IMMUNE TOLERANCE FOR INSULIN IN DEVELOPING B LYMPHOCYTES

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DEDICATION

To my parents, Greg and Elaine Henry, and my sister, Nikki Henry, for all of your love, support, and encouragement during my journey

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LIST OF ABBREVIATIONS

125Tg	anti-insulin BCR composed of IgH and IgL Tg
2-ME	2-mercaptoethanol
Ab	antibody
Ag	antigen
Anti-GPI	anti-glucose-6-phosphate isomerase
Ars/A1	Ars/A1 BCR transgenic mouse model
autoAb	autoreactive antibody
autoAg	autoantigen
BCR	B cell receptor
Cκ	κ constant region
DH	diversity region of IgH
DMEM	Dulbecco's modified eagle medium
ER	endoplasmic reticulum
ES cell	embryonic stem cell
FBS	fetal bovine serum
FWD	forward, or 5', primer in PCR
HBSS	Hank's balanced salt solution
HEL	hen egg lysozyme
Ig	immunoglobulin
IgH	Ig heavy chain
IgHELMD4Tg	anti-hen egg lysozyme BCR transgene (MD4)

Igκ	Igκ light chain
IgL	Ig light chain
Igλ	Igλ light chain
IL-7	Interleukin-7
JH	joining region of IgH
Jκ	joining region of Igκ
kb	Kilobase pair(s)
LPS	lipopolysaccharide
mAb	monoclonal Ab
MACS	magnetic-activated cell separation
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
NFAT	nuclear factor of activated T cells
NOD	non-obese diabetic mouse
PCR	polymerase chain reaction
RAG	recombination activating gene
RBC	red blood cell
REV	reverse, or 3’, primer in PCR
rIL-7	recombinant IL-7
RS	recombination sequence
RSS	recombination signal sequence
SD	standard deviation
SEM	standard error of the mean

SLE	systemic lupus erythematosus
T1D	type 1 diabetes
Tg	transgene or transgenic
TLR	Toll-like receptor
VH	variable region of IgH
VH125Tg	IgH Tg from anti-insulin mAb125
Vκ	variable region of Igκ
VL	variable region of IgL
WT	wild-type

CHAPTER I

BACKGROUND AND RESEARCH GOALS

Introduction

The constant threat of microbial invasion and pathogenesis forces the host to depend on a powerful army: the immune system. Whereas innate immunity provides rapid protection, adaptive immunity generates responses that are highly specific to individual pathogens. Mounting effective immune responses against an unrelenting and changing sea of infectious agents requires broad repertoire specificity potential. This diversity comes at a price however, as many of the randomly generated specificities may recognize self. Such autoreactivity, if not appropriately controlled, can result in autoimmune disease development. Regulation or "tolerance" is thus imposed on immune cells to keep their destructive potential from being unleashed on the host, while preserving anti-microbial recognition. A failure to adequately maintain tolerance underlies the presence of autoimmune diseases that manifest in a diverse range of pathologies that plague the human population. Available therapeutics for many of these diseases often show limited efficacy and are frequently ineffective at reversing immune-mediated destruction. The goal of this thesis work is therefore to better understand how tolerance mechanisms play a role in culling autoreactive B cell specificities that occur in the developing B cell repertoire and thus provide insight into possible avenues for medicinal intervention.

B Cell Development and Function

B Cell Developmental Progression

In 1958, Nossal and Lederberg showed that a single B cell produces a single antibody (Ab) specificity (134). Subsequently, F. Macfarlane Burnet proposed the clonal selection theory in 1959 (25). Monospecificity of the B cell receptor (BCR), which interacts with antigens (Ags), is a key element that allows B cells to participate in adaptive immune responses. B cell development proceeds in an ordered, highly regulated fashion which aids in the generation of monospecific B cells, a process known as allelic exclusion (146). B cells begin life in the bone marrow as pluripotent progenitor cells, and differentiate from hematopoetic stem cells to multilineage progenitors to common lymphoid progenitors to pre-pro-B cells, where they are first committed to the B cell lineage (70).

Stages of early B cell development are predominantly defined by immunoglobulin (Ig) gene rearrangement. Ig heavy (IgH) and light (IgL) chain gene rearrangement is a highly ordered and regulated process. At the pro-B cell stage, a diversity region of IgH (DH) segment joins to a joining region of IgH (JH) segment, followed by rearrangement of a variable region of IgH (VH) to DH-JH through a process called V(D)J recombination (6, 185). Deletion of either recombination activating gene (RAG)-1 or RAG-2 proteins involved in Ig recombination results in failure of the B cell to progress beyond the pro-B cell stage, and ultimately the absence of B and T cells (124, 175). Once the IgH gene segments are successfully rearranged, IgH pairs with the surrogate light chain and is expressed on the surface of the B cell as the pre-BCR, which defines the transition into

the pre-B cell stage (6). The surrogate light chain is encoded by λ5 and V_{pre}-B (91, 166). Expression of the rearranged IgH allows progression to the pre-B cell stage (48), and a loss of surrogate light chain and endogenous light chains results in a block at the pro-B cell stage (141, 144). Signaling from the pre-BCR negatively regulates RAG expression (65) and surface expression of the pre-BCR reduces chromatin accessibility of the IgH gene segments to RAG (39, 183), both of which function as a mechanism for maintaining allelic exclusion. Asymmetric demethylation of one of the IgL alleles also likely enhances allelic exclusion by increasing chromatin accessibility of the demethylated allele to RAG (126).

Upon successful expression of IgH, cells undergo proliferation at the large pre-B cell stage and then enter the small pre-B cell stage and re-express RAG (49, 101, 162). IgL gene rearrangement then initiates, and involves the joining of variable region of IgL (VL) and joining region of IgL (JL) gene segments (188). A successfully rearranged IgL can pair with the IgH to form a mature BCR that is expressed on the surface of the cell, denoting transition into the immature B cell stage (49, 101). However, it has been shown that light chain rearrangement can also take place in μMT mice that lack membrane-bound μ chains (86), and a proportion of wild-type (WT) $CD43^+$ pro-B cells show rearranged VL-JL genes prior to VH-DH-JH gene rearrangement (49, 136), suggesting that a functionally rearranged IgH is not required for the initiation of IgL rearrangement. An experimental model in which BCR expression is inducibly diminished results in "back-differentiation" of the cells, such that pro-B cell genes are re-expressed and V(D)J recombination genes are up-regulated (190).

An appropriate signal from the BCR on the surface of the immature B cell is required for continued development. If this signal is absent, due to faulty pairing of the IgL with the IgH and thus insufficient BCR surface expression (21, 89), or if autoantigen (autoAg) engagement triggers BCR down-regulation, receptor editing, involving rearrangement of novel VL and JL gene segments, may be initiated (61, 152, 171, 187). Recombinational machinery activity is subsequently sustained to facilitate continued IgL rearrangement (31, 187), and decreased BCR surface expression has been shown to correlate with increased RAG expression (125, 171, 204). Upon successful surface expression of a BCR, immature B cells exit the bone marrow and enter the spleen as transitional B cells, and further develop into the mature B cell stage after 4 days (5, 139). Transitional 1 (T1) cells undergo apoptosis, whereas T2 B cells proliferate following BCR signaling, due to BAFF receptor expression in T2 B cells which leads to enhanced survival signaling (12, 148). The various stages of B cell development can be identified through differential cell surface marker expression, which is depicted in Table 1-1 and 1-2.

Immunoglobulin Transgenes Alter B Cell Developmental Progression

BCR Ig transgenic (Tg) models have produced key insights into B cell physiology, as they facilitate the study of how Ag interaction influences B cell biology by restricting the polyclonal repertoire to a single, known Ag specificity. Monospecificity is accomplished by providing functionally rearranged IgH and IgL Tgs, which, due to their completed rearrangement status, are dominantly expressed over endogenous IgH and IgL, which would require successful V(D)J recombination to occur for expression. While

Table 1-1. Identification of B Cell Developmental Stages Present in Bone Marrow Using Differential Cell Surface Marker Expression

Cell Surface Marker	Pro	Pre	Immature	Mature Recirculating
CD19	+	+	+	+
B220	+	+	+	++
CD43	+	-	-	-
IgM	-	-	+	+
AA4.1	-	-	+	-
CD21	-	-	-	+
CD23	-	-	-	+

Table 1-2. Identification of B Cell Developmental Stages Present in Spleen Using Differential Cell Surface Marker Expression

Cell Surface Marker	Transitional	Follicular	Marginal Zone
CD19	+	+	+
B220	+	+	+
CD43	-	-	-
IgM	+	+	+
AA4.1	+	-	-
CD21	+	++	+++
CD23	+	+++	++

such Tgs greatly expand the array of questions that can be tested, some alterations to normal B cell development occur and are important to note. Functionally rearranged IgH and IgL Tgs expedite B cell developmental progression to the IgM^+ immature B cell stage (120). Whereas knocking out RAG-1, RAG-2, or μ heavy chain arrests B cell development at the pro-B cell stage (87, 124, 175), the presence of a functionally rearranged IgH Tg alleviates this developmental block and allows B cells to reach the pre-B cell stage, and the additional presence of a functionally rearranged IgL Tg completely alleviates the developmental block (182, 202).

Mechanisms that Generate B Cell Receptor Specificity Diversity

V(D)J recombination is estimated to generate $>10^{11}$ specificities, due to the myriad of potential combinations of VH, DH, and JH gene segments for the IgH, or VL and JL segments for the IgL, termed combinatorial diversity, as well as junctional diversity arising from differences in the joints between the gene segments (reviewed in 80). P- and N-nucleotide additions also enhance diversity at gene segment joining regions, as do nucleotide deletions (reviewed in 80). Terminal deoxynucleotidyl transferase (TdT) is the enzyme responsible for adding non-templated N-nucleotides at the DH-JH and VH-DH junctions during IgH rearrangement (92), whereas palindromic P-nucleotides result from RAG-mediated cleavage of the hairpin loops (115). Additional diversity and enhanced Ab affinity is achieved through somatic hypermutation, a process which introduces point mutations into the germ line encoded DNA of Ig genes to result in affinity maturation (reviewed in 103).

B Cells and their Role in Generating Immune Responses

The mature B cell pool is made up of many different B cell subsets. B-2 B cells reside in the spleen, and are subdivided into follicular and marginal zone B cells (reviewed in 95). Follicular B cells are located in the follicle next to the T cell zone, and clonal interaction with T cells promotes the formation of germinal centers (reviewed in 95). Marginal zone B cells are present in the marginal zone and are ideally positioned to encounter blood-born pathogens (113). B-1 B cells are located in the pleural and peritoneal cavities and are thus ideally located to encounter gut and peritoneum Ags, and are responsible for generating the majority of circulating Ab (83, 113). B-1 and marginal zone B cells can generate rapid, T-independent responses to Ag (137), and thus function in an innate-like capacity (112). B-1 B cells are thought to have self-renewal capacity, and marginal zone B cells are long-lived; in contrast, the follicular B cell pool depends on continual influx of immature B cells from the bone marrow for homeostatic maintenance of this population (29, 69).

The Effects of Different Antigen Characteristics on Immune Activation of B Cells

Many characteristics of Ag engagement of the BCR influence the activation outcome of the cell. Increased Ag concentration and avidity, enhanced affinity of the BCR for the Ag, as well as the presence of co-stimulatory signals enhance the activating potential of the Ag (reviewed in 73). A classic example of how the nature of a given Ag can modify the manner in which the immune system responds is the immunogenic or "tolerogenic" effects of aggregated or de-aggregated IgG (35, 45). T cell help (37), "danger signals", which emanate from pattern recognition receptors such as Toll-like

Receptors (TLRs) (107), or stimulation through complement receptors (28, 55) all enhance the stimulatory potential of a given Ag by signaling that it is associated with a pathogenic invader. The timing of when Ag is encountered during B cell development also influences whether a B cell is activated; BCR triggering during development typically elicits tolerance, whereas cellular activation is more likely to occur if Ag is encountered once the cell has matured (reviewed in 73).

