Altered Kinetics of Non-Homologous End Joining Mediated DNA Repair in Mouse Models of Aging and Leukemia

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ABSTRACT

DNA encodes the genetic instructions for the development and function of organisms and hence maintaining genomic integrity is essential for the propagation of life. However, DNA molecules are under constant threat of metabolic and environmental insults resulting in DNA damages including DNA double strand breaks (DSB), which are considered as a serious threat to cell survival. The majority of these DSB are repaired by Non-homologous end joining (NHEJ). Unrepaired DSB can lead to genomic instability resulting in cell cycle arrest, apoptosis, and mutations. Thus, delineating this DNA repair process is important in understanding the molecular mechanisms of aging and malignant progression. B lymphocytes undergo physiological DNA breaks and NHEJ-mediated DNA repair during their bone marrow differentiation and peripheral class switch recombination (CSR), thus lending them as a good model system in which to delineate the DNA repair mechanisms. To determine the effect of aging on NHEJ, B lymphocytes from old mice were analyzed. The results showed compromised DNA repair in cells from old mice compared to cells from adult mice. These results suggest that NHEJ is compromised during aging and might play critical roles in the aging process and age-associated conditions. To delineate the role of a CT in regulating the immune system, transgenic mice expressing *NUP98-HOXD13* (*NHD13*) were analyzed for B lymphocyte differentiation, peripheral development, CSR, and antibody production. The results showed impaired B cell development and antibody production, which worsened with antigenic stimulation, suggesting the role of *NHD13* in immune regulation. These studies explored the possibility of altered NHEJ-

mediated DNA repair as a contributing reason for aging process and age-associated conditions. Also, the results from *NHD13* study suggested that a primary CT can result in impaired NHEJ and regulate immune cell development and function. Furthermore, the results pointed to the possibility that a primary CT may lead to secondary mutations through altered NHEJ. Thus, these studies shed insight into the molecular mechanisms of altered NHEJ and may help in developing preventive or therapeutic strategies against accumulation of DNA damage, aging process and secondary mutations.

DEDICATION

Dedicated to my wife, Feby, and our daughters Ayisha and Fathima.

PREFACE

“The roots of education are bitter, but the fruit is sweet”

Aristotle (384-322 BC)

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CHAPTER 1

INTRODUCTION

Deoxyribonucleic acid (DNA), composed of polymers of purine and pyrimidine nucleotides, is the key molecule for the existence of life. As DNA encodes the genetic instructions for the development and function of organisms, maintaining genomic integrity is essential for the propagation of life. However, DNA molecules are under constant threat of metabolic and environmental insults including reactive oxygen species, reactive nitrogen species, ultra-violet rays, and other physical and chemical agents. Studies have proposed that there can be up to one million molecular lesions in each dividing eukaryotic cell DNA per day (Holmquist, 1998). These damages have to be repaired immediately, or cells will undergo cell cycle arrest leading to cellular senescence, apoptosis, or acquired mutations. Of the different types of DNA damages, DNA double strand breaks (DSB) are considered the most serious threat for cell survival, which can occur at an estimated rate of ten DSB per dividing cell per day (Lieber et al., 2003). These DSB are repaired by homologous recombination, non-homologous end joining (NHEJ), or by alternative end joining (AEJ) (Kasparek and Humphrey, 2011). Choice of the repair mechanism depends on various factors including the type of cells affected, the nature of the break, phase of the cell cycle, and availability of repair factors. Homologous end joining occurs between homologous DNA fragments during the resting stage of the cell cycle and when the complementary strand is available to complete the repair. DSB occurring during the G1 stage of the cell cycle are repaired through NHEJ. Delineating the mechanisms of DNA breaks and repair is important in understanding the molecular pathways involved in the maintenance of genomic integrity, which have wide implications in embryonic development, aging, immunity, and oncogenesis.

Non Homologous End Joining

NHEJ is an evolutionarily conserved DNA DSB repair mechanism among eukaryotes and is the predominant DNA DSB repair mechanism in mammals (Hentges et al., 2006). Though NHEJ occurs mainly during G1 phase of the cell cycle, it can occur at any other stage of the cell cycle considering that it does not require the proximity of a complementary DNA stand (Chen and Cerutti, 2011). During NHEJ, DNA DSB are recognized through cellular pathways, which involves proteins such as Ataxia Telangiectasia Mutated (ATM) and histone 2A (H2AX) (Lieber, 2010). During repair, the break ends are protected and brought together by Ku proteins (Ku70 and Ku80) (Xu and Powers, 2010). DNA Protein Kinase catalytic subunit (DNA-PKcs) is recruited to the site (Teo and Jackson, 1997; Yaneva et al., 1997), which acts as a scaffold for other repair factors including Artemis, DNA ligase 4, XRCC4, and Cernunnos (XLF) (Boskovic et al., 2003; Ogiwara and Kohno, 2011; Ropars et al., 2011; Wu et al., 2011). Artemis cleaves the temporary hair pin, which is formed immediately after the DSB (Kurosawa and Adachi, 2010). Next, Ligase 4–XRCC4 complex mediates the actual ligation process. Finally, Cernunnos stimulates the terminal ligation step and reactivates the Ligase 4–XRCC4 complex (Ropars et al., 2011). NHEJ often utilizes microhomologies, i.e., short DNA fragments, as templates, and so some nucleotides might be added or removed at the repair sight. Thus, NHEJ is an efficient DSB repair mechanism utilizing multiple factors sequentially, leading to the prompt repair of DNA breaks with no, or minimal damage to the genome.

Impaired NHEJ can result in a wide variety of complications including impaired immunity, altered lymphocyte development, and malignant transformations. For example, in Ligase 4 deficiency syndrome, patients have reduced T and B cells in circulation and compromised immunity due to impaired immunoglobulin gene recombination events (O'Driscoll et al., 2001). Likewise, in the SCID mouse model, PKcs deficiency results in reduced VDJ and CSR

mechanisms and results in severe immunodeficiency (Blunt et al., 1996). Hyper IgM syndrome patients have ineffective CSR; a fraction of patients have ineffective NHEJ as the reason for this condition (Imai et al., 2003). Defective NHEJ is embryonically lethal due to its essential role in neurogenesis (Frank et al., 2000). Taken together, all of these studies point to the crucial role(s) of NHEJ in maintaining genomic integrity during embryogenesis, lymphocyte differentiation, and antibody production, as well as preventing malignancies.

Alternative End Joining (AEJ)

Reduced availability or altered patterns of NHEJ can result in the activation of AEJ rather than classical NHEJ, leading to substantial deletion of DNA fragments. Studies using conditional knockout mouse models for various NHEJ factors have shown that some types of gene recombination can happen even in the absence of Ligase 4, Xrcc4 or Artemis (Han and Yu, 2008; Rivera-Munoz et al., 2009; Soulas-Sprauel et al., 2007a). Further studies using *in vitro* and murine models lead to the discovery of other possible DNA repair factors such as DNA ligase 1, Ligase 3 and Xrcc1 (Boboila et al., 2010a; Saribasak et al., 2011). However, DNA repair mediated through these factors have more errors with an increased chance for misrepairs and malignant transformations (Sallmyr et al., 2008). Recent studies propose that increased AEJ can be the reason for neoplastic transformation at least in some cases of myeloid leukemia (Fan et al., 2010).

NHEJ in Lymphocytes

Apart from DNA break repair due to metabolic and environmental assaults, NHEJ is crucial in physiological DNA repair during lymphocyte differentiation and antibody production. Lymphocytes are a group of white blood cells that form critical components of both the innate and adaptive immune system (Rock et al., 2011; Speirs, 1963). They differentiate from

hematopoietic stem cells within the liver during fetal development, and from bone marrow following birth. Depending on the function and primary tissue in which they differentiate, lymphocytes are classified into T cells, B cells and Natural Killer (NK) cells (Kiessling et al., 1976). NK cells are non-specific cytotoxic cells and form a part of the innate immune system providing protection against foreign cells and tumors (Herberman and Ortaldo, 1981). T and B cells are considered to be part of the adaptive immune system responsible for specific immune responses against antigens. T cells mature in the thymus and comprise one of the most abundant cells in the human body. They recognize antigens through specific cell surface receptors, often referred to as T cell receptors or TCRs, and are responsible for cell mediated immunity. On the other hand, B lymphocytes directly play important roles in the humoral immune response by producing antigen specific antibodies and by acting as antigen presenting cells (Chesnut and Grey, 1981). Lymphocyte surface receptors, TCRs and B cell receptors (BCR) acquire this vast antigen recognition property through gene recombination mechanisms mediated by NHEJ. Given the role in lymphocyte repertoire development, NHEJ is critical for the diversity of the antigenic receptors on lymphocytes and thus in immunity.

B lymphocytes

B lymphocytes or B cells develop from hematopoietic stem cells of bone marrow or fetal liver and pass through a common lymphoid progenitor stage before B lineage commitment (Nossal and Pike, 1973). Postnatal development of B cells is reviewed in Figure 1-1 and can be divided into: 1) bone marrow differentiation to produce immature B cells, 2) peripheral development in secondary lymphoid organs to form naïve B cells, and 3) clonal expansion to form antibody producing plasma cells following antigenic stimulation. Bone marrow differentiation of B cells follows a tightly controlled sequential pattern and includes rearrangement of the Variable (V),

Diversity (D) and Joining (J) fragments of immunoglobulin genes, also known as VDJ recombination (described in detail under VDJ recombination, page no. 14). Recombined VDJ is unique to each B cell and will be subsequently expressed on the cell surface, known as the BCR. Peripheral development in secondary lymphoid organs involves maturation of B cells where they modify surface markers and prepare to interact with antigens. Once these naïve B cells interact with antigens and cofactors, they will undergo proliferation (known as clonal expansion) and another set of gene recombination events (CSR) (described under Class switch recombination, page no. 19), to produce antigen specific immunoglobulin molecules or antibodies. Depending on the isotype, these antibodies are either released to the surrounding medium or retained in the cell surface. These antibodies are responsible for specifically targeting the antigens and neutralizing or containing them effectively, thus providing immune protection to the body.

Bone marrow differentiation of B cells

Hematopoietic stem cells within the bone marrow differentiate and produce common lymphoid progenitors (CLP), which in turn become lineage committed Pro B cells. Pro B cells further differentiate to Pre B cells and immature B, which leave the bone marrow and populate secondary lymphoid organs. The bone marrow differentiation of lineage committed B cell progenitors can be divided into different developmental stages depending on the cell surface markers (Hardy et al., 1991). Thus depending on the presence and abundance of IgM, IgD, CD41, B220, CD24 and BP1, bone marrow B cells are divided into **Pro B** ($B220^+$ $CD41^+$) and **Pre B** ($B220^+$ $CD41^-$) cells. Pro B cells are further subdivided into fractions **A** ($BP1^-$ $CD24^-$), **B** ($BP1^-$ $CD24^+$), **C** ($BP1^+$ $CD24^{high}$) and **C'** ($BP1^+$ $CD24^{low}$), A being the earliest Pro B fraction and C' being the last pro B fraction. Pre B cells are divided into fractions **D** (IgM^-IgD^-), **E** (IgM^+

IgD^-) and **F** (IgM^+ IgD^+); D being the earliest Pre B fraction or large Pre B cell and F being the final Pre B fraction or immature B (Hardy et al., 1991). Bone marrow differentiation of B cells is regulated by multiple transcriptional factors, epigenetic modifications and signaling cascade events (Hystad et al., 2007). Important transcription factors include Pax5 (Medvedovic et al., 2011), Ikaros (Reynaud et al., 2008), cKit (Santos et al., 2011) and Flt3 (Dolence et al., 2011). Cell signaling occurs during differentiation mainly through BCR (Li et al., 2012), and IL-7 receptors (Kosan et al., 2010). Immature B cells formed within bone marrow enter circulation, and based on a wide variety of chemokine and growth factor signaling, they populate secondary lymphoid organs where they undergo further maturation.

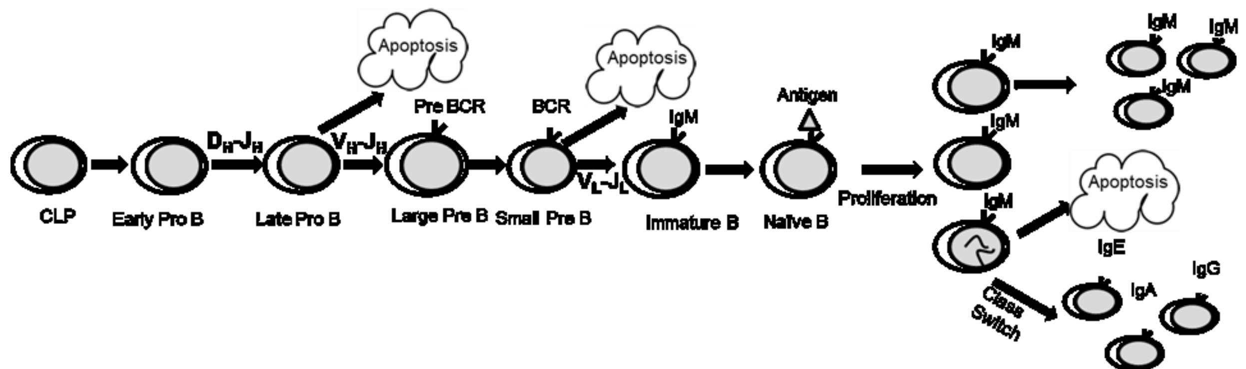

Figure 1-1. Schematic diagram showing various stages of B cell development.
Hematopoietic stem cells give rise to common lymphoid progenitors (CLP) which in turn can become Early Pro B lymphocytes depending on the transcriptional regulation and extrinsic signaling. Pro B cells undergo sequential D_H to J_H recombination followed by V_H to J_H recombination, completing VDJ recombination as they progress from Pro to Pre- B stage. During early Pre B stage (small Pre B), recombined light and heavy chains assemble to form B cell receptors (IgM). When these naïve B cells encounter with antigens in secondary lymphoid organs, they undergo proliferation and class switch recombination, leading to the production of different isotypes of antibodies.

VDJ Recombination

The Immunoglobulin genes in B lymphocytes consists of multiple Variable (V), diversity (D) and Joining (J) fragments, which are sequentially cleaved and rejoined together by a mechanism known as VDJ recombination (Weterings and Chen, 2008). The mammalian immunoglobulin gene consists of close to 65 V regions, 6 J regions and 27 D regions. During bone marrow differentiation, the Immunoglobulin gene is cleaved sequentially at V, D and J regions and recombined, which theoretically can result in approximately 10^8 different combinations and give rise to 3-9 million antigen specific CDR3 regions (Arnaout et al., 2011). During the bone marrow development of Pro B stage fraction B to fraction C, Recombination Activation Gene 1 and 2 (RAG 1, 2) are activated, which in turn cleaves the immunoglobulin heavy chain, leading to the first D to J recombination (Schatz and Ji, 2011). During the development of Pro B to Pre B cells, the immunoglobulin gene undergoes another set of recombination events known as V to DJ rearrangement where a Variable fragment is cut and joined to the already recombined DJ fragment, thus completing the VDJ recombination. In a similar mechanism, V and J fragments in the immunoglobulin light chain are recombined during the same time. The cleaved DNA fragments during VDJ recombination are classically ligated by NHEJ (Soulas-Sprauel et al., 2007b). At the Pre B fraction E stage, light chains 1) assemble with the recombined VDJ heavy chain fragment and, 2) become translated and expressed on the cell surface as the BCR or IgM. Impaired NHEJ can block D to J or V to DJ recombination, leading to cell cycle arrest and apoptosis (Soulas-Sprauel et al., 2007b).

Peripheral Development of B Lymphocytes

As the cells differentiate within bone marrow, B lymphocytes progressively lose their dependency to surrounding stromal cells and are detached at the immature B cell stage.

Immature B cells in circulation populate secondary lymphoid organs such as spleen, lymph nodes, and Peyer's patches where they undergo progressive maturation. These sequential steps include progression from an early stage, referred to as the transitional stage 1 (T1) to T2 and then to T3. T3 cells can become B1 B, marginal zone, or the follicular zone cells depending on the type of external stimuli (Hardy et al., 1991). The naïve B cells with BCR on their surface (IgM) leave the secondary organs and enter the peripheral circulation. Once B cells interact with an antigen and are co-stimulated with ligands such as CD154 (CD40L) and cytokines (illustrated in Figure 1-2), they will proliferate in secondary lymphoid organs (clonal expansion) and produce antigen specific antibody. At this stage, the variable region of immunoglobulin gene undergoes point mutations to produce antibodies of different affinity, a mechanism known as affinity maturation (Victora and Nussenzweig, 2012). This can give rise to highly specific and effective antibodies against antigens. Further, during this clonal proliferation stage, CSR occurs where the constant region of immunoglobulin gene undergoes NHEJ-mediated gene recombination resulting in the production of antibodies with the same VDJ region, but with different constant regions. Different isotypes of antibodies specifically bind to antigens and prompt a cascade of events resulting in the destruction of the antigen or antigen carrying organisms (Chen and Cerutti, 2011).

Immunoglobulins/ Antibodies

Antibodies or Immunoglobulins are protein molecules produced by B lymphocytes that function in identifying and neutralizing foreign molecules or antigens. Immunoglobulins are Y-shaped large globular proteins with antigen binding sites at the tip of their arms (Figure 1-3) (Huber, 1980). Antibodies are made up of two large heavy chains and two small light chains giving them an approximate molecular weight of 150 kDa. The heavy chain consists of three to four Constant regions (C_H) and one Variable region (V_H) whereas the light chain is formed by one C_H and one V_H. The light chain can be kappa or lambda subtype. The chains are linked together by disulfide bonds. Arms of the Y shaped immunoglobulin are also known as F_{ab} (Fragment antigen binding) that is composed of one variable and one constant region from both light and heavy chains. The variable fragment, F_V (antigen binding site/paratope) is located at the amino-terminus of the arms

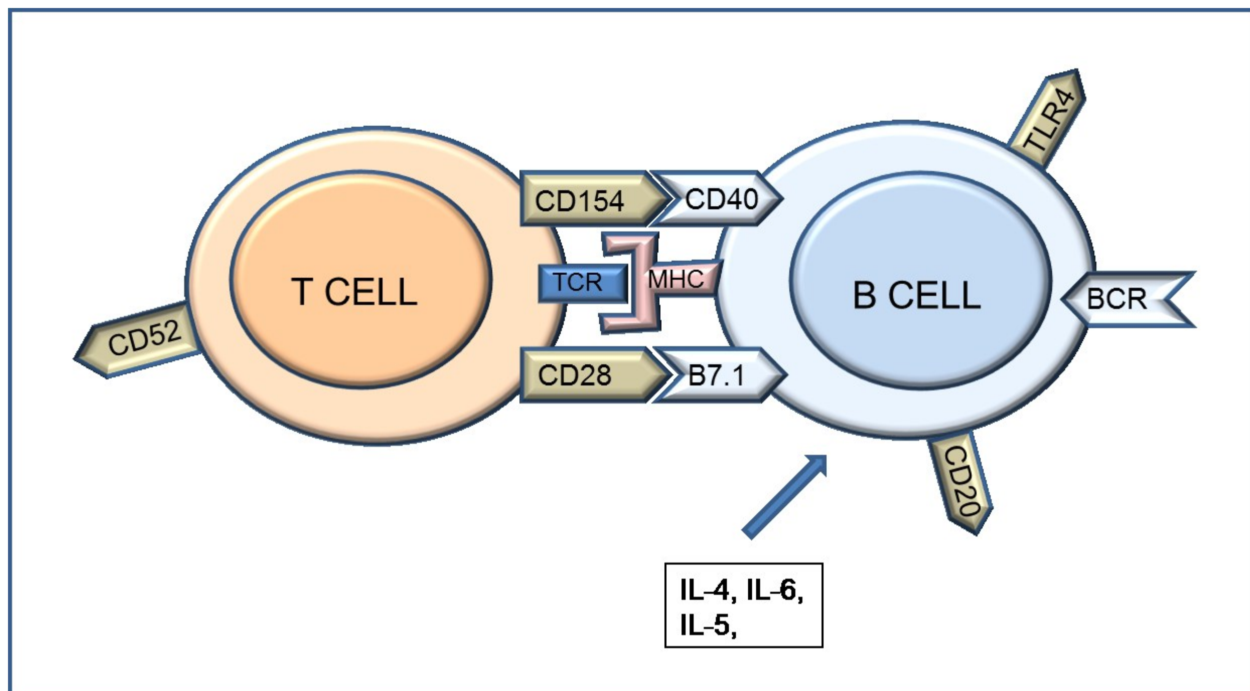

Figure 1-2. Mechanism of B cell activation. B cells activation through MHC, T lymphocyte ligands and cytokines. Activated B cells undergo proliferation and class switch recombination to produce antibodies.

and is formed by variable domains from both light and heavy chains. More precisely, the antigen binding domain is formed by variable loops of three β strands from each heavy and light chain (Vettermann and Schlissel, 2010). These loops are also known as cluster differentiating regions (CDRs) and their variability provides enormous antigen recognition ability. VDJ recombination during bone marrow differentiation is responsible for this immunoglobulin variability. Due to immense variability of the CDR, these regions provide recognizing capability to virtually any antigenic molecule (Davis, 2004).

Isotypes

Antibodies bind to antigen molecules and evoke a wide variety of immune response depending on the isotype of antibody, which in turn depends on the constant region of immunoglobulin. Immunoglobulins in mammals are categorized into five different isotypes, IgM, IgD, IgG, IgE and IgA depending on the constant region present on the immunoglobulin heavy chain molecule. IgM is the first antibody to appear during an antigenic challenge. IgM can occur both as a membrane bound form and as a free form in body fluids. IgM in solution occurs in a pentameric form, and due to its heavy size, can act as an excellent complement activating antibody (Brandtzaeg, 1981). IgD

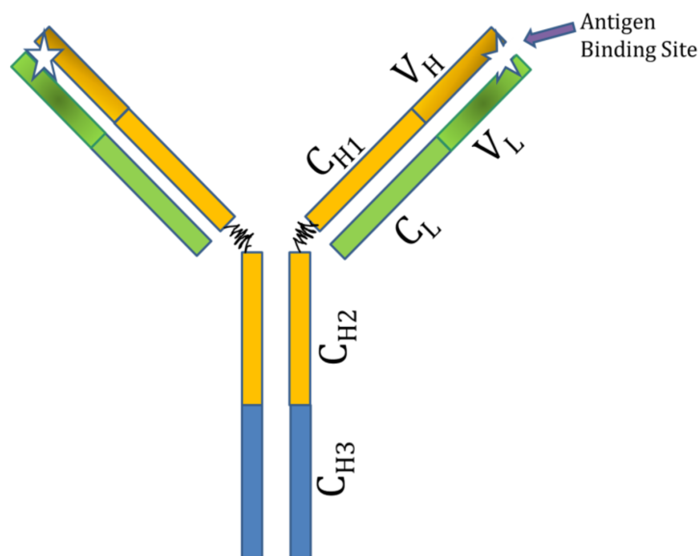

Figure 1-3. **Structure of Immunoglobulin Molecule**
C_H indicates Constant regions of Heavy chain, V_H are the variable regions of heavy chain, C_L are Constant regions of Light chain and V_L are the variable regions of light chain. These chains are assembled in a Y shaped pattern with antigen binding site at the tip of the arms.

molecules are always found attached to the B cell surface whereas IgG, IgE and IgA molecules are often released into plasma (Spiegelberg, 1989). IgA molecules are found in the mucosal membranes of respiratory, gastro-intestinal, and urogenital tracts (Macpherson et al., 2012). These molecules function by attaching to the invading organisms, thus preventing their adherence to mucosa and colonization (Brandtzaeg, 1981). IgD molecules are shown to activate basophils and mast cells to produce antimicrobial factors during an immune response (Chen and Cerutti, 2011). IgE molecules are released mainly during parasitic or allergic reactions, which in turn bind to mast cells and basophils to release histamines. IgG molecules occur in five different subtypes, IgG1, IgG2a, IgG2b, IgG3 and IgG4 and functions by binding to antigen molecules (Anthony et al., 2012). Effector functions of IgG molecules include antigenic agglutination, complement activation, opsonization for phagocytosis and neutralization of toxins.

Class Switch Recombination (CSR)

Naïve B lymphocytes upon antigenic stimulation undergo proliferation and CSR (Kataoka et al., 1980; Kracker and Durandy, 2011). CSR is a tightly controlled process and is regulated by various cytokines, growth factors, cell signaling cascades, and transcriptional factors (Radbruch et al., 1986). CSR occurs as a result of a unique intra-chromosomal deletional recombination at the Guanosine rich switch regions of Immunoglobulin heavy chains (Dunnick et al., 1993; Stavnezer et al., 2008). The extrinsic stimuli, such as antigenic interactions, will result in the expression of Activation Induced Cytidine Deaminase (AID), which in turn deaminates cytosines at switch regions (Muramatsu et al., 2000; Pavri and Nussenzweig, 2011). Deaminated cytosines are converted to urasils, that are excised by Urasil DNA Glycosylase (Chaudhuri et al., 2003). This point is nicked by apurinic apyramidinic endonuclease and leads to DNA DSB (Christmann et al., 2003; Shen, 2007). The recombined IgH can have different constant regions depending on

the signal transduction and repair pathways. Based on the new constant region immediately following the VDJ region, i.e., μ, γ, α, ε, δ, the immunoglobulin molecule can be either IgM, IgG, IgA, IgE, or IgD subtypes respectively. The new recombined heavy chain is translated and expressed on the surface of the B cells or released into circulation depending on the isotype (Radbruch et al., 1986). The new isotypes of antibodies produced will have more diverse and effective functions to clear the antigenic load. During CSR, the majority of the immunoglobulin DNA breaks are repaired by NHEJ; however, homologous end joining and AEJ may also participate in DNA repair process. Aberrant CSR has been found to be associated with chromosomal translocations leading to malignancies such as lymphoma and leukemia (Edry and Melamed, 2007; Puebla-Osorio and Zhu, 2008).

NHEJ and Chromosomal Translocations

Chromosomal translocations (CT) are abnormalities caused by rearrangements of segments from non-homologous chromosomes (Aplan, 2006). CT are associated with a number of different malignancies including leukemia and are considered as a hallmark feature of hematologic malignancies (Caudell and Aplan, 2008; Rabbitts, 1994). Investigations into the molecular pathogenesis of CT have revealed four important paradigms (Lin and Aplan, 2004). First, specific CTs are typically associated with a particular leukemic phenotype. Secondly, CT may either produce a novel chimeric protein as in the case of BCR-ABL (Zhang and Rowley, 2011) or they lead to aberrant expression of protooncogenes such as *MYC-IGH* as observed in lymphomas (Busch et al., 2007). Third, CTs often include parts of genes that encode for either transcription factors or proteins associated with signal transduction pathways (Druker et al., 1989). Finally, genes affected by CTs can also be associated with point mutations (Osato, 2004).

A majority of CT belong to class II mutations, which require secondary collaborating mutations for malignant progression (Gilliland, 2002). However, the role of a primary translocation in inducing the secondary mutations is under debate (Sallmyr et al., 2008). Given that mutations occur as a result of DNA damage and misrepairs, it is reasonable to suspect that the primary mutation could induce DNA damages and/or prevent proper repair of DNA damages. A number of transgenic animal models have been developed with human CT to delineate the molecular mechanisms underlying the malignant progression (McCormack et al., 2008). For example, transgenic mice expressing the leukemic fusion gene *NUP98-HOXD13 (NHD13)* develop characteristic features of class II mutation, i.e., impaired differentiation of bone marrow cells and progression to leukemia after acquiring secondary mutations (Choi et al., 2008; Slape et al., 2007). Given the requirement of secondary mutations for malignant progression, *NHD13* mice are a good model system in which to delineate the role of primary translocation in DNA repair mechanisms.

Based on the current understanding, there is an emerging need to delineate the role of DNA DSB and NHEJ during aging and leukemic progression. Understanding the molecular pathways associated with DNA break induction and repair mechanisms will reveal potential therapeutic targets against malignancies and conditions associated with aging. As physiological DNA breaks and NHEJ-mediated recombination can be induced in B lymphocytes, they can be used as a tool to further delineate the altered NHEJ-mediated DNA repair mechanisms.

The central hypothesis for this PhD thesis is that **NHEJ-mediated DNA repair is impaired during aging and leukemic progression.**

The objective of the study was to determine the DNA DSB and NHEJ-mediated repair patterns in B lymphocytes during CSR. Also, the effect of CT in bone marrow differentiation and

antibody production were analyzed. To test the hypothesis, a multi-model approach was employed.

To test the hypothesis, the following specific aims were proposed

1. Aging is an irreversible physiological process characterized by reduced immunity and reduced response to infectious agents and vaccines. Previous studies have proposed the potential role of impaired DNA repair mechanisms as a reason for aging and age related conditions (Lombard et al., 2005). However, these studies have not documented the fine regulation of DNA repair mechanisms and their efficiency under physiological conditions. To delineate the efficiency of NHEJ during aging, CSR in B lymphocytes from old and adult mice was evaluated. Laboratory mice have an average lifespan of 3 years and mice more than 2 years of age are considered old. Naïve splenic B lymphocytes from old (2 years) and adult (1 year) mice were antigen stimulated to induce physiological DNA breaks and DNA repair pathways were analyzed. *In vitro* plasmid ligation efficiency assay was used to quantitate the NHEJ mediated repair efficiency in B cells from old and adult mice. The details of this study are described in Chapter I of this thesis.
2. Chromosomal translocations are hallmark features of hematologic malignancies. They are considered as Class II mutations, which can impair cell differentiation, but require secondary mutations for malignant transformation. To determine the role of a myeloid leukemic translocation in lymphoid development, *NHD13* transgenic mice were used. *NHD13* mice have been shown to develop leukemia after developing secondary mutations. Further, these mice have impaired lymphocyte development. Bone marrow B lymphocyte differentiation, peripheral development, and antibody production patterns of

B lymphocytes in *NHD13* transgenic mice were used to delineate the role of the fusion gene in impairing B lymphocyte development. In vitro CSR assay was performed to determine the role of fusion gene in B lymphocyte mediated immune response and antibody production. The results showed that the *NHD13* fusion gene impaired B cell development by partially impairing VDJ and CSR mechanisms. Details of these experiments and results are described in Chapter II.

3. Although secondary mutations are required for malignant transformation, the role of a primary translocation in inducing secondary mutations is not clearly understood. As mutations occur as a result of DNA damage and subsequent misrepairs, CT might play a critical role in the DNA repair mechanism. *NHD13* transgenic mice had impaired VDJ and CSR mechanisms in their B lymphocytes, thus creating a model system in which to delineate the role of a chromosomal translocation in DNA repair. To understand the role of chromosomal translocation in NHEJ-mediated DNA repair, we used in vitro CSR mechanisms to analyze the DNA break induction pattern following antigenic stimulation and the gene recombination mechanisms, cell cycle kinetics, and gene expression profiles. Experimental design, results and conclusions are detailed in Chapter III.

The study on the effects of aging and leukemia on DNA repair revealed patterns of compromised NHEJ in stimulated B lymphocytes. Further studies are necessary to delineate the underlying mechanisms of impaired DNA repair during these stressful conditions. Results and conclusions are summarized in Chapter VI. An outline of future directions including experimental design and alternative approaches are provided in Chapter VI.

Conclusion

Understanding the DNA repair mechanisms is important in delineating its role in various conditions such as aging and leukemic development. Furthermore, exploring pathways using lymphocytes will help in elucidating oncogenic mechanisms directly or indirectly related to hematopoietic differentiation. Multiple factors such as altered transcriptional regulation, aging, presence of translocations, and individual genetic makeup could interfere with the classical NHEJ-mediated DNA repair mechanism. This could ultimately compromise the immune function by impairing cell cycle, leading to apoptosis, or result in acquisition of lethal and non-lethal mutations. As NHEJ is having critical roles in embryonic development, immune cell differentiation, antibody production, aging, and malignant transformations, delineating the underlying molecular pathways of NHEJ is important in developing novel therapeutic strategies. The choice of using VDJ and CSR for NHEJ-mediated repair offers the advantage of assessing the DNA repair efficiency under normal physiological conditions. The results herein shed insight into the altered molecular pathways under various cell-stress conditions and these findings might help in developing better strategies for improved patient care and survival.

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CHAPTER 2

LITERATURE REVIEW

Non Homologous End Joining: Lessons Learned from Animal Models

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Abstract

Non-homologous end joining (NHEJ) is a critical DNA repair mechanism implicated in both physiological and pathological DNA double strand breaks. This repair process is important in a number of biological processes including immune reactions, malignancies, aging, and embryonic development. Classical NHEJ (C-NHEJ) is facilitated by a controlled sequential action involving multiple transcriptional factors, proteins, and cofactors including Ku70, Ku80, DNAPKcs, Artemis, DNA Ligase 4, XRCC4 and Cernunnos. Since these factors are evolutionarily conserved across mammals, mouse models have provided valuable insight into delineating the role and function. Several knockout, conditional deletion, and knock in mouse models have revealed how these factors modulate the NHEJ mechanism. Animal studies with depletion of one or more of C-NHEJ genes exhibit variable degrees of end joining, which may be, in some instances, mediated by error prone Alternative End Joining (AEJ). Recent studies have proposed Ligase 3, XRCC1 and Ligase 1 as probable candidate factors involved in AEJ. In this review, we focus on recent advancements in research of NHEJ and AEJ using mouse models.

Key words: Alternative end joining, classical non-homologous end joining, DNA double strand breaks, mouse models

Introduction

Cells are under constant threat of DNA damage, which can compromise their genomic integrity and viability. Damage occurs frequently from endogenous sources such as replication stress, lymphocyte gene recombination events, or from metabolic products such as reactive oxygen and reactive nitrogen species. Additionally, extrinsic sources such as ionizing radiations and physical and chemical agents can cause DNA damage. One of the most harmful types of DNA damage is DNA double strand breaks (DSB). Previous studies estimate that 10 DSBs occur in each dividing cell per day (Lieber et al., 2003). These breaks must be repaired immediately for cell cycle progression, as well as proper cellular development and differentiation. DNA breaks are repaired by one of two distinct cellular mechanisms: homologous recombination (HR) and non-homologous end joining (NHEJ). Homologous recombination occurs when the corresponding sister chromatid is available; this process takes place using the sister chromatid as a template for repairing the DNA break. Homologous recombination can result in repair with perfect joining or with exchange of chromosome segments; the latter is responsible for "crossing over" during meiotic cell division and subsequent genetic variability between offspring. In contrast, NHEJ is the major DSB repair process and is the preferred mechanism for DSB repair in mammalian cells, which does not require a DNA template for repair. NHEJ can be achieved through at least two different pathways. The primary pathway, classical NHEJ, results in minimum end processing of DNA fragments and conserves the integrity of the genome. The second pathway, alternative end joining (also called microhomology mediated end joining), often results in misrepairs and can lead to deletion of a considerable DNA fragment. Choice of the repair mechanism is largely dependent on the cell cycle phase, though other factors including the nature of the DNA breaks, availability of repair factors, and cell type affected are important. The HR

takes place during and shortly after DNA replication in the G2 and S phase of the cell cycle, where sister chromatids are more easily available. In contrast, NHEJ takes place mainly during the G1 phase of cell cycle though it can occur in any other phase of the cell cycle. Absence, reduction, or inefficiency of DNA damage repair can lead to accumulation of DNA breaks, and subsequent cell cycle arrest, apoptosis, mutations, and malignant transformations. This review will focus mainly on the NHEJ mechanisms and selected animal models developed to study NHEJ.

Classical Non-Homologous End Joining

Classical non-homologous end joining (C-NHEJ) is a major evolutionarily conserved DNA DSB repair mechanism in mammalian cells and occurs in the absence of homologous templates (Cui and Meek, 2007; Symington and Gautier, 2011). NHEJ results in direct binding of broken DNA ends and occurs mainly during G1 phase of the cell cycle when homologous chromosomal counterparts are not available (Konishi and de Lange, 2008; Symington and Gautier, 2011). *In vitro* studies using human cells suggest that NHEJ can also occur during other phases of the cell cycle such as synthesis (S) and growth-2 (G2) (Mao et al., 2008). During DNA repair, NHEJ may use short homologous sequences known as microhomologies to bridge the gap between the DNA fragments. Seven different critical proteins for C-NHEJ have been identified and include Ku70 (Tomimatsu et al., 2007), Ku80 (Casellas et al., 1998), DNA Protein Kinase catalytic subunit (DNA-PKcs) (DeFazio et al., 2002), exo-endonuclease Artemis (Li et al., 2005), DNA Ligase 4 (Chistiakov, 2010), X-ray cross complementing factor 4 (XRCC4) (Soulas-Sprauel et al., 2007a), and XRCC4 like factor (XLF/ Cernunnos) (Riballo et al., 2009). In addition, there are many proteins and cofactors, which are associated with NHEJ. These include MRE11, Rad50, Nbs1, ATM, H2AX, 53BP1, and X family DNA polymerases. These factors act in a

highly regulated manner to facilitate an efficient NHEJ process with minimum damage to the break region (Figure 2-1). When a DNA DSB occurs, Ataxia telangiectasia mutated (ATM) kinase is activated, which in turn promotes the accumulation of damage response factors at the site of DNA breaks. Histone H2AX, one of the ATM targets, becomes phosphorylated and stabilizes DNA ends (Burma et al., 2001). Ku proteins (Ku70 and Ku80) form a heterodimer and attach to DNA break regions, protecting the fragments from exo-nucleolytic attacks. DNA-PKcs attach to the dimer, and the resultant trimer (Ku70/Ku80/DNAPKcs) in turn activates protein kinase activity of DNA-PKcs (Collis et al., 2005). DNA-PKcs also acts as a scaffold for other NHEJ factors such as Artemis, DNA ligase4 and XRCC4 (Llorca and Pearl, 2004). Non-compatible DNA break ends (hanging ends) are cleaved by activated Artemis prior to the actual ligation process. X family DNA polymerases such as polymerase μ and polymerase λ synthesize nucleotides to fill the break gap if needed. Ligation is effected by DNA ligase 4 and its cofactor XRCC4 (Lees-Miller and Meek, 2003; Stavnezer et al., 2008). Finally, Cernunnos (also known as XRCC4 like factor or XLF) readenylates Ligase 4 and reactivates it for further ligation (Ahnesorg et al., 2006). Thus, NHEJ is a tightly regulated process involving multiple factors and effective NHEJ is key for cellular integrity and progression through the cell cycle.

