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Elucidating Cellular Signaling Pathways that Contribute to the Immunopathogenesis of Aplastic Anemia

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**ELUCIDATING CELLULAR SIGNALING PATHWAYS THAT CONTRIBUTE
TO THE IMMUNOPATHOGENESIS OF APLASTIC ANEMIA**

A Dissertation Presented

by

CHRISTINA ARIETA KUKSIN

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

DOCTOR OF PHILOSOPHY

September 2014

Program in Animal Biotechnology and Biomedical Sciences

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DEDICATION

To my loving and supportive family

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The writing of this dissertation has caused long periods of reflection of my time and accomplishments at UMass Amherst, and the phrase, “it takes a village” keeps flashing through my mind. I would like to take this time to thank my ‘village,’ without whom I would have never completed this work. First, I would like to first thank my advisor, Dr. Lisa M. Minter, for giving me the drive and inspiration to complete this research. She was the first to teach me to love science, and to embrace the full meaning and purpose of our work. Under her tutelage, I have grown into a mature scientist and woman, and I know that without her mentorship, guidance, and encouragement, I would not be where I am today. I would also like to thank the other members of my committee, who I am proud to count as not only my mentors, but also my friends. Thank you to Dr. Barbara A. Osborne for her encouragement and mentorship. She has been a great sounding board for ideas and experiments, and she is wonderful role model for a successful female scientist. I would also like to thank Dr. Wilmore Webley. It was in his class that I first met Lisa, and also where I fell in love with Immunology. He has always been a wonderful teacher and mentor, and he was the first to encourage me to go into science. I am forever grateful for his support and his helpful critiques. And finally, thank you to Dr. Juan Anguita. He was the first person to challenge my ideas, and teach me how to critically examine my work and the work of others. I appreciate his mentorship and support, and his critical insights into this work. He is greatly missed at UMass.

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ABSTRACT

ELUCIDATING CELLULAR SIGNALING PATHWAYS THAT CONTRIBUTE TO THE IMMUNOPATHOGENESIS OF APLASTIC ANEMIA

SEPTEMBER 2014

CHRISTINA ARIETA KUKSIN, B.A., UNIVERSITY OF MASSACHUSETTS AMHERST

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Directed by: Professor Lisa M. Minter

Aplastic Anemia (AA) is a rare bone marrow failure disease that is hallmarked by hypocellular bone marrow and peripheral pancytopenia. Studies have shown that the disease is driven by aberrant T helper type-1 (Th1) responses that destroy bone marrow stem and progenitor cells. Although 70% of patients respond to therapy, others are refractory or relapse after initial treatment and eventually succumb to disease. In an effort to discover other therapeutic modalities for non-responsive patients, we sought to elucidate signaling pathways within T cells that could be contributing to the immunopathogenesis of AA. Using a mouse model of disease, our lab has previously shown that both the Notch1 signaling pathway and PKC θ signaling pathway is necessary for AA development. In this work, we expand our knowledge of both pathways and show that active PKC θ (pPKC θ) is highly expressed in AA mice compared to irradiation controls and active PKC θ is needed for Notch1^{IC} activation and IFN γ production in T cells. Also, we found that PKC θ differentially regulates Notch1^{IC} and IFN γ in CD8⁺ T cells compared to CD4⁺ T cells. Also, pPKC θ is highly expressed in peripheral blood mononuclear cells collected from treatment-naïve AA patients and treating patient

samples with the PKC θ inhibitor, rottlerin, reduced both NOTCH1^{IC} expression and IFN γ production.

Furthermore, we were able to show that NF- κ B signaling was necessary to drive AA through multiple pathways. For example, we were able to decrease T cell activation and expansion and Th1 cytokine production in our mouse model when we blocked NF- κ B signaling. We were also able to show that NF- κ B signaling regulates CXCR4 expression, and AA induced mice have higher levels of CXCR4 present on their T cells. Surprisingly, we were further able to reduce CXCR4 expression and motility in response to its chemokine ligand, SDF-1 by blocking NF- κ B signaling.

Finally, we were able to delineate a role for the microRNA, miR-155 in our mouse model of AA. We found that miR-155 expression was increased in AA induced mice, and its inhibition was able to ameliorate disease symptoms and ablate infiltration of T cells into the bone marrow. We identified a potential target of miR-155 in CD4⁺ T cells called programmed death ligand 1 (PD-L1) and show that mice induced with miR-155-deficient cells have increased iTregs in the bone marrow and spleen. Taken together, these data illuminate multiple pathways that contribute to AA which could be further investigated for alternative treatments of the disease.

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CHAPTER 1

INTRODUCTION

1.1 T Cell Function and Immunity

1.1.1 T Cell Activation

Successful activation of naïve T cells occurs through a two-step signaling process. The first signal is provided by antigen presenting cells (APCs) that present foreign peptide or antigen loaded on Major Histocompatibility Complexes (MHC) to the T cell receptor (TCR; reviewed in Smith-Garvin et al., 2009). The second signal is provided by engagement of the co-stimulatory receptor, CD28, which is expressed on T cells (reviewed in Bour-Jordan et al., 2011). CD28 will bind to its ligands CD80 and CD86 (also known as B7.1 and B7.2, respectively) on the APCs to induce a positive activation signal. Both signals are imperative for successful T cell activation; without both signals, T cells can become anergic to protect against aberrant T cell signaling. T cell signaling can also be dampened through negative co-stimulatory molecules such as CTLA-4 and PD-1 which can compete with CD28 for binding to CD80 and CD86 to block T cell activation (Parry et al., 2005).

After successful ligation of the TCR, a cascade of phosphorylation events on the activating ITAM motifs causes the recruitment of proteins to the TCR to form the immunological synapse (Reth, 1989). The immunological synapse is characterized by

controlled movement of membrane receptors to specific subcellular sites to facilitate interaction with ligands on interacting cells (Yokosuka and Saito, 2010). The movement of the IS through the lipid bilayers of the membrane also facilitates the recruitment of important cytosolic adaptor proteins that mediate the signaling downstream of the TCR, such as LCK and Zap-70. Additionally, the recruitment of Zap70 to the TCR further recruits other adaptor signaling molecules, such as LAT and SLP76 (Bubeck Wardenburg et al., 1996; Zhang et al., 1998). The formation of the immunological synapse allows for the activation and expression of key transcription factors important in T cell survival: activated protein-1 (AP-1), nuclear factor of activated T cells (NFAT) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). It is through these pathways that expression of key survival proteins, like the T cell survival cytokine IL-2, can be expressed. An overview of TCR signaling can be found in Figure 1.1.

1.1.2 T Cell Differentiation and Subsets

CD4⁺ T cells have the ability to express and produce different cytokine signatures needed to mediate defense against different pathogens through a process called T cell differentiation. CD4⁺ T cells can differentiate into many different T helper (Th) type subset depending on the cytokine milieu present during their activation (these include: Th1, Th2, Th17, iTreg, Th9, Th22, and Tfh). T helper type 1 (Th1) cells are responsible for protection against intracellular pathogens such as viruses and activate CD8⁺ cytotoxic T cells. Th1 cells express the lineage-specific transcription factor, T-BET, and secrete the Th1 associated cytokine, IFN-γ (Szabo et al., 2002). T helper type 2 (Th2) cells are

required for humoral immunity and are responsible for allergic reactions and removal of extracellular pathogens. Th2 cells secrete IL-4, IL-5, and IL-13, and require GATA3 expression for differentiation (Zheng and Flavell, 1997). T helper-17 (Th17) cells provide protection against nematodes and fungal infections, require TGF β and IL-6 for generation, secrete IL-17 and IL-23, and express ROR γ t (Langrish et al., 2005; Yang et al., 2008). Th17 cells also are one of the major cell types that mediate the autoimmune disease multiple sclerosis (Jager et al., 2009). Inducible T regulatory cells (iTregs) are regulated by the transcription factor FOXP3 and produce TGF β (Chen et al., 2003; Fu et al., 2004; Rao et al., 2005). These cells, unlike their counterparts, are responsible for suppressing inflammation.

Cytotoxic T lymphocytes (CTL) are CD8⁺ T cells that are activated similarly to CD4⁺ T cells, except they are presented antigen through MHC type I complexes (present on most nucleated cell types) instead of MHC type II complexes (present only on professional APCs). This gives CTLs the unique ability to identify intracellularly infected cells and induce programmed death pathways to reduce the spread of infection. CTLs induce apoptosis of target cells most often through the release of lytic granules, such as perforin and granzyme, (reviewed in Barry and Bleackley, 2002). Perforin is a pore forming protein that is found in the cytolytic granules released from CTLs. Although its exact function in inducing cell death is debatable, studies in perforin knockout mice have shown that it is required to facilitate granzyme mediated killing (Kagi et al., 1994; Kojima et al., 1994; Lowin et al., 1994). Granzyme proteins are a family of distinct serine proteases that are found in cytolytic granules released from CTLs and activate cell death pathways. The most common of these proteins is granzyme B, which mediates killing

through activation of caspase pathways and DNA fragmentation (Heusel et al., 1994). Granzyme B is important for efficient cell death, as CTLs deficient in granzyme B kill at a slower rate (Pardo et al., 2004). Although lytic proteins released in granules are the most common form of CTL killing, CTLs have been shown to induce death through the Fas/Fas ligand pathway and through the production of cytokines such as IFN γ and TNF, that can cause cell toxicity to neighboring cells. Figure 1.2 outlines the different T helper subtypes and cytotoxic T cells.

1.1.3 T Helper Type 1 Driven Autoimmunity

Despite the extensive regulation of immune responses to protect against aberrant reactions, clones of T or B cells that recognize self-antigens as foreign do arise and cause autoimmunity. Some autoimmune diseases are mediated by auto-antibodies (such as systemic lupus erythematosus); however, other autoimmune reactions are driven by self-reactive T cells that become activated, instead of tolerized or anergic, in response to self-antigen. This leads to the production of cytokines and cellular lysis pathways that cause extreme damage in the affected organ or tissues that express the self-antigen. Some autoimmune disease with a strong T helper type 1 component include rheumatoid arthritis, multiple sclerosis, insulin-dependent diabetes mellitus, and aplastic anemia. Research into the inciting antigens and mechanisms driving these diseases can help in the development of more effective therapies.

1.2 Aplastic Anemia

1.2.1 Symptoms and Etiologies

Severe aplastic anemia (AA) is a rare bone marrow failure disease that mainly affects children and young adults (Young et al., 2008). At the time of diagnosis, patients often present with low levels of circulating platelets, low white and red blood cell counts in the periphery, and a characteristic hypocellular bone marrow. Because of the severe pancytopenia associated with the disease, patients experience cachexia, hypoxia, are highly susceptible to bleeding episodes, and are often vulnerable to multiple infections. Additionally, patients with AA will often times either have coexisting or co-evolving hematological diseases, such as paroxysmal nocturnal hemoglobinuria (PNH) or myelodysplasia (MDS; Young et al., 2008). If left untreated, AA is uniformly fatal (Dezern and Brodsky, 2011).

The etiology of aplastic anemia is unclear in many cases. Paul Ehrlich first described AA in 1888 in a pregnant woman, and while no strong correlation exists, some cases of AA have developed after pregnancy especially after episodes of eosinophilic fasciitis (Choudhry et al., 2002; Young, 2006). While five to ten percent of AA cases follow an episode of seronegative hepatitis (Lu et al., 2004), no definitive infectious agent has been shown to cause disease. Some cases are associated with chemical or drug exposure, such as benzene, pesticides, and chloramphenicol; overrepresentation of drug

metabolizing glutathione-S-transferase gene deletions have been observed in some patient cohorts (Dufour et al., 2005; Sutton et al., 2004; Young et al., 2008).

One third of aplastic anemia patients have shortened telomeres in their white blood cells (Young, 2006). Upon closer assessment of genomic DNA, some patients, even older adults, were shown to have mutations in *TERC* (Fogarty et al., 2003), *TERT* (Yamaguchi et al., 2005), and *TERF1* (Savage et al., 2006). Family members who share these mutations but have adequate blood counts, do have shortened telomeres, but also have hypocellular bone marrows and reduced CD34 population (Fogarty et al., 2003). Based on these findings, it seems that mutations in genes that encode proteins that repair telomeres confers a genetic risk factor to patients since hematopoietic stem cells with already-shortened telomeres may not be able to withstand the immune mediated damage associated with disease (Young, 2006). Despite evidence of some etiological correlation, the cause of AA is still unknown in most cases.

1.2.2 Evidence for Aplastic Anemia as an Autoimmune Disease

Because many cases of AA are idiopathic, it has been hard to properly study the underlying mechanisms that contribute to the early pathogenesis of the disease. However, evidence points to an inciting self-immune reaction as the driving impetus of disease development. It has been shown in early cases that conditioning regimens to dampen the immune system before bone marrow transplants were critical even when recipients received syngeneic bone marrow (Appelbaum et al., 1985; Hinterberger et al., 1997). However, the most compelling evidence that AA is an immune mediated disease

is the responsiveness of patients to immunosuppressive therapies such as anti-thymocyte globulin (ATG) and cyclosporin A (CsA; Young, 2006). Some patients are refractory to immunosuppressive therapies, and this information could infer a non-immune modality of disease. However, these patients characteristically present with severe stem cell depletion, a “spent” immune response, or immunological mechanisms causing the resistance to immunosuppressive therapies (Young et al., 2008).

Because of the strong immune mechanism associated with AA and the idiopathic nature of the disease, AA has been characterized as an autoimmune bone marrow failure disease. Because of this, there has been a search for the inciting autoantigens responsible for disease. There have been a few autoantigens identified by screening patient blood samples against a peptide library. One study identified kinectin, a widely expressed protein that is highly expressed by hematopoietic cells in the bone marrow, being bound by autoantibodies from 40% of patient sera. Although kinectin-reactive cytotoxic T cells can be generated *in vitro* to inhibit hematopoietic colony formation, no anti-kinectin T cells can be found in patients peripheral blood or bone marrow samples (Hirano et al., 2003). Another autoantigen found in a minority of patients was diazepam-binding related protein-1, an enzyme essential in the oxidation of unsaturated fatty acids that is broadly distributed in tissues. Unlike kinectin, a putative T-cell epitope derived from diazepam-binding related protein-1 could stimulate one patient’s cytotoxic T cells (Feng et al., 2004). A few other hematopoietic associated proteins (and not stromal associated proteins) have been identified as targets of autoantibodies in AA patients using serological identification of antigens by recombinant cDNA expression cloning (SEREX), however it is still unclear as to how identifying these autoantibodies can be

used therapeutically, and how the targets of the autoantibodies may correlate to the T cell-mediated pathogenesis of AA (Goto et al., 2013).

1.2.3 Immunopathogenesis of the Bone Marrow

The severe pancytopenia associated with AA is the result of an extreme defect in hematopoiesis in the hypocellular bone marrow of patients. At first it was believed that there was a defect solely in the patients' hematopoietic stem cells that caused this pancytopenia. However, early *in vitro* analysis of patient bone marrow showed that T cells mediated the pathogenicity in the bone marrow and limited the colony formation in a clonogenic assay. When lymphocytes were depleted from diseased bone marrow, colony formation was restored. Addition of the AA lymphocytes to normal bone marrow samples inhibited hematopoiesis (Gorski et al., 1979; Kagan et al., 1976; Torok-Storb et al., 1980). Early assessment of the suppressive lymphocytes characterized them as primarily activated cytotoxic T cells (Maciejewski et al., 1994). Molecular analysis of patients' CD8⁺CD28⁻ cells using flow cytometric analysis for T cell receptor (TCR) V β subfamilies length and sequencing analysis of the CDR3 region show an oligoclonal expansion of CD8⁺CD28⁻ cells upon disease diagnosis, depletion of these clones with immunosuppressive treatment, and re-emergence of the clones upon relapse (Risitano et al., 2004). It is unclear how these T cells are activated; however, overrepresentation of HLA-DR2, an MHC Class II molecule which is also highly expressed in multiple sclerosis patients, suggests a role for antigen recognition by T cells (Maciejewski et al., 2001; Nakao et al., 1994).

Further analysis of circulating T cells from AA patients point to a Th1 mechanism driving disease. Early studies described the pathogenic lymphocytes as producing T helper type-1 cytokines, such as IFN γ and TNF (Sloand et al., 2002; Tong et al., 1991; Zoumbos et al., 1985). Polymorphisms in the tumor necrosis factor- α (TNF2) promoter at -308 (Demeter et al., 2002), IFN γ (Dufour et al., 2004), and in IL-6 genes (Gidvani et al., 2007) are prevalent in patient cohorts. Additionally, AA patient T cells have increased levels of the Th1 master regulator transcription factor T-BET (Solomou et al., 2006), as well as intracellular NOTCH1, which directly regulates T-BET expression (Roderick et al., 2013). The regulatory T cell population, whose purpose is to suppress autoreactive T cells, is decreased in patients with aplastic anemia (Solomou et al., 2007) and studies using animal models of AA have been able to ameliorate disease with an infusion of regulatory T cells (Chen et al., 2007).

The mechanism by which the cytotoxic T cells mediate destruction of the hematopoietic stem cells of the bone marrow is through different apoptosis-inducing pathways. Bystander death of the hematopoietic stem cells and stromal cells is caused by the high levels of cytokines expressed in the bone marrow which inhibits the ability of the cells to proliferate and differentiate, and causes them to upregulate the Fas receptor (Maciejewski et al., 1995a; Maciejewski et al., 1995b; Selleri et al., 1996). Because of the upregulation of the Fas receptor on the hematopoietic stem cells of the bone marrow, direct T cell mediated killing through Fas/Fas ligand interactions has been documented (Ismail et al., 2001; Killick et al., 2000; Maciejewski et al., 1995b). Cytotoxic T cell-mediated killing also directly mediates destruction of the hematopoietic and stromal cells of the bone marrow through the release of the cytotoxic granules, perforin and granzyme,

which are upregulated in patients with aplastic anemia (Kook et al., 2001; Xu et al., 2003). An overview of the immunopathogenesis of the bone marrow in AA can be found in Figure 1.3.

1.2.4 Current Treatments

Currently, there are three types of treatment for Aplastic Anemia: hematopoietic stem cell transplantation (HSCT), immunosuppressive therapy (IST), and supportive care. In children, the most effective treatment for AA is HSCT using bone marrow from a histocompatible matched sibling donor as the source for hematopoietic stem cells (Scheinberg, 2012). A retrospective study done by the Center for International Blood and Marrow Transplant Research (CIBMTR), reported a 5 years survival rate of 82% for those under 20 years of age, compared with approximately 50% for those over the age of 40 (Gupta et al., 2010). Because of the rising risk of graft-versus-host disease (GVHD) and other mortality associated risks with older patients, 40 is the upper age limit for HSCT in the clinic (Maury et al., 2009; Peinemann et al., 2011).

For patients with no available HSCT donor, or for patients over the age of 40, the best mode of treatment is immunosuppressive therapy (IST). The standard IST for patients in the United States, Europe and Japan is administration of horse ATG and CsA, which produces positive hematologic responses in 60%-75% of cases (Young, 2006). Children have a higher survival rate with IST than adults (75% vs. 50%; Scheinberg, 2012). IST is routinely continued for 6 months, at which point efficacy of treatment and alternate treatment modalities are explored if there is IST failure.

For patients who are refractory to the initial HSCT or IST treatments, there are additional options. For patients without a matched sibling donor, there has been an increase in unmatched donor HSCT, with considerable success in children (Scheinberg, 2012). In patients for whom HSCT is not a preferable option, a small study reported some success with a second round of IST using rabbit ATG, rather than horse ATG, or Alemtuzumab, a monoclonal antibody specific for the mature lymphocyte antigen, CD52, with Alemtuzumab being better-tolerated than rabbit ATG (Risitano et al., 2010). In patients who relapse after initial successful IST treatment, a common practice is to reintroduce CsA for a period of 2 to 3 months, and taper off treatment to low doses (Scheinberg and Young, 2012).

Supportive care is important in the treatment of AA and, although not curative, it is essential to the management of disease symptoms. Red blood cell (RBC) or platelet transfusions are often necessary to maintain quality of life; however, the risk of alloimmunization to RBC antigens or HLA antigens is a considerable risk. Restrictive transfusion policies for those who are candidates for HSCT is required (Hochsmann et al., 2013). Patients who have been given numerous RBC transfusions are also at risk for an iron overload. Therefore, iron chelation therapy is needed.

Because of a severely suppressed immune system, prevention of infection in AA patients is paramount. Neutropenia is extremely common in AA patients, leaving them susceptible to infection, especially fungal infections. Therefore, many patients are given prophylactic antibiotic or antitmycotic drugs. Although viral infections can be severe in immunosuppressed patients, prophylactic antiviral drugs are not widely used; therefore, patients must be monitored closely for signs of infection.

1.3 Protein Kinase C Theta in T Cells

1.3.1 Protein Kinase C Family

Members of the protein kinase C (PKC) family are serine and threonine kinases that phosphorylate numerous target proteins in the cell. This family of 10 isozymes is divided into three groups depending on the homology of their structure and mode of activation. The members of the conventional PKC group (cPKC) consist of PKC- α , PKC- β I, PKC- β II, and PKC- γ . cPKC members are activated by Ca^{2+} and diacylglycerol (DAG). The novel PKC (nPKC) family consists of PKC- δ , PKC- ϵ , PKC- η and PKC- θ and are activated by DAG or PMA alone because they lack the critical Ca^{2+} binding motifs in their C2 domain. The atypical PKC (aPKC) family contains PKC- ζ and PKC- ι/λ and are not activated by DAG/PMA or calcium. They lack a calcium-sensitive C2 domain, while their C1 domain binds PIP_3 or ceramide (not DAG or PMA). PKC θ shares a similar conformation to members of the nPKC family (shown in Figure 1.4A) with the N-terminus of PKC θ comprising the regulatory region while the C-terminus of the protein comprises the catalytic region (Baier et al., 1993; Chang et al., 1993). As explained above, the C2 domain of PKC θ is similar to other PKC family members, however, it does not bind Ca^{2+} . The two tandem cysteine-rich domains of C1 bind to DAG, with the C1b domain having a higher affinity for DAG than C1a (Melowic et al., 2007). The C1 domain is flanked by two variable regions, V1 and V3. V3 has been shown to be involved in an indirect association of PKC θ with CD28, probably through interaction with LCK (lymphocyte-specific protein tyrosine kinase). This

interaction leads to the translocation of PKC θ to the immunological synapse in T cells (Kong et al., 2011).

When PKC θ is in its inactive state, the N-terminal regulatory region binds to the substrate binding region in the catalytic domain on the C terminus and blocks the interaction of the catalytic domain with its substrates (House and Kemp, 1987). PKC θ becomes activated through a two step process (Seco et al., 2012). After activation of the T cell through the TCR and CD28 stimulation, DAG binds to the C1 domain to 'open' the activation loop (Melowic et al., 2007). After exposure of the activation loop, PKC θ can be phosphorylated by germinal center kinase-like kinase (GLK) on Thr538 in the activation loop, which results in catalytic activation (Chuang et al., 2011). PKC θ is phosphorylated at six sites: Y90, T219, T538, S676, S685, and S695, which are important for activation and downstream signaling (reviewed in Wang et al., 2012).

1.3.2 The Role of PKC θ in T Cell Activation

When T cells are stimulated through the TCR and CD28 co-receptor, the immunological synapse is generated at the area of contact between the T cell and APC (Rao et al., 1999). Part of the synapse is called the supramolecular activation complex (SMAC) which includes different signaling molecules important to T cell signaling, such as LCK. After successful T effector cell stimulation, PKC θ is recruited to the SMAC through association with the cytoplasmic tail of CD28 (Kong et al., 2011; Schaefer et al., 2004). In the SMAC, PKC θ is phosphorylated by LCK at Y90 which allows for its subsequent phosphorylation and autophosphorylation at T219 (Bauer et al., 2001; Bi et

al., 2001; Freeley et al., 2005; Lee et al., 2005; Liu et al., 2002; Liu et al., 2000; Thuille et al., 2005). Phosphorylation is necessary for retaining PKC θ in the IS, and without PKC θ , IS formation cannot be sustained, thus blocking successful T effector cell signaling (Valitutti et al., 1995). In T regulatory cells, however, PKC θ is sequestered away from the IS in the distal pole complex, suggesting differential roles for PKC θ in T effector cells and T regulatory cells (Zanin-Zhorov et al., 2010).

After activation of PKC θ through phosphorylation at T538 by GLK, PKC θ can mediate downstream T cell signaling (Chuang et al., 2011). PKC θ phosphorylates the membrane-associated guanylate kinase, Carma1, also known as Card11 (Blonska and Lin, 2009). Phosphorylation of Carma1 induces the formation of the CBM complex, a scaffolding complex comprised of Carma1, Bcl10 and Malt1, which activates the NF- κ B signaling pathway (Thome and Weil, 2007). PKC θ also activates the AP1 transcription factor by phosphorylation of the ste20-family kinase SPAK and is important for NFAT transactivation (Li et al., 2004; Pfeifhofer et al., 2003).

To further explore the role of PKC θ in T cell signaling, knockout mice were developed by replacing the exon encoding the ATP-binding site of the kinase domain with the neomycin resistance gene (Sun et al., 2000). Using these mice, it was shown that that PKC θ ^{-/-} CD3⁺ T lymphocytes have a severe reduction in proliferation and IL-2 secretion (Sun et al., 2000). Based on this evidence, PKC θ is an integral driver of T cell activation, and Figure 1.4B summarizes its role in T cell activation.

1.3.3 The Role of PKC θ in Disease and Autoimmunity

How PKC θ may contribute to T helper cell-mediated processes remains controversial. Reports suggest PKC θ is required for Th2 and Th17 responses, but it has been shown to be dispensable for generating both Th1-mediated antiviral and memory T cell responses (Kwon et al., 2012; Marsland et al., 2005; Marsland et al., 2004). Accumulating evidence supports a function for PKC θ in mediating autoimmune disorders, such as experimental autoimmune encephalomyelitis, rheumatoid arthritis, and myosin-induced autoimmune myocarditis (Healy et al., 2006; Marsland et al., 2007; Salek-Ardakani et al., 2005; Tan et al., 2006). Furthermore, in a mouse model of graft-versus-host disease (GVHD), which has a strong Th1 component, PKC θ was required to induce GVHD pathology, but not to clear viral pathogens or mediation of graft-versus-leukemia processes (Valenzuela et al., 2009). These data suggest that inhibiting PKC θ in T cells may abrogate their allopathogenic activity, while preserving their ability to respond appropriately to infectious stimuli. As such, PKC θ may represent an attractive target for modulating immune-mediated conditions, including autoimmune diseases.

1.4 Notch Signaling Pathway

1.4.1 Notch Receptors and Their Ligands

The Notch protein was originally discovered in *Drosophila melanogaster* in 1914 when deletion of the gene encoding this protein produced a “notched” wing phenotype.

Since the discovery of Notch, it has become evident that it plays a pivotal role in cell fate decisions throughout different systems. Mammals express four Notch receptors (Notch1, 2, 3, and 4) and their differing structures are summarized in Figure 1.5A. Notch contains three major domains: the extracellular domain of Notch is comprised of epidermal growth factor (EGF)-like repeats, the transmembrane domain contains a cysteine rich lineage domain (LIN) and a hetero-dimerization domain which prevents ligand-independent activation, and an intracellular domain. The intracellular domain contains a RAM domain (RBP-J κ -associated molecule domain), an ANK domain (ankyrin repeat domain) that mediates protein-protein interactions, two nuclear localization sequences (NLS), a transcriptional activation domain (TAD; only present in Notch1 and Notch2), and a PEST domain (proline-glutamate-serine and threonine-rich domain (PEST) that regulates protein stability (Osborne and Minter, 2007).

Notch can be activated by association with its ligands: Delta-like (DLL1, DLL3, and DLL4) and Jagged (Jag1 and Jag2) ligands (Figure 1.5A). All Notch ligands contain a conserved DSL sequence (Delta/Serrate/Lag2), which is important for receptor binding, and EGF-like repeats. Jagged ligands also contain a cysteine rich (CR) region close to the plasma membrane. To promote ligand binding, Notch can be glycosylated on its extracellular domain by the Fringe glycosyltransferases, Manic fringe, Lunatic fringe, and Radical fringe . In most cases, Fringe glycosylation promotes binding by the Delta-like ligands (Radtke et al., 2010).

1.4.2 Notch Signaling Pathway: Canonical and Non-Canonical

For activation of the Notch signaling pathway, the protein must undergo a series of proteolytic cleavages to create its active form (intracellular Notch, or Notch^{IC}). Notch is produced in the Golgi, and in the trans-Golgi, it undergoes its first cleavage event by furin-like proteases, creating a non-covalently linked, heterodimeric receptor (Logeat et al., 1998). The heterodimeric form of Notch translocates to the plasma membrane where it can then interact with its ligands. After ligand interaction, Notch is cleaved by an ADAM metalloprotease, TNF α -converting enzyme (TACE), which causes the release of the extracellular portion of the protein (Brou et al., 2000). Notch is then endocytosed and the intracellular portion of the protein is ubiquitinated, allowing for the final cleavage of Notch by γ -secretase (De Strooper et al., 1999). This final cleavage releases Notch from the membrane and allows for its signaling within the cytoplasm and the nucleus. The canonical signaling pathway of Notch results in gene transcription through interaction with its binding partner RBP-J κ (murine homolog), also called as CSL (CBF-1 in mammals, suppressor of hairless in *Drosophila melanogaster* and Lag 1 in *Caenorhabditis elegans*). RBP-J κ exists in the nucleus as a transcriptional repressor; however, binding of Notch^{IC} to RBP-J κ results in the recruitment of co-activators, such as p300 and MAML (Mastermind-like). The building of this complex leads to transcription of Notch target genes, such as *Hes* (Hairy/enhancer-of-split) or *Hey* (Hairy/enhancer-of-split related).

