

EFFECTS OF OVEREXPRESSING ALTERNATIVE ISOFORM PAX-5E

IN VIVO

A Thesis

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The Faculty of the Department of Biology
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Master of Arts

by

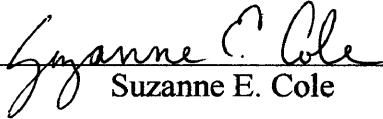
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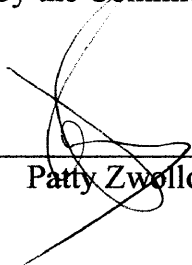
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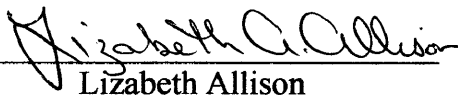
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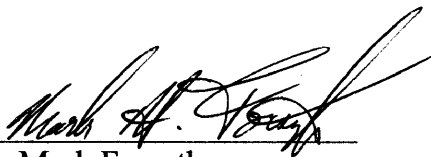

Mark Forsyth

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ABSTRACT

The transcription factor Pax-5 is a member of the *pax*, or paired box, family of genes and is a key regulator in the development, activation and proliferation of B lymphocytes. The *pax-5* gene is alternatively spliced resulting in four isoforms: the full-length isoform Pax-5a and three alternative isoforms, Pax-5b, Pax-5d and Pax-5e. Alternative isoform Pax-5e has been shown to enhance the activity of Pax-5a in a dose-dependent manner, resulting in increased cell proliferation *in vitro*. Based on this information, we hypothesized that if Pax-5e were overexpressed in a murine system that we would see increases in B cell proliferation as well as possible changes in Pax-5a target gene expression.

We generated two lines of transgenic mice overexpressing Pax-5e, TG5e-7 and TG5e-49. Analysis of RNA expression of the transgene indicated that TG5e-7 mice express the transgene in B cells only, while TG5e-49 expresses the transgene in both B and T cell lineages. Cultured bone marrow cells from both strains of mice revealed increases in cell proliferation as compared to negative controls. We also showed that after immunization, TG5e-7 mice have increased numbers of CD19⁺ B cells and IgG-secreting cells as well as an overall increase in secreted IgG and IgM. TG5e-49 mice displayed decreases in CD19⁺ B cells as well as CD3⁺ T cells after immunization. This strain also showed a decrease in the levels of secreted IgG. No change in the number of Ig-secreting cells was observed but a shift toward smaller ELISPOTS was evident. Immunized TG5e-49 mice, but not TG5e-7, also revealed overexpression of Pax-5a target gene, *CD19*, by “real time” PCR.

Our data supports the hypothesis that overexpression of Pax-5e increases the activity of Pax-5a thus resulting in changes in target gene expression and increased cell proliferation. Immunization of transgenic mice reveals a more striking phenotype than found in unimmunized mice indicating that development and activation of lymphocytes are greatly affected by overexpression of Pax-5e. Our *in vivo* data have provided evidence that Pax-5e plays an important role in the regulation of Pax-5a during B cell development, activation and proliferation.

THE EFFECTS OF OVEREXPRESSION ALTERNATIVE ISOFORM PAX-5E
IN VIVO

CHAPTER I

Literature Review

The immune system is essential for defense against foreign antigens as it mediates the removal of those antigens from the body. B lymphocytes are unique immune cells in that they express surface immunoglobulin (Ig), or B cell receptors (BCR), which bind to antigen with extreme specificity. Once an antigen has bound to a BCR, this triggers a cascade of events leading to the activation of the cell, followed by the proliferation and differentiation into Ig-secreting plasma cells and memory cells.

Many mechanisms control development and activation of B cells. Developmental cues include extracellular growth factors and cell-cell contacts as well as stage-specific intracellular control by transcription factors. Combinatorial regulation of transcription factors dictates the developmental stage of the B cell, as well as whether the cell should proceed to the next stage or arrest and undergo apoptosis. The realm of B cell development and function has been under investigation for decades as these cells are crucial to immune function. The use of mice carrying targeted mutations has allowed scientists to manipulate molecular pathways within the B cell and has led to a better understanding of the roles of transcription factors essential for proper B cell development and function. Uncontrolled B cell populations can lead to leukemia, lymphoma, autoimmune disease, or immune deficiency thus, the treatment of many diseases relies on

understanding how B cell transcription factors function within the cell. The focus of this research was to determine the role(s) of transcription factor Pax-5e, an alternative isoform of the Pax-5a factor, in B cell development and proliferation *in vivo*.

B Lymphocyte Development

B lymphopoiesis involves a complex network of transcription factors and extracellular signaling molecules that work together to transform a hematopoietic stem cell into a mature B cell capable of producing antigen-specific surface immunoglobulins (Ig) (Figure I.1). This process occurs within the fetal liver and adult bone marrow of mammals and is antigen-independent (Busslinger, 2004; Bartholdy & Matthias, 2004; Smith & Sigvardsson, 2004). Hematopoietic stem cells commit to a lymphoid lineage through the expression of the Ets family transcription factor PU.1 and will give rise to one of three types of lymphoid cells including B, T and Natural Killer (NK) cells. The *Ikaros* gene isoforms are also crucial to lymphoid development as mice carrying targeted disruption of this gene fail to produce B cells and show significant disruption in T cell differentiation (Reya & Grosschedl, 1998). *Ikaros* and family members, *Helios* and *Aiolos*, may also play a role in gene silencing and chromatin remodeling.

The transcription factors E2A and early B cell factor (EBF) are necessary for the induction of *pax-5* gene expression in B cell progenitors (Bain et al., 1994; Lin & Grosschedl, 1995). The combination of E2A, EBF and Pax-5 regulate the proliferation and differentiation of lymphoid progenitors into the earliest form of B cell, the progenitor B cell (pro-B cell) (Busslinger, 2004; Bartholdy & Matthias, 2004; Smith & Sigvardsson, 2004). Early pro-B cells express the transmembrane protein tyrosine kinase B220 (or

CD45R) and surface antigens CD43 and CD19, and their Ig genes remain in germline configuration (Busslinger, 2004; Smith & Sigvardsson, 2004). It is late in this stage that Ig gene rearrangement, the hallmark of B cell development, is initiated resulting in the rearrangement of D_H - J_H segments of the IgH locus (Figure I.1; Nutt et al., 1997; Reya & Grosschedl, 1998; Bartholdy & Matthias, 2003; Busslinger, 2004; Smith & Sigvardsson, 2004). Gene rearrangement results from the expression of the recombinase proteins RAG-1 and RAG-2 which splice out segments of the Ig loci at recombination signal sequences. Leading into the early precursor B cell (pre-B I cell) stage, the cell completes V_H - DJ_H rearrangement of the IgH locus and a pre-BCR is expressed on the cell surface. The pre-BCR consists of a fully rearranged IgH chain, surrogate light chains $\lambda 5$ and VpreB as well as receptor accessory peptides $Ig\alpha$ (*mb-1*) and $Ig\beta$ (B-29).

During the pre-B I cell stage, cells express the surface antigens CD20, CD22 and CD72, as well as intracellular signaling molecules such as B lymphoid kinase (Blk), which are all involved in pre-BCR signaling (Figure I.1; Liberg and Sigvardsson, 1999). Later in the pre-B II segment of the pre-B cell stage, CD19, an accessory protein involved in pre-BCR signaling, is expressed on the cell surface. Pre-BCR signaling influences positive selection of those pre-B cells that possess a functional IgH gene rearrangement, while those cells that fail to accomplish this undergo programmed cell death or apoptosis (Melchers et al., 1995). The Ig light chain (IgL) locus undergoes V_L - J_L rearrangement later in this stage, also called the pre-B II stage, to produce functional λ and κ light chains (Figure I.1; Liberg and Sigvardsson, 1999; Maier & Hagman, 2002). Following the completion of IgL gene rearrangement, the pre-B cell can now form a functional BCR and enter the next developmental stage, the immature B cell stage.

Immature B cells, defined as expressing fully functional membrane-bound IgM with particular antigen specificity, leave the bone marrow and follow the blood stream to the secondary lymphoid organs such as spleen and lymph nodes. During this time, they undergo negative selection for autoreactivity (Liberg and Sigvardsson, 1999). Those cells which respond to self antigens can initiate receptor editing of their IgL, and if they are still autoreactive after editing, development is arrested and these cells are eliminated by apoptosis (Levine et al., 2000). Once a cell has passed the selection process and expresses membrane-bound IgD, it has reached the mature B cell stage capable of responding to antigen.

Mature B cells migrate to the secondary lymphoid organs, namely the spleen and lymph nodes, where they remain in a resting state until activated by a foreign antigen (reviewed in Rudin & Thompson, 1998). Each B cell contains BCRs with specificity to a certain sequence of peptides or antigen, and when this particular antigen binds the BCR, the cell becomes activated. B cells can become activated via T cell-dependent or T cell-independent pathways. T cell-dependent activation requires crosslinking of the B cell antigen receptor complex. Activation of B cells also occurs in the absence of T cell activity (so called T cell-independent activation) by means of direct stimulation by extracellular cytokines released in the presence of B cell-specific mitogens (i.e. lipopolysaccharide). Activated B cells form germinal centers in secondary lymphoid organs where they undergo a number of transcriptional changes that lead to cell proliferation, Ig isotype switching, affinity maturation through somatic hypermutation, and generation of plasma effector cells and/or memory B cells (Liberg & Sigvardsson, 1999; Hagman et al., 2000).

Differentiation of an activated B cell into a plasma cell is dependent on expression of different transcription factors including B lymphocyte-induced maturation protein-1 (Blimp-1) (Figure I.1; Shaffer et al., 2002). Plasma cells and their precursors, the plasmablasts, are characterized by their ability to secrete immunoglobulin molecules. Upon differentiation of an activated B cell into a plasmablast, these cells migrate from the germinal centers to the splenic pulp or bone marrow where they continue to mature and secrete antibodies (Calame et al., 2003). Terminally differentiated plasma cells display an increase in surface expression of Syndecan-1 and tumor necrosis factor family receptor OX40L, as well as enlarged rough endoplasmic reticulum ideal for mass production of secreted proteins. Though plasma cells possess immunoglobulins, they lack a BCR and other signaling molecules including CD19, CD21, CD 22 and CD45.

Activated B cells can also take on a memory cell fate. Although there is evidence that activated B cells possessing a BCR of extremely high affinity to the specific antigen will favor differentiation into a plasma cell, expression of CD40 favors a memory cell fate (Calame et al., 2003). Memory cells are long-lived cells, surviving in the spleen and tonsils for months and possibly years. They are characterized by expression of surface receptor CD27 as well as a diverse selection of affinity matured membrane-bound IgG isotypes (Agematsu et al., 2000). Differentiation of an activated B cell to a memory B cell within germinal centers is influenced by many extracellular cytokine signals or receptor ligands (e.g., CD40L) (Arpin et al., 1995). In time, these cells may encounter their target antigen again resulting in rapid activation, proliferation and differentiation into plasma cells thus mounting a more efficient “secondary” immune response.

Pax-5

The *Pax* gene family. Though there are many transcription factors that direct B lymphopoiesis, B cell specific activator protein (BSAP) or Pax-5 is one of the master regulators from the pro-B cell through the plasmablast stage (Figure I.1; Hagman et al., 2000). Due to the importance of Pax-5 in B cell development and proliferation, understanding its role was the focus of this thesis research. Pax-5 is a member of the paired box-containing family of transcription factors (TF) which produces TFs that regulate many aspects of organogenesis, morphogenesis and development in both vertebrates and invertebrates (Chi & Epstein, 2002). The *Pax* gene family includes Pax proteins 1-9, all of which contain a highly conserved N-terminal sequence of 128 amino acids that comprises a DNA-binding domain, the “paired domain” (Walther et al., 1991; Chi & Epstein, 2002). Based on both sequence and structural similarity, as well as on the presence or absence of the octamer and homeodomain regions, the Pax proteins have been grouped into four subgroups (Figure I.2; Dahl et al., 1997).

All members of the four groups contain the highly conserved N-terminal paired domain; however, they may vary in other structural aspects (Figure I.2). The octamer sequence is found in groups I-III. In Pax-5, this sequence is known to interact with Grg4, a member of the Groucho family of corepressors, to mediate Pax-5 target gene repression (Eberhard et al., 2000). The homeodomain, which defines these *pax* genes as homeobox genes, is found in its complete form in groups III and IV, and in partial form in group II (Balczarek et al., 1997; Dahl et al., 1997). Each of these transcription factors are involved in regulating normal development of vertebrates. Development of the central nervous system requires regulation by Pax-1-3, 5, 7-9 (Dahl et al., 1997; Schwarz et al.,

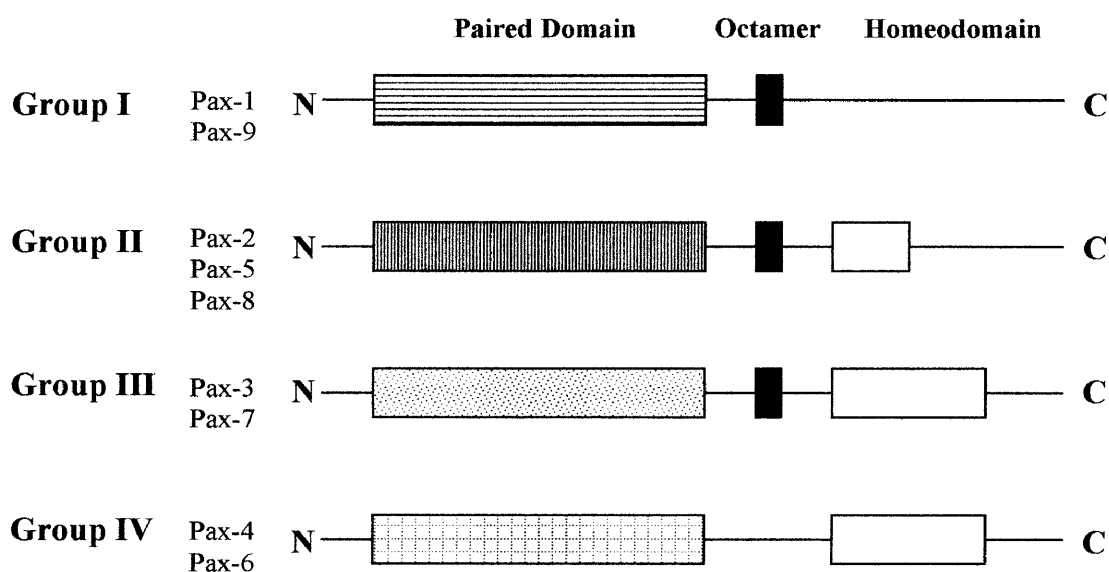


Figure I.2: Structures of protein members of the Pax family of genes. The nine *pax* genes give rise to different proteins which are all related by structure. These structures have allowed these genes and their products to be classified into four distinct groups shown above with a list of the particular gene products found within the group. All groups contain a highly conserved DNA-binding motif, the paired domain. The paired domain in each group is depicted by a different color pattern due to the fact that binding specificity is different for each group. The homeodomain region is found in groups II, III and IV, with group II containing only a partial homeodomain. The octamer (or octapeptide) sequence is found in groups I-III; this domain may have a role in protein-protein interactions.

1997) with Pax-2 and 8 playing fundamental roles in kidney development as well (Chi & Epstein, 2002). Pax-4 plays a crucial role in endocrine development and function, while Pax-6 regulates eye morphogenesis.

Members of Group II, containing Pax-2/5/8, share such extremely high sequence homology in the paired domain motif (90-95%), that it may be possible these proteins can bind many of the same DNA target sequences and thus may potentially create redundancy within the subgroup (Walther et al., 1991). Evidence for this is provided by studies using targeted mutations in one or the other protein and analysis of brain phenotypes of mice expressing different levels of each protein which show conserved function between Pax-2 and Pax-5 in midbrain and cerebellum development (Schwarz et al., 1997). This could explain why all three perform similar roles during CNS development as well as why both *Pax2* and *Pax8* control kidney development.

Pax-5: origin and function. B cell-specific activator protein (BSAP) was first described in a B cell line as a mammalian homolog to the previously described tissue-specific activator protein (TSAP), a transcription factor responsible for different developmental processes in the sea urchin (Barberis et al., 1990). BSAP was found to be expressed in all B cell stages except plasma cells. Further characterization and homology screening of BSAP revealed that it is encoded by the *pax-5* gene in mammals (Adams et al., 1992). Since then, Pax-5 (now known as Pax-5a) and its role in B cell development have been extensively studied, as it is the only member of the Pax family involved in hematopoiesis.

The Pax-5 protein is a 53kDa transcription factor with a complete 128 amino acid N-terminal paired DNA-binding domain which forms a bipartite structure comprised of

two helix-turn-helices (Maier & Hagman, 2002). This motif allows Pax-5 to interact with the bases of the major groove of the DNA within a promoter region. A serine/threonine-rich C-terminal *trans*-activating domain contributes to Pax5's ability to activate or repress target genes, while the partial homeodomain and complete octapeptide sequence mediate protein-protein interactions (Adams et al., 1992; Maier & Hagman, 2002).

Although expressed in the developing CNS as well as the adult testis, Pax-5 expression is more pronounced in the B-lymphoid tissues (Adams et al., 1992). Pax-5 is expressed during all stages of B cell development through maturity and activation; however, it is down regulated once an activated B cell has committed to a plasma cell fate (Barberis et al., 1990). Repression of Pax-5 during plasma cell development is controlled via B lymphocyte induced maturation protein-1 (Blimp-1) (Lin et al., 2002). Experiments using Pax-5-deficient mice revealed a complete block of B lymphopoiesis at the end of the pro-B stage resulting in the absence of immature and mature B cells (Urbanek et al., 1994). These mice are unable to mount a humoral immune response and lack germinal centers within secondary lymphoid tissues. Also, in the absence of Pax-5, pro-B (Nutt et al., 1998) as well as pre-B cells (Mikkola et al., 2002) will default to a myeloid or T cell lineage. Both experiments conclude that Pax-5 must be continuously expressed in order to maintain commitment to a B cell lineage.

Pax-5 maintains B cell lineage commitment by acting as a “master regulator” of many important B cell-specific genes. Pax-5 binding sites have been identified on promoters of these putative target genes: *CD19*, *mb-1*, *PD-1*, *LEF-1*, *N-myc*, *c-myc* (Nutt et al., 1998), immunoglobulin joining chain (*J chain*) (Wallin et al, 1999), *XBP-1* (Reimold et al, 1996), *RAG-2* (Kishi et al., 2002) and *notch-1* (Souabni, et al., 2002).

Pax-5 also regulates the transcription of pre-BCR components such as VpreB and $\lambda 5$ of the surrogate light chain (Tian et al., 1997). Before rearrangement of the IgL kappa gene, a transcription of the gene does occur, producing what is called a sterile transcript (Lennon & Perry, 1990). This is a germline-configuration transcript of the κ light chain gene which is not translated until complete rearrangement of the gene has occurred. Pax-5 is responsible for sterile transcription of the κ light chain during IgL rearrangement (Sato et al., 2004).

What makes Pax-5 interesting is its ability to act as either a transcriptional activator or repressor. Among some of the target genes repressed by Pax5 are *M-CSFR*, *notch-1* and *XBP-1*. During early pro-B development, expression of such transcription factors as E2A and EBF are not sufficient to commit to a B cell lineage. Commitment to the B lineage by Pax-5 involves preventing a response to the myeloid cytokine M-CSF as well as T lymphoid specific notch-1 ligands, by repression of M-CSFR (Busslinger, 2004) and Notch-1 (Souabni et al., 2002), respectively. During B cell development, Pax-5 also appears to repress XBP-1 which is essential for driving plasma cell differentiation as the emergence of Pax-5-repressor Blimp-1 relieves repression of XBP-1 (Shaffer et al., 2002).