NHEJ and Immunity

B and T lymphocytes are integral parts of the immune system, which are responsible for antibody-mediated and cell-mediated immune responses against invading pathogenic organisms and antigens. The vast antigenic recognition property of lymphocytes is due to the variability in the antigen receptors located on their cell surfaces. During early lymphocyte development, Variable (V), Diversity (D) and Joining (J) regions of immunoglobulin genes undergo DNA

break and NHEJ mediated gene recombination. Similar VDJ recombination in T cell receptor genes α, β, γ, and δ chains occurs during T cell lymphocyte development, eventually resulting in a diverse repertoire of antigen receptors on their surface. During early B and T lymphocyte differentiation, transcriptional regulation and IL-7 receptor mediated signaling leads to expression of recombination activating genes *RAG1* and *RAG2.* RAG1/2 forms a hetero tetramer and cleaves at the recombination signal sequences (RSS) flanking V, D and J regions of receptor genes. These fragments are subsequently recombined by the NHEJ mediated break repair process, leading to the formation of novel VDJ fragments. C-NHEJ is the major recombination pathway during VDJ rearrangement, as the RAG post-cleavage complex directs V, D, and J fragments towards the C-NHEJ pathway rather than the error prone alternative end joining pathway (Corneo et al., 2007; Lee et al., 2004). Following recombination, VDJ fragments become translated and expressed as surface receptors. Owing to the variability in recombination, receptor repertoire varies widely giving enormous antigenic recognition property to the cell. As VDJ recombination involves DNA DSBs and strictly C-NHEJ mediated recombination, it is not surprising that defective NHEJ can result in severely impaired B and T lymphocyte differentiation and immune deficiency.

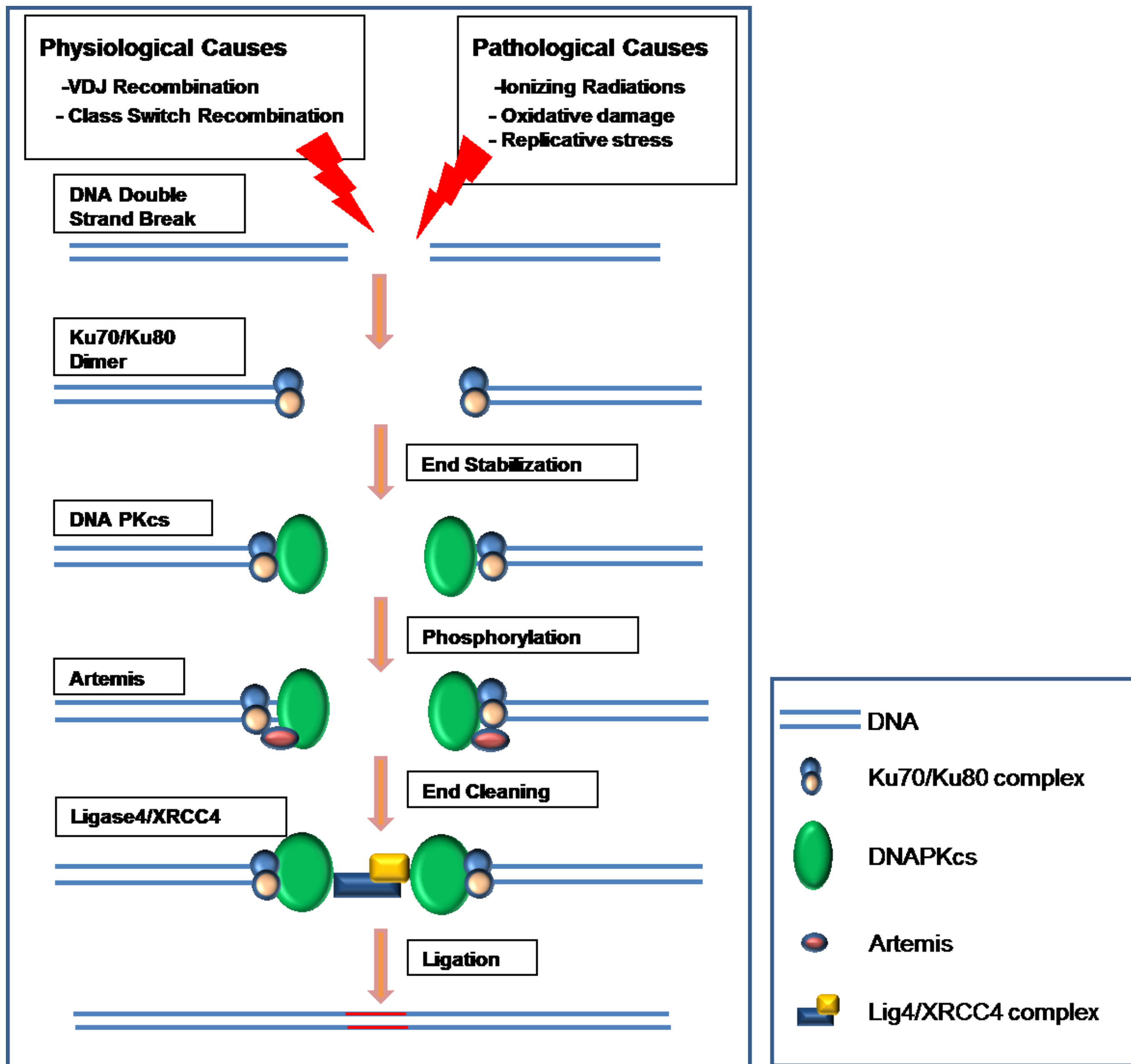

Figure 2-1. Schematic representation of NHEJ-mediated DNA break repair. Physiological or pathological factors result in DNA double strand breaks, which are identified cellular machinery. Once the cell detects a DSB, Ku70/Ku80 heterodimer is recruited to the site which in turn binds to the DNA break ends and stabilizes them. DNAPKcs attaches to Ku70/Ku80 dimer and acts as a scaffold for other repair factors. Further, DNAPKcs phosphorylates Artemis, which in turn cleaves the hairpin loops formed immediately after the DSBs. Ligase4/XRCC4 complex attaches to the site and Ligase 4 completes the ligation process. Cernunnos readenylates and reactivates the Ligase4 for further DNA ligation.

Naive lymphocytes undergo an additional set of gene recombination events upon antigenic stimulation. When encountered with antigens and stimulated with appropriate co-stimulatory molecules, B lymphocytes undergo proliferation and class switch recombination (CSR). CSR is the mechanism whereby IgM^+ lymphocytes undergo Activation Induced cytidine Deaminase (AID) mediated DNA DSBs and recombination in their immunoglobulin heavy chain (*IgH*) gene, subsequently resulting in the production of different isotypes of antibodies with the same original antigenic specificity (Bothmer et al., 2011). AID along with Uracil DNA glycosylase (UNG) lead to DNA breaks at switch regions flanking the μ, γ, ε, and α regions of the immunoglobulin heavy chain. Following DNA repair, the VDJ fragment of *IgH* is followed by either γ, ε, or α Constant (C_H) region fragments. The new isotype of this antibody will be produced depending on the C_H fragment immediately following the VDJ region. Isotype switching provides multiple ways of activating the immune system and effectively controlling the antigens. Defective NHEJ has been shown to alter or abolish the CSR mechanisms in different animal models and human patients, compromising immune function (Boboila et al., 2010a; Bosma et al., 2002; Casellas et al., 1998; Manis et al., 1998). Defective NHEJ has been attributed in a number of different immune-compromised conditions. First, many patients with hyper IgM syndrome, a condition in which B lymphocytes are incapable of undergoing class switch recombination, have reduced expression of NHEJ factors (Imai et al., 2003). Second, patients with Ligase 4 syndrome, characterized by reduced Ligase 4 expression, have impaired VDJ and CSR of lymphocytes apart from developmental impairments (Chistiakov, 2010). Third, many severe combined immune-deficient mice (SCID) have DNA-PKcs mutations, which compromises their lymphocyte development and function, resulting in a severely compromised immune system in these mice (Nonoyama and Ochs, 1996). Given their role in lymphocyte

development, antigen repertoire diversity and class switch recombination, NHEJ is essential for normal lymphocyte development and immune function.

NHEJ and Malignancies

Defective NHEJ predisposes cells to genomic instability, impaired development, and apoptosis and may result in malignant transformation. As previously noted, dividing mammalian cells are subjected to approximately ten DNA DSBs per day (Lieber et al., 2003). Absence or impairment in DNA repair can result in growth arrest at various cell cycle checkpoints and subsequently induce apoptosis. Deficiency of one or more critical NHEJ repair factors results in either less efficient joining with deletion of a considerable DNA fragment or fusion of two unrelated genes (translocations), thus setting the stage for neoplastic progression (Han and Yu, 2008). Several lines of evidence from human patient studies highlight the pivotal role of NHEJ in neoplastic transformation. First, many cancer patients have gross chromosomal abnormalities such as translocations, which occurred as a result of DNA double strand breaks and their misrepairs (Lieber et al., 2006). Second, during physiological VDJ recombination in T lymphocytes, recombination of a TCR gene with a protooncogene gene can occur, resulting in a *TCR* gene translocation as observed in many cases of T cell acute lymphoblastic leukemia (Tsai et al., 2008). Third, translocations have been reported in B lymphocytes during VDJ recombination and CSR whereby an immunoglobulin gene is recombined with an oncogene such as *MYC* or *CCND1* resulting in neoplastic transformation (Hwang et al., 2011; Yan et al., 2007). Fourth, analysis of large B cell lymphoma samples showed frequent deletions in *IgG* switch regions and subsequent misrepairs suggestive of aberrant NHEJ as a key mechanism underlying lymphoma development (Lenz et al., 2007). Fifth, patients with gene polymorphisms for the NHEJ factor *LIGASE4* showed an increased risk for developing multiple myeloma when these polymorphisms

result in a change in one or more amino acids (Roddam et al., 2002). Finally, analysis of mature B cell lymphoma patient samples showed missense mutations in the Cernunnos gene, an accessory factor for NHEJ, and lead to chromosomal translocations and malignancy (Du et al., 2012). These findings show that alterations in the NHEJ factors can result in serious implications and lead to a wide variety of malignant conditions.

NHEJ and Aging

Aging is an irreversible physiological process characterized by increased risk of disease, reduced effectiveness of vaccines, and decreased immune responses (Boraschi et al., 2010; Frasca and Blomberg, 2011). Present paradigms of aging include 1) exhaustion of stem cell reserve (Rossi et al., 2008); 2) loss of replicative power associated with reduced telomerase function (Akbar and Vukmanovic-Stejic, 2007; Nalapareddy et al., 2008); and 3) accumulation of unrepaired DNA damages (Hoeijmakers, 2009; Rube et al., 2011). Of these, DNA damage accumulation has been validated in a variety of tissues including stem cell components during aging (Freitas and de Magalhaes, 2011). Accrual of DNA damage may be due to increased free radical production in old age, as well as increased radiosensitivity associated with aging (Ames et al., 1993; De Bont and van Larebeke, 2004; Kato et al., 2011). Further, diminishing levels of anti-oxidant defenses, especially those provided by superoxide dismutase (Rybka et al., 2011), glutathione peroxidase (He et al., 2009), and glutathione reductase, have been associated with aging in humans (Venkateshappa et al., 2012), making geriatrics more prone to oxidative cell damage. A second explanation is that ineffective DNA repair mechanisms contribute to the accumulation of DNA damage during old age (Puthiyaveetil and Caudell, 2012). Further understanding of the aging mechanism is important to developing strategies for slowing down the aging process, reducing age-associated immunodeficiencies, and in improving the productive life span of individuals.

A number of animal models for aging have diminished NHEJ activity in their cells. For example, NHEJ in neurons from old rats is severely compromised (Sharma, 2007; Vyjayanti and Rao, 2006), suggesting age-related down-regulation of NHEJ activity. Recent studies using embryonic fibroblast cells showed that increased DNA repair activity is directly correlated with replicative life span of these cells (Park et al., 2011), which also suggests diminished DNA repair in old age as a possible reason for reduced replicative potential of cells. Patients with premature aging syndrome have reduced DNA repair factors in their cells, further validating the role of DNA repair in the aging process (Martin and Oshima, 2000). Mice with defective NHEJ have diminished hematopoietic stem cell (HSC) proliferative potential and exhibit a functional exhaustion in an age-dependent manner. This suggests that DNA damage accumulation in these models may result in stem cell exhaustion and serves as a key mechanism during aging (Rossi et al., 2007). Also, studies from old C57BL/6 mice showed that developing T cells from thymus had increased number of DSBs, with no corresponding increase in DNA repair factors (Hesse et al., 2009). Additionally, results from our laboratory suggest that stimulated primary B cells from old mice have reduced NHEJ efficiency compared to B cells from adult mice (Puthiyaveetil and Caudell, 2012). Taken together, these reports suggest a less efficient activation of NHEJ mechanism in response to DNA damage, which subsequently results in increased apoptosis and reduced lymphocyte repertoire observed in old age (Hesse et al., 2009).

NHEJ and Embryonic Development

Embryonic development involves selective proliferation, differentiation, and apoptosis of cells. NHEJ has been shown to be critical during embryonic neuronal development. Knockout mice for NHEJ factors *Ligase 4* (*Lig4*$^{-/-}$) and *Xrcc4* (*Xrcc4*$^{-/-}$) are embryonically lethal, not because of compromised immunity, but rather due to neuronal apoptosis (Frank et al., 2000). However, the

precise role of NHEJ in neuronal development is not clearly understood. Prominent theories for DNA break occurrence in neurons are 1) those resulting from specific cleavage of DNA fragments as in VDJ and CSR mechanisms and 2), non-specific DNA damage by metabolic reactive oxygen species (Barzilai et al., 2008). Some studies suggest that somatic DNA rearrangement may occur in neurons similar to lymphocyte gene recombination (Chun and Schatz, 1999). This is based on the observation of highly diverse odorant receptors (Buck and Axel, 1991); however, sequence analyses were inconclusive for the possibility of NHEJ mediated gene recombination events in neurons (Hasin et al., 2008; Linardopoulou et al., 2005). During embryonic neurogenesis, a predominantly non-replicating layer of cells, known as the intermediate zone, may suffer DNA damage due to yet-to-be identified reasons. Mouse models defective in NHEJ factors have neuronal apoptosis in the intermediate zone, often resulting in embryonic mortality as observed in DNA *Ligase 4*$^{-/-}$ and *Xrcc4*$^{-/-}$ models (Frank et al., 2000). These phenotypes could be rescued by deleting *p53* (*Lig4*$^{-/-}$/*p53*$^{-/-}$ or *Xrcc4*$^{-/-}$/*p53*$^{-/-}$), suggesting that the apoptosis is mediated by *p53* (Frank et al., 2000; Orii et al., 2006). These findings also support the requirement of intact NHEJ mechanisms for neuronal development and that deficiency of NHEJ factors can lead to *p53* mediated apoptosis of neurons and result in embryonic mortality or retarded growth. Though the source of DNA breaks in neuronal development is not well understood, the role of C-NHEJ and probably, alternative end joining, is essential for proper neuronal and embryonic development.

Mouse Models for NHEJ

Genetically engineered animal models have been widely used to study DNA repair mechanisms and their subsequent alterations under various physiological and pathological conditions affecting both humans and animals. Many knockout and deficiency mouse models have been

developed to delineate the role of classical NHEJ factors and their interactions *in vivo*. Here we will discuss selected animal models used for classical NHEJ and AEJ studies. A summary is provided in Table 1.

Ku Proteins

The formation of DNA DSBs activates ATM, which in turn recruits DNA repair factors including Ku proteins, Ku70 and Ku80, at the break site. Owing to their hollow basket shape, Ku proteins bind around the DNA break ends and stabilize them from nucleolytic damage. Ku proteins are involved in recruitment of DNA-PKcs, as well as possess binding sites for XLF, another DNA repair factor. Recent studies involving end processing mechanisms showed that Ku proteins are responsible for excision of abasic nucleotides at the DSB site, and help in effective DNA repair (Roberts et al., 2010). Finally, Ku proteins are involved in telomere maintenance in addition to their role in NHEJ. Ku deficiency can result in defective proliferation of cells including B lymphocytes, probably due to its role in telomere maintenance (Casellas et al., 1998; Manis et al., 1998).

Knockout models for Ku70 and Ku80 have been widely used to characterize the role of Ku proteins during NHEJ and other conditions. *Ku70*$^{-/-}$ and *Ku80*$^{-/-}$ mice have impaired VDJ recombination, arrested lymphocyte development, growth retardation, and increased radiosensitivity (Nussenzweig et al., 1996). Analysis of RSS junctions in VDJ regions of Ku70$^{-/-}$ mice revealed increased microhomologies, suggesting the presence of microhomology-mediated DNA repair mechanism in the absence of *Ku70* (Gu et al., 1997; Weinstock and Jasin, 2006). Introduction of pre-rearranged VDJ fragments in *Ku70*$^{-/-}$ and *Ku80*$^{-/-}$ mice rescued the phenotype and resulted in the presence of mature IgM^{+} B lymphocytes. However, upon antigenic stimulation with LPS and IL-4, these cells failed to undergo efficient CSR, suggesting that Ku

proteins are essential for VDJ and CSR (Casellas et al., 1998). Furthermore, *Ku70*$^{-/-}$ and *Ku80*$^{-/-}$ mouse embryos showed defective embryonic neurogenesis as a result of increased neuronal apoptosis (Gu et al., 2000), suggesting that NHEJ mediated neuronal development is impaired in these mice. Mice with deletion of *Ku80* (*Ku80*$^{-/-}$) resulted in early onset of senescence (Vogel et al., 1999), underlining the role of altered NHEJ in aging. Recent studies using a conditional *Ku70* knockout under a tetracycline on/off system in mature B cells from mice revealed a novel role for this protein in phosphorylating *p53* (Tomimatsu et al., 2007). Analysis of telomeres in *Ku70*$^{-/-}$ with telomere protein TRF2 deficient mice showed increased frequency of sister telomere exchanges mediated by homologous recombination (Celli et al., 2006). This suggested that *Ku70* prevented unequal exchange and probable shortening of telomeres, a key mechanism implied during genomic instability and aging. B lymphocytes from mice with combined absence of *Ku70* and *Ligase 4* (*Ku70*$^{-/-}$ *Lig4*$^{-/-}$) when stimulated *in vitro* showed considerable class switch recombination towards IgG1, but not towards other antibody isotypes. Production of comparable IgG1 suggested the presence of one or more alternative end joining mechanisms during CSR in the absence of C-NHEJ (Boboila et al., 2010b). Finally, studies from *Ku70*$^{-/-}$ mice showed increased sister chromatid exchange in their fibroblasts and a higher frequency for spontaneous malignant transformation to T cell lymphomas (Li et al., 1998). These studies using genetically modified animal models suggest that Ku proteins are essential for the NHEJ mechanism and help in cell proliferation, DNA repair, and telomere maintenance.

Protein Kinase

DNAPKcs acts as a scaffold for DNA repair factors after binding with Ku70/Ku80 dimer, and also activates Ligase 4, Artemis and Cernunnos through phosphorylation (Yaneva et al., 1997). A variety of animal models have been developed by either knocking down the gene or by

mutating the kinase activity, with varying results between these two groups. Studies in DNAPKcs knockout (*DNAPKcs*$^{-/-}$) mice showed that the deficiency can result in early onset of aging with a higher incidence of infections (Espejel et al., 2004). These mice also had impaired VDJ and CSR, in addition to a higher frequency of T cell lymphomas. When *DNAPKcs*$^{-/-}$ mice were introduced with pre-rearranged immunoglobulin heavy and light chains (*DNAPKcs*$^{-/-}$*Ig*) using a knockin strategy, they had proper lymphocyte development with comparable germ line transcript and Aid induction (Franco et al., 2008; Manis et al., 2002). When these naïve B cells were antigen stimulated, class switch efficiency was severely compromised to all subclasses, except for IgG1 (Manis et al., 2002). Results from *DNAPKcs*$^{-/-}$*Ig* and *DNAPKcs* kinase mutant models were inconsistent; null models had impaired CSR whereas SCID mutant model had comparable CSR (Cook et al., 2003; Kiefer et al., 2007). This could be explained by the probable non-essentiality of kinase activity of DNA-PKcs, or that the activity is replaceable with similar proteins, and that DNAPKcs acts only as a synapse complex mediator during CSR (DeFazio et al., 2002). *DNA-PKcs* point mutations lead to variable effects in CSR, whereas complete deletion leads to abolition of CSR (Bosma et al., 2002; Manis et al., 2002). However, T lymphocyte engraftment in *DNAPKcs* kinase deficient mice resulted in CSR and increased plasma antibody concentration (Kiefer et al., 2007).

Artemis

Artemis is an exo-endonuclease enzyme, which acts along with DNA-PKcs during NHEJ. This enzyme cleaves the 5' to 3' over hang ends and linearizes the hairpin loop formed after the DSB, as in RAG-mediated breaks (Ma et al., 2002). Approximately 50 different types of Artemis mutations have been reported in patients, which include mis-sense and non-sense mutations, deletions and insertions (Pannicke et al., 2010). Mutations in Artemis lead to impaired VDJ

recombination, increased radiosensitivity, and loss of B and T lymphocytes (Li et al., 2005). Although bone marrow transplantation is required for rescuing the lymphopenia that occurs in patients with Artemis mutations, graft rejection mediated by functional NK cells has been reported in a subset of patients (Shizuru et al., 1996). Recent studies in *Artemis* (*mArt*$^{-/-}$) deficient mice have suggested that using anti-NK cell antibodies might be a potential therapy to prevent NK cell-mediated graft rejection in these patients (Xiao et al., 2012). Mouse models with hypomorphic mutations in Artemis have been shown to have aberrant VDJ recombination events with T and B cell mediated immune deficiency (Huang et al., 2009). Upon deletion of *p53* in *Artemis* hypomorphic (Art-p70) mice, the mice exhibited increased predisposition for chromosomal translocations and thymic lymphomas (Jacobs et al., 2011). Homozygous knockout mice for *Artemis* resulted in severely reduced lymphocyte counts in mice suggesting that C-NHEJ is necessary for VDJ recombination (Rivera-Munoz et al., 2009). Insertion of rearranged Immunoglobulin heavy and light chains in *Artemis* deficient mice (*Artemis*$^{-/-}$) rescued the B lymphocytes resulting in faithful CSR, suggesting that *Artemis* might not be essential for class switch recombination (Rooney et al., 2005). However, conditional knockout of *Artemis* in mature B lymphocytes under a Cre-/LoxP system showed lower IgG3 class switch recombination and IgA antibody production, suggesting that CSR is compromised in the absence of *Artemis* (Rivera-Munoz et al., 2009). Though Artemis is essential for VDJ recombination, its reduced dependence during CSR might be due to widely varied types of DNA breaks occurring at switch regions, with less frequent hairpin formation at break ends.

Ligase 4

DNA *Ligase 4* is one of the four core NHEJ factors as it mediates the actual ligation process. Targeted disruption of *Ligase 4* in mice resulted in embryonic lethality with extensive p53

mediated apoptosis in neuronal tissue (Barnes et al., 1998). Knocking out *p53* along with *Ligase 4* resulted in live pups, though the pups were smaller with growth retardation. Further, these mice (*Lig4*$^{-/-}$*p53*$^{-/-}$) had impaired B and T cell development and succumbed to lymphomas due to fusion of *IgH* to oncogenic genes (Frank et al., 2000). Conditional knockout of *Ligase 4* in mature B cells showed less efficient CSR mechanisms, further suggesting its requirement for isotype switching in the periphery (Han and Yu, 2008). Due to the embryonic lethality in complete absence of *Ligase 4* (*Lig4*$^{-/-}$), deficient mouse models are created with hypomorphic mutations. A recently described mouse model for *Ligase 4* deficiency syndrome with knockin of *Lig4* R278H mutation showed characteristic features of human Ligase 4 syndrome with growth retardation and immunodeficiency. Reduced *Ligase 4* function in this model resulted in a partial block of B and T cell development, reduced lymphocyte repertoire, and genomic instability (Rucci et al., 2010). Further, these mice had a higher incidence of thymic tumor development (Rucci et al., 2010), underlying the fact that impaired NHEJ can result in malignant progression apart from immunological impairment. Though studies have shown that even low levels of *Lig4* is sufficient for CSR (Windhofer et al., 2007), its reduction in mice resulted in observable immunodeficiencies. A different mouse model with hypomorphic *Ligase 4*, Lig4Y288C, exhibited features of aging such as progressive loss of hematopoietic stem cells, immunodeficiency and severely impaired stem cell function (Nijnik et al., 2007). Similar to other NHEJ deficient mice, they showed impaired neurogenesis with increased apoptosis in the intermediate zone of the brain (Gatz et al., 2011). These studies validated the absolute necessity of Ligase 4 for the classical NHEJ pathway.

Table 1. Summary of selected animal models for NHEJ and AEJ mechanisms showing their genotypic and phenotypic features.

Gene/Protein	Mouse Model	Immunological phenotype	Cancer Phenotype	Other Features	Reference
Ku70	$Ku70^{-/-}$	Impaired VDJ recombination	Thymic and T cell lymphoma	Growth retardation, defective neurogenesis, radiosensitivity, GI abnormalities	Nussenzweig et al., 1996; Weinstock et al, 2006, Sfeir et al., 2012
	$Ku70^{-/-}$ IgH/IgL KI	Reduced CSR			Casellas et al., 1998
Ku80	$Ku80^{-/-}$	Impaired VDJ recombination		Growth retardation, defective neurogenesis, radiosensitivity	Gu et al., 2000; Vogel et al., 1999, Sfeir et al., 2012
	$Ku80^{-/-}$ IgH/IgL KI	Reduced CSR			Yan et al., 2007; Manis et al., 1998
DNAPKcs	$DNAPKcs^{-/-}$	Impaired VDJ and CSR	Lymphoma	Accelerated aging	Espejel et al., 2004
	$DNAPKcs^{-/-}$ IgH/IgL KI	Impaired CSR except for IgG1			Franco et al., 2008, Manis et al., 2002
	DNAPKcs point mutation	Impaired VDJ and CSR (SCID)		Compromised immunity	Cook et al., 2003
Ligase 4	$Ligase4^{-/-}$-$p53^{-/-}$	Impaired VDJ recombination	Lymphoma	Neuronal apoptosis	Frank et al., 2000
	Lig4R278H, Lig4Y288C	Partial VDJ block	Thymic tumors	Genomic instability, accelerated aging	Rucci et al., 2010; Nijnik et al., 2007
Xrcc4	$Xrcc4^{-/-}$-$p53^{-/-}$	Impaired VDJ recombination	Pro-B leukemia		Frank et al., 2000; Gao et al., 2000
	$Xrcc4^{c/c}$	Impaired CSR	IgH-cmyc translocation	immunodeficiency	Sprauel et al, 2007
Artemis	$Art^{-/-}$	Impaired VDJ recombination		Genomic instability	Xiao et al., 2012
	Artemis hypomorphic	Aberrant VDJ recombination	Thymic lymphoma		Jacobs et al., 2011
	Art-/-IgH/IgL KI	Normal CSR			Rooney et al., 2005
Cernunnos	$Xlf^{-/-}$	Progressive lymphopenia	B cell lymphoma	Genomic instability	Li et al., 2008; Zha et al., 2007
Ligase 3	$Ligase3^{c/c}$	Normal NHEJ and AEJ		neuronal apoptosis, reduced mitochondrial repair	Gao et al., 2011
	$Ligase3^{-/-}Ligase4^{-/-}$				Boboila et al., 2012
Xrcc1	$Xrcc1^{c/c}$	Normal NHEJ and AEJ			Boboila et al., 2012

Abrreviations: VDJ= Variable Diversity Joining recombination, CSR= Class switch recombination, SCID= severe combined immunodeficiency, NHEJ= nonhomologous end joining, AEJ= alternative end joining, KI= knock-in

XRCC4 (X-Ray Cross Complementing factor 4)

XRCC4 is another core component of NHEJ pathway, which acts as a cofactor for Ligase 4 during the ligation process. Studies in mice have shown that deficiency of *Xrcc4* or *Ligase 4* resulted in impaired VDJ recombination, leading to a block in B and T lymphocyte differentiation (Gao et al., 1998). Deletion of either *Ligase4* or *Xrcc4* is embryonically lethal in mice (Barnes et al., 1998; Frank et al., 1998). As impaired NHEJ results in *p53*-mediated apoptosis, deletion of *p53* rescues the embryonic lethality phenotype observed in *Ligase4*$^{-/-}$ and *Xrcc4*$^{-/-}$ models. Despite the fact that *Xrcc4*$^{-/-}$ *p53*$^{-/-}$ mice are born alive, they develop Pro-B leukemia immediately after birth (Frank et al., 2000; Gao et al., 2000). However, conditional knockout of *Ligase 4* or *Xrcc4* in the mature B lymphocytes had less effect on CSR, probably due to alternative pathways for gene recombination during CSR (Soulas-Sprauel et al., 2007a; Yan et al., 2007). This conditional knockout of Xrcc4 gene under CD21- Cre/loxP recombination system in mice resulted in immunodeficiency and increased occurrence of peripheral B cell lymphomas (Gao et al., 2000), further suggesting the oncogenic potential of impaired NHEJ mechanisms (Mills et al., 2003). Combined deficiency of NHEJ factors such as *Ligase 4*$^{-/-}$ and *Xrcc4*$^{-/-}$ or *Ligase 4*$^{-/-}$ and *Ku70*$^{-/-}$ in mice promoted alternative end joining during CSR, with increased risk for *IgH* translocations and malignant progression (Boboila et al., 2010a; Boboila et al., 2010b).

Cernunnos/ XLF

Cernunnos/XLF is an accessory component of the *Ligase4/ Xrcc4* complex, which promotes adenylation of the ligation complex and helps in completing the ligation process (Riballo et al., 2009). *Ku* proteins possess the binding site for XLF and hold it in place for effective reactivation of *Ligase 4*. *XLF* deficiency leads to progressive lymphopenia rather than a complete

development block as observed in mice with a SCID phenotype (Li et al., 2008). Mice with an *XLF* exon deletion showed reduced peripheral lymphocyte counts, without typical VDJ recombination deficiency as observed in other NHEJ deficiency/knockout models (Li et al., 2008). Though VDJ recombination is not directly affected in XLF deficient mice, DNA repair and genomic instability is compromised in these mice (Zha et al., 2007). These studies suggest that though XLF participates in C-NHEJ mechanism, its deficiency may not compromise the integrity of NHEJ.

Other Factors

Though not regarded as a key NHEJ factor, Ataxia telagienctasia mutated (ATM) kinase is another protein kinase involved in NHEJ, which shares many kinase functions with DNAPKcs. *ATM* mutations cause ataxia-telangiectasia in humans, a condition characterized by cerebellar degeneration, immunodeficiency, genomic instability and cancer predisposition (McKinnon, 2004). ATM has been shown to phosphorylate H2AX, Ku proteins, Ligase 4, Artemis, XRCC4 and XLF (Gapud and Sleckman, 2011). Combined deletion of ATM and DNAPKcs is embryonically lethal (Gurley and Kemp, 2001). ATM is an early responder to DNA DSBs and is responsible for the phosphorylation of histone H2AX and recruitment of DNA repair factors at the break site (Burma et al., 2001). Phosphorylated H2AX stabilizes DNA break ends until it is repaired. ATM deficiency has been shown to result in incomplete repair of DSBs and progression of cells with DNA damage towards mitotic stage. DNA damage response factor, H2AX, has also been shown to be critical in DNA damage repair, which becomes phosphorylated and acts as a marker for DNA DSBs. H2AX$^{-/-}$ mice showed increased radiosensitivity, immunodeficiency and growth retardation with impaired recruitment of DNA response proteins such as Nbs1, 53bp1 and Brca1 (Celeste et al., 2002). 53bp1, another DNA

damage response factor is essential for CSR; its absence during VDJ recombination leads to loss of T cell receptor integrity, increased apoptosis and lymphopenia (Difilippantonio et al., 2008). *In vitro* studies using a *Mdc1* knock down model system suggested that Mdc1 directly binds to H2AX and accelerates NHEJ mediated DNA repair (Dimitrova and de Lange, 2006). Knockout mice for DNA polymerase μ have immunodeficiency, as well as abnormal B cell differentiation with aberrant immunoglobulin light chain recombination (Bertocci et al., 2003). Taken together, these studies show that multiple factors regulate and direct the NHEJ process.

Alternative End Joining (AEJ)

Though C-NHEJ is the dominant DNA DSB repair pathway, other types of DNA repair can occur, especially in the absence of one or more NHEJ factors, with significantly more errors and possible translocations (Weinstock et al., 2006). This less efficient mechanism(s) is ascribed as Alternative end joining (AEJ) or microhomology mediated end joining (MMEJ). NHEJ proteins *Ku70, Ku80, XRCC4* and *Ligase 4* are evolutionarily conserved in their role of NHEJ mediated repair in mammalian cells and considered as core NHEJ factors. Oncogenic translocations observed in *Ligase 4*$^{-/-}$ and *p53*$^{-/-}$ knockout mice or *XRCC4*$^{-/-}$ and *p53*$^{-/-}$ mice could have resulted from gene fusions mediated through an alternative pathway. These less efficient recombinations are characterized by increased frequency of microhomologies at the joining region. Nussenzweig and colleagues have suggested the possible presence of more than one alternative end joining pathway, as depicted in Figure 2-2. Due to their variability in probable participating proteins and factors, alternative end joining can be defined as any form of end-joining happening in the absence of core NHEJ factors. Upon DNA breaks, protein factors such as Nbs, Mre11 and CtIP have been shown to direct the end joining towards alternative pathway by mediating the end processing (Della-Maria et al., 2011; Deriano et al., 2009). Extra-chromosomal plasmid ligation

assays, biochemical analysis, and chromosomal endonuclease sites suggests involvement of DNA ligase 3 and its cofactor XRCC1 in alternative end joining (Audebert et al., 2004; Della-Maria et al., 2011; Simsek et al., 2011). Based on these analyses, DNA ligase 3 and its cofactor XRCC1 are considered key components in AEJ; however, their role in AEJ is under debate. Given that AEJ is implicated in multiple diseases, delineating its precise mechanism is important to developing therapeutic strategies for patients with those conditions.

Ligase 3

Ligase 3 family is a third type of DNA repair protein found exclusively in vertebrates (Ellenberger and Tomkinson, 2008). There are two subtypes of Ligase 3 enzymes, *Ligase 3α* found in both nucleus and mitochondria (Lakshmipathy and Campbell, 1999), and splice variant Ligase *3* β found in germ cells (Mackey et al., 1997). Nuclear *Ligase 3α* in association with *XRCC1* completes Base Excision Repair (Frosina et al., 1996), single strand break repair (Okano et al., 2005) and Nucleotide Excision Repair pathways (Moser et al., 2007). *Ligase 3* knockout mice were embryonically lethal due to impairment in mitochondrial DNA repair mechanisms (Puebla-Osorio et al., 2006; Simsek et al., 2011). However, complementing expression of *Ligase 3*, *Ligase I* or viral or bacterial ligases in mitochondria could rescue the phenotype (Simsek et al., 2011), suggesting that requirement of *Ligase 3* could be circumvented by alternative enzymes. Conditional deletion of *Ligase 3* in mouse nervous system showed increased neuronal apoptosis and defective mitochondrial DNA repair, while Xrcc1-LigaseIII mediated nuclear DNA repair was not affected (Gao et al., 2011). Finally, studies with depletion of both *Ligase 3* and *Ligase 4* have been shown to have normal levels of alternative end joining (Boboila et al., 2012), suggesting that Ligase III may not be an essential factor for alternative end joining.

XRCC1

In vitro studies using nuclear extracts have suggested that XRCC1 acts as a cofactor for Ligase III in alternative end joining, in the place of XRCC4 in NHEJ. Also, studies using heterozygous *Xrcc1 (Xrcc1$^{+/-}$)* mice showed normal CSR with less frequent microhomology at switch regions, suggesting that *Xrcc1* is involved in alternative end joining (Saribasak et al., 2011). However, animal studies with *Xrcc1* knockout and core NHEJ knockout mice showed some degree of end joining, suggesting the dispensability of *XRCC1* for the alternative end joining. Recent studies using conditional deletion of *Xrcc1* along with C-NHEJ factor *Xrcc4* showed that the effect of *Xrcc1* loss was negligible for CSR and *IgH* translocations, further suggesting that the role of *XRCC1* in alternative end joining is less evident (Boboila et al., 2012).

Ligase 1

Ligase 1 is an important enzyme during DNA replication, where it serves a crucial role in the ligation of okazaki fragments formed during cell division (Applegren et al., 1995). Knockout mice for *Ligase 1* are embryonically lethal (Bentley et al., 1996), validating that *Ligase 1*, similar to *Ligase 3* and *Ligase 4*, is essential for embryonic development. However, its role in AEJ is still under debate. Recent studies showed that AEJ can occur in the combined absence of *Ligase 4* and *Ligase 3*, suggesting that *Ligase 1* might be involved in this mechanism as it is the only other known Ligase enzyme in vertebrates (Boboila et al., 2012).

