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The Roles of Notch1 and PKC-Θ in Immune Mediated Bone Marrow Failure

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THE ROLES OF NOTCH1 AND PKC-Θ IN IMMUNE MEDIATED BONE MARROW FAILURE

A Dissertation Presented

by

JUSTINE E. RODERICK

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2011

Program in Animal Biotechnology and Biomedical Science
THE ROLES OF NOTCH1 AND PKC-Θ IN IMMUNE MEDIATED BONE MARROW FAILURE

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DEDICATION

To anyone who has ever lost a loved one to aplastic anemia, your stories have been the inspirations for this research.
ACKNOWLEDGMENTS

It takes the support of an entire department, friends and family to make it through the pains of graduate school. With that said there are many people I need to thank. First and foremost I need to thank my mentor Lisa Minter she has provided all of the tools I needed to become a confident scientist, a far cry from the insecure undergraduate I was when I entered the program. In addition to being my advisor she has been like a second mom to me, which I will always be grateful for. I also need to thank my second PI Barbara Osborne. I’ve been lucky to have her presence in my scientific career, I’m glad that she had faith in me when I was unsure of myself. The success of this research would not have been possible without the support of my thesis committee and the entire VACSI department. Although I began my career as an only child so to speak I now enjoy and appreciate the company of my lab mates. I especially need to thank Tina, she has been an excellent lab mate, friend and punching bag. In all seriousness she has become my little sister and I will miss her dearly. Gaby has been a tremendous asset and instrumental in completing my experiments and is by far the best “friend” checker I have ever had the pleasure of knowing. All members of “Osguiter” quadrant have been great friends and distraction makers. Who better to commiserate with than your fellow graduate students?! Remember Minter lab “GO BIG OR GO HOME!” Last but certainly not least I need to thank my family for all of their love and support including, but not limited to, my mother, father and brother. They have listened to all of my relentless whining and scientific findings over the past five years. I also have to thank my boyfriend Tim and our cohort of furry children. I must say that no one has had to steady my hand more times or provide more moral support than him; this body of work is proof of that.
ABSTRACT

THE ROLES OF NOTCH1 AND PKC-Θ IN IMMUNE MEDIATED BONE MARROW FAILURE

May 2011

JUSTINE E RODERICK, B.S., UNIVERSITY OF MASSACHUSETTS AMHERST
Ph.D., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Professor Lisa M. Minter

We sought to evaluate the individual contributions of Notch1 and PKC-Θ to disease progression in a mouse model of immune-mediated bone marrow failure and to define a mechanism for their potential cellular cooperation. We transferred parental bulk splenocytes into F₁-hybrid recipients to induce a robust immune-mediated bone marrow failure (BMF) that we could partially rescue by administering a pharmacological inhibitor of Notch activation. Transferring splenocytes from PKC-Θ⁻/⁻ animals did not induce disease, and treating animals with a pharmacological inhibitor of PKC-Θ also provided full protection from disease. We found that inhibiting Notch1 resulted in PKC-Θ down-regulation, and blocking PKC-Θ reduced Notch1 activation, possibly within a positive feedback loop. Our data suggest that both Notch1 and PKC-Θ contribute to disease progression in our mouse model of immune-mediated bone marrow failure. Furthermore, additional findings from the lab demonstrated physical interactions between Notch1, members of the T cell signalosome and PKC-Θ that are essential to mediating full activation of T cells following signaling through the TCR and CD28. Notch1 and/or PKC-Θ may represent novel therapeutic targets in the treatment of bone marrow failure.
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CHAPTER 1

INTRODUCTION

The adaptive immune system has developed evolutionarily into a complex system that maintains self-tolerance while shielding the body against foreign invaders. Key players in the regulation of the adaptive immune system are T cells. T cells are a subset of lymphocytes which develop in the thymus and are known for their ability to identify a diverse number of pathogens. Defects in self-tolerance can arise when there are malfunction in the regulation of T cell development. In order to preserve the essential machinery responsible for self-tolerance the adaptive immune system has developed a series of selection phases to remove autoreactive T cells. Loss of self-tolerance can lead to the activation and expansion of autoreactive T cells. As a result of this abnormal activation of the immune system, autoimmunity may develop. Autoimmune disorders result in the destruction of many different tissues by one’s own immune system.

For hundreds of years the thymus was an organ of unknown purpose, in fact there were many studies which concluded that it had no immune function at all (Bilingham et al. 1962). Today we know that the thymus is an essential organ in the immune system that is responsible for T cell development. T cells begin their life in the bone marrow as a result of hematopoiesis. Once they become common lymphoid progenitors (CLPs), T cells migrate to the thymus where they develop into mature T cells. Part of the maturation process is the elimination of autoreactive T cells. Autoreactive T cells are removed through two processes, positive and negative selection. In the thymus T cells encounter
antigen presenting cells such as epithelial cells, dendritic cells and macrophages, which present antigens, in the context of the major histocompatibility complexes, to the T cells. During positive selection immature T cells are probed for their ability to bind self MHC. T cells that lack the ability to bind self MHC will receive a signal to undergo apoptosis. T cells that survive positive selection then undergo negative selection, which is vital to maintaining self-tolerance. During negative selection T cells that have high-affinity receptors for self-MHC with or without self-antigen will undergo apoptosis. These processes effectively remove any T cells that responded to self, thus preventing the development of an autoimmune disorder. There is one exception in this process that leads to the development of regulatory T cells. The exact mechanisms and time line of positive and negative selection are not completely defined, but it is believed that in some cases they occur simultaneously.

T cell-mediated autoimmune disorders are believed to arise after an initiating event. An example of an initiating event is the exposure to a virus, which presents an antigen that leads to development of autoreactive T cells. It is also hypothesized that patients that develop autoimmune disorders have an underlying predisposition which contributes to the disease development. Each autoimmune disorder is unique in its target organ, such as the beta cells in diabetes or the central nervous system in multiple sclerosis. Although each of these diseases varies in origin and targeted tissue destruction, T cells appear to play an important role in mediating disease.

The objective of this dissertation is to dissect T cell activation and polarization as they occur in the context of autoimmune disorders, such as Aplastic Anemia (AA). Although there are many mechanism that are important in T cell activation and
polarization, the focus of these experiments is on molecules that have been documented
to play a role in the development of a $T_H1$ phenotype. The results from these studies may
potentially lead to the discovery of new therapeutics that can inhibit T cell activation,
dampening immune responses which, in turn, reduce self-mediated destruction in
autoimmune disorders.

1.1 T Cell Function and Differentiation

1.1.1 T Cell Activation

Mature T cells become activated when there is engagement of their T cell receptor
(TCR) by a foreign peptide. The peptide is presented to the T cell in a self-MHC
molecule that is recognized by either the CD4 or CD8 receptor. To achieve robust
activation T cells require a co-stimulatory signal. The major co-stimulatory receptor on T
cells is CD28. CD28 binds to its co-stimulatory ligand, CD80/CD86, on antigen
presenting cells (APC) (Chambers and Allison, 1999). Once the T cell receives the
appropriate signals there is a cascade of phosphorylation events that leads to the
activation of downstream genes, such as $il2$. In this cascade Zap70 signals to several
adaptor molecules, including LAT, SLP76, Vav, Grb7, PI3K, and PLCγ-1 (Lin et al.,
2004). Ultimately these signals aid in mediating the formation of the immunological
synapse which is required for sustained TCR engagement with antigen presenting cells
(Valittui et al., 1995). T cell activation regulates several key transcription factors such as
activated protein-1 (AP-1), nuclear factor of activated T cell (NFAT) and nuclear factor
kappa-light-chain-enhancer of activated B cells (NF-κB). These transcription factors are
vital to maintaining T cell expansion through the production of the cytokine interleukin-2 (IL-2).

1.1.2 T Cell Subsets

T cells are divided into two major groups based on the MHC class type that they recognize. CD4+ T helper cells express the receptor CD4 which recognizes class II MHC that is present on APCs. CD4+ T cells can develop into a series of different types of T helper cells, outlined in Figure 1.1, including but not limited to Th1, Th2, Th17, Tregs. CD8+ cytotoxic T cells express the CD8 receptor which recognizes class I MHC molecule that is expressed on the majority of cell types.

Once activated T helper cells mediate immune responses by secreting cytokines that activate B cells and CD8+ cytotoxic T cells. T helper cells are characterized by the cytokines that they produce as effector cells. Different CD4+ T cell subsets arise as a result of the cytokine milieu which is present during their activation. Th1 cells are responsible for the recognition of intracellular viruses and bacteria; they are also the dominating cell type in the development of autoimmune bone marrow failure syndromes (Solomou et al., 2006). Th1 cells secrete IFN-γ and are regulated by the transcription factor tbx21 (T-bet) (Rangarajan et al., 1996; Szabo et al., 2000; Mosmann, 2006; Abbas, 2006). Th2 cells are required for humoral immunity and the removal of extracellular pathogens. They produce IL-4 and IL-5 and are regulated by the transcription factor gata3 (Zheng, Flavell, 1997). Th17 cells are a relatively newly-defined T cell subset that produces proinflammatory cytokines such as IL-17 and IL-22 (Liang et al., 2006). They aid in the clearance of extracellular bacteria and fungi. Additionally Th17 cells have been
shown to play a role in autoimmune disorders such as multiple sclerosis (Mai et al., 2010). T\textsubscript{H}17 cells are polarized under the transcription factor \textit{rorc} (Ivanov et al., 2006). T regulatory (Treg) cells differ from the other CD4\textsuperscript{+} helper subsets because their function is to dampen immune responses that are associated with excessive inflammation and autoimmunity (Kleinschek et al., 2010; Mai, 2010). Tregs are regulated by the transcription factor \textit{foxp3} (Hori et al., 2003; Fontenot et al., 2003, Khatti et al., 2003).

Recently there has been the discovery of additional CD4\textsuperscript{+} T helper cells such as, T\textsubscript{follicular} cells, T\textsubscript{H}9 and T\textsubscript{H}22.

CD8\textsuperscript{+} cytotoxic T lymphocytes (CTL) arise in a fashion similar to CD4\textsuperscript{+} cells but have a completely different function. Unlike CD4\textsuperscript{+} cells CTLs have a lytic ability. CD8\textsuperscript{+} cells are crucial to the recognition and destruction of altered self-cells that are infected with intracellular bacteria, virus or protozoa. CTLs have also been shown to mediated tissue damage in autoimmune disorders, such as diabetes (Wang et al., 1991, Deckert et al., 2010). Once CTLs are activated they have the capacity to release cytotoxic granules and kill infected cells.

Within cytotoxic granules there are two proteins that regulate cell mediated destruction. Granzymes are a family of distinct serine proteases that mediate cell death. Granzyme B is the most abundant and, therefore, much of the research in the literature is focused on this family member. Granzyme B in particular has been shown to be necessary for effective CTL function; in its absence CTL mediated killing occurs at a much slower rate (Heusel et al., 1994; Pardo et al., 2004). Granzyme B mediates apoptosis through two mechanisms, one is through direct activation of caspase 3 (Adrain et al., 2005; Cullen et al., 2005; Martin et al., 2007; Cullen \textit{et al} 2008) and another is
through the release of cytochrome C (Barry *et al.*, 2000; Sutton *et al.*, 2000; Almonti *et al.*, 2001).

The second important member of CTL-mediated destruction is the pore forming protein, perforin. Perforin is also found in the granules of CTLs. Upon degranulation perforin is released. It then inserts itself into the plasma membrane of the target cell, thus forming a pore. In the absence of perforin there are major defects in CTL-mediated killing by granzyme B (Kagi *et al.*, 1994; Lowin *et al.*, 1994). Thus granzyme and perforin play dual roles in the destruction of target infected cells.

### 1.1.3 T\_H1 and CTLs in Autoimmunity

Autoimmune disorders such as multiple sclerosis, rheumatoid arthritis and aplastic anemia have been associated with a T\_H1 phenotype. In the context of autoimmune disorders T\_H1 CD4\(^+\) T cells react to a self-peptide, resulting in systemic or tissue specific targets. Once activated, T\_H1 cells produce an abundance of inflammatory cytokines such as IL-2, TNF and IFN-\(\gamma\). These cytokines lead the generation of additional, activated T\_H1 cells and also to the clonal expansion of the CD8\(^+\) T cells. T\_H1 cell are essential in mediating the recruitment of CD8\(^+\) T cells, which then facilitate tissue destruction. The presence of inflammatory cytokines create short lived effector CD8\(^+\) T cells (SLEC) that mediate destruction and then die off, as opposed to becoming long lived memory CD8\(^+\) cells (Stemberger *et al.*, 2007; Rubinstein *et al.*, 2008; Obar *et al.*, 2008). This pathway is believed to mediate bone marrow destruction in the autoimmune disorder aplastic anemia.
1.2 Notch1 Signaling

1.2.1 Notch Signaling Pathway

The Notch family of proteins was originally discovered in *Drosophila* in 1914, and was given the name Notch after mutations in the gene led to a notched wing phenotype. Since that time, Notch has been found to play a pivotal role in cell fate decisions throughout many cellular events. There are four mammalian Notch receptors, Notch 1-4, that are diagrammed in Figure 1.2. To activate Notch there and two families of Notch ligands, Jagged (1, 2) and Delta-like (1, 3 & 4). The Notch receptor is a transmembrane protein that undergoes a series of cleavage events to create its active form (intracellular Notch). The first cleavage event of Notch occurs in the trans-golgi by a network of furin-like convertases, which generate the mature heterodimer form of Notch (Blaumueller *et al.*, 1997; Logeat *et al.*, 1998). In its processed, heterodimer form Notch then translocates to the plasma membrane where its extracellular portion waits for engagement with Notch ligands. Once a ligand binds to the extracellular portion of Notch there is the second cleavage mediated by an ADAM metalloprotease, TNF-α-converting enzyme (TACE), which releases the extracellular portion of Notch (Brou *et al.*, 2000). After the second cleavage a third site is exposed for the third and final cleavage by gamma-secretase (De Strooper *et al.*, 1999). Upon this final cleavage the active intracellular portion of Notch is released from the cell membrane and translocates to the nuclease. Notch signaling is outlined in Figure 1.3.

Notch initiates gene transcription by acting as a molecular switch to turn CSL (CBF-1, SuH, Lag-1) from a transcriptional repressor to an activator. When CSL is
activated it recruits co-activators, such as p300 and Mastermind-like-1, to regulate a plethora of genes (Osborne and Minter, 2007). Additional studies have provided evidence that Notch may also initiate gene transcription independently of CSL through a pathway involving NF-κB (Osborne and Minter, 2007).

1.2.2 Notch1 and T Cells

Notch1 has been shown to be an important regulator of T cell development, activation and differentiation. In the absence of Notch there is a skewed development of B cells and abnormal thymic development (Radtke et al., 1999; Han et al., 2002). Notch signaling was first implicated in T cell activation when it was discovered that intracellular Notch1 was upregulated in T cells upon stimulation with anti-CD3 and anti-CD28 antibodies (Palaga et al., 2003; Adler et al., 2003). Recently there has been a number of studies that have shown Notch1 to be important in the development of T\textsubscript{H}1, T\textsubscript{H}2, T\textsubscript{H}17 and Tregs (Minter et al., 2005; Fang et al., 2007; Amsen et al., 2007; Samon et al., 2008; Keerthivasan et al., 2011 submitted). In polarized T\textsubscript{H}1 cells inhibition of Notch resulted in decreased IFN-γ production. It was also shown that Notch was able to decrease IFN-γ production in conjunction with NF-κB through its regulation of T-bet (Minter et al., 2005, Shin et al., 2006). In addition to playing a role in T\textsubscript{H}1 polarization Notch1 has also been named as a regulator of T\textsubscript{H}2 fate via Gata-3 and IL-4 production (Fang et al., 2007; Amsen et al., 2007). However, additional studies have now demonstrated that there is also Notch1-independent differentiation of T\textsubscript{H}2 cells, through a β-catenin-mediated pathway (Yu et al., 2009). Data currently being revised for publication has also suggested that T\textsubscript{H}17 cells require Notch1 for their differentiation,
because in its absence there is decreased rorc (ROR-γt) transcription and decreased production of IL-17 (Keerthivasan et al., 2011 submitted). Additionally, Notch1 was shown to be necessary for the development of T regulatory cells through regulation of Treg transcription factor foxp3 (Samon et al., 2008). Overall, these data support the hypothesis that Notch1 regulates differentiation into many subtypes of T helper cells. The many conflicting reports in the literature are most likely a result of the different methods by which Notch signaling is abrogated. The distinction of how Notch1 chooses which fate may be based on environmental factors such as cytokines, or perhaps the initiating ligand and/or the signal strength which activates Notch1 that determines what subset Notch1 will support in the race of T cell differentiation (Amsen et al., 2004).