The role of Pax-5 in B cell proliferation. Pax-5 has been shown to have a distinct role in B cell proliferation. By transfecting splenic B cells with a BSAP expression plasmid and incubating the cells with one of three proliferative stimuli (LPS, CD40 ligand or anti-IgD), Max et al (1995) showed that increased amounts of transfected BSAP caused an increase in proliferation as measured by ^3H -thymidine incorporation. Data from electrophoretic mobility shift assays (EMSA) revealed increased BSAP activity that

correlated with the increase in proliferation. To address whether down-regulated Pax-5 would show a decrease in proliferation, splenic B cells were incubated with the same three proliferative stimuli as well as varying concentrations of BSAP antisense oligonucleotides (oligo) or control sense oligonucleotides. Increasing antisense oligo concentrations showed a significant decrease in BSAP activity. Similarly, B cell proliferation was severely impaired by increasing amounts of the antisense oligo, as compared to no significant effect from the control sense oligo. Finally, transfection of plasmacytoma cell line MOPC315 with a BSAP expression construct also caused an increase in proliferation, suggesting that Pax-5/BSAP is sufficient to stimulate proliferation in non-B cells (Max et al., 1995).

Among the genes positively regulated by Pax-5 is the B-lymphoid tyrosine kinase (*blk*) gene which contains Pax-5 binding sites in its promoter region (Liberg & Sigvardsson, 1999). Blk has also been shown to play a dynamic role in B cell activation and proliferation (Malek et al, 1998). Blk is a member of the Src tyrosine kinase subfamily of proteins associated with the T or B cell receptor; Blk is specifically expressed in the B cell lineage. Activation of the Blk protein, or family members Fyn and Lyn, is triggered by stimulation of the BCR (Saijo et al, 2003). This may play a role in NF- κ B activation, which is associated with B cell activation after binding to antigen as was shown in mice deficient for Blk/Fyn/Lyn. Knockout of these three genes was necessary as mice deficient for each individual protein showed no change in phenotype from wild-type mice. This suggests that Blk, Fyn and Lyn play redundant roles in NF- κ B induction and B cell activation (Saijo et al., 2003). Transgenic mice overexpressing a constitutively active form of the Blk protein, in B and/or T cell lineages, produced tumors

originating from lymphoid progenitor cells in the spleen and bone marrow (Malek et al., 1998). The transgenic construct also caused Blk expression in T cells, which initiated tumor formation in the thymus. This suggests that Blk has a role not only in B cell activation but also in B cell proliferation during development.

Alternative Isoforms of Pax-5. In 1997 it was discovered that the *pax5* gene is alternatively spliced into four isoforms; Pax-5a, Pax-5b, Pax-5d and Pax-5e (Figure I.3) (Zwollo et al., 1997). Though each alternative isoform is important for B cell function, the focus of this research was to determine the *in vivo* role of Pax-5e in B cell development and proliferation. The full-length variant Pax-5 or BSAP is now known as Pax-5a. The other variants code for proteins with a variety of deleted or novel segments. Pax-5b and -5e transcripts contain incomplete DNA-binding domains lacking exon 2 (nt 47-212). Pax-5d and -5e transcripts have spliced out exons 6-10 (the C-terminal transactivating domain and partial homeodomain), which have been replaced by a novel sequence of unknown origin. The position of this novel sequence in the *pax-5* gene has yet to be determined however; in Pax-5d and -5e transcripts it is comprised of 128 nucleotides and shares homology with the coding region in a number of oxidoreductases. Each of the four proteins is expressed at different levels during B cell development; Pax-5a is the most prominent isoform. Pax-5d and -5e are expressed at very low levels, but this also varies during B cell development as Pax-5e levels increase after 4-6 day stimulation of splenic cells with LPS (Lowen et al., 2001).

To form alternatively spliced isoforms, a gene (or mRNA) requires a splice site (or stop codon) to inform transcriptional (or translational) machinery where one isoform

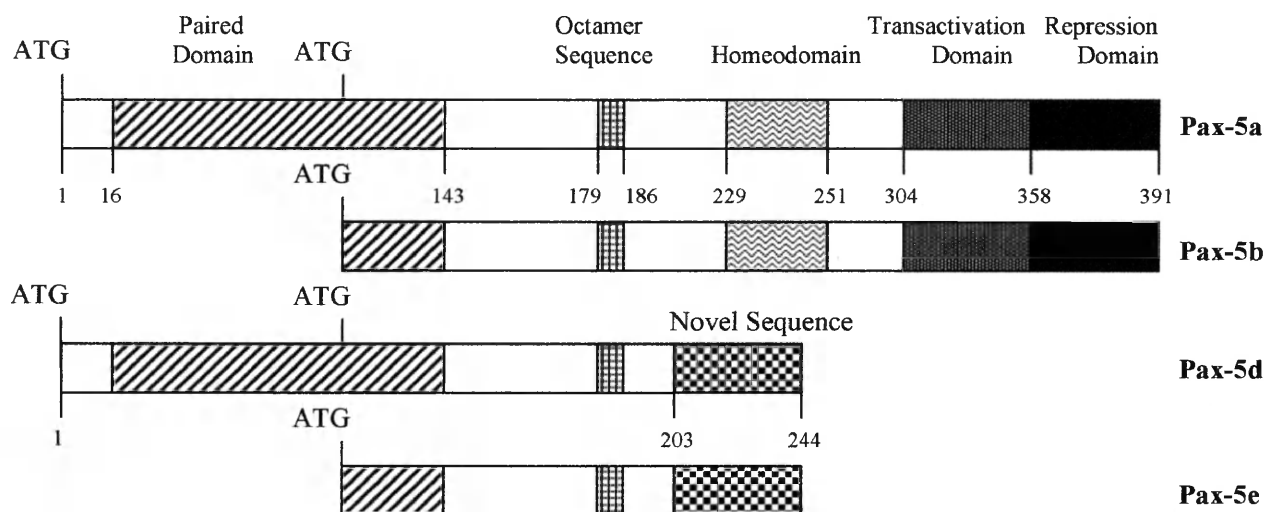


Figure I.3: Alternative isoforms of Pax-5. The gene for the transcription factor Pax-5 generates four isoforms: main isoform Pax-5a (53kDa), and three alternative isoforms Pax-5b (41kDa), Pax-5d (31kDa) and Pax-5e (19kDa). Here, Pax-5/BSAP, the full length protein, is referred to as Pax-5a. Each of the other isoforms contains a different combination of the domains found in Pax-5a. *Diagonally striped boxes* show the DNA-binding domain, called the paired domain (aa 16-143). Pax-5b and 5e each have a partial paired domain as these proteins are produced from the second translational start site (indicated by ATG-2). *Hatched boxes* represent the Octamer sequence (aa 179-186) which is conserved in all isoforms. *Wavy line boxes* indicate the homeodomain (aa 229-251); *Shaded boxes* indicate the transactivation domain (aa 304-358); *Black boxes* indicate the repression domain (aa 358-391). Both Pax-5d and 5e contain a novel sequence (*Checkered boxes*, aa 203-244) of unknown origin and lack the homeodomain, transactivation and repression domains on their C-terminus.

stops and another starts. The *pax-5* gene contains a splice site between nucleotides 606 and 607 which also corresponds to the intron-exon boundary flanked by exons 5 and 6 (Zwollo et al., 1997). Exon 5 encodes the octapeptide sequence (nucleotides 535-558) which is found on all Pax-5 proteins, while exon 6 encodes the partial homeodomain (nucleotides 685-753) found only on Pax-5a and -5b. This splice site produces isoforms Pax-5d and -5e which lack the partial homeodomain yet still retain the octapeptide sequence. Retaining the octamer sequence may allow Pax-5d or -5e to interact with members of the Groucho corepressor family, as Pax-5a interacts with Grg4 through the octamer (Eberhard et al., 2000)

Pax-5e: characteristics and possible roles. The Pax-5e protein is predicted to be 18-19kDa based on amino acid sequence; however, western blots reveal that this protein preferentially runs at 27-29kDa (Lowen et al., 2001). Based on this information, it is hypothesized that Pax-5e either forms a secondary structure in the cell, forms homodimers, or associates with another small protein. To answer these questions, a mutant form of Pax-5e was created using PCR-directed site-specific mutagenesis. In the mutant, called Pax-5e.mC5, the most C-terminal of the three cysteine residues was replaced by a structurally similar serine, making the mutant constitutively reduced. The Pax-5e.mC5 was transfected into NIH3T3 cells and, when followed by western blot analysis, the mutant Pax-5e ran completely at the 19kDa position. This indicates that Pax-5e may normally be in an oxidized form while the mutant lacking the third cysteine residue remains reduced. If Pax-5e does interact with another small protein, it is possible

that only the oxidized form of 5e can create this heterodimer, as the 27-29kDa band was not present when the mutant protein was used.

Transcription factors and other proteins can be regulated in several ways; in particular oxidation/reduction (or redox) modifications can alter protein activity states. Redox regulation occurs by changing the oxidation state of cysteine residues found on the protein; oxidized cysteine residues are bound to one another in a disulfide bond, while reduced cysteines remain unbound (Tell et al., 2000). Proteins such as Ref-1 and thioredoxin (TRX) are enzymes capable of reducing their substrate during episodes of oxidative stress (Hirota et al., 1997). Thus, proteins reduced by Ref-1/TRX will continue to function in a heavily oxidized environment.

Previous studies have shown that Pax-5a interacts with the redox factor Ref-1, indicating that there is the possibility for redox regulation of Pax-5a (Tell et al., 2000). This was accomplished by treatment of cells with low doses of H₂O₂ which resulted in decreased Pax-5 DNA-binding activity. This suggests that Pax-5a functions most efficiently in a reduced form (Tell et al., 2000). These data were supported by initial studies done using recombinant Pax-5a and Pax-8 paired domains that revealed evidence for redox regulation through the conserved cysteine residues present in that region of the protein (Tell et al., 1998). Thioredoxin (TRX) is a small 12kDa protein that also interacts with Ref-1 as well as NF- κ B/p50 (Hirota et al., 1999), which makes it a good candidate for a role in redox regulation of Pax-5a, possibly by interactions with Pax-5e.

Our lab has hypothesized that the 27kDa Pax-5e complex is a heterodimer of Pax-5e and TRX. This hypothesis is supported by immunoprecipitation experiments showing that TRX coimmunoprecipitates with Pax-5e (Lowen et al., 2001). These samples were

analyzed by western blot and probed with a catfish monoclonal anti-TRX (IgA) antibody. The anti-TRX antibody also detected a 27kDa TRX-like species in LPS-activated small resting B cells (SRB) nuclear extracts collected on day 0, 4, 6, and 8 after activation. More convincingly, the level of TRX increases over time with a peak at day 6 after LPS activation and a slight decrease on day 8, which corresponds to the expression pattern of 5e. Metabolically labeled LPS-activated small resting B cell (SRB) nuclear extracts from day 2 and day 6 were immunoprecipitated with anti-Pax-5 antibody ED-1 serum, 6G11 supernatant, or anti-TRX supernatants. A 27kDa complex is absent in day 2 nuclear extracts but present on day 6 when immunoprecipitated with anti-Pax-5 (ED-1), anti-Pax-5e (6G11), or anti-TRX antibodies. This provides strong evidence for the interaction of Pax-5e with a TRX-like protein.

The role of each of the Pax-5 isoforms remains under investigation. Pax-5e appears to enhance the activity of Pax-5a in a dose-dependent manner (Lowen et al., 2001). Transient transfection of the NIH3T3 cell line with a chloramphenicol acetyltransferase (CAT) reporter construct (containing multiple Pax-5a binding sites) as well as with a Pax-5a and Pax-5e revealed that Pax-5a activity is enhanced, as shown by an increase in percent CAT conversion. Proliferation experiments in cell lines have also shown that increasing amounts of transfected Pax-5e in mature B cell lines A20/2J and B17.10 lead to increased cell proliferation at earlier time points (Beth Jones, unpublished data).

Based on these data, we hypothesize that Pax-5e regulates Pax-5a by increasing Pax-5a activity. Pax-5e's interaction with a protein such as thioredoxin suggests a possible redox mechanism of Pax-5a regulation (Figure I.4). Conceivably Pax-5e and 5a

normally remain in an oxidized, or inactive, state. After stimulation of the B cell a thioredoxin-like protein then interacts with and reduces Pax-5e, breaking existing disulfide bonds. Once reduced, Pax-5e then induces reduction of Pax-5a which in turn allows Pax-5a to bind target gene promoters, resulting in expression or repression of target genes. In this model, increasing amounts of Pax-5e should lead to increased 5a activity and thus increased expression or repression of target genes. In the case of genes such as *blk* or *c-myc*, this may also cause increased proliferation of cells overexpressing Pax-5e.

Research Questions

Based on the model proposed above, the objective of this thesis research was to determine the effects on B cells of overexpressing alternative isoform Pax-5e *in vivo*, using a murine model. While investigating these effects, several questions were addressed. First, does Pax-5e increase the activity of Pax-5a leading to increased proliferation of pro/pre-B, (im)mature B, and/or plasmablasts? Proliferation ELISAs were used to detect a change in proliferation rate of cultured bone marrow or spleen cells. LPS-activation of these cells was also used to determine which cell populations were most affected. Secondly, will using flow cytometry reveal any changes in the size and distribution of lymphoid populations such as pro/pre B cells, mature B cells or T cells in the bone marrow or spleen? This analysis allowed determination of whether there are increases in B cell populations that would indicate increased proliferation.

Finally, if overexpression of Pax-5e increases Pax-5a activity, are there changes in Pax-5a target genes such as *CD19*, *J chain* or *blk*? If target gene expression is altered,

Model of Pax-5e activity in B cells

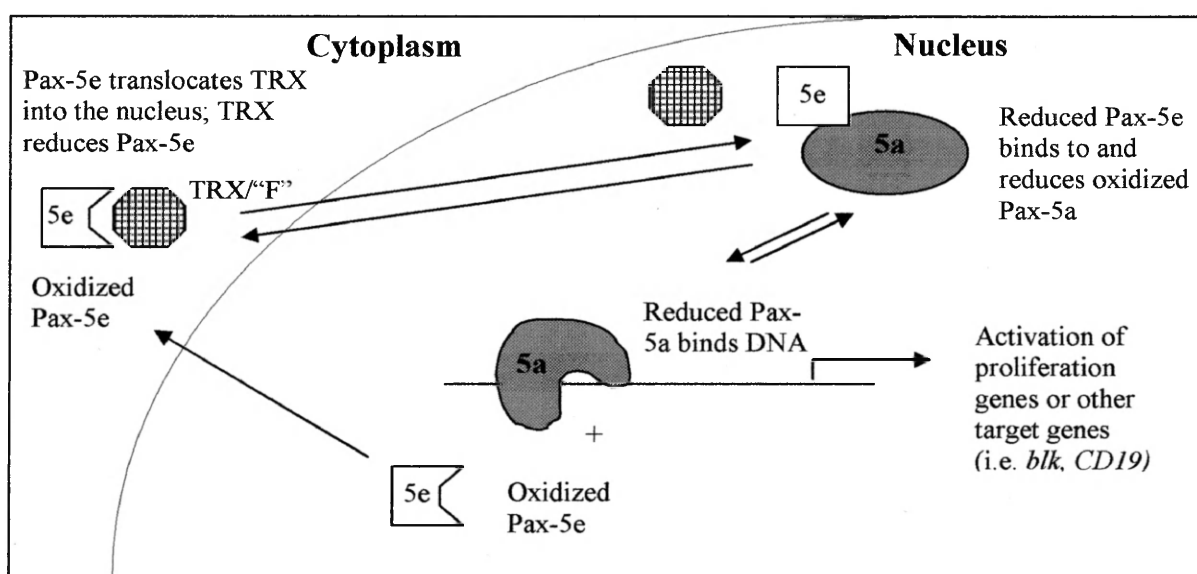


Figure I.4: Model of possible interactions between Pax-5a, Pax-5e and thioredoxin in B cells. As was proposed by Lowen et al. (2001), oxidized Pax-5e interacts with TRX or undefined factor "F" (hatched octagon), inducing both a translocation of TRX/"F" into the nucleus and reduction of Pax-5e. Reduced Pax-5e (square) can then interact with and lead to the reduction of Pax-5a. Once reduced, Pax-5a (irregular shaded shape) is able to bind DNA and initiate transcription of proliferation genes and other target genes. Pax-5e reverts back to an oxidized state, exits the nucleus and is able to bind TRX again.

how would this affect the mice when challenged by immunization? “Real time” PCR was used as a tool in determining possible overexpression of *CD19* or *blk*, as well as potential over-repression of *J chain*, using RNA extracted from spleen and bone marrow cells in both immunized and non-immunized mice. In answering these questions, the *in vivo* functions of alternative isoform Pax-5e have begun to be elucidated.

CHAPTER II

Methods and Materials

Transgenic Mice. In collaboration with the University of Pennsylvania Transgenic Mouse Facility, transgenic mice were developed that overexpress alternative isoform, Pax-5e in a construct flanked by the murine H-2K promoter and the Ig-mu intronic enhancer (Pircher et al., 1989) in a 6.0kb *XhoI-XhoI* fragment (ref) (see figure III.1). The transgenic mice (TG5e) were developed using a B6/SJL strain. The F1 generation was produced by crossing transgenic founders with wild-type SJL mice. Three-five generations of mice were produced before separating negative and homozygous lines for each strain. All mice were housed in semi-sterile conditions with sterilized food, water and bedding.

DNA extraction. At 3 weeks of age, tail biopsies were collected and digested at 55°C for 16 hrs in 700µl of 50mM Tris (pH 8.0)/100mM EDTA/0.5% SDS and 35µl of Proteinase K (10mg/ml). DNA extraction was done using one phenol (equilibrated with Tris-HCl to pH 8.0) and one phenol/chloroform extraction. DNA was precipitated for one hour in 1/10 of a volume of 3M sodium acetate (pH 6.0) and 2 volumes of ethanol. Immediately after precipitation, the samples were spun 30 seconds and pellets washed with 1ml of 70% ethanol, agitated briefly, and centrifuged for 1 minute. DNA pellets were resuspended in 100µl of TE (10mM Tris-HCl (pH 8.0)/1mM EDTA). Optical densities

were read using the Gene Quant II (Amersham Pharmacia Biotech) at 260nm.

Southern Blot. For each sample, 5µg of DNA was added to the appropriate volume of sterile water to a final volume of 75µl. 10 µl of 1.0mg/ml Bovine serum albumin, 10 µl *Bam* H1 buffer and 5µl *Bam*H1 restriction endonuclease were then added to each sample and allowed to digest at 37°C overnight. After the digest, 10 µl of each sample was run on a diagnostic 0.8% agarose gel to ensure proper digestion of the DNA. Samples were then precipitated using 1/10 volume of 3M sodium acetate (pH 6.0) and 2 volumes of 100% ethanol and incubated at -20°C for one hour. Following the incubation, the samples were centrifuged for 25 minutes at 17,900 x g and the supernatants were discarded. The samples were washed with 200µl of 70% ethanol and centrifuged for 5 minutes. The pellets were resuspended in 15µl TE plus 3µl of 6X loading dye (25% xylene cyanol, 25% bromophenol blue, 30% glycerol) and separated by gel electrophoresis on a 0.8% agarose gel. A Polaroid photograph was taken of the gel after ethidium bromide staining. The samples were transferred for 16 hrs onto a positively charged nylon membrane (Roche) in an alkaline buffer (0.4M NaOH). After transfer, the filter was rinsed in 2X SSC (20X stock= 3M NaCl, 300mM sodium citrate, pH 7.0) for 2 minutes then allowed to dry before UV cross-linking. Prehybridization in 5X SSC/5XDenhardt's solution/1% SDS/0.1mg/ml single-stranded Salmon testes DNA was carried out at 65°C for 2 hours. The TG5e (*Xho*I-*Xho*I fragment, in Figure III.1) probe was created utilizing the Random Primed DNA Labeling Kit (Roche) according to manufacturer's instructions and using 5µl of 3000Ci/mmol of [³²P]α-labeled dCTP (Amersham Pharmacia Biotech). The specific activity was $\geq 5 \times 10^8$ CPM/µg. The TG5e probe was added to the hybridization solution (same as the prehybridization) at a

concentration of 1×10^6 CPM/ml and the membrane was incubated on a Little SHOT Hybridization Oven (Boeckel Scientific) for 16-24 hours at 65°C . After hybridizing, the membrane was washed twice for 5 minutes in $2\text{X SSC}/0.1\%$ SDS at room temperature, twice for 5 minutes in $0.2\text{X SSC}/0.1\%$ SDS at 42°C , and finally for 10 minutes in $0.1\text{X SSC}/0.1\%$ SDS at 65°C . The membrane was then exposed to a phosphor screen for 1 hour and read on the STORM 840 Phosphorimager (Amersham Pharmacia Biotech). Band intensities were quantified using a STORM Scanner Control Version 5.01 (Molecular Dynamics, Inc.).