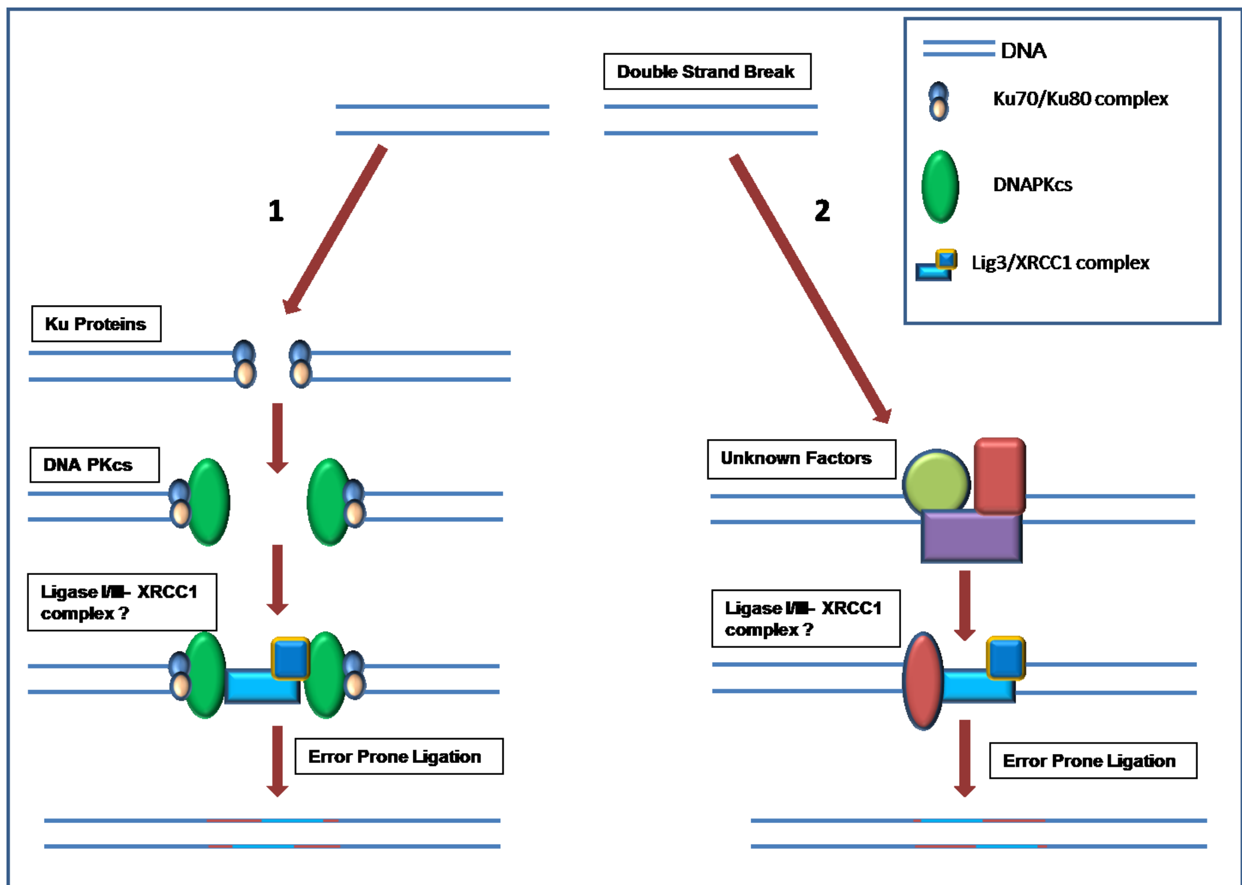

Figure 2-2. Schematic representation of Alternative End Joining pathways
Alternative end joining takes place in the absence of one or more core C-NHEJ factors. More than one pathway may be involved to complete this error prone ligation process. The first probable pathway (1) utilizes C-NHEJ factors Ku complex and DNAPKcs and the ligation is mediated by either LIG3-XRCC1 complex or by Ligase 1. A second pathway (2) has also been proposed, i.e., in the absence of both Ku proteins and Ligase4/XRCC4 complex, cells can still repair DNA breaks with the help of still unidentified factors; ligation is mediated by LIG3-XRCC1 or LIG1, with significant errors.

Miscellaneous Models

Mouse models for various conditions have altered NHEJ in addition to their primary phenotype. The *FLT3*/ITD Mouse model for myeloproliferative disease has a partial block in B lymphocyte development. Further analysis of this model showed that Pro B cells had reduced *Ku* proteins which might impair the classical NHEJ (Li et al., 2011). Furthermore, AEJ factor, *Parp1* has been found to be elevated, explaining the reason for increased misrepair frequency observed in this mouse model during VDJ recombination (Li et al., 2011). A mouse model for myelodysplastic syndrome, *NUP98-HOXD13* (*NHD13*) has impaired lymphocyte development (Choi et al., 2009; Puthiyaveetil et al., 2012) apart from MDS and gradual progression to leukemia after acquiring secondary mutations (Slape et al., 2008). Alternatively, the knock in mouse model for both *FLT3/ITD* and *NHD13* resulted in increased penetrance and leukemia at an earlier stage, suggesting co-operative action of impaired NHEJ and collaborating mutations (Greenblatt et al., 2012). Mouse models with mutated *Huntingtin (Htt)* gene impair with *Ku70*, leading to increased DNA breaks in their neurons with neurodegeneration and behavioral issues. Supplementing the *Htt* mice with *Ku70* reverses the neurodegeneration and abnormal behavior suggesting that restoration NHEJ could rescue the phenotype (Enokido et al., 2010). Finally, mice with NRAS/BCL2 mutations develop lesions similar to myelodysplastic syndrome, and demonstrate an increased frequency of NHEJ misrepair activity and increased marrow blasts (Rassool et al., 2007). Though altered NHEJ results in mutations and malignant transformation, many transformed cells have highly effective NHEJ mechanism as a survival method. Interfering with NHEJ mechanism along with chemotherapeutic agents has been shown to be effective in some tumor treatments. For example, when chemotherapeutic agents were supplemented with

siRNA for *RAD51*, the DNA breaks produced by the agents were not repaired properly, resulting in the apoptosis of tumor cells (Kiyohara et al., 2011).

Conclusion

Mouse models have provided tractable systems for studying the biochemical and sequential steps necessary for C-NHEJ and AEJ mechanisms. Studies have shown that Ku70, Ku80, DNAPKcs, Artemis, Ligase 4, XRCC4 and Cernunnos as the key components of C-NHEJ mediated DNA DSB repair mechanism. However, despite the new data emerging on the crucial factors necessary for AEJ from *in vitro* and *in vivo* studies there remains considerable debate on the exact function of this mechanism during DNA DSB repair. Understanding the DNA repair mechanisms is important in sequentially targeting the defects in immune responses, malignant progression, aging and embryonic development. Future studies utilizing more sophisticated animal model systems will delineate the underlying molecular mechanisms of AEJ to a greater detail. Finally, insight gained through the use of animal models will serve as highly translational platforms for developing novel therapeutic strategies for patients with inherent or acquired DNA DSB misrepair.

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CHAPTER 3

Non Homologous End Joining Mediated DNA Break Repair is Impaired in B lymphocytes of Aging Mice

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Abstract

Aging is an irreversible physiological process characterized by increased risk of diseases, reduced effectiveness of vaccines, and decreased immune responses. One of the most prominent paradigms of aging and age related conditions is the progressive accumulation of un-repaired DNA breaks leading to apoptosis and exhaustion of stem cells. Here we hypothesized that B lymphocytes from old mice have reduced DNA repair mechanisms as a contributing factor for DNA break accumulation. We analyzed Class Switch Recombination (CSR) of naïve B lymphocytes from old and adult mice to delineate the DNA double strand repair mechanisms during aging. In vitro CSR assays and DNA break analysis by labeling phosphorylated Histone H2AX showed that old mice had significantly reduced DNA repair efficiency following DNA breaks. Functional efficiency analysis of DNA break repairs using plasmid ligation method showed that B lymphocytes from old mice had poor repair efficiency and increased misrepair of linear plasmid. Diminished DNA repair in old age can contribute to reduced immune cell repertoire and impaired immunity; increased occurrence of cancer; and reduced stem cell reserve.

Key words: Aging, class switch recombination, B lymphocyte, DNA repair

Abbreviations

CSR- Class switch recombination

Introduction

Aging is an irreversible physiological process characterized by increased risk of diseases, reduced effectiveness of vaccines and decreased immune responses (Boraschi et al., 2010; Frasca and Blomberg, 2011). Both humoral and cellular immunity have been found to be diminishing as age progresses (Linton and Dorshkind, 2004; Zhou and McElhaney, 2011). Present paradigms for the reduced immune status in old ages include exhaustion of receptor repertoire on the T and B lymphocytes (Blackburn et al., 2009), the loss of replicative power associated with reduced telomerase function (Akbar and Vukmanovic-Stejic, 2007) and accumulation of unrepaired DNA damage (Effros et al., 2005). Reduced immunity in old age has also been shown to be associated with slower signal transduction (Sadighi Akha and Miller, 2005). Thus, aging is also associated with reduced immune surveillance, resulting in the persistence, and sometimes proliferation of, abnormal cells with genetic mutations resulting in malignant transformation (DePinho, 2000). DNA double strand breaks (DSBs) are considered as the most serious type of DNA damage, and are produced mostly by intrinsic oxidative stress or by external agents like ionizing radiation or chemicals (Hoeijmakers, 2009). Despite the fact that most of these cellular insults are repaired by non-homologous end-joining (NHEJ) and homologous recombination mechanisms, recent studies have shown that the DNA DSB frequency in stem cells increases with age (Rube et al., 2011). Hence, understanding the critical repair mechanisms is important in addressing many of the problems associated with aging.

Diminished antibody mediated immune response is a critical factor underlying the increased susceptibility for infectious disease in elderly people (McElhaney and Effros, 2009). Moreover, the duration of protective immunity diminishes as age progresses (Steger et al., 1996), suggesting multiple levels of compromised immunity. Naïve B lymphocytes in peripheral

circulation will undergo proliferation and rearrangement of their immunoglobulin heavy chain at constant regions (C_H), known as class switch recombination (CSR) following antigen presentation. CSR involves DNA DSBs mediated by activation induced deaminase (AID) which are repaired mostly by NHEJ mechanism. Recent studies have shown that old age results in reduced B cell diversity (Gibson et al., 2009), probably due to inefficient NHEJ mediated VDJ recombination and reduced output of bone marrow, or by the pathological expansion of selected B cell clones (Cancro, 2005; Ghia et al., 2004). Moreover, studies in old mice have shown that B lymphocytes have reduced class switch recombination efficiency (Frasca et al., 2008; Frasca et al., 2004). Considering the role of NHEJ in class switch recombination of B lymphocytes, it is reasonable to suspect that NHEJ is compromised in these lymphocytes, and possibly in other lymphocyte subsets, during aging.

DNA double strand breaks induced by physiological processes as in VDJ or CSR recombinations or by extrinsic agents often produce 3' overhangs. These overhang ends are processed by XRCC1-XPF to produce blunt ends (Schrader et al., 2004) and the DNA DSBs are joined by the classical non-homologous end joining mechanism. Proteins Ku70 and Ku80 bind to the DNA ends and act as a scaffold for the recruitment of other repair factors (Casellas et al., 1998; Manis et al., 1998). Artemis, an endonuclease enzyme in association with the DNA protein kinase catalytic subunit cleaves the DNA hairpin loop which is formed immediately after the end processing (Ma et al., 2002). DNA ligase4- XRCC4 complex helps in the ligation of donor and acceptor fragments of the immunoglobulin gene (Rooney et al., 2004) and completes the class switch recombination (Stavnezer et al., 2008). Several other factors including ATM (Falck et al., 2005), H2AX (Reina-San-Martin et al., 2003), Cernunnos (Buck et al., 2006; Hentges et al.,

2006), mediator of DNA damage check point 1(Mdc1) (Stewart et al., 2003) and 53BP1 (Pryde et al., 2005) have been described to play roles in the non-homologous end joining mechanism as well.

Given this understanding on DNA repair, we hypothesized that NHEJ is impaired in B lymphocytes from old mice and may serve as a contributing factor in reduced CSR. To test our hypothesis, we used primary B lymphocytes from mice aged 12 months (Adult) and 24 months (Old) to delineate the effects of aging on class switch recombination and the non-homologous end joining mechanism. We selected 12 month old mice as the control group to understand the differences in fine regulation of CSR and NHEJ in adult and old age groups. Our results show that as age progresses, B lymphocytes have reduced NHEJ efficiency and class switch recombination.

Materials and Methods

Animals

Up to five 12-month (Adult/control) and 24-month (Old) old FVB mice were used for each experiment. The mice were bred and maintained at the AAALAC accredited core laboratory animal facility at Virginia Maryland Regional College of Veterinary Medicine, Virginia Tech. All experiments were carried out as per NIH guidelines with the approval from the Virginia Tech Institutional Animal Care and Use Committee (IACUC).

Class Switch Recombination Assay

Splenic B lymphocytes were harvested using anti-mouse IgM magnetic beads and magnetic assisted cell sorting (MACS) system (Milteny Biotec, Auburn, CA). Cells ($2x10^{5)}$ were treated with 5 µM CFSE and cultured in triplicate in 96 well culture plates (BD Biosciences, San Diego,

CA) with media containing *E coli* Lipopolysaccharide (25 μg/ml)(Sigma Aldrich, St Louis, MO) and IL-4 (25ng/ml) (Sigma Aldrich,) and incubated at 37ºC for 96 hours. At the end of the incubation, the LPS + IL-4-treated cells were washed three times and labeled with fluorescently tagged anti-mouse IgG1 and IgE antibodies (e-Biosciences, San Diego, CA) (Puthiyaveetil et al., 2012).The percentage of cells undergoing class switch recombination events was assessed by flow cytometry.

DNA Break Analysis

Confocal Microscopy: Splenic B lymphocytes were harvested using MACS system and cultured 10^6 cells/ml in RPMI (ATCC, Manassas, VA) containing β Mercaptoethanol (Thermo scientific, Rockford, IL), *E coli* LPS, and IL-4. Cells were harvested at 0, 24, 48, 72 and 96 hours and cytospun onto charged slides. Cells were fixed using 4% paraformaldehyde (Thermo scientific) for 10 minutes, washed with TBS (Fisher Scientific, Pittsburg, PA) with Tween20 (Fisher Scientific) and blocked with TBS containing 4% Fetal bovine serum (FBS) for 1 hour at room temperature. Cells were incubated with rabbit anti-mouse γH2AX (Cell Signaling, Danvers, MA) at 1:500 dilution in TBS with 1% FBS followed by incubation with Alexafluor 488 conjugated anti-rabbit antibody (Cell Signaling) at 1:1000 dilution in TBS with FBS. After labeling with DAPI (Cell Signaling), cells were visualized using LSM700 Carl Zeiss confocal microscope (Carl Zeiss Microimaging, Thornwood, NY) and LSM900® software (Carl Zeiss).

Flow Cytometry: Splenic B lymphocytes cultured in the presence of LPS (Sigma) and IL-4 (Sigma) were harvested at 0, 24, 48, 72 and 96 hours as described. Cells were fixed with 70% ethanol in PBS at -20C for 24 hours and permeabilized with 0.1% Triton-X (Sigma Aldrich) in TBS. Cells were then incubated with rabbit anti-mouse phosphoH2AX antibody (Cell Signaling) at 1:500 dilution in TBS with FBS at 4ºC for 30 minutes followed by incubation with Alexa-

fluor 488 conjugated anti-rabbit antibody (Cell Signaling) at 1:1000 dilution. Cells were analyzed using FACScan flow cytometer (BD Biosciences).

Cell Cycle Analysis

Mouse B lymphocytes from five 12 months and 24 months were harvested and cultured in the presence of LPS and IL-4. Cells were incubated with propidium iodide (Sigma Aldrich) at 5µg/ml in PBS containing RNAaseA (5 Prime, Gaithersburg, MD) at room temperature for 20 minutes and kept on ice. Cells were analyzed by flow cytometry.

Gene Expression Analysis

B lymphocytes from adult and old mice were cultured in the presence of LPS+IL-4 and harvested at 0, 24, 48, 72 and 96 hours. Total RNA was harvested using Qiagen RNeasy kit as per manufacturer's protocol and first strand cDNA was synthesized using Superscript III (Invitrogen). Gene expression was determined in triplicate by RQPCR using an iQ™5 Multicolor Real Time PCR Detection System (BioRad) for the following genes *DNA ligase4* (5' ATTGAAGCCACGAGATTAGGT 3'; 5' ACTGAATCGGACACCCAACT 3'); *Ku80/86* (5' AATCCTGTTGAAAACTTCCGTT 3'; 5' GGAAGCTGTTGAAGCGCTG 3'); *KU70/XRCC6* (5' CCGCTTCACATACAGGAGCGAC 3'; 5' GGATTATAACCTGGAGGATAG 3'); *DNAPKcs* (5' GAGAGTGGGCTTTCAGAAGA 3'; 5' ATTTCCTCTGTCTGTCAGAAAT 3'); *p53* (5' CTCTGAGTAGTGGTTCCTGGCC 3'; 5' AAGTAGGCCCTGGAGGATAT 3'); *Ligase 3* (F 5' GCGGTCTATTCATCGTGCGGG 3''; 5' ATGCGGCACACGCCCTTTAC 3'*;* *Gapdh* control (5' GCACAGTCAAGGCCGAGAAT 3'; 5' CCTTCTCCATGGTGGTGAA 3'). Expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and results were normalized to corresponding *Gapdh* internal control.

DNA Ligation Efficiency Assay

Splenic B lymphocytes were harvested from mice using IgM magnetic beads and magnet assisted cell sorting system (Milteny Biotec). Cells ($2X10^6$) were checked for the purity by flow cytometry and cultured in RPMI (ATCC) medium containing *E. coli* Lipopolysaccharide, β Mercaptoethanol and Penicillin- Streptomycin. pUC18 (Roche Applied Sciences, Indianapolis, IN) plasmid was linearized using *Sma1*(New England Biolabs, Ipswich, MA) enzyme to produce blunt ends and gel purified. The plasmid (0.5µg) was introduced into the nucleus of B lymphocytes by nueclofection ® using Amaxa NucleofectorII (Lonza, Walkersville, MD) using program Z001 as per manufacturer's instructions. After 12 hours, cells were harvested, washed with PBS and plasmid was harvested using Qiaprep spin mini kit (Qiagen, Valencia, CA) using modified protocol for plasmid extraction from mammalian cells. Plasmid (200ng each) was transformed into chemically competent DH5-α cells (MaxEfficiency® - Invitrogen, Carlsbad, CA) and cultured on duplicate LB agar plates containing IPTG (5 Prime) and X-Gal (5 Prime) at 37ºC for 16 hours. Colonies were counted and repair efficiency was calculated depending on the number of colonies. Percentage of misrepair was calculated; blue colonies being correctly repaired and white colonies being misrepaired.

Data and Statistical Analysis

Flow cytometric data were analyzed using FlowJo software (FlowJo, Ashland, OR). Confocal images were analyzed using Zen 2009® software (Carl Zeiss Microimaging). Data was analyzed with GraphPad Prism 5.0® (Graphpad Software, La Jolla, CA), using two tailed t-test or ANOVA and Bonferroni posttest; a *P* value <0.05 was considered significant.

Results

B lymphocytes from old mice have reduced Class Switch Recombination efficiency

Consistent with previously published data in C57/B6 mice (Frasca et al., 2004), our results showed that B lymphocytes from old FVB mice had reduced class switch recombination efficiency when compared with adult mice. FVB mice are commonly used in non-clinical drug discovery and development. Due to their prominent pronuclei in fertilized eggs and large litter size(Taketo et al., 1991), they are especially useful in generating transgenic lines (Frese and Tuveson, 2007). Despite the utility in genetic studies, few studies have characterized their immune system. Naïve B lymphocytes from old mice, when cultured in the presence of LPS and IL-4, failed to undergo efficient CSR to form IgG1 and IgE positive lymphocytes (Fig 3-1. A, B). Analysis of the proliferation pattern using CFSE labeling of lymphocytes (Parish, 1999) from old mice showed reduced proliferation as indicated by fewer cells in subsequent generations when compared with cells from adult mice (Fig 3-1 C).

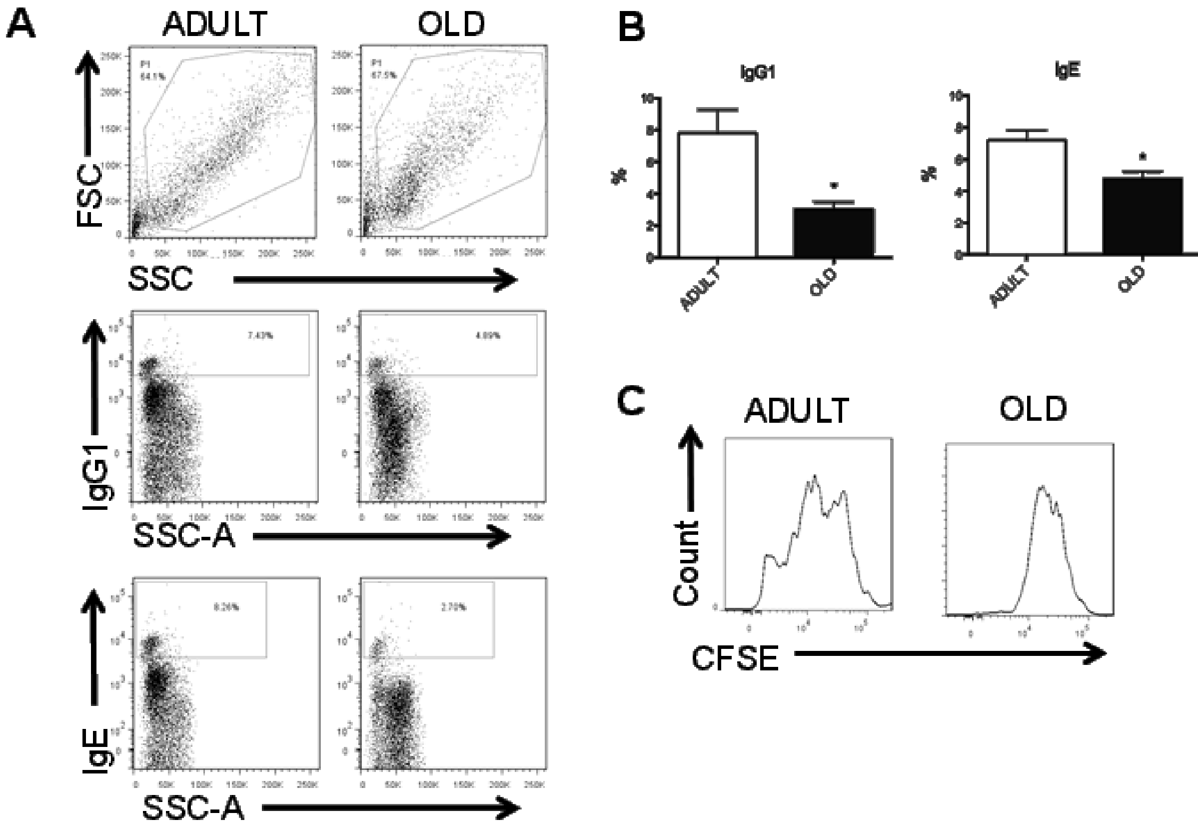
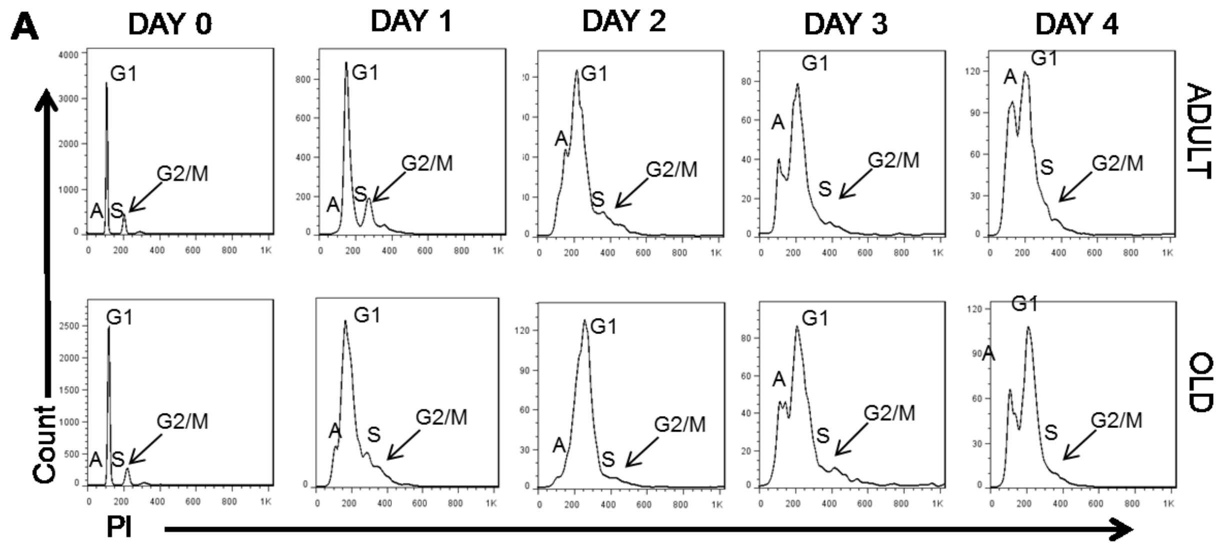


Figure 3-1. Naïve B lymphocytes from old FVB mice have reduced class switch recombination. B lymphocytes from old and adult mouse spleens were harvested by positive magnetic sorting and cultured in RPMI with LPS and IL-4. **A.** Representative flow cytometric plots of CSR pattern of B lymphocytes from old and adult mice showing reduced number of IgE/ IgG1 cells following CSR **. B.** Proliferation pattern analysis of IgE^+ and $IgG1^+$ cells showed that cells from old mice had decreased proliferation, as evidenced by altered pattern and fewer cells in later generations. **C**. Statistical analysis showed that B lymphocytes from old mice had significantly lower IgG1 and IgE CSR when compared with B lymphocytes from adult mice. n=5, *=p<0.05.



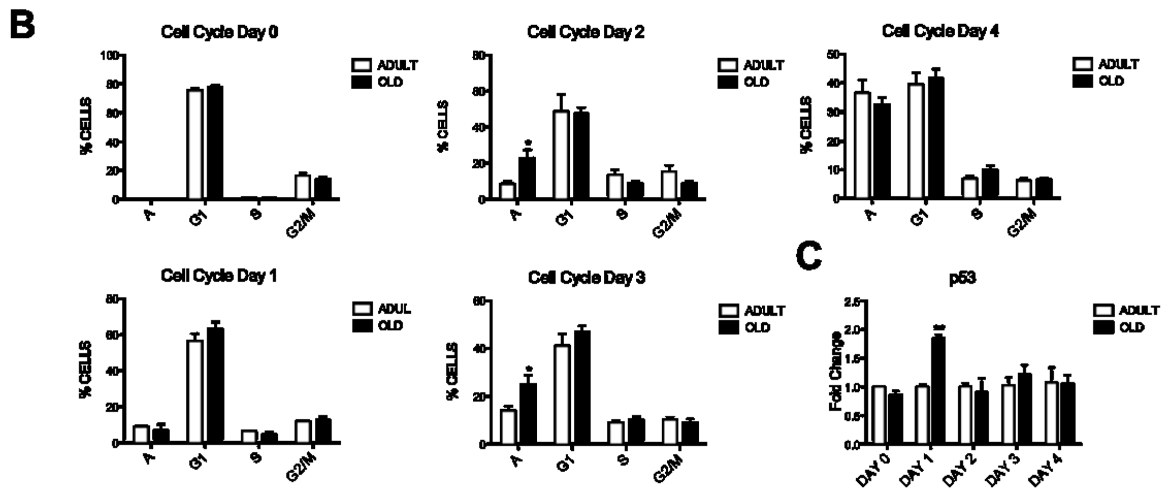


Figure 3-2. B lymphocytes from old FVB mice have increased apoptosis in vitro.
A. Representative cell cycle plots of B lymphocytes cells from adult and old mice stimulated in vitro at days 0, 1, 2, 3 and 4 days. **B.** Analysis of different cell cycle phases showed a significant increase in apoptosis on days 2 and 3 in B lymphocytes from old mice compared to adult mice. **C**. RQPCR analysis of B lymphocytes showed that *p53*, a pro-apoptotic gene, was upregulated in B lymphocytes from old mice on day 2. (A= apoptotic, G1=cells in growth phase1, S= Synthetic phase, G2/M= cells in growth phase 2 or mitotic cells) n=5, *=p<0.05, **=p<0.01.

Old and Adult mice have comparable cell cycle kinetics

Based on our preliminary results, we considered the possibility that cell cycle arrest and/or increased apoptosis are the contributing mechanisms for reduced CSR. To determine this, we stimulated naïve splenic B lymphocytes with LPS and IL-4 to induce CSR. Cells were harvested prior to, and at 24 hour intervals following stimulation and labeled with Propidium iodide (PI). Cell cycle patterns were analyzed by flow cytometry. As shown in Fig 3-2A, old and adult mice had similar cell cycle patterns at the time of stimulation (day 0) and day 1, with a lower percentage of apoptotic (A) and synthetic (S) stage cells. However, on days 2 and 3 following stimulation, old mice showed an increase in the percentage of apoptotic cells (Fig 3-2A, B). Considering the role of p53 as a pro-apoptotic molecule leading to cell cycle arrest and apoptosis(Rappold et al., 2001), we analyzed its expression at five time points (days 0, 1, 2, 3 and 4) by reverse quantitative PCR (RQPCR). Our results showed that cells from old mice had increased expression of p53 at 24 hours following stimulation (Fig 3-2C). However, the expression of p53 was comparable between old and adult mice at all other time points tested (Fig 3-2C). These results suggest that induction of CSR resulted in increased expression of p53 in cells from old mice, and a significant increase in apoptosis.

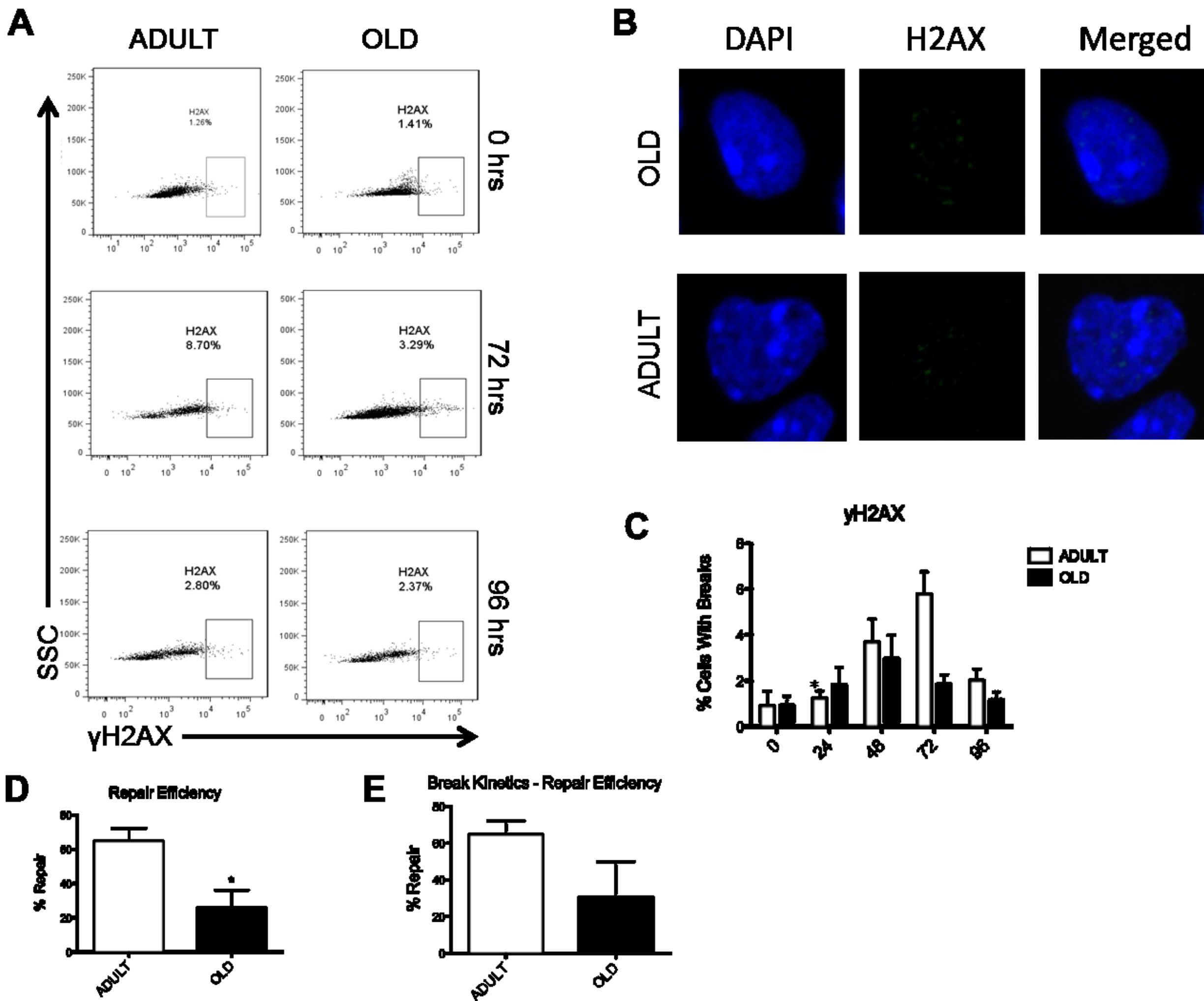


Figure 3-3. DNA breaks and repair efficiency are reduced in B lymphocytes from old FVB mice. DNA breaks were visualized by labeling with anti-mouse γH2AX and by flow cytometry. **A.** Representative flow cytometric plots showing DNA breaks in B lymphocytes from adult and old mice at 0, 24 and 72 hours of stimulation. **B.** Cells from culture were labeled with anti-mouse γH2AX and DAPI and analyzed by confocal microscopy. Confocal analysis showed that adult mice had an increased number of DNA breaks at 72 hours following stimulation. **C**. Statistical analysis of DNA breaks from flow cytometry data showed significantly lower induction of DNA breaks in old mice at 72 hours. **D.** DNA repair efficiency calculated based on the percentage of repair of DNA breaks from 72 hours to 96 hours showed that old mice had significantly lower DNA repair ability. **E.** B cells from old mice have reduced DNA repair efficiency as calculated based on break induction kinetics. As the old and adult mice reached peak DNA breaks at two different time points (48 hours and 72 hours respectively), repair efficiency was calculated for the next 24 hours immediately following the peak breaks. n=5,*=p<0.05.

B lymphocytes from Old mice had reduced DNA breaks and repair following stimulation

Class switch recombination involves DNA double strand breaks in Immunoglobulin C_H switch regions and gene recombination events to form different antibody isotypes (Stavnezer et al., 2008). DNA damage response involves active recruitment of ATM (ataxia telangiectasia mutated) which results in the phosphorylation of serine 139 of histone H2AX molecules adjacent to the break points (Burma et al., 2001; Rogakou et al., 1998). Therefore, Phosphorylated H2AX (γH2AX) is used as a reliable marker for the detection of DNA double strand breaks (Petersen et al., 2001). To study the induction pattern of CSR, we measured the frequency of DNA double strand breaks by fluorescent labeling of γH2AX and flow cytometry at different time points (Fig 3-3A). Our results showed a comparable frequency of double strand breaks in B lymphocytes from adult and old mice at 0, 24 and 48 hours. However, the percentage of B lymphocytes with DNA breaks was significantly higher in adult mice at 72 hours, whereas in old mice, it remained at a lower level (Fig 3- 3C). We also assessed the DNA repair efficiency of B lymphocytes by comparing the percentage of DNA breaks repaired during the time frame between 72 and 96 hours. Our results showed that more than 65% of the cells with DNA breaks were repaired in adult mice from 72 to 96 hour time frame whereas in old mice, only 35% cells were repaired (Fig 3-3D). As the old and adult mice B cells reached peak breaks at different time points, 48 and 72 hours respectively (Fig 3-3C), we also calculated the repair efficiency for the next 24 hours after acquiring peak amount of breaks. Similar to 72 -96 hour time window, our results showed that the old mice had reduced DNA repair efficiency for with a p value of 0.0983. Fluorescent labeling and confocal microscopy of stimulated cells at different time points confirmed the pattern of DNA breaks and repair (Fig 3-3B). These results indicated a slower or reduced

induction of DNA breaks on the IgH heavy chain and significantly reduced DSB repair efficiency in B lymphocytes from old mice.

Old and Adult mice have altered expression of NHEJ factors

Considering the pivotal role of NHEJ in DNA double strand repair, we analyzed the expression of core NHEJ factors by reverse quantitative PCR (RQPCR). Total mRNA was harvested at 1, 24, 48, 72 and 96 hour time points following stimulation and reverse transcribed to produce cDNA. Gene expression analysis revealed that the expression levels of core factors for NHEJ, *DNA Ligase 4* and *Ku70* were comparable at all the time points tested. Expression of *Ku80* was significantly higher at 0 hours and lower at 96 hours in B lymphocytes from old mice (Fig 3-4A). Expression levels of *Ku80* were comparable between cells at other time points tested. DNA protein kinase catalytic subunit (*DNAPKcs*) was significantly higher in cells from old mice 24 hours following stimulation (Fig 3-4A).

However, the expression level of *DNAPKcs* was comparable between adult and old mice at all the other time points tested. Given that the expression of NHEJ factors was not compromised in old mice, we considered the possibility that alternative end joining, another critical mechanism for DNA double strand break repair, might be altered. To test this, we analyzed the expression of alternative end joining factor, *DNA Ligase 3α* by reverse quantitative PCR. Our results showed that *Ligase 3α* was significantly higher in old mice 24 hours following stimulation (Fig 3-4B). However, *Ligase 3α* expression was comparable at the other time points tested. These data show that expression of NHEJ factors and alternative end joining factor were significantly altered in B lymphocytes from old mice.