Not surprisingly studies have also found Notch1 signaling to be important in the development and activation of CD8+ T cells. Notch1 was shown to be a regulator of the transcription factor eomesodermin (Eomes), which is responsible for the expression of granzyme B and perforin, shown in Figure 1.1. Eomes is a member of the T-box family of transcription factors and is similar to T-bet. Inhibition of Notch1 through pharmacological or genetic (Notch1 antisense mice) approaches resulted in decreased expression of Eomes and, subsequently, decreased granzyme B and perforin production (Cho et al. 2009). This study also showed that inhibiting Notch1 led to decreased CTL function in an OVA-specific model. Additionally, Notch1 activation was shown to be necessary for the expression of T-bet (Minter et al., 2005). Tbet has been shown to mediate the production of SLEC (Joshi et al., 2007; Intlekofer et al., 2007), which are essential in cell-mediated tissue destruction. During viral infections this population of CD8+ T cells rapidly expands and then involutes after viral clearance. During a viral
response these cells possesses lytic capabilities, tissue migration, but limited self-renewal and decreased lifespan. This population differs from memory precursor effector T cells (MPEC); which have increased self-renewal capabilities and are vital during secondary infection. Altogether these data show that Notch1 signaling plays a critical role in the development of several T helper cell subsets and effector T cells.

1.2.3 Gamma Secretase Inhibitors

The third cleavage event in the Notch signaling pathway is mediated by gamma-secretase (De Strooper et al., 2000), Figure 1.3. The gamma secretase complex is made up of four proteins presenillin1/2, APH1, Pen2 and nicastrin. All four of these proteins need to be present in order for the complex to have proteolytic capabilities (Edbauer et al., 2003; Kimberly et al., 2003; Takasugi et al. 2003). Gamma-secretase was shown to regulate intramembrane proteolysis of the amyloid precursor protein (APP) (Haass and Selkoe, 1993) In addition to APP gamma-secretase mediates the cleavage of E-cadherin, CD44, Notch and other type-one proteins. Gamma-secretase inhibitors (GSI) were created to prevent the subsequent activation of Notch and other known targets. There are several types of GSIs that act either through molecular mimicry or as competitive inhibitors. Although gamma-secretase may mediate the cleavage of several proteins in the immune system, Notch is believed to be its main target in T cells. GSIs are approved in for use in clinic trials and are currently being used in the treatment of Alzheimer and several types of cancer including, prostate, breast, pancreatic and lung cancer, melanoma and T cell acute lymphoblastic leukemia (http://clinicaltrials.gov/, 2011).
1.3 PKC-θ: Regulator of T Cell Life and Death

1.3.1 Protein Kinase C

Protein Kinase Cs are a subfamily of kinases which specifically phosphorylate serine and threonine residues. This kinase family has been found to have non-redundant roles in T cell biology. The family of 10 isozymes is divided into three groups based upon their mode of activation. Conventional PKC family members are activated by calcium and a second message from diacylglycerol (DAG), this subfamily consists of PKC-α, β and -γ. PKC-θ is a member of the novel PKC family which is not dependent on calcium; this subfamily also includes PKC-ε, δ and -η. The last subfamily contains the atypical PKCs which are not activated by calcium or DAG, this includes PKC-ζ and -λ.

PKC-θ shares a similar structural configuration with other novel members. This includes an NH₂ terminus, C2-like domain, pseudosubstrate (PS), DAG binding domain (C1), unique V3, and kinase domain. In the C2 domain there is a tyrosine residue which is phosphorylated by Lck during T cell activation (Liu et al., 2000). The PS domain binds to the kinase domain which keeps PKC-θ in an inactive state. In response to DAG, which is generated downstream of PLCγ1 signaling PKC-θ undergoes a conformational change into an active state whereby it can then activate its substrates. PKC-θ structure is diagrammed in Figure 1.4. The exact mechanism of these changes is not completely understood, but it is hypothesized that phosphorylation of tyrosine by Lck is necessary.
1.3.2 PKC-θ in T cell Activation

Data in the literature have shown PKC-θ to be expressed predominantly in T cells, muscle cells and platelets. Due to its specific expression and its essential role in T cell activation it is an attractive target for therapeutics to dampen immune responses. PKC-θ in T cell signaling is outlined in Figure 1.5. Although there are other PKC members expressed in T cells, PKC-θ differs from them because of its location and rapid recruitment to immunological synapse (IS) upon T cell stimulation (Bi et al., 2001; Burack et al., 2002). The IS is the interface between the APC and the T cell during activation. In order for the IS to form there are a series of conformational changes that must occur at the cell membrane (Grakoui et al., 1999). In the absence of PKC-θ the IS cannot be formed, thus there is no sustained T cell activation (Valitutti et al., 1995). Part of the IS involves the recruitment of the CBM complex, which is made up of the scaffolding protein Carma1 together with Bcl10 and MALT-1 (Thome et al., 2007). PKC-θ has been shown to positively regulate the activation of NF-κB through the formation of the CBM complex. Without this signaling cascade there is no transcription the il2 gene, Figure 1.5.

In order to truly understand what an integral role PKC-θ plays in T cell development the creation of a knockout mouse was of utmost importance. PKC-θ knockout mice were generated by inactivating the PKC-θ gene by homologous recombination in embryonic stem cells. Within the gene, the ATP-binding site of the kinase domain was replaced with a neomycin cassette. Disruption of the gene resulted in the abrogation of PKC-θ protein production. The loss of PKC-θ had no effect on the development of immature T cells, but there were serious defects in mature T cell
responses. PKC-θ-deficient cells had insufficient signaling through NF-AT, AP-1 and NF-κB, upon stimulation (Sun et al., 2000). The disruption in signaling cascades also led to impaired IL-2 production and reduced expression of the IL-2 receptor. This defect eventually results in apoptosis. Supplying exogenous IL-2 to stimulated T cells was able to partially overcome activation defects and prolong the cells’ survival, but not their proliferation (Sun et al., 2000; Manicassamy et al., 2006). PKC-θ is needed to antagonize apoptotic signals like BAD (Bertolotto et al., 2000; Villalba et al., 2001). Without PKC-θ, T cells also fail to upregulate the anti-apoptotic signals, Bcl-2 and Bcl-xL, which results in accelerated apoptosis during T cell stimulation, compared to wild type cells (Manicassamy et al., 2006). PKC-θ knockout mice show normal Th1 responses to viral infections, but showed defective Th2 responses (Giannoni et al., 2006; Marsland et al., 2004; Salek-Ardakani et al., 2004; Mowen and Glimcher, 2004; Salek-Ardakani et al., 2005; Stevens et al., 2006). PKC-θ knockout mice have been shown to have decreased disease severity or no development of disease in autoimmune models of EAE, allergic asthma, experimental colitis and type II collagen-induced arthritis (Salek-Ardakani et al., 2005; Tan et al., 2006; Salek-Ardakani et al., 2004; Nagahama et al., 2008; Healy et al., 2006). These studies highlight the potential benefit of using PKC-θ inhibitors in the treatment of autoimmune disorders.

1.3.3 Rottlerin is an Inhibitor of PKC-θ

Rottlerin (mallotoxin) is a kinase inhibitor that was isolated from Mallotus philippomesis, which is also known as the monkey-face tree. Rottlerin was originally discovered as an inhibitor of members of the novel family of protein kinases C,
specifically PKC-δ (Gschwendt et al., 1994). Since then, additionally studies have revealed that rottlerin is in fact not an effective inhibitor of PKC-δ, but is an inhibitor of PKC-θ (Soltoff, 2007) and other kinases such as CaMII kinase. Rottlerin is believed to inhibit PKC-θ by competing for the ATP binding site (Gschwendt et al., 1994), this in turn prevents its kinase activity and in turn phosphorylation of its substrates, such as Carma1 (Sommer et al., 2005; Matsumoto et al., 2005). Human T cells (CD4 and CD8) stimulated in the presence of rottlerin show decreased proliferation, which is attributed to decreased IL-2 production and CD25 (IL-2 receptor) expression. Addition of IL-2 to stimulated cultures was not able to restore the proliferation of T cells (Springael et al., 2006). Additionally, rottlerin was shown to inhibit the development of T_H1cells by decreasing T-bet expression and IFN-γ production (Solomou et al., 2006). Unlike PKC-θ knockout mice, treatment with rottlerin did not affect calcium mobilization during T cell activation (Springael et al., 2006). From these studies it would appear that rottlerin, or other PKC-θ inhibitors would be excellent candidates for treating autoimmune disorders by inhibiting T cell activation and inducing cell death.

1.4 Notch1 and PKC-θ: Friends of T Cell Activation

1.4.1 Notch1 and PKC-θ Interact

The exact relationship between Notch1 and PKC-θ remains unknown; however, we do know that the absence of either protein results in decreased activation of the other (Osborne & Minter unpublished data). Using immunofluorescence microscopy Notch1 and PKC-θ were found to co-localize at the immunological synapse at the time of T cell
activation (L. Minter, unpublished data). Additionally, it was discovered that the two proteins could be immunoprecipitated together as part of the CBM complex (Shin et al., in preparation).

1.4.2 Notch1 and PKC-θ both Regulate NF-κB Activity

The NF-κB signaling pathway regulates the transcription of many downstream genes. One of the most-recognized in T cell activation is il2. Both Notch1 and PKC-θ have been shown to directly mediate the activation of NF-κB. The potential mechanisms that allow cross-talk between Notch1 and PKC-θ are diagrammed in Figure 1.6. NF-κB is sequestered in an inactive state in the cytoplasm by IκB. IκB must be degraded to free NF-κB and allow it to enter the nucleus. The IKK (IκB kinase) complex mediates the degradation of IκB and subsequent activation of NF-κB. The IKK complex becomes activated in response to T cell stimulation and inflammatory cytokines (Beinke, 2004). NF-κB is regulated in a negative feedback loop since its activation leads to subsequent transcription of IκBα. Once synthesized, IκBα can translocate into the nucleus to sequester NF-κB back to the cytoplasm and once again prevent its activation (Arenzana-Seisdedos et al., 1997; Rodriguez et al., 1999; Hoffmann et al., 2002; Nelson et al., 2004).

The formation of the CBM complex requires both Notch and PKC-θ. PKC-θ is rapidly recruited to the immunological synapses after T cell activation; it then recruits Carma1, Bcl10, and MALT-1. Notch has also been shown to bind with Carma1, as part of the CBM complex. The assembly of this complex leads to the activation of IKK and degradation of IκB, allowing the activation of NF-κB. Notch also has been shown to have
an additional role in maintaining NF-κB in the nucleus by competing for IκBα binding to the subunit p50, thus retaining NF-κB subunits in the nucleus (Shin et al., 2006).

Although the exact mechanism of how Notch and PKC-ζ act on one another is unknown they both appear to have essential functions in the same pathways, as outlined in Figure 1.6.

1.5 Immune Mediated Bone Marrow Failure Syndromes: An Autoimmune Disorder

1.5.1 Aplastic Anemia

Immune-mediated bone marrow failure syndromes develop as a result of the loss of self-tolerance in the T cell compartment or genetic mutations. Autoreactive T cells mediate the destruction of one or all progenitor cells that are responsible for making the components of the blood. Some examples of immune-mediate bone marrow failure include Aplastic Anemia, Paroxysmal Nocturnal Hemoglobinuria and Myelodysplasia. Aplastic Anemia (AA) is a rare disease with only 900 new cases diagnosed each year in the United States. Age distribution of patients diagnosed with AA fall into two groups 15-25 years and over 60 year. AA is known as a disease of the young, since most cases are found in the younger group (Scheinberg et al., 2008). AA develops following the loss of self-tolerance and the expansion autoreactive T cells, which infiltrate and destroy the bone marrow. The antigen which triggers this response remains unknown. Currently, there have been some autoantibodies that have been identified in AA patients. Kinectin is a widely expressed protein, to which autoantibodies have been found in approximately 40% of patients. Another autoantibody which was found in a much smaller subset of
patients is diazepam-binding related protein-1, an enzyme that regulates the oxidation of unsaturated fatty acids (Young et al., 2006). The relevance of these autoantibodies to disease is still unclear. T cell-mediated destruction of stem and progenitor cells results in hypocellular bone marrow (BM). The consequence of a hypocellular BM is the inability to replenish the components of the blood, resulting in peripheral pancytopenia. Subsequently deficiency in platelets, red and white blood cells leads to an increased risk of bleeding and infection in AA patients.

1.5.1 T Cell-Mediated Destruction

Autoreactive CD4+ and CD8+ T cells mediate the destruction of stem and progenitor cells through three different mechanisms. The first documented mechanism that leads to destruction in the BM is direct killing by CD8+ T cells. Cytotoxic CD8+ T cells mediate the destruction through the actions of perforin and granzyme B. Perforin is a pore forming protein, which binds to the target cell and allows the entrance of granzyme B, which mediates target cell death. Both perforin and granzyme B are increased in patient with AA (Xu et al., 2003; Franzke et al., 2006). Another well-documented mechanism of death mediated by T cells is Fas/Fas ligand interaction (Maciejewski et al., 1995; Callera et al., 1998; Ismail et al., 2001). T cells upregulate Fas ligand and induce cell death through Fas receptor expressed on the stem and progenitor cells. The third mechanism of death is through increased inflammatory cytokine production by the infiltrating T cells. Hematopoietic stem cells (HSC) exposed to the cytokines IFN-γ and TNF undergo apoptosis through bystander cell death (Maciejewski et al., 1995; Selleri et al., 1996; Young et al., 2006; Bacigalupo, 2007). The increased
levels of cytokines secreted by the infiltrating T cells also inhibit the HSCs’ and progenitor cells’ ability to proliferate. These mechanisms by which T cells induce death in the HSCs and progenitor cells are outlined in Figure 1.7.

1.5.3 Aplastic Anemia is a Th1-Mediated Disease

Some cases of Aplastic Anemia develop from the chemical exposure, pregnancy or other conditions that can be traced to an initiating event. However, the majority of cases of AA are idiopathic. The clinical observation that AA patients respond to immunosuppressive therapy is the most concrete evidence that AA is an autoimmune disorder (Young et al., 2006). Analyses of T cells from AA patients have demonstrated that AA is a Th1-mediated autoimmune disorder. This conclusion was made based on the observation that T cells from patients had increased production of Th1 hallmark cytokines, IFN-γ, TNF-α and IL-2 (Zombous et al., 1984; Young et al., 1987; Zombous et al., 1985; Sloand et al., 2002; Giannakoulas et al., 2004; Young et al., 2006; Bacigalupo, 2007). CD4+ T cells from patients have increased expression of T-bet (tbx21), the transcriptional master regulator of Th1 cells (Solomou et al., 2006). Increased protein expression of T-bet in patients with AA correlated with increased levels of IFN-γ and IL-12 receptor expression (Solomou et al., 2006). Based on this evidence AA is believed to be a Th1-mediated disease.