B Cell Receptor Signal Transduction

B Cell Receptor Signaling

Work by many groups as well as work presented in this thesis demonstrates that B cells are exquisitely sensitive to Ag engagement of the BCR and are finely tuned to respond in a multitude of ways depending on subtle changes that are both quantitative and qualitative in nature. The network of molecules involved in diverse signaling cascades is highly complex and interwoven, and for simplicity and relevance, much of this will not be discussed. Engagement of the BCR results in ER Ca^{2+} release from the ER (reviewed in 59). Depletion of the ER Ca^{2+} store leads to extracellular Ca^{2+} influx, which promotes a sustained increase in intracellular Ca^{2+} (142). This can lead to calmodulin and calcineurin activation of nuclear factor of activated T cells (NFAT) (reviewed in 41).

Differences in Immature and Mature B Cell Signaling

B cell maturation triggers important differences in how the cell responds to Ag engagement, and these responsive changes are pivotal in balancing effective B cell

immunity with tolerance to self. In 1975, differential sensitivity of immature (neonatal-spleen derived) versus mature (adult spleen-derived) B cells to IgM crosslinking was observed, providing the first evidence for differential sensitivity of immature and mature B cells (153, 177). Immature B cells showed irreversible IgM down-regulation and higher sensitivity to the concentration of IgM, whereas this down-regulation was reversible in splenic B cells upon anti-IgM removal (153, 177). Following identification of cell surface markers which identify developing B cells, such studies confirmed that IgM crosslinking of the BCR induces cell death in immature B cells, whereas mature B cells proliferate (32, 133, 201). Specifically, this change has been shown to take place between the T1 and T2 transitional B cell stages in the spleen (148).

Despite up-regulating cyclin D2 and cyclin dependent kinase 4 (CDK4) to promote G1 entry, BCR crosslinking of immature B cells fails to up-regulate cyclin E, which thus impairs formation of cyclin E/CDK2 complexes required for cell cycle progression (26). In addition to stimulating proliferation, BCR stimulation of mature B cells results in up-regulation of the activation markers CD69, CD86, and major histocompatibility (MHC) class II, whereas immature B cells show no up-regulation of these molecules (14). Immature B cells are more sensitive to BCR signaling, as lower increases in intracellular calcium are necessary to induce RAG induction in immature B cells than to up-regulate activation markers such as CD86 and MHC class II on mature B cells (14).

Autoreactivity in the B cell Compartment

Prevalence of Self-Reactivity in Developing B Cells

Each day, ~20 x 10^6 cells are produced in the bone marrow of a mouse (138). V(D)J recombination ensures that a tremendous potential for generating repertoire diversity exists, however the cost of this diversity is that autoreactive as well as useful specificities are generated. In humans, 55-75% of newly formed B cells are estimated to be autoreactive (193). Additional evidence in mice for a rather high propensity towards generating autoreactive specificities in the bone marrow is that only 10-20% of immature B cells formed in the bone marrow survive the maturation process long enough to emigrate from the bone marrow, and only 3% survive as mature B cells in the periphery (5, 57, 69, 164). This deletion is specific, as the repertoire of specificities is altered between immature and mature B cell populations, suggesting a selection event (66, 153, 168). The induction of tolerance in the nascent B cell compartment is known to occur upon BCR engagement; immature and transitional B cells undergo tolerance induction in the absence of additional signals, such as T cell help (4, 27, 58).

The Role of B Cells in Autoimmune Disease Development

Preservation of tolerance in developing and mature B cell compartments is critical for the prevention of autoimmune disease. B cells that escape tolerance can generate pathogenic Abs, which have been demonstrated to play a role in autoimmune diseases such as systemic lupus erythematosus (SLE), Goodpasture's syndrome, hemolytic anemia, Graves' disease, thrombocytopenic purpura, myasthenia gravis, and pemphigus

vulgaris (111, 127). Detection of autoantibodies (autoAbs) is also common in patients with autoimmune diseases which result from T cell-mediated destruction, such as Hashimoto's thyroiditis, type I diabetes (T1D), and rheumatoid arthritis (127). Rituximab, which targets B cells, has been approved for the treatment of rheumatoid arthritis, suggesting an indirect role for B cells in disease (reviewed in 118). In addition to showing efficacy for rheumatoid arthritis, Rituximab treatment has also shown promise for treating hemolytic anemia and thrombocytopenic purpura (47, 74, 75, 147). Rituximab clinical trials for T1D efficacy are currently underway. The ability of B cells to mediate autoimmune disease is not restricted to the generation of pathogenic autoAbs. Additional B cell functions, such as presenting of autoAgs to autoreactive T cells, have also been attributed to disease pathogenesis (174).

Multiple checkpoints exist in healthy patients that serve to remove autoreactive specificities generated in the bone marrow; however these checkpoints are dysregulated in patients with SLE, thus permitting escape of autoreactive cells into the periphery (167, 205). These findings suggest that central tolerance defects may promote the development of human autoimmune disease (167, 205), thus highlighting the importance of understanding how these mechanisms function or fail in healthy or diseased individuals, respectively. In addition to mediating systemic autoimmune diseases, such as SLE, autoreactive B cells have also been shown to play a role in organ-specific autoimmune diseases, such as T1D (3, 78, 132, 173, 179).

Importance of B Cells in the Development of T1D in the NOD Mouse

According to the American Diabetes Association, diabetes is characterized by blood glucose levels in excess of 200mg/dL, elevated fasting blood glucose, and impaired oral glucose tolerance. Patients often experience weight loss, thirst, polyuria, polydypsia, visual blurring, and weight loss. This results from destruction of β cells in the pancreas, which impairs the ability of the pancreas to produce insulin in response to elevated blood glucose. By the time T1D patients present with overt disease, ~90% of beta cell function has already been lost, thus highlighting the importance of disease prediction (reviewed in 44). The presence of insulin (140), glutamic acid decarboxylase (GAD) (8, 9), and/or protein tyrosine phosphatase-related IA-2 molecule (IA-2) (36, 143, 151, 180) islet cell autoAbs have all been found to be predictive of T1D development, and are the first indicators of β cell-targeted autoimmunity. Insulin autoAbs are predictive of disease development in T1D-prone non-obese diabetic (NOD) mice in addition to humans, and thus represent an important risk factor (203). T cell-mediated destruction of insulin-secreting beta cells in pancreatic islets results in uncontrolled elevation of blood glucose levels found in T1D patients (18, 19, 62). B cells do not directly mediate beta cell destruction; however they play an indirect role in the progression of diabetes, as T1D-prone NOD mice that lack B cells (μMT) are protected against developing T1D (3, 173), and treatment with anti-IgM Ab is also protective (132). B cells have additionally been shown to play indirect roles in disease, such as in T1D, in which they likely function to present autoAgs to autoaggressive T cells (174). Restricting the B cell repertoire with the use of B cell Tgs that bind irrelevant (non-self) Ags also protects against T1D (78, 179). However, mice which possess an anti-insulin BCR IgH Tg (VH125Tg) or IgH and IgL

Tgs (125Tg) develop T1D (78), thus providing a tractable model in which the role of Ag-specific B cells in disease development can be addressed.

B Cell Tolerance Mechanisms

B cell tolerance maintenance is critical because autoreactive specificities are continually generated. Checkpoints therefore exist that exert control throughout all stages of B cell maturation. Central tolerance describes the mechanisms that act during B cell development in the bone marrow, and include receptor editing and deletion (61, 128, 130, 152, 187), and, as demonstrated in Chapter II, anergy. Receptor editing "edits" the autoreactive BCR (and maintains allelic exclusion) by rearranging a novel IgL, which replaces the autoreactive IgL. "Deletion" of the autoreactive clone from the repertoire through programmed cell death ensures that tolerance is maintained if receptor editing fails (68). Anergy results in functionally impairing various functions of the B cell which preclude it from actively participating in immune responses (64).

Due to the high degree of autoreactivity that originates in the developing B cell compartment (193), central tolerance represents a critical point of control. In the event that central tolerance fails to adequately censor autoreactive B cells, peripheral mechanisms of tolerance also exist to further limit the dangerous potential of self-reactive clones. As additional mechanisms of diversity such as somatic hypermutation function in the mature B cell compartment to create further possibilities for the generation of autoreactive receptors (15, 194), such peripheral checkpoints are critical in preventing potentially harmful immune responses. Mechanisms of peripheral B cell tolerance include anergy and deletion (64, 128, 130).

B Cell Anergy

Allowing autoreactive B cells to remain in the mature B cell repertoire may promote autoimmune disease, however, overly stringent removal of potentially dangerous specificities risks reducing repertoire diversity and thus could impair effective pathogen clearance. A solution to this problem involves a tolerance mechanism called anergy, in which autoreactive B cells remain, thus preserving repertoire diversity, but they are functionally impaired, thereby limiting their ability to promote destruction of self. Under appropriate conditions that suggest immunogenic necessity for the specificity (discussed below), anergy is reversible, allowing functional reinstatement of the B cell.

Such functional unresponsiveness is characterized by the reduced ability to proliferate or differentiate into Ab-secreting plasma cells following BCR stimulation (43, 64). Anergic B cells are functionally unresponsive to further BCR stimulation, have a shortened lifespan in the periphery, and are excluded from B cell follicles (43, 155). The presence of competitor B cells is required for this exclusion, as autoreactive B cells transferred into B cell-deficient animals do not display follicular exclusion (170). Survival of autoreactive B cells transferred into B cell deficient animals is enhanced by the presence of T cells, compared with those transferred into RAG-1$^{-/-}$ animals deficient for both B and T cells (170), suggesting that T cells may play a supportive role in the preservation of autoreactive specificities in lymphopenic animals, such as the NOD T1D model. Active participation in immune responses is limited, as anergic cells do not produce significant levels of circulating Ab, even upon immunization (64, 131, 160). Tg B cells specific for hen egg lysozyme (HEL) (IgHELMD4Tg) or insulin that encounter soluble Ag *in vivo* are rendered anergic (64, 160).

Anergic B cells can regain functionality when they are provided appropriate secondary signals, such as complement receptor engagement (which could be triggered by a complement-fixed pathogen) (108), or anti-CD40 or IL-4 stimulation (mimics of T cell help) (1, 40). Chronic Ag engagement is also required to maintain this state, as transferring HEL-specific B cells into an HEL-free mouse (63), or competitive removal of the Ars/A1 model autoAg by arsonate hapten (13, 60) restores B cell functionality. Further evidence is presented in Chapter II to show that continued insulin exposure is necessary to maintain functional impairment in immature anti-insulin B cells.

A BCR Transgenic Model Facilitates the Study of Immune Tolerance for Insulin

Our lab utilizes a B cell Tg model (125Tg) in which anti-insulin IgH (VH125Tg) and IgL (Vκ125Tg) Tgs are randomly integrated into the genome to generate B cells, >95% of which bind insulin (160). The 125Tg anti-insulin BCR has different affinities for insulin derived from different species (172). Insulin is a highly conserved molecule, and this system allows the effects of altered affinity to be studied with minimal changes to the overall Ag structure. We have demonstrated that the BCR of these cells is continuously occupied by circulating insulin *in vivo*; thus 125Tg animals are a useful BCR Tg model in which a physiologically relevant BCR:Ag interaction can be studied (160). VH125 encodes an anti-insulin IgH, whereas VH281, which differs from VH125 by two amino acids that confer insulin-binding, does not measurably bind to insulin when paired with anti-insulin Vκ125 (186). VH125Tg and VH281Tg mice possess a rearranged IgH Tg that is randomly integrated in the genome, which pairs with endogenous IgLs to produce a BCR, which generates a polyclonal repertoire (186).

Table 1-3 outlines all of the Ig Tg genotypes studied in this dissertation as well their Ag specificities.

Classically, hormones are expected to induce clonal ignorance, as they are soluble proteins present at low concentrations and thus may be "ignored" by the B cell due to insufficient stimulatory potential. Surprisingly, 125Tg B cells are not clonally ignorant, but rather are rendered anergic *in vivo* (160), despite the fact that circulating insulin is present at very low concentrations (up to 1-5 ng/mL post-prandially (56)), insulin is a small, soluble protein and does not appreciably crosslink the BCR, and the affinity of the interaction is modest (7×10^6 M^{-1}) (172). This form of anergy is characterized by impaired Ca^{2+} mobility following acute stimulation in anti-insulin (125Tg) B cells as compared with non-tolerant B cells isolated from the spleen, and basal IP_3 levels are reduced (2). Tolerant 125Tg B cells do not show elevated intracellular Ca^{2+} or impaired tyrosine phosphorylation (2), as has been shown for the IgHELMD4Tg model (40, 72, 191). Anti-IgM stimulation of 125Tg spleen B cells results in decreased endoplasmic reticulum (ER) release of intracellular stores of Ca^{2+}, and they are more sensitive to thapsigargin-induced ER Ca^{2+} store depletion, suggesting that sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA) pump expression and/or activity may be enhanced in these cells (2). 125Tg B cells show decreased basal levels of NFATc1 and impaired NFATc1 nuclear translocation following BCR or ionomycin stimulation (2). Anti-insulin B cells fail to proliferate in response to stimulation with anti-IgM and they do not respond to T cell-dependent immunization (1, 160). This state of functional inactivation also limits the contribution of Tg B cell-derived Abs to the pool of circulating Ab (160).

