 or TGF-β signaling, were compromised strong inflammatory T-cell responses can commence (Welte et al., 2003; Smith et al., 2009). This can lead to an overactivated Th1-cell mediated response and subsequently chronic mucosal inflammation such as Crohn’s disease.

Differentiation of the Th1 subset is promoted by the activation of IFNγ/STAT1 in antigen-stimulated naïve T cells. The IFN-γ gene in resting naïve CD4 T cells is switched off but the required level of IFN-γ needed to promote the differentiation of these T helper cells can initially be produced and secreted by cells of the innate immune system, such as macrophages. STAT1 also induces the expression of another transcription factor, T-bet, which activates IFNγ transcription and also induces the expression of the IL-12 receptor. These T cells are now committed to becoming Th1 cells. The cytokine IL-12, also produced macrophages, can then act through this T cell expressed receptor, initiate a signaling pathway that will promote the expansion and differentiation of Th1 cells therefore polarizing the system to a Th1 mediated cytokine profile. These cells will then start to produce copious amounts of IFN-γ thus reinforcing the signal for the differentiation of more Th1 cells. These cells would not be activated if it weren't for the signals received from the innate immunity. These data demonstrated that macrophages from bone marrow specific STAT3-deficient mice still secrete the normal levels of IFNγ, therefore they can contribute to the initiation of a Th1 cell polarized response.

The CD4+ Th17 cells are distinguished by their ability to produce the cytokine IL17, and they have recently been recognized as distinct effector lineage
that is contributing to the Crohn’s disease phenotype. Naïve CD4+ T cells become polarized Th17 cells when stimulated by both IL-6 and transforming growth factor (TGF)-β that are secreted by macrophages along with IL-9 (Elyaman et al., 2009). These cells express the receptor for the cytokine IL-23, rather than the receptor for IL-12 expressed by the Th1 cells. In these studies the systemic production of IL-23 is within normal limits in the STAT3-deficient model (data not shown). However, within Btie2Cre*STAT3FF macrophages the IL-23 production is slightly reduced in comparison to wild-type and STAT1-deficient mice. The presence of IL-23 maintains the Th17 cells population, but the differentiation is enhanced by a potent IL-9 amplification loop that also protects the cells from apoptosis. Therefore this loss in STAT3 activity highlights a very important role it plays in IL-9 induced differentiation of Th17 cells and the prevention of T cell apoptosis (Demoulin et al., 1996; Yang et al., 2007).

A unique CD4+ T cell population that induces an abnormally large amount of IL-9 and IL-10 production with no regulatory effects has previously been described (Dardalhon et al., 2008; Elyaman et al., 2009) and is capable of causing tissue inflammation. This cell population would explain the cytokine profile, increased IL-9 and IL-10, which is present in these STAT3-deficient mice. It would also explain the reason for an unclassified cytokine profile that is demonstrated as neither Th1, Th2 nor Th17 within the system. Regardless of the systemic changes my studies indicate that the innate immunity plays a more important role in the pathogenesis of CD-like inflammation as in this model.
Data in this study indicate that STAT3-deficient mice produce severely dysfunctional macrophages that produce an abnormal cytokine profile and subsequently lead to a dysregulated mucosal immunity. These data demonstrated significant decreases in GM-CSF and MCP-3 which are both important regulators for macrophages differentiation, proliferation and maturation, which support the theory of immature macrophages in STAT3-deficiency that may be producing an abnormal cytokine profile. Additionally, there is an increased local and systemic expression of IL-5, an eosinophil differentiation activation factor, which contributes to an increase in eosinophil numbers. This elevation in can also contribute to an elevation of IL-9, which promotes eosinophil viability and CD44 activity and therefore can contribute to tissue damage and destruction. These data indicate that the functional loss in macrophage activity also correlates with a severely altered macrophage cytokine profile. Together these data suggest that the dysfunction in innate immunity is prevalent and necessary for the development of Crohn's disease-like pathogenesis.

Macrophages and the entire innate immunity are important to maintain intestinal homeostasis and mucosal immunity, any interruption to these functional capacities can lead to chronic inflammation and disease. Therefore, future studies are needed to understand the interaction of macrophages and T cells within this STAT3-deficient model. It is necessary to identify if these macrophages are able to present antigenic materials to the adaptive immunity and therefore mediate the subsequent response. Meanwhile it is still necessary to analyze the macrophage produced chemokines, responsible for T cell migration, and the cytokines,
responsible upregulating T cell polarization. For that reason, it is also important to examine microarrays of macrophage mRNA to truly understand cytokines are being transcribed and trapped or destroyed in the cell, or if there is a dysregulation of cytokine transcription. My data supports the theory that macrophages are a crucial cellular component within mucosal immunity; dysregulation of these cells due to a loss of STAT3 activity in hematopoietic stem cells results in Crohn’s disease-like pathogenesis. Therefore macrophages should be targeted for therapeutic intervention and further studies within Crohn’s disease.


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