Polymerase Chain Reaction. Using DNA collected from the tail biopsies, $0.1\mu\text{g}$ DNA was amplified over 35 cycles to determine if the mice contained the TG5e transgene. Sense primer TG5e-1-S ($5'$ -GAAGTTCTCAGGATCCTCTAGAG- $3'$ covering a region of the MHC class I promoter $\sim 70\text{bp}$ upstream from the of Pax-5e cDNA sequence – see Figure III.1) and anti-sense primer Pax-5d/kpn-AS ($5'$ -GACTGGTACCCTAGGACCCTGGG AAGCC- $3'$ covering nts 717-735 at the $3'$ end of Pax-5e) were used to amplify the TG5e transgene insert. This amplifies a 575nt fragment which was visualized after ethidium bromide staining on a 2% agarose gel.

RNA extraction and Northern Blot. Total RNA was extracted from spleen or thymus using the Qiagen RNeasy Mini- or Midi-Prep according to the manufacturer's instructions. A total of $20\mu\text{g}$ of RNA was precipitated with $1\mu\text{l}$ of transfer RNA, 1/10 volume of RNase-free 3M sodium acetate (pH 6.0) and 2 volumes of ethanol for 16 hrs at -20°C . The samples were centrifuged for 25 minutes at 4°C and $17,900 \times g$ and the supernatant was discarded. Next, the samples were washed with $100\mu\text{l}$ of 70% ethanol and centrifuged for 5 minutes at 4°C and $17,900 \times g$. The pellets were resuspended in

2.75 μ l diethyl pyrocarbonate (DEPC) treated water, 1.25 μ l 10X MOPS (0.4M MOPS, 0.1M sodium acetate, 0.01M EDTA, and DEPC water to pH 7.0)/2.25 μ l 37% formaldehyde, and 6.25 μ l formamide for a total volume of 12.5 μ l. The samples were then incubated for 15 minutes at 55°C followed by the addition of 2.5 μ l of RNA loading dye (1mM EDTA [pH 8.0], 0.25% bromophenol blue, 0.25% xylene cyanol, 50% glycerol) and loaded onto a 1% agarose gel containing 1X MOPS and 6.7% formaldehyde. The samples were separated by gel electrophoresis in a buffer of 1X MOPS. The gel was then stained with 0.5 μ g/ml of ethidium bromide in 0.5M ammonium acetate for 15 minutes and destained in DEPC water for 2 hours. The gel was viewed on a UV transilluminator and a Polaroid photograph was taken of the gel to determine relative sample concentrations. The gel was washed in 0.05M NaOH/1.5M NaCl for 15 minutes, then in 0.5M Tris/1.5M NaCl for 15 minutes and finally in 10X SSC for 15 minutes all at room temperature. The gel was transferred onto a positively charged Roche nylon membrane according to manufacturer's instructions and RNA allowed to transfer for 16 hours in a solution of 10XSSC. The membrane was then UV cross-linked and prehybridized in a solution of 5X SSC, 5X Denhardt's solution, 1% SDS, 0.1mg/ml denatured salmon testes DNA and DEPC water at 65°C for 2 hours. The hybridization solution was the same as that for the prehybridization; however, 1x10⁶ CPM/ml of ³²P-labeled denatured TG5e probe was added to the mixture and the membrane was incubated in a Little SHOT Hybridization Oven (Boekel Scientific) for 16-24 hours at 65°C. Following the hybridization, the membrane was washed twice for 5 minutes in 2X SSC/0.1% SDS, twice for 5 minutes in 0.2X SSC/0.1% SDS (both at room temperature) and finally for 15 minutes in 0.2X SSC/0.1% SDS at 42°C. The membrane was then

exposed overnight to Kodak X-OMAT-AR film and developed on the Konica SRX-101 X-ray processor.

B and T cell Purification. B or T cells were purified from splenic cells using the Spin-Sep Murine B or T Cell Enrichment Kit (Stem Cell Technologies) according to the manufacturer's instructions. In brief, cells were purified via negative selection using antibody cocktails against CD4, CD8, CD11b, TER119 and Ly-6G (for B cell purification) and CD11b, CD45R, TER119, and Ly-6G (for T cell purification). Purified B or T cells (at a yield of $1-5 \times 10^6$ cells for each) were processed for RNA using the RNeasy Mini Prep (Qiagen).

Reverse Transcriptase PCR. cDNA was prepared from RNA samples using the GeneAmp RNA PCR kit (Applied Biosystems) according to the manufacturer's instructions and using 0.3 μ g RNA. Sense primers Pax-5-164-S (5'-CCAGGCAGCTTCGGGTCA GCC-3' covering nts 164-184 of Pax-5d) and Pdcon1-S (5'-ACCATGTTGCCTGGGAG-3' covering nts 322-339 of Pax-5d and 5e) and Pax-5d/kpn-AS (5'-GACTGGTACCCTAGGACCCTG GGAAGCC-3' covering nts 717-735 of Pax-5d and 5e) were used to amplify the TG5e cDNA to determine expression of the transgene. For Pax-5d, a 571nt fragment was amplified and for Pax-5d and 5e a 413nt fragment was amplified.

Cell Purification and Culture. Spleen, bone marrow and thymus tissues were collected from transgenic mice and negative littermate controls and disrupted in complete RPMI medium (Fisher) using a 5ml syringe. Cells were forced through a 40 μ m nylon cell strainer (Falcon) to create a single cell suspension then centrifuged for 10 minutes at 170 x g and 4°C. The supernatant was poured off and the cells were resuspended in 10ml

HBSS (0.14M NaCl, 5.0mM KCl, 1.5mM Na₂HPO₄ – 7H₂O, 5.5mM Dextrose, 20mM Hepes, pH 7.05) and counted using a Brite-Line Hemacytometer (Hausser Scientific).

Cells to be used for ELISA or ELIspot were plated on 96-well tissue culture plates (Corning) at 1×10^6 cells/well and incubated at 37°C in a CO₂-Incubator (5.0% CO₂).

Nuclear Extract Preparation. 500µl of lysis buffer (10mM Hepes, pH 7.9, 10mM KCl, 0.1mM EDTA, 0.4% Igepal [Sigma]) was added per 10^7 cells and incubated for 15 minutes on ice. The samples were then centrifuged at 4°C, 17,900 x g for 1 minute. Supernatants were saved as cytoplasmic fractions and frozen at -80°C. The remaining nuclei were washed with 250µl of ice cold lysis buffer and centrifuged for 30 seconds. The nuclear pellet was then resuspended in 250µl of ice cold extraction buffer (20mM Hepes, pH 7.9, 0.4M NaCl, 1mM EDTA) and agitated for 20 minutes at 4°C. The samples were then centrifuged for 10 minutes at 17,900 x g and 4°C. Nuclear extract aliquots were collected and stored at -80°C. All buffers contained the following mixture of protease inhibitors: 1mM dithiothreitol, 0.5mM phenylmethylsulphonyl fluoride, 10mg/ml aprotinin, 12.5mg/ml leupeptin, 1.25mg/ml pepstatin A, 0.5mg/ml trypsin inhibitor). Protein concentrations were determined using the Bradford assay (Bio-Rad) according to the manufacturer's instructions.

Western Blot. Nuclear extracts or whole cell lysates from tissues were resuspended in complete 2X loading dye (50mM Tris-HCl, pH 6.8/5% 2-mercaptoethanol/10% glycerol/1% SDS), separated on a 12% denaturing SDS/polyacrylamide gel and electrophoretically transferred onto an Immuno-Blot PVDF Membrane (Bio-Rad) for 1 hour at 100V at 4°C. Following transfer, the membranes were incubated for 1 hour in blocking solution (5% dry milk in PBS). The primary antibody, an anti-Pax-5 anti-serum

called ED-1 (Zwollo *et al.*, 1998), was added at 1:2000 in blocking solution to detect Pax-5a and -5e and incubated for 1 hour. The membranes were then washed 3 times for 10 minutes in PBS. A donkey anti-rabbit horseradish peroxidase-conjugated IgG secondary antibody (Amersham Pharmacia Biotech) was added in blocking solution at 1µg/ml and incubated for 1 hour. The filters were then washed 3 times for 10 minutes in PBS before developing with an enhanced chemiluminescence kit (ECL, Amersham Pharmacia Biotech). The bands were detected on Kodak X-OMAT-AR film.

Flow Cytometry. Single cell suspensions were loaded onto 96-well round bottom plates at 10^6 cells/well and incubated with the antibody cocktail (0.5µg antibody/million cells of each of three fluorescent antibodies in PBS) for 30 minutes on ice in the dark.

Fluorescent antibodies used were: CD45R/B220-PerCP, CD3-RPE, CD69-FITC, CD19-RPE, IgM-FITC, CD43-RPE (all from BD Biosciences/Pharmingen). After incubation the plates were centrifuged for 3 minutes at 1200rpm and the supernatants were discarded. The cells were washed in 200µl of PBS and centrifuged for 3 minutes at 1200rpm and supernatants were discarded. Each sample was resuspended in 200µl of PBS and transferred to a 2.5ml flow cytometry tube. Samples were analyzed on the FACStarPlus flow cytometer (Becton-Dickinson) using 50-70,000 events/sample.

Proliferation Assay. Spleen and bone marrow cells were plated on a 96-well flat bottom plate at a concentration of 10^5 and 10^4 cells/well (in duplicate). Proliferation was measured using the Cell Proliferation ELISA, BrdU Kit (Roche) according to the manufacturer's instructions. Briefly, 10µl of BrdU labeling solution was added to each well and incubated for 18 hours in a CO₂ incubator (37°C and 5% CO₂). Cell suspensions were then centrifuged for 10 minutes at 1000rpm and the supernatants were

discarded. Cells were dried using a standard hairdryer for 10 minutes then permeabilized for 30 minutes at room temperature (using manufacturer's reagents). After removal of the fixing solution, an anti-BrdU-POD solution was added and incubated for 60 minutes at room temperature. Cells were then rinsed and the substrate was added and allowed to react for 2-5 minutes until a clear color change was observed. The reaction was stopped using 1M H₂SO₄ and the optical densities (at 450nm) were obtained using a Microplate Reader (Bio-Rad). Time points included: day 0, day 2 and day 3.

Immunizations. Transgenic mice and negative littermate controls were each immunized intraperitoneally on day zero with 50µg of TNP-BSA in Freund's Complete Adjuvant (Sigma). On day 14, the mice each received a booster of 50µg of TNP-BSA in Freund's Incomplete Adjuvant (Sigma). Blood was collected from the tail vein on day 21 and allowed to clot for 4 hours at room temperature followed by an overnight incubation at 4°C. The blood clots were centrifuged for 10 minutes at 1700 x g and the serum was collected for ELISA. The mice were euthanized on day 23 and spleen, bone marrow and thymus collected for analysis.

ELISA. Cell samples were cultured in quadruplicate in complete RPMI medium on 96-well plates, at 10⁶ cells/well for 24 hours at 37°C in a CO₂ incubator (5.0% CO₂). Supernatants were collected and pooled for each sample and frozen at -20°C until needed. 96-well PRO-BIND Flat-bottom Assay Plates (Falcon) were coated with PBS containing 0.05% sodium azide and either 1µg/ml TNP-LPS, anti-mouse IgM, or anti-mouse IgG for 24 hours at 4°C. After incubation, the plates were washed three times with ddH₂O and incubated for 1 hour with 250µl/well of blocking buffer (0.05% Tween 20, 1mM EDTA, 0.25% BSA, 0.05% NaN₃, 0.015M Na₂B₄O₇-10H₂O and 0.12M NaCl, pH 8.0). The

plates were then rinsed 3 times in ddH₂O and samples (serum or supernatants) were added in duplicate to the plate in dilutions of 1:4, 1:16 and 1:64 and incubated for 2 hours at room temperature. The plates were then rinsed 3 times with ddH₂O and 100µl of horseradish-peroxidase conjugated secondary antibody (goat anti-mouse IgG or goat anti-mouse IgM; Zymed) in blocking solution (at 2µg/ml) was added to each well and incubated for 2 hours at room temperature. Finally the plates were rinsed 5 times in ddH₂O and 50µl of developer (4.8ml citrate buffer, 5µl 30% H₂O₂, 200µl 1% 2,2'-azino-bis[3-ethylbenz-thiazoline-6-sulfonic acid]) (Sigma) was added to each well. The optical density (at 405nm) of each sample was read after 5 or 10 minutes using a Microplate Reader (Bio-Rad).

ELISPOT. Immuno-Blot PVDF membranes (Bio-Rad) were coated with either 1µg/ml of murine IgM or IgG, or 100µg/ml of TNP-LPS on a Biodot Apparatus (Bio-Rad) and incubated at room temperature for 2 hours on a platform rocker. After removing the excess fluid, the membranes were blocked with post coating buffer (0.15M NaCl, 50mM Tris, 1mM EDTA, 0.15M Sucrose, 40g Casein) and incubated on a platform rocker for 1 hour. Membranes were then washed 3 times for 5 minutes with 250µl PBS/well. Cells in complete RPMI (10% FBS) were diluted in a 96-well plate in duplicates of 10⁵ and 10⁴ cells/well for each sample then loaded onto a Bio-Dot apparatus (Bio-Rad). The samples were then incubated for 4 hours at 37°C, 5% CO₂. After incubation, the apparatus was disassembled and the membranes were washed for 1 minute (3 times) in PBS. On a platform rocker, the membranes were incubated with post coating buffer for 1 hour then rinsed 3 times for 5 minutes with PBS. Next, the membranes were incubated with 2µg/ml goat anti-mouse secondary antibody conjugated horseradish peroxidase (either

anti-IgM or –IgG) in PBS for one hour. The membranes were then washed 3 times for 10 minutes in PBS before adding the AEC detection reagent (20ml of substrate buffer: 1.75ml glacial acetic acid, 5.73g NaOAc, sterile ddH₂O [pH 5.0]; for 100µl of substrate: 0.2g 3-amino-9-ethylcarbazole in 5ml dimethylformamide; 20µl of 30% H₂O₂).

Membranes were incubated for 10-20 minutes or until a clear color change was visible. The membranes were allowed to dry for 16 hrs in the dark on filter paper and the filters were analyzed by CTL Cellular Technology, Ltd. Immunospot Analyzers.

Quantitative “Real Time” PCR. cDNA was prepared from splenic RNA samples using the GeneAmp RNA PCR kit (Applied Biosystems) according to the manufacturer’s instructions and using 0.3-0.5µg RNA. Approximately 15-25ng of cDNA was then amplified in a multiplex fashion in iQ Super Mix (Bio-Rad) using primers for β-Tubulin [Tubb5-s-q (5’ ATCTTCAGACCAGACAACCTTCGTT 3’) Tubb5-as-q (5’ CCTTGCAGGCAATCACAGCT 3’) Tubb5-PROBE (5’ /5HEX/TCCGCCTCCTTCCGCACCACATCC/3BHQ-1/ 3’)] and Ig J chain [Jchain-s-q (5’ CCCGATTCTTGCTACCTTGACT 3’) Jchain-as-q (5’ ACCCTGGTTTCAAATTCATTAGCA 3’) Jchain-PROBE (5’ /56-FAM/AGCCACTCACCATGCAGCTCGTTG/3BHQ-2/ 3’)] or β-Tubulin and CD19 [Cd19-s-q (5’ AGTGTCCCTGGGTCCTATGG 3’) Cd19-as-q (5’ AGGTTGGAGTCGTTCTCATAGAAT 3’) Cd19-PROBE (5’ /56-FAM/CCCTCCTCGCTGTCTGGCTCTTCA/3BHQ-2/ 3’)]. Amplification occurred using 15 sec denaturing at 95°C, 30 sec annealing at 60°C, and 1 min extension at 72°C over 40 cycles using the iCycler (Bio-Rad). Threshold values of the experimental gene

(CD19 or J chain) were expressed as a ratio of the experimental gene to β -Tubulin to account for within-sample variation.

Statistics. A student's T-test was performed for ELISA, ELISPOT, qRT-PCR and flow cytometry data using a two-tailed method to determine significance. A value of 0.03 was designated as the p value; samples below which would be considered significant.

CHAPTER III

Results

Generation and Expression Analysis of TG5e Mice. To investigate the *in vivo* role of Pax-5e in B cell development and activation, we developed transgenic mice that overexpress Pax-5e. A construct containing full length Pax-5e flanked by the murine H-2K promoter and the Immunoglobulin HC intronic enhancer was cloned (Figure III.1A), and transgenic mice were generated in collaboration with the University of Pennsylvania Transgenic Mouse Facility. This construct was known to be expressed exclusively in B and/or T lymphocytes (Malek et al., 1998). One would expect the MHC-class 1 H-2K promoter to be expressed in all cell types, however this is limited by the Ig HC intronic enhancer which is restricted to expression in B lymphocytes. It is unclear as to why this construct can be expressed in T cells (Malek et a., 1998).

Ten TG5e founder mice (TG5e-7, 31, 32, 35, 36, 40, 49, 51, 58 and 62) were obtained, 2 of which died at the Univ. of Pennsylvania transgenic facility before shipping (TG5e-36 and 58) by the age of 4 weeks. Founders TG5e-40, 32, 35 and 51 never produced transgenic offspring, while founder 62 died of unknown causes shortly before delivering her first litter. Founder TG5e-31 successfully delivered one litter of transgenic offspring yet died of squamous cell carcinoma before more offspring could be produced. Thus, 40% of the TG5e transgenic founders died within 2 months of birth. The remaining founders, TG5e-7 and TG5e-49, successfully bred with wild-type SJL females and

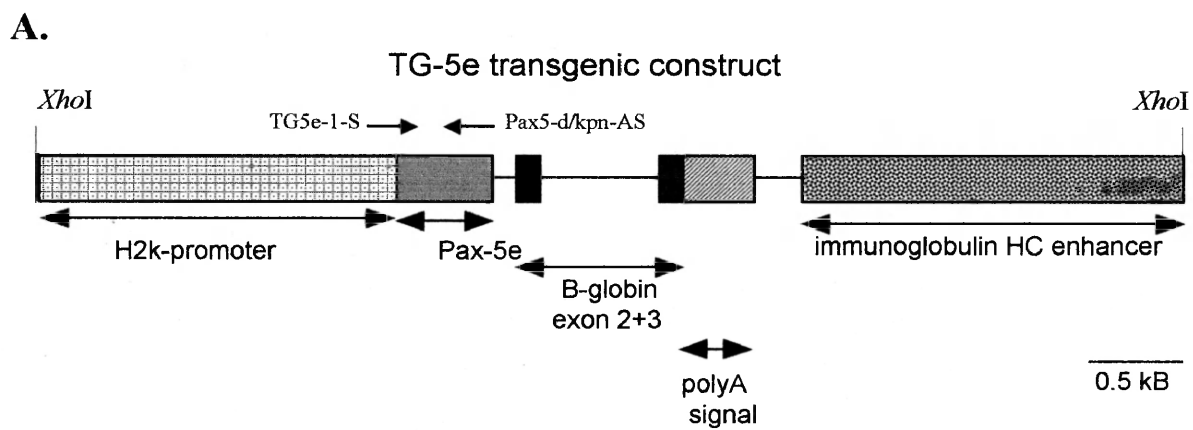


Figure III.1A: TG5e Transgenic construct. The construct contains a copy of the Pax-5e coding sequence flanked by the H2k-promoter and the Ig HC enhancer. This construct was cloned into the pHSE3' vector (a gift from Dr. Steve Desiderio).

homozygous transgenics and negative littermate control lines were developed.