1.5.4 Current Treatment for Aplastic Anemia

When treating Aplastic Anemia patients there are three levels of care: supportive treatments, immunosuppressive treatment (IST) and bone marrow transplants (BMT).
BMT is the first choice of treatment, but many factors can affect the outcome, first and foremost is the availability of a HLA-matched donor. Siblings are the best candidates, but matches can sometimes be found through a bone marrow donor registry. Children are better candidates for bone marrow transplant than adults, because they are less likely to have complications from graft-versus-host (GVH) disease and show better overall survival (Scheinberg *et al.*, 2008). BMT is a superior form of treatment; however, in many cases a matched donor is not available within the necessary time frame. While waiting for a donor match patients will receive a standard IST regimen. The current protocol for IST is antithymocyte globulin (ATG) and Cyclosporine (CsA) (Scheinberg *et al.*, 2008). ATG treatment relieves disease symptoms by killing off the T cells that are mediating the destruction of the bone marrow. ATG is administered intravenously for 8-12 hours for four days, although these regimens vary between hospitals. CsA inhibits the autoreactive T cells ability to become activated; CsA is administered orally in either liquid or a pill. The dosing of CsA is dependent on the patient’s weight since a certain concentration is essential to maintain bioactivity of CsA, but also minimize side effects. When used alone ATG has the ability to improve blood count only about half the time, but when used with CsA approximately 70% of patients respond to treatment. Full benefits from the treatment can take up to 9 months. After that period of time 30% of patients will relapse and will need another course of treatment.

1.6 Significance and Hypothesis

Current treatments for Aplastic Anemia are limited due to the available information about the antigen which causes the development of autoreactive T cells and
the subsequent destruction of the bone marrow. Immunosuppressive therapies provide rescue of disease symptoms, but some patients do not respond to the treatments currently available. The aim of these studies are as follows: i) to create a murine model that recapitulates AA symptoms; ii) to use this model to identify novel molecules that are important in the pathogenesis of disease; iii) to discover therapeutics to inhibit these novel molecules. Using our murine model of bone marrow failure we sought to evaluate the role of two key players in T cell differentiation and activation, Notch1 and PKC-ζ. Our hypothesis is that both Notch1 and PKC-ζ play important roles in disease initiation in a urine model of BMF syndrome. These targets regulate the activation and polarization of a CD4$^+$ T cell towards a T$_{H1}$ phenotype. They also play an important role in the maturation and activation of effector CD8$^+$ T cells. Therefore, we believe that inhibiting Notch1 and PKC-ζ should result in decreased disease severity in mice induced with Aplastic Anemia. Our hypothesis and disease mechanism are outlined in Figure 1.8.
Figure 1.1. T-cell Subsets

A schematic that outlines the different CD4\(^+\) and CD8\(^+\) T cell subsets, including their required transcription factors and signature cytokines.
Figure 1.1. Notch Receptors

There are four mammalian Notch receptors. The extracellular portion of all four contains epidermal growth-factor-like repeats, which bind to signaling ligands Jagged (1&2) or Delta-like (1, 3&4). There are three LIN repeats. The intracellular portion contains a RAM domain (RBP-J-associated molecule) that binds CSL and 7 Ankyrin repeats that are used for protein binding including CSL and NF-κB. At either end of the Ankyrin repeats there are nuclear localization signals, two for Notch 1-3 and one for Notch 4. The transcriptional activation domain activates downstream targets; there is no TAD domain in Notch 3 or 4. The PEST (C-terminal Pro Glu Ser Thr) domain regulates degradation of Notch proteins.
Figure 1.2. Notch Signaling

After engagement with one of its ligands (Jagged 1, 2; Delta-like 1, 3 & 4), Notch undergoes cleavage events mediated through TACE and gamma-secretase, as designated by the lighting symbol. Notch then translocates to the nucleus where it binds to CSL to activate downstream targets.
**Figure 1.3. PKC-θ Structure**

A diagram showing the domains that make up PKC-θ. The C2 domain contains the tyrosine residue that is phosphorylated by Lck during T cell activation. The PS domain binds to the kinase domain to keep it in an inactivated state. C1 is the binding domain for DAG and allows for the conformation change in PKC-θ to an active form.
Figure 1.4. T Cell Signaling Cascade

After engagement of the T cell receptor and CD28 there are a series of phosphorylation events that ultimately lead to the recruitment of PKC-θ to the immunological synapse as shown here. PKC-θ phosphorylation leads to the activation of IKK complex which degrades IκB complex and subsequently releases NF-κB. NF-κB then regulates the transcription of il2.
Figure 1.5. Notch and PKC-θ in NF-κB Activation

Notch1 can act as a scaffolding protein during the formation of the CBM complex along with PKC-θ. Additionally, Notch1 can maintain nuclear retention of NF-κB.
Figure 1.6. Three Mechanisms Result in Death of HSCs and Progenitor Cells

T cell mediated destruction through three documented mechanisms. 1) The direct killing by CTLs through granzyme B/perforin. 2) The upregulation of Fas ligand on T cells and Fas receptor expression on HSCs and progenitor cells. 3) The production of inflammatory cytokines, IFN-γ and TNF-α, which not only kill the HSCs and progenitor cells, but also severely inhibit their proliferation capabilities.
Figure 1.7. An Overview of Mechanisms Leading to Bone Marrow Destruction.

Bone marrow damage in Aplastic Anemia develops from the initial activation of CD4+ T_H1 cells. Notch1 and PKC-ζ signals activate CD4+ T cells and subsequently activate NF-κB and Tbet. We can inhibit signaling through Notch and PKC-ζ with GSI or Rottlerin, respectively. In the absence of the inhibitors, activation of T_H1 cells results in the clonal expansion of CD8+ T cells and the production of inflammatory cytokines. These mechanisms then lead to death of the stem and progenitor cells. (Adapted from NS Young, 2006.)
CHAPTER 2

DEVELOPMENT AND CHARACTERIZATION OF APLASTIC ANEMIA

MURINE MODEL

2.1 Introduction

The antigen which initiates Aplastic Anemia remains unknown; therefore, there is no true autoimmune model of AA. To recapitulate AA using animal models, researchers use acute graft-versus-host disease models (GVHD). These types of GVHD models are induced by the infusion of major or minor histocompatibility-mismatched leukocytes into an F1 progeny (Shlomchik, 2007). These GVHD models develop disease symptoms that are similar to those observed in patients with AA. Disease-induced mice have T cell infiltration into the bone marrow, where they mediate the destruction of stem and progenitor cells. The loss of stem and progenitor cells leads to hypocellular bone marrow and peripheral pancytopenia (Mori et al., 1998; Bloom et al., 2004). These models recapitulate disease symptoms accurately, and the current treatment using cyclosporine A and anti-thymocyte globulin has been validated using a GVHD model (Bloom et al., 2004).

The model which we generated was developed using a combination of two previously published protocols used to induce BMF. From the first protocol, we used the same MHC mismatch of H2b cells in to an H2b x H2d F1 progeny, although we used a different mouse sub-strain of Balb/c (Bloom et al., 2004). To create this AA model we also used a previously reported method of using bulk splenocytes as the initiating cell source (Mori et al., 1998). Preliminary data from our lab (Emily Roberts, unpublished
observations) showed that we were able to induce disease with intraperitoneal (IP) injections of bulk splenocytes, just as effectively as intravenous (IV) injections. In fact we were able to produce more consistent results with the IP injections compared to the IV injections, most likely due to the technical ease of IP injections.

Generation of F1 hybrids is achieved by crossing a Balb/c (H2b) female with a C57BL/6 (H2d) male. These F1 hybrids have a major histocompatibility complex of H2bd, as outlined in Figure 2.1. Then an IP infusion of C57BL/6 splenocytes is used to induce disease after light irradiation (3Gy). Initial experiments were performed to determine the necessary cell number to induce disease. In the literature there are several reports of different models of GHVD. Some studies have demonstrated that the initiating T helper subtype will dictate an organ specific GVHD (Yi et al., 2009). While establishing the model we did not observe any special phenotype based on T cell subsets, but what was discovered was the importance of the number of cell to induce disease.

Using a lesser number of total splenocytes (3x10^7) resulted in a lethal GVHD response which caused mice to succumb to disease on average at day 14 with no signs of bone marrow destruction. Interestingly, mice which received more splenocytes (5x10^7) survived longer periods of time (18-26 days). Mice who succumbed to disease at earlier time points showed a GVH response that was directed towards the gut, while mice that were induced with a greater number of splenocytes induced destruction mediated towards the bone marrow. Once we determined that 5x10^7 splenocytes provided reproducible disease symptoms, we used that number to characterize this AA model. Our induction protocol and disease time course is outlined in Figure 2.1, with an in-depth description provided in the Materials and Methods.
2.2 Results

2.2.1 Murine Model of AA Recapitulates Human Pathology

Once patients are diagnosed with aplastic anemia there is already destruction of the bone marrow cavity. Usually patients are diagnosed once they have developed complications from peripheral pancytopenia. Once patients present with symptoms of AA a bone marrow aspiration is performed to confirm diagnosis. When sacrificed on day 17, mice induced with AA showed a dramatic decrease in the number of cells present in the bone marrow cavity as determined by trypan blue counts, compared to irradiation controls (Figure 2.2 A). We also evaluated cellularity in the sternums of irradiation controls and AA-induced mice. Sternums were fixed, paraffin-embedded and then H&E stained. As expected, disease-induced mice had an extensive loss of cellularity compared to un-induced control mice Figure 2.2 B. Peripheral blood was harvested from animals using cardiac puncture and complete blood counts (CBC) were obtained by running samples through a veterinary hematology analyzer. White and red blood cells where significantly reduced in diseased mice (Figure 2.2 C & D). Patients with AA experience cachexia, a chronic wasting which is associated with dramatic weight loss. At the time of harvest mice induced with disease presented with decreased activity, shallow breathing, unkempt coat and robust weight losses based on a percent weight change, Figure 2.2 E. As with patients, we also observed increased circulating levels of inflammatory cytokines. Using a cytometric bead array we were able to quantify the amount of circulating IFN-γ and TNF in mice with AA, whereas little to no cytokines were present in our irradiation controls (Figure 2.2 F). Although IL-2 and IL-17 (Gu et al., 2007) have
been found at increased levels in patients with AA, we saw no increases in the levels of either cytokine in AA mice.

2.2.2 Disease is Mediated by Donor T cells that Infiltrate into the Bone Marrow

Although there is a small resident population of mature T cell in the bone marrow, a major infiltration of them is not typical. Patients with AA have a massive infiltration of T cells into the bone marrow cavity that wreaks havoc on the sensitive microenvironment and leads to the death of stem and progenitor cells. AA mice have massive infiltration of T cells into the bone marrow cavity as evaluated by flow cytometric analysis (Figure 2.3A). To distinguish between donor and host T cells we used C57BL/6 GFP+ mice as the lymphocyte donors. Using this approach allowed us to track and monitor the location and expansion of donor T cells. In the bone marrow the vast majority of infiltrating CD4+ and CD8+ T cells was donor-derived (Figure 2.3 B). Additionally we saw expansion of T cells, over the disease time course, in the lymph nodes, spleen and peripheral blood of induced mice (Figure 4.3).

2.2.3 Genes that are Dysregulated in Aplastic Anemia Patients are also Dysregulated in Our Mouse Model

To determine whether AA mice truly recapitulate the mechanisms that mediate disease symptoms in patients with AA, we isolated T cells from the spleen and BM of AA mice. Using q-PCR when then evaluated several genes that were found to be dysregulated in AA patients. Using positive selection, CD4+ and CD8+ T cells were isolated from AA mice and irradiation controls. Due to the small number of T cells in AA
mice, 3-6 mice per cohort were pooled and RNA was isolated. cDNA was prepared and analyzed using SYBR green qRT-PCR. Gene expression was evaluated by relative expression and was analyzed using the delta, delta CT method. Target genes were normalized to β-actin and relative expression was based on T cells isolated from an irradiated control. Using qRT-PCR we found similar gene dysregulation in AA mice that have also been documented in AA patients (Franzke et al., 2006; Solomou et al., 2006). We observed increased transcript levels of the following genes: *ifng*, *tnf*, *prf-1*, *gzmb*, *tbox21*, *il12rb2* and a decrease in *pf4* expression, Figure 2.4 A-G.

### 2.3 Discussion

Although there are limits to using animal models for research they are a tremendous resource. They have led to the discovery of novel molecules, mechanisms, cell types and therapeutics that have been found to be relevant in the diagnosis and treatments of a variety of human diseases. This mouse model of AA is another excellent resource for studying T cell activation, polarization and T-cell-mediated destruction of stem and progenitor cells in the bone marrow. This model faithfully replicates the disease symptoms and characteristic of AA in humans, Figures 2.2 and 2.3. In addition to this, T cells isolated from AA mice have a transcriptional phenotype similar to T cells isolated for patients with AA, Figure 2.4.

The extensive characterization of this model shows that although it is technically a GVHD model, it recapitulates the symptoms and gene dysregulation seen in humans with AA. We presume there must be pathways in both responses that function in relatively similar ways which lead to identical outcomes in AA patients and in AA mice.
(T cell-mediated destruction of the BM). Thus this model of AA provides a suitable source with which to ask questions about T cell activation, polarization and mechanisms of disease progression.
Figure 2.1. AA Disease Induction and Time Course

A schematic outlining generation of F1 progeny and disease induction (A). Time line for disease induction through time of harvest (B).
Figure 2.2. AA Induced Mice Show Disease Symptoms Similar to AA Patients

Mice were harvested 17 days after disease induction. Bone marrow cells were flushed and leukocytes were counted using trypan blue staining (A). Cellularity was also evaluated in the sternum with paraffin-embedded sections stained with H&E (B). Peripheral pancytopenia was evaluated by peripheral blood values using a hematology analyzer which provides white and red blood cell counts (C, D). To determine percent weight change, mouse weights were recorded on the day of disease induction and day of harvest (E). Circulating cytokine levels were quantified by flow cytometry using a cytometric bead assay to detect IFN-γ and TNF (F) in the plasma from the peripheral blood.

*P<0.05, **P<0.01, ***P<0.001
Figure 2.3. Mice Induced with AA have Infiltrating T Cells that are Donor Derived

After flushing the bone marrow cells they were stained for CD4 and CD8 and analyzed by flow cytometry (A); each plot represents one animal. Using FACS analysis we also quantified the percent of BM infiltrating CD4 and CD8 T cells that were GFP-positive (B).
qRT-PCR analysis of gene expression in T cells isolated from BM of spleen of AA mice compared to T cells isolated from the spleen of an irradiation controls. Values for each gene were normalized to β-actin and relative expression was determined by normalizing to CT values from irradiation controls. Transcripts shown to be up regulated in patients with AA correlated well with transcripts upregulated in AA mice: IFN-γ (A), TNF (B), perforin (C), granzyme B (D), T-bet (E), and IL-12Rβ2 (F). Similar to patients with AA platelet factor 4 was down regulated in AA mice (G).

Figure 2.4. Gene Dysregulation Seen in Patients with AA is Recapitulated in AA Mice
CHAPTER 3

NOTCH1 PLAYS A ROLE IN THE DISEASE PROGRESSION IN APLASTIC ANEMIA MICE

3.1 Introduction

Notch1 has been shown to be essential for the polarization of T cells toward a T\textsubscript{H}1 phenotype (Minter et al., 2005). Therefore we hypothesized that Notch1 was involved in AA, which is believed to be a T\textsubscript{H}1-mediated disease (Zombous et al., 1984; Young et al., 1987; Zombous et al., 1985; Sloand et al., 2002; Giannakoulas et al., 2004; Young et al., 2006; Bacigalupo, 2007). To address if Notch1 played a role in disease progression, we investigated Notch1 activation in AA induced mice.