Lately, there have been descriptions of transcription of Notch genes that do not require RBP-J κ , and this has become known as non-canonical Notch signaling. It has

been shown previously that Notch and NF- κ B exist in a complex on the *Ifng* promoter even in the absence of RBP-J κ (Cho et al., 2009; Shin et al., 2006). Also, T cell activation and differentiation was found to be Notch1 and NF- κ B dependent, but RBP-J κ independent (Dongre et al., 2014). Interestingly, NF- κ B binding sites on DNA contain a nested RBP-J κ consensus binding sequence. (Minter and Osborne, 2012). Other than NF- κ B mediated Notch signaling, other binding partners have been described in Notch signaling that are independent of RBP-J κ , such as PI3K, Akt, T-bet and GATA-3. These signaling events happen not just in the nucleus, but also in the cytosol (Minter and Osborne, 2012). An outline of canonical Notch signaling is found in Figure 1.5B.

1.4.3 Notch Signaling in T Cells

Notch signaling is critical for the regulation of T cell development, activation, and differentiation. Notch1 is required for T cell lineage commitment, and deletion of Notch in developing thymocytes redirects them towards a B cell fate (Pui et al., 1999). Conversely, overexpression of Notch1 in bone marrow progenitors initiates T cell leukemia (Radtke et al., 1999). Notch signaling was also shown to be important in T cell activation. When T cells are activated through TCR and CD28 ligation, cleaved Notch1 leads to expression of CD25 (the high affinity IL-2 receptor) and the secretion of IL-2, while inhibition of Notch signaling using a γ -secretase inhibitor (GSI) blocks peripheral T cell activation, proliferation, and IFN- γ production (Adler et al., 2003; Palaga et al., 2003).

How Notch signaling drives the differentiation of T cells towards different helper subtypes is an area of intense investigation. Inhibition of Notch signaling using GSIs blocks the ability of T cells to differentiate into Th1 cells (but not Th2) through a reduction in T-bet expression (Minter et al., 2005). Using mice deficient in RBP-J κ or expressing a dominant negative Mastermind-like (DNMAML), it has been surmised that Notch signaling regulates Th2 fate via GATA3 and IL-4 expression; however, since Notch was not specifically deleted in these studies, it is unknown if other RBP-J κ or MAML binding partners could be driving Th2 differentiation (Amsen et al., 2007; Fang et al., 2007). Inhibition of Notch activation using GSI has also been shown to regulate Th17 differentiation, and Treg differentiation through regulation of FoxP3 expression (Keerthivasan et al., 2011; Samon et al., 2008). Notch signaling has also been shown to play an important role in CD8⁺ T cell signaling. Inhibition of Notch results in a decrease in the expression of the CD8⁺ T cell associated transcription factor, Eomes, and thus results in the reduction of granzyme B and perforin production (Cho et al., 2009). Altogether, this evidence suggests that Notch signaling plays pleiotropic roles in T cell activation and differentiation.

1.5 NF- κ B Pathway

1.5.1. NF- κ B Signaling Pathway

The NF- κ B family of transcription factors comprises five homo- or heterodimers: c-Rel, p65 (RelA), RelB, p50 (NF- κ B1) and p52 (NF- κ B2). p50 and p52 are processed

from the precursors p105 and p100, respectively. Each homo- or heterodimer has distinct expression patterns and regulatory functions, depending on the cell type (Gilmore, 2006). All NF- κ B subunits contain a 'Rel homology domain' (RHD) near the N-terminus which contains the sequences needed to bind to DNA (κ B elements), allows interaction between the subunits and the inhibitory I κ B proteins, and facilitates NF- κ B entry into the nucleus. c-Rel, p65, and RelB possess transcriptional transactivation domains (TADs), as shown in Figure 1.6A. Usually, dimers containing these subunits are associated with active transcription (Hoffmann et al., 2006). Control of target gene transcription by p50 or p52, two members lacking TAD, causes repression of transcription at κ B sites, either by recruiting histone deacetylases to promoters or by blocking binding by other NF- κ B subunits that contain TADs. However, the subunits p50 or p52 can also promote gene expression by interacting with co-activators (Hoffmann et al., 2006).

The NF- κ B signaling pathway can be activated through either the canonical or non-canonical route. In the canonical pathway, NF- κ B exists in an inactive state sequestered in the cytosol of the cell by inhibitor of κ B (I κ B). When the pathway is stimulated, I κ B kinase- β (IKK β) is activated and phosphorylates I κ B causing its subsequent ubiquitination and degradation. NF- κ B is then released and translocates into the nucleus where it can mediate transcription of downstream target genes. The predominate subunits involved in the canonical pathway are p65 and p50 (Hayden and Ghosh, 2008). In the non-canonical pathway, NF- κ B inducing kinase (NIK) directly phosphorylates and activates IKK α , which in turn phosphorylates p100, and causes its processing to p52. p52 then goes on to form a heterodimer with RelB and translocate to the nucleus to mediate gene transcription (Sun, 2011).

1.5.2 NF- κ B Signaling and T cell activation

When T cells become activated by antigen recognition through the TCR, a series of signaling events downstream of the TCR and CD28 causes activation of PKC θ (described above) and activation of NF- κ B through the classical pathway. PKC θ phosphorylates Carma1 at S552 and S645 early and transiently after TCR signaling, and these phosphorylation steps are critical to induce the conformational change in Carma1 that allows the binding of Bcl10 and Malt1, to form the CBM complex (Matsumoto et al., 2005; Sommer et al., 2005). Phosphorylation of Carma1 at a third site S649 is inhibitory to Carma1 activation. This phosphorylation event is delayed after TCR signaling but is sustained for a longer duration than the early phosphorylation steps (Moreno-Garcia et al., 2009). After assembly of the CBM complex, IKK γ is polyubiquitinated, likely by TRAF6, while IKK β is phosphorylated by the protein kinase, TAK1 (Thome et al., 2010; Wang et al., 2001). Once phosphorylated, IKK β is able to phosphorylate I κ B and trigger its proteasomal degradation, allowing NF- κ B to translocate into the nucleus and mediate downstream transcription of genes involved in T cell survival, proliferation and effector functions. A summary of NF- κ B signaling after T cell activation is shown in Figure 1.6B.

In activated T cells, NF- κ B has been shown to control the expression of IL-2, CD25, IL-2R α , and IFN γ (Vallabhapurapu and Karin, 2009). NF- κ B has also been shown to be important in T cell differentiation. Transgenic mice which express non-degradable I κ B in T cells have impaired Th1 responses but not Th2 responses (Aronica et al., 1999). RelA, c-Rel and RelB are needed for IFN γ production, however, only RelB deficiency

has been shown to cause a reduction in T-bet expression (Balasubramani et al., 2010; Corn et al., 2005; Hilliard et al., 2002). CD4⁺ cells with a deficiency in p50 can still express T-bet and produce IFN γ . However, p50 has been shown to be necessary for GATA3 expression, and p50^{-/-} deficient mice have defective allergic airway inflammatory responses (Das et al., 2001). c-Rel has also been shown to play a major role in thymic and peripheral Treg differentiation and FoxP3 expression (Long et al., 2009; Zheng et al., 2010). c-Rel- and p65-deficient T cells have defective IL-17 production and Th17 differentiation (Ruan et al., 2011). c-Rel deficient mice are also resistant to experimental autoimmune encephalomyelitis (Hilliard et al., 2002). Based on this evidence, it is clear that NF- κ B subunits have differential roles in T cell activation and differentiation.

1.5.3 NF- κ B in Disease

Because NF- κ B is implicated in many cell survival pathways throughout different systems, it has long been associated with disease. Its role in the proinflammatory response, anti-apoptotic pathways, and cellular growth is prominent; therefore, its dysregulation has been implicated in many cancer models (Baldwin, 2001). NF- κ B is also used by HIV as its transcriptional regulator to propagate new virus (Ghosh et al., 1998). Other viruses, such as Epstein Barr virus and Herpes Simplex virus, also activate NF- κ B in an attempt to induce proliferative and pathogenic responses in their host cell (Mosialos, 1997). Chronically activated NF- κ B has been implicated in a number of

autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus, type I diabetes, multiple sclerosis, and inflammatory bowel disease (Sun et al., 2013).

Because of its widespread involvement in disease, many attempts to target the pathway have been studied. Many classes of drugs have been shown to block different parts of the NF- κ B pathway, such as glucocorticoids, NSAIDs, immunosuppressive agents (such as CsA), and cyclopentenone prostaglandins (Yamamoto and Gaynor, 2001). Clinical trials are currently underway in many different diseases using broader inhibitors that are known to target the NF- κ B pathway, such as curcumin which down-regulates expression of NF- κ B, along with other transcription factors, to cause apoptosis, and Bortezomib, a broad proteasome inhibitor that blocks the degradation of I κ B. Because of the wide scope of NF- κ B signaling in disease, many specific inhibitors are being developed, but they are still in early stages of study (Sun et al., 2013).

1.6 Chemokine Receptor 4

1.6.1 The CXCR4/SDF-1 signaling axis

Chemokines are small molecules that have selective chemoattractant properties depending on the tissue microenvironment. They can have a broad range of activities because of the multiple signaling pathways induced by the binding of chemokines to their receptors. Chemokine receptors are seven-pass-transmembrane receptors coupled to heterotrimeric G proteins (Viola et al., 2006) and through binding to their selective chemokines, cells can be directed to migrate to sites of infections or to different lymphoid

organs. Stromal derived factor-1 (SDF-1), also known as CXCL12, is the sole ligand for CXCR4, and after the binding of SDF-1, CXCR4 undergoes dimerization to activate signaling pathways (Percherancier et al., 2005; Vila-Coro et al., 1999). When CXCR4 becomes activated, the receptor is able to activate the heterotrimeric G-protein and activate downstream signaling pathways important to gene transcription, actin polymerization, cytoskeleton rearrangement and cell migration (Holland et al., 2006; Vila-Coro et al., 1999).

CXCR4 and SDF-1 deficient mice have a lethal phenotype, showing that CXCR4 and SDF-1 are important during embryonic development. CXCR4 is expressed on progenitor cells, and this allows for the migration of embryonic stem cell progenitors where they will differentiate into adult organ and tissues (Wong and Korz, 2008). CXCR4 is expressed on hematopoietic stem cells, and SDF-1 has a role in the quiescence for long-term HSC maintenance (Sugiyama et al., 2006). The main source for SDF-1 in the adult is the bone marrow. CXCR4 is also expressed on endothelial cells, smooth muscle cells, and endothelial progenitor cells, and it has been shown to promote angiogenesis (Gupta et al., 1998; Yamaguchi et al., 2003; Zerneck et al., 2005). SDF-1/CXCR4 has an important role in neurogenesis, and mice deficient in both SDF-1 and CXCR4 have abnormal development, both of the cerebellum and hippocampus (Miller et al., 2008). In the late 1990's, CXCR4 was shown to serve as a co-entry receptor for HIV-1 on CD4⁺ T cells (Feng et al., 1996). CXCR4 expression has also been shown to be overexpressed in many cancers, including human breast cancer cell lines and primary and metastatic breast tumors, ovarian cancer, prostate cancer and melanoma (Hall and Korach, 2003; Kim et al., 2008; Muller et al., 2001; Taichman et al., 2002).

1.6.2 CXCR4 and T cell Signaling

CXCR4 is constitutively expressed on T lymphocytes, and CXCR4's main role in T cells is to drive migration along gradients of SDF-1. CXCR4 also acts as a costimulator of T cell proliferation and T cell differentiation through interaction with the TCR (Kumar et al., 2006; Molon et al., 2005). When SDF-1 initiates CXCR4 signaling and activates G proteins downstream of T cell activation, there is an increase in TCR-activation associated kinases and adaptor proteins, such as phosphatidylinositol 3-kinase, ZAP-70, Fyn, Lyn, and SLP-76 (Chernock et al., 2001; Ganju et al., 1998; Smith et al., 2013; Ticchioni et al., 2002). Downstream of the TCR, activation of CXCR4 also increases the activity of Akt and the JAK/STAT signal transduction pathway (Ganju et al., 1998; Tilton et al., 2000). Also, for CXCR4 signaling to activate Ras and the ERK pathway, ZAP-70 expression is required (Kremer et al., 2003; Kumar et al., 2006).

Although CXCR4 depends on TCR signaling, CXCR4 engagement does not simply recapitulate TCR signaling pathways. For example, while the TCR can stimulate NF- κ B and NFAT activity, multiple studies have proven that CXCR4 cannot (Kumar et al., 2006; Molon et al., 2005; Nanki and Lipsky, 2000). Also, stimulation of CXCR4 with SDF-1 does not increase the phosphorylation of the TCR or ZAP-70, suggesting that CXCR4 utilizes pre-existing, constitutively-phosphorylated TCR-ZAP-70 complexes (Kumar et al., 2006). Because CXCR4 activation increases ERK signaling and ERK enhances the half-life of AP-1 subunits, CXCR4 signaling after SDF-1 binding most likely enhances the survival of resting T cells by activating several anti-apoptotic genes (Muller et al., 2001; Suzuki et al., 2001). Also the activation of AP-1 through CXCR4

helps to upregulate the production of AP-1 target genes, such as CD69, IL-10, and IL-2 (D'Ambrosio et al., 1994; Jain et al., 2005; Wang et al., 2005). CXCR4 effects on IFN γ production seem to be context dependent. If SDF-1 is added to cultures at the time of TCR ligation using costimulatory antibodies, IFN γ production is not increased; however, when SDF-1 is added to culture 2 hours prior to TCR ligation, or after APC-mediated T cell stimulation, it can significantly increase IFN γ production by T cells (Kumar et al., 2006; Molon et al., 2005; Nanki and Lipsky, 2000). Based on this evidence, it seems that CXCR4 can be used as a costimulatory molecule to help promote T cell survival with or without TCR signaling in environments in which SDF-1 is present. CXCR4 signaling in T cells is summarized in Figure 1.7.

1.6.3 Therapeutic Benefits of Altering CXCR4 Signaling

As explained above, CXCR4 overexpression has been described in many primary and metastatic cancers and other autoimmune disease, such as multiple sclerosis and lupus (Domanska et al., 2013). Also, there have been reports of an upregulation of CXCR4 and SDF-1 after chemotherapy with anti-angiogenic drugs such as bevacizumab, (Kioi et al., 2010; Shaked et al., 2008). Because of this, CXCR4 inhibition has been studied as a potential therapy for these diseases. Studies of the CXCR4 inhibitor AMD3100 shows that it mobilizes CD34⁺ human hematopoietic stem and progenitor cells from the bone marrow into the peripheral blood (Broxmeyer et al., 2005). Therefore, AMD3100 has been approved by the FDA for stem cell mobilization into the peripheral blood for the purposes of transplantation, and together with G-CSF (granulocyte-colony

stimulating factor), as therapy in Non-Hodgkin's lymphoma and multiple myeloma patients. Currently, multiple Phase I and Phase II trials are underway to assess the efficacy of CXCR4 inhibitors in multiple cancers (with or without corresponding chemotherapy), and other autoimmune diseases (Domanska et al., 2013).

1.7 Interaction of Signaling Pathways in T cells

1.7.1 Cross talk between the NF- κ B and PKC θ pathways

Considerable evidence has been gathered that PKC θ activation is crucial for stimulation of the NF- κ B pathway. For example, a constitutively active mutant of PKC θ induces activation of both an NF- κ B reporter gene, and activation of the CD28 response element encoding for IL-2 (Coudronniere et al., 2000; Lin et al., 2000). While inhibitors specific for PKC α , β , and γ had no effect on NF- κ B nuclear translocation after T cell stimulation, addition of rottlerin (a PKC θ and δ specific inhibitor) to T cells was able to abrogate NF- κ B translocation (Coudronniere et al., 2000). This data was confirmed by a study wherein expression of kinase-dead PKC θ or addition of PKC θ antisense RNA blocked NF- κ B activation after TCR stimulation (Lin et al., 2000). A loss of PKC θ in CD4⁺ T cells from PKC θ deficient mice causes a severe reduction in I κ B degradation and NF- κ B activation following TCR stimulation, a decrease in IL-2, and a decrease in T cell stimulation (Sun et al., 2000). PKC θ regulation of the NF- κ B pathway is limited to TCR mediated NF- κ B activation (canonical pathway), as stimulation of NF- κ B by either TNF or IL-1 (through the non-canonical pathway) is preserved (Sun et al., 2000). PKC θ

directly interacts with the NF- κ B pathway through phosphorylation of Carma1 (described above). This action allows for formation of the CBM complex, and the subsequent downstream events in canonical NF- κ B signaling.

1.7.2 Cross talk between the Notch and NF- κ B pathways

Several studies have demonstrated interaction between Notch and NF- κ B pathways in T cells. When Notch signaling is abrogated using GSI, NF- κ B activity is reduced in peripheral T cells (Palaga et al., 2003). Cytosolic Notch1^{IC} has been shown to physically interact with Carma1 and the nucleated CBM complex, and blocking Notch using shRNA causes inhibition of the formation of the CBM complex (Shin et al., 2014). Additionally, Notch has been shown to physically interact with NF- κ B (specifically p50 and c-Rel), and Notch regulates NF- κ B activity by sustaining its nuclear localization (Shin et al., 2006). Also, data from our lab has shown that when a non-nuclear form of Notch1^{IC} was expressed, NF- κ B activity remains high in T cells (Shin et al., 2014). Furthermore, Notch3 transgenic mice show constitutive NF- κ B activity in T regulatory cells and T-ALL models (Barbarulo et al., 2011; Vacca et al., 2006). As mentioned above, chromatin immunoprecipitation experiments have shown that Notch1 and NF- κ B subunits can be found on the *Ifng* promoter and this interaction could be abolished with GSI treatment, suggesting that Notch1 and NF- κ B work in tandem to promote IFN γ expression (Shin et al., 2006). In addition, Notch1 and NF- κ B could also be found in a complex on the promoters of *Eomes*, *Granzyme b* and *Perforin*, and this interaction could also be abrogated with GSI treatment (Cho et al., 2009).

1.7.3 Cross talk between Notch and PKC θ pathways

Although the exact mechanism by which the Notch and PKC θ pathways interact is unclear, their interaction seems inevitable in T cells because of the important role they both play in the NF- κ B pathway. For example, the formation of the CBM complex requires both Notch and PKC θ , as described previously; however, data from our lab suggests that these two proteins may interact with each other as well. Using microscopy and biochemical approaches, our lab has shown that Notch and PKC θ co-localize at the immunological synapse and physically interact as part of the CBM complex (Shin et al., 2014). Additionally, earlier reports have shown synergistic activities between PKC θ and Notch^{IC} in T-ALL models induced by cells containing activating Notch^{IC} mutations (Felli et al., 2005; Giambra et al., 2012). Because it is unclear when and why during T cell activation PKC θ and Notch interact, elucidation of these signaling pathways can shed light on diseases in which these pathways are dysregulated. A summary of how these pathways interact can be found in Figure 1.8.

1.8 MicroRNAs

1.8.1 Biogenesis and Modulation of Protein Expression

MicroRNAs (miRNAs) are a class of evolutionarily conserved, single-stranded, non-coding RNAs that control protein expression at a post-transcriptional level. They

have been detected in mammals, plants, and viruses, and have been described to affect multiple signaling pathways in these systems (Bartel, 2004). To date, miRNAs have been predicted to control the expression of 30% of genes in the mammalian genome, and deregulation of microRNAs have been implicated in numerous human diseases including many autoimmune diseases (Filipowicz et al., 2008).

MicroRNAs can be found in gene clusters (like miR-17-92 cluster) or in single units scattered across the genome (like miR-155). MicroRNAs are mainly grouped in what was previously thought of as “junk DNA” or the introns and intergenic portions of the DNA. However, some miRNAs have been found to be encoded in gene exons that do not encode for proteins (such as miR-155 encoded in the *Bic* gene). Once miRNAs are encoded, primarily by RNA Polymerase II, primary transcripts that are about 80 nucleotides long are generated and folded into hairpin structures that have a 5'-cap and a 3'-polyA tail. The primary structures are then processed into approximately 70 nucleotides-long pre-miRNA stem loop structures in the nucleus by a nuclear microprocessor that contains the RNase III enzyme, Droscha, and a double-stranded-RNA-binding protein DGCR8 (Digeorge syndrome critical region gene 8). The pre-miRNA stem-loop structures are then transported to the cytoplasm through the exportin 5 complex (Denli et al., 2004).

Once in the cytoplasm, the pre-miRNA structures are then processed further by the endonuclease, Dicer, along with its cofactors ,TAR RNA-binding protein (TRBP) and PKR-activating protein (PACT), which cleave the pre-miRNA structure into 21–24 base pair duplex miRNA, containing 2 nucleotides that overhang the 3' of each strand (Chendrimada et al., 2005). One strand is then loaded onto the RNA induced silencing

complex (RISC) containing Argonaute (AGO) proteins (Diederichs and Haber, 2007). Once the miRNA is loaded onto the RISC complex, miRNAs pair with their target mRNA based on the complementarity of the microRNA seed sequences (nucleotides in positions 2–8 from the 5' end of an miRNA) to the 3' UTR (untranslated region) of the mRNA target. If there is perfect complementarity between the seed sequence and the 3' UTR target sequence, the mRNA will be degraded. If there is imperfect complementarity, there will be translational repression of the mRNA. The ability of miRNAs to bind with different affinity to a multitude of different targets allows for a fine-tuning of gene expression that can affect signaling. Figure 1.9 outlines the biogenesis of miRNAs.

1.8.2 MicroRNA-155 and the Immune System

MicroRNA-155 (miR-155) is a highly evolutionarily conserved microRNA that is encoded in the second exon of the *Bic* (B-cell integration cluster) gene, which is responsible for a subset of avian leukosis virus integration-induced lymphomas (Tam, 2001). It has been previously shown that miR-155 can transform B cells, resulting in lymphoma in mouse models (Costinean et al., 2006). miR-155 is expressed in both myeloid and lymphoid progenitors at different levels, depending on the cell type. During erythrocyte maturation, miR-155 expression decreases (Masaki et al., 2007). Also, transducing a K562 cell line with miR-155 significantly reduces their ability to differentiate into erythroid or megakaryocyte lineages (Georgantas et al., 2007). miR-155 expression is increased in a human monocytic cell line when the cell line was stimulated with LPS (Taganov et al., 2006). Also, addition of PolyI:PolyC (*polyriboinosinic*–

polyribocytidylic acid) or TNF can induce miR-155 expression in macrophages through JNK pathway activation (O'Connell et al., 2007).

The expression of miR-155 is necessary for B cell immunity. When BIC/miR-155 deficient mice were developed, they were found to have a severe defect in B cell IgM production and switched antigen-specific antibodies when immunized with tetanus toxin fragment C protein (Rodriguez et al., 2007). In a study utilizing a separate BIC/mir-155 deficient strain, miR-155 deficient mice had reduced germinal center function, cytokine production, and T cell-dependent antibody response (Thai et al., 2007). In antigen-stimulated B cells, miR-155 targets expression of PU.1 driving B cell maturation and IgG class switching, and there is a highly conserved target sequence in the 3' UTR region of PU.1 (Vigorito et al., 2007). Also, miR-155 targets and represses expression of the enzyme activation-induced cytidine deaminase, or AID (Dorsett et al., 2008; Teng et al., 2008). AID is important for the process of somatic hypermutation that is required for high-affinity IgG antibody repertoire in antigen-activated B cells by deaminating cytosine residues and introduces U:G mismatches in DNA.

1.8.3 miR-155 and T cell function

miR-155 is important in many T cell signaling processes, from stimulation to differentiation. Upon stimulation through the TCR, miR-155 expression is upregulated (Dudda et al., 2013; Gracias et al., 2013; Thai et al., 2007). Most T cell lineages in miR-155 deficient mice develop normally, however, CD4⁺ T cells differentially skew to Th2 *in vivo* with higher amounts of IL-4 produced under neutral stimulation conditions

(Rodriguez et al., 2007; Thai et al., 2007). Since c-Maf has been shown to be a target of miR-155, T cells deficient in miR-155 would have higher c-Maf production, and thus have higher IL-4 production (Rodriguez et al., 2007). miR-155 is also important for CD8⁺ effector responses through the regulation of type 1 interferon signaling and through the regulation of SOCS1 and STAT5 signaling (Dudda et al., 2013; Gracias et al., 2013). miR-155 is also important in Tregs through its repression of SOCS1, and miR-155 deficient animals have reduced absolute numbers of Treg cells (Lu et al., 2009). miR-155 deficient T cells also have a defect in IL-17 production with less Th17 cells produced (Murugaiyan et al., 2011; O'Connell et al., 2010; Yao et al., 2012). This defect in IL-17 production is due to the inability of miR-155 deficient cells to respond to IL-23 through the transcription factor Ets1 (Hu et al., 2013).

Because of the important role that miR-155 plays in T cell stimulation and differentiation, its dysregulation is evident in many autoimmune diseases. High levels of miR-155 have been found in patients with multiple sclerosis, rheumatoid arthritis, and atopic dermatitis (Junker et al., 2009; Sonkoly et al., 2010; Stanczyk et al., 2008). Additionally, mice deficient in miR-155 do not develop EAE or collagen-induced arthritis (Blumel et al., 2011; Hu et al., 2013; O'Connell et al., 2010; Yao et al., 2012). Additionally, miR-155 deficient CD8⁺ cells were ineffective at controlling tumor growth while overexpression of miR-155 enhances the anti-tumor response (Dudda et al., 2013). miR-155 has also been shown to regulate T cell response to infectious pathogens, such as the response to viruses (Lind and Ohashi, 2014).

1.9 Programmed Death Receptor Signaling

1.9.1 The Programmed Death Signaling Pathway

The Programmed Death-1 (PD-1; CD279) receptor is an inhibitory receptor that is found on the cell surface. It is made up of a single immunoglobulin (Ig) superfamily domain and a cytoplasmic domain containing two tyrosine-based signaling motifs: a tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (Ishida et al., 1992). PD-1 has two receptors: PD-L1 (B7-H1, CD274) and PD-L2 (B7-H2, CD273), and both ligands have Ig-V-like and Ig-C-like extracellular domains and a short intracellular domain (Dong et al., 1999; Latchman et al., 2001; Tseng et al., 2001).

PD-1 is expressed on natural killer T (NKT) cells, NK cells, activated monocytes, some subsets of dendritic cells (DCs), and T and B cells. PD-1 is upregulated on T and B cells after TCR or BCR stimulation, and sustained antigen stimulation sustains high PD-1 expression (Freeman et al., 2006). Cytokines such as IL-2 and interferons can also potentiate PD-1 expression on T cells (Kinter et al., 2008). PD-L1 is broadly expressed throughout cell subtypes, while PD-L2's expression is more restricted. PD-L1 is constitutively expressed on not only hematopoietic lineages, such as T and B cells, DCs, macrophages, mesenchymal stem cells and bone marrow-derived mast cells, but it is also expressed on many non-hematopoietic lineages (Freeman et al., 2000; Yamazaki et al., 2002). These include vascular endothelial cells, epithelial cells, muscle cells, hepatocytes, placental cells, pancreatic islet cells and stromal cells of the bone marrow

(Eppihimer et al., 2002; Liang et al., 2003). Although PD-L1 is broadly expressed, inflammatory molecules, such as IFN γ , can induce the upregulation of PD-L1 expression. PD-L2, conversely, has a more limited expression on hematopoietic cell subtypes, such as DCs, macrophages, bone marrow-derived mast cells and on peritoneal B1 B cells as well as on germinal center B cells (Zhong et al., 2007). PD-L2 expression is not as inducible as PD-L1 expression, but IL-4 and GM-CSF are the strongest known stimuli for inducing PD-L2 expression (Eppihimer et al., 2002; Latchman et al., 2001; Loke and Allison, 2003; Schreiner et al., 2004; Yamazaki et al., 2002).

1.9.2 PD-1/PD-L1 Signaling in T cell function

The major role of PD-1 signaling in T cells is the inhibition of T cell activation by the engagement of the PD-1 receptor on the T cell by PD-L1 on the APCs. Certain subsets express a high level of PD-1, such as CD4⁺Foxp3⁺ regulatory T cells and “exhausted” CD8 cells (Baecher-Allan et al., 2003; Wherry et al., 2007). After ligation of PD-1 in the presence of TCR signaling, the receptor transduces an inhibitory signal through phosphorylation of the tyrosine residue on the ITSM region of its cytoplasmic tail. This leads to the recruitment of SHP-2 (SH2-domain containing tyrosine phosphatase 2) and possibly SHP-2, to the cytoplasmic domain of PD-1. This then downregulates PI3K activity mediated by CD28, thus downregulating the activation of Akt and T cell activation as shown in Figure 1.10 (Parry et al., 2005). PD-1 ligation has also been shown to inhibit phosphorylation of CD3, ZAP70 and PKCs, thus inhibiting signaling directly downstream of the TCR (Parry et al., 2005). PD-L1 can also inhibit T cell stimulation

when expressed on T cells by binding the CD80 molecule (B7-1) and blocking the engagement of CD28 with CD80, thus inhibiting proper T cell stimulation (Butte et al., 2007).

Other than affecting T cell activation downstream of the TCR, PD-1 activation also effects downstream effector signaling pathways, either during migration to the site of inflammation, or in the target tissue (Keir et al., 2008; Riella et al., 2011). PD-1 activation dramatically decreases the production of IL-2, IFN γ , and TNF, thus affecting T cell proliferation (Keir et al., 2008; Latchman et al., 2001). PD-1 activation also decreases the expression of the effector T cell associated transcription factors, GATA-3, T-BET, and Eomes (Nurieva et al., 2006). Additionally, PD-1 signaling has been shown to promote iTreg development, and PD-L1 engagement of its receptors on naïve T cells promotes iTreg development by inhibiting mTOR/Akt signaling (Francisco et al., 2009). Amarnath and colleagues have also shown that PD-L1:PD-1 signaling can be used in a human-into-mouse GVHD model to convert human Th1 cells into iTregs in vivo (Amarnath et al., 2011). It is clear from this evidence that PD-L1:PD-1 signaling is important in the maintenance of proper T cell function and the protection against aberrant T cell signaling.