Analysis of tail biopsy DNA by PCR and Southern blot allowed us to determine which mice were positive for the TG5e transgene. TG5e “+/-” or “+/+” mice of both strains were determined through PCR amplification, yielding a 575bp DNA fragment, while negative littermate controls “-/-” yielded no band (Figure III.1B). Results were confirmed using southern blot analysis; TG5e mice yielded bands of approximately 4500bp that were absent in negative mice. Furthermore, using Southern blots, homozygous transgenics yielded bands of approximately twice the intensity of known heterozygotes (Figure III.1C), as determined using Phosphorimager analysis.

Expression of the TG5e transgene was examined at the RNA transcript level using both reverse transcriptase PCR (RT-PCR) and northern blot. Using RNA from the spleen, TG5e mice showed a PCR product of 413bp and a specific band on the northern blot; a band which represents both the transgenic and endogenous Pax-5e transcripts (Figure III.2A). The transgenic pHSE3' construct has previously been used for overexpression of T cell receptor and *blk* genes and has been shown to be expressed in B and/or T cell lineages (Malek et al., 1998). To determine which lymphocyte population was expressing TG5e, we purified B or T cell populations from spleen or thymus of each founder line. Total RNA was extracted from each of the two lymphoid cell fractions, and analyzed via RT-PCR. TG5e-7 offspring expressed TG5e in B cells only, lacking any bands in samples containing T cell cDNA, while TG5e-49 offspring expressed TG5e in both B and T cell populations (Figure III.2B). Negative littermate controls showed a very faint band representing endogenous Pax-5e transcripts; however, at a much lower intensity than the transgenics (Figure III.2B).

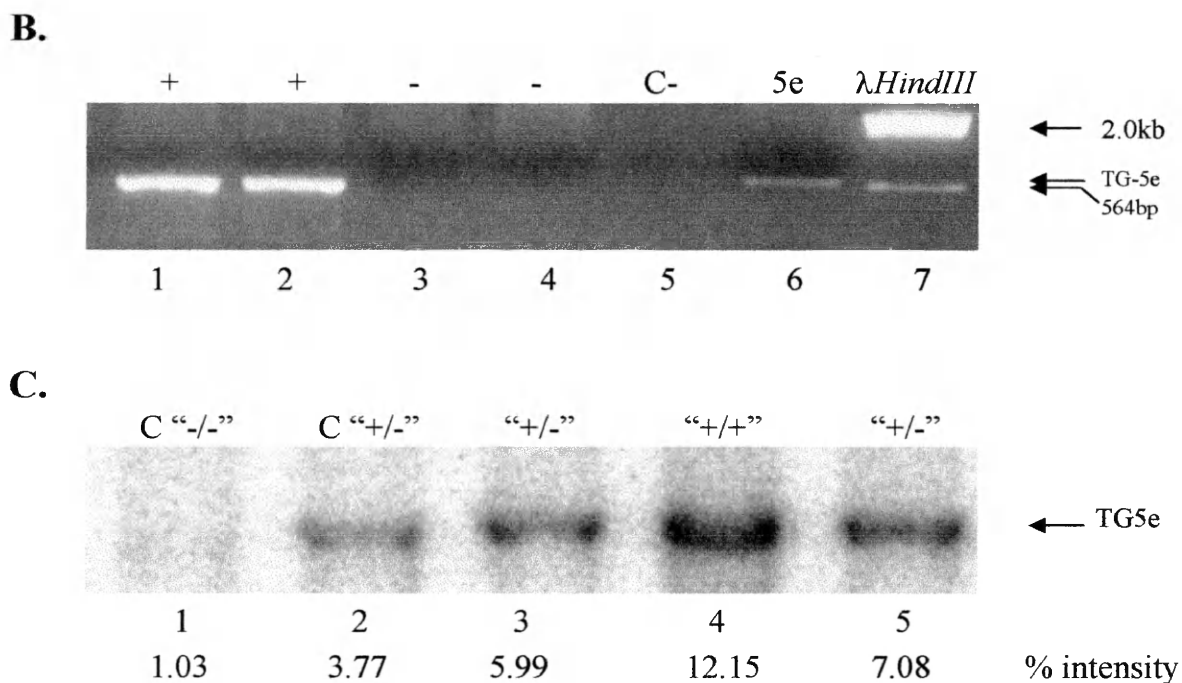


Figure III.1B and III.1C: Screening of founder TG5e founder progeny for genomic incorporation of the TG5e DNA construct. Tail biopsy DNA from TG5e offspring was analyzed for the presence of the TG5e construct. (B.) The samples were evaluated by PCR revealing a band of 575bp if the mouse was positive for the transgene (lanes 1 and 2) and no band if the mouse was negative (lanes 3 and 4). The negative control was in lane 5 while positive control of pcDNA.5e was used to indicate the size of the TG5e band in lane 6. Size marker λ HindIII was used in lane 7. (C.) Southern blot analysis was used to confirm PCR results, as well as to quantify the amount of TG5e present in the sample to determine if the mouse was homozygous, heterozygous or negative. Lane 1 is negative, lanes 2, 3 and 5 are heterozygous and lane 4 is homozygous for TG5e. The values for percent band intensity were based on background intensity and acquired from Phosphorimager analysis.

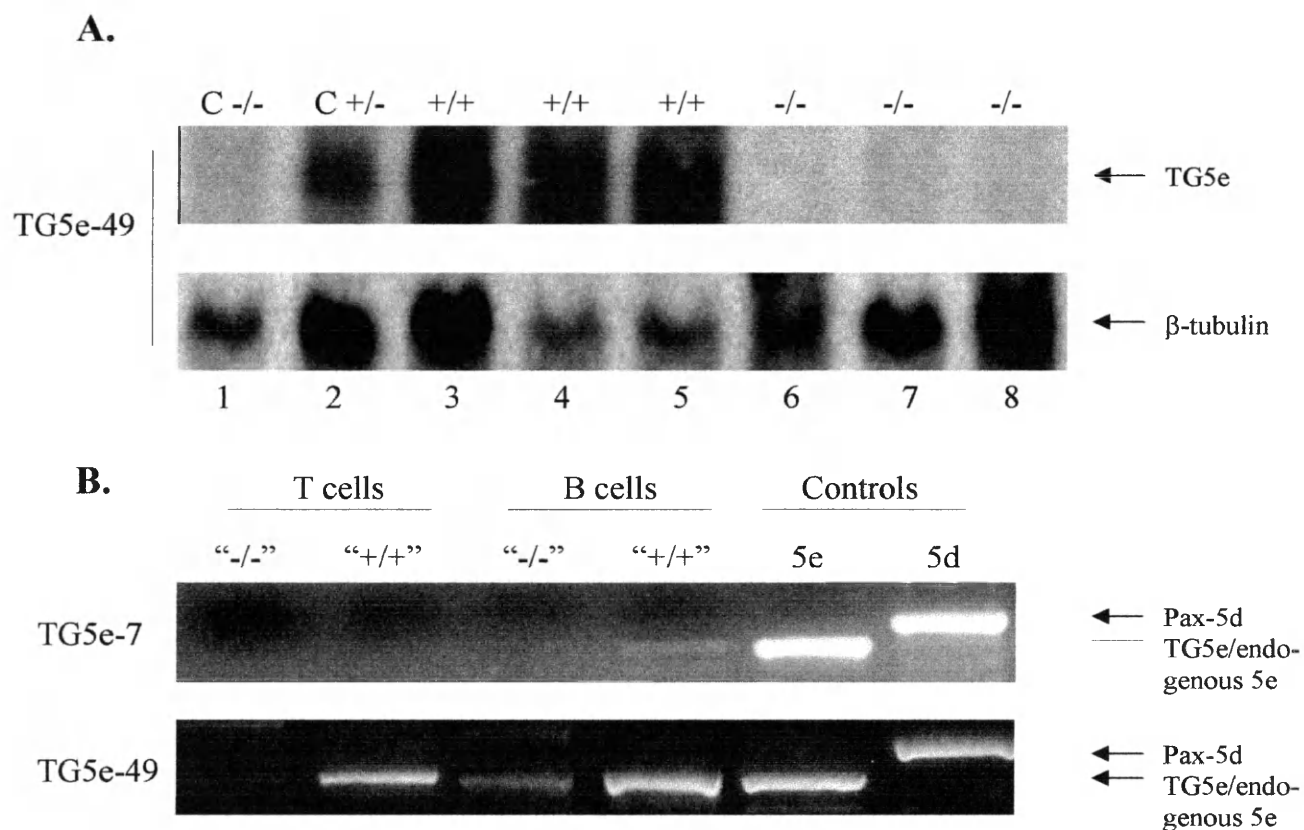


Figure III.2A and III.2B: Analysis of TG5e transcripts in TG5e-7 and -49 progeny. Total RNA was extracted from spleen or thymus tissue and analyzed via northern blot to verify expression of the TG5e transgene in positive mice in (A.) Spleen RNA from TG5e-49 and negative progeny (a representative experiment is shown). Lanes in the northern blot are as follows for both panels: lane 1 negative control, lane 2 heterozygous control, lanes 3-5 homozygous progeny, lanes 6-8 negative progeny. The lower panel represents a β -tubulin control indicating total RNA concentrations in each lane. (B). RT-PCR analysis of purified B and T cell populations from each strain (TG5e-7 upper panel, TG5e-49 lower panel). Lane 1 represents "-/-" T cell, lane 2 "+/+" T cell, lane 3 "-/-" B cell, lane 4 "+/+" B cell and lane 5 and 6 are controls samples pcDNA.5e and .5d, respectively.

Splenic nuclear extracts from both homozygous and negative littermate controls of TG5e-7 and -49 overexpression of Pax-5e protein was analyzed in western blot. Blots were probed with anti-Pax-5d/5e monoclonal antibody 6G11 (Anspach et al., 2001). Both transgenic and negative mice yielded a band of 19kDa, characteristic of the endogenous Pax-5e protein (Lowen et al., 2001). However, homozygous mice of TG5e-7 (Figure III.2C) and TG5e-49 (Figure III.2D) revealed bands of greater intensity indicating overexpression due to the transgene. Since RT-PCR had shown TG5e to be expressed in the T cells of strain TG5e-49, we also analyzed nuclear extracts from the thymus of that strain (Figure III.2E). Due to the presence of a 19kDa band on the western, we concluded that the Pax-5e protein is also overexpressed in the thymus of TG5e-49. Pax-5e has also been reported to run at 27kDa. This slower mobility band is hypothesized to be a heterodimer of Pax-5e with a thioredoxin-like molecule (Lowen et al., 2001). Due to variation between experiments, the 27kDa band shows no consistent pattern. Overexpression of Pax-5e was then confirmed in all blots by probing with a control anti-TFIID antibody, which was expressed equally in all samples.

Analysis of Lymphocyte Populations. To determine if overexpression of Pax-5e affected size and distribution of lymphoid populations, splenic or bone marrow cells were collected from 6 week old homozygous and negative littermate controls from each strain and analyzed via flow cytometry (Figure III.3A and 3B). Using markers PerCP-conjugated B220 (CD45R), PE-conjugated CD19 and FITC-conjugated IgM, as well as the progenitor marker PE-conjugated CD43, we identified specific B cell subpopulations within the spleen and bone marrow (Table III.1).

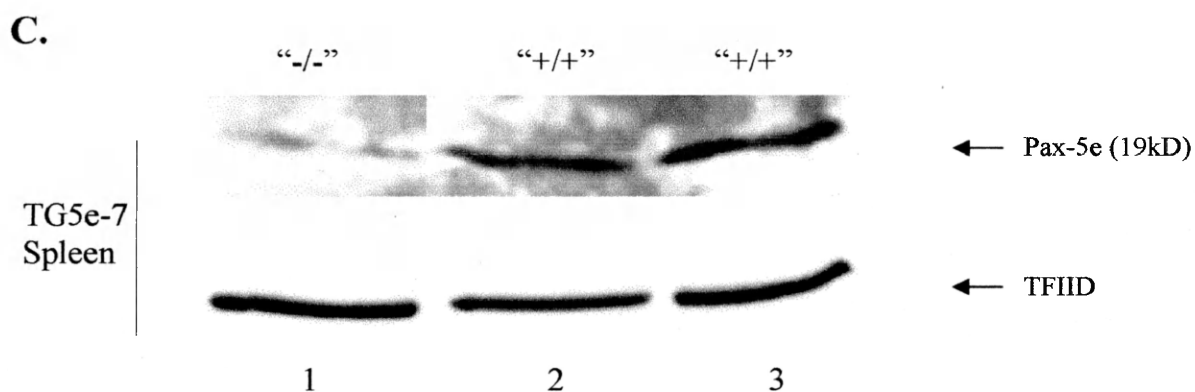


Figure III.2C: TG5e protein expression in TG5e-7. Splenic nuclear extracts were prepared for TG5e-7 with age matched littermate controls and separated by SDS-PAGE then transferred to a PVDF membrane for western blotting. The blot was probed with novel-sequence (Pax-5d/5e) specific 6G11 antibody. Overexpression of Pax-5e in TG5e-7 progeny (“+/+”) is shown. Anti-TFIID antibody was used to indicate the amount of total protein in each sample.

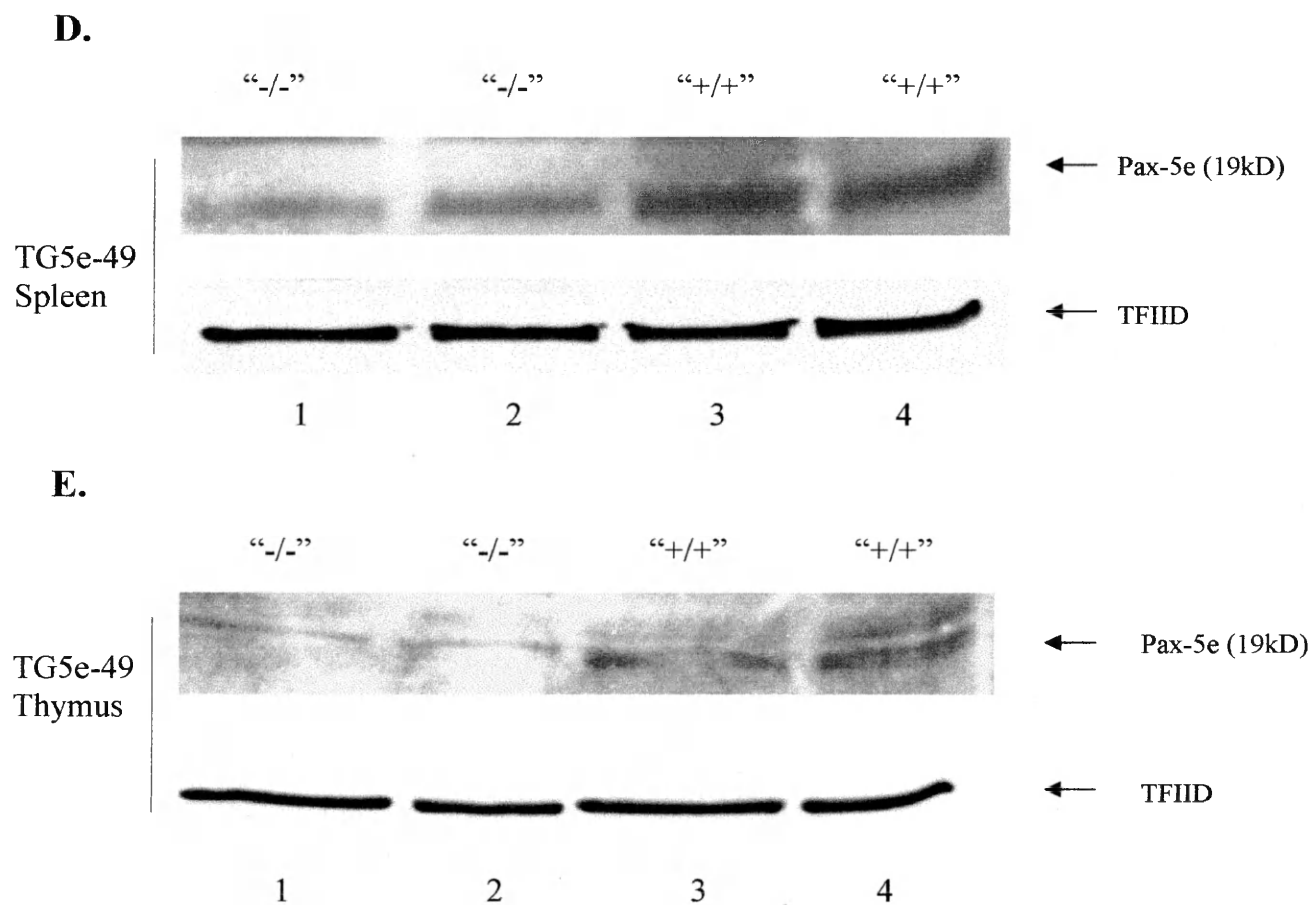


Figure III.2D and III.2E: TG5e protein expression in TG5e-49. Nuclear extracts were prepared for TG5e-49 with age matched littermate controls and separated by SDS-PAGE then transferred to a PVDF membrane for western blotting. The blot was probed with novel-sequence specific 6G11 antibody. (D.) Analysis of spleen nuclear extracts reveals overexpression of Pax-5e in TG5e-49, while (E.) shows the same overexpression in the thymus. Anti-TFIIID antibody was used in each experiment to determine the amount of total protein in each sample.

-----B cells----- ----T cells----

| Cell surface marker | Pro-B early Pre-B | Late Pre-B (Im)mature B | Plasma- blast | Plasma Cell | All T cells |
|---------------------|----------------------|----------------------------|------------------|----------------|----------------|
| B220 | + | + | +/- | - | - |
| CD43 | + | - | - | - | - |
| CD19 | -/+ | + | +/- | - | - |
| IgM | - | + | +/- | - | - |
| CD3 | - | - | - | - | + |

Table III.1: Flow cytometry markers. Shown above is a list of cell surface markers used to differentiate different lymphoid populations. (+) indicates the marker is present at that stage/cell type, (-) indicates the absence of the marker.

For the TG5e-7 progeny we acquired data from a total of 7 transgenic and 7 negative age-matched mice used over 3 independent experiments. TG5e-7 progeny exhibited no consistent changes in any lymphoid populations in the spleen or in the bone marrow as compared to negative littermate controls (Figure III.3A). In the first experiment we saw a decrease in B220+ cells in the transgenics; however, the second experiment revealed the opposite trend, and the third showed no change. This pattern was also seen in CD3+ cells, leading us to the following two conclusions: 1) there is considerable variation in lymphoid populations in TG5e-7 and 2) there are no significant changes in lymphocyte populations in TG5e-7 progeny.

Analysis of TG5e-49, with a total of 9 transgenic and 9 negative mice used over 3 independent experiments, revealed decreases in CD3+ T cells. First, TG5e-49 progeny showed a significant decrease in CD3+ T cells (CD3+B220-) in the bone marrow ($p=0.015<0.03$) (a representative experiment is shown in Figure III.3B). Negative littermate controls contained an average of 3.7% T cells in the bone marrow while TG5e-49 mice only showed an average of 2.2% T cells in the same tissue; a decrease of 41%. This significant decrease in CD3+ T cells was also apparent in the spleen ($p=0.017<0.03$). Splenic CD3+ T cells (CD3+B220-) comprised an average of 36% of non-transgenic mouse lymphoid populations; however, TG5e-49 mice showed only 30.2%; a decrease of 16.7%. This provides evidence that, when expressed, Pax-5e can affect the development, proliferation or apoptosis rate in T cells. No significant change was observed within any thymic cell population. In the bone marrow there was a slight decrease in pro-B cells (B220+CD43+); however, these data were not significant as only 5 out of 9 TG5e-49 progeny exhibited this phenotype ($p=0.36>0.03$).

A.