In order to determine if Notch1 was an important protein in disease onset, we used two approaches to abrogate Notch1. First, we targeted Notch1 genetically using conditional Notch1 knockout mice. These Notch1-floxed mice were then crossed to an Mx-Cre expressing strain. Notch1 was deleted in peripheral T cells following a course of polyinosinic: polycytidylic acid (poly IC) administration. Our second method to target Notch1 activation was using a gamma-secretase inhibitor (LY411, 575). GSI prevents the third and final cleavage of Notch1 and this cleavage is necessary to release the active portion of Notch1. In addition to asking if Notch1 is essential in disease progression, additional studies were done to validate GSI as a possible therapeutic to treat Aplastic Anemia.

In an effort to establish GSI as a possible therapeutic we also wanted to evaluate its effect on hematopoiesis. Notch has been found to be important in many cell fate
decisions including hematopoiesis. To ensure that GSI had no ill effects on hematopoiesis we performed a series of long-term treatment studies consisting of reconstitution assays. These studies are essential in validating GSI as a possible therapeutic for AA.

3.2 Results

3.2.1 Notch1 is Upregulated in T Cells of AA Induced Mice

When T cells become stimulated and polarized there is an increase in the amount of active intracellular Notch1 that can be detected (Palaga et al., 2003; Adler et al., 2003). Therefore, we wanted to determine if Notch1 was activated in the T cells of AA induced mice. Using intracellular staining and flow cytometry, the expression of the intracellular portion of Notch1, N1^ic, was determined by mean florescence intensity (MFI). Compared to splenic T cells of irradiated controls, we observed increased N1^ic in T cells that were isolated from both the spleen and BM of AA induced mice (Figure 3.1 A). Upon closer analysis we observed that there was a significant upregulation of N1^ic in CD4^+ T cells and only a modest increase in CD8^+ T cells, Figure 3.1 B. Furthermore, there was an increase in notch1 transcripts in T cells isolated from the spleen and BM of AA mice, compared to splenic T cells from irradiation controls, Figure 3.2 A. T cells isolated from AA mice were also evaluated for their expression of other Notch family members. No robust difference was detected in the amount of notch2 expression. We observed only a slight increase in the expression of notch3 in T cells from AA mice (Figure 3.2 B&C). There was also a marked increase in the amount of rbpj (also known
as CSL) transcript in T cells isolated from the spleens of AA-induced mice, Figure 3.2 (D).

3.2.2 Conditional Notch1 Knock Out Mice

To validate the role of Notch1 in our model, we used Notch1 conditional knockout mice (N1KO). N1KO transgenic mice were created using floxP sites on either side of the notch1 gene (Yang et al., 2004). N1KO mice were crossed with MxCre mice, and were bred back to homozygosity for the N1 transgene, N1KOfl/fl MxCre+/-. Notch1 was floxed, in vivo, with PolyIC treatment. MxCre floxing has been shown to target gene deletion in the liver, spleen, bone marrow and thymus (Kuhn et al., 1995). As outlined in the schematic, Figure 3.3A, N1KOfl/fl MxCre+/-(N1KO) mice received five intraperitoneal (IP) injections of PolyIC every other day and then rested for three weeks. To control both for the leakiness of the Cre and treatment with PolyIC, we used N1KOfl/fl MxCre-/- mice untreated (without PolyIC treatment, Cre controls) and N1KOfl/fl MxCre-/- mice treated with PolyIC (PolyIC controls).

To validate the deletion of Notch1 after PolyIC treatment, CD4+ and CD8+ splenic T cells were isolated and stimulated with plate-bound anti-CD3 and anti-CD28 for 24 hours for qRT-PCR analyses, and for 48 hours for protein expression by flow cytometry. N1KO mice had significantly less notch1 expression by qRT-PCR compared to T cells isolated from Cre and PolyIC controls. N1KO mice also had considerably less expression of N1ic as determined by intracellular staining both of CD4+ and CD8+ T cell subsets, Figure 3.3 B&C. To ensure that N1KO mice had comparable percentages of T cell subsets in the spleen, we also evaluated total percentages of CD4+ and CD8+ T cell in the
spleens of these mice. Both CD4\(^+\) and CD8\(^+\) cell percentages were normal and did not differ from control mice, Figure 3.3 D. After this validation N1KO mice were used as splenocyte donors to induce disease in F1 recipients.

### 3.2.3 Notch1 Plays a Role in Disease Severity

F1 hybrid mice were induced with bulk splenocytes from N1KO, Cre or PolyIC control animals. Mice that received N1KO splenocytes showed decreased disease severity compared to mice induced with bulk splenocytes from Cre or PolyIC control mice. N1KO induced animals had significantly higher BM cellularities compared to either control group of induced mice (Figure 3.4 A). Although the values do not reach significance, we observed trends that mice receiving N1KO splenocytes had decreased weight loss and less peripheral pancytopenia compared to both control groups (Figure 3.4 B-D). Plasma was collected from all three groups of induced mice and the levels of IFN-\(\gamma\) and TNF were quantified by CBA. CBA analysis revealed that there were no significant differences in the circulating levels of IFN-\(\gamma\) or TNF, as shown in Figure 3.4 E&F.

BM and splenic T cells from AA induced mice were stained for CD4\(^+\), CD8\(^+\) and N1\(^{ic}\); N1KO-derived T cell had reduced capability to infiltrate the BM cavity of F1 progeny compared to either control (Figure 3.5 A). N1\(^{ic}\) levels were also decreased in the F1 recipients that receive N1KO splenocytes, as evaluated by MFI (Figure 3.5 B). Additionally mice that received N1KO splenocytes were able to survive longer than control induced mice (Figure 3.5C). In these experiments induced mice were monitored daily as disease developed and were sacrificed when they were determined to be moribund, unable to take food and water. N1KO mice had a (median) survival time of 25
days, Cre controls 21 days and Poly IC controls 19 days. Although not significant this data still strongly suggests that Notch1 plays a role in the disease progression of AA induced mice.

### 3.2.4 Inhibition of Notch with a Gamma-Secretase Inhibitor Results in Disease Rescue

Gamma-secretase has many different targets; one of the most prominent targets in the immune system is believed to be Notch. Since we determined that Notch1 is important in disease progression, we hypothesized that treating mice with a gamma-secretase inhibitor (GSI) should also result in disease rescue. GSI (LY 411,575) was delivered to mice using two routes of administration: orally, in rodent chow and intraperitoneally, dissolved in DMSO. Preliminary studies were done employing a pretreatment regimen with GSI chow beginning two weeks before disease induction or with IP injections beginning three days pre-induction. Validation of GSI chow pretreatment, showed that mice fed GSI chow for two weeks had T cells that expressed less intracellular Notch1 than mice fed control chow. N1IC levels were determined following *ex vivo* stimulation with anti-CD3 and anti-CD28 for 24 hours (Figure 3.6).

Once GSI was validated we began pretreatment studies in which mice received one of the following treatments, daily: i) the GSI (chow or IP) at 5mg/kg, ii) control chow, or iii) DMSO vehicle control. As expected, mice receiving either GSI treatment had increased bone marrow cellularity and less weight loss, compared to control-treated mice (Figure 3.7 A&B). CBC analysis revealed that GSI-treated animals had significantly higher levels of circulating white and red blood cells with both routes of administration.
GSI treatment reduced the T cells’ ability to infiltrate the bone marrow, resulting in few infiltrating T cells (Figure 3.8 A). T cells in the bone marrow had decreased levels of intracellular Notch1 based on MFI, as shown in Figure 3.8 B. Circulating levels both of IFN-γ and TNF in mice treated with GSI chow were reduced to irradiation control levels, as determined by CBA analysis (Figure 3.8 C). In addition to having an overall decrease in disease severity mice treated with GSI chow were able to survive significantly longer than mice that did not receive GSI (Figure 3.8 D). Mice receiving GSI chow had a median survival of 67 days, control chow fed mice had a median survival of only 21 days. These studies demonstrate that GSI has the ability to be a potential therapeutic in the treatment of AA, by decreasing the expression of Notch1.

To make our treatment more clinically relevant, we chose to begin GSI treatment five days after disease induction. To date, the only successful treatment course published in the literature began one hour post-disease induction (Bloom et al., 2004). Figure 3.9 A-D, shows that mice treated with GSI (IP) beginning at day +5 have increased cellularity, decreased weight loss, and increased circulating red and white blood cells. Treated mice also had significantly less T cell infiltration into the bone marrow cavity (Figure 3.9 E). Mice treated with GSI starting at day +5 also had decreased levels of circulating cytokines IFN-γ and TNF, Figure 3.9 F &G. Currently, additional studies are being conducted to determine the survival of these treated mice. Taken together, GSI treatment is effective at decreasing the disease severity of AA-induced mice, even when treatment is begun at day +5 post disease induction.
3.2.5 Notch1 and GSI have Slightly Different Targets

There are a multitude of genes that are upregulated in response to Notch1 activation through its binding to CSL and other co-activator molecules. To determine what targets were affected by inhibition of Notch1, in vitro T<sub>H</sub>1 polarizations were utilized to evaluate responses of T cells from N1KO mice compared to GSI-treated wild-type T cells. Experimental conditions and samples used are illustrated in Figure 3.10. After a period of 72 hours, cells were analyzed for protein expression using flow cytometry analysis or gene expression by qRT-PCR. Since reports in the literature indicate several different genes are dysregulated in AA, we wanted to explore genes that were also Notch1 regulated. Using flow cytometry we first validated that there was in fact a decrease N<sub>1</sub><sup>lc</sup> in N1KO mice and in wild-type T cells treated with GSI 50μM (3.11 A&B), both in CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets. Flow cytometric analysis based on MFI showed that T cell subsets were altered in absence of Notch1. Expression of T<sub>H</sub>1 effector markers T-bet, CD25 and IFN-γ were analyzed in CD4<sup>+</sup> T cells. There was a significant reduction in T-bet expression when Notch1 activity was abrogated either by GSI treatment or conditional deletion (Figure 3.12 A). We observed a robust reduction of CD25, but only in GSI-treated samples (Figure 3.12 B). After T cells were restimulated with anti-CD3, T-bet expression was still decreased in the absence of Notch1 (Figure 3.12 A). However, CD25 expression in T cells was refractory to the effects of GSI since addition of GSI at the time of restimulation had no effect on CD25 expression (Figure 3.12 B). IFN-γ levels were completely abrogated in the T cells polarized in the presence of GSI, and were reduced in T cells with deletion of Notch1 (3.12 C). Upon examination of CD8<sup>+</sup> T cells we demonstrated there was also decreased expression of T-bet (Figure
3.13 A), which has been implicated as an important transcription factor in the generation of short lived effector cells (SLEC). SLEC have been shown to arise in response to IL-12, IFN-γ and T-bet. Granzyme B is associated with CTL function and was significantly reduced in the absence of Notch1 (Figure 3.13B). Similar to what we observed in CD4⁺ T cells, there was decreased CD25 expression in the presence of GSI but there were no differences in the N1KO polarized cells. Treating cells with GSI at restimulation did not affect CD25 expression (Figure 3.13 C). IFN-γ was abrogated in the presence of GSI and was reduced in N1KO CD8⁺ T cells (Figure 3.13 D). When GSI was added at the time of restimulation, a reduction in IFN-γ was not observed in any of the conditions analyzed in this set of experiments.

In these studies the percentages of cells expressing TH1-associated proteins were also evaluated. CD4⁺ and CD8⁺ T cells from N1KO and GSI-treated conditions had decreased Notch1 expression compared to DMSO controls, Figure 3.14 A&B. The percentage of T cells expressing T-bet, or IFN-γ were evaluated in the CD4⁺ T cells and showed that their percentages were significantly decreased only in response to GSI treatment (Figure 3.15 A & B). CD25 percentages were unaffected by abrogation of Notch1, Figure 3.15 B. Upon the restimulation N1KO CD4⁺ cells showed significant decrease in the percent of cells expressing T-bet, Figure 3.15 A. The CD4⁺ T cells expressing IFN-γ were reduced in cells only upon GSI treatment, Figure3.15 C. Analysis of CD8⁺ T cells showed similar results with decreased percent of cells expressing T-bet, Granzyme B and IFN-γ (Figure 3.16 A,B &D). There was no difference in the percentage of cells expressing CD25 in the absence of Notch1, Figure 3.16 C. These findings help to explain the findings in our model, that mice receiving GSI treatment had better survival.
rates and decreased disease symptoms compared to mice receiving N1KO splenocytes to induce disease.

To address how Notch1 and GSI affect protein expression we harvested RNA from our 72 hour time point to look at differences in gene expression of Notch1-regulated genes. Relative expression of transcripts was determined using the delta, delta CT method. Samples were normalized to β-actin expression and relative expression was based on CT values of naive CD4+ and CD8+ T cells. The genes examined were chosen based on previously reported genes that are dysregulated in AA and genes that are believed to be Notch1 regulated. In Figure 3.17 we evaluated notch1 (A), notch2 (B), tbox-21(C), and hes1 (D), gzmb (E), ifng (F) and pf4 (G). Surprisingly we saw no differences in any of the genes analyzed, with the exception of Notch1 in N1KO cells (Figure 3.17 A). These data suggest that there is some other way in which the loss of Notch1 affects expression of these target proteins.

3.2.6 Long-Term Treatment with GSI Does Not Affect Hematopoiesis

Notch proteins have been shown to be important in many cell fate decisions during hematopoiesis (Duncan et al., 2005). Although these studies suggest a more important role for Notch2, rather Notch1, GSI inhibits activation of all members of the Notch family. To ensure that long-term GSI treatment would not affect normal hematopoiesis, long-term GSI treatment studies were done. These experiments are outlined in Figure 3.18. After six months on control or GSI chow (5mg/kg), bone marrow cells were isolated and used to reconstitute lethally-irradiated recipients, of these mice some were continued on chow during the period of engraftment. Mice were reconstituted
for a period of four months. After four months all mice were sacrificed for analysis of engraftment and stem cell repopulating capabilities. Bone marrow cells were harvested and again used to reconstitute another group of lethally-irradiated mice. These mice were allowed to reconstitute for 2 months. During both the first and second reconstitution, mice that received BM either from control chow or GSI chow-fed animals had no differences in their survival rates. At the time of harvest, mice fed GSI had similar BM cellularities after 6 months (Figure 3.19 A). Mice that were serially transplanted (4 and 2 months) also had similar cellularities regardless of which type of chow they received (Figure 3.19 B-C). BM cells from both control and GSI chow treated groups showed similar capacity to differentiate along all lineages in clongenic assays performed after 6 months on GSI chow, or after serial transplantations (4 and 2 months). Colonies were quantified, shown in Figure 3.20 A-C. All GSI recipient mice had similar percentages of lineage positive cells in the bone marrow compared to their control fed counter parts, at all-time points (Figure 3.21 A-C). Hematopoietic stem cell values were also equivalent between GSI and control fed mice (3.22 A-E). HSC percentages were determined by gating on lineage negative CD34−LO cells. Percentages of cells positive for the markers Sca-1, c-kit and HSCs (double positive cells for Sca-1 and c-kit) were then analyzed. These studies conclude that long term treatment with GSI does not impact normal adult hematopoiesis after six months of treatment. Moreover, administering GSI to mice during the reconstitution period appeared to have no effect on the stem cells’ ability to engraft the lethally-irradiated hosts.
3.3 Discussion

Unlike many other published reports these data, for the first time, identify targets of Notch1 through its genetic deletion. Much of the published data in the field have used several ways of abrogating Notch including GSI treatment and dominant negative forms of master mind. These data suggest that although GSI inhibits Notch1 expression there appear to be additional targets that are not mediated by Notch1. Differences in the effects of GSI and genetic deletion of Notch1 on downstream targets may result from how Notch1 is inhibited. GSI inhibits Notch1 at the level of the protein activation, while conditionally deleting Notch1 prevents its production at the transcription level. Regardless of the exact mechanism of how Notch1 inhibition affects the proteins examined here, it is clear that Notch1 plays a role in the disease progression of our murine model of AA.