B lymphocytes from old mice have reduced NHEJ mediated ligation efficiency

Based on our DNA break analysis and end joining factor gene expression assays, we sought to specifically analyze the NHEJ mechanism during aging. To quantitate the NHEJ mediated DNA repair efficiency, we performed an *in vitro* ligation efficiency assay on stimulated B lymphocytes as depicted in Figure 3-5A. Plasmid pUC18 was linearized using *Sma1* restriction enzyme to produce blunt ended DNA, mimicking actual DNA breaks in mammalian cells (Sallmyr et al., 2008). Purified linear plasmid was introduced into the nucleus of stimulated B lymphocytes using the Nucleofection® method. Plasmids were harvested from the cells after 16 hours of incubation, transformed into competent *E. coli*, and cultured overnight on LB plates containing IPTG and X-Gal (Brady et al., 2003; Sallmyr et al., 2008). Colonies were screened for blue and white color, where blue indicated correct repair and white indicated misrepair. Presence of plasmid was validated by quantitative PCR reaction using specific primers. Our results showed that old mice have reduced efficiency of NHEJ mediated DNA double strand breaks (Fig 3-5B). Moreover, cells from old mice had more white colonies, suggesting that the frequency of misrepair was higher in old mice (Fig 3-5C).

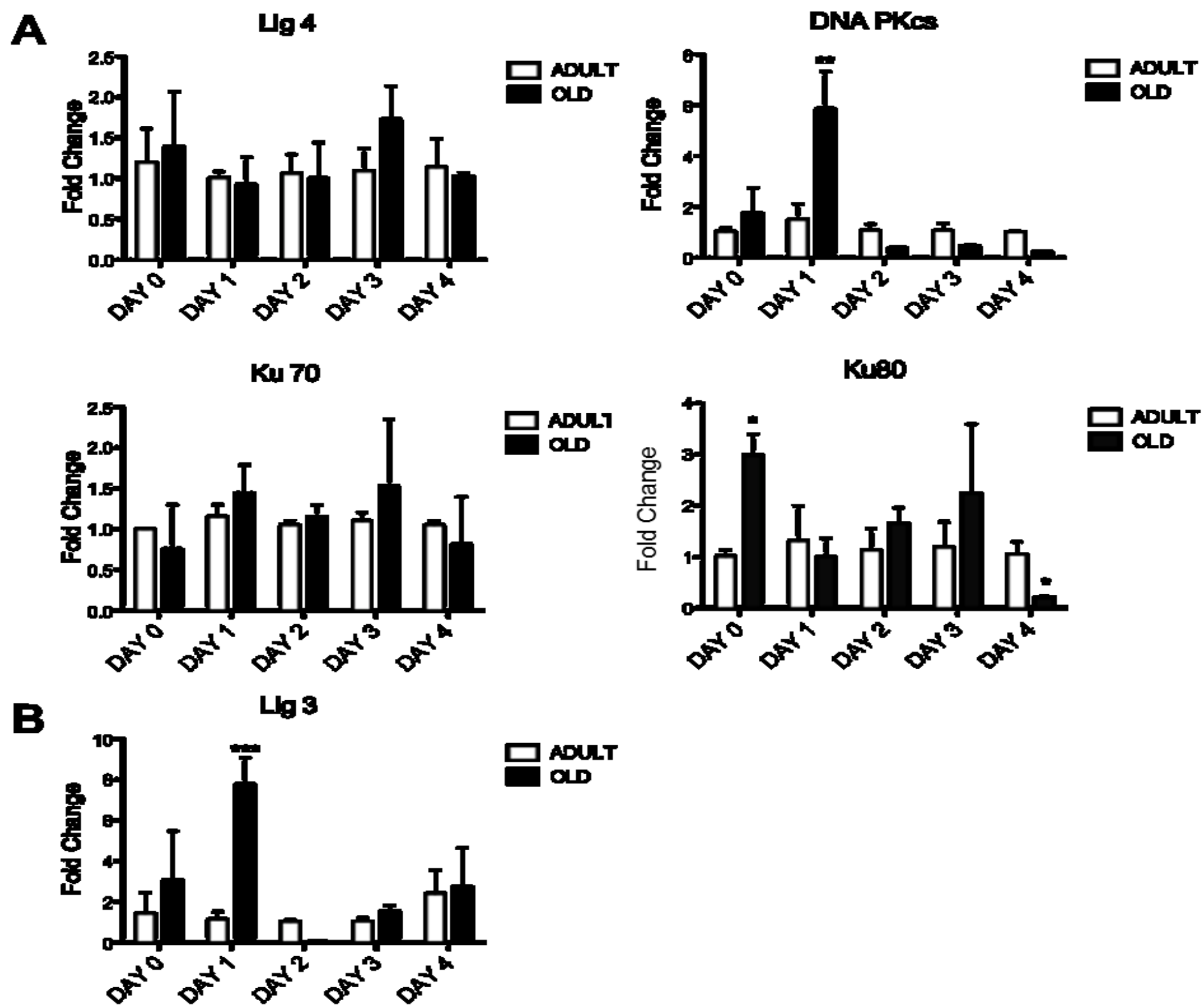


Figure 3-4. Old FVB mice have increased expression of alternative end joining factor in stimulated B lymphocytes. mRNA from B lymphocytes was harvested at 24 hour intervals and analyzed for expression of NHEJ and alternative end joining factors by RQPCR. **A.** Expression of NHEJ core factors DNA ligase 4, Ku70, and Ku80 were comparable at all the time points (day 0, 1, 2, 3 and 4) tested. However, expression of DNA PKcs was upregulated in cells from old mice on day 1 following stimulation. **B.** Analysis of alternative end joining factor Ligase 3α following in vitro stimulation, showed that it was significantly upregulated on day 1 in old mice compared to adult mice,. n=5, *=p<0.05, ***=p<0.001.

Discussion

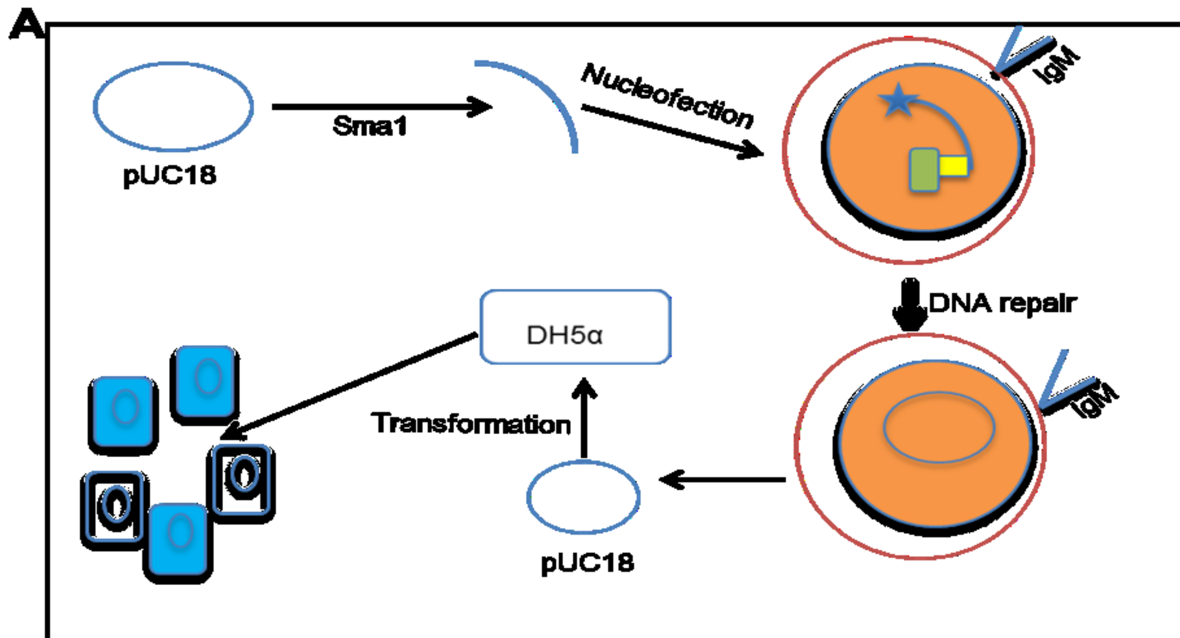
Accumulation of DNA damage is a predominant mechanism underlying both physiological and pathological aging. Hence, delineating the details of this molecular mechanism holds much promise into understanding the process of aging and age-associated compromised immunity. Accumulation of DNA damage consists of two processes, 1) increased occurrence of DNA damage as a result of increased production of reactive oxygen species (Fraga et al., 1990) and, 2) ineffective repair mechanisms of the existing DNA damage (Lombard et al., 2005). Among the various types of damage, DNA DSBs are considered as the most serious threat for cell survival (Shrivastav et al., 2008). Understanding the DNA repair mechanism is as important as the mechanism of damage occurrence. Impaired DNA double strand break repair can trigger the p53 dependent pathway resulting in cell cycle arrest at G1 stage and subsequent apoptosis (Nelson and Kastan, 1994; Rappold et al., 2001). Among the different repair mechanisms, the choice of damage response depends on many factors including the species of organism, type of cell, and stage of cell cycle (Shrivastav et al., 2008; Xu and Price, 2011). Non-homologous end joining is an important DNA repair mechanism responsible for the repair of different types of DNA double strand breaks (Soulas-Sprauel et al., 2007; Wang et al., 2009); reduced NHEJ has been proposed as a reason for the aging process (Hasty, 2008; Vyjayanti and Rao, 2006). Furthermore, previous studies in rat neurons have demonstrated that NHEJ mediated DNA breaks are impaired during aging (Vyjayanti and Rao, 2006). In our study, we used adult mice of 12 months of age and old mice (24 months) to delineate the difference in CSR and end joining mechanisms during aging. Our results show that NHEJ is compromised in old mice under physiological conditions.

As previously published in the C57/BL6 strain (Frasca et al., 2004), our results show that B lymphocytes from old FVB mice have reduced antibody class switching efficiency when cultured in the presence of LPS and IL-4. This can be partly due to the reduced expression of

E47 and AID, key molecules involved in the induction of DNA breaks, as previously suggested (Frasca et al., 2004). In our studies, analysis of the cell cycle pattern showed that cells from old mice have reduced proliferation compared to cells from adult mice following stimulation (Fig 4-1). This could be due to the accumulated DNA damage, possibly due to impaired DNA repair mechanism during CSR (Hamada et al., 2009; Xu, 2006). In fact, analysis of the cell cycle pattern showed that apoptosis was higher in B lymphocytes from old mice at 48 and 72 hours post-stimulation. Further, the number of cells from old mice entering in G2/M stage was less compared to adult mice on day 2 and day 3 (not statistically significant). Increased expression of p53 (Fig 4-2C) following stimulation may be due to accumulated DNA damage(Rappold et al., 2001), which eventually leads to increased apoptosis in the subsequent days (Hamada et al., 2009) (Fig 4-2 B).

Flow cytometric and confocal analysis for the extent of DNA damage by labeling γH2AX foci showed that B lymphocytes from old mice had a significantly lower frequency of double strand breaks at 72 hours following stimulation. DNA double strand break during CSR occurs as a result of *Aid* mediated Cytosine deamination to form Uracil. The reduced frequency of DNA breaks observed in B cells from old mice could be due to reduced activity of Aid and E47 as previously described (Frasca et al., 2004). Break analysis at 96 hours showed that more than 65% of the breaks were repaired in the adult mice whereas only 35% breaks were repaired in the cells from old mice. We also considered the possibility of slower and persistent induction of DNA breaks in cells from old mice leading to induction of DNA breaks for an extended period of time (Sadighi Akha and Miller, 2005). This could also be due to reduced NHEJ activity leading to reduced DNA repair efficiency. However, our analysis for the expression of core NHEJ factors by quantitative PCR showed that most of the factors were not compromised in old mice. In fact,

the *DNAPKcs* level was significantly high in B cells from old mice at 24 hours. Reduced CSR efficiency could be due to increased levels of *DNAPKcs* in old mice as previous studies have shown that reduced *DNAPKcs* levels favors CSR mechanism and antibody production (Cook et al., 2007). Increased level of Ku80 at 0 hour could be due to inherently altered gene expressions associated with aging (Edwards et al., 2007). Reduced Ku80 expression on cells from old mice at 96 hours may have less influence in DNA repair as majority of gene recombinations occur prior to this time frame (Hodgkin et al., 1996). Analysis of the alternative end joining factor DNA ligase 3α showed that its expression was significantly higher at 24 hours post stimulation in old mice. Increased expression of ligase 3α has been described to promote misrepair; and it is implicated in many cancer conditions including leukemia (Li et al., 2011; Sallmyr et al., 2008; Simsek et al., 2011). Further, recent studies in mouse cell lines showed that deficiency of nuclear ligase 3 reduced the occurrence of translocations, implicating that alternative end joining leads to an increased occurrence of chromosomal translocations (Simsek et al., 2011).Thus increased expression of ligase 3α might lead to impaired NHEJ and promoted error prone alternative end joining, thus reducing CSR efficiency. Furthermore, AEJ can reduce the correctness of DNA break repairs and thus increase the chance for chromosomal translocations and subsequent malignant transformations.



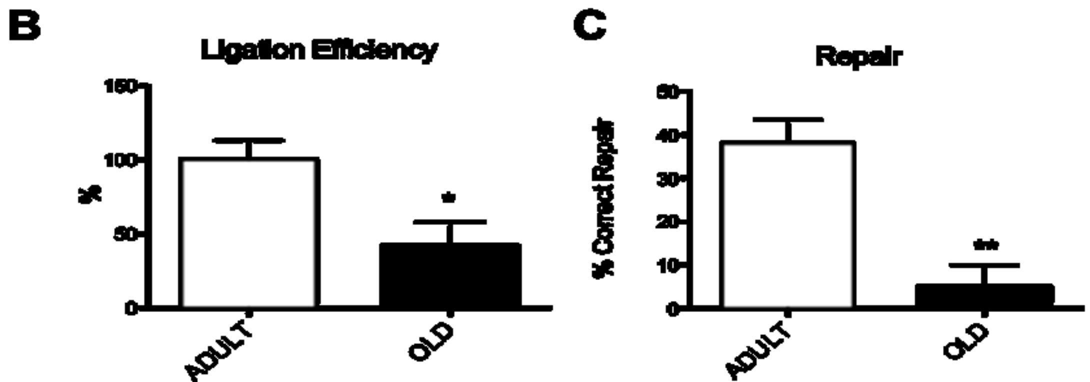


Figure 3-5. Stimulated B lymphocytes from old FVB mice have reduced DNA repair efficiency. **A**. Diagrammatic representation of in vitro DNA repair assay. Linearized plasmid pUC18 was introduced into stimulated B lymphocyte nuclei by Nucleofection® and incubated at 37ºC overnight. Plasmids were harvested, transformed into competent DH5α cells and grown in LB plates containing Ampicillin, IPTG and X-gal. Colonies were analyzed for colony counts and blue/ white screening. **B.** Ligation efficiency was calculated based on the number of colonies and expressed as percentage of colonies. B lymphocytes from old mice had significantly reduced numbers of colonies, indicating diminished DNA repair efficiency. **C.** Chance for misrepair, as calculated based on the proportion between blue and white colonies, was significantly higher in lymphocytes of old mice compared to adult mice. n=5, *=p<0.05, **=p<0.01.

Given the comparable expression of NHEJ factors, we considered functional inefficiency of NHEJ as a possible reason for the impaired CSR and reduced DNA damage repair. Ligation efficiency assay, which mimicked DNA strand break with blunt ends and prompted NHEJ mediated repair (Li et al., 2011), showed that B lymphocytes from old mice had reduced efficiency for DNA repair. Based on our results, we propose that increased expression of ligase 3α leads to competition between alternative and non-homologous end joining, which in turn lead to increased error prone alternative repair. Error prone and or reduced repair could be the reason for the increased expression of p53 and apoptosis. Finally, analysis of the correctness of the repair showed that cells from old mice produced more incorrectly repaired colonies, validating that NHEJ mediated DNA repair is impaired in old ages.

Elderly patients are more prone to infectious diseases due to their reduced immune cell repertoire, and subsequently have lower immune surveillance. Furthermore, there is a strong correlation between some types of cancer occurrence and age progression (Henry et al., 2011; Sprenger et al., 2010). Accumulation of DNA damage and mutations as a result of impaired DNA repair mechanisms can lead to neoplastic transformation (Helleday, 2011). Our results show that NHEJ is compromised in B lymphocytes from aged mice. Given the conservation in the DNA repair mechanisms among vertebrate species, we propose that mechanisms that limit DNA repair activity might be a widespread phenomenon and may apply to other cell types and humans. Further investigations are needed to understand the underlying molecular mechanisms of altered NHEJ mechanisms as it applies to the aging process.

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CHAPTER 4

A *NUP98-HOXD13* Leukemic Fusion Gene Leads to Impaired Class Switch Recombination and Antibody Production

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Abstract

Myelodysplastic syndrome (MDS) is a clonal process characterized by ineffective hematopoiesis and progression to acute leukemia. Although many MDS and leukemic patients have compromised immunity, the role of underlying mutations in regulating immune function is poorly understood. Recent studies show that *NUP98-HOXD13 (NHD13)* fusion gene results in MDS and impairs lymphocyte differentiation in transgenic mice. In our studies, we sought to elucidate the mechanism by which *NHD13* affects B lymphocyte development and function. Based on our preliminary findings that transgenic mice had increased levels of IgM and reduced IgG1 and IgE, we hypothesized that the fusion gene might impair class switch recombination (CSR). Mice were immunologically challenged with Dinitrophenol (DNP). *NHD13* mice showed a marked reduction in B lymphocyte differentiation in their bone marrow and spleen following DNP stimulation and had reduced production of DNP specific antibodies. Spleen follicles from these mice were small and hypocelluar indicating failure of clonal expansion. When isolated *NHD13* B lymphocytes were stimulated *in vitro* using *E.coli* Lipopolysachharide (LPS) or LPS+IL-4, they failed to undergo sufficient CSR and proliferation. Taken together, our findings show that expression of *NUP98-HOXD13* impairs CSR and reduces the antibody-mediated immune response, in addition to its role in leukemia. Further delineation of the *NUP98-HOXD13* transgene may reveal novel pathways involved in CSR.

Key words: *NUP98, HOXD13*, class switch recombination, MDS, B lymphocyte

Introduction

Myelodysplastic syndrome (MDS) is a malignant process characterized by ineffective hematopoiesis, dysplastic BM, peripheral blood cell cytopenias, including lymphopenia, and progression to acute myeloid leukemia (AML) and acute lymphoid leukemia (ALL) (Disperati et al., 2006; Heaney and Golde, 1999; Jaffe et al., 2001; Komrokji and Bennett, 2003; Nimer, 2008). The role of B cells in the pathogenesis of MDS is under considerable debate; however, several lines of evidence support the role of lymphoid progenitor cell involvement in the pathogenesis of MDS and other myeloid malignancies including Chronic Myeloid Leukemia (Lam et al., 2007; Li et al., 1999; Zheng et al., 2009).

Some patients with MDS are reported to have lymphopenia. In fact, analysis of the $CD34^+$ progenitor cells from low risk MDS patients showed that absolute numbers of B cells were low (Sternberg et al., 2005). A subset of MDS and AML patients showed a significant increase in B lymphocyte apoptosis suggesting that modulation of immune system are associated with progression of MDS (Amin et al., 2003). Also, $CD34^+CD38^-$ bone marrow progenitor cells from some MDS patients with 5q deletion (5q-) revealed that large pro-B cell had same deletion; suggesting a lymphoid-myeloid progenitor cell is the target of this mutation (Nilsson et al., 2000). Furthermore, 19% of high risk MDS patients showed an increase in B cells that correlated with poor survival (Hilbe et al., 1994). Moreover, progression from MDS to ALL has been demonstrated in some patients with nearly half of these cases being B-ALL (Bonati et al., 1986; Raghavachar et al., 1987; Shi et al., 2004). This may be due to 1) the requirement of unique cooperating mutations required for B cell transformation; 2) the target lymphoid precursor that undergoes a block in differentiation may be rare or short-lived (Disperati et al., 2006); or 3) the nonhematopoietic cells in the marrow may exert an extrinsic effect on lymphoid progenitors differently than on myeloid progenitors in the context of clonal expansion (Disperati

et al., 2006; Labrie et al., 2004). Also, impaired lymphopoiesis occurs in MDS (Amin et al., 2003; Fujii et al., 2003; Iwase et al., 1995; Srivannaboon et al., 2001) and studies show B cells from MDS patients stimulated with phorbitol myristate acetate failed to incorporate significant levels of tritiated thymidine suggesting an inability to proliferate, or produce adequate immunoglobulin levels (Tsuchida et al., 1991). Based on these findings, it is evident that B cells play contribute to the evolution of MDS either as a component of immune modulation (Barrett and Sloand, 2009), direct targets of mutations resulting in ALL, or as cells that influence microenvironment in which a myeloid clone predominates (Amin et al., 2003; Disperati et al., 2006; Sternberg et al., 2005).

NUP98 forms chromosomal translocations with at least 28 genes including *HOXD13*, which was first observed in patient with AML (Raza-Egilmez et al., 1998). Recent studies in mice have shown that expression of *NUP98-HOXD13* (*NHD13*) results in MDS and progression to acute leukemia (Pineault et al., 2003; Slape et al., 2008). Choi and colleagues showed that this fusion gene also impairs murine B and T lymphocyte development at 6-7 months of age (Choi et al., 2009). *NHD13* transgenic mice had reduced V to DJ recombination in thymocytes and subsequent increased apoptosis, resulting in reduced peripheral T lymphocyte population (Choi et al., 2009). In this study, we sought to expand the role of the *NHD13* transgene during B lymphocyte development prior to the onset of clinical leukemia. B lymphocyte development includes bone marrow differentiation of hematopoietic stem cells to ProB cells and Pre B cells. Immature B cells thus formed enter peripheral circulation and develop further to form follicular or mantle zone cells in secondary lymphoid organs such as lymphnodes and spleen. Once the naïve B cells encounter antigenic stimulation, they undergo the final development to form plasma cells, which produce antigen specific antibodies and contribute to immune response. Our

initial observations of altered IgG and IgE plasma antibody concentrations in the presence of increased IgM levels, led us to hypothesize that the *NHD13* transgene could result in faulty class switch recombination. Here, we show that the leukemic fusion gene impairs class switch recombination thereby leading to an impaired antibody-mediated immune response. Further delineation of this defective pathway in B lymphocyte differentiation and function could reveal greater insight into the mechanisms underlying fusion proteins, and importantly, reveal therapeutic targets.

Materials and Methods

Use of *NHD13* transgenic mice

Eight to twelve-week-old (average age: 10 weeks) clinically healthy *NHD13* transgenic mice and age-matched wild type (WT) mice maintained on an FVB background were used for each experiment. Five *NHD13* and WT were used in each set of experiments unless otherwise stated. The mice were bred and maintained at the core laboratory animal facility at the Center for Molecular Medicine and Infectious Diseases, Virginia Tech. All the experiments were carried out as per NIH guidelines with the approval from the Virginia Tech *Institutional Animal Care and Use Committee (IACUC)*.

Peripheral blood evaluation

Animals were anesthetized using 2.5% Isoflurane gas and approximately 100µl of blood was collected via the retro-orbital method. The blood was used for WBC and lymphocyte analysis, as well as for harvesting plasma. The blood was analyzed using a Veterinary Differential™ hematology system (Heska).

Basal antibody measurement

Plasma from *NHD13* and WT mice was used to determine the concentrations of IgM, IgG1, IgG2a, IgG2b, IgG3 and IgE using ELISA. ELISA plates (Nunc Maxisorp 96 well) were coated with 2µg/ml of goat anti-mouse IgG1 or IgG2b (Southern Biotech), or rat anti-mouse G3 or IgM (BD Pharmingen) in TBS or PBS overnight at 4°C. Plates were blocked for 1 hour at room temperature with TBS or PBS supplemented with 1% BSA. The plates were then incubated with serial dilutions of plasma and standards (Southern Biotech or BD Pharmingen) in PBS/TBS with 1% BSA at room temperature. After 5-7 washes with PBS/TBS with 0.05% Tween, plates were incubated with Biotin conjugated anti-goat IgG1 and anti-goat IgG2b- (Southern Biotech) or Alkaline Phosphatase conjugated anti-rat IgM and anti-rat IgG3, (BD Pharmingen) antibodies for 1 hour at room temperature. For AP conjugated antibodies, PNPP substrate (Fisher Scientific) was added. For Biotin conjugated antibodies, 100µl Horse Radish Peroxidase–Streptavidin (Thermo Fisher Scientific) at a dilution of 1:10,000 was added and incubated at room temperature for 1 hour followed by addition of ABTS substrate (Southern Biotech). The optical density was measured at 405nm using a Spectramax microplate reader (Molecular Devices)

In vivo antigen stimulation

On day zero, Dinitrophenol–Keyhole Limpet Hemocyanin (DNP-KLH) (Calbiochem, San Diego, CA) suspended in Alum (Thermo Scientific) according to manufacturer's recommendations and 50µg total was injected intra-peritoneally into 8-12 week old transgenic and wild type mice. Blood was collected on the same day to determine the total white counts and lymphocyte counts. Plasma was harvested for purposes of determining basal serum antibody levels. A booster injection with the same antigen was given on day 21. On day 28, blood was collected for WBC and lymphocyte counts, flow cytometry, and plasma antibody levels followed

by humane euthanasia. Bone marrow and spleen were used for flow cytometric analysis and sorting of B lymphocyte fractions. Spleens were used for histopathology evaluation.

DNP specific antibody determination

ELISA plates were coated with 1μg/ml DNP in PBS with 1% BSA and incubated overnight at 4°C. The plates were blocked by blocking buffer PBS with 1% BSA for 1 hour at room temperature. Serum was diluted in blocking buffer and incubated at room temperature for one hour. Isotype specific detection antibodies, rat anti-mouse IgG1 and IgG2b (Southern Biotech) were diluted at a concentration of 1ug/ml and incubated at room temperature for 1 hour. Horse Radish Peroxidase Streptavidin (Thermo Fisher Scientific) at a dilution of 1:10,000 was added and incubated at room temperature for 1 hour followed by addition of ABTS substrate (Southern Biotech) mixed with hydrogen peroxide. For Alkaline Phosphate conjugated anti-mouse IgM and anit-mouse IgG3 antibodies (BD Pharmingen) TBS was used instead of PBS and PNPP (Thermo Fisher Scientific) was added as the enzyme substrate. The optical density was measured at 405nm using Spectramax Microplate reader (Molecular Devices)

Flow cytometry

Bone marrow cells were harvested and RBCs were lysed using ACK lysing solution. Single cell suspensions were washed and stained with Allo-Phycocyanin (APC) conjugated anti-mouse B220, Flourescein Isothiocyanate (FITC) conjugated mouse anti-CD43, Phycoerythirn (PE) conjugated anti-mouse BP1, and PECy5 or PerCP conjugated anti-mouse CD24 for the ProB cell fractions and APC conjugated anti mouse B220, FITC conjugated anti-mouse CD43, PE conjugated anti-mouse IgD, and PECy5 conjugated anti-mouse IgM for PreB cell fractions. Single cell suspensions of splenocytes were incubated with Peridinin Chlorophyll Protein Complex (PerCP710) conjugated anti-mouse IgM, FITC conjugated anti-mouse AA4.1, PE

conjugated anti-mouse CD23, and APC conjugated anti-mouse CD21. RBCs from the peripheral blood was lysed and single cell suspensions were labeled using FITC conjugated anti-mouse B220, and PerCep710 conjugated anti-mouse IgM. All antibodies were obtained from eBioscience (eBioscience). Flow cytometry was performed at that College of Veterinary Medicine Flow Cytometry Core Facility using a FACScan flow cytometer (Becton Dickenson). Flow cytometry data was analyzed using FlowJo Software (FlowJo).

Histopathology

At the time of necropsy, spleens were harvested and fixed in 4% formaldehyde followed by routine processing and paraffin embedding. Four micron thick tissue sections were stained with Hematoxylin and Eosin (H&E), and visualized by light microscopy using a Nikon Eclipse 50i microscope. At least five splenic follicles were identified from each sample and tissue morphometry of the spleen was calculated by measuring the area of splenic follicles using the NIS-Elements D software 3.10 (Nikon).

In vitro Class Switch Recombination Assay

Splenic B lymphocytes from 8-12 week old transgenic and wild-type mice were harvested using anti-mouse IgM magnetic beads and Magnetic Assisted Cell Sorting system (Milteny Biotec). A total of $2x10^5$ cells were treated with 5 μM CFSE and cultured in duplicate in 96 well culture plates (BD Biosciences) with RPMI1640 media (ATCC, Manassas, VA) + 10% FBS containing either *E coli* Lipopolysaccharide (LPS) (25 ug/ml), or LPS and IL-4 (25ng/ml) and incubated at 37ºC/ 5% CO_2 for 72 hours (Hodgkin et al., 1996). At the end of the incubation, the LPS + IL-4-treated cells were washed three times and labeled with fluorescently tagged anti-mouse IgG1 and IgE antibodies; LPS-treated cells were labeled with fluorescently tagged anti-mouse IgG2b and IgG3. The percentage of cells undergoing class switch recombination events was assessed by

flow cytometry. Proliferation was calculated by staining cells from culture with Trypan blue and counting the live cells.

Statistical Analysis

Data was analyzed with GraphPad Prism, 5.0 using Students *t*-test or ANOVA and Bonferroni posttest; a *P* value <0.05 was considered significant.

Results

Mice that express an NHD13 leukemic fusion gene have lymphopenia and impaired plasma antibody production.

Whereas Choi and colleagues (Choi et al., 2009) reported decreased white blood cell (WBC) and lymphocyte counts in seven-month-old mice, we found the defect in normal B lymphocyte development to occur in mice as young as 8-12 weeks of age suggesting that the transgene effect occurs much earlier than previously reported. Consistent with previous observations in older mice (Choi et al., 2009), WBC and lymphocyte analysis of peripheral blood revealed a significant reduction in both of these parameters in 8-12-week-old mice (Figure 4-1A). Though the total number of peripheral B lymphocytes was lower in *NHD13* mice, it was not significant (Figure 4-1B). Because of these subtle alterations in *NHD13* mice prior to the onset of leukemia, we sought to understand the functional status of the immune system. Base line parameters were established for plasma immunoglobulin concentrations IgM, IgG1, IgG2a, IgG2b, IgG3 and IgE from *NHD13* and WT mice using quantitative sandwich ELISA (HogenEsch et al., 1999). Our results showed that plasma IgG1 levels were significantly lower in the transgenic mice compared to WT controls (Figure 4-1C) ($p<0.05$). The IgG2a concentration was higher in *NHD13* mice, and IgG2b and IgG3 concentrations were lower compared to WT controls, although they were not statistically significant (Figure 4-1C). Interestingly, plasma IgM concentration was

significantly higher in *NHD13* mice compared to WT mice (Figure 4-1C) (n: TG=10, WT= 10, $p<0.001$). These results indicate that the *NHD13* fusion gene leads to lymphopenia and impaired antibody production in mice.

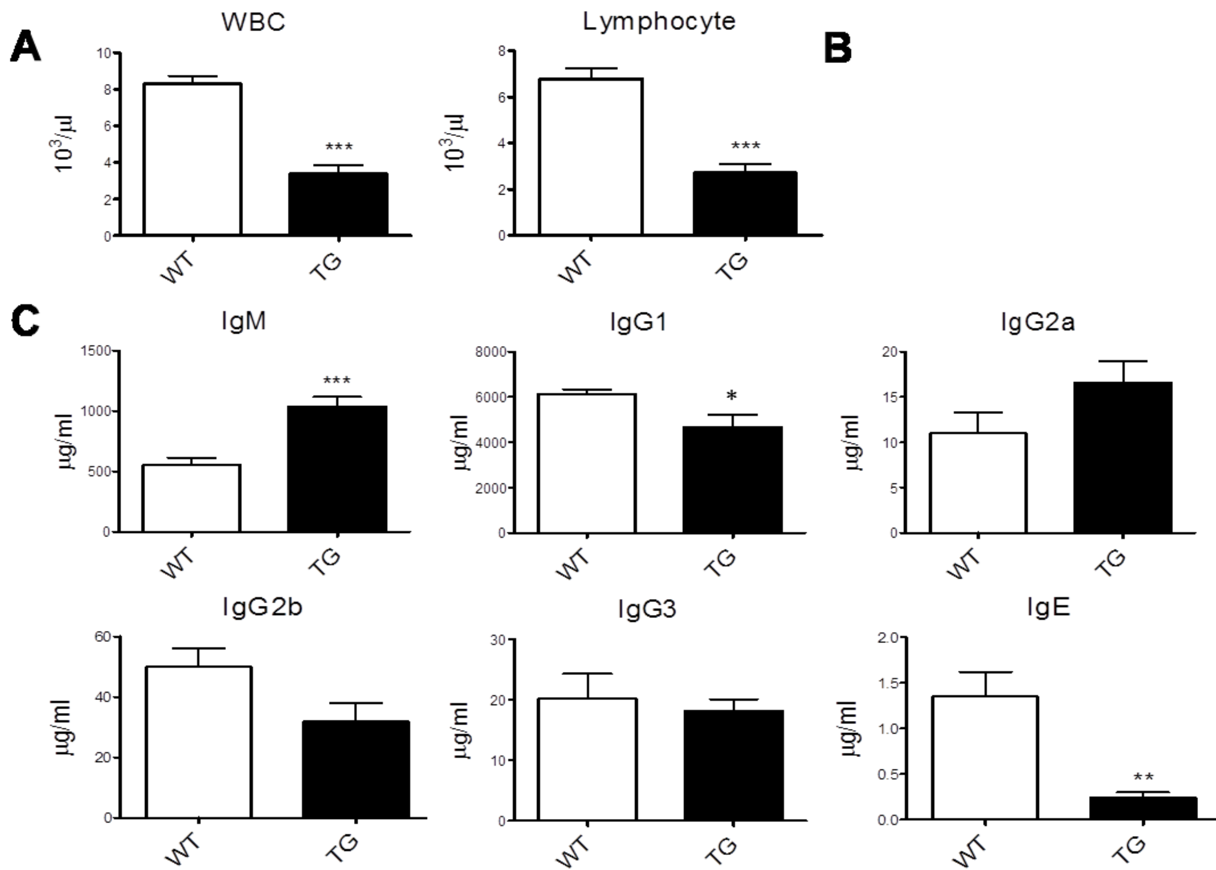

Figure 4-1. Partially impaired differentiation of B lymphocytes in *NHD13* mice.
(A) Peripheral blood analysis of 8-12-week-old *NHD13* (TG) and wild type (WT) littermate controls. *NHD13* mice had significantly lower WBC and lymphocyte counts compared to WT controls. **(B)** *NHD13* mice had lower $B220^+IgM^+$ mature B lymphocytes in circulation. **(C)** Quantitative sandwich ELISA was used to determine immunoglobulin levels in plasma from TG and WT mice (n=10). The TG mice had lower concentrations of IgG1 and IgE compared to WT mice. Basal antibody concentrations of IgG2a, IgG2b and IgG3 showed comparable levels between TG and WT mice. n=5 for TG and WT unless otherwise specified, (*=p<0.05, **=p<0.01, ***=p<0.001).

The NHD13 fusion gene leads to impaired B lymphocyte development in bone marrow and spleen.

We analyzed the bone marrow and splenic development of B lymphocytes using stage specific combination of surface markers labeling and flow cytometry. Evaluation of bone marrow differentiation using Hardy fraction method (Figures 4-2, A and B) showed an altered pattern of B cell differentiation in *NHD13* mice. The total amount of Pro B and Pre B fractions were comparable (Figure 4-2B), but analysis Pre B sub-fractions showed a significantly high fraction D and significantly lower fraction F. After observing impaired B lymphocyte differentiation in the bone marrow (Choi et al., 2009), we raised the question that the *NHD13* transgene might have a significant effect on B lymphocyte development in the spleen. To determine the effect of the transgene on B lymphocyte development we analyzed B lymphocytes from the spleen using flow cytometry using the markers IgM, CD21, CD23, and AA4. Specific compartments were analyzed accordingly T1 The percentage of cells in transitional stages 1 ($IgM^{+}CD23^{-}AA4^{+}CD21^{-}$), and 3 (IgM^{lo}, $CD23^{+}$, $AA4^{+}CD21^{-}$) and advanced stages Fo (IgM^{+}, $CD23^{+}$, $AA4^{-}CD21^{-}$), B1 (IgM^{+}, $CD23^{-}$, $AA4^{-}CD21^{-}$) and MZ (IgM^{+}, $CD23^{-}$, $AA4^{-}$ $CD21^{+}$) populations were comparable between transgenic and WT mice (Figure 4-2C). However, the percentage of cells in transitional stage 2 (T2) (IgM^{hi}, $CD23^{+}$, $AA4^{+}CD21^{+}$) from *NHD13* mice showed a statistically significant reduction suggesting a block in maturation (Figure 4-2C). No difference was noted in either bone marrow or splenic development of B lymphocytes from mice of different age groups. Our results indicate that the *NHD13* fusion gene impairs B lymphocyte development in both bone marrow and spleen in pre-clinical transgenic mice.

The NHD13 transgene impairs an antibody-mediated immune response in transgenic mice. Considering that impaired immunity is an important finding in some patients with leukemia and many other hematological diseases, we sought to define the mechanism by which the *NHD13* transgene resulted in B lymphocyte development and decreased antibody production. To determine the effect that the *NHD13* transgene might have on immune response, we challenged *NHD13* and WT mice with a comprehensive immunogen, DNP-KLH (HogenEsch et al., 1999). Animals were euthanized on day 28 following a booster injection with DNP-KLH on day 21. At the time of euthanasia, peripheral blood analysis showed a significant reduction in WBC and lymphocyte counts (Figure 4-3A) ($n=5$, $p<0.001$), as well as a significant reduction in $IgM^{+}/B220^{+}$ lymphocytes (Figures 4-3, B and C). The plasma DNP specific antibody concentration was measured by ELISA. Intriguingly, the DNP specific IgM levels were comparable between *NHD13* and WT mice. However, quantification of the IgG subclasses for DNP specific Immunoglobulins, including IgG1, IgG2a, IgG2b and IgG3 were significantly lower in *NHD13* mice compared to WT controls (Figure 4-3D). As shown in Figure 4-3D, there was almost a 4 fold reduction in IgG1 (average 594.4 titers/ml vs 153 titers/ml, $p<0.001$); a 5 fold reduction in IgG2b (330 titers/ml vs 63 titers/ml, $p<0.01$); and a 6 fold reduction in IgG2a and IgG3 levels (1550 titers/ml vs 180 titers/ml, $p<0.01$ and 24 titers/ml vs 4 titers/ml, $p<0.001$). Our results show that antigenic stimulation with DNP-KLH failed to evoke a strong antibody-mediated immune response in *NHD13* mice when compared with WT mice.