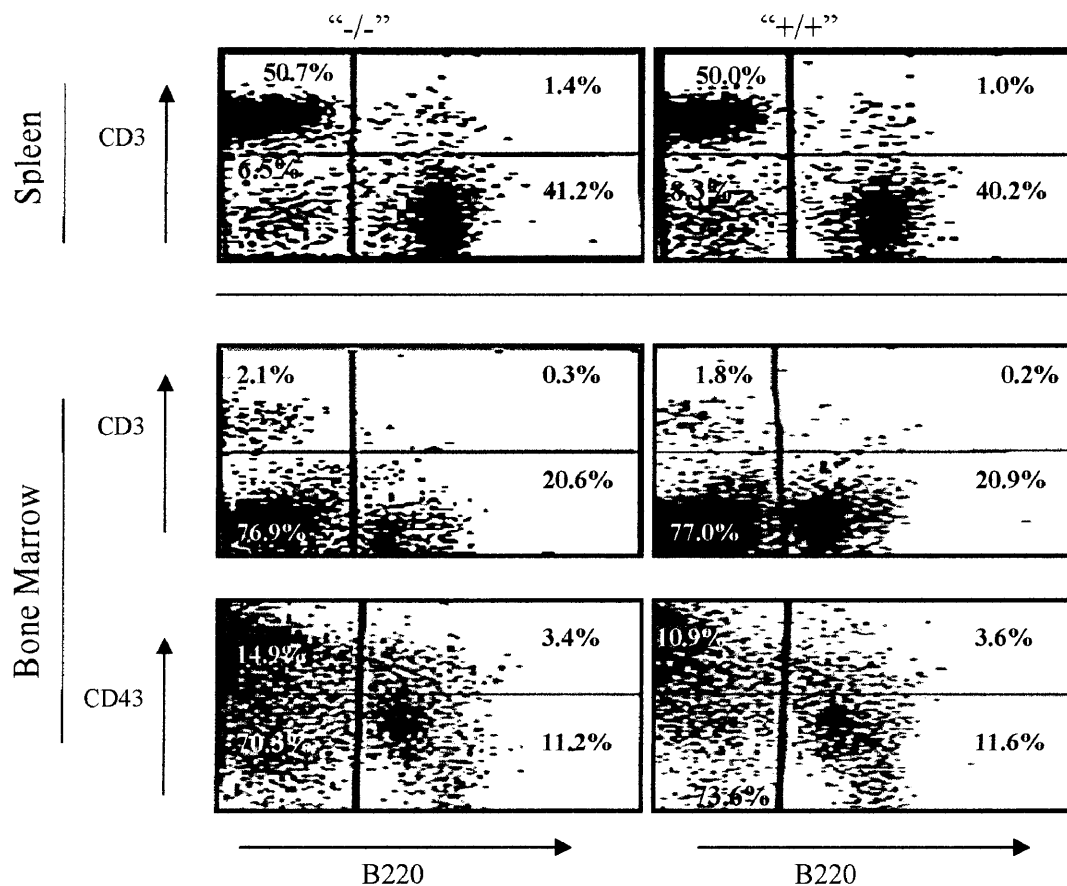


Figure III.3A: Analysis of lymphoid populations for TG5e-7 progeny using flow cytometry. Spleen and bone marrow of 6 week old transgenics and negative littermate controls were collected and analyzed via double staining with the combinations illustrated above. TG5e-7: top panels include PE-CD3 (T cells) vs. PerCP-B220 (B cells) for the spleens of both negative and transgenic mice. The lower panel contains representative samples from the bone marrow comparing PerCP-B220 (x-axis) against PE-CD3 (middle) or PE-CD43 (progenitors, bottom). Percent gated cells are labeled in each quadrant.

B.

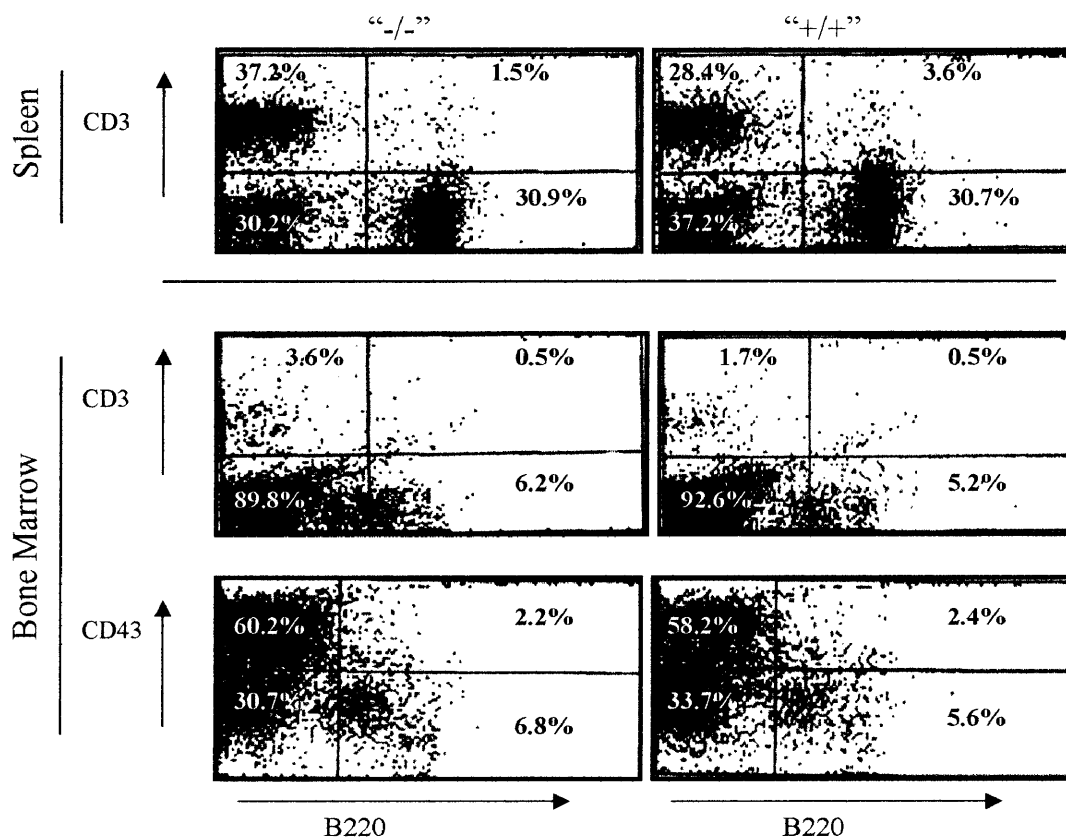


Figure III.3B: Analysis of lymphoid populations for TG5e-49 progeny using flow cytometry. Spleen and bone marrow of 6 week old transgenics and negative littermate controls were collected and analyzed via double staining with the combinations illustrated above. TG5e-49: top panels include PE-CD3 (T cells) vs. PerCP-B220 (B cells) for the spleens of both negative and transgenic mice. The lower panel contains representative samples from the bone marrow comparing PerCP-B220 (x-axis) against PE-CD3 (middle) or PE-CD43 (progenitor cells, bottom). Percent gated cells are shown for each quadrant.

In summary, TG5e-7 progeny showed no significant changes in any lymphoid populations in non-immunized mice. On the other hand, TG5e-49 progeny showed decreases in CD3⁺ T cell in both the spleen and bone marrow but not in the thymus. Frequencies of B cell populations in either strain were unaffected by the transgene.

Effects of TG5e on cell proliferation in culture. As indicated by previous research, overexpression of Pax-5a leads to increased B cell proliferation (Max et al., 1995). Our lab had also demonstrated that: 1) isoform Pax-5e can enhance the activity of Pax-5a in mature B cell lines (Lowen et al., 2001), and 2) ectopically expressed Pax-5e can increase cell growth in B cell lines (Beth Jones, unpublished data). Therefore, we decided to examine the effects of B (and T) lymphocyte proliferation in the TG5e mice as compared to negative littermate controls. Cultured bone marrow and resting B and T lymphocytes (70% Percoll separated) from both strains were grown in the presence or absence of lipopolysaccharide (LPS). This mitogen stimulates B cell activation and proliferation as well as differentiation into plasma cells. Proliferation was measured through BrdU incorporation and was analyzed via a colorimetric proliferation ELISA (Roche).

Freshly isolated, unstimulated bone marrow cells of TG5e-7 progeny exhibited a 25% increase in proliferation on day 0, as compared to negative littermate controls ($p=0.0001<0.03$) (Figure III.4A). We also recorded proliferation on day 2 and 3 for unstimulated bone marrow cells. Interestingly, no difference was observed between transgenic and negative mice on day 2 ($p=0.13>0.03$), but a 43% decrease in proliferation in TG5e-7 cells was detected on day 3 ($p=0.0008<0.03$) (Figure III.4A). Unstimulated,

resting mature splenocytes (“70%”) showed no significant change in proliferation state on day 0 ($p=0.09>0.03$) (data not shown), although significant variation was observed.

LPS-activated bone marrow cells of TG5e-7 progeny showed no significant change in proliferation on day 3, as compared to negative littermate controls ($p=0.39>0.03$). Also on day 3, LPS-activated TG5e-7 splenic cells revealed no significant change in proliferation ($p=0.17>0.03$) (Figure III.4B).

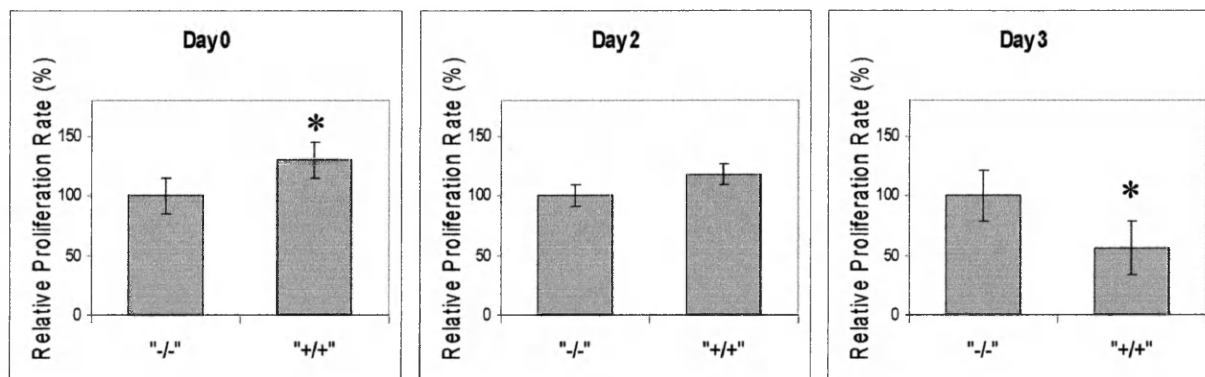
In summary, TG5e-7 bone marrow cells exhibit an increase in proliferation without LPS stimulation on day 0, and then show a significant decrease in proliferation after 3 days. On day 0, neither stimulated bone marrow cells nor unstimulated spleen cells displayed a significant change in proliferation as compared to negative littermate controls. Finally, stimulated spleen cells revealed no change in proliferation rate after LPS induction on day 3.

TG5e-49 progeny exhibited no significant change in proliferation in unstimulated bone marrow cells on day 0 ($p=0.5>0.03$) (Figure III.4C). However, on day 3, unstimulated bone marrow cells demonstrated a 19.5% increase in proliferation ($p=0.01<0.03$) (Figure III.4C). Unstimulated splenic cells displayed no significant change in proliferation as compared to negative littermate controls on day 0 ($p=0.45>0.03$) (data not shown). Stimulation of bone marrow cells with LPS for 3 days revealed a 21% increase in proliferation rate for the TG5e-49 mice compared to negative controls ($p=0.019<0.03$) (Figure III.4D). LPS-activated TG5e-49 splenic cells showed no significant change in proliferation ($p=0.04>0.03$) (Figure III.4D).

In summary, the presence of the transgene in TG5e-49 results in a significant increase in proliferation for both stimulated and unstimulated bone marrow cells on day 3.

A.

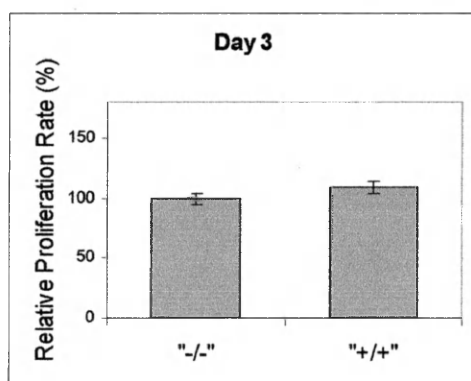
TG5e-7 Unstimulated Bone Marrow Cell Proliferation



B.

TG5e-7 LPS-Activated

Bone Marrow Cell Proliferation



Spleen Cell Proliferation

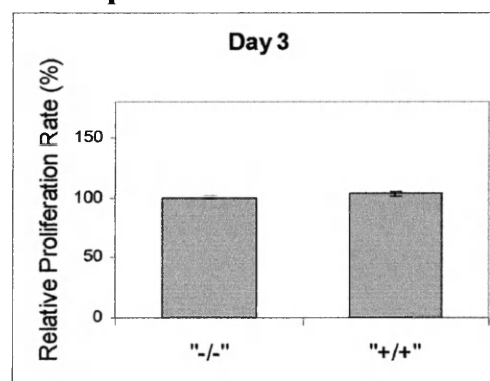
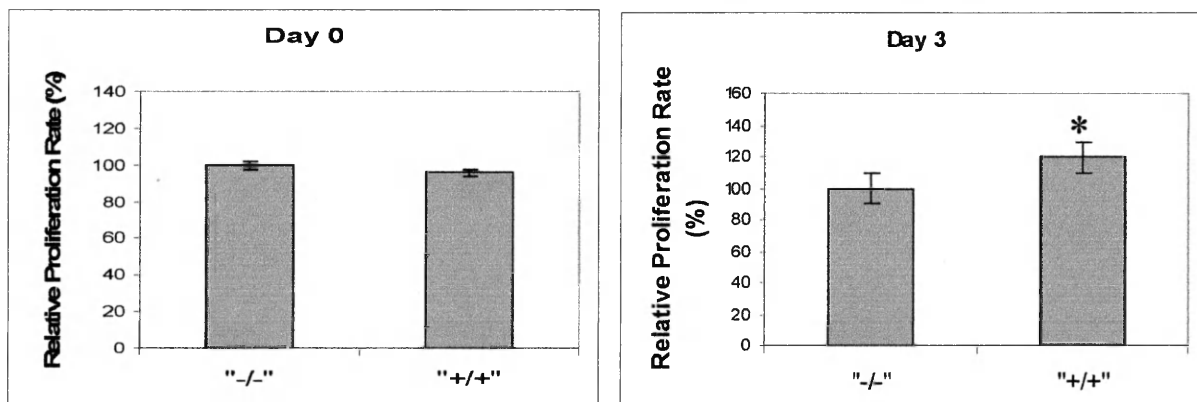


Figure III.4A and III.4B: Effect of TG5e on bone marrow and spleen cell proliferation in TG5e-7. Unstimulated bone marrow cells were diluted to 10^5 cells/well and incubated with BrdU for 18 hours before colorimetric analysis of BrdU incorporation with experiments spanning (A.) day 0, 2 and 3 for unstimulated bone marrow cells and (B) 3 days after LPS-activation of bone marrow or spleen cells. Measurements were read by a Microplate Reader (Bio-Rad). Relative proliferation rate of the negative littermate controls were set at 100% and the transgenics were indicated as a percent change in proliferation, N=9 over 3 independent experiments (* represents p value < 0.03).

C.

TG5e-49 Unstimulated Bone Marrow Cell Proliferation



D.

TG5e-49 LPS-Activated Bone Marrow Cell Proliferation and Spleen Cell Proliferation

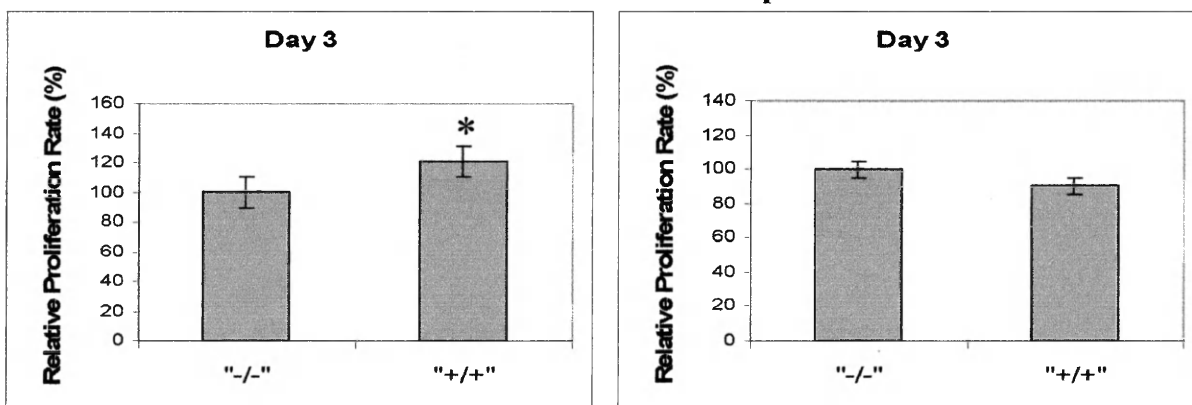


Figure III.4C and III.4D: Effect of TG5e on bone marrow and spleen cell proliferation in TG5e-49. Unstimulated cells were diluted to 10^5 cells/well and incubated with BrdU for 18 hours before colorimetric analysis of BrdU incorporation with experiments spanning (C.) day 0 and day 3 for unstimulated bone marrow cells and (B) 3 days after LPS-activation of bone marrow or spleen cells. As in previous figure, the negative controls represent 100%, N=9 over 3 independent experiments (* represents p value < 0.03).

However, no change in proliferation was seen in either unstimulated spleen or bone marrow cells on day 0, nor was a change in proliferation evident in LPS-activated spleen cells on day 3.

Changes in Ig secretion in immunized TG5e mice. TG5e offspring from both strains and age-matched littermate controls were immunized with TNP-conjugated Bovine Serum Albumin (BSA) in order to examine the effects of the TG5e-transgene on the immune response. Supernatants from splenic, bone marrow and thymic cells were analyzed for both TNP-specific and total IgG and IgM levels by ELISA. Additionally, live cells were cultured on immunoblot membranes coated with anti-IgM or -IgG antibodies to determine the number of Ig-secreting cells by the ELISPOT technique.

After 24 hours in culture, supernatants from 10^6 spleen or bone marrow cells were collected and diluted 1:4 and 1:16 for analysis by ELISA. Standards ranging from 1000ng-0.1ng of IgG (or IgM) were also plated in order to generate a standard curve and to evaluate actual Ig concentrations of each sample. TG5e-7 progeny showed a significant increase in total IgM and IgG levels in the spleen (Figure III.5A). The transgenic mice produced an average of 7800ng IgG/ 10^6 cells, an increase of 43.6% over the negative littermate control average of 4400ng IgG/ 10^6 cells ($p=0.01<0.03$). Total IgM for TG5e-7 also showed a significant increase over the negative mice. TG5e-7 mice generated almost 2000ng IgM/ 10^6 cells versus only 1500ng IgM/ 10^6 cells from the negative mice, a difference of 25% ($p=0.01<0.03$). Total IgM produced in the bone marrow showed no significant change ($p=0.09>0.03$) (Figure III.5A). Total IgG originating in the bone marrow showed no change compared to negative littermate

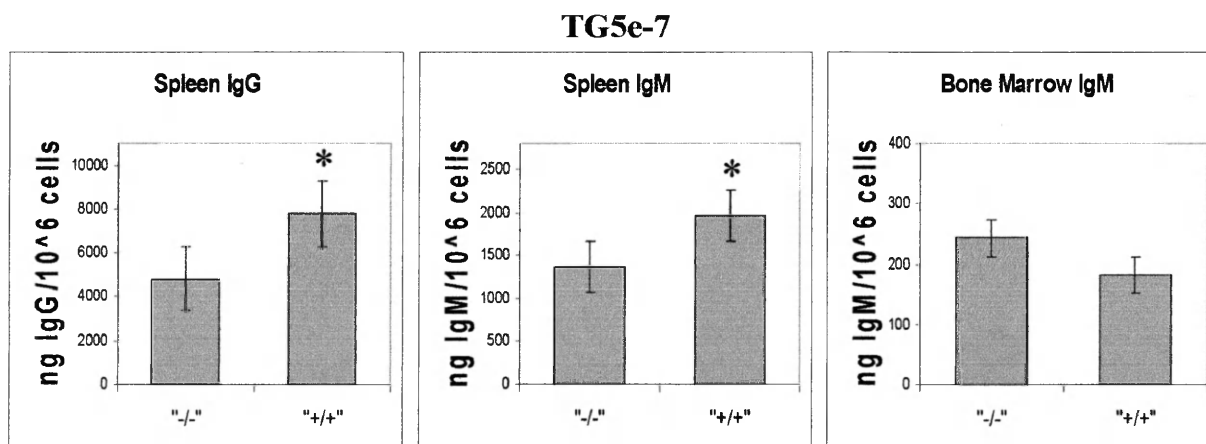
controls (data not shown). IgG antibodies specific to the hapten carrier TNP were also analyzed for both tissues; however, values were too low to be considered reliable.