1.9.3 The PD-1/PD-L1 Signaling and Disease Regulation

Because of the important role of PD-1 signaling in T cells, its dysregulation has been linked to many autoimmune diseases. Mice that are deficient in PD-1 develop autoimmune disease over time because of the loss in peripheral tolerance. The type of

autoimmune disease they develop depends on the strain of mouse. For example, mice deficient for PD-1 develop a lupus-like glomerulonephritis and arthritis on a C57BL/6 background, while they develop dilated cardiomyopathy on a BALB/C background (Nishimura et al., 2001). PD-L1 expression on parenchymal cells have also been shown to protect against autoimmune diabetes (Keir et al., 2006). Single-nucleotide polymorphisms in the gene *PDCDI* (gene encoding for PD-1), has been found in various kinds of autoimmune diseases, including SLE, type I diabetes, multiple sclerosis, rheumatoid arthritis, Grave's disease, ankylosing spondylitis, and aplastic anemia (James et al., 2005; Nielsen et al., 2003; Prokunina et al., 2002; Wu et al., 2013). Although dysregulation of PD-1 signaling is evident in many autoimmune diseases, no agonist for PD-1 or its ligands is currently in clinical trials.

In normal immune function, PD-1 is responsible for attenuating the primary immune response during acute infection. Using a model of adenovirus-induced hepatitis, PD-1 deficiency causes a significant decrease in the proliferation and accumulation of effector T cells in the liver. This also causes a rapid clearance of the virus; however, mice deficient in PD-1 conversely develop severe hepatitis, probably since PD-1 is responsible for dampening the cytokine storm that leads to excess tissue damage (Iwai et al., 2003). After chronic viral infection (such as with LCMV clone 13), viral specific PD-1^{hi}CD8⁺ T cells become anergic and unresponsive, or "exhausted." They lose their ability to produce TNF, IFN- γ , and IL-2. Although this population is not functionally competent, they contain memory cells that can re-expand after secondary infection with acute forms of the virus (Barber et al., 2006).

Clinically, PD-1:PD-L1 signaling has been mostly scrutinized in cancer models. The PD-1:PD-L1 pathway plays a major role in dampening the immune surveillance against tumors. Overexpression of PD-L1 on plasmacytoma cell inhibits cytolytic activity of CD8⁺ T cells through engagement of PD-1, and enhances the growth and invasiveness of the plasmacytoma (Iwai et al., 2002). Additionally, high expression of PD-L1 on clinical samples of tumors is associated with poor prognosis (Thompson et al., 2004). Also, blocking PD-1:PD-L1 interaction through antibody inhibition or genetic manipulation has been shown to accelerate the eradication of tumors in various experimental models (Blank et al., 2004; Curiel et al., 2003; Hirano et al., 2005; Strome et al., 2003). Currently, there is a fully humanized mAb to PD-1 (also known as nivolumab) that has made it through phase I clinical trials with little toxicity, as well as a mAb to PD-L1 (BMS-936559 or MDX-1105) that has also made it through some clinical trials, both with a potential for strong antitumor activity (Brahmer et al., 2010; Brahmer et al., 2012; Topalian et al., 2012). Although its efficacy in cancer treatment may prove high, it has not yet been determined the effect that blocking PD-1 signaling may have on worsening GVHD after bone marrow transplantation, which is a treatment used for some cancers.

1.10 Significance and Hypothesis

Although most patients respond to standard therapy (BMT or IST) given to Aplastic Anemia patients, there is still a large cohort of patients who do not respond to this therapy and succumb to disease. Because no autoantigen to this disease has been

found, elucidating the mechanisms driving the pathogenic T cell response is paramount to developing other treatments for patients who are refractory to treatment or relapse.

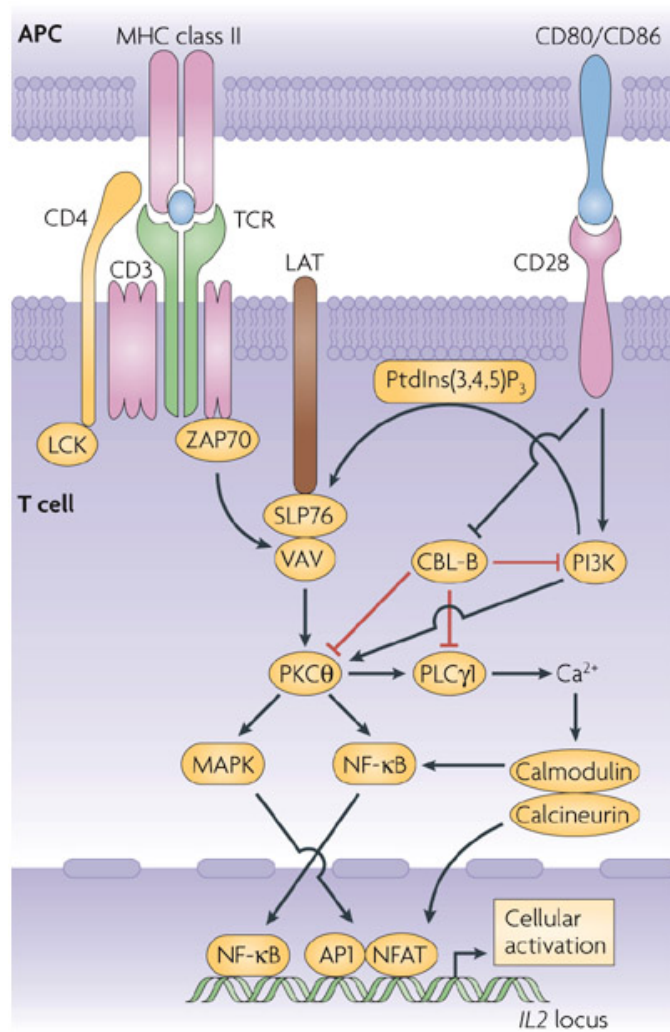
Therefore, through the following specific aims, we intend to extend the understanding of pathogenic T cell signaling pathways driving Aplastic Anemia:

Specific Aim I: Determine if PKC θ works in conjunction with Notch1 signaling to drive AA pathogenesis.

Specific Aim II: Elucidate the role NF- κ B plays in the disease pathology of Aplastic Anemia.

Specific Aim III: Determine if miR-155 drives disease severity of AA, and understand the mechanism of its signaling.

Using our murine model of AA and patient samples, we sought to evaluate these aims to find pathways that could prove to be novel targets for therapy. We hypothesize that PKC θ works in conjunction with Notch signaling to drive AA both in the mouse model and patient samples. Also, we predict that targeting NF- κ B using small molecule inhibitors can ameliorate disease symptoms through the down regulation of CXCR4 expression and suppression of T cell activation. Also, we hypothesize that miR-155 expression is increased in mice with AA compared to controls, and genetically deleting miR-155 in our mouse model mitigates disease severity through an increase in PD-L1 expression on T cells. The findings of this work can not only be used to further clinical translational research for Aplastic Anemia, but also for any autoreactive T cell signaling driving autoimmune diseases



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Figure 1.1 T Cell Receptor Signaling

Signals delivered by the engagement of the T-cell receptor and co-stimulatory molecules induce different signaling pathways that result in the activation of multiple transcription factors. Ligation of the TCR by peptide–MHC complexes triggers the recruitment of signaling molecules, such as Zap70, LAT, and SLP76. Downstream signaling activates the NF-κB pathway, NFAT pathway, and AP-1 pathway to promote a program of gene expression that leads to interleukin-2 (IL-2) production and T cell activation. Adapted from Miller et al., 2007.

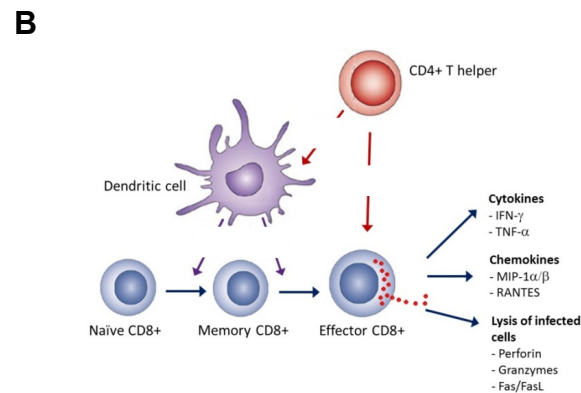
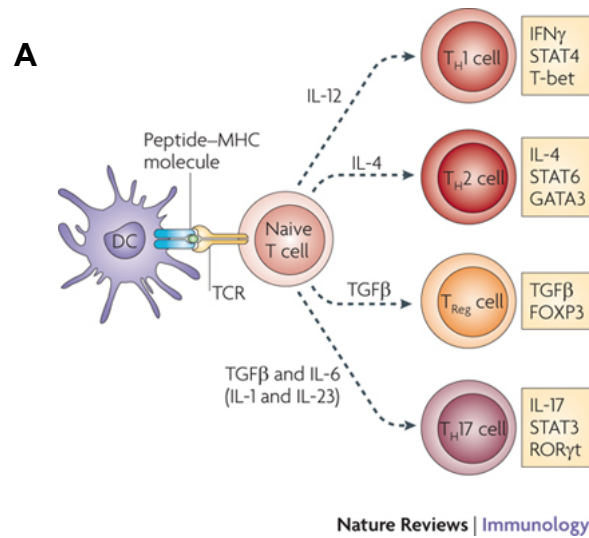


Figure 1.2 T Cell Subsets

A. Following activation by APCs, naive CD4⁺ T cells can be polarized into different effector T cell subsets depending on the local cytokine environment. The differentiation of each of these effector T cell subsets is controlled by distinct sets of transcription factors. Adapted from Zou and Restifo, 2010. B. After activation of naïve CD8⁺ by APCs, CD4⁺ T cells or MHC I presentation, CD8⁺ effector cells produce chemokines, cytokines and apoptotic proteins in order to eliminate infected cells. Adapted from De Haes, et al., 2012.

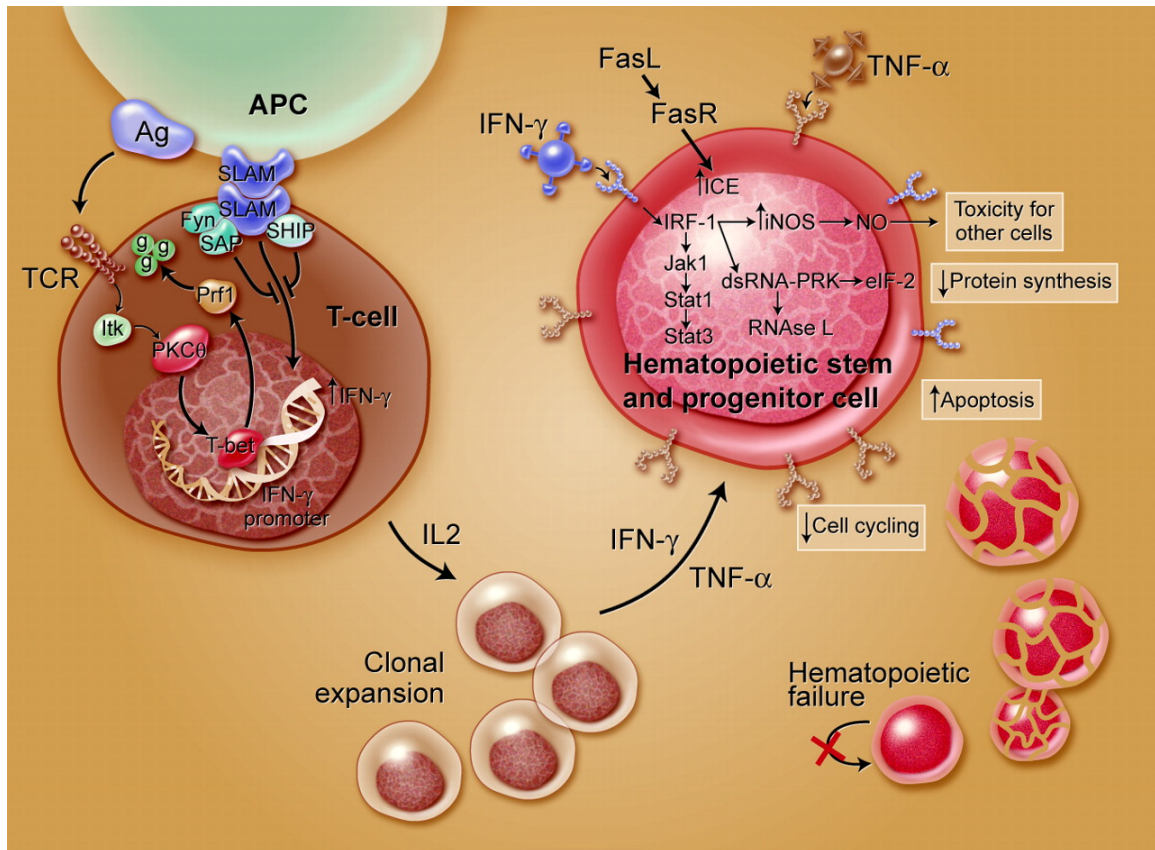


Figure 1.3 Immunopathogenesis of the bone marrow in Aplastic Anemia

Unknown self-antigens are presented to T lymphocytes by antigen presenting cells (APCs), which trigger T cells to activate and proliferate. Increased production of IL-2 leads to polyclonal expansion of T cells, and the upregulation of T-bet causes differentiation of T cells to a Th1 program. IFN γ and TNF upregulate other T cells' cellular receptors and also the Fas receptor. The expression of these proteins activate apoptosis programs in hematopoietic stem cells resident in the bone marrow, and leads to the pancytopenia that is associated with AA. Adapted from Young, 2006.

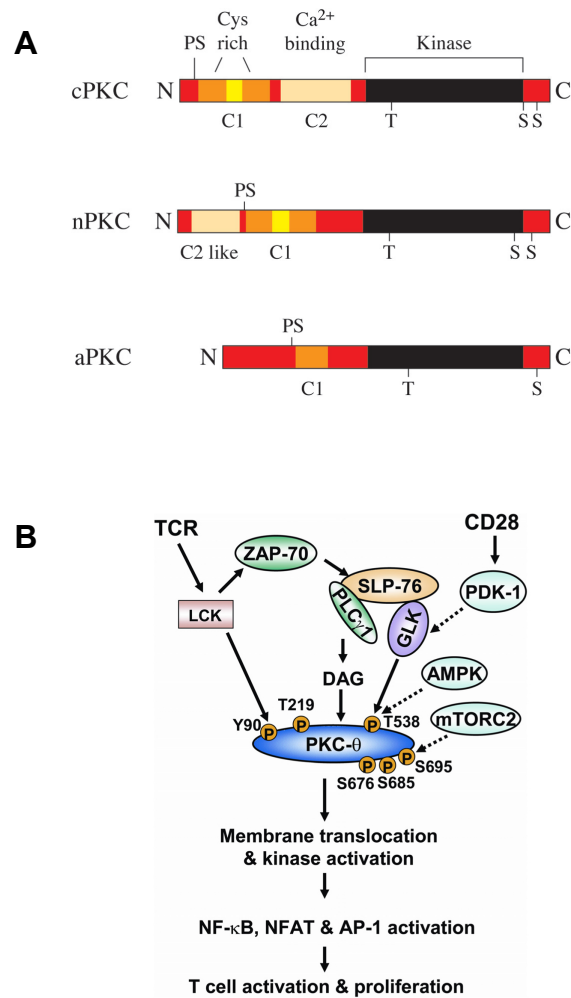


Figure 1.4 PKC Family Members

A. Structure of PKC proteins. PKC proteins are classified into conventional PKCs (cPKC; α , β , and γ), novel PKCs (nPKC; ϵ , δ , θ , and η) and atypical PKCs (aPKC; ζ and ι). All families have the kinase region and C1 region (Constant 1 region), while only cPKCs have a Ca^{2+} binding region. Adapted from Pfeifhofer-Obermair et al., 2012. B. Activation of PKC θ . Ligation of the TCR induces the activation of tyrosine kinase LCK, ZAP-70, induces phosphorylation of SLP-76. SLP-76 directly interacts with and activates PLC γ 1 which cleaves a phospholipid, generating the second messenger DAG. The binding of DAG with PKC- θ induces a conformational change; T538 of PKC- θ is then phosphorylated by GLK, leading to catalytic activation of PKC- θ . The CD28 costimulatory activates PDK-1, which facilitates PKC- θ T538 phosphorylation. Catalytic activation and membrane translocation of PKC- θ lead to the activation of transcription factors NF- κ B, NF-AT, and AP-1, and to subsequent T cell activation. Adapted from Wang et al., 2012.

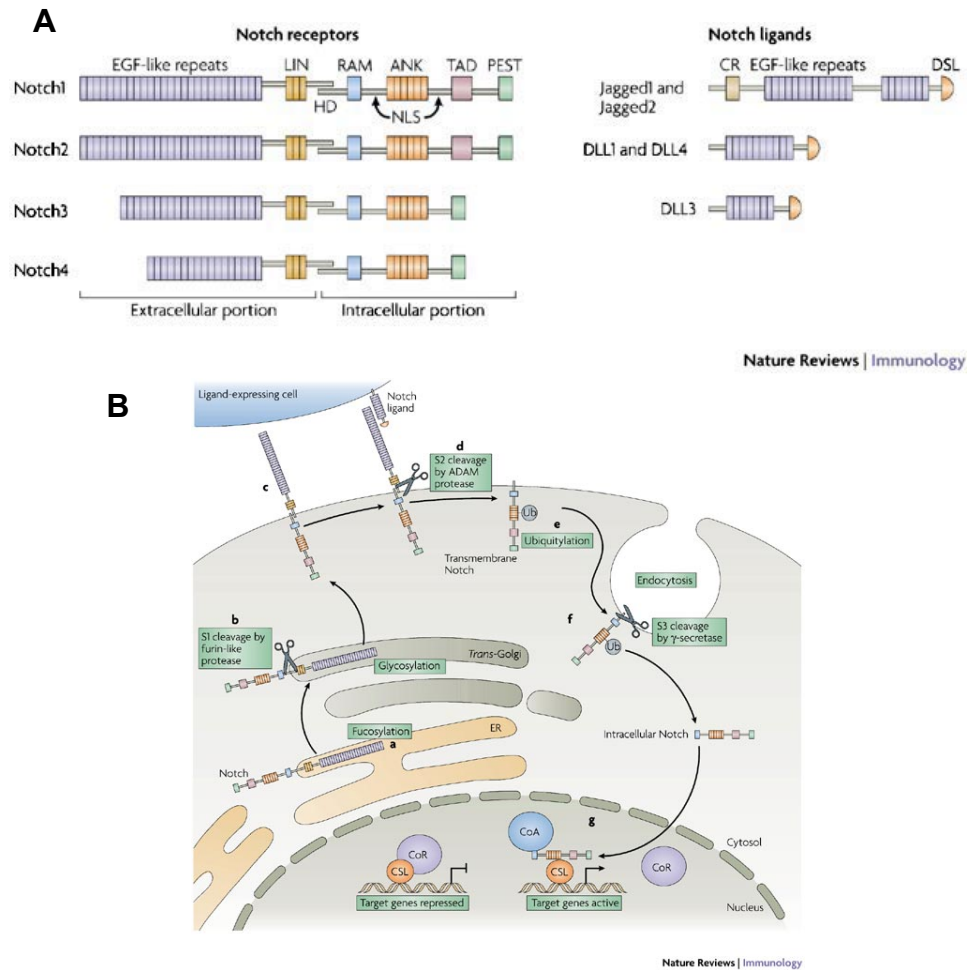


Figure 1.5 Notch Signaling Pathway

A. Structural representation of Notch receptors and their ligands. All receptors contain an extracellular domain with EGF-like repeats, the LIN domain for heterodimerization, RAM domain and ankyrin repeats for binding proteins and PEST domain for protein degradation. Notch3 and Notch4 lack a transcriptional activation domain (TAD). All ligands contain EGF-like repeats and a conserved DSL sequence. Jagged ligands have an additional cysteine rich (CR) domain. B. Canonical Notch Signaling. After fucosylation in the ER, Notch transits into the Golgi where it is cleaved at the S1 site by a furin-like protease. This leads to the expression of a non-covalently linked hetero-dimeric receptor on the cell surface. Following ligand binding, a second cleavage at the S2 site by an ADAM protease leads to the shedding of the extracellular domain. Following ubiquitination, the rest of the receptor is endocytosed and cleaved at the S3 site by a γ -secretase. This releases the intracellular, active domain of Notch, which migrates into the nucleus and interacts with CSL/RBP-J κ , previously associated with co-repressors (CoR). Recruitment of co-activators (CoA) converts CSL to an activator of transcription leading to transcription of target genes. Adapted from Osborne and Minter, 2007.

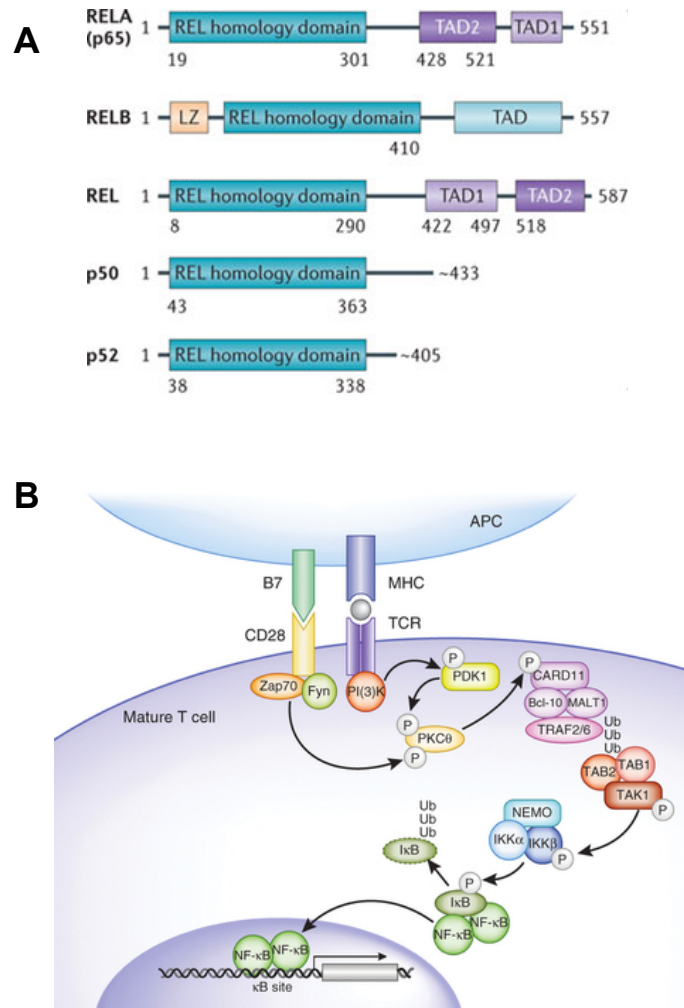


Figure 1.6 NF- κ B Signaling Pathway

A. Structure of NF- κ B subunits. All subunits share an approximately 300 amino acid-long DNA binding and dimerization domain that is termed the REL homology domain (RHD). RelA, RelB and c-Rel all contain carboxy-terminal transactivation domains (TADs), and RelB has an amino-terminal leucine zipper (LZ)-like motif. p52 and p50 are derived from proteolysis of their precursor proteins p100 and p105, respectively (not shown). Adapted from Perkins, 2012. B. TCR signaling pathway used in mature T cells to activate NF- κ B. Antigen-MHC and CD80 or CD86 binding to the TCR and CD28, respectively, engage and activate kinase signaling cascades (Fyn-PI(3)K, PDK1 and PKC θ) that through the recruitment of a specific signaling adaptor network involving the CMB complex, TRAF (TRAF2 and TRAF6) and TAB (TAB1 and TAB2) proteins, activates the canonical NF- κ B pathway via the TAK1-dependent phosphorylation of IKK β . Adapted from Gerondakis et al., 2014.

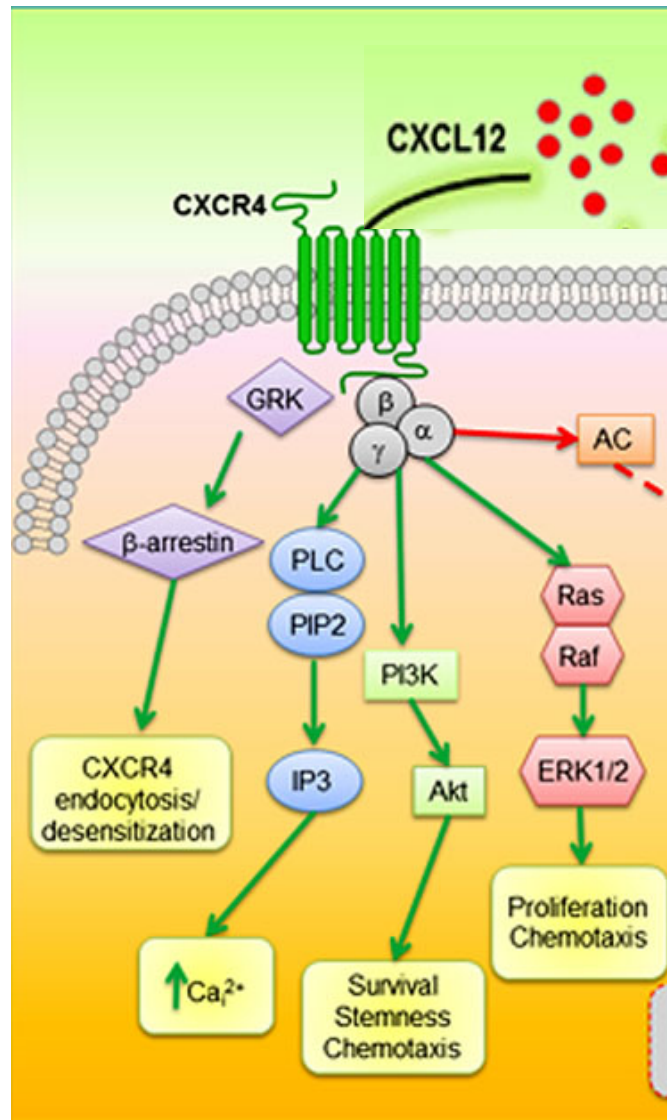


Figure 1.7 CXCR4 Signaling Pathway

CXCR4 signaling in T cells. When CXCR4 binds to CXCL12 (or SDF-1), it induces a conformational change of CXCR4/G-proteins and triggers GPCR signaling through PI3K/Akt, PLC/IP3, and ERK1/2 pathways, thus regulating cell survival, proliferation, and chemotaxis. Beta-arrestin pathway can be activated through GRK to internalize CXCR4. Adapted from Wurth et al., 2014.

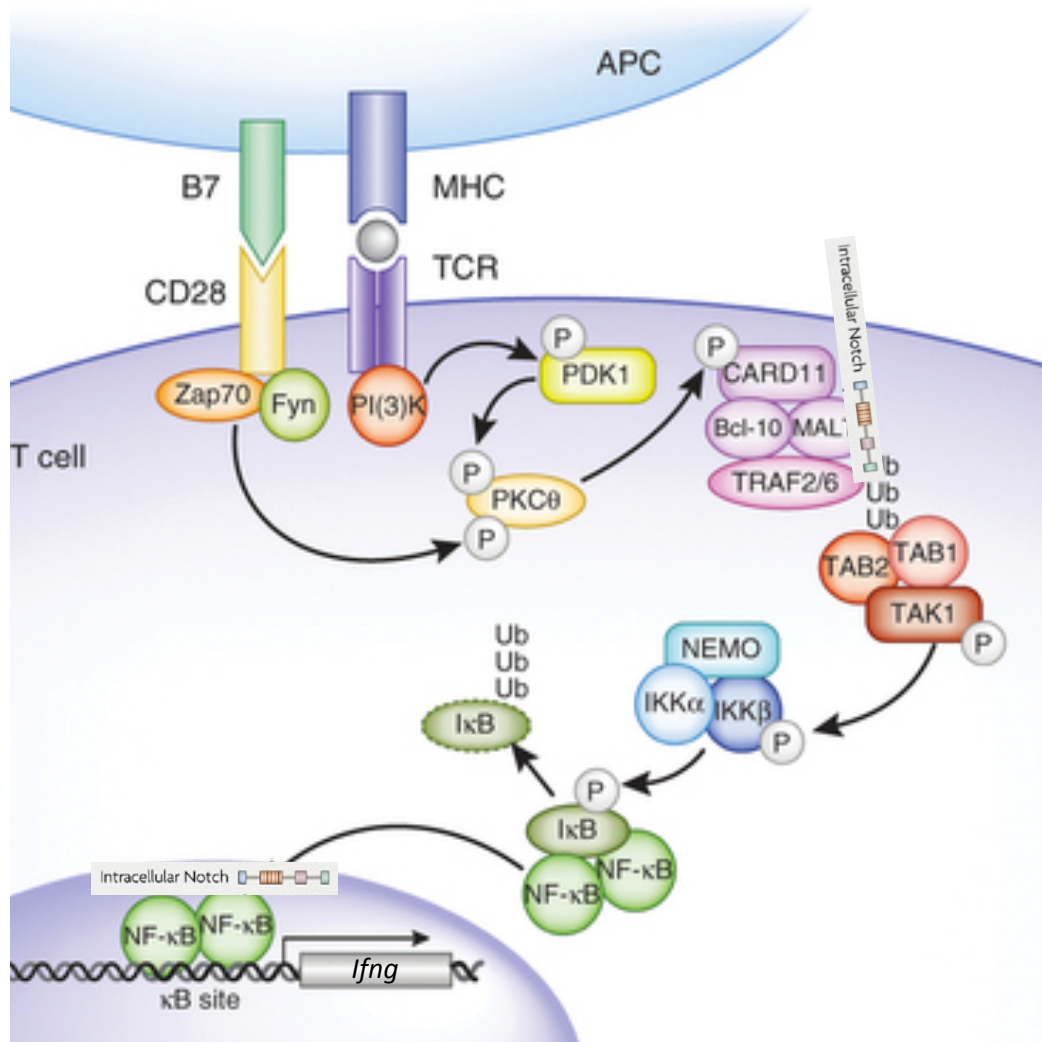


Figure 1.8 Signaling Pathway Cross Talk in T cells

An overview on potential steps downstream of TCR signaling where the Notch, NF-κB, and PKCθ pathways are interacting. Adapted from Gerondakis et al., 2014 and Osborne and Minter, 2007.