Mice that received N1KO splenocytes had delayed disease onset and decreased disease severity compared to controls. Similarly, we were able to recapitulate this disease rescue when treating AA mice with GSI by two routes of administration, even when treatment was started five days post disease induction. Treatment with GSI resulted in a consistently more robust rescue than that of the Notch1 conditional knock-outs. After our in vitro analysis we believe this could be explained by several observations. First is through the decrease in CD25+ and IFN-γ expression that was observed in GSI-treated cells as opposed to Notch1 deficient cells. Additionally cells treated with GSI showed not only decreases in protein expression per cell as determined by MFI, there was also a significant decreases the percent of cells expressing each protein with GSI treatment but not when N1KO T cells. This observation suggests that another target of GSI is being
affected or perhaps another Notch (2-3) is compensating in the loss of Notch1 although this unlikely since we did not observe robust changes in Notch2 or 3.

To ask the question of how Notch1 is effecting disease progression we performed \textit{in vitro} polarization assays which determined that inhibition of Notch1 led to a decrease in the protein expression of T-bet, Granzyme B and IFN-\(\gamma\). Inhibition of Notch1 through genetic means or with the treatment of GSI leads to slightly different patterns of protein expression. Although there are dramatic differences in protein expression, qRT-PCR revealed that there were no significant alteration in the gene expression when Notch1 was conditionally deleted or inhibited with GSI. The mechanism which leads to decreased protein expression remains unclear although we speculate it could be through the regulation of microRNAs.

The delayed disease onset in mice in which Notch1 is inhibited is consistent with an observation made by Anushka Dongre (unpublished) that, at 24 hours, there are apparent defects in IL-2 production, IL-2 receptor expression (CD25) and expression of CD69 (very early antigen). However after 72 hours this defect is no longer observed, suggesting that there is an initial delay in T cell activation, but it is able to recover after extended stimulation. This delay in T cell activation may be a result of defective NF-\(\kappa\)B signaling in the absence of Notch1. This defect may be due to decreased nuclear retention of NF-\(\kappa\)B by Notch, or insufficient formation of the CBM complex when Notch acts as a scaffolding protein. These possibilities correspond with another observation made in our lab (Christina Arieta and Gaberila Gonzalez-Perez) that inhibition of NF-\(\kappa\)B through genetic and biochemical approaches also lead to delayed disease onset in AA mice.
A recent publication implicated Notch as an essential regulator in another GVHD model (Zhang et al., 2010). In this publication Notch was abrogated using a dominant-negative mastermind mouse. This study showed that dominant negative mastermind donor cells became less activated (by CD25 expression) and had decreased expression of IFN-γ, granzyme B, perforin and IL-2, by qRT-PCR. These results vary from what we saw in our studies looking at the gene transcription regulated by Notch1. These discrepancies can be attributed to different methods of inhibiting Notch. It is important to note that a decrease in Notch activation was not validated in these studies. This report also demonstrated that there was no reduction in T-bet levels, in donor dominant negative mastermind T cells. This observation is consistent with our results showing there is no difference in T-bet expression by gene transcription when Notch1 is inhibited. Our data clearly demonstrates that in the absence of Notch1 we dramatically decrease the protein expression of the master Th1 regulator, T-bet.

Our studies concluded that long-term treatment with GSI had no detrimental effects on hematopoiesis. Hematopoiesis was determined not to be affected based on the stem cells ability to engraft lethally irradiated mice, clongenic assays and flow cytometry analyses of lineage differentiation and HSC population. Each of these tests showed no significant difference in the repopulation capacity of BM cells from mice that received control or GSI chow. This suggests that GSI could be used as an immunosuppressive to treat of AA with no detrimental effects on hematopoiesis or engraftment if patients were then eligible to receive a BM transplant. Additionally, inhibition of Notch1 in a BMT model was shown to decrease GVH response. This observation suggests that continued
GSI treatment after a BMT would decrease the likelihood of GVH response (Zhang et al., 2010), but still allow for complete engraftment of stem cells.
Figure 3.1. Notch1 Activation is Increased in T Cells in AA Induced Mice

Bone marrow cells and splenocytes were isolated and stained with antibodies specific for CD4, CD8 and intracellular Notch1. Increased N1ic expression can be observed by the shift in MFI represented in the histogram overlay (A). Quantification of N1ic by MFI expression in each T cell subset is collated in the graph (B).

**P<0.01
Figure 3.2. Upregulation of Notch in AA Mice, Specifically Notch1

Isolated CD4 and CD8 T cells from the spleen and bone marrow of diseased mice showed significant increase in the expression of notch1 (A), but not notch 2 (B) or notch3 (C). Additionally, the transcriptional activator of Notch1-regulated genes, rbpj, was increased in splenic T cells compared to control T cells (D). All genes were normalized to β-actin and their relative expression was based on CD4 and CD8 T cells isolated from the spleens of irradiation-control mice. Values were determined using the delta, delta CT method.
Figure 3.3. Validation of Notch1 Conditional Knock-out Mice

A schematic demonstrating our floxing protocol and experimental methodology used to validate Notch1 deletion (A). qRT-PCR demonstrated that Notch1 gene expression was significantly decreased following our floxing protocol in splenic T cells isolated from N1KO mice compared to Cre and Poly IC controls (B). Decreased protein expression of $N1^{ic}$ was also observed using flow cytometry (C). CD4 and CD8 percentages in N1KO mice and controls showed no significant differences by flow cytometry analysis (D).

* $P<0.05$, ** $P<0.01$, *** $P<0.001$
Figure 3.4. Mice that Receive Notch1 Deficient Splenocytes have Intermediate Disease Phenotype

Mice that received N1KO splenocytes had significantly higher BM cellularities at the time of harvest on day 17 (A). These mice also presented with less weight loss and increased white and red blood cells compared to control induced mice (B-D). CBA analysis revealed that Notch1 presence or absence in bulk splenocytes had no affect on the amount of circulating IFN-γ and TNF (E&F).

*P<0.05, **P<0.01
Figure 3.5. Notch1 Deficient T cells Fail to Infiltrate the Bone Marrow which Leads to Increased Survival

Donor-derived T cells from N1KO mice were significantly reduced in the BM by FACS analysis, compared to control induced animals (A). The T cells that did infiltrate the bone marrow in N1KO recipient F1 mice had decreased intracellular Notch1 levels based on MFI expression (B). N1KO recipient mice succumbed to disease later than control induced animals. (C) Median survival times for recipients were 19 days for Cre controls, 21 days for PolyIC controls and 25 days for N1KO induced mice.

*P<0.05, **P<0.01
Figure 3.6. Notch1 is Decreased *in vivo* after Treatment with GSI chow

Mice were fed GSI chow (5mg/kg) for two weeks after which time splenocytes were harvested. Splenocytes were stimulated with plate bound anti-CD3 and anti-CD28 for 24 hours. Splenocytes were then stained for CD4, CD8 and N1^ic^ and analyzed by flow cytometry. MFI's are represented by a histogram looking at intracellular Notch1 in CD4 (upper) and CD8 cells (lower panels). CD4 cells had 24.8% less N1^ic^ expression compared to control fed mice and CD8 cells had 26.3% less N1^ic^ expression. Data are representative of one experiment.
Figure 3.7. GSI Treatment Ameliorates Bone Marrow Failure

Mice were treated with GSI (LY575411) by two routes, orally (in rodent chow) or by intraperitoneal injection. Treated mice had increased bone marrow cellularities and increased circulating levels of white and red blood cells (A-C). Mice treated with GSI also had decreased amounts of weight loss (D). Values for all controls and AA-induced mice are shown in the graphs on the left and values for AA-induced treated or untreated mice are shown in the graphs on the right.

*P<0.05, **P<0.01, ***P<0.001
Figure 3.8. GSI Treatment Decreases T cell Infiltration and Increases Survival Time

GSI treatment by either route of administration resulted in significant reduction of infiltrating T cells into the BM (A). GSI chow-treated mice had decreased intracellular Notch1 levels compared to control fed mice (B). CBA analysis of INF-γ and TNF showed significant reduction in the level of these circulating cytokines when treated with GSI chow (C&D). Decreased disease severity led to increased survival time of mice treated with GSI chow, compared to mice fed control chow (E).

*P<0.05, **P<0.01, ***P<0.001
Figure 3.9. Therapeutic Administration of GSI Attenuates Disease

Mice treated with GSI, starting at day 5 post disease-induction show rescue of BM cellularity, weight loss and peripheral pancytopenia (A-D), and reduced T cell infiltration into the bone marrow (E). Mice treated beginning at day 5 also had slightly decreased levels of inflammatory cytokines (F&G)

**P<0.01, ***P<0.001
Figure 3.10. Experimental Outline of in vitro Studies

Bulk splenocytes were isolated and CD4⁺ and CD8⁺ T cells were separated using the IMAG system. Co-cultured CD4 and CD8 T cells were stimulated under Th1 polarizing conditions for 72 hours. At that time some cells were re-plated and stimulated with anti-CD3 for an additional 5 hours, in the presence or absence of GSI. All samples were then stained and analyzed by flow cytometry.
Figure 3.11. Successful Reduction of Notch1 Protein Expression

Levels of N1^c were significantly reduced in CD4 and CD8 T cells that were treated with GSI or isolated from N1KO mice (A&B).

**P<0.01, ***P<0.001
Figure 3.12. Decreased T<sub>H</sub>1-Associated Proteins in CD<sub>4</sub><sup>+</sup> T cells

CD<sub>4</sub><sup>+</sup> T cell were gated on and the MFI levels of T-bet (A) CD25 (B) and IFN-γ (C) were determined. GSI-treated and N1KO T cells showed significant reduction of T-bet levels. CD25 expression was decreased when GSI was added at time 0, but not prior to the restimulation. IFN-γ was reduced in the absence of Notch1 both in GSI-treated and in N1KO cells (C).

*P<0.05, **P<0.01, ***P<0.001
Figure 3.13. Decreased CTL Associated Proteins in CD8\(^+\) T Cells

CD8\(^+\) T cell were gated on and the levels of T-bet (A), Granzyme B (B), CD25 (C) and IFN-\(\gamma\) (D) were analyzed by MFI. GSI and N1KO T cells showed significantly reduced levels of T-bet and Granzyme B. CD25 expression was altered in the presence of GSI at time 0, but not prior to the restimulation. IFN-\(\gamma\) was reduced in the absence of Notch1 both in GSI-treated and N1KO T cells.

\(*P<0.05, \,**P<0.01, \,***(P<0.001\)
Figure 3.14. Decreased Percentages of T cells Expressing Notch1 After its Abrogation

Flow cytometric analysis of CD4 (A) and CD8 (B) T cells showed a reduction in the percent of T cells that were expressing intracellular Notch1 after 72hrs stimulation and a 5hr restimulation. Adding GSI at the time of the restimulation had no effect on Notch1 expression.

*P<0.05, **P<0.01, ***P<0.001
CD4⁺ T cell were gated on and the percentages of cell expressing T-bet (A), CD25 (B), and IFN-γ (C) were analyzed by flow cytometry. GSI treatment decreased percentages of cell expressing T-bet and IFN-γ; however, N1KO T cells did not show robust differences in the percentage of cells expressing T-bet or IFN-γ. Percentages of cells expressing CD25 were not altered under any conditions.

*P<0.05, ***P<0.001
Figure 3.16. Reduced Percentages of CD8+ T cells Expressing CTL Associated Proteins

CD8+ T cells were gated on and the percentages of T-bet (A), Granzyme B (B) CD25 (C), and IFN-γ (D) were analyzed by flow cytometry. GSI treatment resulted in decreased percentages of cells expressing T-bet, Granzyme B and IFN-γ; however, N1KO T cells did not show robust differences. Percent of cells expressing CD25 were not altered under any conditions.

*P<0.05, **P<0.01, ***P<0.001
After 72 hours, RNA was isolated from CD4\(^+\) and CD8\(^+\) T\(_H1\) polarized cells. The following genes were then analyzed in triplicate: Notch1 (A), Notch2 (B), T-bet(C), Hes1 (D), Granzyme B (E), IFN-\(\gamma\) (F) and platelet factor four (G). These genes were chosen based on previous analyses of dysregulated genes in AA mice.

\(**P<0.01\)
Mice were fed GSI or control chow for 6 months beginning at 6 weeks of age. Mice were sacrificed and BM cells were isolated. Total BM cells were used to reconstitute a group of lethally-irradiated animals. Of this group, some mice were maintained on GSI chow during the reconstitution period of 4 months. After 4 months mice were sacrificed and total BM cells were isolated and used to reconstitute a second group of lethally irradiated mice. The second group of animals was allowed to reconstitute for 2 months. Of this group, some mice were maintained on GSI chow during the reconstitution period of 2 months.

**Figure 3.18. Experimental Outline of Long-Term GSI Treatment Studies**
After 6 months on control or GSI show mice showed no difference in the number of nucleated cells in the bone marrow (A). Mice that were reconstituted from the 6 month group also showed no differences in cellularity between control and GSI recipients. Additionally mice maintained on GSI during the reconstitution period did not have altered cellularity (B). The second group of recipient mice was reconstituted for 2 months, at which time there was no difference in mean cellularity between groups.
Figure 3.20. HSC from GSI-Treated mice can Repopulate Clonogenic Assays

Clonogenic assays were performed according to the manufacturer’s protocol and were plated in duplicate. Colonies were counted after 12-14 days of culture. HSCs from both control- and GSI-fed mice were able to produce equal colony numbers (A). The first groups of reconstituted mice were also able to repopulate clonogenic assays, even when maintained on GSI chow during engraftment (B). The second transplanted group showed similar capabilities to repopulate clonogenic assays and had equal number of colonies.
Figure 3.21. Long Term GSI Treatment does not Affect Development of any BM-derived lineages

Bone marrow cells from each group and condition were isolated and stained for flow cytometric analysis using a lineage kit (BD). There were no differences in the percentages of each lineage analyzed including B220, CD3, CD11B, Ly-6G, Ter119 or total amount of lineage positive cells between control- or GSI chow-fed mice. Samples analyzed were from mice fed either control or GSI chow (A), four month reconstituted mice from donors that received control or GSI chow (B), or mice that received bone marrow from the “First Reconstitution” group (C).
Figure 3.22. GSI does not Perturbed Stems Cell Percentages

BM cells were isolated and stem cells were quantified using flow cytometric analysis. Bone marrow cells were stained for the lineage markers, CD34, c-kit and Sca-1. Stem cells (HSC) were determined to be lineage-, CD34⁻/lo, c-kit⁺ and Sca-1⁺. These cells were then quantified for each group including 6 month treated (A), 4 month reconstituted (“First Reconstitution”) (B) and 2 month reconstituted animals, (“Second Reconstitution) (C). There were no deficiencies or differences in stem cell percentages under any of the GSI-treated conditions.
CHAPTER 4
PKC-Θ IN A MURINE MODEL OF APLASTIC ANEMIA

4.1 Introduction

Therapies targeted towards the prevention of T cell activation, such as cyclosporine A, when used in combination with ATG, have proved to be effective in decreasing disease severity of AA. Therefore, we wanted to determine if PKC-Θ, a key player in T cell activation, was involved in the disease progression in our model of AA. PKC-Θ was first implicated in AA when one study discovered that treating CD4⁺ T cells from AA patients with rottlerin (a PKC-Θ inhibitor) resulted in down regulation of T-bet (Solomou et al., 2006). Rottlerin was also shown to dramatically decrease human CD4⁺ and CD8⁺ T cells’ ability to become activated using an in vitro system (Springae et al., 2007). Rottlerin is believed to inhibit PKC-Θ by competing with ATP binding. Subsequently, this leads to inhibition of PKC-Θ phosphorylation and reduces its activity on its substrates. Based on PKC-Θ’s essential role in T cell activation it is likely to play an important role in mediating T cell activation, proliferation and tissue destruction in our model of AA.