Samples from TG5e-49 were analyzed under conditions identical to those of TG5e-7, but were also evaluated for antibody production from thymic B cells. TG5e-49 progeny revealed a 46.8% decrease in total splenic IgG ($p=0.004<0.03$); decreasing from 1400ng IgG/ 10^6 cells to 750ng IgG/ 10^6 cells (Figure III.5B). Conversely, no change was found in splenic IgM ($p=0.5>0.03$); however, in the bone marrow TG5e-49 mice displayed a 63.4% decrease in total IgM; a drop from 142 to 52ng IgM/ 10^6 cells ($p=0.004<0.03$) (Figure III.5B). Furthermore, no changes were observed in bone marrow IgG and, as with TG5e-7, the level of TNP-specific IgG in the bone marrow or spleen was too low for measurement (data not shown). Concentrations of both IgM and IgG found in the thymus were also measured; however, the transgenics showed no significant change over negative control mice (data not shown).

To determine whether changes observed in secreted antibody levels were due to altered plasmablast/plasma cell populations, we performed ELISPOT. Using a dot-blot apparatus containing membranes coated with IgM, IgG or TNP-KLH, dilutions of 10^6 or 10^5 cells were plated and allowed to secrete Ig on the membranes for 4 hours. After washing, the IgM membranes were probed with goat anti-mouse IgM-HRP while the TNP-KLH and IgG membranes were probed with goat anti-mouse IgG-HRP. The membranes were then shipped to Cellular Technologies Ltd. where the number and size of spots in each well were analyzed by computer-based software.

TG5e-7 progeny show significant increases of 2 and 3 fold for spleen ($p=0.006$) and bone marrow ($p=0.004<0.03$) for total IgG-secreting cells, respectively (Figure

A.



B.

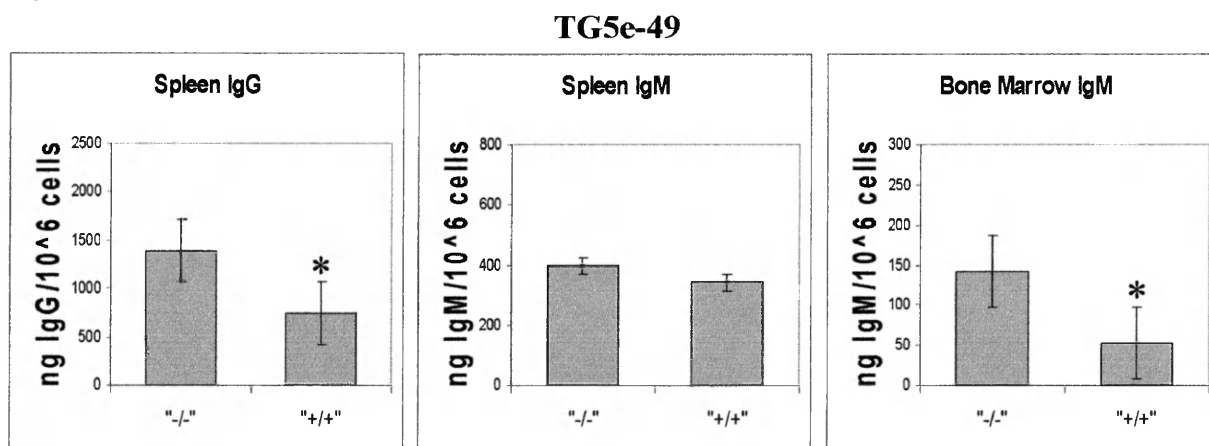


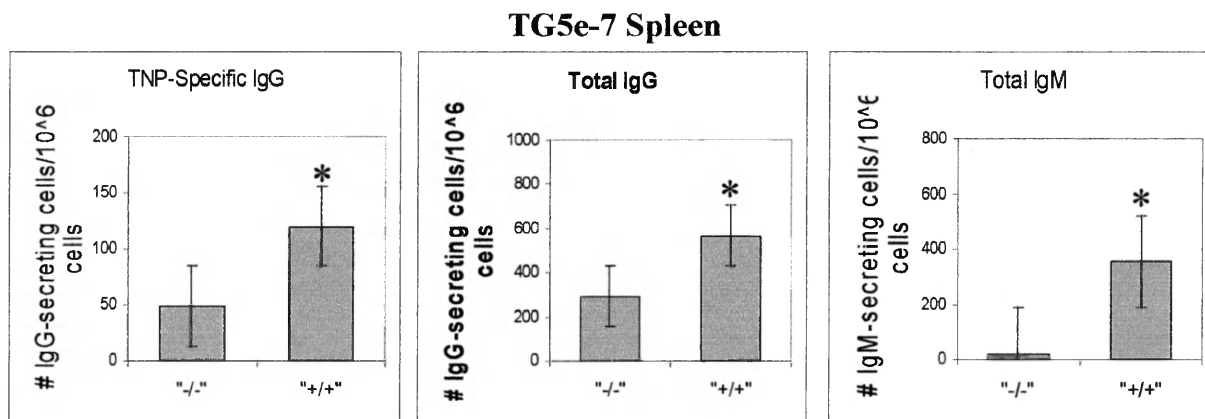
Figure III.5A and III.5B: The effects of TG5e on Ig-secretion in the spleen and bone marrow. Spleen and bone marrow cells of A) TG5e-7 and B) TG5e-49 and negative mice were collected and grown in culture for 24hrs. Supernatants were analyzed by ELISA against a set of standards of known Ig concentration for the presence of total IgM or IgG. Spleen IgM and IgG and bone marrow IgM levels are shown. N=7 over 2 independent experiments (* represents $p < 0.03$).

III.6A & B). Transgenics also show a significant 3-fold increase in the number of TNP-specific IgG-producing cells in the bone marrow ($p=0.016<0.03$); however, no significant trend was seen in the spleen ($p=0.04>0.03$) as there was too much variation within counts from both dilutions (data not shown). Membranes analyzed for changes for IgM-secreting cells also showed a significant increase in cell numbers in the spleen as compared to negative control mice ($p=0.000002<0.03$) (Figure III.6A). No significant changes were observed in the bone marrow ($p=0.08>0.03$) (data not shown). We conclude that TG5e plays a role in increasing the number of Ig-secreting cells in the bone marrow and spleen from immunized transgenic mice.

Similar trends were seen in TG5e-49 progeny although none of the changes were significant for total number of IgG-secreting cells in the spleen ($p=0.7>0.03$) or bone marrow ($p=0.3>0.03$) (Figure III.6C and III.6D). There were also no changes in the number of TNP-specific IgG-secreting cells in the bone marrow ($p=0.6>0.03$), while the number of TNP-specific IgG-secreting cells in the spleen increased significantly by 5-fold over the negative control mice ($p=0.015<0.03$). Due to limited resources, we were unable to analyze changes in IgM-secreting cells for this strain.

Changes in lymphoid populations in transgenic mice after immunization using flow cytometry. Following immunization of transgenics from both strains and negative littermate controls, 2×10^6 cells were stained with the same fluorescent antibodies used for non-immunized mice then fixed in 2% paraformaldehyde until needed. Cells were then thawed at 37°C and analyzed by flow cytometry. We analyzed spleen and bone marrow for TG5e-7 and TG5e-49, as well as thymus for TG5e-49.

A.



B.

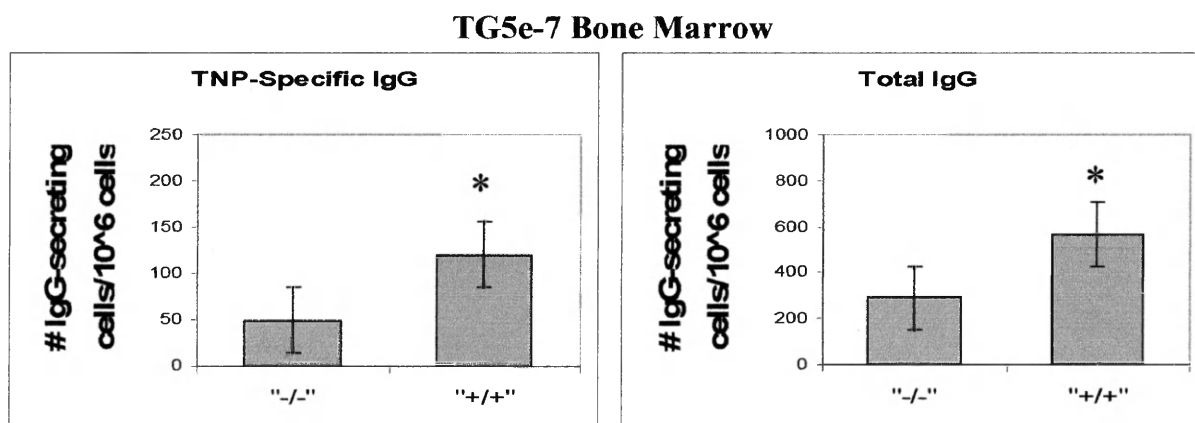
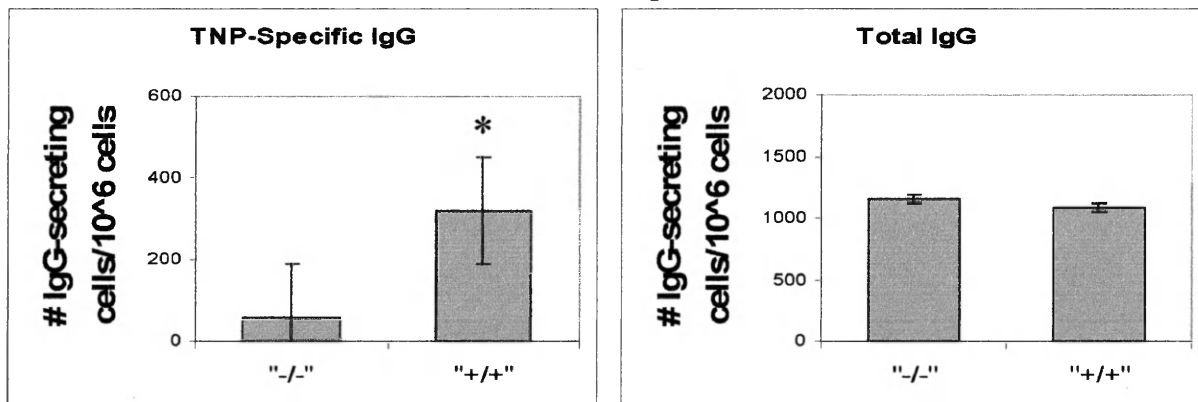


Figure III.6A and III.6B: Effects of TG5e on the number of IgG-secreting cells in TG5e-7 as determined by ELISPOT. Bone marrow and spleen cells from TG5e-7 were cultured on membranes coated with TNP-KLH or IgG on a dot-blot apparatus (Bio-Rad) separately in dilutions of 10^6 cells/well. Membranes were probed with goat anti-mouse IgG-HRP and sent to Cellular Technologies Ltd. for computer-based counting of spots. These data allowed for more accurate determination of total number of IgG-secreting cells or TNP-specific IgG-secreting cells present in the tissue, N=8 (4 mice done in duplicate) (* represents p value < 0.03 , all others $p > 0.03$).

C.

TG5e-49 Spleen



D.

TG5e-49 Bone Marrow

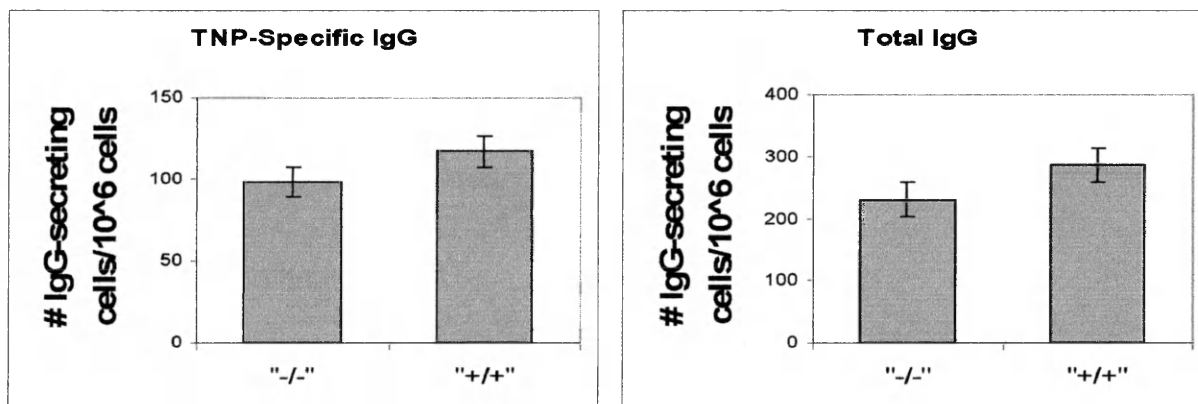


Figure III.6C and III.6D: Effects of TG5e on the number of IgG-secreting cells in TG5e-49 as determined by ELISPOT. Bone marrow and spleen cells from TG5e-49 were cultured and probed as described in Figures III.6A and III.6B, N=6 (3 mice done in duplicate) (* represents p value < 0.03 , all others $p > 0.03$).

After acquiring 70,000 events (or number of cells), all TG5e-7 progeny revealed significant increases in pre-B and (im)mature B cells (B220+CD19+) ($p=0.007<0.03$) and in (im)mature B cells (B220+IgM+) ($N=8$, $p=0.002<0.03$) in the bone marrow (Figure III.7A). The total number of B220+CD19+ cells in the transgenics increased an average of 18.7% over a total of 4 negative littermate controls and similarly, a 21% increase was seen in B220+IgM+ cells in the transgenics. This suggests that TG5e has some effect on immature and mature B cell stages in the bone marrow, and maybe pre-B cells as well, possibly causing excess proliferation during an immune response. No effects were seen in the T cell populations or progenitor cell populations in the bone marrow. Unfortunately, the fixation process adversely affected the condition of the spleen cell samples and thus we were unable to obtain reliable counts.

TG5e-49 displayed a strikingly different phenotype after immunization as compared to pre-immunization. There was a significant decrease in B220+CD3- cells (B cells) in the spleen ($p=0.027<0.03$) by 33.1% over the negative control mice (Figure III.7B). There was also a 34.8% decrease in CD3+ T cells in the spleen ($p=0.006<0.03$) (Figure III.7B). Though there was a slight decrease in CD3+ T cells in the thymus, this was not significant ($p=0.05>0.03$) (data not shown). Within the splenic B cell populations we found a significant decrease of 21.3% in IgM+CD19+ B cells ($p=0.007<0.03$). This decrease suggests that during an immune response, overexpression Pax-5e leads to increased (im)mature B cell death. The bone marrow of TG5e-49 displayed a 46.5% increase in progenitor B cells (B220+CD43+) (Figure III.7B). During an immune response, the bone marrow increases immune cell production so proliferation is to be expected. Regarding the TG5e-49 mice, it is possible that overexpression of Pax-

A.

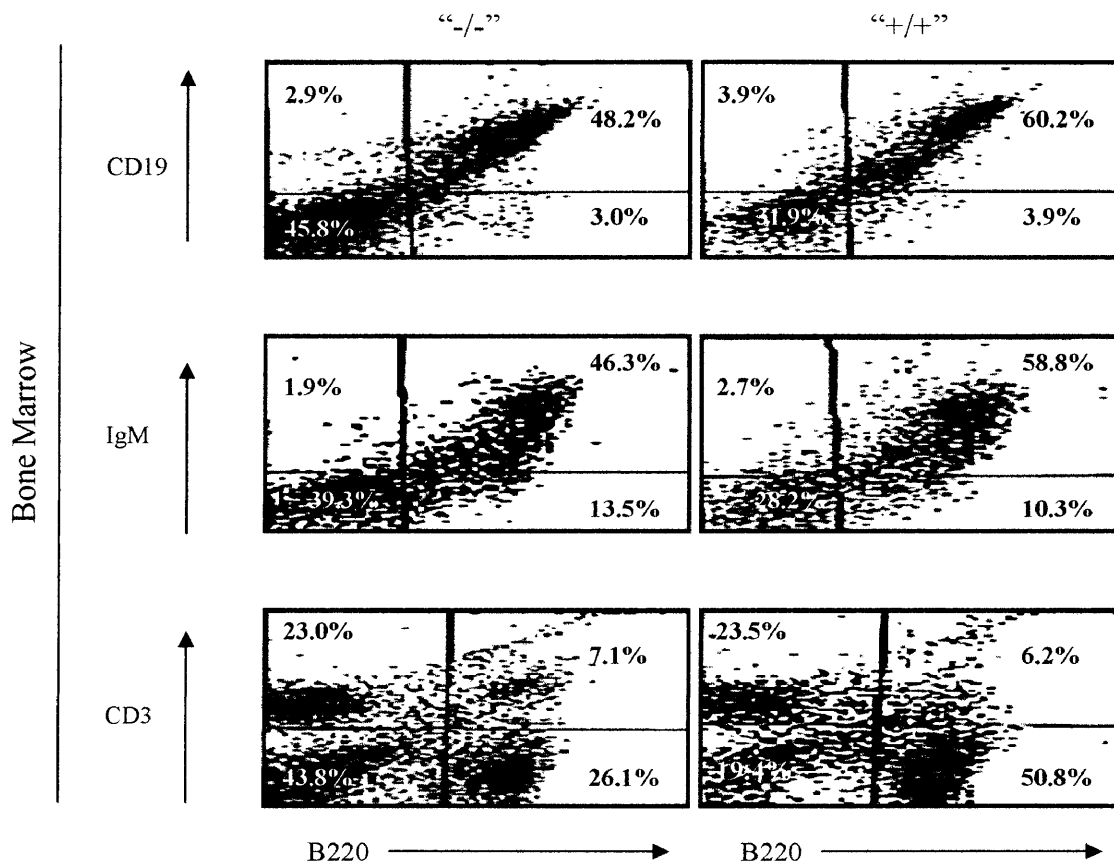


Figure III.7A: Changes in lymphoid populations in TG5e-7 after immunization.

Analysis of bone marrow from 10 week old immunized transgenics and negative littermate controls after double staining with PE-CD19/PerCP-B220 (top panel) or FITC-IgM/PerCP-B220 (middle panel) or PE-CD3/ PerCP-B220 (bottom panel) are shown above. Percent gated cells are shown for each quadrant.

B.