Table 1-3. Nomenclature and B Cell Antigen Specificities of Heavy and Light Chain Transgenes Present in Genotypes Studied

Genotype	Heavy Chain Transgene	Light Chain Transgene	Antigen Specificity in Repertoire
WT	None - endogenous	None - endogenous	Polyclonal
VH125Tg	Non-targeted, anti-insulin	None - endogenous	Insulin/Polyclonal
VH281Tg	Non-targeted, non-insulin-specific	None - endogenous	Non-insulin/Polyclonal
VH125Tg/Vκ1ki	Non-targeted, anti-insulin	Targeted, anti-DNA	Non-insulin/Polyclonal
VH125Tg/Vκ4ki	Non-targeted, anti-insulin	Targeted, anti-DNA	Non-insulin/Polyclonal
VH125Tg/Vκ8ki	Non-targeted, anti-insulin	Targeted, anti-DNA	Non-insulin/Polyclonal
VH125Tg/Vκ125ki	Non-targeted, anti-insulin	Targeted, anti-insulin	Insulin/Polyclonal
VH281Tg/ Vκ125ki	Non-targeted, non-insulin-specific	Targeted, anti-insulin	Non-insulin/Polyclonal
125Tg	Non-targeted, anti-insulin	Non-targeted, anti-insulin	Insulin-restricted
IgHELMD4Tg	Non-targeted, anti-HEL	Non-targeted, anti-HEL	Hen Egg Lysozyme-restricted

HEL: Hen Egg Lysozyme

Anti-insulin B cells make up the majority (95-98%) of the mature repertoire, yet anti-insulin Abs are a minor fraction of the total circulating Ab; rather, the bulk of circulating Abs are derived from the small (2-5%) population of endogenous B cells that do not express the anti-insulin Tgs (160).

Evidence for Immature B Cell Anergy

It was shown in 1980 that culture of flow cytometry-purified IgM^- cells with anti-IgM impaired the ability of the cells to differentiate to become Ab-forming cells *in vitro*, thus providing evidence that functional silencing triggered through BCR stimulation may begin early during B cell development (149). Immature Ars/A1 B cells which harbor an anti-arsonate BCR which also recognize self Ags show impaired Ca^{2+} mobilization in the presence of autoAg (13), and show impaired Ca^{2+} mobilization and homing following stromal cell-derived factor-1 (SDF-1) stimulation, which is SHIP-dependent (22). Much of the underlying mechanism that maintains anergy, as well as the ultimate functional consequences that occur in anergic immature B cells are still poorly understood. Work presented in Chapter II seeks to further address this important question.

The Discovery of Receptor Editing

During B cell development, an IgL is rearranged, and if it does not pair well or produces an autoreactive specificity when paired with the IgH, a new IgL is rearranged which can replace the initial IgL in a process called receptor editing. The first evidence for IgL receptor editing involved the observation by several groups that secondary IgL rearrangements were present in B cell lines (53, 88, 96). This process was shown to be

stimulated by anti-BCR Ab, consistent with a mechanism for altering autoreactive specificities (96). It was observed in B cell Tg mouse models that allelic exclusion of the Tg IgL was not tightly conserved, again hinting at the potential for a new mechanism of tolerance (158, 165).

Deletion was found to be the predominant tolerance mechanism in autoreactive B cells which possessed a non-targeted IgL Tg and recognized highly crosslinking autoAgs (71, 128, 130). In 1993, it was shown that a small fraction of the B cells escaped deletion by rearranging an endogenous (non-autoreactive) IgL, and thus demonstrated that such Ags could induce receptor editing in a physiologically relevant setting (61, 152, 187). The use of such non-Ig-targeted BCR Tg models however does not allow direct study of receptor editing as it would normally proceed, because the IgL Tg is randomly integrated into the genome, and thus is not subject to replacement attempts occurring at the IgL loci.

Later work with Ig-targeted BCR Tg models, which represent a much more physiologically relevant model for the study of receptor editing, indicated that anti-MHC class I or anti-HEL B cells were predominantly censored through receptor editing following membrane-bound cognate Ag encounter, and that clonal deletion only occurred when receptor editing was not possible, due to RAG-1 deficiency or deletion of the Jκ region, both of which are required for novel light chain rearrangements to occur (68, 76). Studies show that ~25% of mature B cells in the human polyclonal repertoire show evidence of secondary IgL rearrangement, which suggests that IgL receptor editing plays an important role in shaping the mature B cell repertoire (31).

The Mechanism of Receptor Editing

In humans, many developing B cell specificities are autoreactive (193). The ability to "edit" an autoreactive BCR to an innocuous specificity therefore represents a key salvage pathway that can rescue cells otherwise destined for anergy or deletion. Receptor editing is a critical tolerance mechanism that acts during B cell development in the bone marrow, and can be stimulated by Ag encounter (61, 152, 187). RAG proteins bind recombination signal sequences (RSS) in DNA to induce double strand breaks to mediate DNA recombination (114). During B cell development, faulty pairing of the IgH with the newly rearranged IgL (38, 54), or pairing that produces an autoreactive specificity can induce continued RAG expression and rearrangement of a new IgL (61, 152, 187). This second IgL then pairs with the existing IgH to form a BCR that will have an altered specificity. Receptor editing involves either inversion or deletion of the DNA that codes for the initial IgL rearrangement to ablate its expression, and a second VL and JL gene segment are joined to create an IgL with a new specificity. Deletion of the κ constant region (Cκ) can occur if a RSS near the κ joining region (Jκ)-Cκ intron or at the 3' end of a non-rearranged variable region of κ IgL (Vκ) gene recombines with the recombination sequence (RS, mice) (46) or the κ-deleting element (humans).

The work in Chapter IV will focus exclusively on IgL receptor editing, however receptor editing is not restricted to the IgL locus, or to B lymphocytes. Replacement of IgH can occur (88, 156), however the ability of Ag to stimulate the BCR to directly induce receptor editing is predominantly affiliated with IgL replacement, as enhanced N nucleotide addition seen in edited IgHs indicates that editing is initiated prior to IgL rearrangement and pairing to result in surface expression of the mature BCR (30, 33,

181). Rearrangement of a second VH to an additional JH is also impaired by the removal of intervening DH segments during the initial VH-DH-JH rearrangement; direct VH-JH rearrangement violates the 12/23 base pair rule, thus cryptic heptamer RSSs are necessary for additional rearrangements to proceed (129). A high frequency of IgH replacement was observed in an anti-DNA transgenic model, in which the pairing of the 3H9 IgH with ~60% of the available IgLs produces an anti-DNA specificity (79). It is more likely that these IgH editing events are enriched in the mature B cell pool of mice that possess a highly autoreactive IgH, and are present due to a selection bias, rather than an actively stimulated process. Although not likely triggered directly by BCR:Ag engagement, this process influences the mature repertoire, as 5-12% of human B cells appear to have undergone IgH replacement (208).

It has been observed that secondary rearrangements can occur at the T cell receptor (TCR) α or δ loci following TCR engagement (110, 116, 192), which are akin to the IgL loci of B cells. T cells may undergo receptor editing to eliminate autoreactive specificities, but they may also edit the TCR to generate sufficient MHC recognition, as blocking interactions with MHC via Abs results in RAG re-expression (20).

Hallmarks of Receptor Editing

Edited B cells can easily be identified in the restricted repertoires of mice possessing Ig Tgs with known Ag specificities by detecting a loss of binding to the specific Ag. Studies of such models have identified a correlation between B cells which have undergone receptor editing and increased usage of distal Jκs (Jκ4 or Jκ5), whereas those which have not undergone receptor editing preferentially rearrange Vκs to upstream

Jκs (152, 196). An increased percentage of B cells expressing λ IgL (Igλ) is also indicative of receptor editing (184), as only ~5% of B cells from a normal murine repertoire express Igλ (184). In the polyclonal repertoire of mice, 47% of Igλ-expressing B cells show RS rearrangements which inactivate κ IgL (Igκ) (157). Thus, enhanced usage of distal Jκ segments and increased Igλ usage can be used as indirect evidence for the presence of receptor editing in a polyclonal repertoire.

Enhanced RAG expression is observed in H-2$K^{k,b}$ (MHC class I)-specific B cells isolated from H-2K^k mice compared with H-2K^d mice (187), as well as in IgHELMD4Tg B cells initiating receptor editing in response to HEL encounter (52). B cells undergoing editing are delayed at least two hours at the pre-BII stage and show increased RAG levels compared to non-editing B cells (31). The increased time spent in the editing-competent pre-B cell compartment results in an observable increase in pre-B cells via flow cytometry analysis, such that in Ig Tg models, the percentage of pre-B cells is increased when autoAg is present, compared with a virtually undetectable population when the autoAg is absent (145).

Receptor editing temporarily, and sometimes permanently, violates the principle of allelic exclusion (61), which normally ensures monospecificity of a B cell (146). Allelic inclusion sometimes occurs in edited B cells, in which expression of a secondary IgL, termed an "editor", is co-expressed with the initial autoreactive IgL (61, 100). The editor may preferentially compete for IgH binding and therefore surface expression over the autoreactive IgL (61), or both IgLs may be expressed on the surface of the cell and the editor may function to "dilute out" the autoreactivity intrinsic to the autoreactive IgL (100).

BCR Transgenic Models of B Cell Receptor Editing

Receptor editing occurs upon engagement of the BCR with membrane-bound Ags, such as membrane-bound HEL, MHC class I, DNA, phosphatidylserine, and type IV collagen (34, 61, 93, 97, 187, 189, 207). The soluble Ag, HEL, was also shown to promote receptor editing, showing that Ags that did not strongly cross-link the BCR could also induce this process (76, 189). However, the IgHELMD4Tg BCR binds HEL with high affinity (5×10^{10} M^{-1}) (11, 94); thus the 125Tg BCR model allows assessment of lower affinity interactions (8×10^{6} M^{-1}) (172) and their effects on central tolerance. The non-targeted 125Tg model presents the same limitations of other non-Ig-targeted models, thus in Chapter IV we develop a new model in which the anti-insulin Vκ125 is targeted to the Vκ locus, to facilitate physiologic study of whether insulin can elicit receptor editing.

Receptor Editing Enhances Autoreactivity in an Autoimmune Strain

Receptor editing is a mechanism for abrogating autoreactivity, but it has also been implicated in increasing autoreactivity (200). High-affinity Ab specificities were generated from low-affinity precursors through receptor editing on the NZB/NZW background, which is a SLE disease model (200). In a non-autoimmune prone strain, the same B cells were targeted for anergy, rather than receptor editing (200).

It is believed that the slow rate of Igλ rearrangement helps to maintain allelic exclusion (154). Allelic exclusion is not absolute, as demonstrated by B cells that express dual IgLs on the surface of the cell, termed “editors” (84, 98). The purpose of the

non-autoreactive IgL presumably "dilutes out" the autoreactive IgL (98). B cells expressing the autoreactive Igλ and the "editor" Igκ are shunted into the marginal zone, which is known to preferentially harbor autoreactive B cells (98). This may prevent the participation of autoreactive cells in active immune responses by physically sequestering them away from the T cell zone (100). This sequestration is defective in MRL/lpr mice, another SLE model, which may help to explain the disease activity in these mice (99).