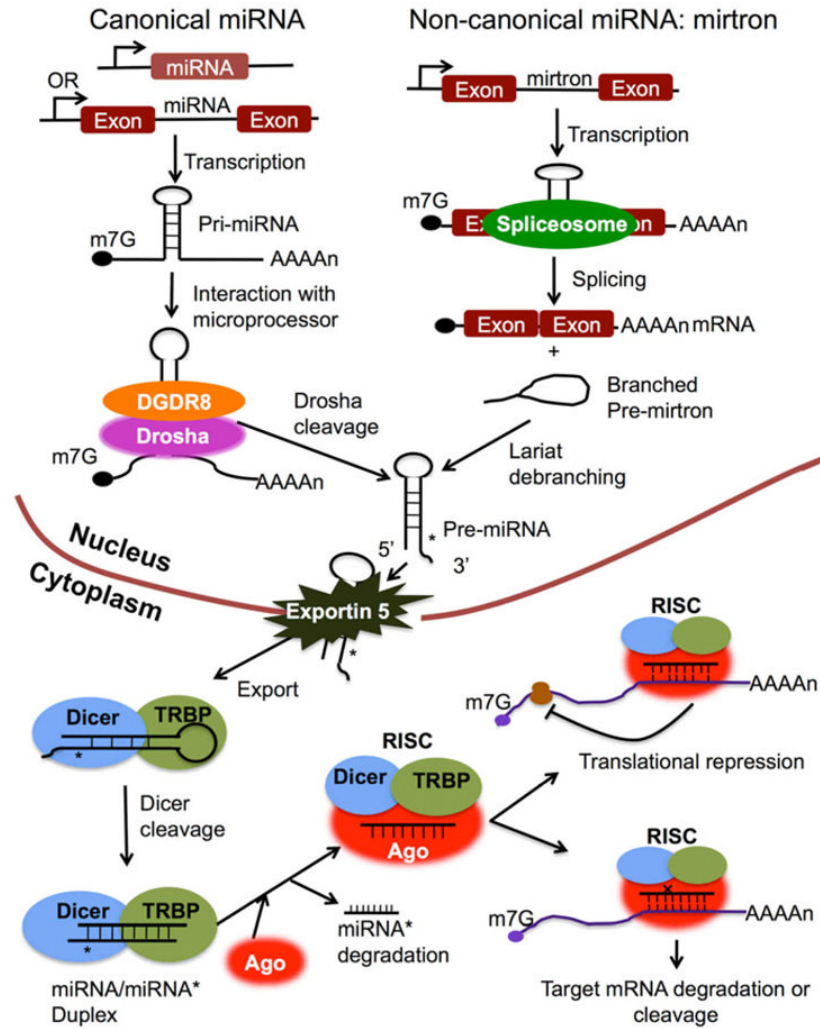


Figure 1.9 Biogenesis of microRNAs

Biogenesis pathways of microRNAs. In the canonical miRNA biogenesis pathway, the pri-miRNA is processed by DGCR8/Drosha microprocessor to generate pre-miRNA. In non-canonical miRNA biogenesis pathway, pre-miRNA is generated from small intronic miRNA, mirtron, through spliceosome splicing and then lariat-mediated debranching. Exportin 5 transports the pre-miRNA that derived from either canonical miRNA or mirtron from the nucleus into the cytoplasm. In the cytoplasm, the pre-miRNA is further processed by Dicer to generate miRNA/miRNA duplex, which is then loaded into Argonaute protein and forms RISC together with Dicer and TAR RNA binding protein (TRBP). miRNA regulates the expression its target genes through either translation inhibition or mRNA degradation/cleavage depending on the complimentary with its target mRNA. Adapted from Dai and Ahmed, 2011.

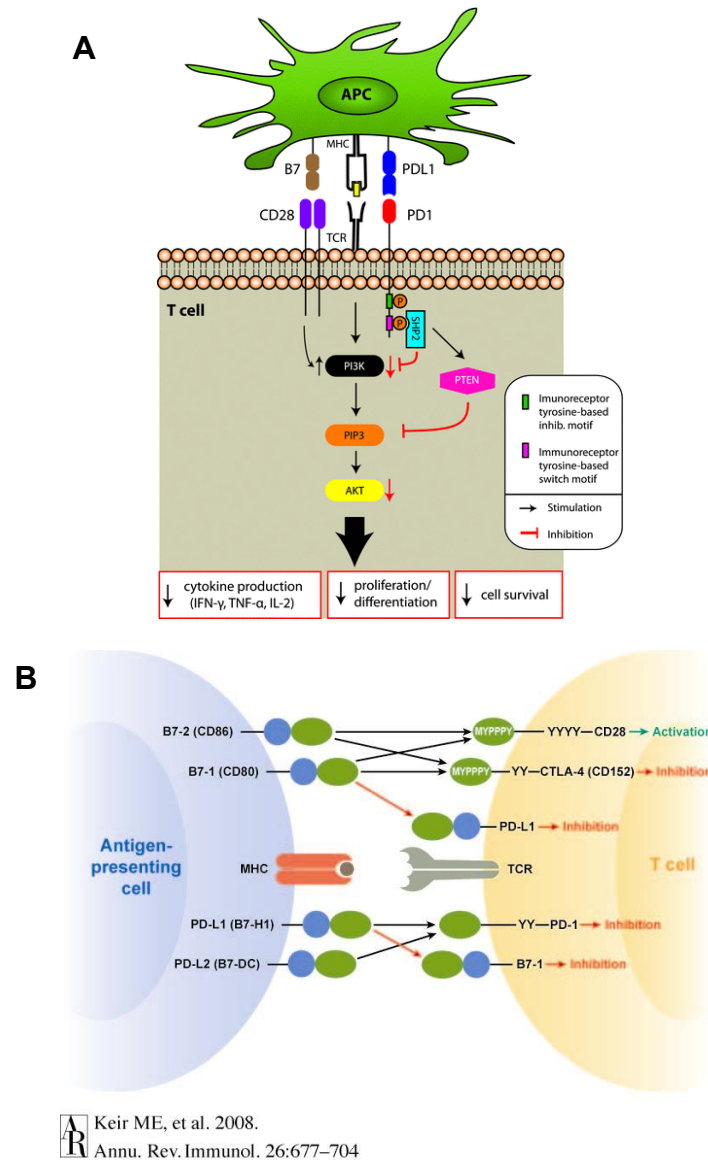


Figure 1.10 PD-1/PD-L1 Signaling Axis

A. PD-1 signaling in T cells. PD-1 signaling dephosphorylates proximal signaling molecules and augments PTEN expression, inhibiting PI3K and AKT activation. The consequences include decreased T cell proliferation, cytokine production and cell survival. Adapted from Riella et al., 2012. B. B7:CD28 family interaction expands pathways in the B7:CD28 family. PD-L1 and B7-1 productively interact on T cells and can deliver bidirectional inhibitory signals. PD-L1:B7-1 binding may not only deliver signals when ligated, but may also serve to segregate binding away from previously identified receptors (PD-1, CD28, CTLA-4). IgV-like regions are depicted in blue and IgC-like regions in green, while tyrosine-containing signaling motifs are depicted by Ys. Adapted from Keir et al, 2008.

CHAPTER 2

PKC θ ACTS UPSTREAM OF NOTCH1 TO CONTRIBUTE TO THE PATHOGENESIS ASSOCIATED WITH APLASTIC ANEMIA

2.1 Introduction

Severe aplastic anemia (AA) is a rare acquired bone marrow failure (BMF) syndrome. At the time of diagnosis, patients often present with low levels of circulating platelets, white and red cells, and a characteristic hypocellular bone marrow (Young et al., 2008). Immune-mediated destruction of hematopoietic stem and progenitor cells, together with compromised stromal cell integrity in the bone marrow, leads to peripheral pancytopenia and leaves patients susceptible to bleeding episodes, infection, and hypoxia (Chen et al., 2005; Young et al., 2008). Most cases of AA are of unknown etiology and, if left untreated, can be fatal (Dezern and Brodsky, 2011).

The clinical observation that the majority of AA patients respond to immunosuppressive therapy underscores an autoimmune mechanism driving disease progression (Young et al., 2006). Evidence points to autoreactive T helper type-1 (Th1) lymphocytes as instrumental in mediating disease. Circulating T cells from patients with AA express increased levels of the Th1 transcriptional regulator, T-BET (Solomou et al., 2006), as well as intracellular NOTCH1, which directly regulates T-BET expression (Roderick et al., 2013). In the diseased bone marrow, elevated levels of proinflammatory, Th1-associated cytokines, IFN γ and TNF, suppress hematopoiesis and damage stromal

cells lining the hematopoietic niches through by-stander effects (Chen et al., 2004; Giannakoulas et al., 2004; Sloand et al., 2002; Young, 1987; Young et al., 1987; Zoumbos et al., 1985). Approximately 30% of newly-diagnosed patients do not respond to a single round of immunosuppressive therapy. Thus, new studies aimed at elucidating the cellular mechanisms at work in AA are critically important to identify novel therapeutic targets.

Full T cell activation is a sequential process. It is initiated when the T cell receptor (TCR) binds antigen displayed on an antigen presenting cell; a second signal is delivered when the T cell co-receptor, CD28, engages its cognate ligand, also found on the APC. Subsequently, cytokines generated in response to antigenic stimulation further act on T cells to drive their proliferation and differentiation. Protein kinase C-theta (PKC θ) is a novel member of the PKC family of kinases. Its activity, which is central to T cell signaling, is Ca²⁺-independent (Ono et al., 1988). Following T cell stimulation, PKC θ is activated through phosphorylation by germinal center kinase (GSK)-like kinase (GLK; Chuang et al., 2011), a process that can be successfully inhibited pharmacologically, using the PKC θ inhibitor, rottlerin (Springael et al., 2007). Physical redistribution of phosphorylated PKC θ within the fluid domains of the cell membrane results in its accumulation at the APC-T cell interface, where it facilitates assembly of a macromolecular signaling aggregate, known as the CARMA1-BCL10-MALT1 (CBM) complex (Sommer et al., 2005; Sun et al., 2000). CBM assembly is an integral step in the signaling process that links TCR engagement to gene transcription critical for T cell survival, proliferation, and differentiation (Matsumoto et al., 2005; Wang et al., 2004).

Together with PKC θ , the transmembrane receptor, NOTCH1, has been shown to be indispensable for assembly of the CBM complex, suggesting PKC θ and NOTCH1 function within intersecting signaling pathways (Shin et al., 2014). In mammals, the NOTCH family comprises four cell surface receptors (NOTCH1-4) that are critical regulators of cell fate acquisition in multiple cell types, including T cells during thymocyte development (Deftos and Bevan, 2000; Osborne and Minter, 2007). In mature T cells NOTCH1 mediates survival, proliferation, differentiation, and cytokine production in response to antigenic stimulus (Adler et al., 2003; Osborne and Minter, 2007; Palaga et al., 2003; Zhang et al., 2011). A role for aberrant NOTCH signaling in autoimmune disease, including AA, is also emerging (reviewed in Palaga and Minter, 2013; Roderick et al., 2013).

How PKC θ may contribute to T helper cell-mediated processes remains controversial. Reports suggest PKC θ is required for Th2 and Th17 responses, but it has been shown to be dispensable for generating Th1-mediated antiviral, as well as memory, T cell responses (Kwon et al., 2012; Marsland et al., 2005; Marsland et al., 2004). Accumulating evidence supports a function for PKC θ in mediating autoimmune disorders, such as experimental autoimmune encephalomyelitis, rheumatoid arthritis, and myosin-induced autoimmune myocarditis (Healy et al., 2006; Marsland et al., 2007; Salek-Ardakani et al., 2005; Tan et al., 2006). Furthermore, in a mouse model of graft-versus-host disease (GVHD), which has a strong Th1 component, PKC θ was required to induce GVHD pathology, but not to clear viral pathogen or residual leukemic cells (Valenzuela et al., 2009). These data suggest that inhibiting PKC θ in T cells may abrogate their pathogenic activity, while preserving their ability to respond appropriately

to infectious stimuli. Our lab has previously shown that PKC θ is necessary for the development of AA in a mouse model of disease (Roderick, unpublished). Also, therapeutically treating mice induced with AA with rottlerin ameliorates disease symptoms (Roderick, unpublished). As such, PKC θ may represent an attractive target for modulating immune-mediated conditions.

We tested the hypothesis that PKC θ is critical driver of AA pathogenesis. Using an established mouse model of AA shown in (Roderick et al., 2013), we demonstrate that phosphorylated PKC θ (pPKC θ) is elevated in spleen- and bone marrow-infiltrating T cells of AA mice, but not of control animals. Mechanistically, we show interfering temporally with PKC θ signaling differentially affects the expression of the intracellular domain of NOTCH1 (NOTCH1^{IC}) in murine CD4⁺ and CD8⁺ T cells. Abrogating PKC θ activity, pharmacologically or genetically, reduced NOTCH1^{IC} and IFN γ expression. Finally, we highlight the clinical relevance of our findings by demonstrating increased levels of phosphorylated PKC θ in peripheral blood samples from AA patients, which we show respond to *in vitro* rottlerin treatment by down-regulating NOTCH1^{IC} expression and IFN γ production. Collectively, our findings reveal PKC θ is an important contributor to AA pathogenesis through its regulation of the NOTCH1 signaling axis.

2.2 Results

2.2.1 Mice with aplastic anemia express high levels of pPKC θ in T cells

Phosphorylated PKC θ (pPKC θ) is required for full T cell activation (Liu et al., 2002). As we began our investigation of how PKC θ might contribute to bone marrow failure, our progress was hampered by the fact that the small number of cells recovered from the bone marrow of AA mice, or even from patients' peripheral blood, limits the use of immunoblotting as a means of evaluating protein expression. Therefore, we used conventional immunoblotting methods to validate a flow cytometric approach for determining levels of pPKC θ (Figure 2.1, A and B), then examined the expression of pPKC θ in T cells that mediate disease in a mouse model of AA (Roderick et al., 2013). Using flow cytometry, we assessed pPKC θ expression in CD4⁺ and CD8⁺ T cells both in the bone marrow and spleens of AA mice. Compared to control mice, which only receive γ -irradiation, there is significantly more pPKC θ expression in CD4⁺ and CD8⁺ cells in the bone marrow of AA mice on day 17 of the disease course (Figure 2.1, C and D). This observation extended to the spleens of diseased mice whose CD4⁺ and CD8⁺ T cells also showed increased pPKC θ , compared to T cells from γ irradiated control mice (Figure 2.1, E and F). These data indicate that T cells infiltrating the bone marrow and spleens of AA mice express high levels of pPKC θ expression at the peak of disease.

2.2.2 PKC θ acts upstream of and is necessary for NOTCH1^{IC} and IFN γ expression in stimulated T cells

Individually, NOTCH1 and PKC θ have been shown to be essential for successful activation of T cells after stimulation through the TCR and the CD28 co-receptor (Adler et al., 2003; Eagar et al., 2004; Helbig et al., 2012; Palaga et al., 2003; Sun et al., 2000). Earlier studies showed treating peripheral blood mononuclear cells (PBMCs) from AA patients, *in vitro*, with the PKC θ inhibitor, rottlerin, decreased T-BET expression, a critical Th1 transcription factor frequently overexpressed in patients with AA (Solomou et al., 2006). We showed previously that intracellular NOTCH1 (NOTCH1^{IC}) is increased in PBMCs from AA patients, and can be detected bound to the promoter of *TBX21*, which encodes T-BET (Roderick et al., 2013). We therefore asked whether PKC θ might function upstream of NOTCH1 during T cell activation. We stimulated WT murine T cells in the absence or presence of rottlerin, or we stimulated PKC θ ^{-/-} T cells alone, and assessed levels of intracellular NOTCH1^{IC} using flow cytometry (Roderick et al., 2013). T cells stimulated in the presence of rottlerin showed significantly reduced median fluorescence intensity (MFI) of NOTCH1^{IC}, both in CD4⁺ (Figure 2.2A) and CD8⁺ T cells (Figure 2.2C). We noted similar decreases in NOTCH1^{IC} when T cells isolated from PKC θ ^{-/-} mice were stimulated under similar conditions (Figure 2.2, B and D).

In the earlier study, treating AA patient samples with rottlerin *in vitro* also reduced their capacity to produce IFN γ (Solomou et al., 2006). Since NOTCH1 has been shown to directly regulate IFN γ expression in murine T cells (Shin et al., 2006), we used IFN γ as a biological readout to ask whether inhibiting PKC θ affects NOTCH1-mediated

IFN γ secretion. Compared to DMSO-treated cells, abrogating PKC θ activity with rottlerin significantly reduced IFN γ in CD4 $^+$ (Figure 2.2E) and CD8 $^+$ T cells (Figure 2.2G), as measured by intracellular cytokine staining. We also observed low levels of IFN γ in stimulated PKC $\theta^{-/-}$ CD4 $^+$ and PKC $\theta^{-/-}$ CD8 $^+$ T cells (Figure 2.2, F and H). Altogether, these results provide evidence that PKC θ acts upstream of NOTCH1 to positively modulate its expression, as well as the production of the NOTCH1-regulated, pro-inflammatory cytokine, IFN γ .

2.2.3 Differential requirements of PKC θ for NOTCH1^{IC} expression in stimulated CD4 $^+$ and CD8 $^+$ T cells

We recently demonstrated a physical interaction between PKC θ and NOTCH1 that occurs within 3 hours of T cell activation (Shin et al., 2014). In the present study, we sought to better understand the extended temporal requirements of PKC θ activity on NOTCH1^{IC} expression. Specifically, we wanted to know whether inhibiting PKC θ expression when T cells are activated affects NOTCH1^{IC} expression. If so, are these effects long-lived and, importantly, for therapeutic considerations, what are the effects on NOTCH1^{IC} expression when we perturb PKC θ signaling well beyond T cell activation? To answer these questions, we isolated WT T cells and cultured them with plate-bound anti-CD3 ϵ and anti-CD28 under one of the following conditions: i) with rottlerin added to culture medium at time of plating, ii) with rottlerin added at the time of plating, but removed from cells when fresh medium was added 24 hours later, or iii) with rottlerin added 24 hours after T cells were seeded into antibody-coated wells. DMSO was

added to control wells under identical conditions as a vehicle control. We harvested cells at 24-hour time points over a 96-hour culture period and utilized flow cytometry to assess NOTCH1^{IC} expression and intracellular IFN γ in CD4⁺ and CD8⁺ T cells. When rottlerin was added at the time of T cell stimulation, NOTCH1^{IC} expression both in CD4⁺ (Figure 2.3A left panel) and CD8⁺ T cells (Figure 2.3B, left panel) was significantly lower, compared to DMSO-treated cells. We observed similarly-low levels of IFN γ in stimulated CD4⁺ (Figure 2.3C, left panel) and CD8⁺ cells (Figure 2.3D, left panel) treated with rottlerin, compared to those cultured with DMSO. These data suggest PKC θ signaling at the time of T cell activation positively modulates NOTCH1^{IC} expression as well as IFN γ production.

We next asked whether inhibiting PKC θ signaling at the time of T cell stimulation had durable effects on NOTCH1^{IC} signaling. We found that even after removing rottlerin-containing medium and replacing it with fresh medium 24 hours later, CD4⁺ (Figure 2.3A, center panel) and CD8⁺ T cells (Figure 2.3B, center panel) exposed to rottlerin during stimulation exhibited significantly less NOTCH1^{IC} compared to DMSO treated controls. CD4⁺ (Figure 2.3C, center panel) and CD8⁺ T cells (Figure 2.3D center panel) cultured under these conditions also produced less IFN γ . These results indicate blocking PKC θ signaling, even for relatively short durations at the time T cells are stimulated, has long-lasting effects on NOTCH1^{IC} expression and its downstream targets, including IFN γ .

Finally, we assessed the extent to which blocking PKC θ signaling as late as 24 hours after T cell activation affects NOTCH1^{IC} expression and IFN γ production. To our surprise, when T cells were stimulated for 24 hours before rottlerin was added to cultures,

we observed a significant decrease in NOTCH1^{IC} in CD8⁺ (Figure 2.3B, right panel) but not in CD4⁺ T cells (Figure 2.3A, right panel). Consistent with its effects on NOTCH1^{IC} expression, blocking PKC θ 24 hours after T cell activation also inhibited IFN γ production in CD8⁺ (Figure 2.3D, right panel) but not in CD4⁺ T cells (Figure 2.3C, right panel). Altogether, these data reveal CD4⁺ and CD8⁺ T cells exhibit a differential requirement for PKC θ signaling during NOTCH1^{IC} induction and IFN γ production. We conclude that CD8⁺ T cells require uninterrupted PKC θ signaling to maintain NOTCH1^{IC} expression. It would seem CD4⁺ T cells do not have this same degree of stringency, since they are able to maintain NOTCH1^{IC} expression as long as PKC θ is present at the time of activation. These data have important clinical ramifications, since they support the notion that interrupting PKC θ signaling in activated CD8⁺ T cells may further disrupt NOTCH1^{IC} expression and IFN γ production, to potentially attenuate disease symptoms.

2.2.4 Peripheral T cells from patients with AA express high levels of pPKC θ which respond to rottlerin treatment by downregulating NOTCH1^{IC} and IFN γ

PBMCs from patients with AA express elevated levels of Th1-associated proteins, including T-BET and Rottlerin treatment decreased T-BET expression and IFN γ secretion (Solomou et al., 2006). Moreover, we previously noted increased expression of NOTCH1^{IC} in peripheral T cells from AA patients, and demonstrated that it was enriched at the promoter that regulates T-BET (Roderick et al., 2013). Intrigued by these overlapping findings, we asked whether Rottlerin treatment also affected NOTCH1^{IC} expression in AA patient samples. We used flow cytometry to evaluate the level of

active, phosphorylated PKC θ (pPKC θ) in unmanipulated PBMCs from a cohort of AA patients who had not received prior treatment. Compared to healthy controls, the PBMCs of patients with untreated AA expressed significantly higher levels of pPKC θ both in CD4 $^{+}$ and CD8 $^{+}$ T cells (Figure 2.4, A and B). We investigated whether these cells would respond to PKC θ inhibition by down-regulating NOTCH1 IC expression, as we had observed with murine T cells. We seeded PBMCs from healthy donors, or from patients with untreated AA, into tissue culture wells pre-coated with antibodies specific for CD3 ϵ and CD28, and cultured cells for 72 hours in the presence of rottlerin or DMSO, as vehicle control. We determined that both in CD4 $^{+}$ and CD8 $^{+}$ T cells of AA patients, NOTCH1 IC levels were significantly lower after rottlerin treatment, compared to those treated with DMSO (Figure 2.4, C and D). This reduced NOTCH1 IC expression could also be seen in healthy PBMC controls suggesting that, although AA patient samples express significantly higher levels of pPKC θ , the PKC θ expressed responds equivalently to the actions of rottlerin.

As a biological readout of NOTCH1 IC activity, we quantified IFN γ production by stimulating PBMCs from healthy controls or from patients with AA, treated with DMSO or rottlerin. PBMCs from both cohorts responded to rottlerin treatment by secreting significantly less IFN γ into culture supernatants (Figure 2.4E), compared to DMSO-treated controls, indicating Rottlerin can effectively reduce the expression of key inflammatory proteins associated with AA pathology. Consistent with the increased expression of pPKC θ we noted in AA mice, we noted pPKC θ is also expressed at high levels in patients with AA who have not received prior IST. Furthermore, PBMCs from

patients are not refractory to Rottlerin treatment but rather respond by down regulating both NOTCH1^{IC} expression and IFN γ secretion.

2.3 Discussion

Phosphorylated PKC θ is elevated in T cells from mice and patients with AA. Furthermore, we identify a specific requirement for PKC θ in CD8⁺ T cells, but not in CD4⁺ T cells to drive IFN- γ . High levels of pPKC θ observed in human patient samples responded to rottlerin treatment by reducing levels of NOTCH1^{IC} expression and IFN γ , two pro-inflammatory proteins that are frequently expressed in patients with AA (Roderick et al., 2013, Solomou et al, 2006).

Our results firmly place PKC θ upstream of NOTCH1^{IC} accumulation, following T cell stimulation. Cross-talk between the NOTCH and PKC families has been reported in multiple systems. Signaling pathways mediated by PKC α and NOTCH4 converge in some instances of endocrine resistant breast cancer (Yun et al., 2013). Earlier reports have shown synergistic activities between PKC θ and NOTCH^{IC} in T cell acute lymphoblastic leukemia models induced by cells containing activating NOTCH^{IC} mutations (Felli et al., 2005; Giambra et al., 2012). However, how PKC θ functions to modulate NOTCH1 activation in normal, mature T cells remains ill-defined. In this study, inhibiting PKC θ signaling at the time of TCR stimulation revealed a previously undescribed requirement for this PKC family member, acting upstream of NOTCH1^{IC} and modulating its expression.

These results extend and inform previous observations by Solomou et al. In that report, treating PBMCs from AA patients with the PKC θ inhibitor, rottlerin, reduced levels of the Th1 transcriptional regulator, T-BET, as well as the pro-inflammatory cytokine, IFN γ . However, pPKC θ levels were not measured directly in that study, so it remained unclear whether and how PKC θ might be acting to modulate T-BET and IFN γ expression. In our present study, we directly measure pPKC θ , and show it is robustly expressed in PBMCs from patients with AA. In agreement with Solomou's observations, patient PBMCs responded to Rottlerin treatment by downregulating IFN γ production. We further demonstrate here, an as yet unreported effect of rottlerin on AA patient samples, which is to attenuate NOTCH1^{IC} expression following stimulation with anti-CD3 ϵ and anti-CD28. We recently showed that NOTCH1^{IC} is elevated in patients with AA, and can be found bound to the *Tbx21* promoter, which codes for T-BET (Roderick et al., 2013). In addition, we previously reported that NOTCH1 binds to and positively regulates the *ifng* promoter in mice (Shin et al., 2006). These observations, together with the results of our current study, prompt us to propose a unifying mechanism whereby PKC θ modulates NOTCH1^{IC} expression which, in turn, acts to regulate T-BET and IFN γ expression. Solomou et al. (2006) also identified T-BET bound to the IFN γ promoter in patients with AA. In this regard, therapeutic strategies which target PKC θ may prove to be exceptionally beneficial in reducing IFN γ expression in AA patients, since the collective data suggest it would decrease both T-BET and NOTCH1 expression, to effectively interrupt *Ifng* transcription.

Accumulating experimental evidence supports the notion that PKC θ signaling is complex and a strict requirement for its activity in CD4⁺ vs CD8⁺ T cells is likely

context-dependent (Marsland and Kopf, 2008). PKC θ is differentially required by CD4 $^{+}$ and CD8 $^{+}$ T cells to convey survival signals, *in vitro*, with PKC $\theta^{-/-}$ CD8 $^{+}$ T cells showing a marked survival defect that only moderately affects PKC $\theta^{-/-}$ CD4 $^{+}$ T cells (Saibil et al., 2007). We demonstrated that using rottlerin to inhibit PKC θ activity in CD4 $^{+}$ and CD8 $^{+}$ T cells 24 hours after stimulation resulted in differential effects on NOTCH1 IC expression. While NOTCH1 IC expression in CD4 $^{+}$ T cells appeared to be less influenced by PKC θ inhibition, CD8 $^{+}$ T cells required uninterrupted PKC θ activity to maintain NOTCH1 IC expression. How exactly PKC θ influences NOTCH1 IC accumulation in CD8 $^{+}$ T cells is under further investigation. TCR engagement, together with signals conveyed through the CD28 co-receptor, triggers an activation cascade in T cells that requires PKC θ and NOTCH1 (Shin et al., 2014). These amplified signals culminate in the nuclear translocation of transcription factors belonging to the NF- κ B family, which are normally sequestered in an inactive complex in the cytosol of resting T cells. Other groups have shown that translocation from the cytosol to the nucleus of one of these transcription factors, c-Rel, is required for CD8 $^{+}$ T cell activation (Deenick et al., 2010). We previously demonstrated that preventing NOTCH IC accumulation, using a pharmacological inhibitor of NOTCH activation, markedly reduced the nuclear accumulation and DNA-binding ability of c-Rel. Inhibiting NOTCH IC in murine T cells also abrogated NOTCH1 and c-Rel binding to the *Ifng* promoter (Shin et al., 2006). Thus, it is intriguing to speculate that inhibiting PKC θ may be acting upstream of NOTCH1 to regulate IFN γ expression through this mechanism. The diminution of IFN γ production by CD8 $^{+}$, but not CD4 $^{+}$ T cells, treated with rottlerin 24 hours after stimulation certainly lends credence to this model.

How PKC θ functions during T helper cell differentiation to mediate specific immune responses has been the subject of debate. PKC θ has been shown to be dispensable for Th1 immune reactions in response to pathogen infection (Marsland et al., 2005; Marsland et al., 2004). On the other hand, PKC θ deficiency is protective against autoimmune models with a pathogenic Th1 component, such as some the mouse models of EAE, Th1 dependent antigen induced arthritis, and autoimmune myocarditis (Anderson et al., 2006; Healy et al., 2006; Kwon et al., 2012; Marsland et al., 2007; Salek-Ardakani et al., 2005; Tan et al., 2006). Studies suggest in some of these disease models, especially EAE, PKC θ is also required to facilitate a pathogenic Th17 response that is responsible for a significant portion of central nervous system destruction (Rostami and Ciric, 2013). Th17 cells have been identified in AA patients; however, their contribution to disease pathology has not been extensively explored (de Latour et al., 2010). Nonetheless, whether PKC θ is important for development of Th1 or Th17 cells, or both, its therapeutic targeting might provide a means of interrupting the differentiation of these pro-inflammatory T cell subsets during AA progression.