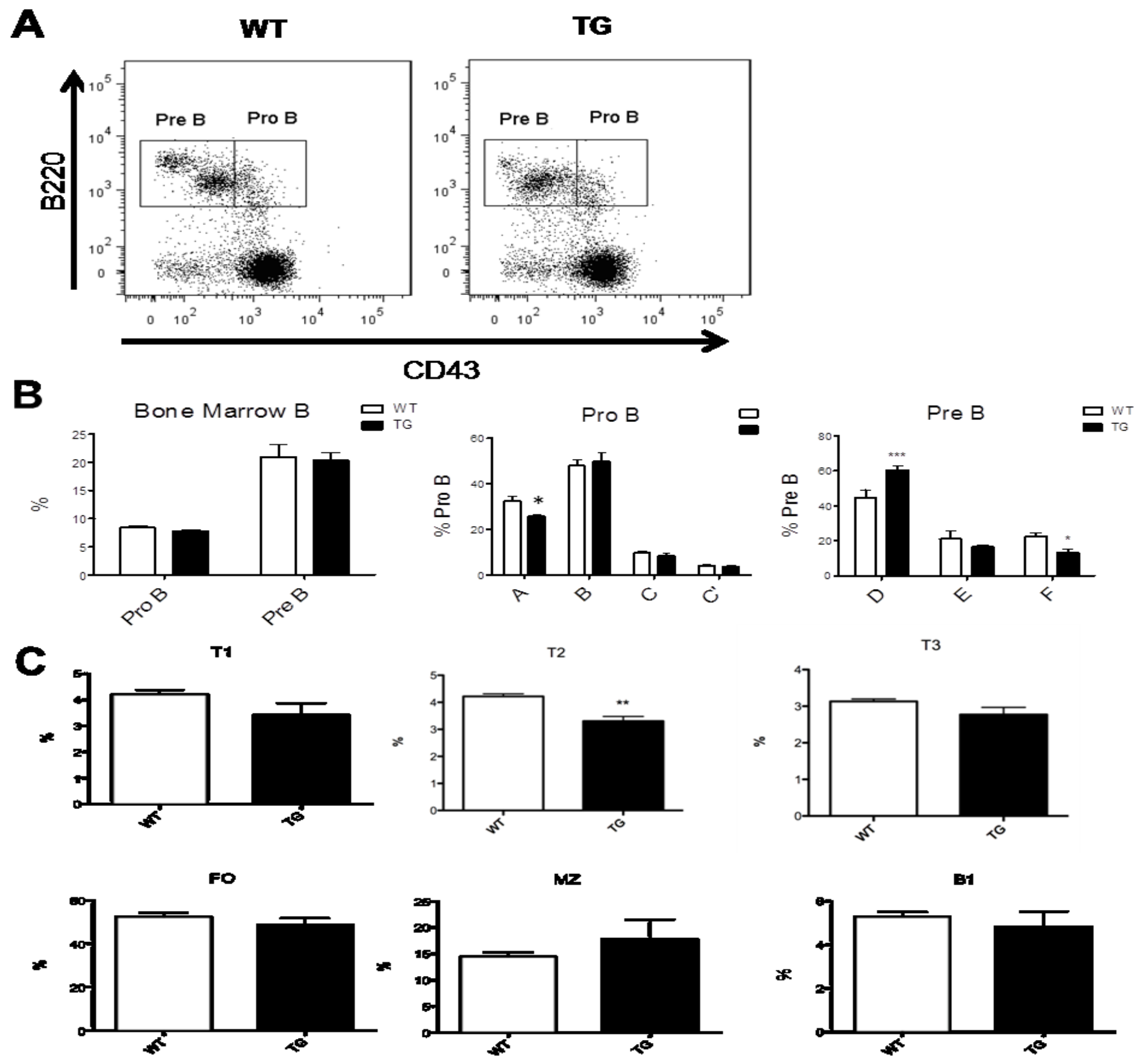

Figure 4-2. B lymphocyte differentiation is impaired in *NHD13* (TG) mice.

Bone marrow was harvested from 8-12-week-old *NHD13* and WT mice and single cell suspensions were labeled with fluorescently-tagged anti-mouse B220, CD43, CD24, BP1, IgM and IgD antibodies and analyzed by flow cytometry. Cells were gated based on their surface marker expression into their respective Hardy fractions. **(A)** Representative FACS plots of BM Hardy fractions from TG and WT mice. **(B)** Flow cytometric analysis using the Hardy method showed comparable numbers of BM B lymphocyte precursors in the Pro and Pre B lymphocytes fractions (expressed as a percentage of total bone marrow cells after RBC lysis). B lymphocytes in fraction A and F of Pro B and Pre B cells respectively were significantly reduced. In contrast, fraction D was significantly increased. There were comparable numbers of B-cells in fractions B, C, C' and E from *NHD13* mice and WT controls. **(C)** Flow cytometric analysis of splenic B cell fractions showed altered peripheral maturation. Transitional stage2 (T2) was significantly lower while change in other fractions were not significant. (n=5 for TG and WT, *=p<0.05, **=p<0.01, ***=p<0.001)

Antigenic stimulation resulted in impaired bone marrow differentiation in *NHD13* mice

After observing reduced mature B cells in circulation and impaired antibody production following antigenic stimulation, we sought to delineate the bone marrow differentiation pattern of B lymphocytes in *NHD13* mice following DNP injection. Flow cytometric analysis for the developmental pattern using Hardy fraction method showed comparable amount of total Pro and Pre B cells in *NHD13* and WT mice (Figure 4-4, A and B). However, subfractions of Pro B showed significantly lower fractions A and B, but significantly higher fractions C and C' (Figures 4-4, A and B). Analysis of Pre B fractions showed significantly increased fraction D and reduced percentage of immature B cells (fraction F). Considering the role of IL-7 receptor (IL-7R) mediated signaling for the Pro B to Pre B cell development, we analyzed the expression of IL-7R on bone marrow B cells. Our results showed comparable expression of IL-7R on both *NHD13* and WT B cells (Figure 4-4C, 4D). These findings suggest pronounced impairment in B cell differentiation within bone marrow of *NHD13* mice following antigenic stimulation.

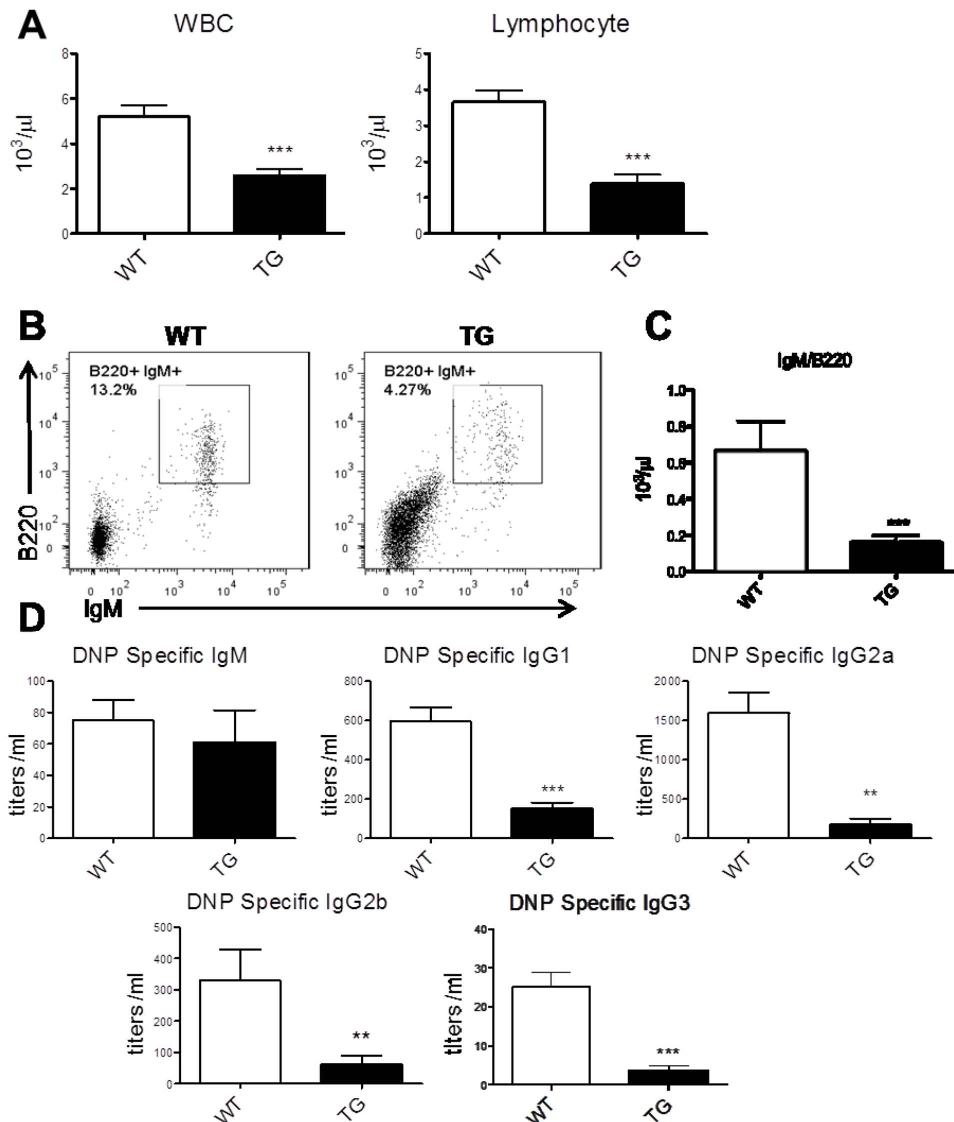

Figure 4-3. Antibody production is impaired in B lymphocytes of *NHD13* mice after stimulation with DNP KLH. *NHD13* and WT mice were sensitized with 100 µg of DNP-KLH on day 0 and boostered on day 21. On day 28 tissues including blood, spleen, and bone marrow were harvested. **(A)** Peripheral blood analysis from *NHD13* (TG) and WT controls. *NHD13* mice had significantly reduced total WBC and lymphocyte counts compared to WT controls. **(B)** Representative FACS plots and **(C)** histogram for mature B cells in peripheral blood following antigenic stimulation with DNP. Flow cytometry of peripheral blood showed significantly lower B220+/IgM+ mature B cells in *NHD13* mice compared to WT controls. **(D)** Quantitative ELISA for DNP specific antibodies showed comparable levels of IgM in TG and WT mice whereas IgG1, IgG2a, IgG2b and IgG3 were significantly lower in *NHD13* mice compared to WT control. (n=5 for TG and WT, **=p<0.01, ***=p<0.001).

The *NHD13* fusion gene impairs clonal expansion in the spleen after antigenic challenge. Given the role of splenic B lymphocyte maturation and clonal expansion in antibody production (Meffre et al., 2000), we considered the impact the fusion gene might have on these important mechanisms in B lymphocyte biology. To gain insight into our concept, we evaluated the spleen from DNP-KLH stimulated mice using histopathology and flow cytometry. Histopathology of spleens from unstimulated *NHD13* and WT mice showed similar morphological features in the follicles (Figure 4-5A TG US and WT US) and (Figure 4-5B). This is in contrast to the findings observed in *NHD13* mice stimulated with DNP. Histopathology of the spleen from these mice revealed considerably smaller, hypocellular follicles with poorly demarcated mantle and marginal zones (Figure 4-5A TG DNP and WT DNP) and (Figure 4-5B). Despite these morphological differences, there was no difference in spleen weights between groups of mice (Figure 4-5C). To gain better insight into development pattern in the spleen, we evaluated the developmental stages of B lymphocytes using the surface markers IgM, CD21, CD23, and AA4 as shown in Figure 4-5D. Our results revealed a significant reduction in B lymphocytes within the transitional 1 (T1) stage (Figure 4-5E, $p<0.05$). Subsequent transitional stages were comparable between *NHD13* and WT indicating that the cells could proliferate and compensate for the reduced numbers in Fraction T1. In contrast, *NHD13* mice showed reduced number of cells within the mature B lymphocyte stages including Follicular (FO), B1 or marginal zone (MZ) compartments (Figure 4-5E). Taken together, our results show that the *NHD13* fusion gene leads to impaired B lymphocyte development and reduced clonal expansion suggesting the antibody mediated immune response is impaired in *NHD13* mice following immune challenge.

Class Switch Recombination is impaired in *NHD13* B lymphocytes.

Finally, we sought to gain insight into the underlying molecular mechanism(s) for impaired B lymphocyte development and function in the *NHD13* mouse model. Our earlier observations indicated that *NHD13* mice had comparable concentration of DNP specific IgM but lower IgG subclasses and impaired clonal expansion in the spleen. This leads to the possibility of impaired class switch recombination as a mechanism for the impaired immunity in the *NHD13* mice. We therefore performed an *in vitro* Class Switch Recombination assay (Ward et al., 2004) on IgM^+ splenic B lymphocytes by stimulating isolated cells with either LPS or LPS and IL-4. When stimulated with LPS and IL-4 to induce IgG1 and IgE class switching, B lymphocytes from *NHD13* mice had a significantly lower class switching to IgG1 and IgE when compared to WT controls (Figure 4-6A, B). Likewise, when B-cells were stimulated with LPS alone in order to induce IgG2b and IgG3 class switching, there was less IgG2b and IgG3 switching from *NHD13* mice in comparison to WT mice (Figure 4-6A, B). The proliferation pattern determined by CFSE labeling and IgG1, IgE, IgG2b or IgG3 indicated impaired proliferation of B cells from *NHD13* mice (Hodgkin et al., 1996) (Figure 4-6C). These findings are in contrast to the WT cells that had most of their cells in their later generations. Total cell yield from culture further suggested lower proliferation efficiency of B cells from *NHD13* under stimulation (Figure 4-6D). These results indicate that impaired antibody production in *NHD13* mice is a result of impaired class switch recombination.

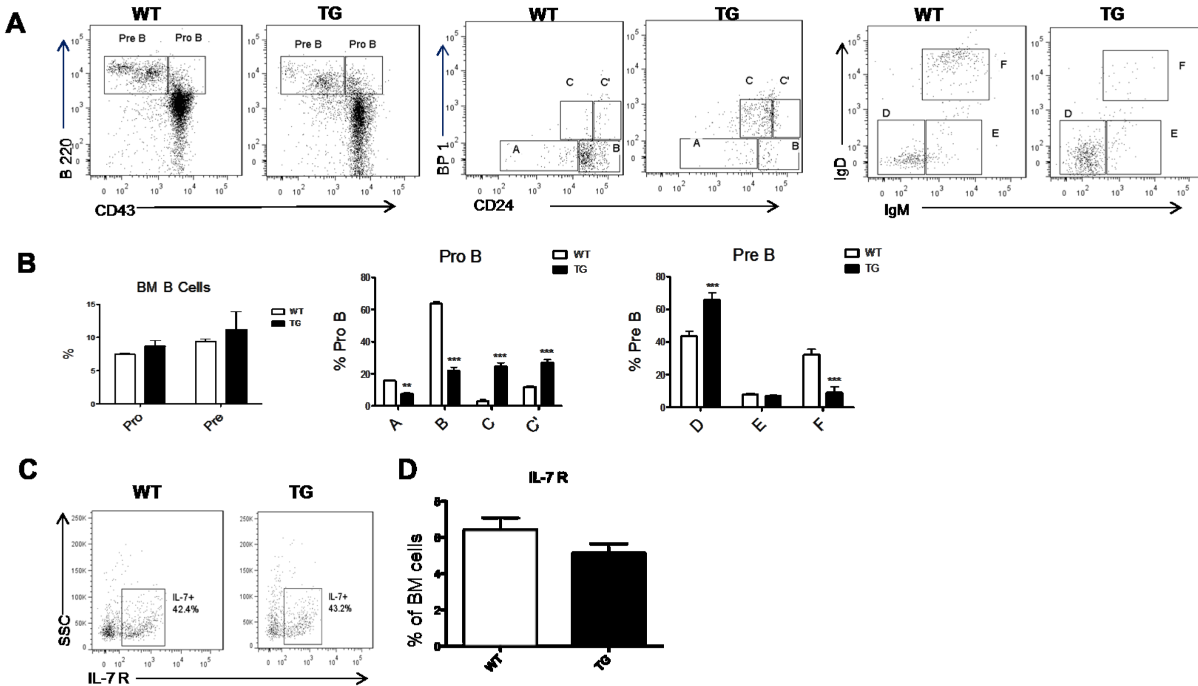

Figure 4-4. B lymphocyte differentiation is impaired in NHD13 mice after DBP-KLH stimulation

Bone marrow was harvested from 8-12-week-old *NHD13* and WT mice and single cell suspensions were labeled with fluorescently-tagged anti-mouse antibodies including B220, CD43, CD24, BP1, IgM and IgD; and analyzed by flow cytometry. **(A)** Representative FACS plots of fractions from TG and WT mice. **(B)** B cell fractions were calculated as a percentage of cells in each population and its parent population. Pro B fractions A and B were significantly lower in the TG mice compared to WT controls, whereas fractions C and C' were significantly higher. Pre B fraction D was significantly higher in *NHD13* mice, but immature B (fraction F) was significantly lower in *NHD13* TG mice indicating differentiation arrest **(C)** Representative FACS plot showing surface expression of IL-7 receptors on BM B cells. **(D)** The expression of IL-7R was comparable between WT and TG mice. n=5 for TG and WT, *=p<0.05, **=p<0.01, ***=p<0.001

Discussion

Chromosomal abnormalities have been associated with impaired immune cell development and antibody production (Kracker et al., 2010; Rucci et al., 2010). However, most studies are conducted using direct loss or mutation in genes directly involved in immune function. Choi and colleagues recently showed that *NHD13* mice have diminished B and T lymphocytes numbers and suggested that expression of this fusion gene lead to a partial block in the development of lymphocytes (Choi et al., 2009). In our studies we show that the *NHD13* fusion gene impairs B lymphocyte development in spleen as well. We report that expression of the fusion gene leads to significantly impaired class switch recombination resulting in ineffective antibody production following antigenic stimulation. Reduced B cell mediated immunity has been implicated in different tumor occurrences (DiLillo et al., 2010; Smyth et al., 2008). Different genetic mutations and leukemia observed in *NHD13* mouse model at advanced age (Slape et al., 2008) may be a result of impaired immunity observed at earlier stages.

Specific antibody production after an immunological challenge using various hapten molecules has been routinely used to elicit specific immune function in numerous animal models (Herzenberg and Tokuhisa, 1982; HogenEsch et al., 1999; Osborne and Katz, 1972). Our initial investigations were to determine the antibody mediated response of the *NHD13* mice given that previous studies had shown a block in the B lymphocyte repertoire. Upon antigenic stimulation, IgM^+ B lymphocytes with matching antigen binding sites will undergo preferential proliferation. During their proliferation, B lymphocytes undergo an additional set of immunoglobulin heavy chain rearrangements with subsequent formation of IgG subclasses, IgA and IgE, wherein the variable antigen binding portion remains constant (Yan et al., 2007), a process known as class switch recombination. Upon antigenic stimulation with DNP-KLH, *NHD13* transgenic mice failed to mount an adequate immune response as determined by assaying plasma

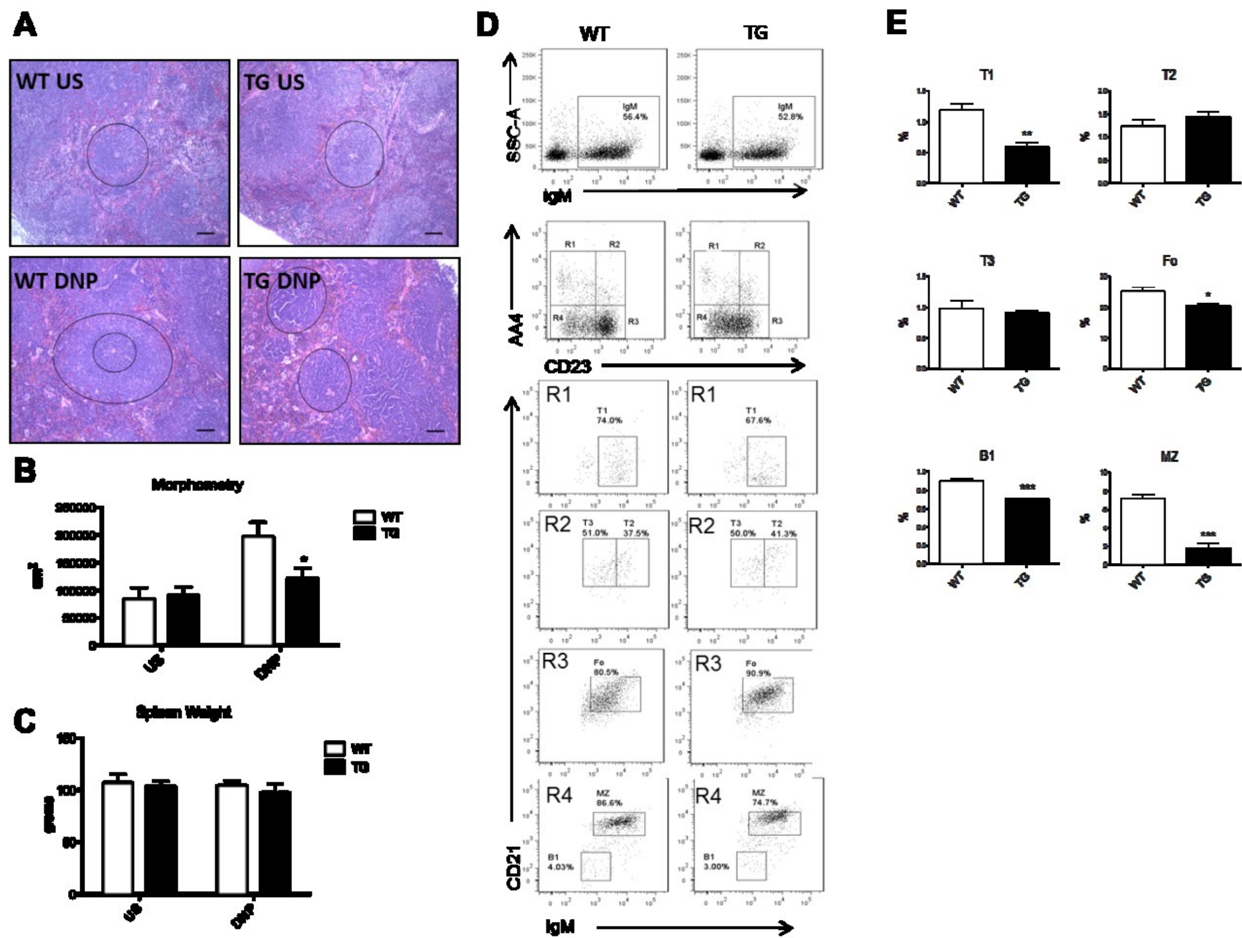

Figure 4-5. Clonal expansion in the spleen is impaired in *NHD13* mice after DNP stimulation
(A) Spleens collected following DNP stimulation were stained with H&E and evaluated using light microscopy. Representative photomicrographs of unstimulated WT spleen (WT US), unstimulated TG spleen (TG US), and WT and TG spleen post DNP stimulation (WT DNP and TG DNP respectively). Histopathology of spleen from TG mice revealed comparable follicular morphology between WT and TG unstimulated mouse spleens. However, after DNP stimulation, TG mice had smaller splenic follicles with poorly demarcated mantle and marginal zones. Follicles are outlined with black circles. (B) Follicles from *NHD13* mice have statistically significant reduction in size following stimulation when compared with WT controls; US= unstimulated, DNP=stimulated (n=5). (C) Analysis of spleen weights from unstimulated and stimulated WT and TG mice showed no significant difference between groups US= unstimulated, DNP=stimulated (n=3). (D) Representative FACS plots

of splenic B cell fractions. Single cell suspensions from spleens were labeled with fluorescently tagged anti-mouse IgM, CD21, CD23 and AA4.1 antibodies and analyzed by flow cytometry. Population R1 was gated for T1 stage; R2 for stages T2 and T3; R3 for follicular stage; and R4 for B1 and Marginal zone cells. **(E)** IgM^+ B cells in the first transitional stage (T1) was significantly lower in TG mice whereas transitional stages T2 and T3 were comparable between TG and WT controls. B-cells in the advanced splenic stages, Follicular (Fo), B1 and Marginal Zone (MZ) cells were significantly lower in TG mice compared to WT controls. (n=5 for TG and WT, *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$)

immunoglobulin levels. Considering that CSR involves DNA double strand breaks in immunoglobulin genes followed by recombination events, we suspected that the *NHD13* fusion gene might be impairing this recombination event.

Prior to antigenic stimulation we noted that *NHD13* mice had significantly higher levels of plasma IgM, compared to the WT controls. It is reasonable to believe that the IgM^+ lymphocytes were arrested during their development and failed to undergo proper class switch recombination, as observed in hyper IgM syndrome in humans. This assumption is further supported by lower IgG1 and IgE concentration in the presence of increased IgM. Comparable levels of MZ and Fo cells in the presence of reduced T2 cells (Figure 4-2 C) could be due to an impartial block or compensatory proliferation of these cells. Peripheral blood analysis by flow cytometry showed that the unstimulated *NHD13* mice had reduced IgM^+B220^+ mature B lymphocyte in circulation, further supporting the hypothesis that the *NHD13* fusion gene resulted in impaired B cell development and function.

Figure 6

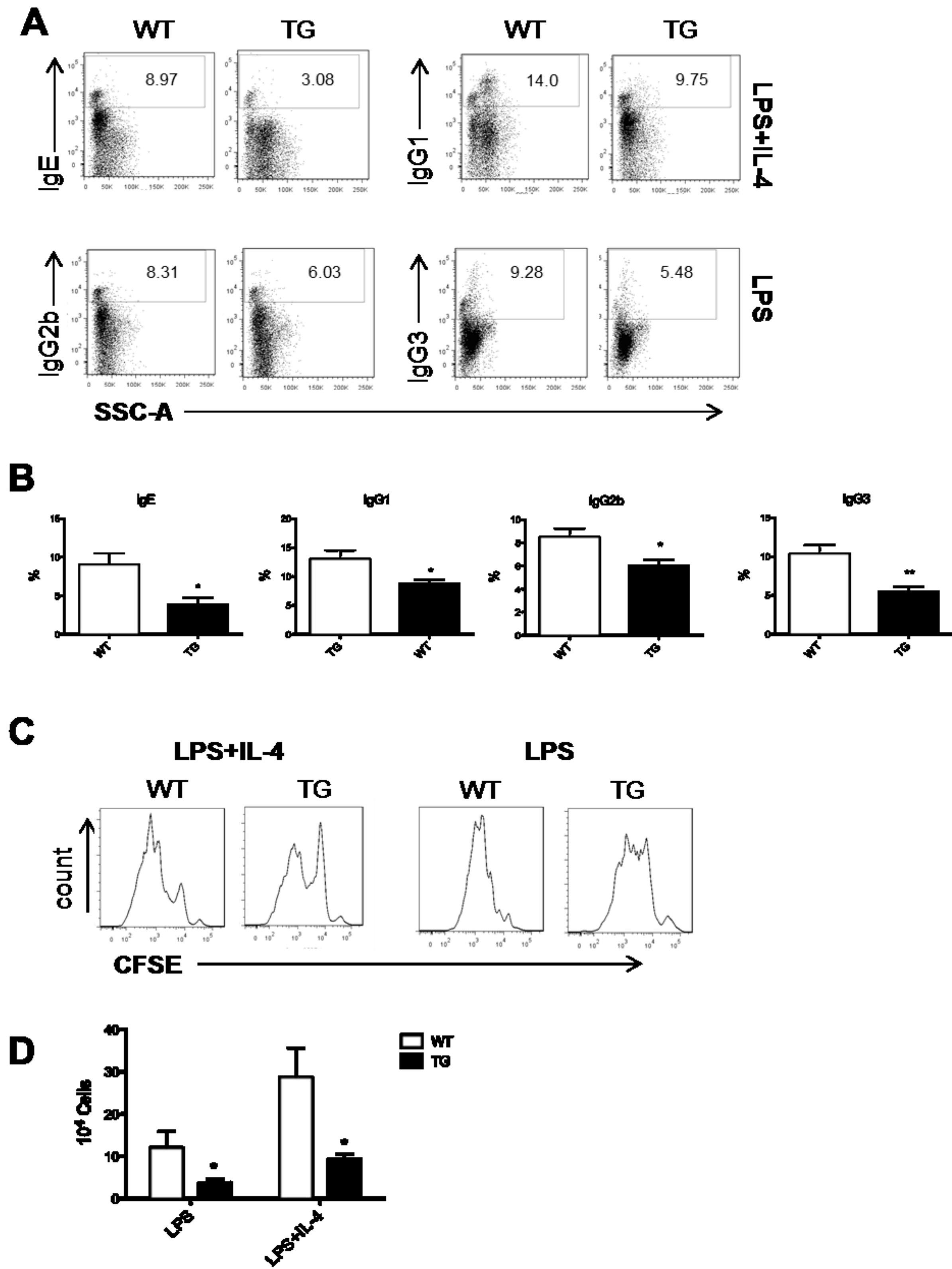

Figure 4-6. Class Switch Recombination is impaired in IgM$^+$ B lymphocytes from *NHD13* mice. IgM$^+$ cells were harvested from *NHD13* and WT spleens using positive selection with anti-mouse IgM antibody. Cells were labeled with CFSE and incubated with either LPS +IL-4 or LPS alone in triplicate wells. After 72 hours, Cells were harvested and labeled with anti-mouse IgG1 and IgE for LPS+IL-4 treated cells or IgG2b and IgG3 for LPS treated cells and analyzed by flow cytometry. **(A)** Representative flow cytometric plots of class switch recombination in IgM$^+$ splenic cells from TG and WT mice. **(B)** Statistical analysis showed significantly reduced class switch recombination in B cells from *NHD13* mice compared to WT controls. **(C)**. Proliferation pattern assessed by CFSE showed that impairment in proliferation of cells from TG mice following stimulation.. **(D)** Proliferation of stimulated cells was determined by counting the total cell yield from the culture. Our results showed reduced proliferation efficiency of B cells from *NHD13* mice. (n=5 for TG and WT, *=$p<0.05$, **=$p<0.01$)

A typical antibody-mediated immune response results in expansion of splenic follicles. Following antigenic stimulation with DNP, we observed that *NHD13* mice had severely reduced follicular development in their spleens (Figure 4-5A). Given our earlier observations in the bone marrow B lymphocyte compartment, it was reasonable to believe that these small, hypocelluar follicles were due to either: 1) deficient numbers of B lymphocyte precursors from bone marrow; 2) a lack of clonal expansion of existing B-lymphocytes; or 3), a combination of both suggesting that the *NHD13* transgene mediated its effect in both primary (bone marrow) and secondary lymphoid (spleen) compartments. Further analysis of the splenic B lymphocytes using flow cytometry indicated that the small follicles we observed histologically were a result of B lymphocytes unable to undergo clonal expansion, as evidenced by decreased follicular and marginal zone populations (Figure 4-5, C and D). When we considered the findings of impaired antibody production and deficient splenocytes, we reasoned that the *NHD13* might somehow impair class switch recombination as a mechanism responsible for our findings. Functional analysis of B-lymphocytes with either LPS or LPS and IL4 to stimulate CSR (Hodgkin et al., 1996) revealed that *NHD13* mice have significantly impaired CSR (Figure 4-6) thus validating our initial *in vivo* observations of reduced clonal expansion of splenic follicles and antibody production. We also considered the possibility that the reduced CSR could be partly due to impaired proliferation observed in *NHD13* B cell in culture (Figure 4-6 C, D). Finally, we acknowledge the role of T cells and related cytokines pertinent to B cell development and antibody production. These findings warrant further investigation into the mechanisms by which NHD13 impairs antibody mediated immune response.

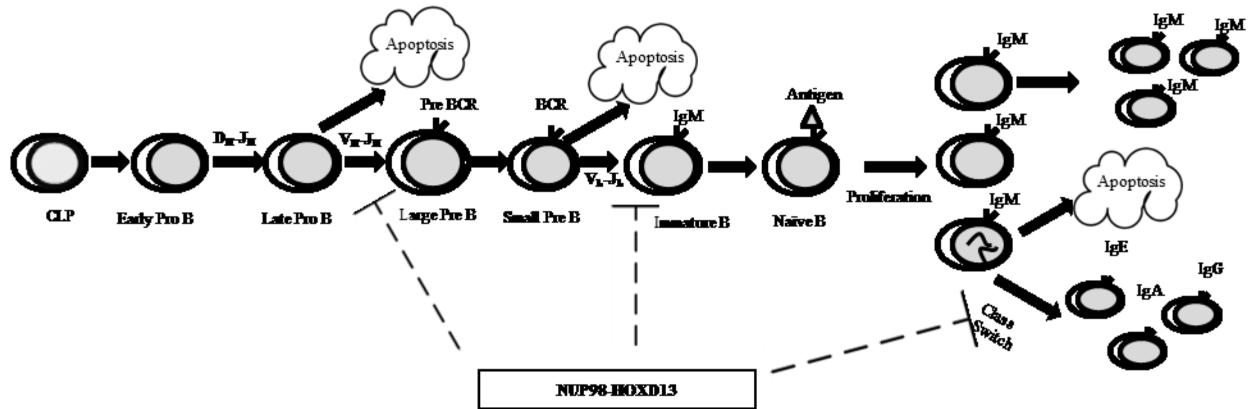

Figure 4-7. Schematic diagram depicting the partial blocks in B lymphocyte development in the bone marrow and spleen. B lymphocytes originate in the bone marrow from a common lymphoid progenitor (CLP) and proceed through early and late pro B stages before entering large and small pre B stages. Immature B lymphocytes exit the bone marrow and populate the spleen as naïve B lymphocytes where they are presented with antigens and undergo clonal expansion. The stages of B lymphocyte differentiation are characterized by Variable, Diversity, and Joining (VDJ) rearrangement in the bone marrow and class switch recombination (CSR) in the spleen. *NUP98-HOXD13* partially blocks late Pro B to large Pre B, small Pre B to immature B development and class switch recombination (indicated in dotted lines).

NUP98 proteins are involved predominately in nuclear membrane transport (Powers et al., 1997). Recent studies have shown these proteins can directly regulate transcription of a variety of genes and participate in mitosis (Capelson et al., 2010; Cross and Powers, 2011; Kalverda et al., 2010; Laurell et al., 2011). In addition, patients bearing these fusions have also been described with a compromised immune status (Kohler and Hurt, 2010). This state of health may contribute, not only to their disease progression, but also to their susceptibility of acquiring additional mutations (Kohler and Hurt, 2010). Studies suggest that reduced lymphocyte repertoire renders the patient susceptible to tumor development (Luo et al., 2011). Additional studies by Palmquist and colleagues involving forced expression of *NHD13* in hematopoietic stem cells identified candidate genes including Igh6 and H2-DMa (Palmqvist et al., 2007). These findings suggest that *NHD13* collaborates with genes known to be involved in signal transduction, lymphocyte proliferation, and immune responses. Our studies suggests a sequential block in B lymphocyte development and function by *NHD13* leading to an impaired immune response (depicted in Figure 4-7). Finally, given the putative role of adaptive immunity through B lymphocytes in controlling different hematopoietic malignancies, it is reasonable to believe that the effects of *NUP98* fusion genes on B lymphocytes will also contribute to disease progression.

In summary, expression of the *NUP98-HOXD13* transgene inhibits B lymphocyte development and function. These studies show that expression of a myeloid leukemic transgene inhibits class switch recombination as the mechanism underlying the impaired antibody mediated immune response. Taken together, these studies indicate that *NUP98-HOXD13* not only interferes with the differentiation of B lineage precursors, but that it also interacts with, as yet unidentified, critical components essential for immunoglobulin production. Further understanding of this

mechanism will shed additional insight into managing patients who harbor these chromosomal mutations, as well as other immunocompromised conditions.

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CHAPTER 5

The Leukemic Fusion Gene *NUP98-HOXD13* Impairs the Non-Homologous End Joining-Mediated DNA Repair Mechanism

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Submitted to Leukemia Research

Abstract

Background: Chromosomal translocations belong to class II mutations and typically impair hematopoietic cell differentiation. Many translocations require secondary mutations for malignant transformation, although the role of primary mutations in the development of collaborating mutations is debatable. Mutations occur as a result of increased DNA damage and or impaired damage repair mechanisms, which might occur as a consequence of the primary mutation. Previous studies have shown that the leukemic fusion gene *NUP98-HOXD13* (*NHD13*) impairs lymphocyte differentiation and class switch recombination in transgenic mice. After acquiring secondary collaborating mutations, these mice also develop leukemia. Physiological DNA break and repair mechanisms were analyzed to delineate the indirect role of the fusion gene in secondary mutagenesis. **Methods:** To determine the DNA break induction pattern and its repair mechanism, *in vitro* class switch recombination of splenic B lymphocytes was selected as a tool. DNA breaks, cell cycle analysis, and gene expression of non-homologous end joining factors in stimulated naïve B lymphocytes were determined. **Results:** Our findings show that the DNA double strand break induction pattern is not altered in *NHD13* B lymphocytes. However, the fusion gene resulted in reduced expression of DNA repair factors, specifically, DNA Protein kinase catalytic subunit (DNAPKcs), DNA ligase 4 and Xrcc4 leading to less efficient repair mechanisms and subsequent cell cycle arrest at the G2/M stage. **Conclusions:** Our results show that leukemic fusion gene *NHD13* results in impaired DNA double strand break repair, by preventing DNAPKcs, DNA Ligase 4 and Xrcc4 activity. Taken together, impaired non-homologous end joining mediated DNA repair might be the reason for the secondary mutations and leukemic transformation observed in this mouse model. Our studies reveal the potential role of a primary leukemic fusion gene in inducing secondary mutations and subsequently contributing to leukemic progression.