The availability of PKC-Θ null mice on a C57BL/6 background allowed us to first validate PKC-Θ through genetic means, by using them as bulk splenocyte donors, which are then infused into our F1 hybrids. PKC-Θ knockout mice were generated by Dan Littman’s group in 2000 (Sun et al., 2000). These mice lead to the discovery that loss of PKC-Θ results in deficiencies in TCR-induced activation in mature, but not immature T cells. PKC-Θ knock-out mice have been well-documented to have normal T_{H}1 responses.
to viral infection, but in the following studies we observed defective T cell responses to the MHC mismatch GVHD used in this model.

In addition to determining if PKC-θ had an important role in mediating disease we also wanted to determine in which cellular subset PKC-θ was necessary to mediate disease and bone marrow destruction. To complete these studies we used add back experiments in which we combined CD4⁺, CD8⁺ and APCs in different combinations from wild-type and PKC-θ⁻/⁻ mice. These combinations of wild-type and PKC-θ⁻/⁻ cells were then added back to recipient mice as outlined in Figure 4.4; disease was then evaluated at day 17.

PKC-θ has been shown to be important in the production of T regulatory cells, which are necessary to maintain immune homeostasis. Because PKC-θ⁻/⁻ mice showed decreased number of Tregs in the peripheral pool (Jeremy Samon, unpublished observation) we asked whether loss of PKC-θ in the recipient mice influenced disease severity in PKC-θ deficient F1 hybrids. Deficiency in the Treg pool has also been observed in patients with AA. This mechanism is driven by the loss of NFAT1 expression in patients with AA that subsequently leads to decreased FOXP3 expression, the master transcriptional regulator of Treg differentiation (Solomou et al. 2007).

Similar to our experiments exploring the role of Notch1 in AA and GSI as a therapeutic, we examined whether rottlerin held potential to be a viable therapeutic option. Therefore, AA mice were treated with rottlerin and disease symptoms were analyzed. We performed therapeutic studies in which we began treatment either 1 hr after disease induction or +10 days after disease induction. Since PKC-θ is vital in T cell activation it has been implicated in disease progression of many T cell mediated
autoimmune diseases and is an excellent therapeutic target due to its specific expression in T cells (Baier and Wagner, 2009).

4.2 Results

4.2.1 Mice that receive PKC-θ−/− Splenocytes Fail to Develop Disease Symptoms

To determine if PKC-θ plays a role in our murine model of AA we first used a genetic approach. To address this in our model we used PKC-θ−/− mice as bulk splenocyte donors and compared disease to F1 recipients that received bulk splenocytes from wild-type donor. Mice that received PKC-θ−/− splenocytes failed to develop any disease symptoms; they exhibited normal BM cellularity, no observed weight loss or decreased peripheral blood counts, unlike their wild-type-induced litter mates (Figure 4.1 A-D). Additionally these mice were able to survive long past wild-type controls to day 31 at which point they were humanly euthanized. At day 31 there were no signs of disease as determined by cellularity, weight loss and peripheral blood values (Figure 4.1 A-D). Additionally there was no T cell infiltration into the bone marrow of mice induced with PKC-θ−/− splenocytes either at day 17 or day 31 post-disease induction (Figure 4.2 A). Finally, there was no increase in the amount of IFN-γ or TNF in the circulation of mice induced with PKC-θ−/− splenocytes compared to WT induced controls Figure 4.2 B.

Using GFP+ PKC-θ−/− mice or GFP+ wild-type allowed us to locate the donor splenocytes, to address the question of whether PKC-θ−/− splenocytes did not survive in recipients or whether they failed to infiltrate the bone marrow. At day 17, there was very little GFP expression in any of the secondary lymphoid organs or circulating in the blood
whereas WT controls had massive infiltration of donor-derived cells in all tissues examined (Figure 4.3 A&B). This finding was suggestive that the cells deficient in PKC-ζ do not survive in the circulation as inactivated T cells, and most likely undergo apoptosis, as had been shown in the literature. To determine the expansion of donor T cells over the disease time course the mice were induced with AA and harvested at the following time points post-induction: days 3, 6, 9, 12, 13, 15 and ending with day 17. We discovered that, unlike wild-type T cells, PKC-ζ−/− T cells failed to expand above 5% at any time point in any of the organs analyzed, with the percent GFP-positive expression peaking around day 12 and decreasing until day 17 (Figure 4.3 B). The control-induced mice had massive expansion of GFP+ donor cells in the circulation and in the lymphatic system beginning at day 13 (Figure 4.2 A). Using flow cytometry, T cell expansion was measured by GFP expression present in the spleen, peripheral blood and BM as well as in the cervical, axillary, mesenteric and inguinal lymph nodes.

4.2.2 PKC-θ Drives Disease in the CD8+ Compartment of T cells

Our original hypothesis was that PKC-θ was essential to initiate disease specifically in the CD4+ T cell compartment. This hypothesis was based on observations in the literature that CD4+ T_{H1} cells are believed to be the initiating cell type in autoimmune disorders such as AA. In order to define which T cell compartment (CD4 or CD8) was essential for mediating disease, we performed a series of add-back experiments consisting of wild-type and PKC-θ−/− T cells in different combination. To distinguish between WT and PKC-θ−/− T cells, we used GFP+ PKC-θ−/− and WT GFP−. Add-back experiments were performed using the combination of T cell subsets and genotypes
shown in the schematic in Figure 4.4. Interestingly, we found that loss of PKC-θ in CD4+ T cells was not sufficient to prevent disease whereas loss in CD8+ T cells was unable to initiate disease. We evaluated disease by BM cellularity weight loss and peripheral pancytopenia. We observed that mice receiving PKC-θ−/− CD4+, WT APCs and WT CD8+ induced mice developed severe hypocellular BM equivalent to control WT-induced mice (Figure 4.5 A). Using this combination of T cells with PKC-θ−/− APCs resulted in similar levels of hypocellularity (Figure 4.5 A). Mice that were induced with WT CD4+ with PKC-θ−/− CD8+, and either PKC-θ−/− or WT APCs, had normal cellularities (Figure 4.5 A) When we isolated both T cell subsets from WT mice and added them to PKC-θ−/− APCs we observed levels of hypocellular BM similar to WT-induced controls (Figure 4.5 A). Mice that received PKC-θ−/− T cells and WT APCs exhibited normal BM cellularities (Figure 4.5 A). We observed similar trends in weight loss for each condition as we with the BM cellularities (Figure 4.5 B). When observing peripheral values we saw marked decreases in the circulating white blood cells in the following three conditions, WT induced, PKC-θ−/− CD4+, WT APCs and WT CD8+ and WT CD4+, WT CD8+, PKC-θ−/− APC (Figure 4.6 A). Similar to what we observed in circulating levels of white blood cells, the same three induction conditions resulted in decreased levels of circulating red blood cells (4.6 B).

Infiltration of cells to the BM in add-back experiments demonstrated a very interesting pattern. Mice that received CD4+ WT cells and CD8+ KO cells (with either source of APCs) failed to develop infiltration into the bone marrow cavity of either CD4 or CD8 T cell subset, similar to that seen with of PKC-θ−/− induced mice (Figure 4.7 A&B). However WT CD8+ T cells were capable of initiating infiltration of both WT
CD8+ cells and PKC-θ−/− CD4+ T cells, but only in the presence of WT APCs (Figure 4.7 A&B). Using the combination of PKC-θ−/− CD4+, WT CD8+ and WT APCs also led to increased CD8+ T cell infiltration. This finding is suggestive that PKC-θ−/− may also play a role in the T cell depleted population. Mice that received T cells of both subtypes, either WT CD4+, CD8+ plus PKC-θ−/− APCs or PKC-θ−/− CD4+, CD8+ and WT APCs, mimicked T cell infiltration in WT-induced or PKC-θ−/−-induced animals, respectively. This observation demonstrated that intact PKC-θ in the CD4 and CD8 T cells takes precedent over PKC-θ expression in the APC subset (Figure 4.7 A&B).

4.2.3 PKC-θ−/− Signaling is only Essential in the Donor T cell Compartment

To determine if decreased Treg pools in recipient mice influence disease severity we created F1 hybrids that were PKC-θ−/−. These mice were generated as outlined in the Materials and Methods. Mice were determined to be PKC-θ−/− as evaluated by genotyping and western blot. Lysates prepared from total splenocytes demonstrated that there was no protein product detected in PKC-θ−/− F1 hybrids, whereas intact PKC-θ was detected in F1xF1 hybrid controls, Figure 4.8. PKC-θ−/− F1 hybrids or F1 controls were induced and monitored for signs of disease until day 17. At that time BM cellularity, weight loss, peripheral pancytopenia, and T cell infiltration was analyzed. Increased disease severity in the PKC-θ deficient recipients was not observed, compared to control recipients, Figure 4.9 A-D. Additionally there were no differences in the levels of circulating cytokines of IFN-γ or TNF between wild type and PKC-θ−/− F1s. Since we observed no clear differences in the two groups additional experiments were not pursued.
4.2.4 Rottlerin is an Inhibitor of PKC-θ

Rottlerin was first described as an inhibitor of PKC-δ (Gschwendt et al., 1994). Later studies revealed this was not the case and identified it as a PKC-θ inhibitor (Soltoff, 2007). Rottlerin has been shown to prevent phosphorylation and subsequent kinase activity of PKC-θ (Springael et al., 2007). To demonstrate rottlerin as an in vivo inhibitor of PKC-θ, we treated mice for 5 days with IP injections of rottlerin at a dose of 10mg/kg. CD4+ T cells were isolated by positive selection and stimulated with plate-bound anti-CD3 and anti-CD28 for 48 hours. Cell lysates were prepared and analyzed by western blot. To ensure that rottlerin was preventing phosphorylation of PKC-θ we probed blots for total PKC-θ and phospo-PKC-θ (Figure 4.10 A). A clear decrease in the phosphorylation of PKC-θ was observed in mice that were treated with rottlerin, documenting its effectiveness in vivo.

4.2.5 Rottlerin Treatment Results in Complete Disease Abrogation

Once we validated rottlerin as an inhibitor of PKC-θ we began toxicity studies to ensure there were no adverse side effects. After a 17 day treatment with rottlerin there was no apparent defect in total T cell percentages in either T cell compartment, data not shown. We then began treating AA-induced mice daily, beginning one hour after disease induction. Mice that received rottlerin treatment had increased BM cellularity and no weight loss, peripheral pancytopenia or T cell infiltration, Figure 4.11 A-E. Using GFP+ mice as donors, we determined there was no expansion of donor-derived T cells in the host, as quantified by flow cytometry (Figure 4.12 A). Also there was no increase in the
levels of IFN-γ or TNF, Figure 4.11 F-G. Treatment with rottlerin resulted in complete disease abrogation, but had no ill effects on non-activated host T cells (Figure 4.12 B).

Mice treated with rottlerin exhibited no signs of disease when treatment was begun 1hr post disease induction. With such robust rescue, we decided to begin rottlerin treatment starting at day 10. This time point was chosen because T cells in the spleen don’t begin expanding until day 9 (Figure 4.3 A). Preliminary studies dosing at 10mg/kg daily did not result in disease rescue, therefore in subsequent studies we administered a dose of 20mg/kg a day of rottlerin. Mice that received rottlerin treatment had increased BM cellularity, decreased weight loss and increased numbers of circulating white and red blood cells (4.13 A-D). Rottlerin-treated mice also had decreased infiltration into the bone marrow cavity (4.13 E), and decreased levels of the inflammatory cytokines, IFN-γ and TNF (4.13 F&G). These experiments outline the excellent reduction in T cell-mediated destruction resulting from using rottlerin administered in a clinically-relevant time frame.

4.3 Discussion

Using our mouse model of AA we have established that PKC-ζ is essential for the initiation of disease. T cells deficient in PKC-ζ fail to expand and proliferate in response to the MHC (H2^b into H2^d) mismatch, as has been documented previously (Lei et al., 2009). These studies demonstrate that PKC-ζ deficient cells undergo apoptosis in our murine model in response to the MHC mismatch, since the percent of T cells in the circulation decreases over the disease course from day 3 to day 17. There are several studies in the literature that have shown that in the absence of PKC-ζ stimulation of T
cells results in accelerated apoptosis. PKC-θ is needed during T cell stimulation to counteract the actions of the pro-apoptotic molecule BAD. It is also required for the upregulation of Bcl-xL and Bcl-2, which is anti-apoptotic molecules (Bertolotto et al., 2000; Villalba et al., 2001; Hindley et al., 2001; Manicassamy et al., 2006). We believe that these mechanisms are also playing a role in inducing apoptosis of PKC-θ deficient cells in our AA model.

Another mechanism that could be responsible for the decreased survival of PKC-θ−/− T cells is defective IL-2 production. Mature T cells from PKC-θ knockout mice have defects in NF-κB-mediated production both of IL-2 and its receptor CD25 (Sun et al., 2000). Additionally, findings that abrogation of NF-κB activity, through genetic or biochemical approaches also resulted in decreased disease severity in our model of AA (Christina Arieta and Gabriela Gonzalez-Perez, unpublished data).

Using add-back studies, CD8+ T cells were found to be the T cell compartment necessary for the development of disease in AA mice. Other GVHD models have established that in acute models, such as the model of AA we created, GVHD is mediated by CD8+ T cells, while models of chronic GVHD are mediated by CD4+ T cells (Yu et al., 2006). In our model of AA we did not observe any signs of chronic GVHD in mice induced with wild-type CD4+ cells. These results could be attributed to several different factors. One possibility is that mice receiving CD4+ wild-type cells were not allowed enough time to develop chronic GVHD and were sacrificed at day 17 before onset of any symptoms. Another possibility is that the number of cells which we use to induce disease is not sufficient to induce a chronic response.
CD8+ T cells have been found to be more efficient in mediating acute GVHD due to their ability to recognize MHC mismatch on both donor and host APCs. On the contrary, CD4+ T cells are only capable of recognizing responses to donor APCs (Yu et al., 2006). The most valuable and interesting finding of our add-back experiments is that the process of T cell activation and expansion in the WT CD8+ T cells allowed for the expansion and infiltration of the CD4+ PKC-0/- T cells. This observation suggests that the secretion of cytokines such as IL-2 may supply this necessary growth factor to allow the expansion of CD4+ PKC-0/- T cells. However these studies also demonstrated that WT CD8+ T cells needed WT APCs (donor derived) to be present to allow for this expansion and infiltration of PKC-0/- CD4+. Although the combination of WT CD8+ and PKC-0/- CD4+ and WT APCs lead to disease symptoms, the combination of WT CD4+ and CD8+ T cells with PKC-0/- APCs had the most robust disease induction compared to WT induced mice. This suggests that although we may have found a role for PKC-0 in the T cell-depleted population, when PKC-0 is present in both T cell subsets PKC-0/- APCs are sufficient to induce disease. In conclusion, these studies demonstrated that PKC-0 expression in CD8+ cells is necessary for the development of disease in our model of AA. However, these studies do not rule out that CD4+ WT cells are not sufficient to induce disease. CD4+-mediated responses may take a longer period to develop than the 17 day time course used here. Additionally, we may have discovered a novel role for PKC-0 in donor APCs.