A curative treatment for patients with AA is a bone marrow transplant (BMT) from a donor who shares high genetic similarity, preferably a sibling. A significant clinical obstacle to wider use of BMT, for treating AA patients as well as patients with other hematological malignancies, is the risk of developing a serious post-transplant condition known as graft-vs-host disease (GVHD). A recent elegant study identified PKC θ as necessary to drive the pathogenesis associated GVHD (Valenzuela et al., 2009). The authors suggested PKC θ may function to lower the threshold of T cell activation. They further showed that in the presence of high affinity peptides or increasing doses of

low affinity peptides, T cells deficient for PKC θ could be fully stimulated. In the absence of PKC θ , T cells were unable to respond to the low affinity of the mismatched MHC/antigens, and this suboptimal stimulation contributed to their inability to expand and mediate disease *in vivo*. However, CD8⁺ T cells were able to bind high affinity bacterial pathogens, presumably enabling them to overcome the lack of PKC θ , and clear the infection. We noted a similar lack of T cell expansion in AA mice whose BMF was induced with PKC θ ^{-/-} splenocytes (Roderick, unpublished). Furthermore, our observation that PKC θ was specifically required in CD8⁺ T cells to mediate BMF is completely consistent with the findings and interpretation provided by Valenzuela and colleagues (Roderick, unpublished).

In this scenario inhibiting PKC θ , even for a relatively short duration, may deprive T cells of the amplifying signal needed to respond productively to the low affinity stimulus of self-antigens. Finally, it is possible that PKC θ may be regulating strength of signal through the activation of NOTCH1, since studies showed NOTCH1 can regulate TCR signal strength in thymocytes, as well as in mature T cells (Dongre et al., 2014; Izon et al., 2001). Additional studies are required to understand the exact mechanisms at work in our model and how they may be further extrapolated to patients with AA.

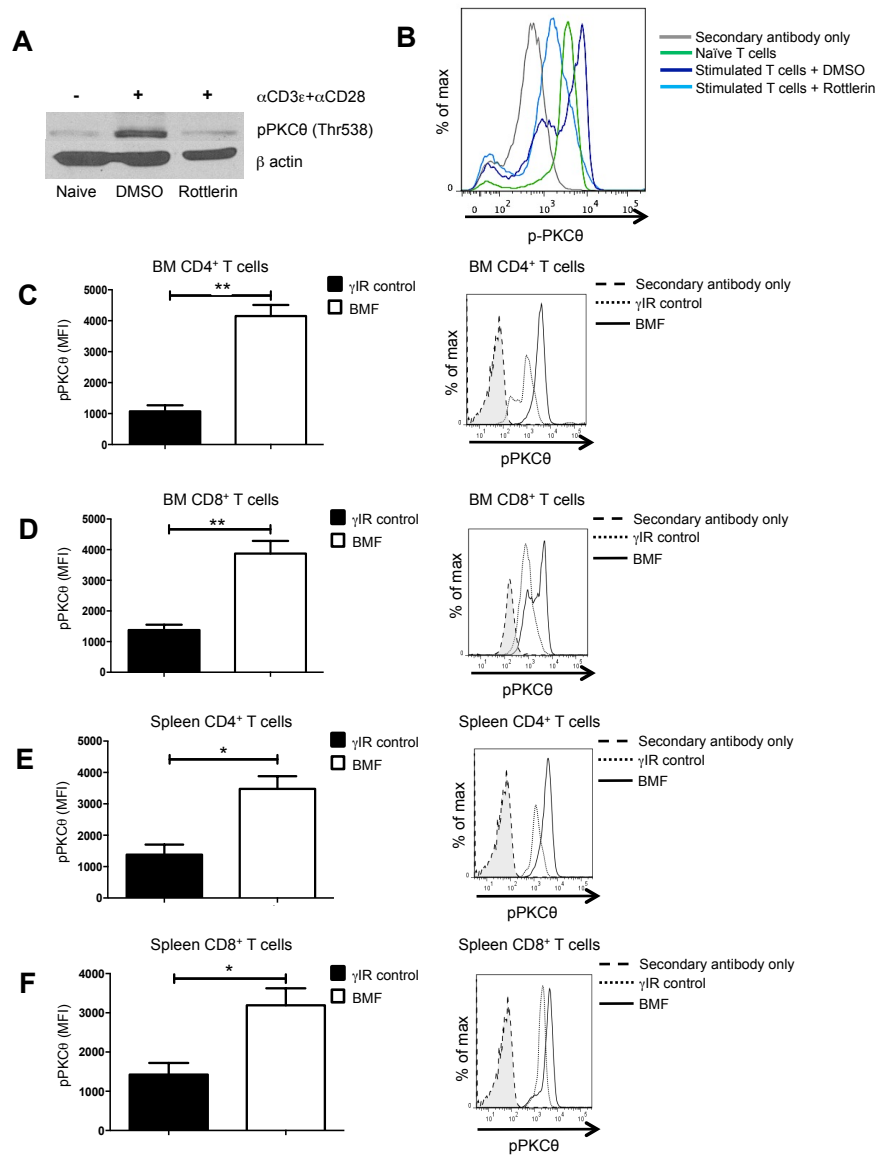


Figure 2.1 T cells from AA mice express elevated pPKCθ

F1 hybrid mice were irradiated only (γ IR controls) or induced with AA (BMF) and harvested 17 days after disease induction. pPKC θ expression was determined in (A, C) CD4⁺ and (B, D) CD8⁺ T cells isolated from (A, B) bone marrow and (C, D) spleen. Data are the mean + SEM of three independent experiments. *P < 0.05, **P < 0.01; unpaired student's *t* test.

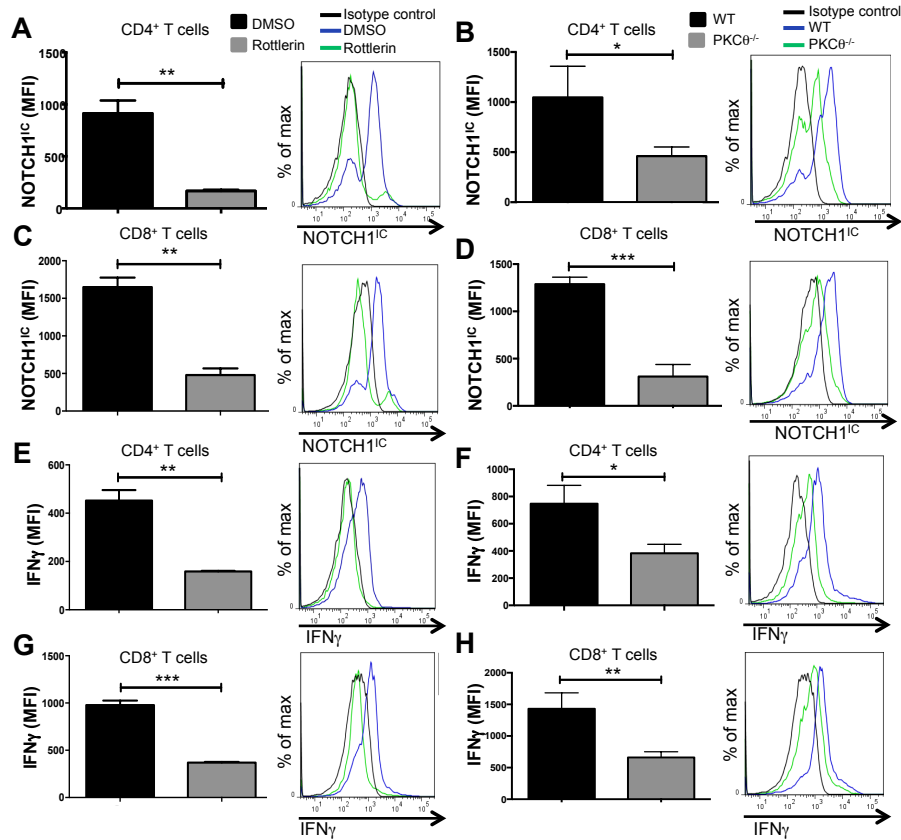


Figure 2.2 PKC θ acts upstream of NOTCH1^{IC} and IFN γ expression

We used flow cytometry to assess NOTCH1^{IC} expression in WT (A) CD4⁺ and (C) CD8⁺ T cells treated with DMSO or rottlerin and in (B) CD4⁺ and (D) CD8⁺ T cells from PKC θ ^{-/-} mice stimulated 48 hours with anti-CD3 ϵ and anti-CD28. IFN γ expression in DMSO- or rottlerin-treated (E) CD4⁺ and (G) CD8⁺ T cells or in (F) CD4⁺ and (H) CD8⁺ T cells from PKC θ ^{-/-} mice was also quantified using flow cytometry 48 hours after stimulation with anti-CD3 ϵ and anti-CD28. Data are the mean + SEM of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001; unpaired student's *t* test.

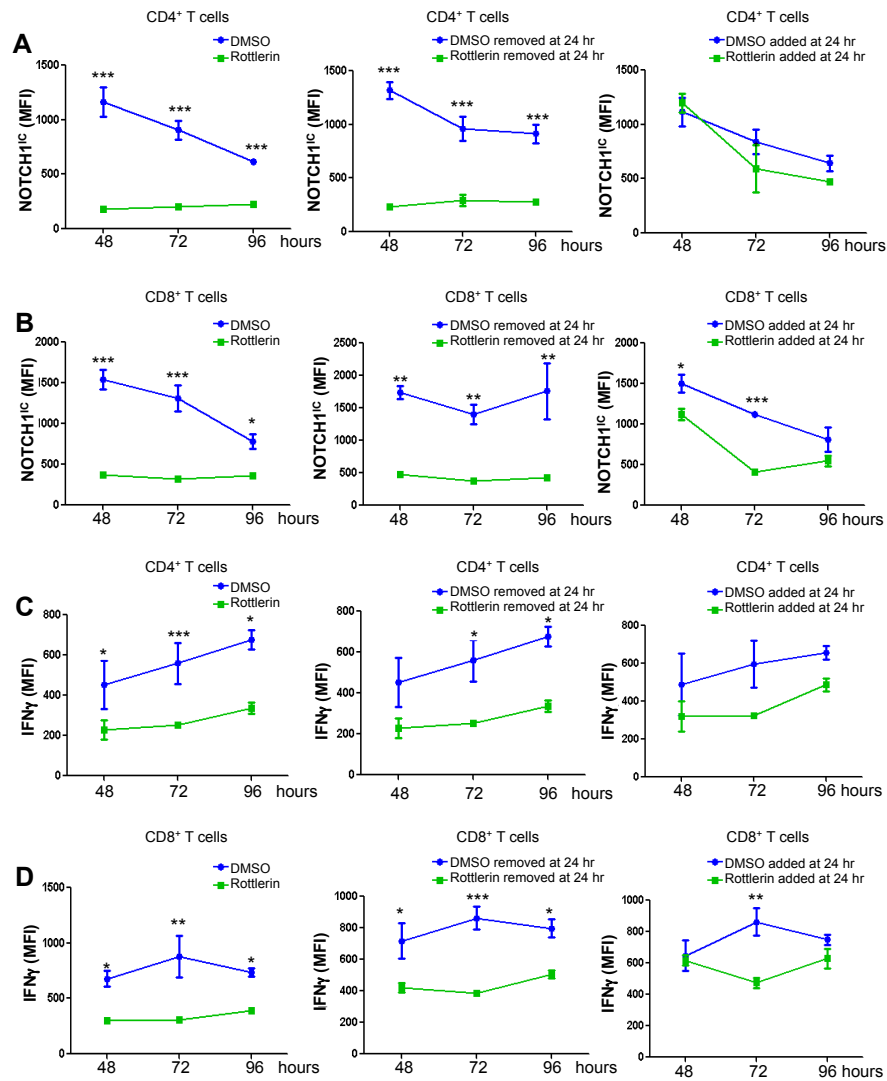


Figure 2.3 CD4⁺ and CD8⁺ T cells show differential requirements for PKC θ

(A-D) WT T cells were stimulated up to 96 hours with anti-CD3 ϵ and anti-CD28 and cultured either continuously in the presence of DMSO or rottlerin (3 μ M; left panels), cultured only for the first 24 hours in the presence of DMSO or rottlerin (center panels), or cultured in the presence of DMSO or rottlerin that was added 24 hours after stimulation (right panels). Using flow cytometry we assessed (A, B) NOTCH1^{IC} expression in (A) CD4⁺ and (B) CD8⁺ T cells at the indicated time points. We also used flow cytometry to quantify (C, D) intracellular IFN γ in (C) CD4⁺ and (D) CD8⁺ T cells at the time points indicated. Data are the mean \pm SEM of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001; two way ANOVA with Bonferroni post-test applied.

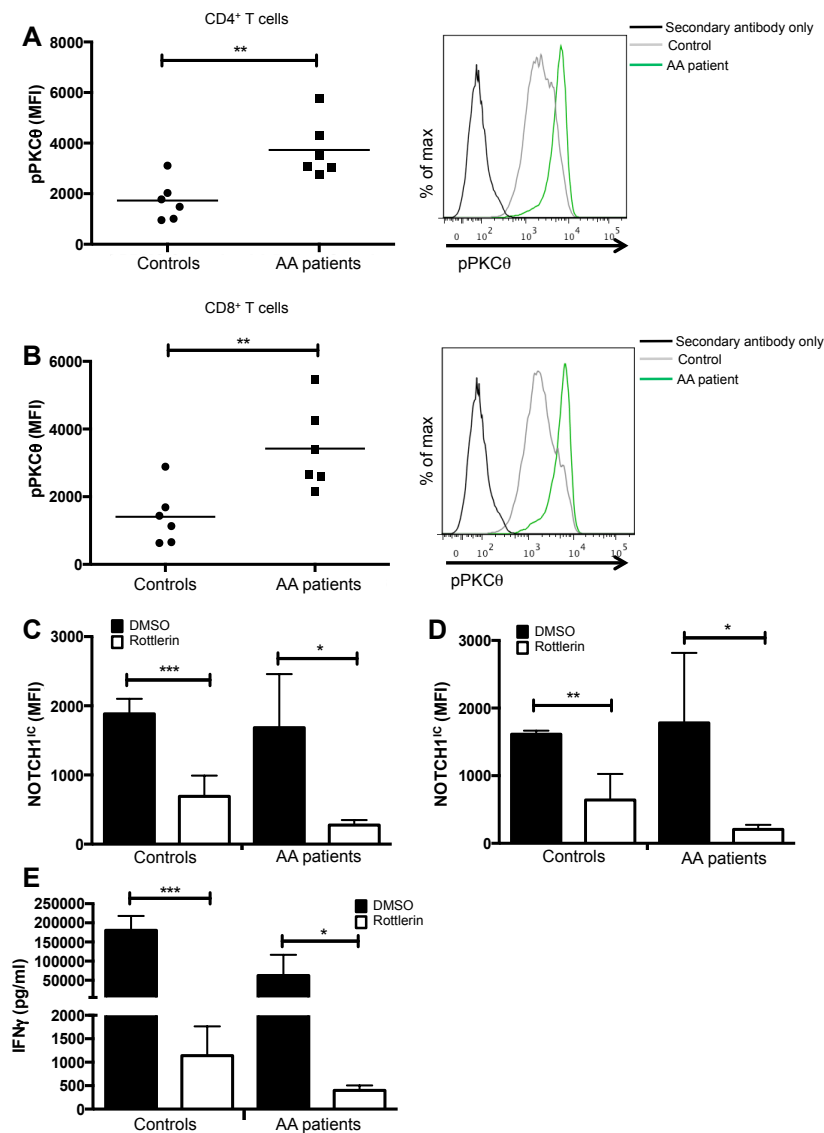


Figure 2.4 T cells from treatment-naïve AA patients express elevated pPKCθ

We used flow cytometry to assess pPKCθ expression in unmanipulated (A) CD4⁺ and (B) CD8⁺ T cells from healthy controls and from patients with AA who had not received prior IST. (C,D) Peripheral blood mononuclear cells (PBMCs) from healthy controls or from patients with AA were pre-treated either with DMSO or rottlerin (3μM) then stimulated for 72 hours with anti-CD3ε and anti-CD28. We evaluated NOTCH1^{IC} levels in (C) CD4⁺ and (D) CD8⁺ T cells using flow cytometry. (E) We used ELISA to quantify IFNγ secretion in supernatants of cultures treated as in C and D. *n*=3-6; Data are the mean + SEM and represent three independent experiments; **P* < 0.05, ***P* < 0.01, ****P* < 0.001; (A, B) unpaired student's *t* test, (C-E) one way ANOVA with Tukey's post-test applied.

CHAPTER 3

NF- κ B PLAYS A ROLE IN DISEASE PATHOLOGY OF APLASTIC ANEMIA THROUGH THE REGULATION OF CXCR4

3.1 Introduction

Severe aplastic anemia (AA) is a rare acquired bone marrow failure (BMF) syndrome where autoreactive T helper type-1 (Th1) lymphocytes are instrumental in mediating disease. Circulating T cells from patients with AA express increased levels of the Th1 transcriptional regulator, T-BET (Solomou et al., 2006), and intracellular NOTCH1, which directly regulates T-BET expression in patient peripheral blood samples (Roderick et al., 2013). Because approximately 30% of newly-diagnosed patients do not respond to a single round of immunosuppressive therapy, it is imperative to investigate other pathways in the autoreactive T cells that drive disease.

The NF- κ B family of transcription factors are a family of homo- or heterodimers that are comprised of 5 subunits: c-Rel, p65 (RelA), RelB, p50 (NF- κ B1) and p52 (NF- κ B2). p50 and p52 are processed by the precursors p105 and p100, respectively. Each homodimer or heterodimer has distinct expression patterns and regulatory functions, depending on the cell type (Gilmore, 2006). In T cells, NF- κ B is activated canonically after T cell stimulation. After T cell receptor activation in T cells, active PKC θ phosphorylates Carma1, which is able to complex with Malt1 and BCL10 to form the CBM complex. After formation of the signaling complex, NF- κ B is released from I κ B following its phosphorylation and subsequent degradation. NF- κ B is able to then

translocate into the nucleus and mediate downstream signaling associated with T cell activation and differentiation such as expression of IL-2, CD25, IL-2R α , and IFN γ (Vallabhapurapu and Karin, 2009). Dysregulation of NF- κ B signaling has been associated with numerous autoimmune diseases, including rheumatoid arthritis, systemic lupus erythematosus, type I diabetes, multiple sclerosis, and inflammatory bowel disease (Sun et al., 2013).

Chemokines are small molecules that are able to influence the motility of cells through binding to their cognate chemokine receptor. CXCR4 is a chemokine receptor that is constitutively expressed on T cells, and its main role in T cells is to drive migration along gradients of SDF-1, its chemokine ligand. CXCR4 can act as a costimulator of T cell proliferation and differentiation through its interaction with the TCR (Kumar et al., 2006; Molon et al., 2005). CXCR4 has also been shown to be highly overexpressed in many cancers, including human breast cancer cell lines and primary and metastatic breast tumors, ovarian cancer, prostate cancer, and melanoma (Hall and Korach, 2003; Kim et al., 2008; Muller et al., 2001; Taichman et al., 2002). Additionally, NF- κ B signaling has been shown to directly regulate CXCR4 expression to promote breast cancer migration and metastasis (Helbig et al., 2003). Since NF- κ B is important in T cell activation and differentiation and has been shown to drive CXCR4 expression and chemotaxis in cancer migration and metastasis, we asked if NF- κ B drove AA pathogenesis through an upregulation of CXCR4.

In this study, we show that NF- κ B signaling is necessary for the development of AA and inhibiting its function ameliorates disease. When we blocked NF- κ B signaling by using p50^{-/-} to induce AA in our mouse model, we found that donor cells were unable to

expand in the bone marrow, spleen and peripheral blood of the recipient mice. Treatment of AA mice with DHMEQ, a nuclear transportation inhibitor of NF- κ B, reduced the levels cytokines associated with AA in the serum. We could also reduce Notch1^{IC} expression in T cells that were infiltrating the bone marrow of AA mice through NF- κ B signaling inhibition. When we examined CXCR4 expression in AA mice compared to irradiation control mice, we found that AA mice had increased expression of CXCR4 on the bone marrow infiltrating T cells and when NF- κ B signaling is blocked in AA mice, CXCR4 expression is reduced. Also, when T cells are treated with Bay-11, a I κ B α degradation inhibitor and stimulated in a mixed lymphocyte reaction (MLR), chemotaxis in response to SDF-1 is severely reduced. Taken together, these data show that NF- κ B drives AA progression through T cell activation and through not only a reduction in T cell activation, but also by upregulation of CXCR4 expression.

3.2 Results

3.2.1 Blocking NF- κ B signaling abrogates AA disease progression

Because NF- κ B has been shown to be important in T cell signaling and activation, and it has been shown to be dysregulated in many autoimmune diseases, we wanted to see if it was necessary to drive pathogenesis of AA (Vallabhapurapu and Karin, 2009). To pharmacologically inhibit NF- κ B signaling *in vivo*, we used two inhibitors of NF- κ B that block signaling a different points in the pathway. The first inhibitor, Bay-11, is an irreversible inhibitor of cytokine-inducible I κ B α phosphorylation, therefore, it acts by

blocking the release of NF- κ B subunits from their cytoplasmic sequestration (Pierce et al., 1997). The other inhibitor, Dehydroxymethylepoxyquinomicin or DHMEQ is a new NF- κ B inhibitor that is a 5-dehydroxymethyl derivative of a novel compound epoxyquinomicin and prevents nuclear translocation of NF- κ B by sequestering it in the cytoplasm (Matsumoto et al., 2000).

For these studies, we induced mice with AA as described previously, and one hour after disease induction we treated mice with DHMEQ (30 mg/kg/day), Bay-11 (5 mg/kg/every other day), vehicle only (DMSO), or we induced recipient mice with p50-deficient donor splenocytes (B6.Cg- *Nfkb1^{tm1Bal}/J*). On day 17 post induction, mice were humanely euthanized to assess disease severity. Pharmacological inhibition of NF- κ B in AA mice, with both DHMEQ and Bay-11 and genetic deletion of the p50 subunit in donor cells resulted in significantly increased bone marrow cellularity when cells were enumerated using trypan blue exclusion (Figure 3.1A) and when assessed by H & E staining (Figure 3.1B). Treatment of AA mice by blocking NF- κ B signaling also increased the numbers of circulating white and red blood cells (Figure 3.1, C and D) compared with AA mice in all cases, therefore ameliorating the peripheral pancytopenia associated with disease.

To see if the amelioration of disease symptoms was due to a reduction of pathogenic T cell infiltration into the bone marrow, we used flow cytometry to look at the percentages of CD4⁺ and CD8⁺ T cells in the bone marrow. Compared to AA mice that have a large percentage of T cells in the bone marrow at day 17 P.I., mice treated with DHMEQ, Bay-11 and p50^{-/-} AA mice have significantly reduced numbers of CD4⁺ and CD8⁺ T cells in the bone marrow, at levels comparable to γ -IR control mice (Figure

3.1E). Next we asked whether therapeutic administration of Bay-11 prolonged the survival of AA mice. For these experiments, we induced mice with AA and treated mice with Bay-11 (5 mg/kg/every other day) or vehicle only (DMSO) from day 5 to 17 post-disease induction at which time treatment was discontinued and disease progression was monitored. Compared with DMSO-treated mice for which the median survival time was 19.5 days, mice treated with Bay-11 showed a remarkable survival benefit with four of seven mice fully rescued from lethal BMF ($P < 0.01$; Figure 3.1F). We also looked at the survival potential of AA mice induced with $p50^{-/-}$ splenocytes. Compared to mice induced with WT splenocytes who succumbed to disease on a median day of 20 post induction, mice induced with $p50^{-/-}$ cells did not succumb to disease and survived indefinitely ($p=0.0339$, Figure 3.1G). Taken together, these data demonstrate that NF- κ B drives disease progression of AA.

3.2.2 Mice induced $p50^{-/-}$ deficient cells do not develop disease because of a failure of cells to expand

Because AA mice induced with $p50^{-/-}$ splenocytes never develop disease, we asked if the $p50$ -deficient donor cells were able to react to alloantigens and expand in the recipient AA mice. To ask this question, mice were induced with AA using WT or $p50^{-/-}$ splenocytes and were harvested on days 10, 13, 15 or 17 post disease induction and donor cells were enumerated by assessing the percentage of $H2kb^{+}H2kd^{-}$ cells in the bone marrow, spleen and peripheral blood by flow cytometry. In the bone marrow, there is a very low presence of donor cells in $p50^{-/-}$ AA mice present throughout the disease course;

however, on day 10 post induction, about 20% of cells in the bone marrow in WT AA mice are donor derived, while by day 17, almost half of the cells in the WT AA mice are of donor origin (Figure 3.2A). There are slightly more donor cells in the spleen (Figure 3.2B) and peripheral blood (Figure 3.2C) over the disease course in p50^{-/-} induced AA mice, however, the amount is significantly reduced compared to WT induced animals. These data infer that p50^{-/-} never become activated enough to clonally expand to a point where they can mediate migration to the bone marrow and drive disease.

3.2.3 Inhibiting NF-κB nuclear localization decreases Th1 associated cytokine secretion in the plasma of AA mice

Plasma from mouse models of AA and human patients contain high amounts of Th1 associated cytokines (such as IFN γ and TNF) compared with healthy controls specimens (Young et al., 2008). NF-κB signaling has been shown to regulate the production of proinflammatory and Th1 associated cytokines in T cells (Vallabhapurapu and Karin, 2009). To see if we could ablate high cytokine levels in the plasma of AA mice by blocking NF-κB activation, we treated AA mice with DHMEQ or DMSO as described above. On day 17 post induction, mice were humanely sacrificed and plasma was collected from induced mice. To measure the concentration of circulating cytokines present in the plasma, a Cytometric Bead Array (CBA; BD Biosciences) was done to measure proinflammatory cytokines (IL-2 and IL-6), Th1 cytokines (TNF and IFN γ), Th2 (IL-4), Th17 (IL-17a) and anti-inflammatory (IL-10) cytokines. When mice were treated with the NF-κB inhibitor DHMEQ, the proinflammatory cytokines (IL-2 and IL-6; Figure

3.3, A and B) and Th1 cytokines (TNF and IFN γ ; Figure 3.3, C and D) were reduced in the serum of AA mice compared with DMSO treated controls. When we examined the levels of cytokines associated with other T helper subtypes, such as IL-4 (Th2), IL-17a (Th17), and IL-10 (Treg/anti-inflammatory), there was no difference in levels of cytokine production between DMSO treated and DHMEQ treated animals (Figure X, E-G). This implies that NF- κ B signaling is needed for the production of Th1 associated cytokines in AA, and blocking the activation of NF- κ B suppresses the cytokine storm associated with disease.

3.2.4 Notch1^{IC} is reduced in CD4⁺ cells infiltrating the bone marrow of AA mice when NF- κ B signaling is targeted

Our lab has shown that Notch1 signaling is necessary for driving AA disease pathogenesis (Roderick et al., 2013). Also, we have also shown that Notch and NF- κ B signaling interact to drive T cell activation (Shin et al., 2006; Shin et al., 2014). Because of this evidence, we hypothesized that blocking NF- κ B signaling would reduce Notch1^{IC} levels in AA mice. In mice treated with Bay-11 or induced with p50^{-/-} splenocytes, the percentage of Notch1^{IC} in both CD4⁺ and CD8⁺ T cells in the bone marrow is significantly decreased compared to AA controls on day 17 post induction (Figure 3.4A). When Notch1^{IC} expression was examined on a per cell basis, however, Notch1^{IC} was decreased in the CD4⁺ gate in the bone marrow only from mice induced with p50^{-/-} splenocytes compared to AA controls, and did not decreased in any mice where NF- κ B signaling was inhibited when CD8⁺ T cells were analyzed (Figure 3.4B). While there was

a decrease in the percentage of Notch1^{IC} expressing cells in Bay-11 treated and p50^{-/-} induced AA mice, DHMEQ treated AA mice have similar or higher levels of Notch1^{IC}, which was not expected (Figure 3.4, A and B). This difference in Notch expression between the groups treated with different NF-κB signaling inhibitor could be due to the fact that various components of the NF-κB pathway are being targeted differently depending on which method is used.

3.2.5 CXCR4 is differentially regulated in mice induced with AA compared to irradiation controls

The role of CXCR4/SDF-1 signaling in the progression and dissemination of hematopoietic malignancies and autoimmune diseases have been well documented (Domanska et al., 2013). However a role for CXCR4 in the pathogenesis of the autoimmune disease Aplastic Anemia has not been previously explored. First, we assessed both the gene and protein expression of CXCR4 in T cells from the spleen and BM of mice induced with AA. On day 17 post-disease induction, the transcript levels of *Cxcr4* in T cells isolated from spleen and BM of AA mice were similar to those found in T cells from spleens of control mice that had only been irradiated (Figure 3.5A). However, the protein expression of CXCR4 was significantly higher in spleen CD4⁺ T cells and BM-infiltrating CD4⁺ and CD8⁺ T cells of AA mice compared with spleen T cells of γ-irradiation control mice (Figure 3.5, B and C). Furthermore, compared with control mice, AA mice expressed CXCR4 at a significantly higher percentage in CD4⁺ and CD8⁺ T cells from both the spleen and the bone marrow (Figure 3.5D). Notably, of

those T cells that migrated to the bone marrow in AA mice, CD8⁺ T cells showed a higher expression of CXCR4 than CD4⁺ T cells (Figure 3.5E). These results document that CXCR4 expression on CD4⁺ and CD8⁺ T cells in mice induced with AA is highly upregulated, especially on CD8⁺ T cells resident in the bone marrow.

3.2.6 Inhibition of NF-κB reduces expression of CXCR4 in T cells and abrogates their chemotaxis to SDF-1α

Studies have shown that NF-κB regulates the expression of CXCR4 to promote migration and metastasis in a variety of malignancies such as breast cancer (Helbig et al., 2003). Therefore, we wanted to see if blocking NF-κB signaling in our mouse model of AA would reduce CXCR4 expression. Therefore, we induced mice with AA and treated them with NF-κB inhibitors as described above. In Bay-11-treated AA mice, we detected CXCR4 in a significantly lower percentage of BM-infiltrating CD4⁺ and CD8⁺ T cells (Figure 3.6A) and the expression of CXCR4 on a per cell basis was significantly reduced in these cells compared with diseased animals (Figure 3.6B). In CD4⁺ infiltrating bone marrow T cells, however, AA mice treated with DHMEQ and p50^{-/-} AA mice had no difference of CXCR4 expression on their infiltrating T cells. Conversely, CD8⁺ bone marrow T cells from mice where NF-κB signaling was inhibited by all methods had a decrease in both the percentage of CXCR4⁺ cells and a decreased of the expression of CXCR4 on a per cell basis (Figure 3.6, A and B). These data show that in CD8⁺ T cells, CXCR4 expression can be reduced by inhibiting NF-κB signaling.