Multiple Factors Influence the Type of Tolerance Induced

The affinity and avidity of the BCR interaction with Ag influences the method of B cell tolerance employed. Strong high-avidity or high-affinity BCR:Ag interactions favor deletion, whereas weak interactions elicit receptor editing or anergy (33, 50, 71, 128, 129, 130, 189). Anti-ssDNA B cells are anergic, whereas anti-dsDNA B cells undergo receptor editing on a non-autoimmune disease-prone mouse strain (198). Enhanced strength and avidity of the BCR interaction with autoAg likely elicits increased intracellular Ca^{2+} changes, and increased intracellular Ca^{2+} is associated with increased RAG induction (119). Stimulation of immature B cells with Ca^{2+} ionophores, such as ionomycin, induces receptor editing, but stimulation with higher doses of ionomycin (1μM) triggered apoptosis rather than receptor editing (14), again suggesting that enhanced signaling strength alters the tolerance mechanism employed.

The developmental state of the B cell, as well as the anatomical location in which the cell encounters Ag also influences the type of tolerance induction. BCR crosslinking of T1 B cells results in apoptosis (deletion), whereas T2 B cells are stimulated to up-regulate activation markers and proliferate, showing that the timing of Ag encounter

during development drastically alters the response of the B cell (148). Anti-MHC class I Tg B cells that initially encounter Ag in the periphery undergo deletion, whereas those encountering Ag during development in the bone marrow undergo receptor editing (187). Transitional B cells cultured in the presence of whole bone marrow are protected from apoptosis, whereas those cultured in the presence of whole spleen still undergo deletion, suggesting that the bone marrow microenvironment protects against deletion and instead stimulates receptor editing, whereas the same autoreactive signal encountered in the spleen induces cell death (168).

In contrast to data generated with a conventional Tg model, studies using an anti-MHC class I Tg targeted to the Igκ locus suggest that receptor editing, rather than deletion, is the main mechanism through which tolerance is maintained for this membrane-bound Ag (68). Autoreactive anti-MHC class I and anti-DNA BCR Tgs induce receptor editing on a RAG-competent background, but they stimulate clonal deletion on a RAG-deficient background to result in the absence of peripheral B cells, demonstrating that deletion may be initiated if receptor editing fails (182, 198). The presence of a *bcl-2* survival gene spares autoreactive cells from undergoing deletion, but anergic programming still remains intact, suggesting that anergy can serve as an additional safety mechanism, should deletion fail (71).

Receptor editing can act as a salvage pathway for autoreactive B cells, however if a satisfactory BCR is not generated during a finite period of time, the cell will likely undergo anergy or deletion (31). B cells can likely undergo 2-3 Igκ rearrangement attempts before initiating Igλ rearrangement, based on experiments using Igκ-null (Igκ enhancer-deleted) mice (7). The length of time during which the B cells can edit their

receptors during development, and potentially the alternative forms of tolerance employed depend on the strength and nature of the B cell interaction with Ag (73). Taken together, these studies suggest that deletion is a last resort, which initiates when other forms of tolerance fail to adequately censor the autoreactive cell.

Research Goals

The goal of this research is to elucidate how receptor editing and anergy play a role in maintaining tolerance in the developing B cell compartment among insulin-reactive B cells. Insulin is a key autoAg in T1D (140) and possesses unique antigenic features to other well-studied autoAgs such as DNA. BCR Tg models that exclude or allow tolerance through receptor editing are utilized to explore how various tolerance mechanisms act on developing anti-insulin B cells.

Anergy is known to function in the periphery to silence mature anti-insulin B cells, but the ontogeny of this critical process has not been identified. Chapter II sought to ascertain whether developing B cells are functionally silenced following insulin encounter, and to what extent they may be impaired. This was accomplished by analyzing functional responses in immature B cells which are known to be impaired in mature, anergic B cells. Immediate and downstream responses of naïve immature B cells following activation were compared with those of cells which had encountered insulin, thus allowing specific isolation of autoAg influence on the functional state of the cells. Study of replacement of the insulin specificity through receptor editing was impaired in this model, as the BCR Tg (125Tg) was non-Ig-targeted.

The role that receptor editing plays in establishing tolerance in a polyclonal repertoire has not been well characterized. Work highlighted in Chapter III thus attempts to provide evidence that insulin can drive this process *in vivo*. Anti-insulin B cells are readily detected in the periphery of VH125Tg NOD mice, in which endogenous IgLs pair with the anti-insulin VH125 to produce a polyclonal repertoire. This same population is not found in VH125Tg C57BL/6 mice, which do not develop type T1D. One potential explanation for this finding is that receptor editing effectively replaces the anti-insulin specificities that emerge in the non-autoimmune strain, but fails to fully abrogate such specificities in the autoimmune prone strain, perhaps due to incomplete tolerance induction.

The anti-insulin Vκ125 is not targeted to the Igκ locus in the current anti-insulin-restricted BCR Tg model used for studies in Chapter II, and the proportion of insulin-binding B cells generated in VH125Tg mice is very low (Chapter III). Ablation of autoreactivity through receptor editing is therefore difficult to study with current models. In Chapter IV, I therefore describe the development and characterization of a new Tg model in which the anti-insulin Vκ125 is targeted to the Igκ locus, and is thus subject to replacement if receptor editing is stimulated. These studies identify the role that insulin plays in initiating receptor editing, a critical manifestation of B cell tolerance.

The body of work presented in this thesis demonstrates a role for multiple arms of B cell tolerance in preventing insulin autoimmunity. The use of different BCR Tg models has provided evidence that a hierarchy of tolerance checkpoints function in healthy animals to abolish or silence insulin-reactive B cells in the developing repertoire, such that if B cells escape receptor editing, anergy serves as a second line of defense

against insulin autoreactivity. Such tolerance redundancy ensures that autoreactive specificities do not functionally participate in immune responses. These studies highlight that a protein hormone, which does not substantially cross-link the BCR and is present at a low circulating concentration range, can drive multiple forms of tolerance, and further characterization of how these checkpoints are dysregulated in autoimmune-prone animals will provide critical insight regarding points at which tolerance may be manipulated to enhance disease protection.

CHAPTER II

INITIATION AND MAINTENANCE OF FUNCTIONAL SILENCING IN IMMATURE ANTI-INSULIN B CELLS

Abstract

Mechanisms of B cell tolerance act during development in the bone marrow and periphery to eliminate or restrict autoreactive clones to prevent autoimmune disease. B cells in the spleens of mice that harbor anti-insulin BCR Tgs (125Tg) are maintained in an anergic state by endogenous hormone, but it is not clear when and where anergy is induced. An *in vitro* bone marrow culture system was therefore used to probe whether small protein hormones, a critical class of autoAgs, could interact with the BCR to induce anergy early during B cell development. Upon exposure to insulin, 125Tg immature B cells show hallmarks of anergy observed in mature spleen B cells, including BCR down-regulation, impaired proliferation to anti-CD40, and diminished Ca^{2+} mobilization following acute stimulation. Inhibition of calcineurin also results in reduced immature B cell proliferation, suggesting a potential mechanism through which reduced intracellular Ca^{2+} mobilization may be altering cellular proliferation. Signs of impairment appear after short-term exposure to insulin, which are reversible upon Ag withdrawal. This suggests that a high degree of functional elasticity is maintained at this stage, and that constant Ag engagement is required to maintain functional inactivation. These findings indicate that tolerance observed in mature, spleen 125Tg B cells is initiated by insulin in the developing B cell compartment, and thus highlight an important therapeutic window for the prevention of insulin autoimmunity.

Introduction

Preservation of tolerance in developing and mature B cell compartments is critical for the prevention of autoimmune disease. B cells that escape tolerance can generate pathogenic Abs to play a direct autoaggressive role, such as in SLE. They have additionally been shown to indirectly mediate disease, such as in T1D, in which they likely present autoAgs to autoaggressive T cells (174). Multiple routes therefore exist through which breaches in B cell tolerance can result in autoimmune disease. B cell tolerance is maintained by the mechanisms of anergy, receptor editing, and deletion (61, 64, 128, 130, 152, 187). Among these, anergy is known to function in the periphery to silence B cells that recognize soluble protein from actively participating in immune responses (40, 131, 160). Anergic B cells are functionally unresponsive to BCR stimulation, show impaired proliferation, compete poorly for follicular entry, have a reduced lifespan in the periphery, and fail to differentiate into Ab-secreting plasma cells (43). Anergy is a reversible form of tolerance that must be actively maintained by Ag availability (60, 63).

B cells in mice that harbor anti-insulin Tgs (125Tg) recognize endogenously circulating insulin and are rendered anergic in the periphery; thus, an Ag that interacts with the BCR with moderate affinity (8×10^6 M^{-1}) and possesses limited BCR crosslinking potential is capable of eliciting tolerance (160, 172). This form of anergy is characterized by impaired Ca^{2+} mobilization upon stimulation and is accompanied by diminished basal NFATc1 levels and defective NFATc1 translocation to the nucleus (2). Mature, anergic anti-insulin B cells show diminished proliferative responses to anti-IgM, anti-CD40, and LPS stimulation, produce little circulating Ab, and do not respond to T

cell-dependent immunization (1, 160). Isolation of naïve 125Tg cells is not possible in this model, as ablation of endogenous insulin would rapidly result in animal morbidity and mortality, due to the essential nature of the hormone. This considerable roadblock to the dissection of insulin-induced B cell tolerance can be overcome using an IL-7-driven *in vitro* culture system (16, 42, 122). Accordingly, this culture system was employed to identify the developmental ontogeny of anergy in anti-insulin B cells and to directly dissect how Ag (insulin) governs tolerance in immature B cells.

While many studies have mechanistically addressed how the anergic state exists in mature B cells, the induction of anergy in immature B cells has not been as well characterized. Previous studies in the Ars/A1 model show that autoreactive immature B cells maintain elevated basal levels of intracellular Ca^{2+} and impaired Ca^{2+} mobilization upon anti-IgM stimulation (13). Immature B cells which have encountered Ag show impaired Ca^{2+} mobilization and homing following stromal cell-derived factor-1 (SDF-1) stimulation, which is SHIP-dependent (22). Whether and how such changes may alter additional downstream functions of autoreactive immature B cells, such as proliferation are unknown, and the mechanism through which anergy is established in immature B cells has not been adequately addressed. Unlike mature B cells, immature B cell engagement with Ag stimulates tolerance, rather than proliferation (133, 195). To identify whether a small protein hormone can induce anergy in immature B cells and to further characterize the extent and nature of unresponsiveness, the phenotype and function of immature 125Tg B cells cultured with insulin were assessed.

We show that *in vitro*-derived anti-insulin immature B cells are naïve and flux Ca^{2+} following acute stimulation with insulin, anti-IgM, and ionomycin, and confirm that

they proliferate upon anti-CD40 stimulation. These responses are reduced in immature B cells that chronically engage insulin *in vitro*, suggesting that they acquire many hallmarks of the anergic phenotype observed for mature B cells. Similar to chronic insulin encounter, inhibition of calcineurin reduces the proliferation of 125Tg immature B cells, highlighting a potential mechanism through which the observed changes in intracellular Ca^{2+} mobilization may be negatively regulating cell cycle progression. These data suggest that anergic programming is initiated when developing B cells encounter insulin and is dynamically regulated by Ag availability. Insulin-reactive B cells are therefore precariously positioned to escape tolerance during physiological fluctuations of insulin and facilitate the development of autoimmunity. These studies highlight key aspects of developing B cell tolerance which must be considered to understand and prevent insulin autoimmunity.

Materials and Methods

Animals

The Ig Tg mice used in this study harbor non-targeted anti-insulin Ig Tgs (125Tg) or anti-HEL Tgs, IgHELMD4 on C57BL/6 backgrounds as described previously (backcrossed >20 generations to C57BL/6) (24, 160). IgHELMD4Tg mice were purchased from the Jackson Laboratory. All mice were housed under specific pathogen-free conditions, and all studies were approved by the Institutional Animal Care and Use Committee of Vanderbilt University.