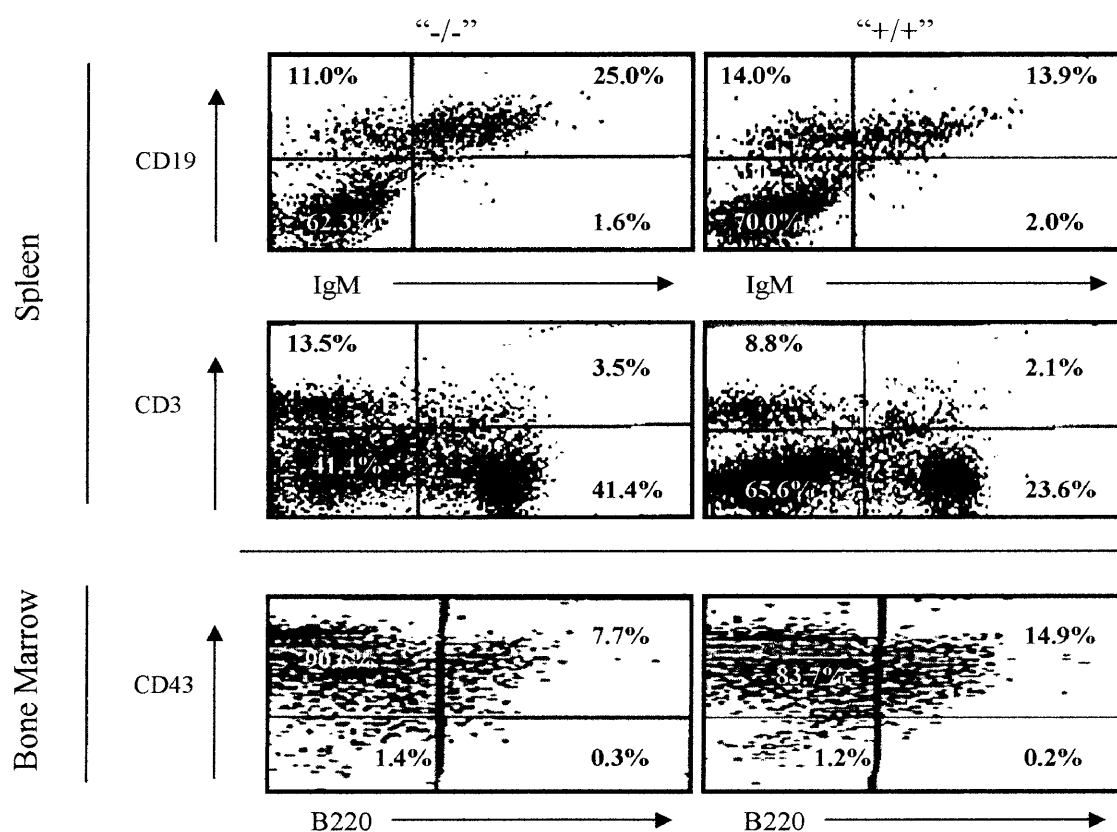


Figure III.7B: Changes in lymphoid populations in TG5e-49 after immunization. Analysis of bone marrow and spleen tissues from 10 week old immunized transgenics and negative littermate controls after double staining with FITC-IgM/PE-CD19 (mature/immature B cells, top panel) or PerCP-B220/PE-CD3 (B/T cells respectively, middle panel) or PE-CD43 (progenitor cells, bottom panel) are shown above. Percent gated cells are shown for each quadrant (one experiment, N=6).

5e is leading to increased cell proliferation in early B and T cell stages by enhancing the effect of Pax-5a on target proliferation genes (e.g. *blk*).

Analysis of Pax-5a target gene expression. Since our previous data support the hypothesis that Pax-5e enhances Pax-5a activity (Lowen et al., 2001; Beth Jones, unpublished data), we wished to test if Pax-5a target genes were affected by the overexpression of Pax-5e. Using quantitative “real-time” PCR (qRT-PCR) we analyzed the transgenic mice of both strains for any change in the expression of Pax-5a target genes CD19 and immunoglobulin joining chain (J chain) in the spleen. Target gene expression was investigated in both immunized and non-immunized transgenics and negative littermate controls.

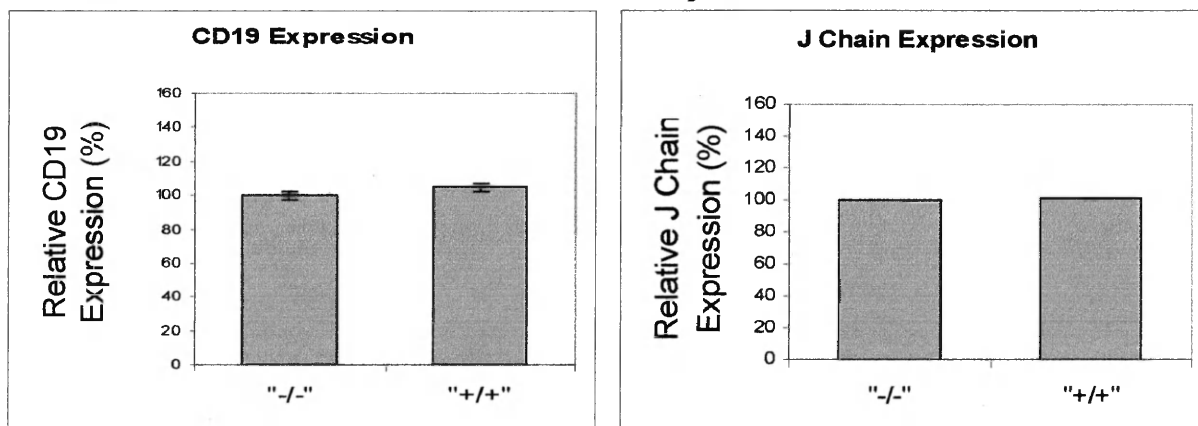
Analysis of 3 age-matched transgenics and 3 negative littermate controls from non-immunized TG5e-7 displayed no significant changes in CD19 ($p=0.2>0.03$) or J chain ($p=0.3>0.03$) expression (data not shown). Similarly with TG5e-49, non-immunized transgenic mice showed no consistent changes in expression of either CD19 ($p=0.9>0.03$) or J chain ($0.7>0.03$) (data not shown). For each strain, 3 independent experiments were run. The data were combined and shown as the negative mice representing 100%, while the transgenics represent a percent change from their negative littermate controls. Based on these data, we conclude that TG5e does not affect the expression of J chain or CD19 in non-immunized transgenic mice.

RNA samples from the spleens of immunized TG5e-7 and 49 progeny were also collected and processed via qRT-PCR. Immunized TG5e-7 progeny revealed no significant change in CD19 ($p=0.2>0.03$) or J chain ($p=0.7>0.03$) expression (Figure

III.8A). Whereas, a significant increase in CD19 expression in TG5e-49 ($p=0.022<0.03$) was apparent. This increase in expression was 20% over the negative control mice. No change was observed in the expression of J chain of the same mice ($p=0.65>0.03$) (Figure III.8B). These results suggest that immunization of TG5e-49 mice has no effect on the expression of J chain but leads to the increased expression of CD19. At the same time, immunized TG5e-7 mice show no significant change from the unimmunized state.

A.

TG5e-7 Immunized Spleen RNA



B.

TG5e-49 Immunized Spleen RNA

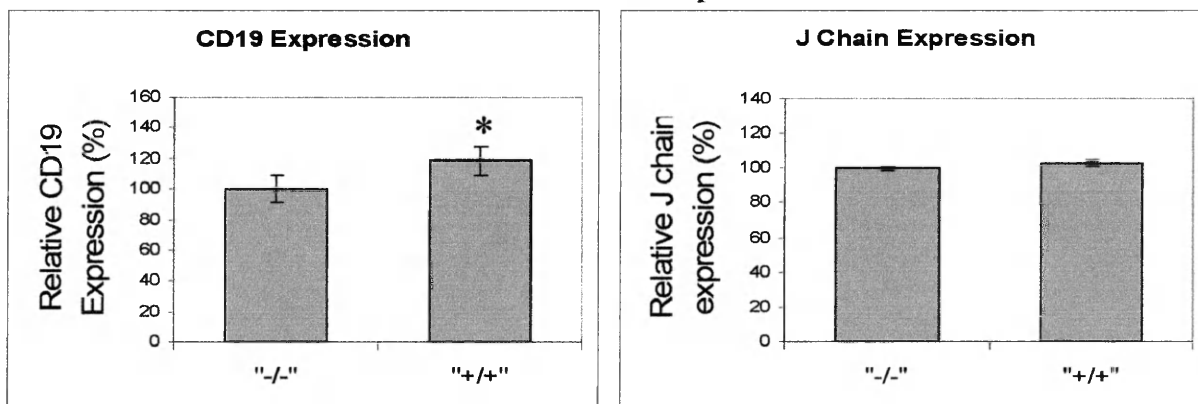


Figure III.8A and III.8B: Changes in Pax-5a target genes CD19 and J chain in immunized transgenic mice. Total RNA was extracted from the spleens of immunized A). TG5e-7 and B). TG5e-9 offspring and negative littermate controls and cDNA was analyzed via quantitative “real time” PCR for changes in CD19 and J chain expression. β -Tubulin was used as an internal control. The values were calculated by normalizing the “-/-” to 100% based on the inverse ratio of the threshold value (Ct) of the target gene to the Ct of the control gene. Values for 3 independent experiments, N=9, * represents $p < 0.03$.

CHAPTER IV

Discussion

The transcription factor Pax-5a is a master regulator of B cell development and proliferation. In this thesis research, we generated transgenic mice overexpressing alternative isoform Pax-5e and acquired two founder mice: TG5e-7 and TG5e-49. RT-PCR revealed that TG5e-7 mice expressed the transgene in only the B cells, while TG5e-49 expressed in both B and T cells. We found an increase in cell proliferation in the bone marrow of both strains. After immunization, TG5e-7 mice showed an increase in the number of antibody-secreting cells as well as an increase in the total amount of secreted IgG and IgM. ELISPOT analysis of TG5e-49 showed no change in the numbers of antibody-secreting cells, but a shift towards smaller ELISPOTs, indicating more plasmablasts and less plasma cells. These mice also showed a decrease in IgG levels. Flow cytometry of immunized mice revealed increases in pre, immature and mature B cells in TG5e-7 in the bone marrow whereas TG5e-49 showed decreases in the same B cell stages as well as decreases in T cell populations. Finally, “real time” PCR indicated overexpression of *CD19* in TG5e-49 mice but not in TG5e-7 mice.

Based on this data, the two transgenic lines display distinct phenotypes although overlapping for bone marrow proliferation. Several conclusions can be drawn from the data collected from the transgenic mice relating to B cell proliferation and changes in Pax-5a target gene expression. This murine model allows us to further investigate the role of Pax-5e.

TG5e affects lymphocyte proliferation

TG5e-7 bone marrow cells undergo increased proliferation. Previous results in our lab have shown that overexpression of Pax-5e *in vitro* leads to increased cell proliferation (Lowen et al., 2001). Based on these data, we hypothesized that increased levels of Pax-5e protein *in vivo* would also lead to increased cell proliferation in both the spleen and bone marrow. By utilizing a proliferation ELISA, we were able to determine the proliferation rates of spleen and bone marrow cells from TG5e-7. Unstimulated, fresh bone marrow cells from TG5e-7 mice showed a significant increase in proliferation on day 0 compared to control mice. This supports our hypothesis that overexpression of Pax-5e will increase cell proliferation. This increase in proliferation was not seen on day 2. Then, on day 3, these cells displayed a significant decrease in proliferation as compared to negative littermate controls. This decrease in proliferation was not predicted by our hypothesis, but is discussed in more detail in the next section.

We know that TG5e-7 expresses the transgene in the B cells; however, we do not know in which B cell population this increase in proliferation is occurring. The H2K promoter/Immunoglobulin HC enhancer combination in the TG5e construct is known to be active at early stages of lymphocyte development (Pircher et al., 1989), including the pro- and/or pre-B cell stages. Normally, during early stages of lymphocyte development in the bone marrow, hematopoietic stem cells and lymphoid progenitors undergo a form of regulated proliferation in order to expand pro- and pre-B cell populations. It is possible that overexpression of Pax-5e in B cells during normal proliferation events leads to increased proliferation rates. Normal proliferation of B cells occurs after checkpoints which ensure the IgH and IgL genes are properly rearranged (Hardy et al., 1991). Pro-

and pre-B cells that have successfully rearranged their IgH gene and express a complete pre-BCR (or after IgL rearrangement, a BCR), will continue to proliferate and carry on to the next stage of B cell development. If Pax-5e enhances the activity of Pax-5a, which regulates these stage-specific proliferation events, this may explain why there is increased proliferation in the bone marrow of the transgenic mice.

Pax-5a is involved in activating the expression of the pre-BCR of the pre-B cell, thus overexpression of Pax-5e may lead to increased production of the pre-BCR components. Pax-5a binding sites are found on pre-BCR components VpreB and $\lambda 5$ of the surrogate light chain (Tian et al., 1997). Pax-5a is also known to regulate the expression of κ sterile transcripts during the rearrangement of the Igk gene (part of the IgL chain) and in the absence of sterile transcript production, the Igk gene will not rearrange (Sato et al., 2004). Thus, Pax-5a is essential for producing a properly formed pre-BCR. Furthermore, studies utilizing transgenic mice that express Pax-5a in thymocytes have shown that Pax-5a is sufficient to induce V-DJ rearrangement of the IgH locus in pre-T cells (CD4⁺CD8⁺) (Hsu et al., 2004). With this in mind, overexpression of Pax-5e may not only enhance the activity of Pax-5a resulting in overexpression of proliferation genes like *blk*, but also increase the rate of IgH locus rearrangement as well as increased sterile κ transcript production or VpreB/ $\lambda 5$ expression, boosting pre-BCR assembly. This could lead to increased proliferation through a more rapid production of pre-BCRs, allowing cells to pass checkpoints more quickly and thus reaching the next stage of development sooner. If cells mature quickly, this may contribute to the increased pre/immature/mature B cell numbers seen in immunized

TG5e-7. Early stage B cells proliferate and mature more rapidly in transgenic mice; therefore more B cells are available for activation by immunization.

Do TG5e-7 bone marrow cells undergo increased apoptosis as a result of increased proliferation? The increased proliferation seen on day 0 in freshly isolated TG5e-7 bone marrow cells most likely indicates what is actually going on in the bone marrow *in vivo*. On the other hand, after day 3 in culture growth *in vitro*, TG5e-7 bone marrow cells showed a significant decrease in proliferation compared to those from wild-type mice. Though this decrease in proliferation may not be indicative of actual *in vivo* events, it is important to note that the cells of the transgenic mice behave dramatically different than the wild-type mice. This drop in proliferation could indicate that the cells are unable to sustain such rapid growth in culture or that apoptosis is induced more readily in these cells. If apoptosis is induced after 3 days in culture, it is possible that this could be brought on by either improper development of these cells due to increased pre-BCR/BCR production as proposed earlier or inappropriate activation of immature B cells.

Overexpression of Pax-5e in B cells could lead to increased IgH rearrangement or increased production of pre-BCR components by increasing the activity of Pax-5a which as mentioned before, has a critical role in these processes. If this leads to more rapid maturity, it is possible that these cells may pass through IgH or IgL rearrangement checkpoints prematurely, leading to proliferation of cells with inherent abnormalities in their pre- or mature BCR. B cells which fail to properly rearrange their IgH or IgL chains, respond to self antigen, or activate before maturity, will normally undergo apoptosis as a part of the negative selection checkpoint (Hardy et al., 1991; reviewed in Liberg and

Sigvardsson, 1999). If overexpression of Pax-5e leads to more rapid production of the pre-BCR, rearrangement of the Ig loci may not be completed properly. These abnormalities would then lead to programmed cell death.

Another hypothesis is that increased Pax-5a activity may increase the likelihood that precursor or immature B cells become activated through stimulation of their pre-BCR or BCR. As part of negative selection, any immature B cell activated by self-antigen or without antigen will undergo caspase-mediated apoptosis (reviewed in de Alboran et al., 2003). If B cells overexpressing Pax-5e increase the production of a mature BCR, some of these cells may show low affinity to self antigen or activate before the mature BCR is established, thus leading to apoptosis. Based on this information, B cells in TG5e-7 mice at any stage of development may undergo rapid expansion as a result of increased Pax-5e levels, but succumb to negative selection during subsequent stages (see model in Figure IV.1).

Do increases in endogenous Pax-5e combined with transgenic overexpression produce an additive effect in LPS-stimulated cells? Endogenous Pax-5e is normally found at very low levels in resting B cell lines (Lowen et al., 2001). Previous experiments in our lab have shown that Pax-5e levels increase 4-6 days after LPS activation of normal resting B cells, indicating that antigenic stimulation results in increased endogenous Pax-5e (Lowen et al., 2001). It is possible that the combination of excess Pax-5e from the transgene with increased expression of endogenous Pax-5e resulted in a more pronounced effect on proliferation than could be seen by each variable

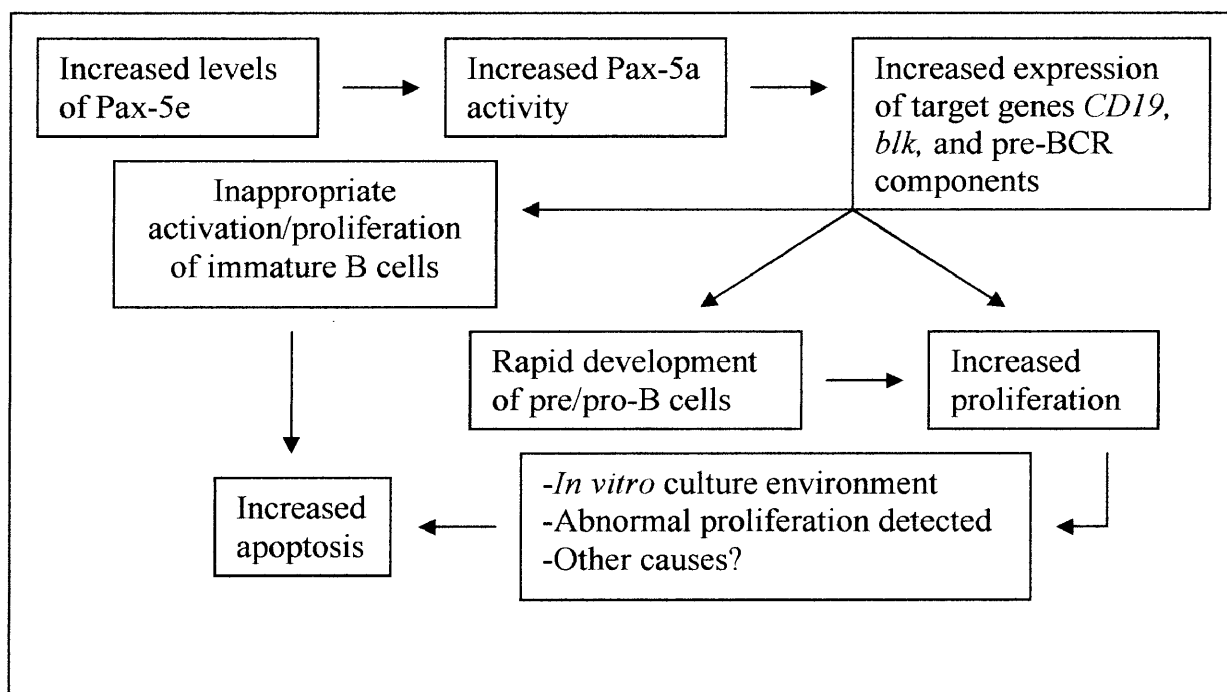


Figure IV.1: A model portraying the effects of TG5e on proliferation and/or apoptosis in developing B cells.

independently. This would explain why the effects of the transgene are more visible after immunization. This was supported by earlier studies in our lab showing that increasing amounts of transfected Pax-5e result in a dose-dependent increase in B cell growth and proliferation (Beth Jones, unpublished data). This suggests that the actual concentration of Pax-5e in B cells is important for cell proliferation. Thus, higher concentrations of Pax-5e result in a greater increase in cell growth compared to lower concentrations of Pax-5e.