We next asked whether inhibition of NF- κ B signaling could result in a reduction of SDF-1 α mediated chemotaxis. To do this, we examined expression of CXCR4 in T cells activated in conditions resembling the interactions occurring *in vivo* between donor and recipient cells in our AA model. We set up a mixed lymphocyte reaction (MLR) culture in which bulk splenocytes from C57BL/6 mice were co-cultured with BMDCs from F1 hybrids (BALB/c X C57BL/6) and treated cultures with Bay-11 (1 μ M) or vehicle only (DMSO) at the time of plating. We then examined the expression of CXCR4 using flow cytometry over a 12 day period. Bay-11-treated CD4⁺ T cells showed significantly lower levels of CXCR4 on days 6 and 8 of culture compared with DMSO-treated T cells (Figure 3.6C) Meanwhile, compared with DMSO-treated T cells, Bay-11-treated CD8⁺ T cells also displayed significantly lower levels of CXCR4 at days 6, 8 and 10 (Figure 3.6D). We then took cells from the MLR cultures on day 8 of culture and subjected those cells to a chemotaxis assay towards SDF-1 α . We found that Bay-11-treated CD4⁺ and CD8⁺ T cells exhibited a significantly lower chemotactic response to SDF-1 α compared with control T cells treated with vehicle only (Figure 3.6, E and F). These findings indicate that inhibition of NF- κ B reduces expression of CXCR4 in CD4⁺ and CD8⁺ T cells both *in vitro* and *in vivo* and affects their ability to migrate in response to SDF-1 α *in vitro*.

3.3 Discussion

For the first time, we describe a role for NF- κ B signaling in driving the immunopathogenesis of Aplastic Anemia. Using our mouse model of AA (Roderick et

al., 2013), we can ameliorate progression of AA using two inhibitors of NF- κ B signaling, DHMEQ and Bay-11, and through genetic ablation of the p50 subunit of NF- κ B in our donor cells. p50^{-/-} splenocytes fail to expand in the AA recipient mice in both the bone marrow, spleen, and peripheral blood. Also, DHMEQ treatment reduces the secretion of Th1 associated cytokines in the serum of AA mice. Besides its classical role in T cell activation and differentiation, NF- κ B has been shown to directly regulate CXCR4 expression in breast cancer, thus increasing their mobility and metastasis (Helbig et al., 2003). Therefore, we looked at the expression of CXCR4 in our mouse model of AA and found that CXCR4 was upregulated on BM-infiltrating T cells from AA mice, and was most highly expressed on the CD8⁺ T cells from the bone marrow. Finally, we could reduce the expression of CXCR4 by blocking NF- κ B signaling both *in vitro* and *in vivo*, and could reduce the SDF-1 α migration potential of T cells using the NF- κ B signaling inhibitor, Bay11.

Our results clearly show that NF- κ B signaling drives the pathogenesis of Aplastic Anemia. We used three different methods to abrogate NF- κ B signaling. We used two pharmacological inhibitors: Bay-11, an inhibitor that blocks the degradation of I κ B and the subsequent translocation of NF- κ B into the nucleus, and DHMEQ, an inhibitor that blocks the translocation of NF- κ B into the nucleus through unknown methods. We also used cells from animals that had a genetic knockout of the p50 subunit of NF- κ B. Not surprisingly we are able to concretely show that signaling through this pathway is necessary for AA pathogenesis by using these three methods. Since NF- κ B signaling is important for T cell stimulation and Th1 differentiation, its important role in AA pathogenesis was expected (Vallabhapurapu and Karin, 2009). Also, because our lab has

shown that both the Notch signaling pathway and PKC θ pathway are necessary for AA disease progression (Roderick et al., 2013), and both these pathways have been shown to interact with NF- κ B to drive T cell activation, our results fit nicely with the notion that aberrant T cell signaling downstream of the TCR in response to self antigen is driving disease (Shin et al., 2006; Shin et al., 2014; Sun et al., 2000).

Since the NF- κ B signaling pathway regulates multiple downstream processes in T cells, we wanted to elucidate which cellular mechanisms NF- κ B could be affecting to regulate disease. NF- κ B signaling has been shown to be responsible for regulating T cell activation and proliferation through direct regulation of cytokine production (Lamhamedi-Cherradi et al., 2003), and we were able to abrogate cytokine production in our model when AA mice were treated with the NF- κ B inhibitor, DHMEQ. Additionally, we show here that genetically deleting the p50 subunit in donor cells ameliorate disease due to a failure of donor cells to expand and proliferate in the recipient while in vitro studies show that p50^{-/-} T cells are able to produce similar amounts of IL-2 and IFN γ when stimulated through the TCR and CD28 co-receptor (data not shown). Studies have shown that while T cells deficient in c-Rel intrinsically have defects in IL-2 production and Th1 cytokine production in a diabetes model, the inability of p50^{-/-} T cells to mediate disease was not through direct cytokine production, but was a through an indirect reduction in autoreactive T cell activation via APC stimulation (Lamhamedi-Cherradi et al., 2003). However, further discovery into the differential roles of the NF- κ B subunits in different cell types responsible in AA development is required.

Interactions between the NF- κ B and Notch signaling pathways have been shown to be important in T cell activation (Shin et al., 2006; Shin et al., 2014). Therefore, we

wanted to see if a decrease in NF- κ B signaling correlated with a decrease in Notch1 activation in our AA mouse model, where Notch signaling has been shown to play an important role (Roderick et al., 2013). We did see a decrease in the percentage of cells expression Notch1^{IC} in both CD4⁺ and CD8⁺ T cells of the bone marrow when we treated cells with Bay-11 or induced mice with p50^{-/-} cells. However, we did not see a decrease in Notch1^{IC} when AA mice were treated with DHMEQ. This could be because of the method with which DHMEQ blocks NF- κ B. It is not well characterized, but DHMEQ is thought to block NF- κ B by blocking its nuclear localization signal (Ariga et al., 2002). Along with NF- κ B, DHMEQ could also be affecting Notch1^{IC} translocation, and somehow causing an accumulation of Notch1^{IC} in the cytoplasm that is unable to be degraded. However, further studies on the mechanism of inhibition of DHMEQ need to be performed to understand the mechanism of inhibition.

The chemokine receptor CXCR4 is responsible for chemotaxis of cells along gradients of SDF-1, which is mainly secreted in the bone marrow (Viola et al., 2006). Many metastatic cancers, which require motility of cancerous cells to sites such as the bone marrow, also have high expression of CXCR4 (Helbig et al., 2003). Since SDF-1 is highly expressed in the bone marrow, it was not surprising that T cells from AA mice that have migrated to the bone marrow express higher levels of CXCR4 compared to splenic resident T cells from irradiated controls. Also, we found that CD8⁺ T cells that infiltrate the bone marrow express higher amounts of CXCR4 compared to CD4⁺ T cells that infiltrate the bone marrow. While we cannot assume that higher expression of CXCR4 contributes to T cell pathogenicity in this context, it is interesting to speculate that the high expression of CXCR4 is one of a multitude of contributing factors that cause CD8⁺

T cells to play such an important role in our model of AA and human disease (Young et al., 2008). It is probably through the aberrant expression of CXCR4 that T cells are able to home to the bone marrow in response to SDF-1 and mediate damage to the resident stem and progenitor cells. However, since CXCR4 has been shown to be a costimulatory molecule during TCR signaling, it could be helping to augment T cell activation in this context (Kumar et al., 2006; Molon et al., 2005).

NF- κ B signaling has been shown in breast cancer models to directly regulate the expression of CXCR4, and this contributes directly to the metastatic and motile tendencies of secondary tumors both *in vitro* and in animal tumor models (Helbig et al., 2003). Notably, in AA mice where NF- κ B signaling was inhibited, we detected a significant reduction of the percentage of BM-infiltrating CD8⁺ T cells expressing CXCR4, and the expression of CXCR4 on these cells was also significantly decreased. We were able to mirror this observation using *in vitro* MLR cultures. Using this method, we were able to see a decrease in CXCR4 expression in both CD4⁺ T cells and CD8⁺ T cells when cultures were treated with Bay-11. We didn't see the same robust decrease in CXCR4 expression on CD4⁺ T cells from the bone marrow of our AA mice, however, this could be due to differing kinetics of expression between the two systems or CD4⁺ T cells in the bone marrow of AA mice could downregulate their CXCR4 expression upon entrance to the bone marrow. Both of these possibilities would have to be further researched.

Using a chemotaxis assay, we are able to show that inhibiting NF- κ B signaling not only decreases CXCR4 expression, but it also reduces the chemotactic ability of these cells in response to SDF-1 α . This fits in with our hypothesis that CXCR4 expression is

causing T cells to mobilize to the bone marrow where this is a large expression SDF-1 α in normal physiological instances. However, levels of SDF-1 have not been explored in AA patient bone marrow aspirates, but it would be interesting to see if they expressed higher levels of SDF-1. This could partly answer why T cells home to the bone marrow to mediate destruction in AA.

Based on these studies, we provide clear evidence that NF- κ B signaling contributes to disease pathology in AA by modulating CXCR4-mediated migration of CD4⁺ and CD8⁺ T cells to the BM. Blocking NF- κ B signaling may not only help decrease CXCR4 mediated migration, but it may also help quell the pathogenic T cell activation that is a hall mark of this disease. The multimodal targeting of NF- κ B signaling inhibitors makes them an attractive therapy. However, studies must be done to see if there would be pan-suppressive effects to the immune system by blocking this important signaling pathway. CXCR4 inhibitors may also be an attractive candidate. However, it may cause pathogenic T cells to home away from the bone marrow, causing possible tissue destruction in other organs through cytokine storm. Further studies on both types of inhibitors could prove invaluable in the search for a cure.

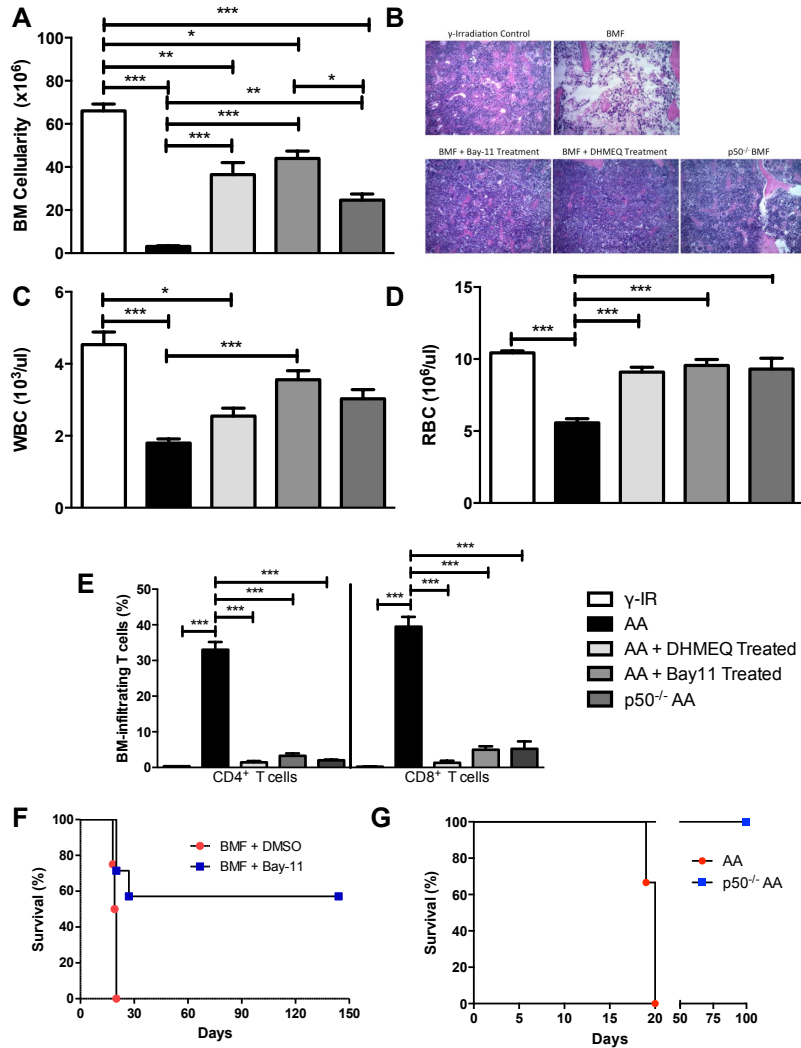


Figure 3.1 Pharmacologically inhibiting NF-κB inhibition in a mouse model of AA attenuates disease

Mice were induced with AA and treated with DHMEQ, Bay-11, or induced with p50^{-/-} splenocytes as described above. On day 17 post-induction, mice were harvested and (A) total bone marrow cellularity was determined using trypan blue exclusion. (B) Representative hematoxylin and eosin staining of sterna of AA mice. Magnification=20x. Circulating (C) white blood cells (WBC) and (D) red blood cells (RBC) were measured. (E) Percentages of CD4⁺ and CD8⁺ T cells infiltrating the bone marrow were determined using flow cytometry. (F) Kaplan–Meier survival estimates of AA mice induced with disease and treated with DMSO (n=4) or Bay11 (n=7) beginning on day 5 post-disease-induction and continuing until day 17 post-disease-induction (p<0.01) and (G) Kaplan–Meier survival estimates of AA mice induced with WT (AA; n=4) or p50^{-/-} splenocytes (p50^{-/-} AA; n=7), p=0.0339. Data are the mean plus SEM and analyzed using one way ANOVA plus Tukey post test or log rank test for survival estimates *, P < 0.05; **, P < 0.01; ***, P < 0.001.

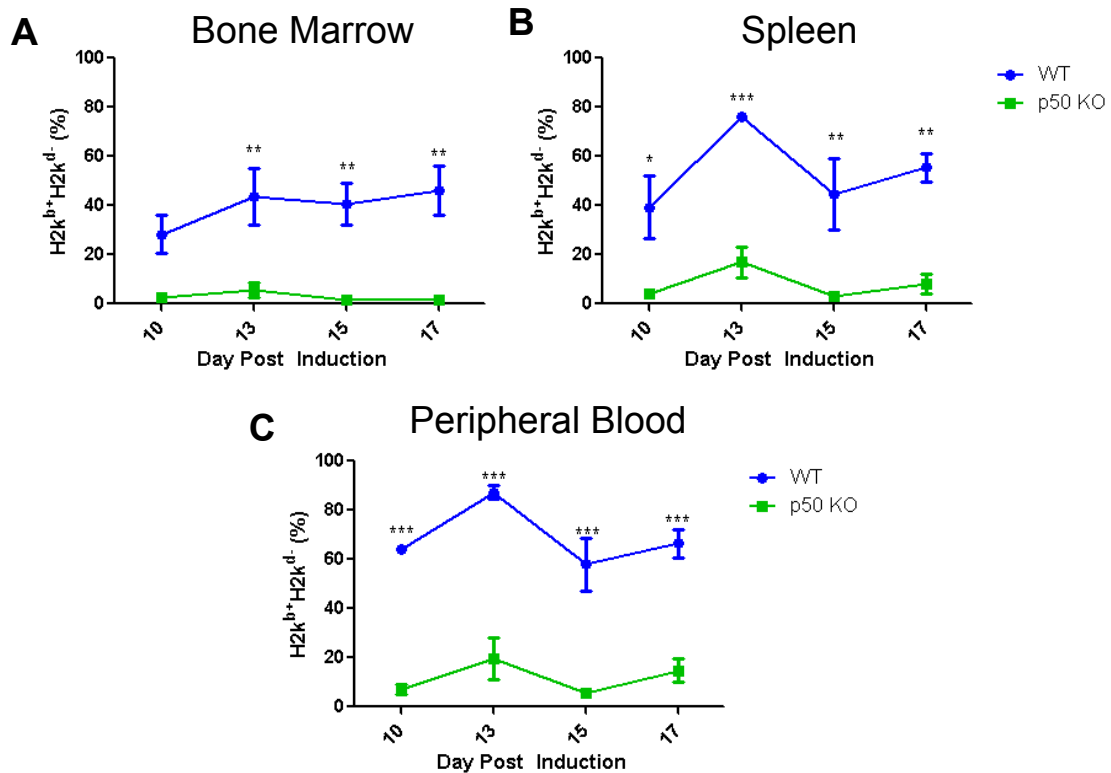


Figure 3.2 Donor cells in $p50^{-/-}$ induced AA mice do not expand compared to donor cells in WT induced AA mice

F1 recipient mice were induced with WT or $p50^{-/-}$ C57BL/6 bulk splenocytes and mice were harvested at day 10, 13, 15, and 17 post induction. Donor cells were tracked by flow cytometry by gating on $H2k^b^+H2k^d^-$ cells in the (A) bone marrow, (B) spleen, and (C) peripheral blood of recipient mice. $n=3$; data are the mean \pm SEM of three independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$; Two Way ANOVA and Bonferroni post test.

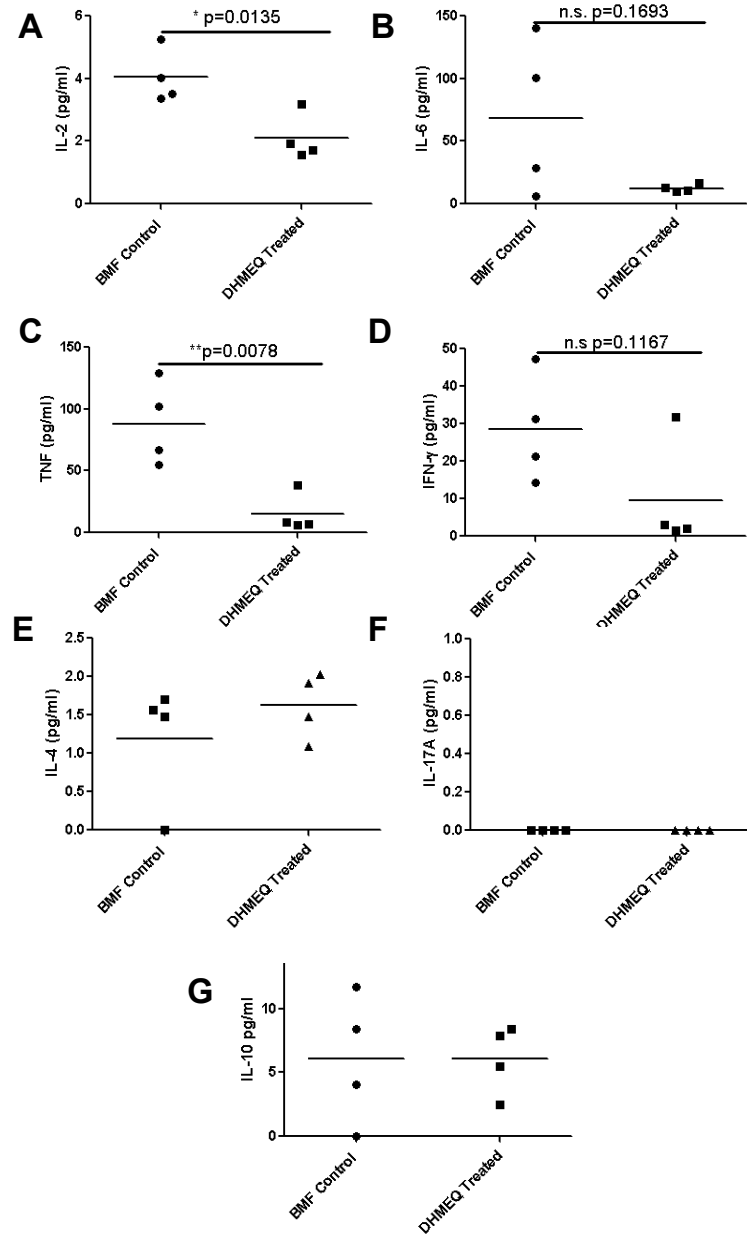


Figure 3.3 Inhibiting NF- κ B nuclear localization decreases Th1 associated cytokine concentrations in the plasma of AA mice

AA mice treated with DMSO (BMF Control) or treated with DHMEQ were induced and circulating cytokines were assessed using the Cytometric Bead Array Multiplex Kit (BD Biosciences). Cytokines assessed: (A) IL-2, (B) IL-6, (C) TNF, (D) IFN γ , (E) IL-4, (F) IL-17A, and (G) IL-10. n=4 in each group. *p<0.05, **p<0.01, ***p<0.001; unpaired student t test.

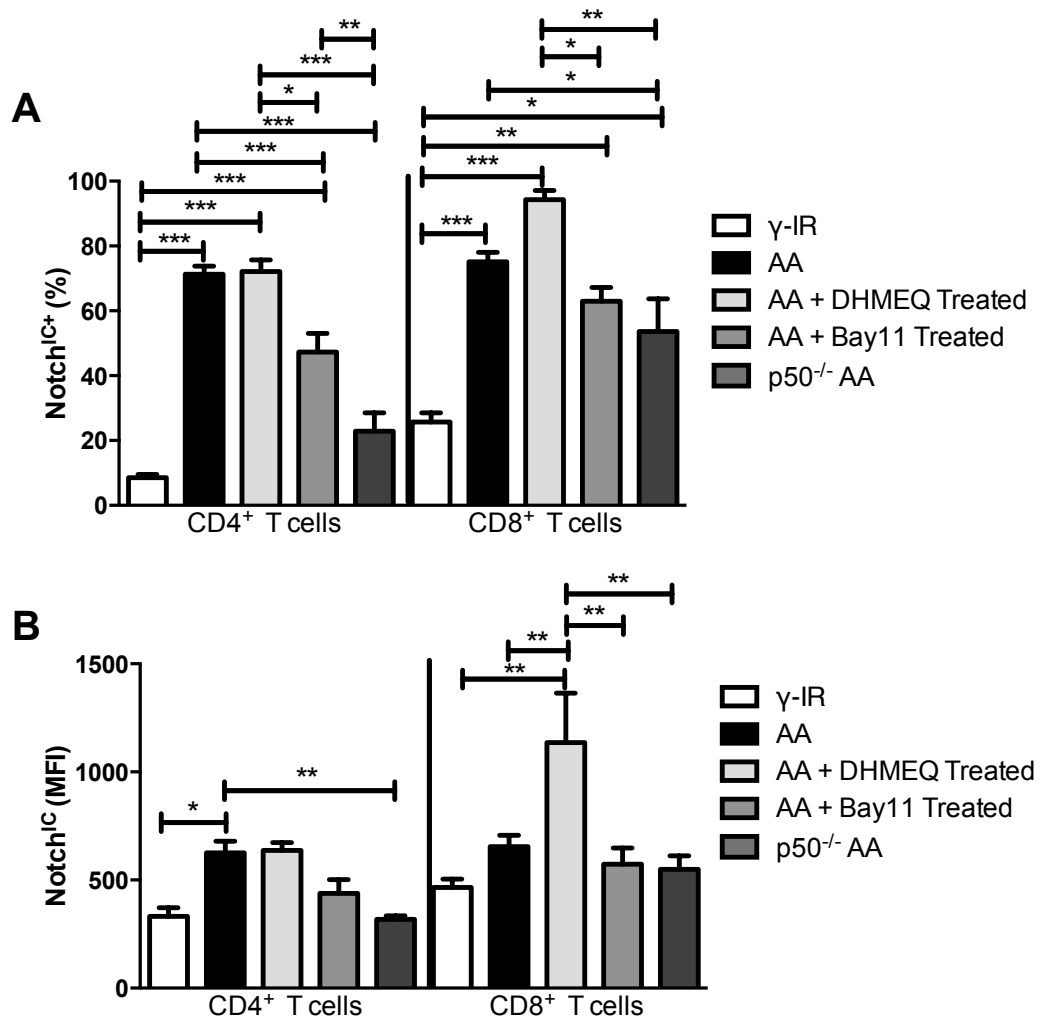


Figure 3.4 Intracellular Notch1 is reduced in the bone marrow bone marrow infiltrating T cells when NF-κB signaling is targeted

Notch1^{IC} were analyzed using flow cytometric methods in CD4⁺ or CD8⁺ T cells from the bone marrow of mice induced with AA. (A) Percentage of Notch1^{IC} and (B) Median fluorescent intensity (MFI) were determined. n=3-15 animals. Data represents the mean plus SEM analyzed with One Way ANOVA and Tukey's post test. *p<0.05, **p<0.01, ***p<0.001.

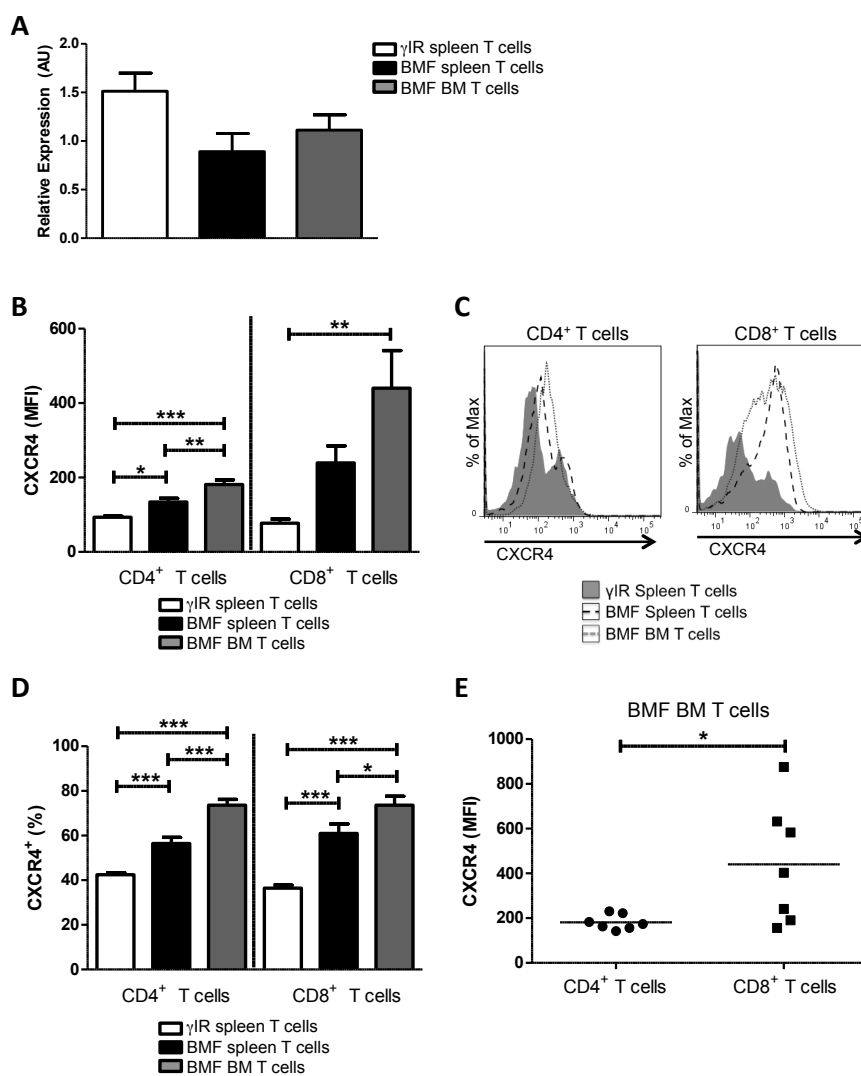


Figure 3.5 CXCR4 protein expression is upregulated on T cells in mice with Aplastic Anemia

Mice were induced with AA and T cells were isolated from the bone marrow and spleen. (A) Relative expression of *cxc4* transcript in spleen and bone marrow T cells from diseased mice was determined by qPCR and compared to expression in spleen T cells from γ IR-treated mice; n=8. (B) Median fluorescence intensity (MFI) of CXCR4 on spleen and bone marrow T cells from diseased animals was determined by flow cytometry; n=8. (C) Representative comparative histogram of CXCR4 MFI of spleen and bone marrow T cells in diseased mice. (D) Percentage of CXCR4 expressing T cells as assessed by flow cytometry in T cells from AA mice. (E) MFI of CXCR4 expression on T cells from the bone marrow of AA mice. Data represent the mean plus SEM and were analyzed by one way ANOVA plus Tukey post test (A,B, and D), or by unpaired student *t* test (E). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

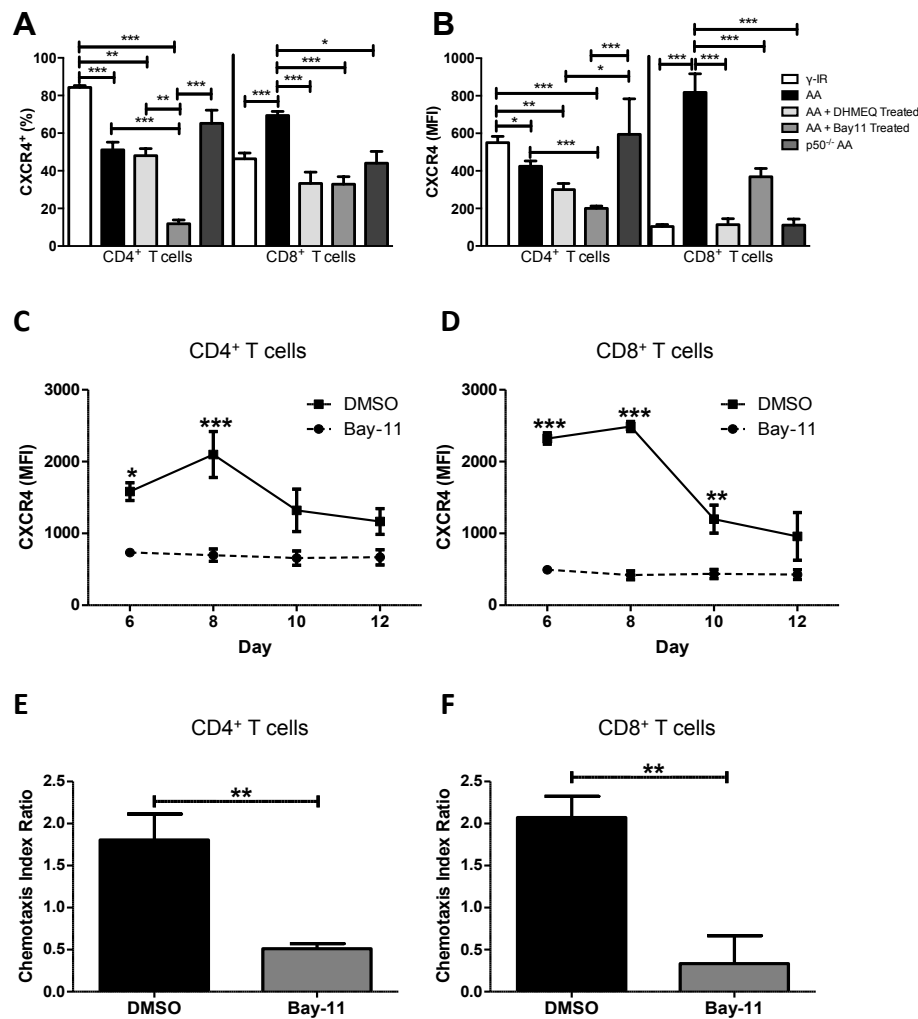


Figure 3.6 Blocking NF-κB decreases CXCR4 expression in T cells and abrogates their chemotaxis

Mice were induced with AA and treated with NF-κB inhibitors as described above. The (A) percentage of CXCR4 T cells and the (B) MFI of CXCR4 on T cells resident in the bone marrow was analyzed by flow cytometry, n=8. Bulk splenocytes from C57BL/6 mice were treated with DMSO or Bay11 (1 mM) and co-cultured with bone marrow-derived DCs from F1 hybrids for 12 days. CXCR4 expression was assessed using flow cytometry in (C) CD4⁺ and (D) CD8⁺ T cells; n=3. T cells isolated on day 8 from co-cultures (as above) were seeded into a chemotaxis assay with 0 ng/ml or 100 ng/ml of SDF-1 was coated onto lower chamber wells. The chemotaxis index ratio was calculated: (number of cells present in the 100 ng/ml SDF-1 chamber/number of cells present in the 0 ng/ml SDF-1 chamber) for DMSO- or Bay11-treated (E) CD4⁺ and (F) CD8⁺ T cells; n=3. Data are the mean plus SEM and were analyzed using Two Way ANOVA with Bonferroni post test (A,B) or two-tailed unpaired student *t* test (C,D). *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.