Cell Culture

Bone marrow was eluted from the long bones of 4-6 wk old animals with Hank's balanced salt solution (HBSS) (Invitrogen Life Technologies) + 10% fetal bovine serum (FBS) (HyClone). Red blood cells (RBCs) were lysed and cells were resuspended at 2 x 10^6 cells/mL in complete bone marrow culture media [Dulbecco's modified eagle medium (DMEM) + 10% FBS (HyClone) + L-glutamine + HEPES buffer + MEM sodium pyruvate + gentamycin + 2 x 10^{-5} M 2-mercaptoethanol (2-ME) (Invitrogen)] + 20% J558L supernatant-derived interleukin 7 (IL-7) (IL-7 expressing J558L cell line kindly provided by John Cambier, National Jewish Medical and Research Center, Denver, CO) or 15 ng/mL human rIL-7 (Peprotech) and cultured for 5 d in a 37°C CO_2 incubator. FBS contains fg/mL amounts of insulin, which is below the threshold necessary to induce any B cell responsiveness (125Tg included) in all assays tested. To remove IL-7 and promote differentiation, 5 d cultures were washed 3 times with HBSS + 10% FBS and re-suspended at 2 x 10^6 cells/mL in complete bone marrow culture media without IL-7 and were cultured with or without cognate Ag [human insulin, (Sigma, cat. # I2643), or HEL, (Sigma)] for 1-3 d (see Fig. 2-2 assay schematic).

Bone Marrow Proliferation Assay

Bone marrow cells were harvested and grown in bone marrow culture media with IL-7 for 5 d as described above. Upon IL-7 removal, 0, 500, or 5 x 10^4 ng/mL human insulin (Sigma) was added to the culture. After 1 d, cells were recounted and plated at 2 x 10^{-5} cells/well in 96-well, flat-bottom plates (Corning) with the indicated concentrations of insulin and anti-CD40 (HM40-3; BD Pharmingen) "stimulation", in the

absence (unless otherwise noted) or presence of 1 μg/mL cyclosporin A (Alexis Biochemicals). In preliminary studies, 0.5 μg/mL anti-CD40 induced maximal immature B cell proliferation. After 1 d, wells were pulsed with 1 μCi of [^{3}H]thymidine (NEN) and grown for 1 d, at which point wells were harvested using a semi-automated cell harvester (Skatron). [^{3}H]Thymidine uptake was measured by scintillation counting after stimulation. Results are reported as the mean ± standard deviation (SD) for the average of triplicate determinations for each of the number of mice indicated.

Calcium Mobilization Assay

Bone marrow B cells derived from the *in vitro* IL-7 culture system described above or magnetic-activated cell separation (MACS)-purified CD43-depleted spleen B cells from WT, IgHELMD4Tg, and 125Tg mice were suspended in HBSS (Invitrogen Life Technologies) and plated on poly-D-lysine-coated glass bottom microwell dishes (MatTek Corporation) for 30 min at 37°C. Cells were loaded with 5 μM of the cell-permeant Ca^{2+}-sensitive dye Fura 2-AM (Molecular Probes). Spleen B cells were incubated for 30 min at room temperature, and bone marrow B cells were incubated for 15 min at 37°C, and were then washed in HBSS. The dishes were mounted on an inverted Nikon TE300 microscope enclosed in a humidified plexiglass chamber maintained at 37°C. Images were collected using a 40X/1.3 NA oil immersion Plan Fluor objective lens and a side-mounted CoolSNAP$_{HQ}$ camera. Fluorescence was monitored using dual excitation wavelengths (340/380 nm) and a single emission wavelength (510 nm). Fluorescence at 340 nm indicates dye bound to Ca^{2+}, while that at 380 nm corresponds to free dye. Basal readings were taken for 45 s prior to stimulation.

Cells were stimulated with the indicated concentrations of anti-IgM [F(ab')$_2$ goat anti-mouse μ-chain, (Jackson ImmunoResearch Labs, Inc.)], ionomycin (Sigma), human insulin (Sigma, I2643), or HEL (Sigma) at 37°C for the times indicated. Metamorph imaging software (Universal Imaging) was used for automated collection of images at either 2 or 5 s intervals over a 5 min period. During this time, cells maintained healthy morphology. Average fluorescence measurements of ≥ 30 cells were determined using Metamorph software (Fig. 2-5, A-D and F-G). Data are expressed as the ratio of bound/free Fura 2-AM fluorescence intensities.

Alternatively, 2 x 10^5 cells were plated per well in 96 well flat bottom black plates (Costar) and were resuspended in 100 μL loading buffer (HBSS + 25 μL DMSO + 1 vial Fura-2AM dye + 25 μL pluronic acid (Invitrogen) + 1 % FBS + 500 mM HEPES) containing the same concentration of insulin that was previously present. Cells were loaded for 30 min at 37°C and were then washed 3X with Wash Buffer (HBSS + 50mM HEPES + 1 % FBS). Cells were resuspended in 100 μL Wash Buffer containing the same concentration of insulin that was previously present. A FlexStation II fluorimeter (Molecular Devices) was used to measure triplicate wells (Fig. 2-5E, 2-7, and 2-8). Measurements were taken at 5 s intervals over a 5 min period, 50 μL 3X Stimuli were added to wells after 45 s of baseline readings were taken.

Flow Cytometry and Antibodies

Bone marrow cells were washed in 1X PBS containing 5% FBS, 0.02% EDTA, and 0.1% sodium azide and stained with the indicated mAb. Ab reagents were reactive with B220 (6B2), IgM^a (DS-1), IgM^b (AF6-78), CD43 (S7), CD21 (7G6), CD23 (B3B4),

AA4.1 (an immature/transitional B cell marker), or 7AAD (BD Pharmingen). Streptavidin reagents (Pharmingen) were used to detect biotinylated reagents. Analysis was performed on a FACSCalibur or LSR II flow cytometer (BD Biosciences). WinMDI 2.8 software (Dr. J. Trotter, Scripps Institute, San Diego, CA) was used for analysis.

Statistical Analyses

Two-tailed, expected mean, one sample t tests or two-tailed, two sample, unequal variance t tests were used as indicated to determine statistical significance.

Results

A homogenous population of immature anti-insulin B cells is generated using an IL-7-driven *in vitro* bone marrow culture system

Pro-B cell proliferation is enhanced by culture with IL-7, while B cells that have differentiated beyond this IL-7-responsive stage die, leaving only Ag-naïve B cells which subsequently differentiate to express surface BCR (16, 42, 122). 125Tg bone marrow cells were therefore cultured with IL-7 for a 5 d period, and B220 and IgM expression were assessed using flow cytometry after an additional 2 d IL-7 withdrawal period (Fig. 2-1). In freshly isolated bone marrow, ~5-10% of the cells are B220$^+$, which include various stages of B cell development (Fig. 2-1A). However, upon culture and subsequent IL-7 withdrawal, 70-80% of the cells recovered are B220$^+$, and > 90% of the B cells are IgMa$^+$ (Fig. 2-1, B-C). B cells generated *in vitro* phenotypically resemble immature B cells, as they are AA4.1$^+$, CD21$^-$, and CD23$^-$ throughout the duration of the culture (Fig. 2-1, D-F). This culture system therefore generates Ag-naïve immature B cells to permit

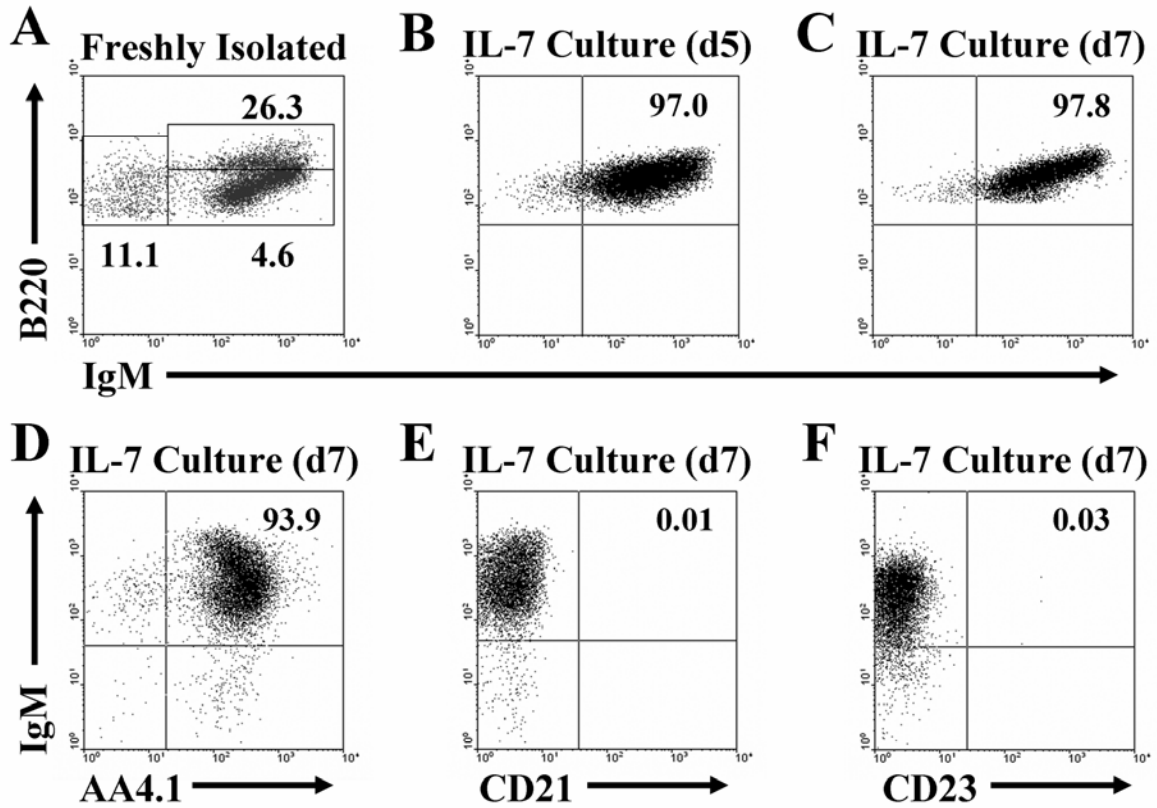


Figure 2-1. Immature anti-insulin B cells are generated *in vitro* using an IL-7-driven bone marrow culture system. Flow cytometry identified expression of B220, IgM, AA4.1, CD21, and CD23 on freshly isolated bone marrow cells (A), cells cultured with IL-7 for 5 d (B), or cells cultured with IL-7 for 5 d, then 2 d in the absence of IL-7 (C-F). (A-F) $B220^+$ $7AAD^-$ cells are shown. The IL-7-driven culture system used to generate naïve 125Tg immature B cells is shown in the schematic in Fig. 2-2A. All data are representative of > 8 experiments. The average ± SD of $B220^+$-gated cells is: (A) Pro and pre ($B220^+$ $IgMa^-$): 14.7 ± 10.6 %, immature ($B220^{mid}$ $IgMa^+$): 61.0 ± 10.2 %, mature recirculating ($B220^{high}$ $IgMa^+$): 24.4 ± 10.2 %, (B) $B220^+$ $IgMa^+$: 95.3 ± 6.1, (C) $B220^+$ $IgMa^+$: 97.9 ± 1.2 %, (D) $B220^+$ $AA4.1^+$: 91.3 ± 2.9 %, (E) $B220^+$ $CD21^+$: 0.03 ± 0.03 %, (F) $B220^+$ $CD23^+$: 0.20 ± 0.17 %.

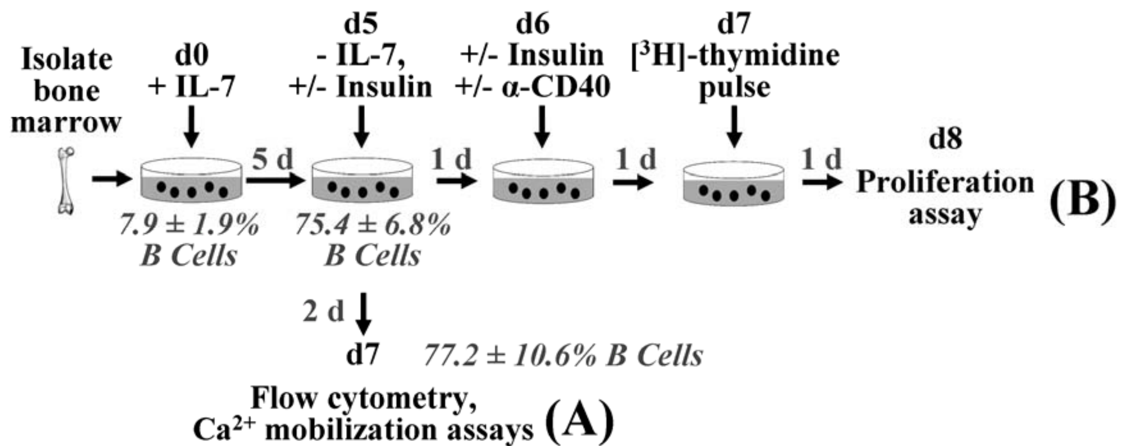


Figure 2-2. Schematic of IL-7-driven culture system and experimental design. Ag-naïve immature B cells are generated by culture with IL-7 for 5 d. IL-7 is then withdrawn and (A) cells differentiate for an additional 2 d in the absence or presence of Ag, after which flow cytometry analysis or Ca^{2+} mobilization assays are performed (Fig. 2-3, 2-5, and 2-6), or (B) cells differentiated in the absence or presence of insulin for 1 d, at which point cells are stimulated with anti-CD40 in the presence or absence of insulin for 1 d. Cells are then pulsed with [^{3}H]thymidine for 1 d, and harvested on d8, and [^{3}H]thymidine incorporation (counts per minute) is measured (Fig. 2-4, 2-7, and 2-8).

investigation of how insulin modulates tolerance. Subsequent assays were performed using IL-7-generated immature B cells, as outlined in Figure 2-2.