Although mice deficient in PKC-0 have been shown to have decreased numbers of Tregs in their peripheral pool (Jeremy Samon, unpublished observation), we observed no signs of accelerated disease when F1 PKC-0/- mice were induced. Compared to F1xF1
hybrid controls, induced PKC-θ−/− mice had decreased BM cellularity, increased weight loss, peripheral pancytopenia, robust T cell infiltration into the bone marrow and increased levels both of IFN-γ and TNF (Figure 4.9 A-G). These results demonstrate that the presence or absence of PKC-θ in recipient mice does not accelerate or inhibit disease progression in our model of AA. This finding suggests that our model may not be a good resource for studying whether T regulatory cells play protective role patients with AA.

Currently PKC-θ inhibitors have been used clinically for the treatment of different cancers (http://clinicaltrials.gov/, 2011). In the literature there have been very few studies looking at the therapeutic benefits of rottlerin in other models of autoimmune disorders. This finding is very interesting considering how many autoimmune models have implicated PKC-θ as playing a role in disease progression (Salek-Ardakani et al., 2005; Tan et al., 2006; Salek-Ardakani et al., 2004; Nagahama et al., 2008; Healy et al., 2006). In this AA model rottlerin was a very effective inhibitor of T cell activation that resulted in complete disease abrogation when delivered 1hr post disease induction and provided substantial rescue even when treatment was begun +10 days after disease induction. Treatment with rottlerin showed no toxicity towards host T cells that were not activated; only T cells that were activated were affected by rottlerin treatment (Figure 4.12 A&B).

These experiments showed for the first time showed the essential role of PKC-θ in the development of BMF in our AA model. These findings also show that the robust expansion which is seen in induced animals can be completely eliminated with treatment of rottlerin. Additional studies with other PKC-θ inhibitors that have been used clinically would further support the use of PKC-θ inhibitor in the treatment of AA patients.
Figure 4.1. Mice that Receive PKC-θ⁻/⁻ Splenocytes do not Develop Disease

Mice induced with PKC-θ⁻/⁻ splenocytes have increased survival; some mice were harvested on day 31. Mice do not exhibit hypocellular bone marrow (A), weight loss (B) or peripheral pancytopenia (C&D)

*P<0.05, **P<0.001
Figure 4.2. PKC-θ⁻/⁻ Fail to Infiltrate the Bone Marrow or Induce Cytokines

Bone marrow cells were stained with antibodies specific for CD4 and CD8 analyzed by flow cytometry to determine the percent of CD4⁺ and CD8⁺ infiltrating T cells. Mice that received PKC-θ⁻/⁻ cells had no infiltration at either of the time points analyzed (A). CBA analysis of the plasma isolated from these mice showed no differences in the levels of circulating cytokines, IFN-γ and TNF, compared to irradiation controls (B).

*P<0.05, ***P<0.001.
Figure 4.3. PKC-θ Deficient T cells Fail to Expand in F1 Recipients

A time course was performed to look at the expansion of donor WT versus PKC-θ⁻/⁻ T cells. Mice were induced either with WT or PKC-θ⁻/⁻ splenocytes, and then sacrificed at days 3, 6, 9, 12, 13, 15 ending with day 17. To track donor T cells GFP⁺ WT or PKC-θ⁻/⁻ mice were used as donors. At each time point T cell expansion was analyzed in the following organs; spleen, lymph nodes (cervical, axillary, mesenteric, inguinal), peripheral blood and bone marrow. GFP expression was quantified by flow cytometric analysis for WT (A) or PKC-θ⁻/⁻ T cells (B). There was no increase in GFP-expressing PKC-θ⁻/⁻ T cells (B) at any of the time points or tissues analyzed, compared to the rapid expansion of WT donor T cells (A).
Figure 4.4 Schematic for Add Back Experiments using PKC-θ⁻/⁻ Subsets

The combination of cell types shown will be used to induce F1 recipients. Disease severity will be evaluated on day 17.
Figure 4.5. Disease Develops only in the Presence of Wild-Type CD8$^+$ T Cells

Add-back combinations which contained wild type CD8$^+$ T cells were necessary for developing bone marrow hypocellularity (A). Although these F1 recipients developed hypocellular bone marrow they appeared to have no significant weight loss compared to mice that received wild-type splenocytes, suggesting decreased disease severity. Weight loss was most robust when WT CD4$^+$ and CD8$^+$ T cells were infused in combination with PKC-θ$^{-/-}$ APCs (B).
Figure 4.6. Add-Back Combinations Require WT CD8⁺ T cells to Induce Robust Peripheral Pancytopenia

CBCs performed on peripheral blood samples revealed that mice that received wild-type bulk splenocytes, WT CD8⁺ T cells, PKC-0⁻/⁻ CD4⁺ and WT APCs, WT CD4⁺ CD8⁺ T cells and PKC-0⁻/⁻ APCs or WT CD8⁺ T cells, CD4⁺ T cells and KO APCs led to robust peripheral pancytopenia both in the white (A) and red (B) cell compartments.
Figure 4.7. Add Back Combinations Reveal a Novel Role for PKC-θ in APCs Resulting in T cell Infiltration

Wild-type CD8$^+$ T cells and APCs are necessary for PKC-θ$^{-/-}$ CD4$^+$ T cells to infiltrate into the BM cavity (A). In the absence of wild-type CD8$^+$ T cells, CD8$^+$ T cells failed to infiltrate the bone marrow cavity (B). Mice induced with CD4$^+$ wild type combinations fail to develop disease symptoms, similar to mice induced with PKC-θ$^{-/-}$ bulk splenocytes (A&B). WT CD4$^+$ and CD8$^+$ T cells with PKC-θ$^{-/-}$ APCs results in CD4$^+$ and CD8$^+$ T cell infiltration in the BM (A&B).

*P<0.05
Figure 4.8. PKC-θ−/− F1 mice Lack PKC-θ Protein

To verify that F1 PKC-θ−/− mice lacked PKC-θ protein expression, cell lysates were generated from splenocytes from F1 controls and F1 PKC-θ−/− mice and run on a western blot that was probed for PKC-θ (Santa Cruz), and β-actin (Sigma) (A). Relative expression was normalized using β-Actin as loading control and values were determine using Image J software (B).
Figure 4.9. F1 PKC-θ⁻/⁻ Mice Develop Disease Comparable in Severity to that of F1 Control Mice

F1 recipient mice that were or were not deficient in PKC-θ developed disease symptoms similarly. Both group of mice had hypocellular bone marrow (A), weight loss (B), and peripheral pancytopenia (C&D). T cells infiltrated the BM with similar penetrance and there were similar amount of circulating IFN-γ and TNF at the time of harvest (E).
Mice were treated with rottlerin for 5 days at a concentration of 20mg/kg per day. CD4\(^+\) T cells were then isolated from DMSO or rottlerin-treated mice and stimulated with plate bound anti-CD3 and anti-CD28 for 48 hours. Samples were lysed and a western blot was run. Blots were incubated with a phospho-PKC-\(\theta\) antibody (Cell Signaling). Mice that received rottlerin treatment had decreased phosphorylation of PKC-\(\theta\).
Figure 4.11. Treatment with Rottlerin Results in Complete Disease Abrogation

Daily treatment with rottlerin at a dose of 10mg/kg/ beginning 1hr post-induction resulted in normal BM cellularity (A), no weight loss (B) and no signs of peripheral pancytopenia (C&D). Treatment also prevented T cell infiltration into the BM cavity (E) and inhibited the production of IFN-γ and TNF (F&G).  

**P<0.01, ***P<0.001
Figure 4.12. Rottlerin Inhibits only Activated T cells

Activated donor T cells are reduced in mice treated with rottlerin based on GFP expression as determined by flow cytometric analysis (A). Additionally mice that were induced with AA and treated with rottlerin had similar percentages of CD4 and CD8 T cells in the spleen as irradiated, untreated controls, demonstrating that rottlerin had no ill effects on the host T cells during treatment (B).
Figure 4.13. Rottlerin has Powerful Therapeutic Potential

Mice were induced with AA and began to receive rottlerin treatment beginning at day 10 post disease-induction at a dose of 20mg/kg daily. Treated mice had increased BM cellularity (A), decreased weight loss (B) and increased level of circulating white (C) and red blood cells (D). Rottlerin treatment decreased T cell infiltration into the BM cavity (E).

*P<0.05, **P<0.01
CHAPTER 5
HUMANIZING THE MODEL

5.1 Introduction

To determine if human T cells respond in a GVHD model of AA in the same way murine T cells do, we created a humanized model of AA. We used the same strategy to induce disease with an HLA mismatch. To create humanized mice we used NOD SCID mice that were also lacking the gamma chain of the IL-2 receptor. These mice are capable of being successfully engrafted with human CD34+ stem cells which leads to the engraftment a human immune system (Shultz et al., 2005). Engraftment of human CD34+ leads to the development of human T cells, monocytes, neutrophils in these mice, but not the development of mature B cells. CD34+ stem cell were obtained from pooled donors leading to mice that have the potential to develop T cells of several HLA types.

To induced disease we used human peripheral mononuclear blood cells at a concentration of 6x10^6 cells, delivered through an IP injection. Since there were variations in HLA compatibility, we also saw variable disease induction. However the development of GVHD which targeted the bone marrow allowed us to conclude that the mechanisms which we found to be essential in the murine model of AA also regulates disease progression in this newly defined humanized model of AA.
5.2 Results

5.2.1 Development of Humanized Mice

At 6-8 weeks of age NOD SCID mice were reconstituted with 2x10^5 human CD34^+ stem cells by IV injection following a light irradiation, outlined in Figure 5.1. Mice were then rested for 16 weeks to allow reconstitution of their immune systems. After 16 weeks mice were bled to determine their percent reconstitution of human cells. Peripheral blood was stained for human and mouse CD45 expression and analyzed by flow cytometry. The percent reconstitution was determined using the formula in Figure 5.2 A. With increased time, we saw an increase in the cellularity of reconstituted mice (Figure 5.2B). We were also able to detect CD4^+ and CD8^+ cells of human origin circulating in the periphery of humanized mice, Figure 5.2 C. Using this previously described technique we were able to successfully reconstitute NOD SCID mice with human hematopoietic stem cells.

5.2.2 AA Induction in Humanized Mice Mimics Phenotypes seen in our Murine Model of AA

Bone marrow failure was observed in humanized mice that were induced AA as described in the Materials and Methods. Induced mice succumbed to disease at various time points (between days 8-10). Although the time line varied, the disease symptoms did not. Mice induced with disease had hypocellular bone marrow, Figure 5.3 A. Flow cytometry analysis of the bone marrow of AA humanized mice showed decreased expression of human CD34^+ cells compared to un-induced controls (Figure 5.3B). Unlike
normal mice humanized mice have a resident population of CD4$^+$ and CD8$^+$ T cells in their bone marrow. Although humanized controls had CD4$^+$ and CD8$^+$ T cells in the bone marrow we were able to detect increased expression of both of these populations in the bone marrow of AA-induced humanized mice, based on MFI (Figure 5.3 C).

5.3 Conclusions

Even though there is variability in HLA types in the humanized mice we were still able to generate a GVH response by injecting human peripheral blood mononuclear cells. This humanized model of disease induction recapitulated disease symptoms that were observed in our murine model of AA, such as decreased bone marrow cellularity, loss of stem cells and increased infiltration into the BM of human T cells. Additional steps such as HLA typing the humanized mice would be valuable when creating the HLA mismatch when inducing AA. Further characterization of this model is necessary to establish if Notch and PKC-θ play a role in disease progression. We could also use this model to validate the therapeutic benefits of GSI and rottlerin on human cells in this AA model. This humanized mouse model would bring murine models one step closer to clinical relevance.
Figure 5.1. Schematic of Humanizing Mice

NOD SCID mice were humanized using pooled CD34+ human cord blood (Stem Cell Technology). Mice were rested for 4-6 months after reconstitution, at which time animals were bled and the percent reconstitution was determined.
Figure 5.2. Mice were Successfully Reconstituted with a Human Immune System

Percent reconstitution was determined based on the percent of human CD45$^+$ cells compared to murine CD45$^+$ cells at two time points (A). Bone marrow cellularities were also evaluated at these time points (B). Of the circulating human CD45$^+$ T cells there were robust percentages that were CD4$^+$ or CD8$^+$ (C).
**Figure 5.3. Induction of AA in Humanized Mice**

Disease was induced using human PBMCs. Mice were then monitored for signs of disease and were harvested when they were considered moribund, between days 10-12. Mice induced with disease had decreased BM cellularity (A) and decreased expression of CD34+ cells (B). Additionally there was an increase in the expression of CD4+ and CD8+ T cells in the BM (C).
CHAPTER 6

CONCLUDING REMARKS

The process that converts T cells from friend to foe is a long mysterious road that remains unclear in the case of aplastic anemia. There are so many molecules and mechanisms that are required to operate properly in order to maintain self-tolerance. When taking all of details into account it is quite impressive that in most circumstances the immune system functions so flawlessly. Continued research in the field of AA will lead to a better understanding of disease pathogenesis and possibly the identification of the causative antigen(s). Since people come into contact with so many different pathogens it is likely that there is no single target antigen that results in AA, therefore the establishment of models in which there is little variation in T cell response, but the pathology of disease is maintained, make excellent tools with which to study mechanisms of disease.

The development of this murine model of bone marrow failure as described in Chapter 2, replicates the disease pathology seen in AA patients. In addition to showing the same disease pathology it is also likely that the T cells mediating the disease progression are programmed to respond to the MHC mismatch in a mechanism that is similar to the production of autoreactive T cells. This theory is validated by the observation that T cells isolated from our AA mice share a gene expression profile that is similar to T cells isolated from AA patients (Figure 2.4). Using a model similar to ours led to the discovery of adjunct immunosuppressive therapies that are used clinically
(Bloom et al., 2004). Thus, these results demonstrate the relevance of animal models to the discovery of clinical therapeutics.

Using our model of AA we have demonstrated the importance of the cell surface receptor Notch1, in disease progression. Notch1 is necessary for robust disease onset and a delayed response is observed when N1KO splenocytes are transferred, this results most likely from impaired T cell activation and polarization. Loss of Notch1 appears to cause this delay by modulating the protein expression of T-bet and Granzyme B, which have both been shown to play a role in disease progression in patients with AA (Solomou et al., 2006, Franzke et al., 2006).

Furthermore, we were able to target Notch1 therapeutically through administration of GSI. Currently GSI is being used in clinical trial in the hopes of alleviating disease symptoms associated with Alzheimer’s Disease and many different forms of cancer. Some clinical studies found that there were complications regarding gut toxicity, but the GSI which was used in these studies (LY411,575) had no detrimental effects on the small or large intestines (Searfoss et al., 2003, Milano et al., 2004, Tosello and Ferrando, 2009). GSI treatment in our model of AA appeared to be superior to genetic inhibition of Notch1 because GSI was able to abrogate additional targets that were not affected by the absence of Notch1 (Figure 3.12-3.16). Studies validating a regimen of GSI in combination with ATG would be a great addition to these studies. Due to the inability to obtain ATG we were not able to evaluate the effects of GSI when used in conjunction with ATG, but these would be excellent studies to highlight GSI as a possible adjuvant therapeutic for AA. Another benefit of GSI as a therapeutic is that long treatment showed no harmful effects on hematopoiesis, or the ability of stem cells to
engraft in a GSI-treated recipient mouse. GSI treatment could be used in BMT patients with the added benefit that decreasing Notch activity also decreases GVH response (Figure 3.19-3.22).