Key Words: Chromosomal translocation, NUP98, HOXD13, DNA double strand break, non-homologous end joining

Introduction

Chromosomal translocations (CT) are the hallmark features associated with many hematological malignancies. The majority of CT are considered class II mutations resulting in impaired hematopoietic cell differentiation but require collaborating secondary mutations for complete malignant transformation (Caudell and Aplan, 2008; Caudell et al., 2010). However, the mechanisms by which the cell acquires secondary mutations are not completely understood. Increased DNA damage and/or impaired DNA repair mechanisms are widely accepted as mechanisms for genomic mutations (Markowitz and Bertagnolli, 2009; Mladenov and Iliakis, 2011). An emerging concept is that CT might increase the chance for further DNA damage or that they can somehow impair the DNA repair mechanisms (Fan et al., 2010; Sallmyr et al., 2008; Skorski, 2007). Of the different types of DNA damage to occur, one of the most important types is the double strand breaks (DSB), which occurs in each dividing cell at an estimated rate of 10 breaks per day (Lieber et al., 2003). These breaks are typically repaired by Non-homologous end joining (NHEJ) which is a tightly controlled process involving multiple factors (Kenter, 2005). Ironically, impaired NHEJ can lead to mutations including the formation of CT. Delineating DNA break induction and repair pathways in cells with primary CT during leukemic progression will identify the potential roles of CT in inducing secondary mutations and potential therapeutic targets.

Transgenic mice expressing the leukemic fusion gene *NUP98-HOXD13 (NHD13)* progress through Myelodysplastic syndrome (MDS) and develop acute leukemia with the occurrence of collaborating mutations (Slape et al., 2008). We have recently shown that expression of *NHD13* in stimulated B-lymphocytes results in: 1) impaired B-cell differentiation at stages in the bone marrow that are dependent on VDJ recombination and 2) impaired class switch recombination (CSR) and antibody production (Puthiyaveetil et al., 2012). During CSR naïve B lymphocytes

undergo DNA breaks on immunoglobulin heavy chain gene and NHEJ-mediated break repair when stimulated with appropriate antigens and cofactors to produce different immunoglobulin isotypes (Stavnezer et al., 2008). DNA DSB results in the phosphorylation of histone H2AX by Ataxia telangiectasia mutated (ATM), which in turn results in the stabilization of break ends. Proteins Ku70 and Ku80 binds to the break ends to form a heterodimer and recruits DNA PKcs. DNAPKcs acts as a scaffold and DNA repair factors DNA ligase 4 and XRCC4 complex. As CSR involves physiological DNA breaks and NHEJ mediated repair, expression of the *NHD13* fusion in B lymphocytes is a suitable system for delineating the mechanism by which CT can induce DNA instability and result in secondary mutations. Based on these observations, we hypothesized that *NHD13* enhances double strand break induction and impairs the DNA repair mechanism as an underlying reason for secondary mutations. Here we used CSR as a tool to induce DNA DSB repair in the presence of *NHD13*. Our results show that *NHD13* fusion gene impairs expression of critical NHEJ repair factors, and impairs DNA repair mechanism resulting in cell cycle arrest.

Results

B cells from *NHD13* mice had reduced DNA double strand break repair

Class switch recombination essentially involves DNA double strand breaks and NHEJ-mediated DNA recombination. To delineate the effect of *NHD13* on the pattern of DNA DSB during CSR, we analyzed stimulated B cells from *NHD13* and WT mice for DNA breaks using flow cytometry and confocal microscopy. Lymphocyte stimulation using *E. coli* Lipopolysaccharide (LPS) and IL-4 results in expression of Activation induced deaminase (Aid), which will initiate DNA DSB. These DNA DSB result in phosphorylation of histone H2AX (γH2AX), and can be employed as a marker for DNA DSB. Flow cytometric analysis for γH2AX revealed a

comparable percentage of cells with DNA breaks in *NHD13* and WT mice at 0, 24 and 48 hours (Figure 5-1 A and B). However, at 72 hours, *NHD13* mice had increased DNA DSB as evidenced by flow cytometric analysis (Figure 5-1 A, and C). We also analyzed the pattern of breaks at the single cell level using confocal microscopy (Figure 5-1 B). The DNA repair efficiency, expressed as the percentage of reduction in break positive cells, was estimated between 48 and 72 hours. Our results showed a significantly lower DNA repair efficiency in *NHD13* B cells (Figure 5-1D). These results indicate that *NHD13* B cells have comparable DNA double strand break induction following stimulation, but have reduced efficiency for break repair.

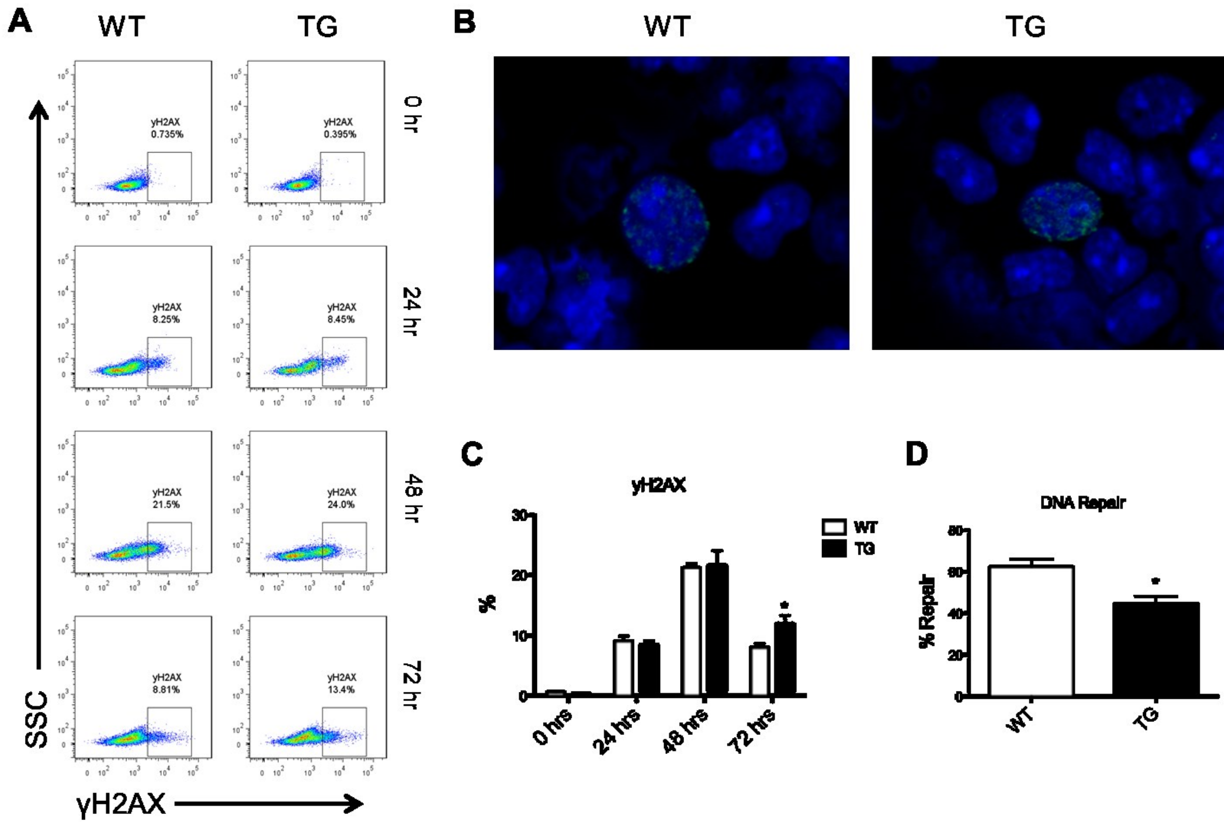

Figure 5-1. B cells from *NHD13* mice have impaired DNA double strand break repair following stimulation. LPS and IL-4 stimulated splenic B cells from *NHD13* and WT mice were harvested at 24 hour intervals and labeled for γH2AX, a marker for DNA double strand breaks. **A.** Representative flow cytometric pattern of DNA double strand breaks at 24 hour intervals. **B.** confocal analysis of DNA double strand breaks showing DNA breaks in *NHD13* and WT B-cells at 72 hours. **C.** Analysis of the percentage of DNA breaks using flow data showed significantly high levels in *NHD13* B-cells at 72 hours following stimulation. **D.** DNA repair efficiency calculated based on the percentage of breaks repaired between 48 and 72 hours showed significantly lower repair efficiency in *NHD13* B-cells. n=5, $p<0.05$

***NHD13* B cells had a G2/M cell cycle arrest following stimulation**

To better understand the impact of *NHD13* on DNA repair, we determined the cell cycle kinetics of stimulated B cells from *NHD13* mice at 24 hour intervals. B cells were stimulated with LPS and IL-4, harvested at 24 hour intervals and labeled with PI for flow cytometric analysis. Our results showed a comparable cell cycle pattern in *NHD13* and WT cells at 0, 24 and 48 hours following stimulation as indicated by a comparable percentage of apoptotic cells and cells in G1, S and G2/M phases (Figure 5-2 A and B). However, at 72 hours of stimulation, *NHD13* mice had a significantly higher percentage of cells in the G2/M stage (Figure 5-2 A and B) indicating cell cycle arrest at this stage. Cells in other stages, apoptotic, G1 and S phase were altered in *NHD13* mice at 72 hours, but not significant (Figure 5-2 B). We assayed expression of the cell cycle regulator gene *p53* and found that its expression was higher at 72 hours in *NHD13* B cells (not statistically significant, Figure 5-2 C). Our results suggest that the *NHD13* fusion gene impairs the DNA break repair mechanism and results in a G2/M check point arrest during the cell cycle in stimulated B-cells.

DNA Ligase 4, XRCC4, and DNAPKcs were down-regulated in *NHD13* B lymphocytes

Considering the essential role of NHEJ during DNA double strand repair, we hypothesized that *NHD13* fusion gene might interfere with the NHEJ mechanism. To test this assumption, we harvested RNA from stimulated B cells at 72 hours of culture and assayed the gene expression of critical genes involved in NHEJ mechanism. Our results showed comparable levels of Ku70 and Ku80 levels in cells from both *NHD13* and WT mice (Figure 5-3A). However, *DNA ligase 4, Xrcc4,* and *DNAPKcs* were significantly lower in *NHD13* B lymphocytes. We also considered the possibility that Alternative End Joining (AEJ) could play a role during the DNA repair process. We evaluated expression of possible AEJ factors including *Ligase I* and *Ligase 3α*

(Figure 5-3 B) and found that there was no statistical significance between transgenic and wild type cells. Our results indicate that expression of *NHD13* fusion gene results in reduced expression of *DNA ligase 4, Xrcc4,* and *DNAPKcs*, leading to reduced DNA repair.

Discussion

Chromosomal translocations are hallmark features of hematopoietic malignancies, which generally require collaborating mutations for malignant transformation (Gilliland, 2002). Recent studies have proposed that CT can induce secondary mutations before malignant transformation (Fernandes et al., 2009; Sallmyr et al., 2008a). A wide variety of factors can cause DNA damage, promote misrepairs and lead to the development of mutations (Lin et al., 2012). Of these etiologies, DNA DSB (Kasparek and Humphrey, 2011) are frequent in mammalian cells as a result of physiological and pathological factors including reactive oxygen species, ionizing radiations or chemicals (Vilenchik and Knudson, 2003; Vilenchik and Knudson, 2000), and are repaired mainly by NHEJ (Mladenov and Iliakis, 2011). Effective repair is essential for the maintenance of genomic stability and cell viability, whereas defective break repair results in cell cycle arrest, apoptosis, mutagenesis and malignant transformation (Sallmyr et al., 2008b; Tseng et al., 2009). Here, we analyzed the effects of a primary leukemic fusion gene, *NHD13,* on the DNA DSB repair pathways. Previous studies in transgenic mice expressing *NHD13* have shown that leukemic progression is accompanied by random mutations in a wide variety of collaborating genes (Slape et al., 2008). Taken together, it is reasonable to believe that the presence of a primary leukemic fusion gene can induce DNA instability by impairing the NHEJ pathway and lead to the formation of secondary mutations.

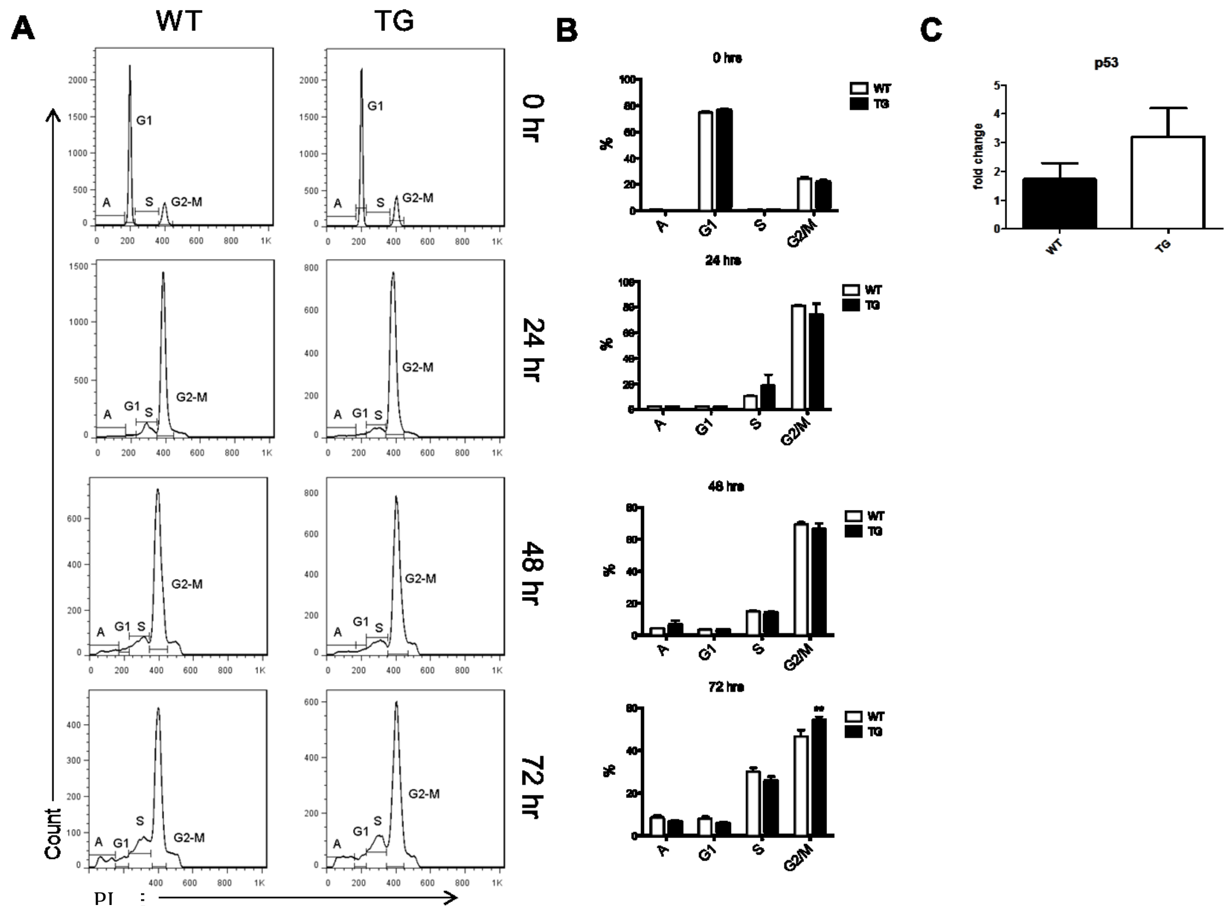

Figure 5-2. Cell cycle is arrested at G2/M phase in B-cells from *NHD13* mice following stimulation. Isolated splenic B cells were stimulated with LPS and IL-4 to induce CSR. Cells were harvested at 24 hour intervals and stained with Propidium Iodide to analyze cell cycle pattern. **A.** Representative cell cycle plots from *NHD13* and WT cells at 24 hour intervals, 0 indicating prior to stimulation. **B.** Statistical analysis of different phases of cell cycle pattern showed comparable kinetics at 0, 24 and 48 hours. However, at 72 hours, cells in G2 phase were significantly high in *NHD13* mice suggesting a cell cycle arrest. **C.** Expression analysis of *p53* showed elevated levels in *NHD13* lymphocytes 72 hours following stimulation. n=5, **=p<0.01

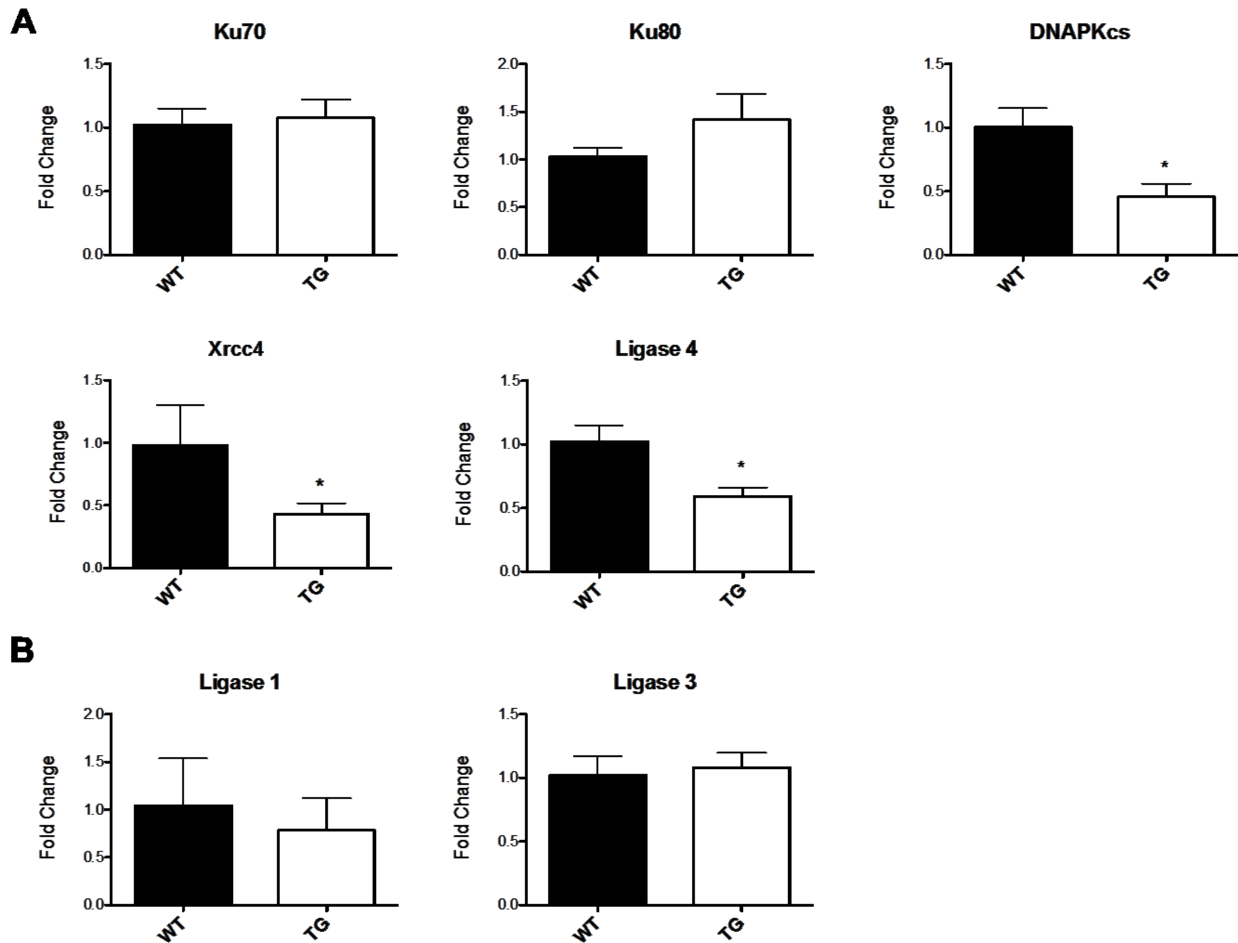

Figure 5-3. DNA ligase 4, Xrcc4 and DNAPKcs expression is downregulated in *NHD13* B cells following stimulation. LPS and IL-4 stimulated splenic B cells were harvested at 72 hours following stimulation and, RNA was harvested for RQPCR. **A.** Expression analysis of critical NHEJ genesf showed that *Ku70*, and *Ku80* were comparable between *NHD13* and WT B cells. However, *DNA ligase 4, Xrcc4* and *DNAPKcs* were significantly low in *NHD13* mice following stimulation. **B.** Expression analysis of AEJ factors *Ligase 1* and *Ligase 3* showed comparable levels in both WT and *NHD13* TG B lymphocytes. n=5, $p<0.05$

Analysis of lymphocyte development and gene recombination pattern in *NHD13* mice suggests that the VDJ recombination mechanism is perturbed in *NHD13* lymphocytes (Choi et al., 2009). Furthermore our previous studies have shown that B lymphocyte differentiation and CSR are altered in *NHD13* mice, with partial blocks occurring during gene recombination events (Puthiyaveetil et al., 2012). Based on these findings, we used CSR as a tool to determine the role of *NHD13* to induce DNA breaks and thereby analyze the NHEJ-mediated break repair pattern. As CSR involves DNA double strand break induction and NHEJ-mediated repair mechanisms, delineating the underlying molecular pathways of impaired CSR can have wider applications (Bothmer et al., 2011). Our current study showed a comparable percentage of cells with DNA breaks prior to stimulation and at 24 and 48 hours following stimulation (Figure 5-1 A, B). However, the percentage of cells with DNA breaks was significantly higher at 72 hours of culture, suggesting an impaired DNA repair mechanism. Single cell analysis for DNA breaks using confocal microscopy showed a comparable amount of breaks in both WT and TG B cells, suggesting that the breaks are induced in a similar pattern. DNA break repair efficiency calculated based on the percentage of repair in a 24 hour time frame (Puthiyaveetil et al, 2013, Molecular Immunology, in press) showed a significantly lower repair in stimulated cells from *NHD13* mice between 48 and 72 hours (Figure 5-1D), suggesting that DNA repair is impaired in *NHD13* mice rather than break induction. Analysis of cell cycle pattern showed a G2/M stage cell cycle arrest in *NHD13* B lymphocytes at 72 hours of stimulation. As previous studies have shown that DNA repair defects will result in cell cycle arrest at G2/M checkpoint (Shibata et al., 2010; Sturgeon et al., 2006), our results further verified that cell cycle arrest is mediated by defective or inefficient DNA repair mechanism in *NHD13* B cells.

Our analysis of expression of DNA repair factors showed comparable levels of *Ku70, Ku80* and, but significantly lower levels of *DNA Ligase 4*, *Xrcc4*, and *DNAPKcs* at 72 hours following stimulation. Reduced expression of repair factors have been shown to impair classical NHEJ pathway, leading to more error prone repair mechanisms, increasing the chance for secondary mutations (Fan et al., 2010). During NHEJ-mediated DNA repair, *Ligase 4* expression is often associated with increased AEJ activity. However, in our model, the C-NHEJ factors *DNA Ligase 4*, *Xrcc4*, and *DNAPKcs* are down regulated without any substantial increase in expression of AEJ factors, i.e., *Ligase1* and *Ligase 3α*. Considering the phosphorylation of H2AX, an early break detection process, is intact but that NHEJ factors are not up-regulated, we propose that *NHD13* impairs DNA break repair induction. In conclusion, our results showed that the myeloid leukemic fusion gene *NHD13* suppressed the expression of *DNA Ligase 4*, *Xrcc4*, and *DNAPKcs*, DNA double strand break accumulation and cell cycle arrest as probable underlying mechanisms for secondary mutations. Further delineating the molecular pathways of *DNA Ligase 4*, *XRCC4*, and *DNAPKcs* regulation by this chromosomal translocation might reveal novel therapeutic targets in the treatment of leukemia and cancer.

Materials and Methods

Animals

Five young (8-12 week) *NHD13* mice on an FVB background were used for each experiment. Five wild type (WT) FVB littermates of the same age were used as controls. Mice were bred and maintained at the AAALAC accredited core laboratory animal facility at Virginia Maryland Regional College of Veterinary Medicine, Virginia Tech. All experiments were carried out as per NIH guidelines with prior approval from the Virginia Tech Institutional Animal Care and Use Committee (IACUC).

In vitro Class Switch Recombination

To induce in vitro class switch recombination, splenic B lymphocytes from transgenic and wild-type mice were harvested using anti-mouse IgM magnetic beads and magnetic assisted cell sorting (Milteny Biotec, Auburn, CA). A total of $2x10^5$ cells were treated with 5 µM CFSE and cultured in 96 well culture plates (BD Biosciences) with media containing *E coli* Lipopolysaccharide (LPS, Sigma Aldrich, St. Louis, Missouri) (25 µg/ml) and IL-4 (PeproTech, Rocky Hill, NJ) (25ng/ml). To verify that CSR had occurred, cells were harvested at 72 hours, labeled with anti-mouse IgG1 and IgE antibodies and analyzed by flow cytometry as previously published (Puthiyaveetil et al., 2012).

DNA Break Analysis by Confocal Microscopy

Splenic B lymphocytes were harvested and cultured to induce CSR as described above. Cells were harvested at 0, 24, 48 and 72 hours of culture and cytospun onto positively charged slides. Cells were fixed using 4% paraformaldehyde (Thermo Scientific, Rockford, IL) and blocked with Tris buffered saline (TBS) containing 4% Fetal bovine serum (FBS). Cells were then incubated with rabbit anti-mouse γH2AX (Cell Signaling, Danvers, MA) at 1:500 dilution in TBS+1% FBS followed by incubation with Alexafluor-488 conjugated anti-rabbit antibody (Cell Signaling, Danvers, MA) at 1:1000 dilution in TBS with FBS. After labeling with DAPI (Cell Signaling), cells were visualized using LSM700 Carl Zeiss confocal microscope (Carl Zeiss Microimaging, Thornwood, NY) and LSM900® software (Carl Zeiss).

DNA Break Analysis by Flow Cytometry

Splenic B lymphocytes cultured in the presence of LPS (Sigma Aldrich) and IL-4 (Sigma Aldrich) were harvested at 0, 24, 48 and 72 hours. Cells were fixed with 70% ethanol in PBS at -

20ºC for 24 hours and permeabilized with 0.1% Triton-X (Sigma Aldrich) in TBS. Cells were then incubated with rabbit anti-mouse phosphoH2AX antibody (Cell Signaling) at 1:500 dilution in TBS with FBS at 4ºC for 30 minutes followed by incubation with Alexa-fluor 488 conjugated anti-rabbit antibody (Cell Signaling) at 1:1000 dilution. Cells were analyzed using a FACScan flow cytometer (BD Biosciences).

Cell Cycle Analysis

B lymphocytes from *NHD13* and WT mice were harvested and cultured in the presence of LPS and IL-4 as described above. Cells were harvested at 24 hour intervals and $2x10^6$ cells were fixed in 70% ethanol in PBS at -20ºC for at least 24 hours. Cells were then incubated with propidium iodide (PI) (Sigma Aldrich) at 5µg/ml in PBS containing RNAaseA (5 Prime, Gaithersburg, MD) at room temperature for 20 minutes and kept on ice. Cells were analyzed by flow cytometry.

Gene Expression Analysis

Lymphocytes stimulated with LPS+IL-4 were harvested at 72 hours of culture, total RNA was harvested using Qiagen RNeasy kit (Qiagen, Valencia, CA) and cDNA was synthesized with Superscript III first strand synthesis system (Invitrogen, Grand Island, NY) as per the manufacturer's protocol. Gene expression was determined by reverse quantitative PCR using gene specific primers, Sybrgreen mastermix (Applied Biosystems) using an IQ5 Real-time PCR detection system (Bio-rad, Hercules, CA). Gene expressions were quantified by 2(-delta delta CT) method using Gapdh as an internal control gene (Livak and Schmittgen, 2001).

Data and Statistical Analysis

Flow cytometric data were analyzed using FlowJo software 7.6 (FlowJo, Ashland, OR). Confocal images were analyzed using Zen 2009® software (Carl Zeiss Microimaging). Data was

analyzed with GraphPad Prism 5.0® (Graphpad Software, La Jolla, CA), using either two tailed t-test or ANOVA and Bonferroni posttest; a P value <0.05 was considered significant.

Competing Interest Statement

The authors declare that they have no competing interests.

Author Contributions

AG designed and performed experiments and prepared the first draft. CR designed experiments and helped in experiments and manuscripts preparation. DC conceived and designed the study and prepared the final draft of manuscript.

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CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

Efficient DNA repair is essential for the maintenance of genomic integrity; impaired repair can lead to cell cycle arrest, apoptosis, and mutations. Of the different types of DNA damages, double strand breaks (DSB) are considered the most serious threat to cell survival, and the majority of them are repaired by non-homologous end joining (NHEJ). Accumulation of DNA damage has been implied in aging and age-related conditions, which can occur as a result of 1) increased DNA damage by environmental and physiological factors, and 2) impaired DNA repair mechanisms. Furthermore, previous studies have proposed that chromosomal translocations, a common mutation identified in a variety of malignancies, require secondary mutations for complete malignant transformation. Due to the presence of multiple mutations in cancer patients at the time of diagnosis, it is reasonable to suspect that the primary mutation might in fact increase the likelihood for subsequent mutations. As genetic mutations and DNA break accumulations can occur as a result of impaired DNA repair mechanisms, deficient NHEJ could be a potential mechanism underlying the DNA break accumulation and altered cell cycle kinetics during the aging process and leukemic progression.

B lymphocytes were used as a tool for analyzing the DNA break induction pattern and repair mechanisms. During bone marrow development, B cells undergo gene recombination in their immunoglobulin gene, known as VDJ recombination. Also, upon antigenic stimulation, mature naïve B cells undergo another set of gene recombination events, class switch recombination (CSR), to produce different isotypes of antibodies. Given that VDJ and CSR involve DNA DSB and NHEJ mediated repair, B lymphocytes are a good model system in which to delineate the DNA break induction and repair mechanisms. To determine the effect of aging and CT on DNA repair, B lymphocytes from mouse models for aging and leukemic progression were analyzed for their developmental pattern, DNA break induction and efficiency of NHEJ-mediated repair.

In order to delineate the DNA break and repair pattern during aging, naïve B lymphocytes were stimulated with LPS and IL-4. Analysis of break induction and repair pattern showed significantly lower repair efficiency in B cells from old mice, thus supporting the hypothesis that NHEJ–mediated DNA repair is compromised during aging. An *in vitro* plasmid ligation assay was used to validate the DNA repair pattern, which further confirmed that NHEJ is impaired in B cells from old mice and resulted in increased misrepairs. Analysis of Alternative end joining (AEJ) factor *DNA ligase 3*α showed increased levels of expression, suggesting that AEJ might play a predominant role in DNA repair during aging. These findings suggested that impaired NHEJ and elevated AEJ could be responsible for the increased accumulation of DNA damage, reduced immunity, and cancer susceptibility during aging.

To delineate the effects of a leukemic CT in hematopoietic cell differentiation, the *NUP98-HOXD13 (NHD13)* transgenic mouse model was used. *NHD13* mice show impaired bone marrow differentiation of lymphocytes and progression to leukemia after acquiring secondary mutations (Choi et al., 2008). Analysis of B cell differentiation patterns showed partial blocks in the bone marrow and peripheral lymphoid development, suggesting VDJ and CSR are impaired. This was further confirmed by an *in vitro* CSR assay, which suggested that the presence of the fusion gene could interfere with the NHEJ-mediated DNA repair.

To better understand the role of the *NHD13* fusion gene in NHEJ-mediated DNA repair, a comprehensive analysis of CSR was performed in B cells from transgenic and wild type mice. Analysis of cell cycle pattern and DNA break induction/repair process showed that the fusion gene in fact compromised NHEJ-mediated DNA repair, leading to cell cycle arrest. Gene expression analyses for core NHEJ factors showed significantly reduced levels of *DNA PKcs, DNA ligase 4*, and *Xrcc4* in transgenic mice, suggesting that the *NHD13* fusion gene impaired

initiation of NHEJ-mediated repair. These results showed that the presence of a primary leukemic fusion gene can result in reduced DNA repair mechanisms and altered cell cycle kinetics.

The study focused on delineating the roles of DNA DSB induction patterns and NHEJ-mediated repair mechanisms during aging and leukemia. The results showed impaired NHEJ during aging, resulting in less efficient DNA DSB repair and reduced CSR. Studies on the role of CT in lymphocyte development in *NUP98-HOXD13* (*NHD13*) mice showed impaired B cell differentiation within the bone marrow and altered DNA break repair and reduced expression of classical NHEJ factors in peripheral B cells, suggesting impaired DNA repair mechanisms. These results suggested that NHEJ-mediated repair is altered under stressful conditions and contributed in the cell cycle arrest, increased apoptosis, and reduced immunity. Finally, the study explored the possibility of impaired NHEJ as a crucial factor in developing misrepairs, leading to mutations and malignant progression.

The analysis of NHEJ-mediated DNA repair in B cells under stressful conditions showed their altered kinetics, compromised cell cycle regulations, and increased apoptosis. As impaired DNA repair is considered one of the major contributing reasons underlying the aging process, this study showed the severity of compromised NHEJ and its role in DNA break accumulation and antibody production. Furthermore, there are people with cryptic primary chromosomal translocations without any clinical symptoms (Chng et al., 2007; Mansour et al., 2007); understanding the mechanisms of secondary mutation development will possibly aid in preventing malignant progression in this subset of people. Thus, these studies shed insight into the mechanisms of altered NHEJ and help in developing preventive or therapeutic strategies against accumulation of DNA damage, compromised immunity and secondary mutations.

Future Directions

The following outline of experiments was submitted in part of a National Institute of Health AREA R-15 Grant.

Stimulated B lymphocytes can be used as a tool to delineate the DNA break induction and NHEJ mediated DNA break repair pathways. Previous studies have shown that expression of *NHD13* fusion gene in mice leads to impaired hematopoietic differentiation and leukemic progression. Further, our analysis herein showed that the fusion gene could interfere with the expression of DNA repair genes and CSR, leading to impaired antibody mediated immune response (Chapter III). This novel role of a myeloid leukemic fusion gene in regulating lymphocyte development and function is important, as the primary fusion gene sets a stage for secondary mutations and immune evasion of tumor cells. Understanding the possible molecular pathways of the *NHD13* fusion gene in regulating DNA repair and CSR will help in developing therapeutic strategies for patients with similar disease conditions. Further understanding of the vital role of the *NHD13* fusion gene is important to the biology of cancer, infectious disease, and immune responses. The proposed studies will expand the understanding on the role of *NHD13* in regulating gene expression, leukemogenesis, and antibody-mediated immune responses. This study could in fact reveal novel mechanisms for *NUP98* and/or its fusion partner *HOXD13* and

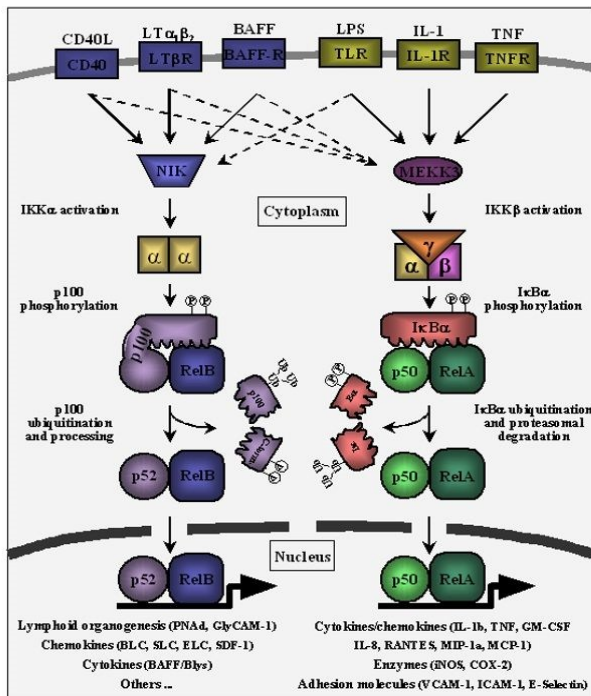

Figure 6-1. Representative diagram of Classical and Alternate NF-kB pathways.

might lead to discovery of novel therapeutic targets.

NF-kB in class switch recombination: Initiation of class switch recombination begins with the activation of NF-kB, which in turn regulates expression of NHEJ factors (Kobayashi et al., 2009). Classical NF-kB signaling (Figure 6-1) occurs through TNFR, IL-1R, or TLR activation. Downstream activation of MAP/ERK kinase kinase 3 (MEKK3) and IKKb eventually results in degradation of IkBa and subsequent translocation of the RelA/p50 homodimer to the nucleus (Blonska et al., 2004). Activation through the alternative NF-kB pathway (Figure 6-1) in B cells begins with signaling through CD40, LTbR, or BAFF-R. This leads to activation of NIK and IKKa, and subsequent translocation of p52/RelB dimers into the nucleus (Baeuerle and Henkel, 1994; Bishop and Hostager, 2001; Gerondakis et al., 1998; Hayden and Ghosh, 2011). The focus of the specific aim is to understand the effects that the *NHD13* transgene on the NF-kB alternate pathway, thus leading to impaired CSR. There is strong evidence in the literature to support the hypothesis that *NHD13* regulates the NF-kB pathway (Nakamura, 2005; Takeda et al., 2010): 1). Studies show that *NUP98* overexpression in cancer cells results in increased nuclear accumulation of NF-kB (Takahashi et al., 2008) and, 2), Nup98 contains FG/GLFG repeats, which can bind DNA and augment transcription (Griffis et al., 2002; Radu et al., 1995); these repeat regions are retained in the *NHD13* fusion (Lin et al., 2005). Taken together it is reasonable to believe that *NHD13* dysregulates NF-kB activity in such a way as to impair NHEJ.