Immature anti-insulin B cells down-regulate B cell receptor surface expression upon interaction with cognate antigen

One hallmark of anergy in mature B cells is down-regulation of BCR surface expression (64). To identify whether anti-insulin 125Tg immature B cells (which are devoid of IgD) down-regulate the BCR upon encounter with insulin, immature 125Tg B cells were generated using the *in vitro* culture system described (Fig. 2-2A). After IL-7 withdrawal, 125Tg cells were cultured in the absence or presence of human or beef insulin, for which the 125Tg BCR has an affinity of 3×10^8 M^{-1} or 3×10^6 M^{-1}, respectively (172), and the relative level of IgM expressed on the surface was measured using flow cytometry. As indicated in Figure 2-3A, immature anti-insulin 125Tg B cells (white bars) show progressive down-regulation of surface BCR, with increasing Ag concentration leading to increased IgM down-regulation. This is BCR-specific and independent of biologic functions of the hormone, as anti-HEL "MD-4" (IgHELMD4Tg) immature B cells (black bars) cultured with insulin show no decrease in surface IgM. A similar reduction in surface Ig is observed in anti-HEL immature B cells cultured with HEL (data not shown). Culture of 125Tg immature B cells with beef insulin (gray bars) produced levels of IgM downregulation comparable to those observed following culture with human insulin (white bars), suggesting that the lower affinity interaction of beef insulin is also sufficient to drive this process (Fig. 2-3B). Importantly, differentiation of 125Tg B cells to the immature B cell stage is not inhibited by the presence of insulin, as the percentage of IgM^{a+} cells was unaffected (data not shown).

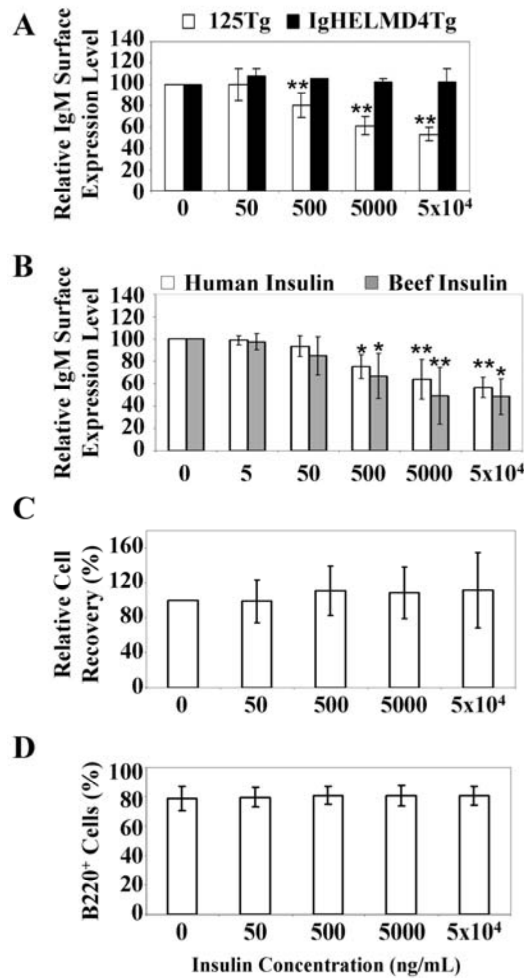


Figure 2-3. Immature anti-insulin B cells down-regulate surface IgM expression upon insulin encounter *in vitro* and do not undergo clonal deletion. (A-B) Flow cytometry to show IgM expression levels. The IgM mean fluorescence intensity (MFI) of cells cultured in the absence of Ag was normalized to 100% and relative IgM expression was determined for cells cultured with insulin (ex. 50 ng/mL insulin IgM MFI ÷ 0 ng/mL insulin IgM MFI x 100% = relative IgM expression). (A) Cells were cultured with human insulin. The average of 3 (IgHELMD4Tg, black) or 15 (125Tg, white) experiments is shown ± SD, (B) The average of 4 mice ± SD is shown, human insulin, white bars; beef insulin, grey bars. (C) Relative cell recovery in bone marrow culture was calculated to minimize experimental differences in IL-7 stimulatory potential by dividing the human insulin-cultured cell % recovery by the % recovery of cells cultured without insulin in the same experiment, % recovery was calculated by dividing the end cell number by the input cell number. The average of 18 experiments ± SD is shown. (D) Histograms showing percentages of $B220^+$ cells present at the end of the culture assessed using flow cytometry. The average of 17 experiments ± SD is shown. Ag-naïve immature B cells are generated as in Fig. 2-2A and cells are harvested after culture with 0 – 5 x 10^4 ng/mL insulin. * $p < 0.05$, ** $p < 0.005$, as calculated by a two-tailed, expected mean, one sample t test.

IgM surface downregulation, a phenotype of anergic B cells (64), is observed in 125Tg immature B cells cultured with insulin, indicating that B cell tolerance may be induced by this protein hormone. In addition to anergy, receptor editing or clonal deletion are also potential tolerance outcomes (61, 64, 128, 130, 152, 187). In the 125Tg model, the Ig Tgs are not targeted to the Ig loci, thus censoring of the autoreactive receptor through receptor editing cannot proceed in a physiological manner; however, deletion is possible. To establish whether clonal deletion was being triggered, total cell numbers were counted upon culture with insulin and the relative cell recovery was determined based on input cell numbers. Neither the relative cell recovery nor the percentage of B cells present upon culture with insulin was altered (Fig. 2-3, C-D); thus, encounter even with high concentrations of insulin does not induce deletion, as occurs with BCR crosslinking by anti-IgM stimulation (not shown). These findings demonstrate that the principal outcome of BCR engagement with insulin is to decrease surface BCR expression in 125Tg immature B cells, a phenotype typical of mature B cells that are rendered anergic.

Cognate antigen encounter impairs CD40-induced proliferation of immature anti-insulin B cells

Whereas BCR crosslinking does not stimulate proliferation of immature B cells (32, 201), other types of stimulation can drive this process. Simultaneous co-stimulation of the BCR and CD40 inhibits immature B cell apoptosis and allows immature B cells to overcome the block to cell cycle entry and are capable of proliferating (164, 169), and CD40 stimulation alone is sufficient to drive immature B cell proliferation (23). These findings suggest that T cell help signals can override negative signals stemming from

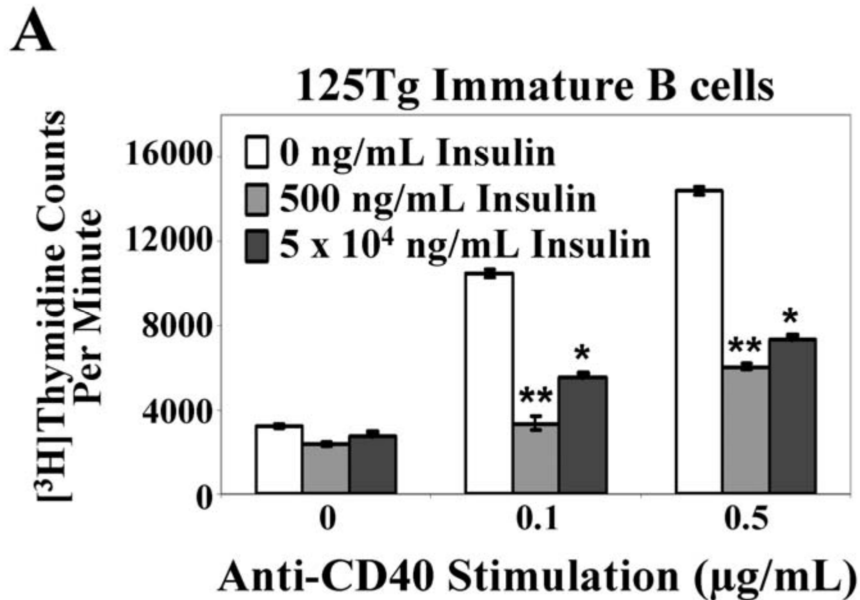
BCR signaling alone and thus may help to maintain relevant specificities in the repertoire for effective pathogen clearance.

125Tg immature B cells that encounter insulin show IgM down-regulation (Fig. 2-3), a phenotype consistent with cells inducing anergy (64). Anti-CD40 stimulation (which emulates T cell help), bypasses the BCR to induce immature and mature B cell proliferation (23), but this response is impaired when mature anti-insulin B cells are exposed to insulin *in vivo* (1). To identify whether a similar diminished responsiveness is evoked by Ag engagement of immature B cells, Ag-naïve immature B cells were generated using IL-7 culture, and were subsequently cultured for 1 d with or without insulin. Cells were then stimulated with anti-CD40 for an additional 2 d, during which the initial insulin concentration indicated remained present (see Fig. 2-2B for experimental design schematic). As shown in Figure 2-4, [^{3}H]thymidine uptake indicated that proliferation of 125Tg, but not control IgHELMD4Tg immature B cells was reduced ~2-3 fold upon stimulation with anti-CD40 in the presence of insulin. These data suggest that even modest concentrations of insulin are capable of downmodulating immature B cell proliferation when compared with naïve cells stimulated in the absence of cognate Ag. This interaction is BCR-specific and independent of any hormonal effects, as the presence of even the highest dose of insulin does not alter IgHELMD4Tg immature B cell proliferative responses.

Calcium mobilization is impaired in immature B cells upon insulin encounter

The intracellular Ca^{2+} concentration in a cell is tightly regulated and can be rapidly modulated by various stimuli to produce dynamic changes in a wide array of

A



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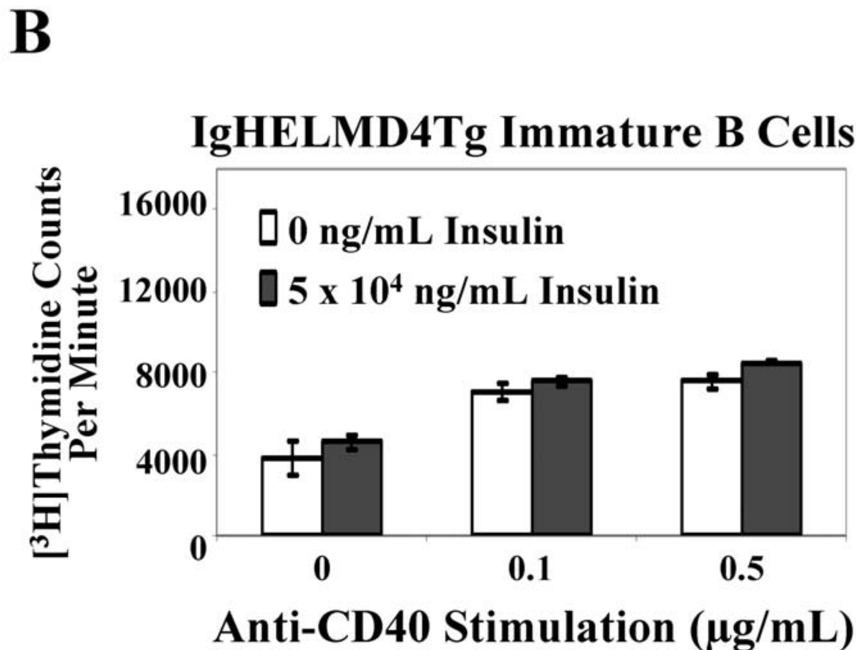

Figure 2-4. Immature anti-insulin B cells chronically cultured with insulin *in vitro* show impaired proliferation to anti-CD40. Immature B cell proliferation to anti-CD40 was assessed. 125Tg (A) or IgHELMD4Tg (B) cells were naïve (white bars) or cultured with human insulin (500 ng/mL, grey bars, or 5 x 10^4 ng/mL, black bars) for 1 d, then stimulated with CD40 (x-axis) in the presence of the indicated concentration of insulin. [^{3}H]thymidine incorporation (counts per minute) was measured. Bars represent the average of triplicate determinations of 3 animals, error bars represent the SD, * $p < 0.03$, ** $p < 0.007$, as calculated by a two-tailed, two sample/unequal variance t test. Immature B cells were generated as in Fig. 2-2B.

cellular processes, and altered Ca^{2+} homeostasis is associated with lymphocyte anergy (67). The 125Tg BCR interacts with insulin with a lower affinity and does not lead to substantial BCR crosslinking compared with other available Tg models of B cell tolerance (11, 172). We therefore tested whether insulin could elicit a sufficiently strong signal to evoke Ca^{2+} flux by measuring Ca^{2+} mobilization. Ag-naïve immature 125Tg B cells fluxed Ca^{2+} following acute insulin, but not HEL stimulation (Fig. 2-5A), and small responses have been observed with as little as 50 ng/mL insulin stimulation (not shown). Naïve IgHELMD4Tg immature B cells mobilized Ca^{2+} when stimulated with HEL as a positive control, but not insulin (Fig. 2-5B). These data suggest that a small protein hormone Ag can interact with the BCR to elicit changes in intracellular Ca^{2+} concentration and confirm that cells generated *in vitro* are naive.