CHAPTER 4

MICRORNA-155 DRIVES THE PROGRESSION OF APLASTIC ANEMIA THROUGH REGULATION OF PD-L1

4.1 Introduction

MicroRNA-155 (miR-155) is a non-coding RNA in an exon that is transcribed from the B-cell Integration Cluster (BIC) gene located on chromosome 21 in humans and chromosome 16 in the mouse genome (Tam, 2001). This region of BIC is conserved across human, mouse and chicken genomes and is highly expressed in lymphoid organs across species, implying an evolutionarily conserved function (Lagos-Quintana et al., 2002). miR-155 signaling has been shown to play important roles throughout the immune system, including roles in hematopoiesis, innate immunity, neoplasia and viral infections (Bhela et al., 2014; Chen et al., 2014; Dudda et al., 2013; Jiang et al., 2014; O'Connell et al., 2008; Romania et al., 2008). Additionally, miR-155 has been implicated in the development of inflammation and other adaptive immune responses (Lodish et al., 2008; O'Connell et al., 2008). For example, miR-155 has been shown to play an important role in T and B cell responses, and miR-155 levels increase upon their activation (Thai et al., 2007). In B cells, miR-155 is important for the formation of germinal centers, class switching of antibodies, and somatic hypermutation (Dorsett et al., 2008; Rodriguez et al., 2007; Thai et al., 2007; Vigorito et al., 2007).

In T cells, miR-155 has been shown to play an important role in influencing the differentiation of naïve T cells into different helper subtypes. T cells deficient in

BIC/miR-155 skew towards a Th2 phenotype through their inability to produce IFN γ and IL-12 (Rodriguez et al., 2007). miR-155 deficiency has also been shown to reduce the number of Tregs in the periphery and thymus of mice, but it does not reduce their suppressive capacity (Lu et al., 2009). Also, miR-155^{-/-} cells produce less IL-17a, and miR-155^{-/-} mice do not develop EAE (O'Connell et al., 2010). Additionally, patients with rheumatoid arthritis (RA) have higher levels of miR-155 in their synovial fluid than patients with osteoarthritis (Spoerl et al., 2013). Also in RA patients, miR-155 has been shown to target CTLA-4, a negative regulator of T cell activation (Spoerl et al., 2013). These data suggest that miR-155 could be acting to enhance T cell activation, and the dysregulation of miR-155 could cause aberrant T cell signaling in autoimmune diseases through faulty regulatory mechanisms.

Because miR-155 plays a role in Th1 differentiation and autoimmune disease, we sought to see if miR-155 plays a role in driving Aplastic Anemia. Using our AA mouse model and miR-155^{-/-} mice as the source of our donor splenocytes, we found that miR-155 was necessary for AA pathogenesis. Also, we found that miR-155 expression is decreases Notch1 activation, and miR-155 targets PD-L1 expression in T cells causing a decrease of iTreg cells in our AA mice.

4.2 Results

4.2.1 miR-155 expression is increased when mice are induced with Aplastic Anemia

Because AA is driven by Th1 cells and miR-155 has been shown to play a role in Th1 differentiation, we hypothesized that miR-155 would be highly expressed in our mouse model of AA. To examine the levels of miR-155, AA was induced in our mouse model as described above. Seventeen days after disease induction, we harvested bone marrow from recipient mice and quantified the miR-155 expression using qRT-PCR. Compared to irradiated controls, AA mice had significantly more miR-155 expressed in the bone marrow (Figure 4.1A). In the spleen, T cells from irradiated controls and spleen cells from AA mice were also analyzed for miR-155 expression. Like in the bone marrow, T cells from mice induced with AA had significantly higher amounts of miR-155 compared to the irradiation controls (Figure 4.1B). We next took peripheral blood samples from AA patients without prior IST treatment and compared the miR-155 expression to T cells from healthy donors. While there was no significant difference in miR-155 expression between the two groups, 2 out of 3 patients samples had increased miR-155 expression compared to the mean of healthy donor samples (Figure 4.1C). These data confirm that miR-155 levels are increased in our mouse model of AA.

4.2.2 miR-155 is necessary for the development of Aplastic Anemia

Because miR-155 plays an important role in autoimmune diseases with a strong Th1 component such as EAE and RA, we wanted to see if miR-155 was necessary to drive AA (O'Connell et al., 2010; Spoerl et al., 2013). To achieve this goal, we used splenocytes from WT or miR-155^{-/-} mice (B6.Cg-Mir155^{tm1.1Rsky}/J; Jackson Laboratories) and induced AA as described above. AA mice induced with miR-155^{-/-} splenocytes had increased bone marrow cellularity as assessed using trypan blue exclusion (Figure 4.2A) and increased white blood cells (Figure 4.2B) and red blood cells (Figure 4.2C) compared to WT BMF controls confirming that miR-155 is required for the development of AA symptoms in our mouse model.

Next, we wanted to see if miR-155 was needed for the autopathogenic T cell response in our model. When we examined T cell infiltration into the bone marrow using flow cytometry, miR-155^{-/-} AA mice had a significantly reduced level of both CD4⁺ (Figure 4.2D) and CD8⁺ (Figure 4.2E) T cells present in the bone marrow, and the T cell levels were comparable to irradiation controls. Finally, we induced AA mice with WT splenocytes or miR-155-deficient splenocytes and performed a survival study. We found that while WT mice succumb to disease at a mean day of 21 post induction, miR-155^{-/-} mice survived indefinitely, and this difference was significant (Figure 4.2F; p<0.01). These data suggest that miR-155 is necessary in driving the pathogenesis of Aplastic Anemia.

4.2.3 AA mice induced with miR-155-deficient cells express less Notch1^{IC} in the T cell compartment.

Notch signaling has been shown to drive the pathogenesis of Aplastic Anemia (Roderick et al., 2013). Therefore, we wanted to see if deleting miR-155 in our donor cells decreased Notch1^{IC} in the infiltrating T cells in our mouse model. To answer this question, we induced our AA mice with WT or miR-155^{-/-} cells and on day 17 post disease induction, we examined the Notch1^{IC} expression using flow cytometry. In the bone marrow of mice induced with miR-155^{-/-} splenocytes, the Notch1^{IC} expression is significantly decreased in the CD8⁺ T cells and are present in these cells at levels similar to irradiation controls (Figure 4.3B). While not significant, Notch1^{IC} is reduced in CD4⁺ T cells infiltrating the bone marrow (Figure 4.3A). In the spleen of AA induced mice, Notch1^{IC} expression in both CD4⁺ and CD8⁺ T cells is significantly reduced compared to the WT BMF induced mice, and the level of Notch1^{IC} in these cells is also similar to irradiation controls (Figure 4.3, C and D). These data suggest that miR-155 is needed for activation of Notch1 in our mouse model of AA, and this could be one mechanism of how miR-155 is driving the pathogenesis of T cells in the disease.

4.2.4 Notch1^{IC} expression is reduced in mir-155-deficient CD4⁺ T cells compared to WT CD4⁺ T cells

To further understand the temporal regulation that miR-155 may have on Notch1 activation, we use MLR cultures as a way to simulate activation of CD4⁺ T cells in our

mouse model of AA. We used BMDC from an F1 hybrid donor and cultured them with CD4⁺ T cells that were isolated from WT or miR-155^{-/-} spleens. We cultured these cells for 8 days and examined Notch1^{IC} expression using flow cytometry. Both WT and miR-155-deficient cultures have the same percentage of Notch1^{IC} positive CD4⁺ T cells (Figure 4.4A) over the entire 8 day cultures. However, in WT CD4⁺ T cells, Notch1^{IC} expression on a per cell basis (MFI) peaks on day 4 and drops back to initial levels on day 6 and 8. In miR-155^{-/-} CD4⁺ T cells, however, Notch1^{IC} levels never peak on day 4, and the day 4 Notch1^{IC} expression is significantly decreased compared to WT CD4⁺ T cells (Figure 4.4B).

To see if miR-155 is upstream of Notch activation, or if Notch activation may also be important in driving miR-155 expression, we set up MLR cultures with F1 derived BMDC and WT CD4⁺ T cells as described above and treated the CD4⁺ T cells with DMSO or GSI 30 minutes prior to plating. We observed that GSI significantly decreased miR-155 expression over the 8 day culture as compared to cultures where CD4⁺ T cells were treated with DMSO (Figure 4.4C). The kinetics of activation, however seemed to be similar between the two cultures. While both cultures have a peak of miR-155 expression on day 4, GSI treated CD4⁺ T cells do not reach the same levels of miR-155 as DMSO treated cells. These data suggest that after T cell activation, both miR-155 and Notch activation regulate each other's expression.

4.2.5 PD-L1 expression is significantly higher in miR-155 deficient CD4⁺ T cells than WT CD4⁺ T cells

Next, we wanted to identify some potential targets of miR-155 that could be important in AA pathogenesis. When miR-155 was put into the microRNA target predictor, TargetScan, one potential target was PD-L1. Two sites in the human 3' UTR of PD-L1 (gene name *CD274*) had 7 sites of perfect complementarity to the miR-155 seed sequence (Figure 4.5.A). PD-L1 can block T cell proliferation on corresponding T cells by engaging its receptor, programmed death ligand 1, or it can also inhibit T cell stimulation intrinsically by binding the CD80 molecule (B7-1) on APCs and blocking the engagement of CD28 with CD80 (Butte et al., 2007). Therefore, we decided to look at the expression of PD-L1 in CD4⁺ T cell MLR cultures using WT CD4⁺ T cells or miR-155-deficient T cells. In the first 2 days of the MLR culture there was no difference in PD-L1 expression between WT or miR-155-deficient CD4⁺ T cells. However, when cultures were analyzed on day 8 of culture, PD-L1 was significantly increased in the miR-155-deficient CD4⁺ T cells, both in percentage of cells expressing PD-L1 and expression on a per cell basis (Figure 4.5, B and C). We also looked at the expression of one receptor for PD-L1 signaling, PD-1. We found that PD-1 expression is significantly decreased in miR-155-deficient CD4⁺ T cells, however the percentage of cells expressing PD-1 is not decreased. (Figure 4.5, C and D). This would suggest that miR-155 regulates expression of PD-L1 in CD4⁺ cells, and this regulation may also change the expression of PD-1 on CD4⁺ T cells.

4.2.6 AA mice induced with miR-155^{-/-} splenocytes have a higher absolute number of iTregs in the spleen and bone marrow compared to WT AA mice

PD-L1 engagement of its receptors on naïve T cells promotes iTreg development by inhibiting mTOR/Akt signaling (Francisco et al., 2009). Additionally, PD-L1:PD-1 signaling can be used in a humanized GVHD model to convert human Th1 cells into iTregs *in vivo* (Amarnath et al., 2011). Therefore, we wanted to see if AA mice induced with miR-155-deficient cells had an increased number of iTreg cells. To do this, we induced mice with AA as described above and used flow cytometry to assess the iTreg population (CD4⁺CD25⁺FoxP3⁺) in the bone marrow and spleen. When we looked at the percentages of iTregs, there was no difference between WT and miR-155^{-/-} induced AA mice in the bone marrow (Figure 4.6A) or the spleen (Figure 4.6C). However, there was a significant increase of iTreg cells in miR-155^{-/-} AA mice when we examined the absolute number of iTreg cells. AA mice induced with miR-155 deficient cells had significantly higher absolute numbers of iTreg cells compared to WT AA mice in both the bone marrow (Figure 4.6B) and the spleen (Figure 4.6D). These results show that miR-155 deficient AA mice have higher numbers of iTreg cells which could be due to the increased PD-L1 expression on the CD4⁺ T cells.

4.3 Discussion

Here we show that miR-155 levels are elevated in mice induced with Aplastic Anemia, and miR-155 is necessary for the development of immunopathogenic T cells

that drive disease symptoms. miR-155 can decrease the levels of activated Notch1^{IC} both *in vitro* after T cell stimulation and in our mouse model of disease, and inhibiting the activation of Notch using GSI decreases miR-155 levels in CD4⁺ T cells. We have identified PD-L1 as a potential target for miR-155 in CD4⁺ T cells; when miR-155 is genetically deleted in these cells, PD-L1 expression is increased. In our mouse model, miR-155-deficient AA mice have an increased number of iTreg cells in both the spleen and bone marrow, perhaps a result of an increase of PD-L1 expression.

miR-155 expression has been shown to upregulated in human diseases that have a strong Th1 component, such as multiple sclerosis, rheumatoid arthritis, and atopic dermatitis when samples are compared to healthy donors (Junker et al., 2009; Sonkoly et al., 2010; Stanczyk et al., 2008). Therefore, it was not surprising that we had elevated levels of miR-155 expressed in the bone marrow and spleen of our AA mice. However, we expected to see a larger difference in levels of miR-155 between our AA patients and healthy controls. The fact that we didn't see an increase in patient samples is probably due to our limited sample size, and our lack of knowledge about the patient's disease status. It has been shown previously that expression of markers of Th1 cells, such as T-BET often correlate with disease severity and an inability to know the disease severity of our patient samples could skew our results (Solomou et al., 2006). Also, miR-155 have been found in exosomes of human saliva and serum (Bala et al., 2012). We may find a higher expression of miR-155 in different types of patient samples, beyond the peripheral blood samples we have been analyzing. Moving forward, having a larger patient cohort with different types of samples may be able to show if there is a true difference in miR-155 expression in AA patients.

Mice deficient in miR-155 do not develop EAE or collagen-induced arthritis, two autoimmune disease with strong Th1 elements (Bluml et al., 2011; Hu et al., 2013; O'Connell et al., 2010; Yao et al., 2012). We were pleased to see that miR-155 was also necessary for AA pathogenesis in our model. Not only were symptoms ameliorated and T cells that infiltration into the bone marrow ablated, mice induced with miR-155-deficient splenocytes were able to survive indefinitely. This evidence points to a T cell intrinsic role that miR-155 is playing in AA. miR-155 has been shown to mediate the inflammatory affects of CD4⁺ cells in a mouse model of EAE, and miR-155 deficient CD8⁺ cells were ineffective at controlling tumor growth (Dudda et al., 2013; O'Connell et al., 2010). Further investigation into the role of miR-155 in both CD4⁺ T cells and CD8⁺ T cells in our model would be interesting.

We observed that miR-155 deficiency results in a decrease of Notch1 activation in T cells, both *in vitro* and *in vivo*. Also, in MLR cultures, using GSI to inhibit Notch activation reduces the miR-155 expression in CD4⁺ T cells. Although neither of these experiments address direct regulation, it does seem that both of these pathways work in tandem after T cell activation. Currently, there has been no studies that investigate the regulation of miR-155 by Notch, or visa versa. However, both Notch1 activation and miR-155 expression have been shown to be regulated by the NF-κB pathway. While there have been studies providing a link between the Notch1 and NF-κB signaling pathway in T cells (Shin et al., 2006; Shin et al., 2014), the studies linking miR-155 expression and NF-κB signaling has been done only in B cells and cancer cell lines (Ma et al., 2011). Therefore, it would be interesting to further understand if Notch and miR-155 work

together to regulate signaling downstream of the TCR and if they interact with NF- κ B signaling to do so.

We next aimed to find a direct mRNA target of miR-155 repression that could be playing a role in Aplastic Anemia. Upon searching the microRNA target database, Target Scan, we were able to identify *CD274* or PD-L1 as a potential target. Since PD-L1 signaling acts to suppress aberrant T cell activation through interaction with PD-1 or CD80 (Butte et al., 2007), we reasoned that high miR-155 expression in AA would lead to a decrease in PD-L1 expression, thus favoring pathogenic T cell activation. We were able to show that miR-155-deficient CD4⁺ T cells have increased levels of PD-L1 compared to WT CD4⁺ T cells. More analysis has to be done into if PD-L1 is a direct target of miR-155, i.e. does miR-155 directly bind to PD-L1 mRNA on its 3' UTR regions to block translation. However, a study pulling down complexes of miR-155 and Argonaute in CD4⁺ T cells was able to identify PD-L1 mRNA present in these complexes (Loeb et al., 2012).

Since PD-L1 engagement of its receptors on naïve T cells promotes iTreg development, and PD-L1:PD-1 signaling can be used in a human-into-mouse GVHD model to convert human Th1 cells into iTregs *in vivo* (Amarnath et al., 2011; Francisco et al., 2009), we hypothesized that miR-155-deficient AA mice could have higher iTreg populations in their bone marrow and spleen compared to WT AA mice. While we did not see a difference in the percentage of iTreg cells, there was a significant difference in the absolute numbers of iTregs in the bone marrow and spleen of miR-155-deficient AA mice. To show that higher PD-L1 expression is necessary for iTreg development that in turn could be ameliorating disease in AA mice, blocking PD-L1 expression in miR-155-

deficient AA mice and assessing both iTreg populations and disease symptoms should be performed. Also, further exploration into the kinetics of PD-L1 expression in AA mice would be helpful in understanding if PD-L1 signaling is turning already differentiated Th1 cells into iTreg cells in our model, or if miR-155 deficiency is influencing naïve T cells to differentiate to iTreg cells instead of Th1 cells. All together, this cumulative evidence shows that miR-155 is important in AA pathogenesis and is functioning, at least in part, through a repression of PD-L1 expression.

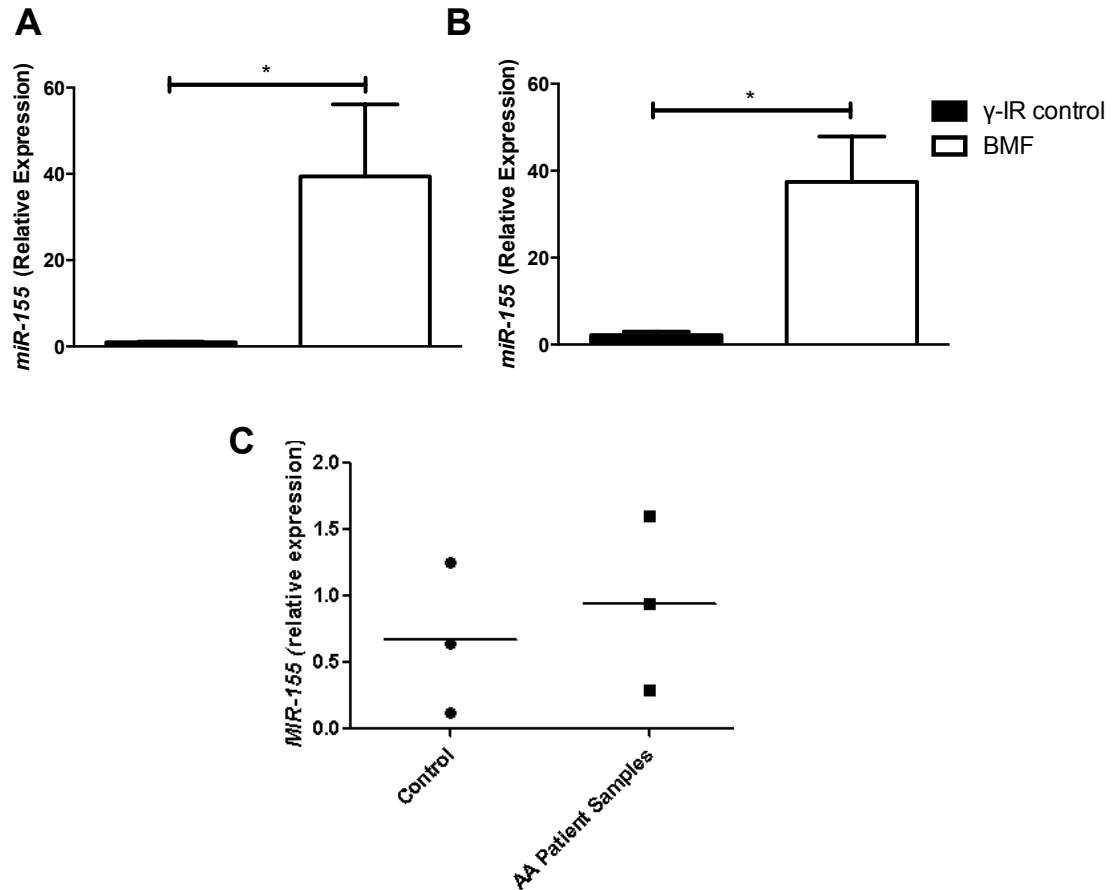


Figure 4.1 miR-155 expression in mice with Aplastic Anemia is higher compared to irradiated controls

Mice sub-lethally irradiated only (IR control) or mice induced with AA (BMF) were harvested on day 17 post disease induction. (A) Whole bone marrow from IR controls and BMF mice or (B) CD4⁺ and CD8⁺ positively selected cells from IR controls and whole splenocytes from BMF mice were analyzed for miR-155 expression with *sno202* used as a reference gene. n=7; Mean + SEM of three independent experiments. (C) CD4⁺ and CD8⁺ cells were positively selected from human PBMC (Control) and whole AA peripheral blood samples (AA Patient Samples) were analyzed for miR-155 using qRT-PCR with *RPS9* used as a reference gene. n=3; line represents mean of two independent experiments. *p<0.05, **p<0.01, ***p<0.001; unpaired student t test.

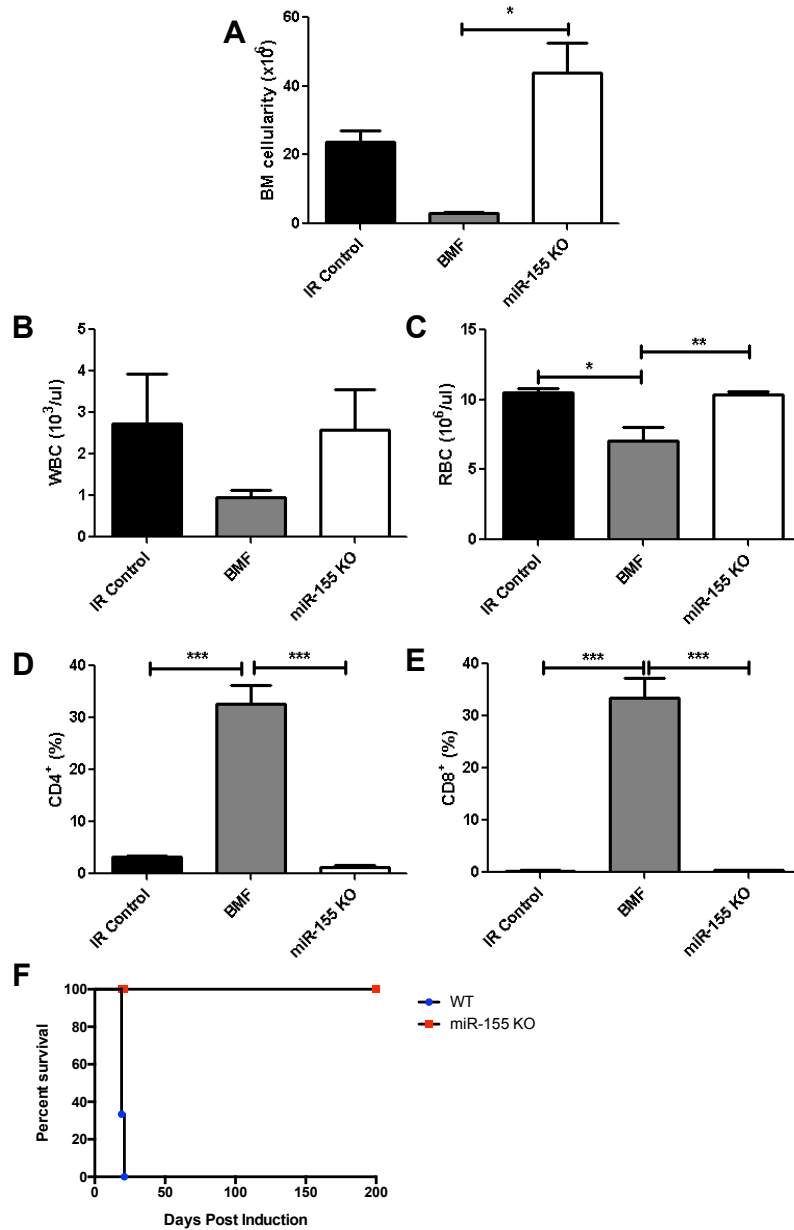


Figure 4.2 Inducing BMF mice with miR-155-deficient splenocytes ameliorates disease symptoms

Mice were induced with AA using WT or miR-155-deficient splenocytes. On day 17 post-induction, mice were harvested and (A) total bone marrow cellularity was determined using trypan blue exclusion. Circulating (B) white blood cells (WBC) and (C) red blood cells (RBC) were measured. Percentages of (D) CD4⁺ and (E) CD8⁺ T cells infiltrating the bone marrow were determined using flow cytometry. (E) Kaplan–Meier survival estimates of AA mice induced with disease and treated with WT (n=4) or miR-155-deficient splenocytes (n=7; p<0.01) Data are the mean plus SEM and analyzed using one way ANOVA plus Tukey post test or log rank test for survival estimates *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001

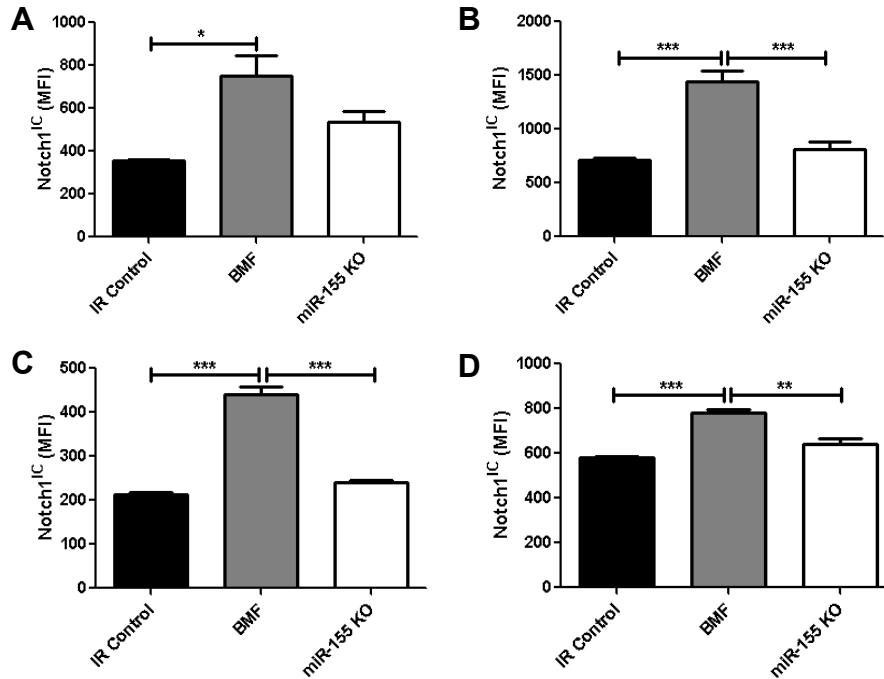


Figure 4.3 miR-155^{-/-} AA mice express less Notch1^{IC} in the T cells in the bone marrow and spleen

Notch1^{IC} was analyzed using flow cytometry in mice induced with AA using WT (BMF) or miR-155-deficient splenocytes (miR-155 KO), or irradiated only (IR control). Data represents the Median Fluorescent Intensity (MFI) of Notch1^{IC} in the (A) CD4⁺ gate (B) and CD8⁺ gate of the bone marrow of induced mice and the MFI of Notch1^{IC} in the (C) CD4⁺ gate (D) and CD8⁺ gate of the spleen. n=3-7; Data represents the mean + SEM of three independent experiments; * p < 0.05, ** p < 0.01, *** p < 0.001; One-Way ANOVA test with Bonferroni post test.