Specific Aim: Determine the effect that *NUP98-HOXD13* exerts on NF-kB signal transduction in B-cells during class switch recombination and NHEJ.

Rationale: Upon antigenic stimulation of naïve B cells, they undergo class switching to produce different antibody isotypes. This process is tightly regulated through different signaling

pathways, including NF-kB (Stavnezer et al., 2008). Activation of the NF-kB results in downstream activation of NHEJ factors including DNAPKcs, Ku70 and Ku80, which are necessary for repairing the DNA breaks during CSR (Castro et al., 2009; Dolcet et al., 2005; Manis et al., 2002; Zhang, 2003). Our preliminary data indicates that activation of naïve B cells from *NHD13* transgenic mice failed to induce proper CSR, compared to wild type controls (Puthiyaveetil et al., 2012). Given the interaction between NHEJ factors and NF-kB, it is possible that the NF-kB pathway might play a role in compromised NHEJ. Analysis of *DNAPKcs* using RQ-PCR showed that indeed, the B-cells from *NHD13* mice have decreased *DNAPKcs* expression, suggesting a possible mechanism by which the *NHD13* transgene exerts its effect on NF-kB during CSR. Furthermore, impaired NF-kB signaling can lead to elevated *p53* (Dolcet et al., 2005; Luo et al., 2005; Sen, 2006), as observed in the *in vitro* CSR study in transgenic B-cells, further supporting the hypothesis that the transgene may exert its effect through NF-kB. Taken together, it is reasonable to believe that the *NHD13* fusion may interfere with NF-kB transduction signaling as a possible mechanism for impaired NHEJ. To define the mechanism by which *NHD13* interacts with the NF-kB in CSR, the expression, localization, and activity of NF-kB following activation in transgenic and WT B-cells will be determined.

Experimental Objective. Determine the activation and function of the NF-kB factors following antigenic stimulation of B cells expressing the *NHD13* transgene.

Task 1. Determine the effect of *NHD13* on mRNA and protein expression of critical NF-kB factors in activated B-cells. Stimulation of naïve B-cells with anti-CD40 and IL-4 leads to change in expression, localization, and binding activity of RelA, RelB, cRel, p50, and p52, the five known mammalian NF-kB factors. (Figure 1). Considering that Nup98 may bind to the NF-

kB complex, it is possible that the NHD13 fusion protein might bind to the NF-kB complex and inhibit the transduction pathways. Naïve transgenic and WT B-cells activated with anti-CD40 and IL-4 (Puthiyaveetil and Caudell, 2012) will be used to test whether or not the expression of NF-kB factors are impaired in transgenic B-cells. The mRNA expression will be assayed using Real-time reverse quantitative transcription (RQ-PCR) and TaqMan primer and probe sets (Applied Biosystems). The expression of the 18S ribosome will be used as an endogenous control. RQ-PCR results will be reported as relative gene expression and the fold change in target genes will be determined by calculating the -ΔCt mean and SE for each sample [$-\Delta Ct = (Ct_{Target} - Ct_{18S})_{Gx} - (Ct_{Target} - Ct_{18S})_{control}$; Ct value = the cycle number that crosses the signal threshold]. Final results for RQ-PCR will be expressed as mean plus or minus SD and tested using a two tailed Student *t*-test as previously published (Caudell et al., 2010; Puthiyaveetil and Caudell, 2012). Likewise, protein expression will be performed for these same proteins using Western blotting techniques as previously published (Caudell et al., 2010)

Task 2. Determine the activation and localization of NF-kB in activated B-cells that express the *NHD13* transgene. To test if the *NHD13* transgene inhibits NF-kB activation, Western blot and immunocytochemistry will be used. In short, naïve B cells from *NHD13* and WT mice will be stimulated with anti-CD40 and IL-4 to induce class switch recombination. After two, four, and eight hours of stimulation, total cytoplasmic and nuclear proteins will be harvested to determine the phosphorylation of NF-kB. Also, NF-kB nuclear translocation will be verified using confocal microscopy as previously published (Peairs et al., 2009). Cells will be visualized on a Carl-Zeiss® confocal microscope located in the core facility at the Fralin Life Sciences

Institute at Virginia Tech using Argon and Helium-Neon lasers and UV light. The confocal images will be analyzed using Zen2009® software.

Task 3. Investigate whether the *NHD13* transgene induces IkB expression following activation. One possible mechanism for the inhibition of NF-kB nuclear translocation is phosphorylation of IkB, a negative regulator for NF-kB pathway. To test whether the *NHD13* transgene induces IkB expression/ activation isolated naïve B-cell from *NHD13* transgene and WT controls stimulated with anti-CD40 and IL-4 will be used. At various time points (15, and 30 minutes, and 1, 2, and 4 hrs), IkB protein (phosphorylated and non-phosphorylated) and mRNA expression will be determined.

Task 4. Determine the DNA binding activity of NF-kB in activated B-cells that express the *NHD13* transgene. Activation of intact NF-kB pathway results in nuclear localization of NF-kB factors, their DNA binding and subsequent transcriptional regulation of NHEJ factors. Here, the DNA binding property of NF-kB complexes in activated B-cells will be determined using Electrophoretic Mobility Shift Assay (EMSA). Naïve B cells will be stimulated as previously explained, and nuclear and cytoplasmic extracts will be harvested at 12 hours of stimulation using the NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Pierce Biotechnology, Rockland, IL). Nuclear extracts will be incubated with custom IR-Dye 700 end-labeled with double stranded DNA oligonucleotides corresponding to known binding domains for NF-kB. Mutant oligonucleotides will be used as competition in the reaction for binding specificity. Binding reactions will be run on a non-denaturing 4% polyacrylamide gel and scanned using the Kodak Image Station 440 (Molecular Imaging). Depending on our results, a supershift assay will

be performed in a similar manner using the NF-kB antibodies (Invitrogen Corporation, Carlsbad, CA) to further confirm the identity of the shifted Protein-DNA complex. These data will provide details of the binding capabilities of the NF-kB complex proteins and demonstrate their functional efficiency in *NHD13* B-cells.

Potential Pitfalls, Anticipated Outcomes and Alternative Approaches for Specific Aim: One of the major drawbacks to *NHD13* model is the *vav* expression vector used in the initial design (Lin et al., 2005). This was used to achieve pan-hematopoietic expression, which is not the typical presentation of naturally occurring chromosomal translocations. The issue is partially addressed using CSR assays *in vitro* following the *in vivo* DNP stimulation studies. Also, the results obtained from this study may or may not correlate with those observed in patients. We are also aware that this study proposes to investigate the role of myeloid fusion gene in B-cell function. While this may be considered a “red herring”, the preliminary data is convincing enough to support further investigations. The proposed studies are based on the preliminary data showing impaired CSR and decreased NHEJ activity in stimulated B cells. We expect reduced expression of one or more NF-kB factors. Depending on the results, co-immunoprecipitation for the dimerization of the NF-kB factors will be performed. Also, it is reasonable to expect reduced nuclear translocation of active NF-kB factors and DNA binding activity in stimulated *NHD13* B cells. These studies will show that the *NHD13* transgene interferes with the activation of NF-kB as a mechanism for deficient NHEJ and impaired CSR. Depending on the results, the physical interaction of the *NHD13* transgene and NF-kB will be determined. Immunocytochemical and immunoprecipitation analyses will be used to investigate the co-localization of *NHD13* transgene on different NF-kB in activated B cells. This will delineate what role, if any, the *NHD13* fusion

gene might have on NF-kB signaling as a possible mechanism for impaired NHEJ and CSR. To delineate the apoptotic mechanisms mediated through NF-kB signaling, the intrinsic pathway of apoptosis in the B cells will be determined by evaluating the translocation of BCL2 and activity of early stage caspases. Also, depending on the results, effect of *NHD13* on post-transcriptional modifications of NF-kB factors such as sumoylation will be determined.

Statistical Analysis: A power analysis was performed for this study, which showed that five mice were required in each experimental group to get an alpha value (confidence interval) of 5%, and a beta value of 50% with the expected standard deviations. Continuous data will be evaluated using Analysis of Variance (ANOVA) and the appropriate posttest (Tukey's or Bonferroni). Where appropriate (e.g., gene expression data), a standard two-tailed Student's T-test will be used. A p value of ≤ 0.05 will be considered as statistically significant. Statistical analysis will be performed using GraphPad Prism®, Version 5.00 (Graphpad Software).

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CHAPTER 7

APPENDIX A

Diverging in vitro Antibody Isotype Switching Preference between C57BL/6 and FVB Mouse B Lymphocytes

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Abstract

Inbred strains of laboratory mice are commonly used in different immunological studies with varying results. However, these variations are often overlooked and the underlying molecular mechanisms are less explored. In this study, we analyzed the differences in B lymphocyte response and mechanisms of antibody production in two commonly used mouse strains, FVB and B6. These two strains of mice vary in mainly in their major histocompatibility complex (MHC) antigens, C57BL/6 having H-2b and FVB having H-2q, along with a multiple cryptic changes. Our results showed that the two strains of mice had altered preference for immunoglobulin isotype switching, though the class switch recombination patterns are similar. Cell cycle patterns and gene expression studies for critical genes showed moderate changes as possible mechanisms for diverse immunological responses observed in these two strains of mice.

Introduction

Inbred strains of laboratory mice are commonly used in a variety of biomedical research settings to elucidate immune-pathogenesis, infectious disease challenge, drug discovery, and cancer biology (Dranoff, 2012). Many of these inbred strains and transgenic models are also employed in immune system characterization studies. Of the inbred strains available, FVB and C57BL/6 (B6) are often used to generate knock-out, transgenic, or other conditionally modified strains of mice to mimic human disease conditions. B6 is the most commonly used mouse strains in biomedical experiments. FVB mice carry homozygous $Fv1^b$ allele for sensitivity of Friend's leukemia virus (Taketo et al., 1991). These two strains vary in their major histocompatibility complex (MHC) antigens; FVB having H-2q subtype (Taketo et al., 1991) and B6 being H-2b subtype (Smith et al., 1997). Also, FVB mice carry polymorphisms in $Tcra\text{-}V_{11.1}$ and genomic deletion of some *Tcrb-V* genes including $Tcrb\text{-}V_{8.2}$ (Osman et al., 1999). In addition to these known genetic differences, there can be multiple cryptic genetic variations between these two strains (Davie et al., 2007). As a result when these mice are immunochallenged, they have variable immune responses, making research findings difficult to translate to the human condition being modeled.

B lymphocytes are an integral part of immune system as they produce antibodies, act as antigen presenting cells and can phagocytize antigens (DiLillo et al., 2011). B lymphocytes develop in the bone marrow from a common lymphoid progenitor (CLP) and proceed through early and late pro B stages before entering large and small pre B stages (Hardy et al., 1991). These immature B lymphocytes exit the bone marrow and populate the spleen and other secondary lymphoid organs where they undergo further development to form mature naïve B lymphocytes. Following interaction with antigens and co-stimulatory molecules, B-cells will proliferate and undergo somatic hypermutations (Maul and Gearhart, 2010), class switching (Kracker and Durandy,

2011), and clonal expansion (Viau and Zouali, 2005) to produce highly specific immunoglobulin molecules against the invading antigens. Finally, they will differentiate into memory B cells or long-lived plasma cells, the later group releases specific antibodies to circulation (Yoshida et al., 2010). The specificity and effectiveness of antibodies are attributed to the Variable, Diversity, and Joining (VDJ) rearrangement in the bone marrow and Class Switch Recombination (CSR) in secondary lymphoid organs. VDJ recombination results in enormous diversity in antigen recognition, somatic hypermutation increases the affinity and class switch recombination results in production of different isotypes of antibodies with varying effector functions. These gene recombination events are initiated by DNA double strand breaks in immunoglobulin gene mediated by Recombination-Activating Gene (RAG) and Activation-Induced Cytidine Deaminase (AID) enzymatic events during VDJ and CSR respectively (Dudley et al., 2005). Next, these DNA double strand breaks are repaired through the break repair mechanisms, predominantly by Non-homologous end joining (NHEJ) (Dudley et al., 2005). Though NHEJ mechanisms are conserved across mammalian species, their functional efficiency and preferences varies between individuals, age groups, genetic strains and species. Previous studies have shown that FVB mice produce significant levels of antigen specific IgE antibody *in vivo* (Whitehead et al., 2003; Zhu and Gilmour, 2009). In this study we sought to compare the class switch recombination ability between FVB and B6 mice. Our studies showed that although these two strains had similar immunoglobulin gene DNA break repair efficiency, they had altered efficiency and preferences for immunoglobulin isotype switching.

Materials and Methods

Mice: Five 20-week-old FVB and B6 mice were used for each experiment. The mice were bred and maintained at the AAALAC accredited core laboratory animal facility at Virginia Maryland

Regional College of Veterinary Medicine, Virginia Tech. All experiments were carried out as per NIH guidelines with the approval from the Virginia Tech Institutional Animal Care and Use Committee (IACUC).

Splenic parameters

Spleens were harvested and evaluated grossly for size comparison. Single cell suspensions were labeled with fluorescently tagged CD4, CD8 and IgM antibodies and analyzed by flow cytometry. Sections of spleen were fixed in 4% formaldehyde followed by routine processing and paraffin embedding. Four micron thick tissue sections were stained with Hematoxylin and Eosin (H&E), and evaluated by a veterinary pathologist using a Nikon Eclipse 50i light microscope. Images of the spleen were acquired using the NIS-Elements D software 3.10 (Nikon).

Class Switch Recombination Assay

Splenic B lymphocytes were harvested using magnetic beads and magnetic assisted cell sorting (MACS) system (Milteny Biotec, Auburn, CA). A total of $2x10^5$ cells were treated with 5 µM CFSE and cultured in triplicate in 96 well culture plates (BD Biosciences, San Diego, CA) with media containing E coli Lipopolysaccharide (LPS) (25 µg/ml)(Sigma Aldrich, St Louis, MO) or LPS and IL-4 (25ng/ml) (Sigma Aldrich,) and incubated at 37ºC for 96 hours (McBride et al., 2008). At 96 hours of incubation, cells were washed three times and labeled with fluorescently tagged anti-mouse IgG1, IgE, IgG2b and IgG3 antibodies (e-Biosciences, San Diego, CA).The percentage of cells undergoing class switch recombination events was assessed by flow cytometry.

Confocal Microscopy

Splenic B lymphocytes were harvested using MACS system and cultured at 10^6 cells/ml in RPMI 1640 (ATCC, Manassas, VA) containing β Mercaptoethanol (Thermo Scientific, Rockford, IL), LPS and IL-4. Cells were harvested at 0, 24, 48, 72 and 96 hours and cytospun onto charged glass slides. Cells were fixed using 4% paraformaldehyde (Thermo scientific), washed with TBS (Fisher Scientific, Pittsburg, PA) with Tween20 (Fisher Scientific) and blocked with TBS containing 4% Fetal Bovine Serum (FBS). Cells were incubated with rabbit anti-mouse γH2AX (Cell Signaling, Danvers, MA) at 1:500 dilution in TBS with 1% FBS followed by incubation with Alexafluor 488 conjugated anti-rabbit antibody (Cell Signaling) at 1:1000 dilution in TBS with FBS. After labeling with DAPI (Cell Signaling, Danvers, MA), cells were visualized using an LSM700 Carl Zeiss confocal microscope (Carl Zeiss Microimaging, Thornwood, NY) and LSM900® software (Carl Zeiss).

γH2AX Flow Cytometry

Splenic B lymphocytes cultured in the presence of LPS (Sigma) and IL-4 (Sigma) were harvested at 0, 48, 72 and 96 hours as described above. Cells were fixed with 70% ethanol in PBS at -20C for 24 hours and permeabilized with 0.1% Triton-X (Sigma Aldrich) in TBS. Cells were then incubated with rabbit anti-mouse phosphoH2AX antibody (Cell Signaling) at 1:500 dilution in TBS with FBS at 4ºC for 30 minutes followed by incubation with Alexa-fluor 488 conjugated anti-rabbit antibody (Cell Signaling) at 1:1000 dilution. Cells were analyzed using FACScan flow cytometer (BD Biosciences).

Cell Cycle Analysis

Mouse splenic B lymphocytes from FVB and B6 were harvested and cultured in the presence of LPS and IL-4 as described above. Cells were fixed in 70% ethanol for 48-72 hours and incubated

with propidium iodide (Sigma Aldrich) 5µg/ml in PBS containing RNAaseA (5 Prime, Gaithersburg, MD) at room temperature for 20 minutes and kept on ice. Cells were analyzed by flow cytometry.

Gene Expression Analysis

B lymphocytes from FVB and B6 were cultured in the presence of LPS+IL-4 and harvested at 0, 24, 48, 72 and 96 hours to prepare total RNA using Qiagen RNeasy kit. First strand cDNA was synthesized using Superscript III (Invitrogen). Gene expression was determined in triplicate by RQPCR using an iQ™5 Multicolor Real Time PCR Detection System (BioRad) for the following genes Pax5 (5'AGTCTCCAGTGCCGAATG3'; 5' TCCGTGGTGGTGAAGATG3'); Aid (5'GCCAAGGGACGGCATGAGACC3'; 5' CAACAATTCCACGTGGCAGCCAGAC TTG3'): DNA ligase4 (5' ATTGAAGCCACGAGATTAGGT3'; 5'ACTGAATCGGACAC CCAACT3') Ku80/86 (5' AATCCTGTTGAAAACTTCCGTT3'; 5'GGAAGCTGTTGA AGCGCTG3'); KU70/XRCC6 (5'CCGCTTCACATACAGGAGCGAC3'; 5'GGATTATA ACCTGGAGGATAG3'); DNAPK (5'GAGAGTGGGCTTTCAGAAGA3'; 5'ATTTC CTCTGTCTGTCAGAAAT3'); p53 (5' CTCTGAGTAGTGGTTCCTGGCC3'; 5'AAGTA GGCCCTGGAGGATAT3'); Gapdh control (5'GCACAGTCAAGGCCGAGAAT-3'; 5'CCT TCTCCATGGTGGTGAA-3'). Expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and results were normalized to corresponding Gapdh internal control.

Data and Statistical Analysis

Flow cytometric data were analyzed using FlowJo software (FlowJo, Ashland, OR). Data was analyzed with GraphPad Prism 5.0® (Graphpad Software, La Jolla, CA), using either a two tailed t-test or ANOVA and Bonferroni posttest; a P value <0.05 was considered significant.

Results

FVB and B6 mice had different spleen features

In order to better understand the immune system of two strains of mice (FVB and B6) were compared splenic parameters. Five mice of the same age group (20 weeks) were euthanized and spleen was harvested. Primary evaluation of the size showed visibly smaller spleens in B6 mice compared to FVB mice (Figure 7-1 A). B6 mice also had a significantly reduced splenic weight (Figure 7-1 B) although histopathological evaluation revealed comparable cellularity of the follicles when compared to the FVB mice (Figure 7-1 C). To better understand the B lymphocyte subsets within the spleen, cells were labeled with fluorescently tagged anti-mouse IgM, CD4 and CD8 antibodies and analyzed by flow cytometry. Our results showed a comparable percentage of total IgM+ cells in the spleens of both strains. CD4+ T lymphocyte population was lower and CD8+ T lymphocyte population was higher (not significant) in B6 mice compared to FVB mice (Figure 7-1 D, E). CD4/CD8 ratio is an important parameter for assessing the immune status and regulation of immune system. Altered CD4:CD8 ratios have been reported in aging (Callahan et al., 1993), viral infections (Tripp et al., 1995), autoimmunity (Zhang et al., 1994), diet (Lee and Woodward, 1996), cancer metastasis (Hernberg et al., 1996) and exposure to sunshine (Falkenbach and Sedlmeyer, 1997). Moreover, strain specific genetic makeup also plays a critical role in CD4:CD8 ratio in lymphoid organs and periphery of inbred mouse (Kraal et al., 1983; Sim et al., 1998). Consistent with previously published reports (Myrick et al., 2002), our analysis showed a significantly lower CD4/CD8 ratio in B6 mice compared to FVB (Figure 7-1 F) suggesting altered production of T lymphocyte subsets in inbred mice with two different genotypes.

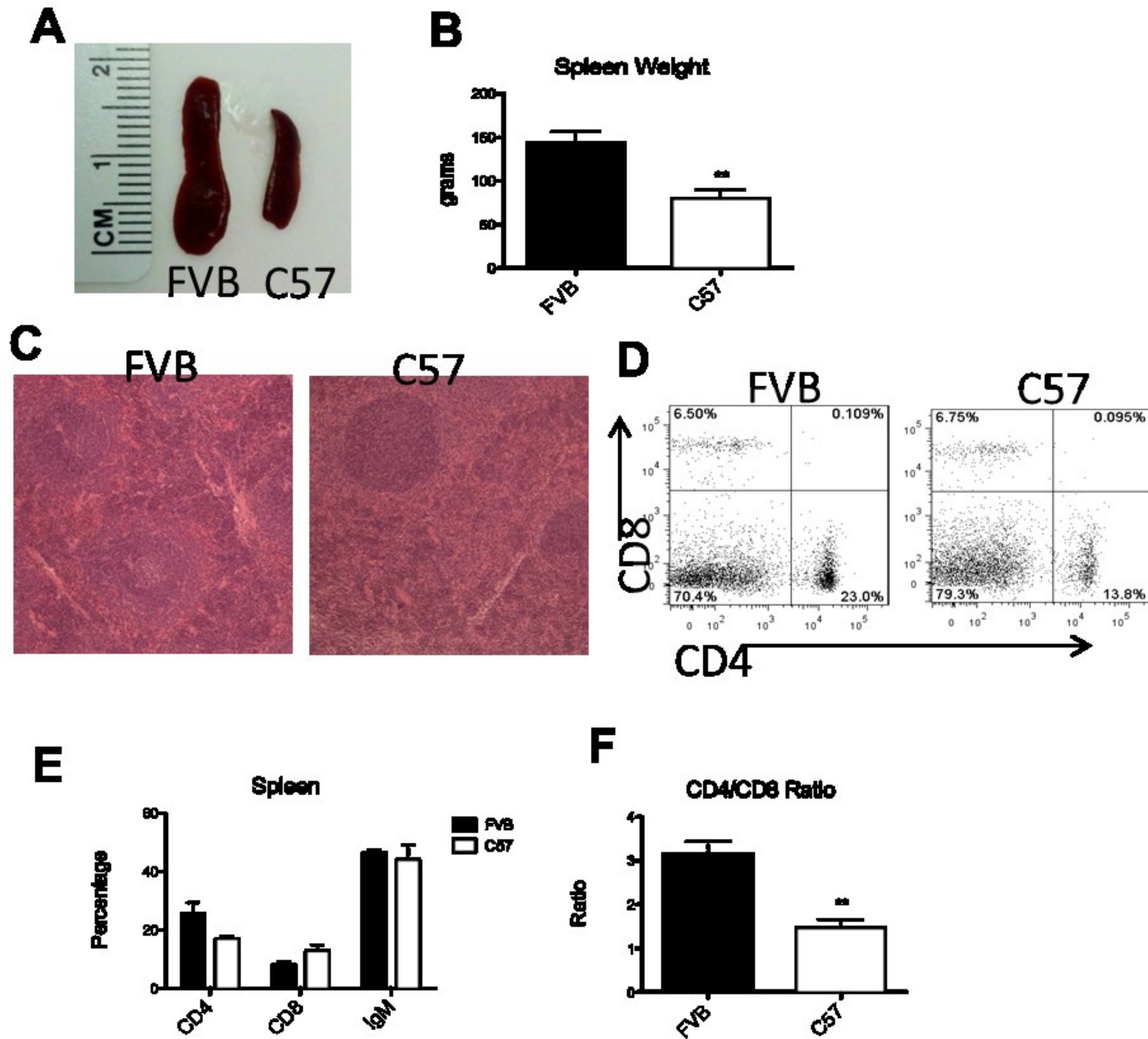


Figure 7-1. Strain difference within spleen. A. Photograph of spleen from FVB and B6 showing a comparatively smaller spleen in B6. B. Analysis of splenic weight showed significant reduction in B6mice. C. Histology of spleen from C57 and FVB showed comparable follicular and peri-arteriolar cellularity between the strains. D. Representative flow cytometric plots for the splenic T lymphocyte fractions showed altered CD4+ and CD8+ cell populations. E. Statistical analysis of T cell fractions and IgM^+ cells showed a lower percentage of $CD4^+$ and a higher percentage of $CD8^+$ cells. Total splenic IgM+ cell percentage was comparable between FVB and B6mice. F. Analysis of CD4/CD8 ratio showed significantly lower ratio in B6mice. n=5, **=p<0.01

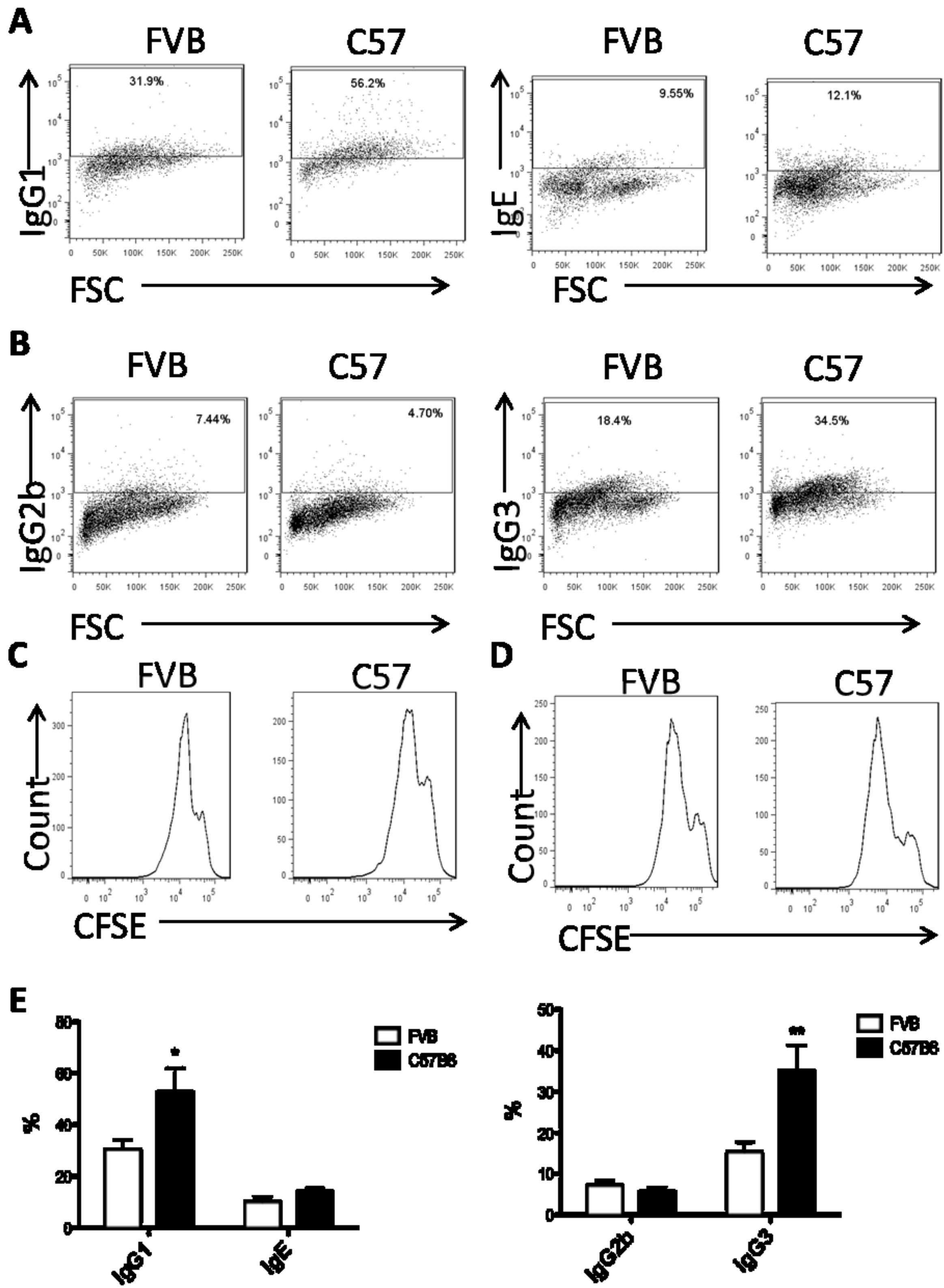


Figure 2
A
FVB
C57
FVB
C57
IgG1
IgE
31.9%
56.2%
9.55%
12.1%
FSC
B
FVB
C57
FVB
C57
IgG2b
IgG3
7.44%
4.70%
18.4%
34.5%
FSC
C
D
FVB
C57
FVB
C57
Count
CFSE
E
%
FVB
C57B6
IgG1
IgE
IgG2b
IgG3

Figure 7-2. *In vitro* CSR assay showed altered recombination efficiency between strains under same conditions. Harvested splenic lymphocytes were cultured in the presence of LPS or LPS+ IL-4 and analyzed for CSR efficiency by flow cytometry. A. Representative flow cytometric plots of cells stimulated with LPS+IL-4 and analyzed for IgG1 and IgE class switching. B. Representative flow plots of cells stimulated with LPS and analyzed for IgG2b and IgG3 C. The proliferation pattern of lymphocytes stimulated with LPS+IL-4 using CFSE labeling showed comparable proliferation between FVB and B6 strains. D. LPS stimulated cells from FVB and B6 mice showed comparable proliferation when analyzed using CFSE labeling. E. Analysis of the CSR efficiency showed higher IgG1, IgE and IgG3 class switching in B cells from B6. However, IgG2b recombination efficiency was significantly lower B6 mice compared to cells from FVB mice. n=5, *=$p<0.05$, **=$p<0.01$

FVB and B6 mice have altered class switch recombination pattern

Splenic IgM+ lymphocytes were harvested and cultured in the presence of LPS or LPS+IL-4 to induce class switch recombination to produce IgG2b and IgG3 or IgG1 and IgE antibodies respectively. Cells were harvested 96 hours following stimulation and analyzed for recombination efficiency and proliferation pattern by flow cytometry. Our results showed that B6 mice had increased efficiency for IgG1 and IgG3 antibody recombination ($p<0.05$ and 0.001 respectively. B6 had a reduced efficiency for IgG2b recombination and a slightly higher efficiency for IgE recombination when compared with cells from FVB mice, though the levels were not significant (Figure 7-2 A, B and E). Evaluation of proliferation pattern of lymphocytes following stimulation using CFSE labeling and flow cytometry showed comparable proliferation in cells from both mouse strains (Figure 7-2 C and D). To better understand the cell proliferation pattern, cell cycle analysis was determined by labeling cells with Propidium iodide and assessed by flow cytometry. Analysis showed comparable cell cycle phases (Apoptotic, G1, Synthetic and G2/mitotic) between FVB and B6 mice at the time of stimulation (Figure 7-3 A, B). In addition, the cell cycle pattern for apoptotic, synthetic and G2/mitotic phases were comparable at 96 hours of stimulation with LPS+IL-4 in cells from both FVB and B6 mice. However, B6 mice had an increased number of cells in the G1 stage at 96 hours following stimulation, suggesting either cell cycle arrest or a strain specific prolonged G1 stage (Figure 7-3 A, B). Because of the prolonged G1 stage in B6 mice, we analyzed the expression of the pro-apoptotic gene, *p53*, and found its expression to be elevated at 24, 48 and 72 hours in B6 mice (Figure 7-3C).

Figure 3

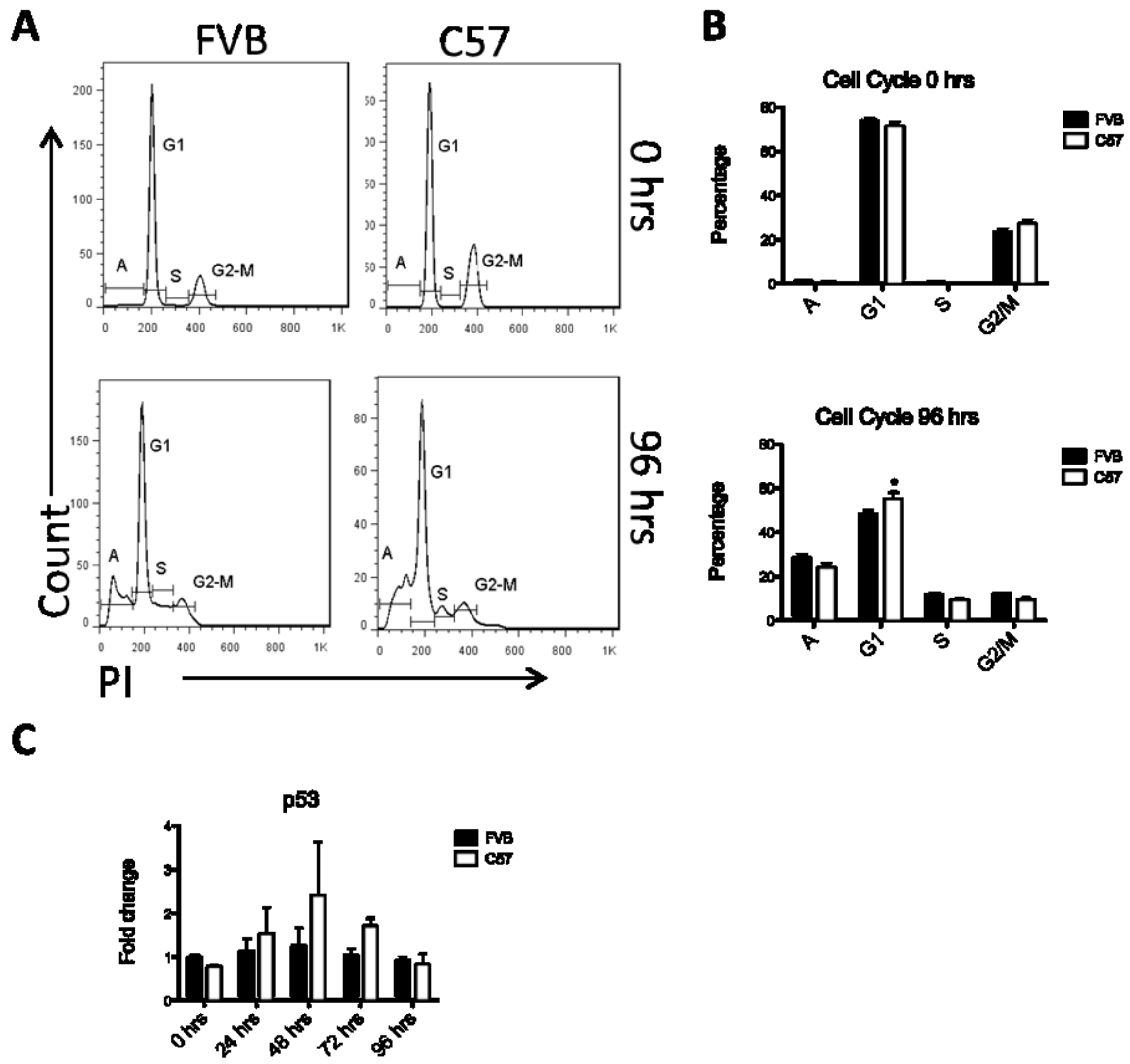


Figure 7-3. Cell cycle kinetics showed growth arrest of B cells at 96 hours post-stimulation. Harvested B lymphocytes from FVB and B6 mice were cultured in the presence of LPS+IL-4 and analyzed for cell cycle pattern using Propidium Iodide labeling. Cells were analyzed for DNA content and divided into A (apoptotic), growth phase 1(G1), S (synthetic) and G2/M (growth phase 2/ Mitotic). A. Representative flow cytometric plots showing different cell cycle stages of stimulated B cells from FVB and B6 mice prior to stimulation (0 hrs.) and 96 hrs post-stimulation. B. Statistical analysis showed comparable percentages of cells in the apoptotic, synthetic and G2/M stage at 0 and 96 hours. However, the percentage of cells in G1 was higher in B6 mice at 96 hours of stimulation suggesting partial cell cycle arrest. C. Analysis of *p53* gene expression showed comparable levels at 0, 24, 48, 72 and 96 hours of stimulation between the two strains of mice. n=5, *=$p<0.05$

Figure 4

A FVB C57

0 hrs 0.623% 0.559%

48 hrs 2.29% 1.96%

72 hrs 12.5% 14.7%

96 hrs 4.29% 4.40%

SSC

yH2AX

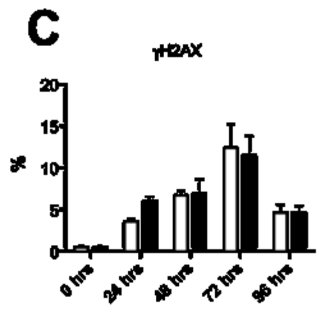
B FVB C57

DAPI

yH2AX

Merged

C



D

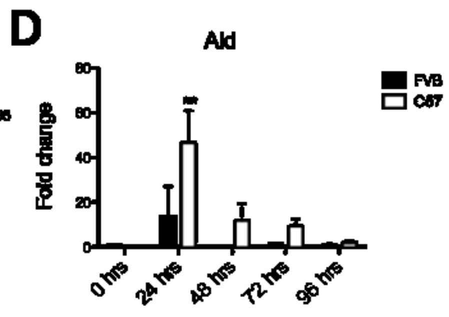


Figure 7-4. FVB and B6mice have comparable DNA break induction and repair pattern

Harvested B cells were cultured in the presence of LPS+IL-4 and harvested at 0, 48, 72 and 96 hours and labeled with anti-mouse γH2AX antibody. Cells were analyzed by flow cytometry and confocal microscopy. A. Representative flow cytometric plots of B cells from FVB and B6 mice at different time points showing DNA breaks indicated by H2AX staining. B. Single cell analysis of the break pattern in FVB and B6 mice by confocal microscopy revealed similar patterns. C. Statistical analysis showed comparable percentage of cells with DNA breaks at all the time points tested. D. Gene expression analysis of *Aid* showed significantly higher levels in B6 mice at 24 hours post stimulation. n=5, *=$p<0.05$, **=$p<0.01$

FVB and B6 mice had comparable DNA break induction and repair pattern

Naive B lymphocytes were harvested from FVB and B6 spleen and cultured in the presence of LPS+IL-4. Stimulation with LPS and IL-4 should result in the expression of activation induced cytidine deaminase (Aid) and lead to DNA double strand breaks in the immunoglobulin heavy chain gene, a critical step in class switch recombination (Mai et al., 2010). DNA double strand break induction results in the phosphorylation of Serine139 of histone H2AX (phosphoH2AX or γH2AX), which can be detected by labeling and immunofluorescence assays (Yin et al., 2009). Flow cytometric analysis for γH2AX showed a comparable percentage of cells with DNA breaks at all the time points tested, viz., 0, 24, 48, 72 and 96 hours post-stimulation (Figure 7-4 A , C). To better understand the DNA break induction at an individual cell level, we labeled cells with fluorescently tagged anti-γH2AX and evaluated the number of breaks by confocal microscopy. Our results showed a comparable amount of breaks at cellular level between both FVB and B6 mice (Figure 7-4 B). Considering the role of *Aid* in DNA break induction, we analyzed its expression by reverse quantitative PCR on cDNA isolated at 0, 24, 48, 72 and 96 hours post-stimulation. Our results showed significantly higher *Aid* expression in B6 mice than FVB mice at 24 and 48 hours; however, the expression was comparable between both strains of mice at other time points tested (Figure 7-4 D).