The anergic phenotype observed in anti-insulin mature B cells includes an impaired ability to mobilize Ca^{2+} upon acute stimulation with ionomycin and suboptimal doses of anti-IgM (2). To determine whether a similar impairment in Ca^{2+} mobilization exists in immature B cells that have previously encountered insulin, Ag-naïve immature 125Tg B cells were generated *in vitro* and were cultured with or without insulin (Fig. 2-2A). Cells were then acutely stimulated with insulin, anti-IgM, or ionomycin and intracellular Ca^{2+} mobilization was measured. Immature 125Tg B cells that have previously encountered insulin showed an impaired ability to mobilize Ca^{2+} upon acute stimulation with insulin (Fig. 2-5D) or anti-IgM (Fig. 2-5E), similar to what is observed in mature 125Tg spleen B cells (Fig. 2-5C, (15)). As immature 125Tg B cells cultured with insulin downregulate surface IgM, the impaired Ca^{2+} flux that is observed may be due to reduced BCR molecules available for Ag engagement. Ionomycin was thus used

to bypass the BCR and stimulate intracellular Ca^{2+} mobilization. 125Tg and control IgHELMD4Tg naïve immature B cells produced a robust response of equivalent magnitude upon stimulation with ionomycin (Fig. 2-5F), which was diminished following culture with insulin (Fig. 2-5G). These findings suggest that immature B cells can be rendered unresponsive to BCR or ionomycin stimulation upon prior encounter with a small protein such as insulin, which has limited BCR crosslinking potential. This tolerant state mimics the state of altered intracellular Ca^{2+} homeostasis in mature tolerant anti-insulin B cells.

Calcineurin inhibition impairs immature B cell proliferation

Elevation of intracellular Ca^{2+} levels leads to enhanced calcineurin activity in mature lymphocytes, which can result in many downstream biological changes, including cell proliferation (67). To probe whether calcineurin activity affects immature 125Tg B cell proliferation, [^{3}H]thymidine uptake of immature B cells grown in the absence of insulin and stimulated with anti-CD40 was assayed (as in Fig. 2-2), in the presence or absence of cyclosporin A, a calcineurin inhibitor. As shown in Fig. 2-6, immature B cell proliferation to anti-CD40 stimulation was diminished in the presence of cyclosporin A. This impairment indicates that calcineurin is required for maximal immature B cell proliferation to anti-CD40. These findings suggest that calcineurin plays a role in 125Tg immature B cell proliferation to anti-CD40, and thus provide a potential mechanistic link between the diminished Ca^{2+} mobilization and impaired proliferation observed in 125Tg immature B cells which chronically engage insulin.

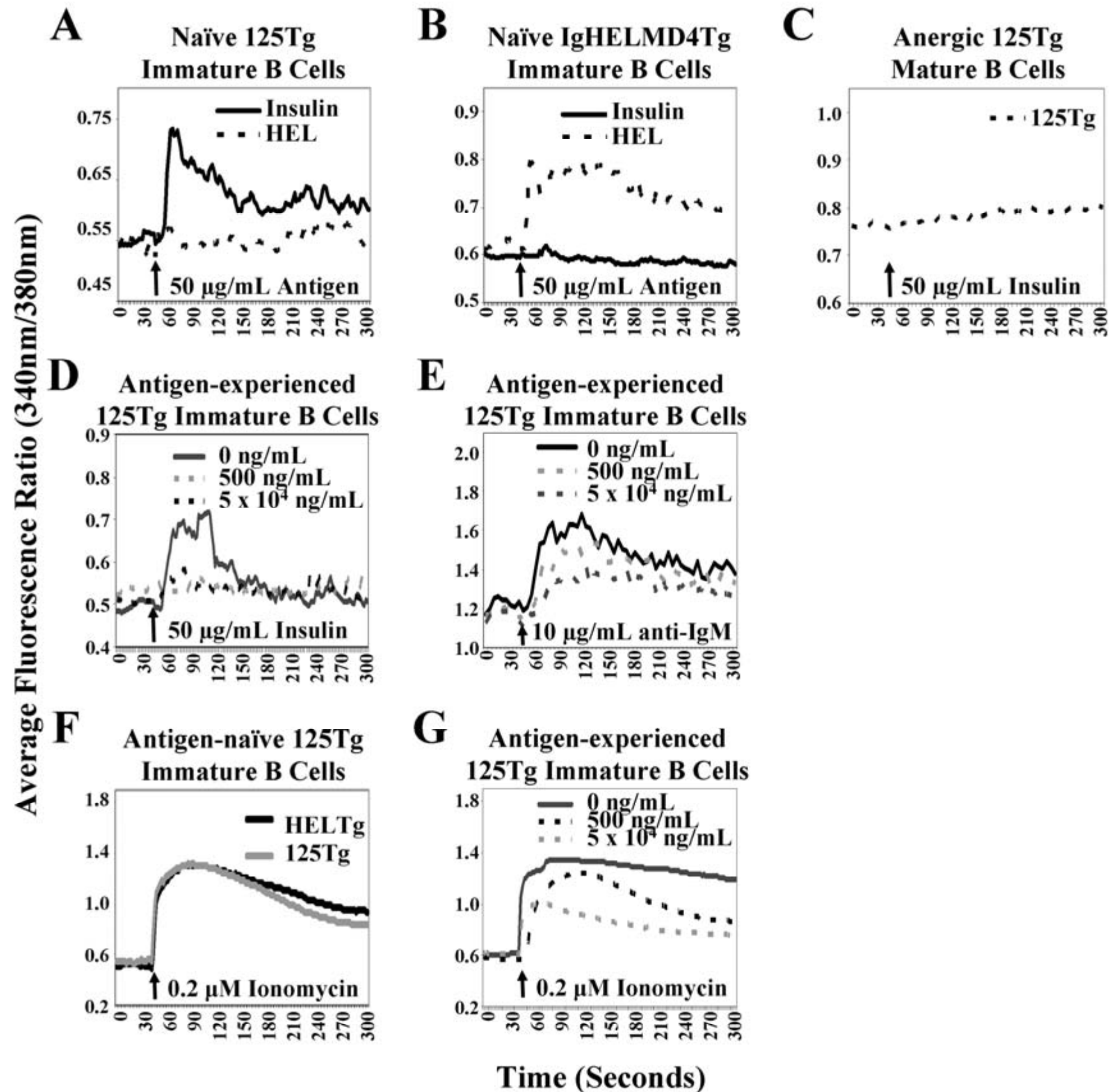

Figure 2-5. Calcium mobilization is impaired in anti-insulin immature B cells upon chronic insulin encounter. Intracellular Ca^{2+} mobilization was measured. Cells were loaded with fura 2-AM. Basal fluorescence readings are taken for 45 s, arrow indicates stimulation. 125Tg (A) or IgHELMD4Tg (B) naïve immature B cells (generated as in Fig. 2-2A) were stimulated with human insulin (solid line) or HEL (dashed line). (C) Mature, anergic 125Tg spleen cells were stimulated with human insulin. 125Tg naïve immature B cells were cultured ± human insulin (0 ng/mL: black solid line, 500 ng/mL: dashed black line, 5 x 10^4 ng/mL: dashed grey line) for 1 d and stimulated with human insulin (D), anti-IgM (E), or ionomycin (G). (F) 125Tg (grey line) or IgHELMD4Tg (black line) naïve immature B cells were stimulated with ionomycin. Data are representative of 3 or more experiments.

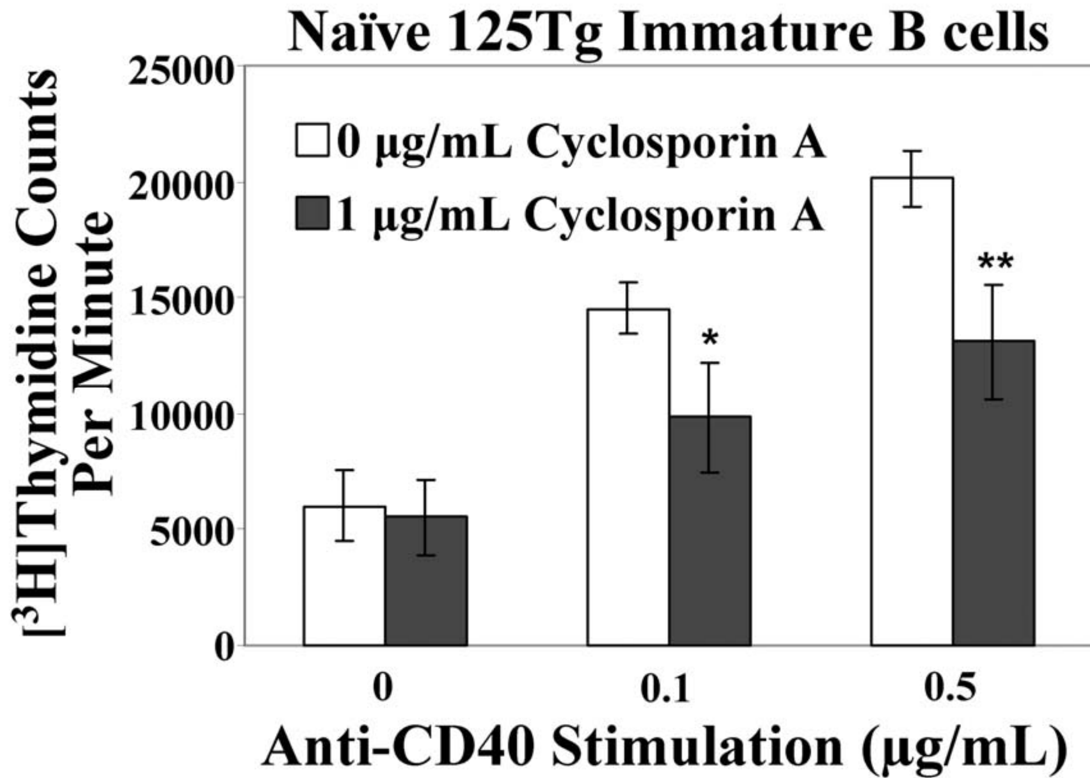

Figure 2-6. Calcineurin inhibition impairs immature anti-insulin B cell proliferation to anti-CD40 stimulation. Proliferation to anti-CD40 was measured. 125Tg immature B cells were cultured in the absence of insulin for 1 d, then stimulated with CD40 (x-axis) in the absence (white bars) or presence of 1 µg/mL cyclosporin A (black bars). [^{3}H]thymidine incorporation (counts per minute) was measured. Bars represent the average of triplicate determinations of 4 animals, error bars represent the SD, * $p < 0.021$, ** $p < 0.0048$, as calculated by a two-tailed, two sample/unequal variance t test. Immature B cells were generated as in Fig. 2-2B.