In summary, TG5e-7 progeny appear to show increased proliferation in the early stages of B cell development which may lead to increased apoptosis. More experiments need to be performed to see if there is, in fact, a decrease in proliferation or an increase in apoptosis or both on day 3. It would also be advantageous to look at individual cell populations to determine at which stage the proliferative expansion is most prominent. The cell populations to consider should include pro-B, pre-B, immature and mature B cells as well as activated B cells, plasmablasts and plasma cells. Finally, any additive effect from the combined levels of endogenous and transgenic Pax-5e during LPS stimulation should be considered.

TG5e-49 bone marrow cells show increased proliferation in culture. TG5e-49 showed distinct increases in proliferation in the bone marrow after day 3 under LPS-stimulated or unstimulated conditions. If we compare these data to that collected from strain TG5e-7, it is possible to hypothesize that TG5e-49 cells are not undergoing the same level of selection or apoptosis seen in TG5e-7, and that more of these cells are surviving after 3 days in culture. It is possible that a longer period of time in culture

would reveal decreases in proliferation, but this would need to be tested. Also, the fact that TG5e-49 expresses the transgene in both B cells and T cells could lead to changes in bone marrow cell population growth. Since progenitor T cells are formed in the bone marrow then migrate to the thymus, any effect on proliferation seen in the bone marrow of TG5e-49 mice would be limited to the pro-T stage and all B cell stages. Assaying for proliferation changes within the thymus of this strain is the next step that should be taken to determine if T cell populations are proliferating as a result of Pax-5e expression.

The role of Pax-5 in proliferation has many applications in cancer research as the *pax* genes as a whole are considered proto-oncogenes (Maulbecker & Gruss, 1993). Translocation of the human *pax-5* is associated with the onset of non-Hodgkin's lymphomas and acute leukemias (Zhang et al., 2003) and is also expressed in neuroblastoma cells which are neural crest-derived tumors (Baumann-Kubetzko et al., 2004). Earlier work has shown that overexpression of Pax-5 increased proliferation in cultured splenic B cells activated by LPS, CD40 ligand or anti-IgD (Max et al., 1995), and as mentioned before, Pax-5e overexpression in mature B cell lines also resulted in an increase in Pax-5a activity as well as an increase in B cell proliferation (Lowen et al., 2001; Beth Jones, unpublished data). Given this information, it is conceivable that overexpression of alternative isoform Pax-5e could also lead to similar tumor growth. As was mentioned in the results, one of the founder mice, TG5e-31, succumbed to squamous cell carcinoma. Also, there were 3 other founder mice that died before 8 weeks of age of unidentified causes.

Immunization enhances effects of TG5e in transgenic mice

TG5e-7 mice show increased Ig secretion and B cell numbers. Immunized TG5e-7 mice revealed a significant increase in IgM and IgG levels secreted by spleen cells, which correlates well with ELISPOT data showing an increase in the number of IgG secreting cells in the spleen and bone marrow. Although the ELISPOT data revealed a significant increase in the number of Ig-secreting cells, the spot-size distribution remained unchanged suggesting that there was no change in the individual populations of plasmablasts or plasma cells (discussed in more detail below). Therefore, overexpression of Pax-5e leads to increased numbers of Ig-secreting cells in spleen and bone marrow, thus resulting in increased levels of secreted IgG and IgM in these organs.

Flow cytometry of immunized mice revealed an increase in pre- through mature B cells (CD19+B220+) and (im)mature B cells (IgM+B220+) in the bone marrow of the transgenic mice. “Real time” PCR (qRT-PCR) showed no change in CD19 expression in the spleen, a characteristic also seen in the non-immunized mice. This could suggest that the average spleen cell may be expressing less CD19 in the transgenic mice than the wild-type mice (maybe as a result of shifted frequencies of B cell subpopulations). An increase in the production of (im)mature B cells (CD19+B220+ or IgM+B220+) in the bone marrow of these mice likely results in the generation of more Ig-secreting cells after immunization, as there are more mature B cells to be potentially activated by antigen. If there is an increase in plasmablast/plasma cells in bone marrow, this would likely be the cause of the increase in total secreted Ig seen via ELISA.

These data also relate to the previously described proliferation assays which suggest that an increase in the level of Pax-5e results in increased B cell proliferation. The question now becomes, what subpopulations of B cells show increased proliferation

during an immune response? Based on the data acquired, we hypothesize that increased levels of Pax-5e have lead to increased proliferation of pro/pre-B cells in the bone marrow, resulting in increased numbers of (im)mature B cells (CD19+IgM+), which in turn may contribute to an increase in plasma cells in the bone marrow and spleen. This is supported by ELISPOT data that show an increase in all IgG secreting cells independent of spot size.

Another possibility is that overexpression of Pax-5e in the plasmablast, the proliferating intermediate between activated B cell and plasma cell, leads to increased proliferation at this stage. Differentiation into CD138- plasmablasts results in extensive proliferation as well as low levels of Ig secretion, before the cells terminally differentiate into plasma cells which cease to divide (Jego et al., 1999). Plasmablasts continue to express low levels of Pax-5a as expression of BLIMP-1, a repressor of Pax-5a, begins to increase, leading to terminal differentiation into a plasma cell and the complete silencing of the *pax-5* gene (Lin et al., 2002). Since Pax-5a is still present at low levels in plasmablasts, excess Pax-5e may be enhancing Pax-5a activity, resulting in continued expression of Pax-5a target genes, leading to increased plasmablast proliferation. Our data show an increase in (im)mature B cells/plasmablasts (CD19+B220+ and IgM+B220+) as well as increases in IgM/IgG expression seen in TG5e-7. From this, we hypothesize that this increase in the number of (im)mature B cells and/or plasmablasts in the bone marrow is due to excess proliferation at pro/pre-B cell or plasmablast stages.

In summary, the data collected on immunized TG5e-7 using several independent approaches, imply that excess Pax-5e results in increased numbers of CD19+B220+ and IgM+B220+ cells as well as increased numbers of Ig-secreting cells in the bone marrow.

It would be interesting to use IgG as a marker in flow cytometry to see if this technique shows an increase in the number of IgG⁺ mature B cells (memory B cells) and if this correlates to the data collected using ELISPOT and ELISA to analyze plasma cell populations. Also, plasma cell markers such as CD138 used in flow cytometry would be helpful to define changes in plasmablast or plasma cell populations to determine if they support ELISPOT and ELISA data directly. Though proliferation assays of LPS-activated spleen and bone marrow cells showed no change in proliferation rate, it is possible that an effect could emerge when immunized mice were used. Also, TNP was the agent used to immunize the mice, not LPS, therefore comparisons between LPS-activated cultures and data from immunized mice should be considered, but not necessarily used as a direct correlation. The data collected thus far from TG5e-7 provide evidence for the hypothesis that increasing levels of Pax-5e enhance the activity of Pax-5a and in turn, lead to increase cell proliferation (Lowen et al., 2001; Beth Jones, unpublished data).

Immunized TG5e-49 mice show decreased B cell numbers and overexpression of CD19. TG5e-49 mice displayed a very different phenotype after immunization compared to TG5e-7 mice. We hypothesize that this difference is due to expression of the transgene in both B and T cells in TG5e-49 as opposed to only B cells in TG5e-7 mice. Progeny of strain TG5e-49 showed significantly decreased levels of secreted IgG in the spleen as well as secreted IgM in the bone marrow (as shown by ELISA). ELISPOT data indicated no change in the number of IgG-secreting cells; however, there appeared to be relatively more smaller spots (cells secreting less IgG) and fewer larger spots (cells

secreting more IgG) in the transgenic mice than in negative control mice. This was true for bone marrow and spleen cell populations. This pattern suggests that even though the number of IgG-secreting cells is the same in TG5e-49 mice, the amount of secreted IgG per cell has decreased in the transgenic mice, resulting in the decrease seen by ELISA. A possible explanation is that in the TG5e-49 there is a shift from plasma cells towards plasmablasts which secrete less Ig and therefore produce smaller ELISPOTs. TG5e-49 mice show increased proliferation in the bone marrow cultures after 72 hours as well as a significant increase in the number of pro-B cells (CD43+B220+) in the bone marrow of immunized mice. This implies that, as with TG5e-7, increased proliferation of pro-B cells produces increased numbers of (im)mature B cells which in turn lead to more plasmablasts. Alternatively, the overexpression of Pax-5e may result in prolonged proliferation of plasmablast populations and subsequent delays in the terminal differentiation of plasma cells.

Quantitative RT-PCR also revealed a significant increase in CD19 expression in the spleen of TG5e-49 mice. An increase in CD19 expression may indicate one of two possibilities: 1) There are more CD19+ B cells in a particular organ or 2) there are the same number of B cells, or fewer, but more CD19 molecules per cell. To answer this question, we utilized flow cytometry to determine changes in the number of CD19+ B cells. Flow cytometry data indicated that there were fewer (im)mature B cells (CD19+IgM+) in the spleen and bone marrow of the transgenics. Thus the second possibility, that there are fewer B cells, but with each B cell overexpressing CD19, seems most likely. If there is overexpression of CD19 in TG5e-49 B cells, this could explain

the increase in prolonged (3 day) proliferation seen in unstimulated and LPS-activated bone marrow cells.

CD19 is a cell surface protein involved in transducing signals from the BCR and is believed to have a role in the proliferative expansion of pre-B I cells (large pre-B) after the completion of the pre-BCR (Otero and Rickert, 2003). Mice lacking CD19 (CD19^{-/-}) show significant decreases in large pre-B cells in the bone marrow. Otero and Rickert's data was supported by BrdU incorporation measured by flow cytometry. The investigators suggest that CD19's role in proliferation may be to augment pre-BCR signaling. Therefore, overexpression of CD19 in our transgenic mice may initially increase proliferation of pre-B cells by means of the pre-BCR, as indicated by increased proliferation in LPS-stimulated bone marrow cultures. This increase in proliferation at early B cell stages may result in increased apoptosis of cells with poorly formed BCRs or inappropriately activated immature B cells, leading to fewer B cells during an immune response. This also suggests that overexpression of Pax-5e could be enhancing 5a activity, resulting in increased amounts of CD19 and other proliferation genes, which may collectively increase the proliferation rate of the cells at all developmental stages whether they are stimulated or unstimulated.

The effects of Pax-5e on B and T cell development in TG5e mice. RT-PCR analysis on purified B and T cells from the spleens of TG5e-49 progeny and negative littermate controls showed that this strain expresses the transgene in both B and T cells. We were also able to show Pax-5e protein overexpression in the thymus of TG5e-49 mice (see Figure II.2E). The latter is not surprising as this construct had been shown to express in

B as well as in T lymphocytes (Pircher et al., 1989; Malek et al., 1998). Flow cytometry revealed a significant decrease in the number of CD3⁺ T cells in the spleen of immunized TG5e-49 progeny and, though there was a decrease in the thymus, these data were not significant. This leads us to believe that overexpression of Pax-5e in T cells has negative consequences for development, specifically during activation of T cells as no changes were seen in unimmunized mice. No further work has been done on this aspect of TG5e-49; however, possible experiments are being considered. Most importantly, the availability of T cells expressing Pax-5e in the absence of Pax-5a will be a valuable tool to study Pax-5a-independent role(s) of Pax-5e in proliferation.

Pax-5a is not normally expressed in T lymphocytes (Adams et al., 1992), therefore there must be some other molecule present in the T cell for Pax-5e to interact with in order to produce the effects seen in the T cell populations of TG5e-49. There are, however, endogenous interaction candidates present in T cells that could interact with Pax-5e. One of those candidates is thioredoxin (TRX) which is up-regulated within T cells during oxidative stress by H₂O₂ (Kondo et al., 2004). The data from this thesis do not prove an interaction between Pax-5e and TRX in T cells, thus the following hypotheses are speculative; inferred from other TRX studies.

TRX interacts with redox factor-1 (Ref-1) to activate the AP-1 transcription factor during oxidative stress or ionizing radiation treatments (Wei et al., 2000). TRX, Ref-1 and AP-1 are active in T cells as well as B cells (Tell et al., 2000; Wei et al., 2000). Previous research in our lab has shown that Pax-5e interacts with a small protein, possibly forming a heterodimer (Lowen et al., 2001). Experiments have revealed that a thioredoxin-like protein co-immunoprecipitates with Pax-5e and is detected by a catfish

monoclonal anti-TRX (IgA) antibody. These immunoprecipitation experiments also show that as Pax-5e levels increase after 4-6 day stimulation of B cell lines with LPS, the level of TRX that co-immunoprecipitates with Pax-5e also increases.

TRX lacks a nuclear localization sequence (NLS) and is thought to be shuttled into the nucleus via another protein, as the exact means of nuclear localization is unknown (Powis et al., 2001). Our lab proposed that interactions with Pax-5e, which does have an NLS, allow for TRX to be shuttled into the nucleus in order to interact with such factors as Ref-1 and AP-1 (Lowen et al., 2001). Based on the 3D structure and physiochemical properties of the Pax-5e novel sequence, bioinformatics and 3D protein structure software have predicted this sequence to be ideal for interaction with TRX (Cuperlovic-Culf et al., 2003). Also, the transcription factor AP-1 interacts with nuclear factor of activated T cell (NFAT) during T cell activation by binding as a heterodimer to composite elements of a promoter region in order to activate genes such as IL-2 and IL-5 (Kel et al., 1999). TRX typically responds to oxidative stress by inducing the activation of NF- κ B or up-regulating intracellular antioxidants like glutathione (Nakamura et al., 1997). As TRX interacts with AP-1, and possibly NF- κ B, it may play a role in regulating apoptosis pathways of activated or oxidatively stressed cells. Therefore, if TRX and Pax-5e do in fact interact, effects of Pax-5e within a T cell are most likely initiated by the activation of the T cell during immunization, as AP-1/NFAT are active during T cell activation. Since Pax-5e is normally absent in a T cell system, this alone may cause perturbations in cell activation, possibly leading to increased apoptosis (see proposed model Figure IV.2).

Interactions of Pax-5e in T cells of TG5e-49

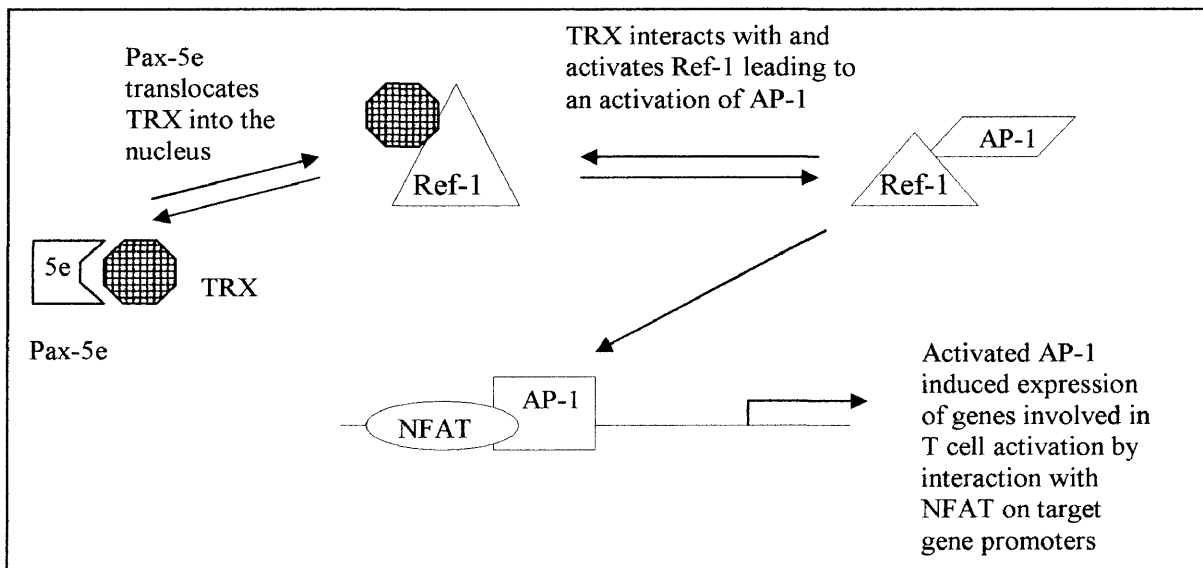


Figure IV.2: Model of Pax-5e interaction in T cells. The presence of Pax-5e in T cells enhances the activation process of T cells during an immune response by shuttling excess TRX (hatched octagon) into the nucleus. Once in the nucleus, TRX interacts with Ref-1 (triangle) which allows Ref-1 to activate AP-1 (inactivated=parallelogram). Activated AP-1(square) binds NFAT (oval) and the heterodimer situates on promoter regions of activation-induced T cell genes causing expression of genes such as IL-2 and IL-5.

Interactions between Pax-5e and TRX have implications for B cell development in both B and T cell overexpressing strains. Studies have suggested that Pax-5a functions most efficiently in a reduced form as treatment of cells with H₂O₂ reduces Pax-5 DNA-binding activity *in vivo* (Tell et al., 2000). These data were supported by initial studies using recombinant Pax-5a and Pax-8 paired domains that revealed evidence for redox regulation through the conserved cysteine residues present in that region of the protein (Tell et al., 1998). This leads us to believe that overexpression of Pax-5e could cause increased reduction of Pax-5a thus leading to increased Pax-5a DNA-binding activity. A proposed model shows Pax-5e interacting with TRX thus inducing both the reduction of Pax-5e and the translocation of TRX into the nucleus. Once in the nucleus, Pax-5e then reduces Pax-5a causing an increase in DNA-binding activity and subsequently an increase in target gene expression, specifically in this case of proliferation genes such as *blk* (refer back to Figure I.4).

Future Directions

Our data provide evidence that Pax-5e enhances the activity of Pax-5a in B cells. This activity results in changes in cell proliferation and activation in normal and immunized transgenic mice and negative littermate controls. We also suggest that Pax-5e has a profound affect on the growth and development of T cells during an immune response, possibly by interacting with endogenous T cell factors such as TRX. Recently, it was discovered that the human *pax-5* gene is alternatively spliced resulting in five novel isoforms (Robichaud et al., 2004). These isoforms differ from the murine isoforms in that all retain the ability to bind DNA, but come about by splicing out different

combinations of exons 7, 8 and 9. It would be interesting to now compare the functions of these structurally different isoforms to the murine Pax-5 isoforms. Do the human Pax-5 isoforms have a role in cell proliferation? Do they interact with one another?

There are many avenues that could be taken to expand on the data presented in this thesis. The most important experiments will need to include isolation of subpopulations of B cells into progenitor, precursor, (im)mature B cells as well as activated B cells, plasmablasts and plasma cells. These subpopulations are critical for analyses in both strains, however T cell subpopulations will need to be purified for TG5e-49. Once we have these populations of cells, we can look at changes in proliferation rates (for both LPS-stimulated and unstimulated cells), as well as changes in specific lymphoid population sizes and distributions via flow cytometry. Some of the necessary markers for flow cytometry include CD4 and CD8 for T cells as well as T cell progenitor markers, pre-BCR components for late progenitor and early precursor B cells, and CD138 for plasma cells.

Due to time and resources, we were unable to investigate the effects of TG5e on the expression of *blk*, one of the key proliferation genes in the B cell. Thus, future studies should include “real time” PCR analysis of *blk* expression in normal and immunized mice of both strains. Since *blk* is a target gene of Pax-5a, if it is overexpressed in our transgenic mice this would provide further evidence for our hypothesis that Pax-5e leads to increased proliferation by enhancing Pax-5a activity. Other target genes of Pax-5a should be considered as well such as *mb-1*, *XBP-1*, and *c-myc* as well as components of the pre-BCR.

Further studies on TG5e-49 may provide insight into Pax-5e interactions with TRX as well as Pax-5a-independent proliferation. Focusing on genes affected by active TRX in T cells may allow for analysis by CAT assays or EMSA to determine if AP-1 binding to target genes is increased transgenic mice. Overexpression of AP-1 target genes may be investigated through the use of “real time” PCR.

Our studies using the murine Pax-5 isoforms will hopefully shed light on the roles of their human counterparts and further develop models for cell proliferative disorders such as lymphoma, leukemias, and multiple myelomas. Though there is still much more work involved in this project, the opportunities of future experimentation are bountiful and the applications are promising.

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