B6 mice had altered DNA protein Kinase expression following stimulation

As our results showed comparable DNA break induction in both FVB and B6 strains, we considered the possible role of transcription factors and DNA repair mechanism as a reason for the altered preference for isotype switching. Splenic IgM+ cells were harvested and stimulated with LPS +IL-4 in culture for 96 hours to delineate the NHEJ during CSR. Cells were harvested at 24 hour intervals, total RNA was prepared and cDNA was synthesized. Previous studies have

shown that *Pax5* is an important regulator for class switch recombination as it serves as master regulator during B cell differentiation and peripheral development (Medvedovic et al., 2011); and regulates Aid expression (Gonda et al., 2003). Expression of *Pax5* in FVB and B6 cells was determined by RQPCR showed comparable levels at all the time points tested. RQPCR analysis for the genes known to be involved in NHEJ mechanism showed comparable expression of DNA Ligase 4, Ku70 and Ku80 at 0, 24, 48, 72 and 96 hours of culture in both strains of mice (Figure 7-5). Previous studies have shown that increased expression of DNA PKcs correlates with reduced class switch recombination efficiency in chicken cell line DT40 (Cook et al., 2007). Likewise, our results showed increased expression of DNA-PKcs in cells from FVB mice at 96 hours (Figure 7-5) and reduced class switch recombination towards IgG1 and IgG3 (Figure 7-2 A, B and E). Expression of DNAPKcs was comparable between FVB and B6 mice at other time points tested (0, 24, 48 and 72 hours). These results suggest that under our in vitro conditions, gene expressions also might have a role in class switch preference.

Discussion

Different inbred strains of mice have been frequently employed for immunological studies interchangeably, overlooking the possible variation between these strains (Darville et al., 2001; Schulte-Herbruggen et al., 2006). Here, we delineate additional molecular changes between two commonly used strains of mice, FVB and B6, during a typical immunological reaction. To understand the mechanisms at lineage specific levels and to avoid possible extracellular effects, we performed our experiments in harvested naïve B lymphocytes. Our results shed additional insight into strain specific immunoglobulin class switch preferences and patterns under identical culture conditions.

Our preliminary results showed changes in the size of spleen between FVB and B6 mice of same age group, though the animal weights were comparable (data not shown). Histopathological analysis revealed comparable morphological features with similar follicular and peri-arteriolar cellularity. Flow cytometric analysis for total T and B cellularity was comparable between the two strains tested; however the CD4/CD8 ratio was skewed in B6 mice, with more CD8+ cells. The ratio of CD4/CD8 is critical in determining the type of immune reactions as well as regulation of B lymphocyte activation and proliferation patterns (Salgame et al., 1991). Previous studies have reported low CD4/CD8 ratio in B6 mice thymus, spleen and lymphnodes (Myrick et al., 2002). This could be due to inherent genetic regulations affecting TCRα locus (Sim et al., 1998) or strain specific altered expressions of genes such as Notch1 (Deftos et al., 1998) and Bcl-2 (Linette et al., 1994) which are known to polarize the T cell differentiation. Genetic variation in different strains could result in an altered expression of linkage related genes and thus alter the CD4/CD8 preference (Myrick et al., 2002; Vocanson et al., 2005). Given that inherently increased CD8+ cells can lead to more of a Th1 type immune reaction, resulting in more cytotoxic type reactions (Vocanson et al., 2006), increased CD8+ population could also be a reason for frequently observed hyper-responsiveness and dermatitis often observed in the B6 mouse strain (Sundberg et al., 2011).

Antibodies are critical components of the humoral immune response. Different subclasses/isotypes of antibodies are produced under different stimulatory conditions and they have distinct functions. IgG1 is the most abundant IgG subclass making 66% of total IgG and functions mainly by complement fixing. IgG2 subclasses IgG2a and IgG2b, are produced in response to bacterial lipopolysaccharides and have low antigenic affinity, though IgG2a is more effective in activating complement. IgG3 has the highest compliment activation ability and it has

high affinity to the antigens. IgE antibodies are known to stimulate allergic hypersensitive reactions and parasitic responses. In regards to strain difference in antibody production, FVB mice have been shown to produce significant amounts of IgE and lead to asthma like air-way responsiveness. This prompted us to explore the antibody isotype switching pattern between these two strains.

Class switch recombination is a process in which IgM positive B lymphocytes undergo gene recombination in their Immunoglobulin heavy chain gene resulting in the production of different isotypes of antibodies with same specificity. To determine the inherent differences in CSR between two different strains, we performed in vitro CSR assay on naïve B cells. Our results showed that B6 mice had an increased efficiency for IgG1 and IgG3 switching, in contrast to a reduced efficiency for IgG2b production. This difference shows a clear preference towards specific recombinations between two mouse strains when immune cells are stimulated under identical conditions. Previous studies have shown that CSR efficiency depends on proliferation of stimulated B cells (Hodgkin et al., 1996). Our analysis showed comparable proliferation of cells between the two strains, suggesting that proliferation may not be the determining factor for recombination preferences in this study.

Figure 5

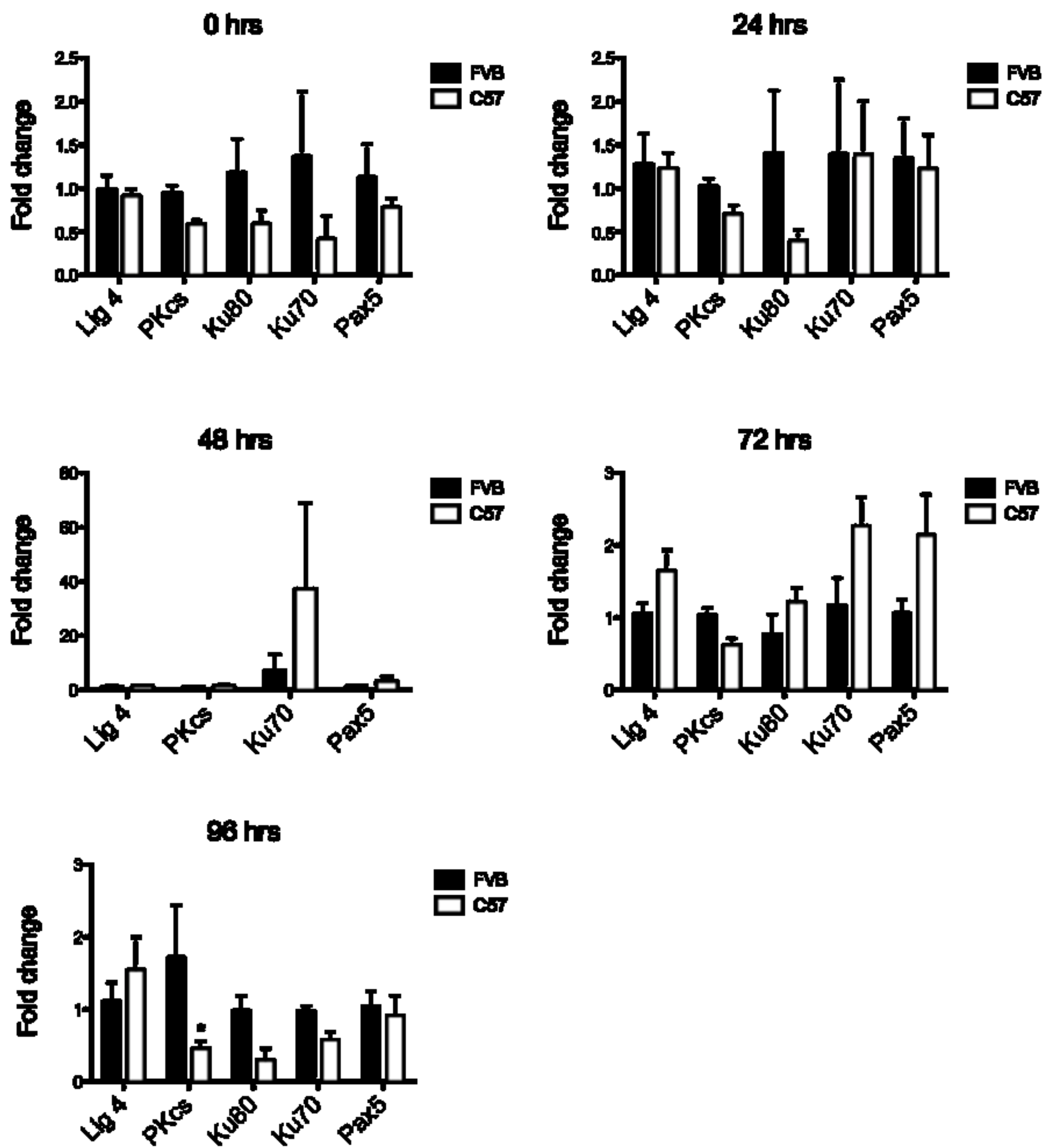


Figure 7-5. Gene expression profiling for NHEJ factors showed altered DNA Protein Kinase expression in B6mice. B lymphocytes from FVB and B6 were harvested and stimulated with LPS+IL-4. Cells were harvested at 24 hour intervals and total RNA was extracted for gene expression by reverse quantitative PCR. Analysis showed comparable levels of *Ligase4, Ku70, Ku80* and *Pax5* at all of the time points tested. However, *DNA PKcs* was significantly lower in B6 mice at 96 hours post-stimulation. n=5, *=$p<0.05$

Recent studies have shown that break induction and fusion during class switching are not entirely random, rather, they are tightly regulated by cellular machinery depending on the type of antigenic stimulation (Kracker and Durandy, 2011; Stavnezer, 2011). Antigenic stimulation leads to Aid mediated DNA double strand breaks at switch regions of immunoglobulin heavy chain (*IgH*). Previous studies have suggested that isotype switching preference is regulated mainly by germline transcription of immunoglobulin switch regions (Stavnezer, 1996). Specificity of *Aid* mediated breaks on *IgH* also depends on binding sites for B cell signaling downstream mediators, Nf-kβ or Stat6 (Kashiwada et al., 2010; Pone et al., 2012). Underlying differences in genetic makeup at switch regions between the two strains of mice or NF-kβ or Stat6 binding sites may contribute to the strain specific isotype switching preference observed in this study. To better understand the DNA break induction pattern, we analyzed the frequency of DNA breaks in stimulated B cells from FVB and B6 mice. Our results showed comparable induction and repair patterns of DNA breaks in FVB and B6 mice, suggesting that induction and repair patterns are similar. Expression of Aid was significantly high at 24 hours of stimulation in B6 mice; however, these cells did not exhibit a higher number of breaks. This could be due to less understood internal cellular mechanisms to prevent the over-activity of DNA damage inducing signals. Single cell analysis for breaks using confocal microscopy showed comparable amount of breaks in break positive cells, thus indicating that the DNA break induction and repair is similar in the two strains of mice tested.

Studies on cell cycle kinetics during class switch recombination suggested that the DNA break induction occurs during the G1 phase of cell cycle (Schrader et al., 2007). The DNA break marker γH2AX is detectable during the G1 and early S phase, but not during G2/M phase of cell cycle (Petersen et al., 2001) suggesting that the DNA break repair starts at S phase. The majority

of NHEJ mediated repair occurs during the G2/M phase (Mao et al., 2008) and therefore, impaired DNA repair results in cell cycle arrest at this stage (Shi et al., 2009). Our analysis of cell cycle kinetics showed an increased number of G1 stage cells in stimulated cells from B6 mice at 96 hours. As CSR involves DNA breaks and repair, any impairment will result in the expression of pro-apoptotic and apoptotic genes, especially p53. Our studies also showed increased p53 in B6 mice at 24, 48 and 72 hours, though the differences were not significant. This slight increase might have resulted in the cell cycle arrest or prolongation at G1 stage at 96 hours. Thus, B6 and FVB mice had different cell cycle kinetics with more cells in G1 stage in B6 mice under identical culture conditions.

As induction of double strand breaks by *Aid* have been shown to be less specific (Staszewski et al., 2011) it might not be the sole reason for isotype preference. We considered the role of NHEJ factors as determining components for class switching preference observed in this study. Previous studies have shown that altered expression or lack of NHEJ factors can impair CSR (Stavnezer et al., 2008). Our results showed that *Ku70* a critical component for the NHEJ mediated DNA break repair (Manis et al., 1998), expression was higher in B6 mice at 48 and 72 hours of stimulation, though the level was not significant. Expression of *Ku80* was lower at 24 hours of stimulation in B6 mice. Also, increased levels of NHEJ factor *DNAPK*cs may reduce the class switching efficiency (Cook et al., 2007). Consistent with this findings, our gene expression analysis showed increased *DNA-PKcs* expression and reduced IgG1 and IgG3 class switch efficiency in FVB mice (Figure 7-5). Expression of *Pax5*, which is considered a master regulator for B cell lineage (Medvedovic et al., 2011), showed comparable levels at all the time

points tested. Taken together, these data suggests that altered expression of critical NHEJ genes might also play a role in the CSR pattern difference between different inbred strains of mice.

Effective CSR is an important determinant of robust antibody mediated immune response. Strain specific differences in isotype switching pattern might contribute to their altered immunity towards various infectious agents and vaccines (Eisenstein et al., 1984; Lyons et al., 2004). Our results show that differences in genetic makeup in the background strains can lead to altered preferences in isotype switching under identical conditions. These preference changes could be due to 1) changes in DNA break sites in immunoglobulin switch regions and/or 2) altered recombination of switch regions as a result of changes in expression of NHEJ factors, resulting in preferential recombination in different strains. Further studies are required to elucidate the molecular mechanisms underlying the class switch preferences in different inbred strains of mice.

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APPENDIX B

Primers used for RQ-PCR (Unless otherwise noted)

Pax5	F	5’	AGTCTCCAGTGCCGAATG	3’
	R	5’	TCCGTGGTGGTGAAGATG	3’
Aid	F	5’	GCCAAGGGACGGCATGAGACC	3’
	R	5’	CAACAATTCCACGTGGCAGCC	3’
DNA Lig 4	F	5’	ATTGAAGCCACGAGATTAGGT	3’
	R	5’	ACTGAATCGGACACCCAACT	3’
Ku 80	F	5’	AATCCTGTTGAAAACTTCCGTT	3’
	R	5’	GGAAGCTGTTGAAGCGCTG	3’
Ku 70	F	5’	CCGCTTCACATACAGGAGCGAC	3’
	R	5’	GGATTATAACCTGGAGGATAG	3’
DNAPKcs	F	5’	GAGAGTGGGCTTTCAGAAGA	3’
	R	5’	ATTTCCTCTGTCTGTCAGAAAT	3’
p53	F	5’	CTCTGAGTAGTGGTTCCTGGCC	3’
	R	5’	AAGTAGGCCCTGGAGGATAT	3’
Gapdh	F	5'	GCACAGTCAAGGCCGAGAA	3'
	R	5'	CCTTCTCCATGGTGGTGAA	3'
Xrcc4	F	5’	ATTACACTTACTGACGGCCATTC	3’
	R	5’	AATGCCGAGACTCCTTAGAAAAG	3’
Lig III	F	5’	GCGGTCTATTCATCGTGCGGG	3’
	R	5’	ATGCGGCACACGCCCTTTAC	3’
Lig I	F	5’	GGATTCCCAAAGCGTCGAACT	3’
	R	5’	AGAGTGTCTTGAATTGTCCGTTT	3’
Hoxa9	F	5’	TGAGAGCGCGGAGACAA	3’
	R	5’	GTCCAGTTCCAGCGTCTGGT	3’
pUC18 (plasmid)	F	5’	GTAAAACGACGGCCAGT	3’
	R	5’	CAGGAAACAGCTATGAC	3’
Rag1	F	5’	CCAAGCTGCAGACATTCTAGCACTC	3’
	R	5’	GTCGATCCGGAAAATCCTGGCAATG	3’
Rag2	F	5’	CACA TCCACAAGCA GGAAGTACAC	3’
	R	5’	CTTAGTAGGAGATGTCCCTGAACC	3’
E2a	F	5’	GAGGATGAGGAAATCGCATCAGTA	3’
	R	5’	GGCCATACGCCTCTCCCGGTC	3’
Ebf1	F	5’	GCCACTATAAACAAGACTCCATGT	3’
	R	5’	TCCTATTCTGTCCATACGAGCTCT	3’
NHD13 (genotyping)	F	5’	TGGAGGGCCTCTTGGTACAGG	3’
	R	5’	GGCTTCTAAGCTGTCTGTGGCC	3’
Scid (genotyping control)	F	5’	GGAAGAGTTTGAGCAGACAATG	3’
	R	5’	CATCACAAGTTATAACAGCTGGG	3’

APPENDIX C

Virginia Tech Graduate Research Development Program Award Winning Proposal

Delineating the DNA Repair Mechanisms in a Mouse Model for Myelodysplastic Syndrome

Submitted by Abdul Gafoor Puthiyaveetil

Awarded in Fall, 2010

Background Information

Myelodysplastic Syndrome (MDS) is a clonal process characterized by ineffective hematopoiesis, dysplastic bone marrow (BM), peripheral blood cell cytopenias, including lymphopenia, and progression to acute myeloid leukemia (AML) and acute lymphoid leukemia (ALL). MDS occurrence increases with age; the median age at presentation is 70 years with an annual incidence of 2-30 cases per 100,000 persons. The prognosis for MDS is poor due to refractory cytopenias resulting in hemorrhage and infections. Identification of the clonal cell of origin (lymphoid or myeloid) has been difficult given the heterogeneity character of MDS. The precise role of B-cell involvement in the pathogenesis of MDS remains elusive. However, it is evident that B-cells play a role in the progression of MDS either as direct targets of mutations resulting in ALL, or as cells that influence the bone marrow microenvironment in which a myeloid clone predominates.

Non-homologous end joining (NHEJ), and the expression levels of NHEJ factors are altered in MDS patients, leading to impaired somatic DNA double strand break repair (DSB). NHEJ functions in Variable (V), Diversity (D), and Joining (J) (VDJ) recombination, a mechanism of antigen receptor gene recombination that occurs in B-cells giving rise to antibody diversity. MDS patients have increased error-prone NHEJ activity. In particular, DNA Ligase IV has been shown to be decreased in BM of adult patients with MDS9. Defective NHEJ predisposes hematopoietic cells to genomic instability, impaired development, and ultimately leukemia.

Transgenic mice that expresses the fusion gene NUP98-HOXD13 (NHD13) develops the typical findings seen in MDS patients10.Studies in NHD13 mice show a significant (4-10X) decrease in

Pre-B cells and B220+ cells in peripheral blood, all in the face of a normal percentage of Pro B cells.

Preliminary data

Our preliminary data on the B cell differentiation in the NHD13 transgenic mouse model showed reduced levels of Hardy flow cytometric fractions C, C', and F, suggesting partial blocks immediately after the Immunoglobulin VDJ gene rearrangements. Antigenic stimulation using DNP KLH resulted in reduced bone marrow fractions B, E and F and splenic fractions Fo, B1 and MZ. DNP specific antibody measurement by ELISA showed reduced IgG subclasses and IgE in the phase of increased IgM suggesting problematic Immunoglobulin class switch recombination (CSR). Histopathologic data also revealed reduced follicular size.

Hypothesis

Since DNA double strand break and NHEJ mediated recombination are the critical steps in the VDJ recombination and CSR, we hypothesize that "NHD13 expression results in defective NHEJ activity leading to impaired B-cell development and function"

To test this hypothesis, we propose in vitro Ligation efficiency assay to measure the DNA double strand break repair efficiency of nuclear proteins from transgenic splenic B cells. Synthetic oligonucleotides of known length will be incubated with nuclear protein extracts from the cells and monitored for the blunt ligation efficiency.

Materials and Methods

Animals

Clinically healthy five 8-12 week old NHD13 transgenic mice and five wild type mice of the same background will be used for this study under the approval of Virginia Tech IACUC. Two

days prior to the experiment, animals will be anesthetized with 2.5% Isoflurane and blood (50ul) will be collected into heparinized tubes by retro orbital method. Blood smear will be prepared for histopathology and whole blood will be analyzed for CBC using Heska CBC machine to ensure that animals are clinically healthy.

Statistics

All the data will be tested by two way ANOVA and two sided student's t test using GraphPad Prism 5.0. We will consult Statistics Department for further assistance if necessary.

Method

Animals will be euthanized by cervical dislocation after isoflurane anesthesia and whole spleen will be harvested and kept on ice. Single cell suspension of splenocytes will be prepared after lysing the RBCs by treating with ACK Lysing solution (Lonza) and straining through 70um nylon cell strainer (Fisher Scientific). Cells will be labeled with CD19 magnetic beads (Milteny BioTech) - a mature B lymphocyte marker- and passed through magnetic column (Milteny BioTech) by positive selection method. CD19 positive cells will be flushed out after washing away the negative cells. Nuclear protein will be extracted from the sorted cells using NXTRACT CelLytic NuClear reagents (Sigma Aldrich) containing Protease inhibitor (Invitrogen) and stored at -800C. Blunt end 40bp long oligonucleotides (Sigma Genosys) will be incubated with the nuclear protein extract at 370C for 1-3 hours. The product will be electrophoresed in a 0.8% agarose (Fisher Scientific) gel containing 0.005% Ethidium Bromide (Promega) at 90V for 1 hour and visualized under Ultra Violet for the band size.

Anticipated results and alternate approaches

If all the DNA ligation factors are present in optimum quantity and the mechanism is intact, the oligonucleotides will get ligated together and we will get bands in multiplications of 40bp.

Inefficient ligation will be indicated by reduced oligonucleotide ligations and the 40bp band will be thicker. Quantitative measurement of ligation efficiency will be determined by real time PCR amplification using primers specific for the nucleotides. We anticipate reduced ligation efficiency in the transgenic splenic B cell nuclear extract consistent with our flow cytometric and ELISA data.

Significance

The study will reveal the DNA double strand break repair ability of splenocytes in the NHD13 mice. Impaired DNA repair leads to cell cycle arrest, apoptosis and cancer. Understanding the mechanisms and efficiency of DNA repair will help to formulate therapies targeting specific pathways in the treatment various disease conditions.

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APPENDIX D

ABSTRACTS

A Nup98-Hoxd13 Fusion Gene Impairs B Lymphocyte Development and Function in a Mouse Model for Myelodysplastic Syndrome

Abdul Gafoor Puthiyaveetil, Bettina Heid, David Caudell

(This abstract won travel awards from American Society of Hematology and Virginia Tech Graduate Student Assembly, 2010)

Transgenic mice which express the fusion gene *NUP98-HOXD13 (NHD13)* have been shown to develop characteristic features of Myelodysplastic syndrome (MDS) including impaired hematopoietic differentiation and peripheral blood cytopenias in the presence of normocellular or hypercellular bone marrow. It is evident that B-cells play a role in the progression of MDS by immune modulation or as direct targets of mutations resulting in ALL, or as cells that influence the bone marrow microenvironment in which a neoplastic myeloid clone evolves. Choi and colleagues suggested a block in differentiation during the early development of B lymphocytes in the bone marrow (BM) of *NHD13* mice leading to lymphopenia consistent with the observation in some MDS patients. In this study, we sought to further delineate the role of *NHD13* on B lymphocytes which escaped the initial differentiation block in the BM. We hypothesized that *NHD13* impairs maturation and function of IgM^+ B lymphocytes contributing to immunodeficiency. To study this, we performed blood smear examination, Complete Blood Counts (CBC), quantitative ELISA for antibody concentrations, and flow cytometric analysis of B cell fractions from the bone marrow and spleen in 8-12 week-old transgenic and wild type (WT) mice. CBCs revealed significant lymphopenia and ELISA showed higher IgM concentrations (n=10, $p<0.001$), reduced levels of IgG1 (n=10, $p<0.05$) and IgE (n=10, $p<0.01$). The IgG2a, IgG2b, and IgG3 antibody levels were comparable to WT counterparts. Flow cytometric analysis of bone marrow and splenic B cell fractions revealed reduced numbers of B cells in Hardy fractions D and F (n=10, $p<0.01$) indicative of impaired differentiation prior to these stages; splenic fractions in *NHD13* mice were comparable to WT controls. Next, to assess

the peripheral maturation and functional efficiency of B lymphocytes in the context of a comprehensive immune stimulation, a cohort of five WT and five preclinical transgenic mice were injected with 100 µg DNP followed by a booster dose on day 21. Animals were euthanized on day 28 and whole blood, spleen, lymphnodes and bone marrow were harvested. CBC evaluation revealed significant lymphopenia in *NHD13* mice (n=5, $p<0.001$). Quantitative ELISA for DNP specific antibodies showed comparable levels of serum IgM and significantly reduced levels of serum IgG1 (n=5, $p<0.001$), IgG2a (n=5, $p<0.001$), IgG2b (n=5, $p<0.01$), IgG3 (n=5, $p<0.001$) and IgE (n=5, $p<0.01$). Flow cytometric analysis of peripheral blood showed reduced numbers of $B220^+$ IgM^+ B cells (n=5, $p<0.01$), but comparable percentages of $CD4^+$ and $CD8^+$ T-cells. Detailed flow cytometric analysis of B-cell fractions in the BM and spleen of DNP-stimulated mice revealed reduced populations of advanced levels of B lymphocytes. The earliest B cell lineage population, Pre-Pro B, was comparable to the WT controls. Hardy Pro B fraction B (n=5, $p<0.001$) and Pre B fractions E (n=5, $p<0.01$) and F (n=5, $p<0.01$) from BM of stimulated mice were significantly reduced in contrast to fractions C and C', which were higher (n=5, $p<0.05$ and $p<0.001$ respectively), indicative of cell growth arrest at these stages. Flow cytometry of splenic B-cell fractions from the DNP-stimulated mice showed significantly lower Transitional 1 (n=5, $p<0.01$), Follicular (n=5, $p<0.05$) and Marginal Zone (n=5, $p<0.001$) populations upon antigenic stimulation suggestive of defective clonal expansion of IgM^+ cells even after escaping the block in the BM. Histopathology of the spleen revealed smaller lymphoid follicles with poorly developed mantle and marginal zone regions in the transgenic mice when compared to WT controls, consistent with the flow cytometric data. This study indicates that when *NHD13* mice are immunologically challenged, B lymphocytes undergo impaired differentiation in the bone marrow and maturation in the spleen, as well as reduced antibody

class switching and subsequently lower antibody production. Analysis of the common mechanisms during the B cell subset differentiation in the BM and specific IgG/IgE antibody production, suggest that transgene expression impairs VDJ gene recombination and class switch recombination, leading to lymphopenia and further mutations.

THE LEUKEMIC FUSION GENE *NUP98-HOXD13* IMPAIRS CLASS SWITCH RECOMBINATION AND ANTIBODY PRODUCTION

Abdul Gafoor Puthiyaveetil, Bettina Heid, Christopher Reilly, David Caudell

(This abstract won the NCRI Prize Award at, 7th Annual Meeting, National Cancer Research Institute, UK. 2011)

Chromosomal translocations occur as a consequence of DNA breaks and misrepair resulting in novel fusion genes often observed in leukemia. Leukemic patients frequently experience compromised immunity; however, the role of chromosomal translocations in regulating immune function is poorly understood. Here we used a transgenic mouse model that expresses the human leukemic fusion gene *NUP98-HOXD13* (*NHD13*) to determine its role during an immune response. *NHD13* mice had significantly altered basal levels of antibodies when compared to WT mice. These mice also had impaired development of their B-lymphocytes in both the bone marrow and spleen. To study the response of B-lymphocytes to a comprehensive antigen, mice were immunized with Dinitrophenol (DNP). Following a booster injection, blood and spleen were harvested and analyzed for antibody production and B-lymphocyte development. DNP specific plasma antibodies determined by ELISA showed that IgM levels were comparable between *NHD13* and WT mice whereas IgG1, IgG2a, IgG2b and IgG3 levels were significantly lower. Histopathology revealed that spleens from *NHD13* mice had smaller hypocellular follicles compared to WT mice. Flow cytometry of spleen showed that follicular, B1 and mantle zone B-lymphocytes were significantly lower. Considering the role of Class switch recombination (CSR) in clonal expansion of B cells and antibody production, we induced in vitro CSR by stimulating splenic B-lymphocytes with LPS or LPS+IL-4. Flow cytometric analysis and proliferation pattern of these cells after 72 hrs in culture showed that CSR was severely impaired in B-lymphocytes from the *NHD13* mice. These results indicate that the impaired antibody production

observed in *NHD13* mice is due to impaired CSR. Our findings shed novel insight into the role of chromosomal translocations indicating that, in addition to their activity in leukemic progression, they can also severely compromise an immune response.

A Nup98-Hoxd13 Fusion Gene Impairs B Lymphocyte Development and Function in a Mouse Model for Myelodysplastic Syndrome

Abdul Gafoor Puthiyaveetil, Bettina Heid, David Caudell

(Submitted to American Society of Hematology Annual Meeting, 2012)

Transgenic mice which express the fusion gene *NUP98-HOXD13 (NHD13)* have been shown to develop characteristic features of Myelodysplastic syndrome (MDS) including impaired hematopoietic differentiation and peripheral blood cytopenias in the presence of normocellular or hypercellular bone marrow. It is evident that B-cells play a role in the progression of MDS by immune modulation or as direct targets of mutations resulting in ALL, or as cells that influence the bone marrow microenvironment in which a neoplastic myeloid clone evolves. Choi and colleagues suggested a block in differentiation during the early development of B lymphocytes in the bone marrow (BM) of *NHD13* mice leading to lymphopenia consistent with the observation in some MDS patients. In this study, we sought to further delineate the role of *NHD13* on B lymphocytes which escaped the initial differentiation block in the BM. We hypothesized that *NHD13* impairs maturation and function of IgM^+ B lymphocytes contributing to immunodeficiency. To study this, we performed blood smear examination, Complete Blood Counts (CBC), quantitative ELISA for antibody concentrations, and flow cytometric analysis of B cell fractions from the bone marrow and spleen in 8-12 week-old transgenic and wild type (WT) mice. CBCs revealed significant lymphopenia and ELISA showed higher IgM concentrations (n=10, $p<0.001$), reduced levels of IgG1 (n=10, $p<0.05$) and IgE (n=10, $p<0.01$). The IgG2a, IgG2b, and IgG3 antibody levels were comparable to WT counterparts. Flow cytometric analysis of bone marrow and splenic B cell fractions revealed reduced numbers of B cells in Hardy fractions D and F (n=10, $p<0.01$) indicative of impaired differentiation prior to these stages; splenic fractions in *NHD13* mice were comparable to WT controls. Next, to assess the peripheral maturation and functional efficiency of B lymphocytes in the context of a comprehensive immune stimulation, a cohort of five WT and five preclinical transgenic mice were injected with 100 µg DNP followed by a booster dose on day 21. Animals were euthanized on day 28 and whole blood, spleen, lymphnodes and bone marrow were harvested. CBC evaluation revealed significant lymphopenia in *NHD13* mice (n=5, $p<0.001$). Quantitative ELISA for DNP specific antibodies showed comparable levels of serum IgM and significantly reduced levels of serum IgG1 (n=5, $p<0.001$), IgG2a (n=5, $p<0.001$), IgG2b (n=5, $p<0.01$), IgG3

($n=5$, $p<0.001$) and IgE ($n=5$, $p<0.01$). Flow cytometric analysis of peripheral blood showed reduced numbers of B220$^+$ IgM$^+$ B cells ($n=5$, $p<0.01$), but comparable percentages of CD4$^+$ and CD8$^+$ T-cells. Detailed flow cytometric analysis of B-cell fractions in the BM and spleen of DNP-stimulated mice revealed reduced populations of advanced levels of B lymphocytes. The earliest B cell lineage population, Pre-Pro B, was comparable to the WT controls. Hardy Pro B fraction B ($n=5$, $p<0.001$) and Pre B fractions E ($n=5$, $p<0.01$) and F ($n=5$, $p<0.01$) from BM of stimulated mice were significantly reduced in contrast to fractions C and C', which were higher ($n=5$, $p<0.05$ and $p<0.001$ respectively), indicative of cell growth arrest at these stages. Flow cytometry of splenic B-cell fractions from the DNP-stimulated mice showed significantly lower Transitional 1 ($n=5$, $p<0.01$), Follicular ($n=5$, $p<0.05$) and Marginal Zone ($n=5$, $p<0.001$) populations upon antigenic stimulation suggestive of defective clonal expansion of IgM$^+$ cells even after escaping the block in the BM. Histopathology of the spleen revealed smaller lymphoid follicles with poorly developed mantle and marginal zone regions in the transgenic mice when compared to WT controls, consistent with the flow cytometric data. This study indicates that when *NHD13* mice are immunologically challenged, B lymphocytes undergo impaired differentiation in the bone marrow and maturation in the spleen, as well as reduced antibody class switching and subsequently lower antibody production. Analysis of the common mechanisms during the B cell subset differentiation in the BM and specific IgG/IgE antibody production, suggest that transgene expression impairs VDJ gene recombination and class switch recombination, leading to lymphopenia and further mutations.

Mechanisms of Impaired Antibody Production in a Transgenic Mouse Model for Leukemia

Abdul Gafoor Puthiyaveetil*, Bettina Heid*, C M. Reilly**, David Caudell*

(Presented at Graduate Student Assembly Annual Symposium, 2012)

Many leukemic patients experience compromised immunity; however, the role of underlying DNA mutations in regulating immune function is poorly understood. We used *NHD13* transgenic mouse model for leukemia to delineate the role of translocations in immune regulation,. Our studies show that total WBC and lymphocyte counts were significantly lower *NHD13* mice prior to the onset of leukemia. Basal levels of plasma antibodies *NHD13* mice were altered. To study the humoral immune response, mice were challenged with Dinitrophenol (DNP). *NHD13* mice failed in splenic response and antigen specific antibody production. As class switch recombination (CSR) is a key mechanism for splenic response and antibody production, we performed *in vitro* CSR assay. Our results showed that CSR is impaired in clinically healthy *NHD13* mice. Our findings suggest that leukemic fusion genes can compromise immune response in addition to leukemic progression.

Mechanisms of Impaired Antibody Production in a Transgenic Mouse Model for Myelodysplastic Syndrome

Abdul Gafoor Puthiyaveetil*, Bettina Heid*, Christopher Reilly**, David Caudell*

Presented at Research Day, National Institute of Health, 2011

Myelodysplastic syndrome (MDS) is a group of hematological disorders characterized by ineffective hematopoiesis. MDS patients frequently experience compromised immunity as a feature of their disease; however, the role of mutations in regulating immune function is poorly understood. Transgenic mice expressing the fusion gene *NUP98-HOXD13 (NHD13)* develop characteristic features of MDS by 6- 7 months of age. To study the role of this fusion gene in immune function, we evaluated 2-3 month old clinically healthy *NHD13* mice and analyzed antibody production, B lymphocyte developmental pattern and histopathology before and after antigenic stimulation. Our studies show that total WBC and lymphocyte counts were significantly lower in clinically healthy *NHD13* mice compared with age matched wild type (WT) littermates. Moreover, basal levels of plasma antibodies in clinically healthy *NHD13* mice are altered in *NHD13* transgenic mice. To study the response of B-lymphocytes to a comprehensive antigen, *NHD13* and WT mice were stimulated with Dinitrophenol-Keyhole Limpet Hemocyanin (DNP-KLH). After 28 days, blood and spleen were harvested and analyzed for antibody production and B-lymphocyte development. DNP specific plasma antibodies determined by ELISA showed that the IgM levels were comparable between *NHD13* and WT mice whereas IgG1, IgG2a, IgG2b and IgG3 levels were significantly lower. Histopathology revealed that spleens from *NHD13* mice had smaller follicles with poorly demarcated mantle and marginal zones. Flow cytometric analysis of spleen showed that mature B-lymphocytes in follicular, B1, and mantle zone compartments were significantly lower. Considering the role of class switch recombination (CSR) in antibody production and B lymphocyte clonal expansion, we performed an in vitro CSR assay. Our results showed that CSR is impaired in clinically healthy *NHD13* mice compared with WT controls. Our findings shed insight into the role of leukemic fusion genes indicating that, in addition to their activity in leukemic progression, they can also compromise the immune response.