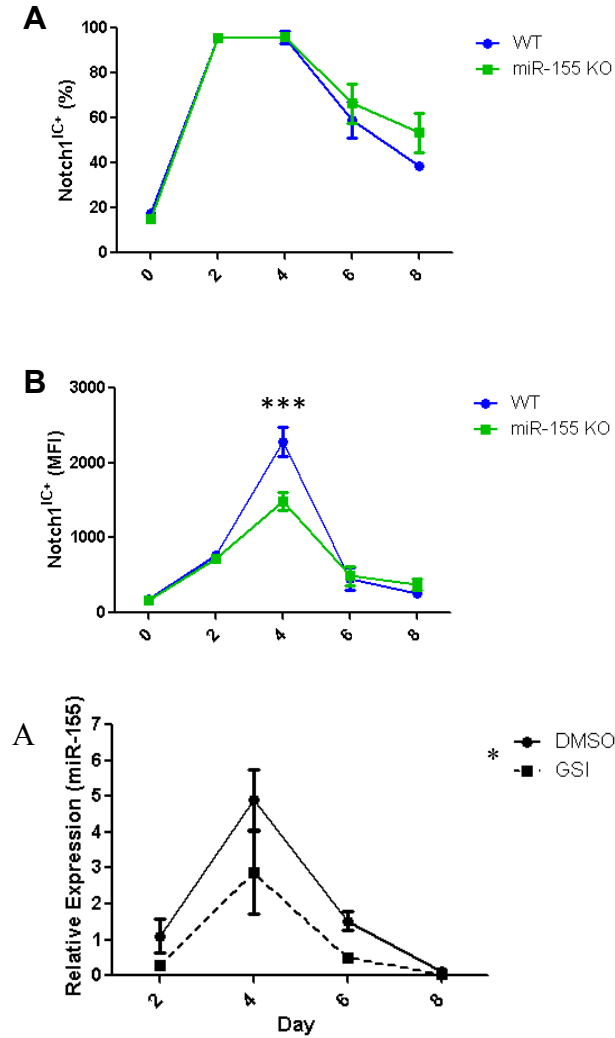


Figure 4.4 The expression of intracellular Notch1 in miR-155-deficient CD4⁺ T cells is reduced compared to WT CD4⁺ cells

A mixed lymphocyte reaction (MLR) culture was set up with F1 derived BMDC cells mixed with positively selected CD4⁺ C57BL/6 WT or miR-155^{-/-} cultured for 8 days. Notch1^{IC} expression was analyzed using flow cytometry. (A) Percentage of CD4⁺ cells expressing Notch1^{IC} and (B) MFI of Notch1^{IC} expression in the CD4 gate are shown. (C) WT CD4⁺ cells were pretreated with DMSO or 50 μ M of GSI for 30 minutes and mixed with F1 derived BMDC in a MLR culture. qRT-PCR was done to determine miR-155 expression using *sno202* as a reference gene. All expression values were calculated relative to naïve CD4⁺ WT T cells. Data are the mean \pm SEM of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; Two Way ANOVA and Bonferroni post test.

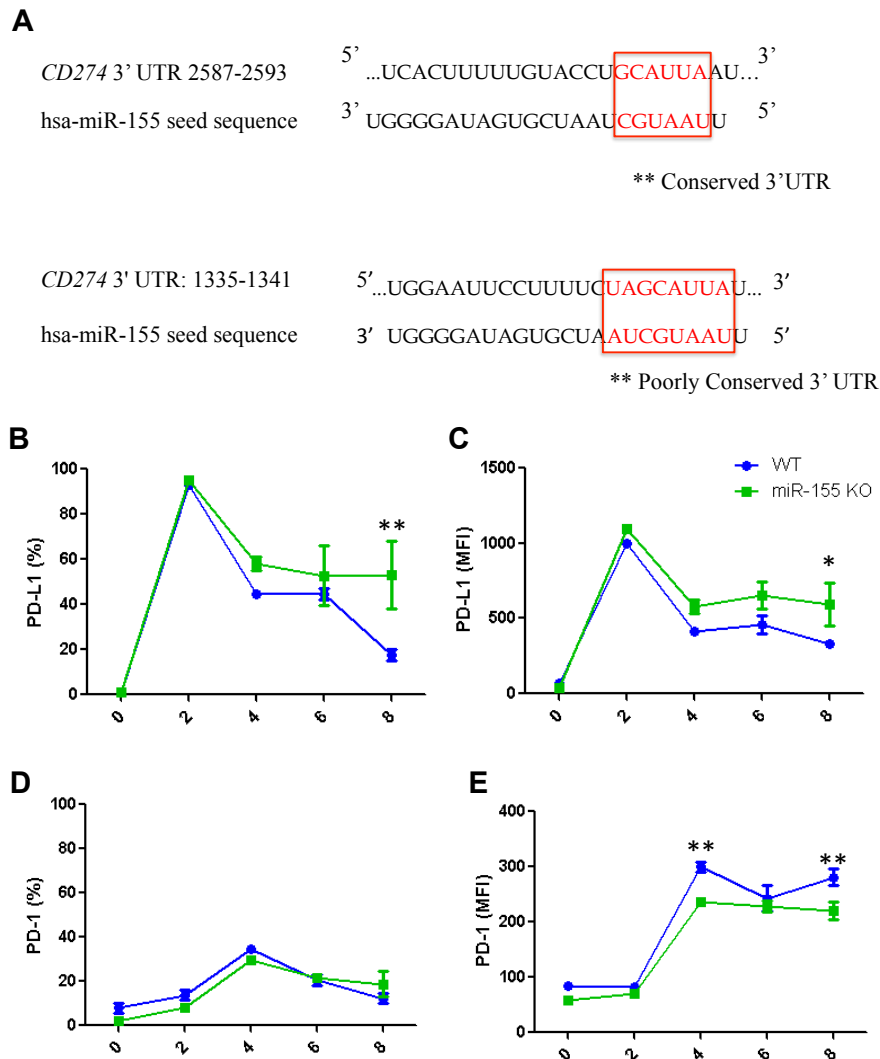


Figure 4.5 PD-L1 expression is increased in miR-155-deficient CD4⁺ cells compared to WT CD4⁺ cells

(A) A schematic of two predicted miR-155 target sequences on the 3' UTR PD-L1 mRNA. Red boxes and red text indicate the complementary sequences. The top miR-155 seed sequence is conserved amongst human and mouse (among others), while the bottom is poorly conserved. These sequences were predicted using the online software, TargetScan (targetscan.org). (B-E) MLR cultures using WT and miR-155^{-/-} CD4⁺ cells were analyzed by flow cytometry. (A) Percent of PD-L1 expression on CD4⁺ cells, (B) PD-L1 MFI of CD4⁺ cells, (C) percentage of CD4⁺ cells that express PD-1, and (D) the MFI of PD-1 expression on CD4⁺ cells. Data are the mean +/- SEM of three independent experiments. *p<0.05, **p<0.01, ***p<0.001; Two Way ANOVA and Bonferroni post test.

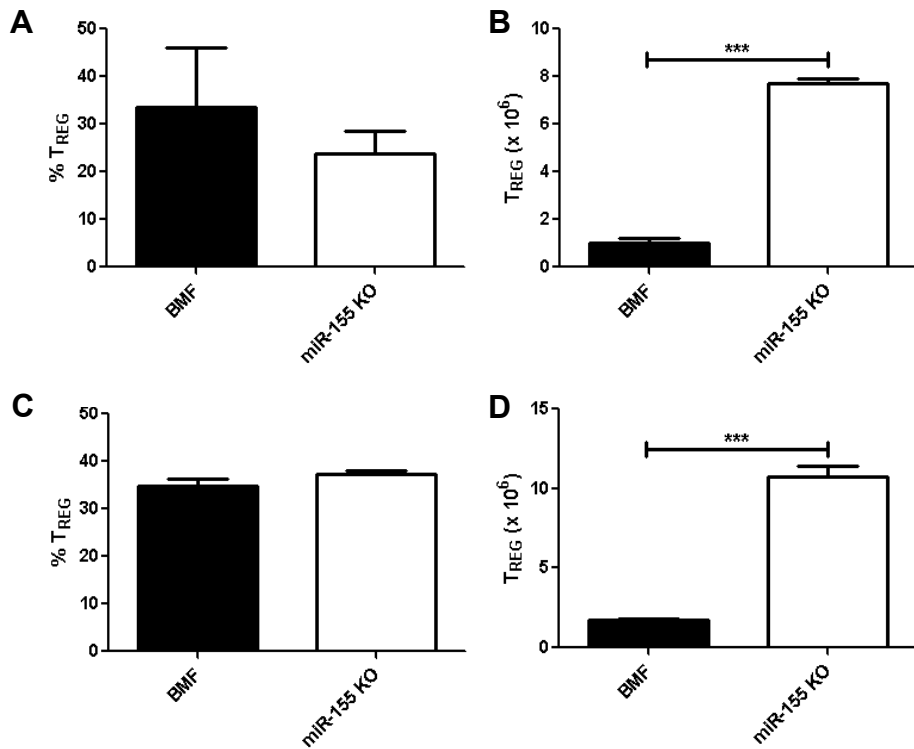


Figure 4.6 miR-155-deficient AA mice have a more iTreg cells in the bone marrow and spleen than WT AA mice

Percentage of iTreg expression was analyzed in mice were induced with AA with WT (BMF) or miR-155^{-/-} (miR-155 KO) splenocytes. iTreg cells were analyzed by FACS analysis on day 17 post induction and gated as CD4⁺CD25⁺FoxP3⁺ cells. Percentage of iTreg cells were determined in (A) bone marrow and (C) spleens of induced mice. Absolute numbers of iTregs were enumerated by multiplying the percentage of iTreg cells by total cell number of (B) bone marrow and (D) spleen cells. n=3-4. Data are the mean + SEM of three independent experiments. *p<0.05, **p<0.01, ***p<0.001; unpaired student t test.

CHAPTER 5

CONCLUDING REMARKS

Aplastic Anemia is a rare bone marrow failure disease that is characterized by bone marrow hypoplasia and severe pancytopenia. Fortunately, there is a low incidence of disease in the United States (less than 6 cases per million population) and the estimated five year survival rate of those who respond to IST and BMT therapies are 75% and 90%, respectively (Scheinberg, 2012). However, there is a large cohort of patients who do not respond to current treatments and eventually succumb to disease.

Understanding the cellular mechanisms that drive AA has long been a focus of research in the hopes of developing new therapies. Unfortunately, samples from patients with the disease are rare. Earlier work focused on discovering the epitopes of serum antibodies and the activating antigen from patient CD8⁺ T cell clones, but researchers were never able to pinpoint inciting antigens that caused the vast immune destruction to the bone marrow in a large cohort of patients (Feng et al., 2004; Goto et al., 2013). It is possible that inciting antigen in each patient is as diverse as the etiologies of the disease. However, this could cause difficulty for basic researchers trying to study the disease.

Because an inciting antigen has never been found, the field has had to rely on MHC haplotype mismatch graft-versus-host disease models for research purposes, and our lab has been able to develop a mouse model that closely resembles human disease (Roderick et al., 2013). Using this model, we investigated the involvement of three important signaling pathways that have been previously implicated in T cell activation and

differentiation in Aplastic Anemia. First, we are able to show that active PKC θ (pPKC θ) is highly expressed in our mouse model, and we were able to observe the same high expression when we looked at pPKC θ levels in patient peripheral blood samples. Previous studies from our lab have also shown that using PKC θ -deficient donor cells in our mouse model or treating AA mice with rottlerin, a PKC θ activity inhibitor, ameliorates disease symptoms (Roderick, unpublished). We were additionally able to show that PKC θ acts upstream of Notch1 to drive T cell stimulation and IFN γ production. Since Notch1 signaling is important in AA pathogenesis (Roderick et al., 2013), we can conclude from this evidence that PKC θ plays an important role in driving Aplastic Anemia and works upstream of Notch1 to drive disease.

We then investigated the role that NF- κ B plays in AA pathogenesis. This work shows that NF- κ B drives AA through multiple mechanisms. In our model, the p50 subunit of NF- κ B was needed for the expansion of donor T cells in our model. While other NF- κ B subunits are also important for mediating donor T cell expansion, we did not have the genetic tools to ask those questions; however, it would be interesting to discern which subunits were important in T cell expansion during disease. We were also able to show that NF- κ B signaling regulates CXCR4 expression on T cells in our mouse model and that CXCR4 drives T cells into the bone marrow to mediate destruction. Although NF- κ B has been shown to regulate CXCR4 expression in cancer models (Helbig et al., 2003), our work is the first to describe direct CXCR4 regulation by NF- κ B in T cells and in Aplastic Anemia.

We finally examined the role of the microRNA, miR-155 in AA. miR-155 is a microRNA that has been widely implicated in the development and maturation of the

immune system, and plays a large role in both T cell activation and differentiation. miR-155 has been shown to be highly expressed in patient samples from many autoimmune diseases, and is necessary for the development of these diseases in mouse model studies. We found that miR-155 is highly expressed in the bone marrow and spleen in our mouse model, and AA mice induced with miR-155-deficient cells do not develop disease. miR-155 regulates Notch1 activation in our AA mice, and although there has been little research linking these two pathways, both miR-155 and Notch1 have been shown to be regulated by NF- κ B signaling. However, *in vitro* studies seem to suggest that not only does miR-155 regulate Notch1 activation, but Notch signaling also regulates miR-155 expression. This could point to the NF- κ B as a linker of the two pathways, since Notch signaling is differentially regulated by NF- κ B at different points post TCR activation (Shin et al., 2006).

We were also able to identify a potential direct target of miR-155 in CD4⁺ T cells. PD-L1 was predicted by the database TargetScan.org as a potential direct target of miR-155 translational repression, and we were able to show that in CD4⁺ cells, PD-L1 is increased when miR-155 is deleted. Further work will have to be done using luciferase assays to see if the 3' UTR region of PD-L1 is indeed a direct translational target of miR-155. Based on a former study where PD-L1 mRNA could be pulled down with Argonaute-miR155 complexes in CD4⁺ T cells, it is a strong possibility that PD-L1 is a direct target of miR-155 (Loeb et al., 2012). Also, It would also be interesting to if disease in miR-155^{-/-} AA mice could be rescued by treating mice with a blocking antibody to PD-L1. This would further confirm that PD-L1 is a target *in vivo*. Also, it would be interesting to see if miR-155 regulation of PD-L1 is limited to CD4⁺ T cells or

if this regulation is found in other cell types.

The three pathways that are investigated in this work are important to T cell stimulation, differentiation, and AA pathogenesis. However, it begs the question: would targeting any of these pathways be a possible therapeutic route for AA? NF- κ B and miR-155 are pathways that are found to have important roles in many cell types, not just those of the hematopoietic origin. Therefore, it is possible that targeting these pathways would not be an improvement on the current IST used for treatment because they would lack specificity to T cells. In fact, they may be more widely suppressive than current treatment. More studies into toxicity and off-target effects must be done before any advancements can be made in these instances.

Targeting the PKC θ pathway, however, could hold more promise. PKC θ expression is fairly limited to a small amount of cell types, and T cells are the only immune cell in which PKC θ is expressed. Therefore, inhibiting this pathway could preserve innate immunity and B cell responses, and in the event that AA patients on PKC θ inhibitors were also infected with a viral or bacterial pathogen, their other immune responses would still be able to fight disease. Additionally, PKC θ seems to be selectively needed only in certain T cell immune responses, further protecting the patient against infectious pathogens. For example, reports suggest that PKC θ is required for Th2 and Th17 responses, but it has been shown to be dispensable for generating both Th1-mediated antiviral and memory T cell responses (Kwon et al., 2012; Marsland et al., 2005; Marsland et al., 2004). Conversely, PKC θ has been shown to play a role in autoimmune diseases with a strong Th1 component, such as experimental autoimmune encephalomyelitis, rheumatoid arthritis, and myosin-induced autoimmune myocarditis

(Healy et al., 2006; Marsland et al., 2007; Salek-Ardakani et al., 2005; Tan et al., 2006). Also, in a mouse model of graft-versus-host disease (GVHD), PKC θ was required to induce GVHD pathology, but not to clear viral pathogens or mediate graft-versus-leukemia processes (Valenzuela et al., 2009). This evidence is extremely important when thinking about treatment for AA. PKC θ is clearly necessary in Th1 associated autoimmune diseases such as AA, however, Th1 responses to intracellular pathogens seems dispensable, making PKC θ inhibitors very attractive for AA. Maybe most importantly, PKC θ inhibiting treatments could be used before BMT or in conjunction with BMT. PKC θ inhibition has been shown to not only block GVHD, but preserve GVL. This would be an improvement on current therapies, which sometimes cause rejection of the BMT. For these reasons, PKC θ inhibiting therapies are perhaps the most compelling in the search for a treatment for refractory and relapsing Aplastic Anemia.

In conclusion, this work investigates multiple pathways that drives the progression of Aplastic Anemia both in our mouse model and in patient samples. While there is still much work to be done to elucidate the full story of how these pathways interact to drive disease, this work presents a strong foundation that can be used in the search for future therapies.

CHAPTER 6

MATERIALS AND METHODS

6.1 Animals

All mouse protocols were approved by the Institutional Animal Care and Use Committee of the University of Massachusetts Amherst. F1 progeny were obtained by crossing BALB/c females with C57BL/6 males, both obtained from the Jackson Laboratory (Bar Harbor, Maine). PKC $\theta^{-/-}$, p50 $^{-/-}$ and miR155 $^{-/-}$ mice were created on a C57BL/6 background and were maintained as homozygous breeding pairs. PKC $\theta^{-/-}$ parental strains were originally received as a gift from Dan Littman, and p50 $^{-/-}$ and miR155 $^{-/-}$ parental strains were obtained from the Jackson Laboratories. Mice between the ages of 7–12 weeks were used in experiments.

6.2 Bone Marrow Failure Induction and Analyses

F1 progeny were conditioned with 3 Gy of total body irradiation using a ^{137}Cs source. 4 to 6 h later, 5×10^7 bulk splenocytes from age- and gender-matched C57BL/6 (WT or knockout) donors were given via i.p. injection. Mice were monitored daily for signs of disease and harvested on day 17 or 31-post disease induction. For survival studies, mice were considered lethally induced on the day they were no longer able to take food or water, at which time they were humanely euthanized. After CO_2

asphyxiation, peripheral blood was obtained via cardiac puncture and sterna were collected for histology. BM cells were recovered from the tibiae and femurs of both legs by flushing the bones with 5% FBS/PBS. Splenocytes were isolated by manipulation through a 40- μ M filter. Red blood cells were lysed in ACK lysis buffer, and the remaining white blood cells were enumerated using Trypan Blue exclusion. White and red cell counts were performed on peripheral blood using a HemaTrue Hematology Analyzer (Heska).

6.3 *In vivo* administration of NF- κ B inhibitors

For NF- κ B inhibition studies, one hour after disease induction mice were treated with 30 mg/kg/day of DHMEQ or 5 mg/kg/every other day of Bay 11-7085 (Calbiochem, hereafter abbreviated as Bay 11) administered via i.p. injection and the treatment was continued until day 16 post-disease induction. Control mice received an equivalent volume of DMSO vehicle. For NF- κ B survival studies, mice were treated with 5 mg/kg/every other day of Bay 11 administered via i.p injection from days 7 to 17 post-disease induction, at which time treatment was discontinued. For survival studies, mice were considered lethally induced on the day they were no longer able to take food or water, at which time they were humanely euthanized.

6.4 Histology

Sterna were harvested on day 17 after BMF induction, fixed in 10% neutral buffered formalin (VWR) overnight, and decalcified in Cal-Rite (Richard Allen Scientific) for 48 hours. Samples were preserved in 70% ethanol at 4°C until they were processed, paraffin-embedded, sectioned, and stained with hematoxylin and eosin.

6.5 T cell isolation and *in vitro* assays

Spleens were isolated and manipulated through a 40- μ M filter (BD Biosciences), and splenocytes were treated with ACK lysis buffer. CD4⁺ and CD8⁺ T cells were then isolated using the anti-mouse CD4 and CD8 magnetic particles (IMag; BD) and separated using the BD IMag system. Cells were plated at $2.25\text{--}3 \times 10^6$ cells/well in 12-well plates precoated with anti-CD3 ϵ and anti-CD28, purified from 145-2c11 and 37N hybridoma cell lines, respectively and cross-linked with anti-Hamster IgG (Sigma). Cells were cultured in a 1:1 ratio of RPMI-1640 and DMEM medium supplemented with 10% FBS (Gibco), 2 mM l-glutamine, 1 mM Na pyruvate, 100 U/ml penicillin, and 100 μ g/ml streptomycin, at 37°C in a humidified atmosphere with 5% CO₂ for the indicated amount of time. Media and supplements were purchased from Lonza unless otherwise specified. In some cases, WT T cells cell were treated with DMSO or 3 μ M of Rottlerin (Sigma) at the time of plating or 24 hours after plating, as specified.

6.6 Patient samples and healthy controls

PBMCs from six patients with severe AA who had not received IST were obtained from the National Marrow Donor Program Research Sample Repository. PBMCs from six healthy donors (STEMCELL Technologies) were included as controls. PBMCs were plated at 10^6 cells/ml in RPMI 1640 medium supplemented with 10% FBS (Gibco), 2 mM l-glutamine, 1 mM Na pyruvate, 0.1 mM nonessential amino acids, 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37°C in a humidified atmosphere with 5% CO₂. Media and supplements were purchased from Lonza unless otherwise specified. Control T cells and AA patient PBMCs were preincubated with DMSO or Rottlerin (3 μ M in DMSO) for 30 min at 37°C before being stimulated with 5 μ g/ml of plate-bound anti-CD3 ϵ (UCHT1) and 2.5 μ g/ml anti-CD28 (clone 37407) for 48 to 72 h. All antibodies were purchased from R&D Systems. IFN γ cytokine from supernatants were determined using a standard ELISA assay (BD Pharmingen).

6.7 Surface and intracellular flow cytometry of murine and human samples

Murine samples were surface stained with PerCP-conjugated anti-CD4 (RM4-5; BD), Pe-Cy7-conjugated anti-CD8a (53-6.7; eBioscience), and APC-conjugated anti-CXCR4 (2B11; eBioscience). For intracellular staining, cells were fixed and permeabilized using the Foxp3 staining buffer set (eBioscience) and stained with PE-conjugated anti-NOTCH1 (mN1A; eBioscience) and PE-conjugated anti-IFN γ (DB-1; BD) according to the manufacturer's protocol. For IFN γ staining, cells were harvested

and cultured in fresh media on anti-CD3 ϵ -coated plates for 5 h in the presence of Brefeldin A (GolgiPlug; BD).

Human samples were surface-stained with APC-conjugated anti-CD4 (RPA-T4) and PeCy7-conjugated anti-CD8 (RPA-T8) antibodies. For intracellular staining, cells were fixed and permeabilized using the Foxp3 staining buffer set (eBioscience) and stained with PE-conjugated anti-NOTCH1 (mN1A). All human antibodies were purchased from eBioscience. Samples were acquired on an LSRII flow cytometer and analyzed using the acquisition software FACSDiva (BD). Analysis of FACS data was performed using FACSDiva or FlowJo (Tree Star) software.

6.8 Validation of phosphorylated PKC θ detection using flow cytometry

To validate flow cytometric analysis of phosphorylated PKC θ , whole cell lysate were made using RIPA buffer (150mM NaCl, 1% IgeCal-CA 360, 0.1% SDS, 50mM Tris, pH-8.0, 0.5% Sodium deoxycholate) and 40 μ g of total protein lysates from DMSO- or Rottlerin-treated, stimulated murine WT CD4 $^{+}$ and CD8 $^{+}$ T cells was resolved on an 8% SDS-PAGE, transferred to nitrocellulose, and probed with an anti-p-PKC θ (Cell Signaling) and anti-actin mAb (AC-40; Sigma-Aldrich) to verify equal loading. The primary antibodies were detected with HRP-conjugated antibody (GE Healthcare) and developed using ECL reagents (Amersham). An aliquot of CD4 $^{+}$ and CD8 $^{+}$ T cells from the same experimental replicate was stained for the surface expression of CD4 or CD8 (described above) and perm/fixed using the BD Cytofix/Cytoperm kit according to manufacturer's instructions. Cells were then incubated with anti-pPKC θ (Cell Signaling)

for 30 minutes, washed, and incubated with Alexa Fluor 488-conjugated anti-rabbit IgG (Cell Signaling) for 30 minutes. Samples were acquired using an LSRII flow cytometer as described above.

6.9 Cytometric bead array

Cytokine levels were determined in plasma using either the Th1/Th2 or Th1/Th2/Th17 cytometric bead array kit (BD) according to the manufacturer's protocol. Sample data were acquired on an LSRII flow cytometer and analyzed using FCAP array software (BD).

6.10 Mixed lymphocyte reaction

To generate BM-derived dendritic cells (BMDC), BM cells from F1 progeny were cultured at 10^6 cells/ml in RPMI 1640 medium supplemented with 10% FBS (Gibco), 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin and 20 ng/ml GM-CSF (R&D systems), and incubated at 37°C with 5% CO₂. On days 2 and 4 post-culture, half of the media was removed and replaced with fresh supplemented media. On day 6 post-culture, non-adherent cells were harvested and cultured in fresh supplemented media for 2 additional days. For the MLR culture, BMDC were co-cultured with bulk splenocytes from age- and gender-matched WT or KO C57BL/6 mice at a 1:10 ratio in a 1:1 mixture of RPMI 1640 and DMEM medium supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 μ g/ml streptomycin in 96-well round-

bottom plates and incubated at 37°C with 7% CO₂ for 12 days. To inhibit NF-κB, 1 μM of Bay 11 or an equivalent volume of DMSO vehicle was added at the time of plating. From days 6 to 12 post-culture, non adherent cells were harvested every other day and analyzed for protein expression by flow cytometry or microRNA expression using qPCR.

6.11 Chemotaxis Assay

On day 8 post-culture, non-adherent cells were harvested from the MLR culture to evaluate their chemotactic response to SDF-1α. Chemotaxis assays were performed in 24-well plate transwell inserts (5 μm polycarbonate membrane, 6.5 mm insert, Corning Costar) where 600 μl of RPMI 10% FBS with or without 100 ng/ml of SDF-1α (R&D systems) were added to the plate wells and 0.5 X 10⁶ cells in 0.1 μl of RPMI 10% FBS were added to the inserts, and incubated at 37°C with 5% CO₂. After 3 hours of incubation, the transwell inserts were carefully removed and the migrated cells were harvested from the wells. The number of CD4⁺ and CD8⁺ T cells was determined by flow cytometry. The results are expressed as chemotactic index which is the number of migrated cells from 100 ng/ml SDF-1α wells divided by the number of migrated cells from 0 ng/ml SDF-1α wells. Media and supplements were obtained from Lonza unless otherwise specified.

6.12 mRNA isolation and quantitative real-time PCR

Total RNA was extracted using the RNAqueous kit (Ambion) according to the manufacturer's protocol. The RNA (1 µg) was reverse transcribed to cDNA using dNTPs (Roche), M-MuLV reverse transcriptase reaction buffer (New England Biolabs, Inc.), oligo-(dT)₁₂₋₁₈ (Invitrogen), RNase inhibitor (Promega), and M-MuLV reverse transcriptase (New England Biolabs, Inc.) on a Mastercycler gradient Thermal Cycler (Eppendorf). Quantitative real-time PCR was performed in duplicate with SYBR Premix Ex Taq (Takara Bio Inc.) using the Stratagene Mx3000P qPCR system (Agilent Technologies). The primer sequences used were: *Cxcr4*: forward, 5'- GAC TGG CAT AGT CGG CAA TG -3'; and reverse, 5'- AGA AGG GGA GTG TGA TGA CAA A -3'; and *Actb*: forward, 5'- GGC TGT ATT CCC CTC CAT CG -3', and reverse, 5'- CCA GTT GGT AAC AAT GCC ATG T -3'. Quantitative real-time PCR conditions were as follows: 95°C for 1 min, 95°C for 25 s, 62°C for 25 s (35 cycles), 95°C for 1 min, 62°C for 1 min, and 95°C for 30 s. The relative expression of *Cxcr4* was determined using the $2^{-\Delta\Delta Ct}$ method. The results are reported as the fold change in gene expression normalized to the housekeeping gene *Actb* and relative to irradiation controls.

6.13 microRNA isolation and quantitative real-time PCR

Total RNA (including microRNA) were isolated using the mirVana Kit (Ambion) according to manufacturer's instruction. The RNA (500 ng) was reverse transcribed to cDNA using dNTPs (Roche), M-MuLV reverse transcriptase reaction buffer (New

England Biolabs, Inc.), oligo-(dT)₁₂₋₁₈ (Invitrogen), RNase inhibitor (Promega), M-MuLV reverse transcriptase (New England Biolabs, Inc.) and miR-155 stem loop primer GGTAGACACAAGCGACACTAACCACCCCT on a Mastercycler gradient Thermal Cycler (Eppendorf). Quantitative real-time PCR was performed in duplicate with SYBR Premix Ex Taq (Takara Bio Inc.) using the Stratagene Mx3000P qPCR system (Agilent Technologies). The primer sequences used were: mmu-miR-155-PCR-FW: GCGGCGTTAATGCTAATTGTG; hsa-mmu-miR-155-PCR-REV: GCGACACTAACCACCCCTATCQ; hsa-miR-155-PCR-FW: GCGGCGTTAATGCTAATCGTG; sno202-FW: GCTGTACTGACTTGATGAAAG; sno202-REV: CATCAGATGGAAAAGGGTTCA; U6-FW: AGAGCCTGTGGTGTCCG; U6-REV: CATCTTCAAAGCACTTCCCT. Quantitative real-time PCR conditions were as follows: 95°C for 1 min, 95°C for 25 s, 62°C for 25 s (35 cycles), 95°C for 1 min, 62°C for 1 min, and 95°C for 30 s. The relative expression of *mir155* was determined using the $2^{-\Delta\Delta Ct}$ method. The results are reported as the fold change in gene expression normalized to the housekeeping gene *sno202* and relative to controls.

6.14 Statistical analysis

Results are expressed as mean \pm SEM. Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software). P values were calculated using an unpaired two-tailed Student's *t*-test, one- or two-way ANOVA with post-tests as indicated. Survival curves were generated using the Kaplan–Meier method and survival differences were

determined with a Mantel-Cox log-rank test. P values of ≤ 0.05 were considered statistically significant.

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