Interest in PKC-θ as a possible player in disease progression of AA developed from the observation that PKC-θ knockout mice had decreased Notch1 expression and impaired production of IFN-γ (Lisa Minter, unpublished data). Therefore we hypothesized that inhibition of PKC-θ would reduce disease severity. However, the loss of PKC-θ not only reduced disease severity but completely abrogated disease. Inhibition of PKC-θ prevented the donor T cells from becoming activated, expanding and infiltrating the bone marrow, as described in Chapter 4.

In addition to investigating the role of PKC-θ in donor splenocytes we furthered these studies by investigating the individual T cell subsets and APCs. We found in these studies that intact PKC-θ was essential in mediating CD8+ T cell destruction. PKC-θ in the CD8+ T cells and APC populations was also critical for the infiltration and expansion of PKC-θ−/− CD4+. In all combinations, intact PKC-θ was needed in both T cell subsets and not necessary in the APC compartment to mediate the same level of disease seen in WT induced mice. Loss of PKC-θ in both T cell subsets, in combination with WT APCs, resulted in no disease. In conjunction with these studies we addressed whether loss of PKC-θ in recipient mice showed any phenotype. We observed no differences in disease pathology between PKC-θ−/− F1 recipients and WT F1 mice.

Loss of PKC-θ resulted in dramatic loss of T cell function, therefore, we also examined the benefits of therapeutically inhibiting PKC-θ. Incredibly, we were able to maintain the same disease inhibition by using an inhibitor of PKC-θ, rottlerin, that we
saw in our genetic studies. Rottlerin was well-tolerated and although it had dramatic effects on the T cells that became activated there were no ill effects on the number of circulating host T cells (Figure 4.12). Rottlerin was effective in disease prevention even when begun at day +10 post-disease induction, which demonstrates its therapeutic capacity. Rottlerin or other PKC-ζ inhibitors could serve as other potential therapeutics that would lead to prevention of T cell activation, expansion and polarization in the treatment of AA and other autoimmune disorders.

Taking models of human disease to the next level can be done by utilizing humanized mice. In the case of blood disorders we can take advantage of the ability to grow and differentiate human blood cells in NOD SCID mice. These mice will allow the engraftment of human stem cells and subsequently human hematopoiesis. Mice that produce these human cells are an excellent model for studying human T cell responses. Taking advantage of such technology has allowed us to begin development of a humanized model of AA, which mimics the acute, BM-specific GVH response we demonstrated in the AA murine model. Preliminary data outlined in Chapter 5 demonstrates that this system would be beneficial in evaluating targets such as Notch1 and PKC-ζ in human T cells. This humanized model would also create an effective way to evaluate human T cell response to therapeutic treatments with GSI or rottlerin. The production of models that are half mouse and half human will combine the clinical relevance of human disease into the convenience of animal models providing researchers with the best of both worlds.

There are still many aspects of T cell activation in response to self-antigen that are not completely described. From these studies we propose that both Notch1 and PKC-ζ
play a role in the T cells’ ability to mediate destruction in autoimmune disease to varying degrees. In conclusion, Notch1 plays a role in the disease progression of AA, while PKC-ζ is indispensable for T cell mediated destruction. Both of these pathways most likely function through the activation of NF-κB but may also have other distinct reinforcing mechanisms. More studies will need to be done to determine the exact mechanisms of how Notch1 and PKC-ζ interact, but it has been suggested that there is a relationship between both molecules that is instrumental in T cell-mediated autoimmune disorders. Further studies will be necessary to determine precisely what this relationship entails.
Figure 6.1. Model of Disease

Both Notch1 and PKC-0 play a role in disease induction. Notch1 appears to aid in disease development, while PKC-0 is required. Both molecules interact through NF-κB activity, but direct interaction between one another remains undefined. Loss of either protein results in decreased disease severity in our murine model of AA.
CHAPTER 7
MATERIALS AND METHODS

7.1 Animals

F1 progeny were obtained by crossing Balb/C female with C57BL/6 male, offspring were used in experiments between 9-12 weeks of age. Notch1 conditional floxed mice were obtained by crossing Notch1\textsuperscript{fl/fl} (Notch1\textsuperscript{tm2Rko}/GridJ) to MxCre\textsuperscript{+/-} (B6.Cg-Tg(Mx1-cre)1Cgn/J) from Jackson Laboratory (Bar Harbor, ME.). Homozygous Notch-1 \textsuperscript{fl/fl} females were mated to Notch1\textsuperscript{fl/fl} x MxCre\textsuperscript{+/-} male mice to maintain breeding pairs. C57BL/6 GFP\textsuperscript{+/-} mice were originally obtained from Jackson Laboratory (Bar Harbor, ME), were maintained by crossing C57BL/6 wild type females with GFP\textsuperscript{+/-} males. PKC-\(\zeta\)\textsuperscript{-/-} were created on a C57BL/6 background and were maintained as homozygous breeding pairs, mice were originally received as a gift from Dan Littman. PKC-\(\zeta\)\textsuperscript{-/-} mice were crossed with GFP\textsuperscript{+/-} to obtain homozygous PKC-\(\zeta\)\textsuperscript{-/-} GFP\textsuperscript{+/-} mice. PKC-\(\zeta\)\textsuperscript{-/-} F1 hybrids were created by crossing PKC-\(\zeta\)\textsuperscript{-/-} male mice with female Balb/C to obtain F1 progeny that was heterozygous for the PKC-\(\zeta\) allele. The second generation was created by brother sister mating. Homozygous PKC-\(\zeta\)\textsuperscript{-/-} knockout mice were haplotyped using for H2\textsuperscript{b} and H2\textsuperscript{d} by flow cytometry. H2\textsuperscript{b} PKC-\(\zeta\)\textsuperscript{-/-} females were crossed with H2\textsuperscript{b} males to create progeny that were homozygous for the PKC-\(\zeta\) allele and had a haplotype of H2\textsuperscript{b} and H2\textsuperscript{d}, these mice are thus referred to as PKC-\(\zeta\)\textsuperscript{-/-} F1 mice. Crosses between haplotyped F1 x F1 hybrids were used as controls in these studies. NOD SCID (NOD.Cg-Prkdc\textsuperscript{scid}IL2rg\textsuperscript{tm1Wjl}/SzJ) breeding pairs were purchased from Jackson Laboratory (Bar Harbor, ME), and maintained as a homozygous colony. In order to keep
mice clear of pathogens, mice were placed on acidified, antibiotic water. All mice were housed in the animal care facility at the University of Massachusetts Amherst in accordance with IACUC guidelines.

### 7.2 Bone Marrow Failure Inductions

Balb/C and C57BL/6 F1 progeny were irradiated with 3 Gy of irradiation from a selenium source. Four to six hours later mice received an IP injection with $5 \times 10^7$ bulk splenocytes from an age- and gender-matched donor. Mice were monitored daily for signs of disease until harvested on day 17 or, in survival studies, mice were sacrificed when they were found to be moribund (not able to take food or water) and were considered lethally-induced.

### 7.3 Bone Marrow Failure Harvest

Mice were euthanized using CO$_2$. At time of harvest, final weight was recorded and peripheral blood was harvested using cardiac puncture through a heparinized 21 gauge needle. Liver, sternum, spleen and small intestines were collected for histology. Bone marrow was pulverized using a mortar and pestle in a PBS 5% FBS solution. Splenocytes were isolated by manipulation through a 40μM filter. Red blood cells were lysed using ACK lysis buffer and white blood cells were counted.
7.4 *In Vivo* Floxing of Notch-1

Notch1^fl/fl^ x MxCre^+/−^ mice and Notch1^fl/fl^ x MxCre^−/−^ mice were given 5 IP injections of Poly(I)-Poly(C), Amersham Biosciences (Piscataway, NJ), at a dose of 12-15 μg per gram of weight every other day. Notch1^fl/fl^ x MxCre^+/−^ control mice were given equal amounts of PBS vehicle. Mice were then rested for 3 weeks after the last injection before being used in experiments.

7.5 *In Vivo* Administration of GSI

7.5.1 Pretreatment Studies

Mice were fed either Harlan-Tekland mouse/rat chow formulated with the gamma secretase inhibitor (LY 411,575) formulated to deliver 5mg/kg/day or control chow. Mice were put on GSI chow beginning two weeks prior to disease induction and maintained to GSI chow until harvest date. In injection studies mice received IP injection of GSI 411,575 dissolved in DMSO at a dose of 5mg/kg/day beginning three days before disease induction and every day after, control mice received an equal volume of 50μL DMSO.

7.5.2 Treatment studies

Mice were given daily IP injections of GSI(LY 411,575) or DMSO starting at day 5 after disease induction until harvested at day 17.
7.6 *In Vivo* Administration of Rottlerin

On the day of induction mice received 10mg/kg/day of Rottlerin (Sigma) IP beginning one hour post disease-induction. Control mice received 50μL of DMSO by IP injection daily. In treatment studies, mice began receiving Rottlerin treatment at day +10 post disease-induction at a dose of 20mg/kg daily.

7.7 GSI Long-Term Treatment and Lethal Irradiation and Reconstitution

C57BL/6 GFP+/− mice were fed control or GSI chow for 6 months beginning at 6 weeks of age. Recipient C57BL/6 mice received 12gys of gamma irradiation from selenium sources split into two doses: 8Gys followed by a three hours rest before the remaining 4Gys. Mice then received an IV injection of 2x10⁶ total bone marrow cells from long-term GSI-treated mice. Recipient mice were maintained on acidified antibiotic water and monitored daily for signs of failed engraftment. The first group of transplanted mice were allowed to reconstitute for 4 months, before being euthanized and transplanted into a second group of mice that were allowed to reconstitute for another two months. During the reconstitution period some mice were placed on GSI chow. Due to complications of irradiation-induced dermatitis some mice were treated with silver nitrate cream.

7.8 Clonogenic Assay

Isolated bone marrow cells were seeded in MethoCult media at concentration of 2x10⁴ per well and plated in duplicate. Plated samples were incubated for 12-14 days at
37° in a humidified chamber. Colonies were counted and identified according to the manufactures protocol (Stem Cell Technology).

### 7.9 Reconstitution of NOD SCID mice

Human CD34+ cells were purchased from StemCell Technologies. Cells were thawed and washed twice in PBS before being injected into NOD SCID mice. Twenty four hours prior to injections mice received 1.3 Gys of irradiation from a selenium sources. Mice received 2x10^5 CD34+ Human stem cells IV injection. Human CD34+ cells engrafted for 4 weeks at which time peripheral blood was collected stained for human and murine CD45+, and analyzed by flow cytometry. Percent of reconstitution was determined using the flowing equation (% human CD45+/ (% human CD45+ % mouse CD45+)).

### 7.10 Induction of Bone Marrow Failure in Humanized Mice

On the day induction mice received 1.3 Gys of irradiation, mice were the rested for 4-6 and then received an IP injection of 6x10^6 Human PBMC’s (StemCell Technology). Mice were monitored for disease progression and sacrificed when mice were determined to be moribund.

### 7.11 Histology

Sternums, spleens, livers and small intestines were harvested on day 17 and fixed in 10% neutral buffered formalin overnight (VWR). Sternums were then decalcified for an
additional two days in Cal-rite (Richard Allen Scientific). Samples were then stored in 70% Et-OH at 4°C until they were sent to Pioneer Valley Life Sciences Institute where they were paraffin-embedded and stained with hematoxylin and eosin.

7.12 Surface and Intracellular Flow Cytometry

Isolated bone marrow and spleen cells were counted and resuspended in 100μL of FACS wash buffer (0.2% BSA in PBS) at a concentration of 1x10^6 per sample or less. Staining was done in a V-bottom plate as follows, surface staining at a dilution at 1:100 dilution for 20 minutes at 4°C protected from light. Cells were washed twice with FACS was buffer cells were then fixed and permeabilized for 30 minutes at 4°C using the Foxp3 intracellular staining kit (BD). Cells were then washed twice with perm wash buffer and then intracellular stained at a dilution of 1:50 in perm wash buffer for 30 minutes at 4°C protected from light. Cells were washed twice more and then resuspended in FACS wash buffer and acquired on LSRII flow cytometer with FACS Diva software (BD). Analysis of FACS data was done using FACS Diva (BD Bioscience) or FlowJo (Tree Star) software. A complete list of antibodies can be found in Table 7.1.

7.13 Cytometric Bead Array

Cytokine levels were determined in plasma using either the Th1/Th2 or Th1/Th2/Th17 cytometric bead array kit (BD Bioscience), following manufactures protocol. Plasma samples were acquired on a LSRII and analyzed using FCAP array software (BD Bioscience).
7.14 *In vitro* T cell Isolation and Activation

Spleens were isolated and manipulated through a 40μM filter; splenocytes were treated with ACK lysis buffer to remove red blood cells. CD4 and CD8 T cells were isolated using CD4/CD8 T lymphocyte IMAG system (BD Biosciences). Cells were then plated at 2.25-3x10⁶ cells in a 12-well antibody-coated plate. Plates were coated with anti-CD3ε and anti-CD28 as follows, anti-hamster IgG (Sigma) incubated at room temp on a rocker for 2 hours. Anti-hamster was removed and replaced with 120μL of anti-CD3ε and anti-CD28 and incubated over night at 4°C on a rocker. Anti-CD3ε and anti-CD28 were purified from 145-2c11and 37N hybridoma cell lines by Becky Lawlor. T-cells were polarized toward a Th1 phenotype using 1ng/mL of murine IL-12 and of anti-IL-4 antibody.

7.15 RNA Isolation and QPCR

RNA was isolated using the RNAqueous kit (Ambion) following the manufacture protocol. Concentration of RNA was determined by nanodrop analysis. RNA sample yielding less than 60ng/μL were concentrated using the RNA easy kit (Qiagen). cDNA was synthesized using 1μg of RNA 1.2μL of dNTPs (Roche Applied Science), 2μL of M-MuLV reverse transcriptase buffer (New England Biolabs), 1μL of olig-DT₁₂₋₁₈ (Invitrogen), 1μL RNase inhibitor (Promega) and 1μL of 200.000 U/mL M-MuLV reverse transcriptase (New England Biolabs). Thermocycler program: 42°C for 45 minutes, 65°C for 10 minutes. qRT-PCR was performed using 1μL of cDNA for *in vivo* generated samples or 1μL of a 1:10 diluted cDNA for *in vitro* generated samples.
Samples were run in duplicate on a MxPRO 3000 (Stratagene) and prepared as follows: 10μL of SYBR green mix (Takara), .4μL of 10μM stock of both forward and reverse primers and 8.2μL of water. All samples were run using the subsequent program: 95° 5 minutes, 35 cycles of: 95° 25 seconds, 62° 25 seconds, 95° 1 minute, 62° 1 minute, 95° 30 seconds. Data was analyzed using MxPro3005p software (Stratagene) and quantified using the delta delta Ct method. β-Actin was used as the housekeeping gene for normalization and relative expression was determined to a naïve control. A table of the QPCR primer can be found in Table 7.2.

7.16 Western Blotting

Western blot were performed using standard techniques. Briefly, lysates were prepared in a presenillin lysis buffer for 30 mins with rocking. Protein concentration was determined using a BSA kit (Pierce) following the manufactures protocol. Samples were run on an 8% gel and transferred to a nitrocellulose blot. Blots were blocked for 1hr at room temp in Blotto. The following antibodies were used for western, β-Actin Ac-40 at 1:7500 (Sigma), PKC-0 212 1:1000 (Santa Cruz) and Phospho-Thr583 PKC-0 1:1000 (Cell Signaling). Blots were incubated with the proper secondary HRP conjugated antibodies for 30 minutes at room temp. Blots were then developed using homemade ECL reagents. Normalization of western blots was done using ImageJ software (Wayne Rasband, NIH).
### Table 7.1. Antibodies used for Flow Cytometry

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