Extended exposure of 125Tg immature B cells to insulin is not required to observe IgM downregulation, impaired calcium mobilization, or diminished proliferation

In the previous experiments, insulin (if added) was present continuously throughout the multi-day culture period. To determine if signs of tolerance were present after short-term culture with insulin, immature anti-insulin (125Tg) B cells were cultured for 0.5-5 h with or without insulin after IL-7 withdrawal and 1 d without insulin. Flow cytometry analysis indicated that IgM expression decreased after 0.5 h of culture with insulin, and continued to decrease with time (grey and black bars, Fig. 2-7A). 125Tg immature B cells also showed impaired Ca^{2+} mobilization upon acute insulin or anti-IgM stimulation following culture with insulin for 3 h (earlier timepoints were not assessed) (Fig. 2-7, B-C). To test whether primary insulin co-exposure with anti-CD40 stimulation is sufficient to impair proliferation, Ag-naïve 125Tg immature B cells were cultured for 1 d in the absence of insulin (Fig. 2-2B). Anti-CD40 and insulin were then simultaneously added and the cells were cultured for an additional 2 d, prior to measuring [^{3}H]thymidine uptake. Proliferation was dampened in cells which had encountered insulin concurrent with anti-CD40 stimulation (Fig. 2-7D), suggesting that an extended period of "anergic reprogramming" prior to anti-CD40 stimulation is not required for insulin to impair proliferation.

Maintenance of immature anti-insulin B cell anergy requires continuous antigen presence

To identify whether anergy in immature anti-insulin B cells requires continuous Ag exposure, 125Tg B cells were cultured with or without insulin (Fig. 2-2A) and relative IgM expression (Fig. 2-8A) and Ca^{2+} mobilization (Fig. 2-8, B-C) were assessed.

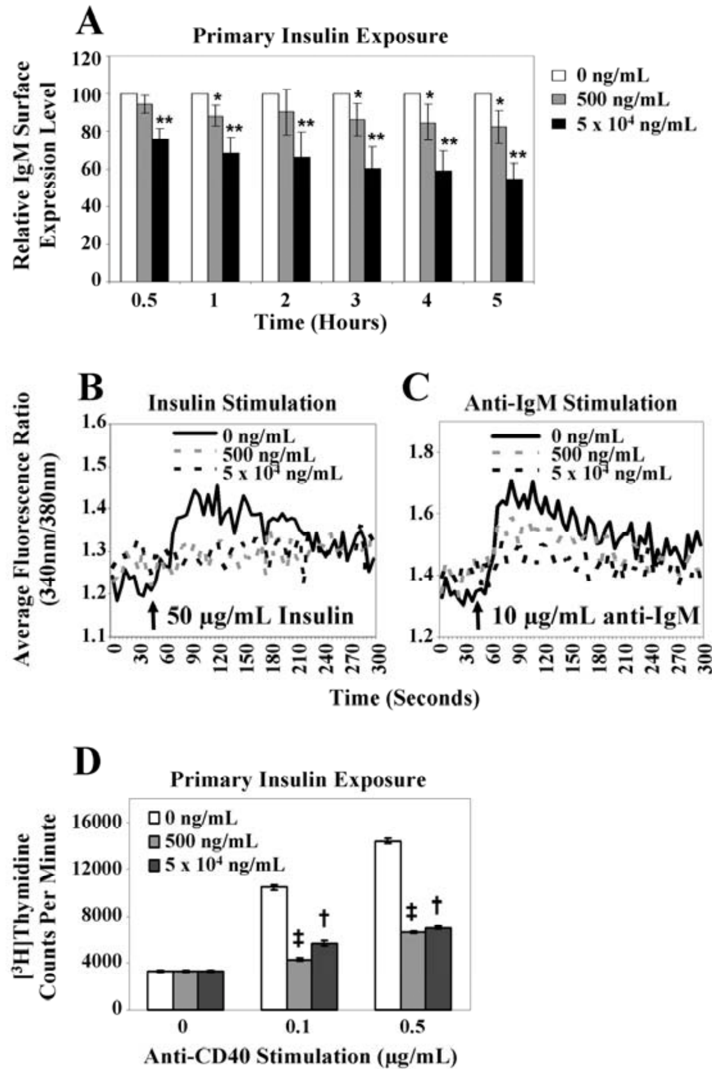

Figure 2-7. Functional impairment is observed in anti-insulin B cells after short-term exposure to insulin. Naïve immature 125Tg B cells were generated (as in Fig. 2-2). (A) Flow cytometric analysis showing relative IgM expression levels on 125Tg cells cultured for 0.5-5 h in the presence of human insulin, as calculated in Fig 2-3A. The average of 7 animals ± SD is shown, * $p < 0.01$, ** $p < 0.007$, as calculated by a two-tailed, expected mean, one sample t test. (B-C) Intracellular Ca^{2+} mobilization was measured in cells cultured for 1 d with no insulin, followed by 0 ng/mL (solid black line), 500 ng/mL (dashed grey line), or 5 x 10^4 ng/mL human insulin (dashed black line) for 3 h and stimulated with human insulin (B) or anti-IgM (C). Data are representative of > 5 animals. (D) [^{3}H]thymidine incorporation (counts per minute) was measured. Following culture without insulin for 1 d, anti-CD40 (x-axis) and 0 ng/mL (white bars), 500 ng/mL (grey bars), or 5 x 10^4 ng/mL (black bars) human insulin were added simultaneously and the cells were cultured for an additional 2 d. The average of triplicate values from 3 animals ± SD is shown, † $p < 0.031$, ‡ $p < 0.001$, as calculated by a two-tailed, two sample/unequal variance t test.

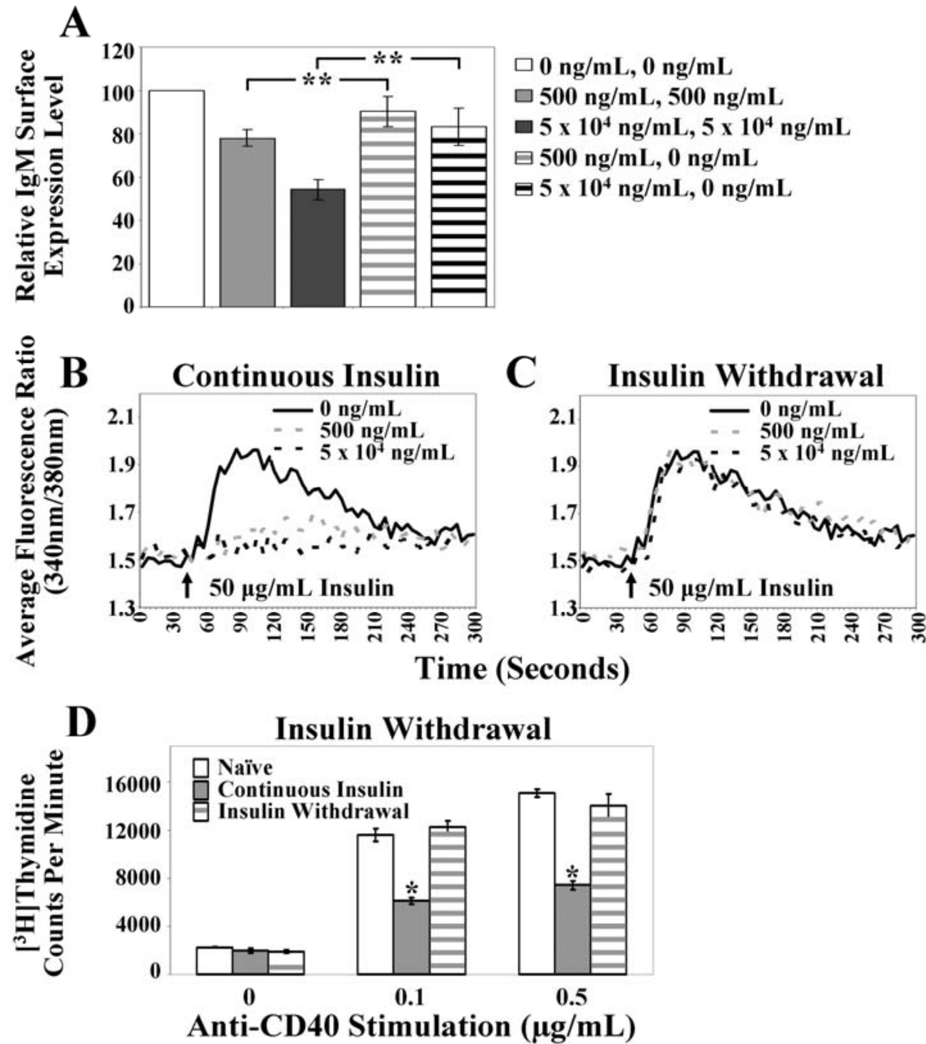

Figure 2-8. Functional impairment of 125Tg immature B cells is highly reversible. 125Tg immature B cells were cultured (Fig. 2) with continuous Ag (d1-d2) or Ag was withdrawn from the culture (Ag added d1, removed d2), IgM down-regulation, calcium mobilization, and proliferation were assessed. (A) Flow cytometric analysis showing relative IgM expression levels, as calculated in Fig 2-3A. The average of 8 animals ± SD is shown. (B-C) Intracellular Ca^{2+} mobilization was measured in cells cultured with 0 ng/mL (solid black line), 500 ng/mL (dashed grey line), or 5 x 10^4 ng/mL human insulin (dashed black line) for 2 d (continuous insulin presence, B), or 1d, followed by antigen removal and 1 d additional culture in the absence of insulin (insulin withdrawal, C) and stimulated with human insulin. Data are representative of 4 animals. (D) [^{3}H]Thymidine incorporation (counts per minute) was measured. 500 ng/mL human insulin was absent for 3 d (solid white bars), continuously present for 3 d (solid grey bars), or added for 1 d and then removed for 2 d (striped grey bars), cells were stimulated with anti-CD40 (x-axis) for the final 2 d period. The average of triplicate values ± SD is shown, data are representative of 2 animals. A two-tailed, two sample/unequal variance t test was used to calculate significance for all bar graphs, * $p < 0.001$, ** $p < 0.0007$.

Continuous Ag was present (grey and black solid bars, Fig. 2-8A, or grey and black dashed lines, Fig. 2-8B) or Ag was removed (grey and black striped bars, Fig. 2-8A, or grey and black dashed lines, Fig. 2-8C) and cells were cultured for an additional day. Removal of Ag for 1 d (striped bars) resulted in restoration of relative surface IgM expression compared to continuous Ag presence (solid bars, Fig. 2-8A). Insulin withdrawal also restored the ability of cells to mobilize Ca^{2+} upon acute stimulation with insulin (Fig. 2-8C). Using a similar approach, withdrawal of insulin upon addition of anti-CD40 resulted in reversion of the level of proliferation to the levels found in naïve cells (Fig. 2-8D). Together, these data indicate that immature B cells retain a high level of functional elasticity, initiating or suppressing the anergic phenotype based on Ag availability.

Discussion

Using IL-7-driven bone marrow culture, we find that naïve immature B cells can be generated that mobilize Ca^{2+} upon insulin stimulation and proliferate upon CD40 engagement. However, immature 125Tg B cells exposed to insulin adopt many of the tolerant phenotypes observed in mature 125Tg spleen B cells, including surface IgM down-regulation, reduced Ca^{2+} mobilization upon stimulation with insulin, anti-IgM, or ionomycin, as well as impaired proliferative responses to anti-CD40 stimulation. This functional impairment is induced in an Ag-specific fashion, and is not due to the hormonal activity of insulin. These findings therefore suggest that a small protein hormone, such as insulin, is capable of altering the biological responsiveness of immature bone marrow B cells in a way that closely resembles the anergic phenotype observed in

mature B cells. This initial phase of anergy induction likely serves as a critical line of defense against autoaggressive B cells present in the developing repertoire.

Tonic signaling emanating from BCR surface expression is critical for B cell development to proceed (90, 117, 176). A lack of positive signaling due to BCR down-regulation induces receptor editing, another arm of developing B cell tolerance (171). The 125Tg non-targeted transgenic model is not ideal to study how this process physiologically proceeds; therefore a targeted model was generated and studies addressing whether receptor editing is involved in maintaining tolerance for insulin are described in Chapter IV. Whether or not frustrated editing attempts are made, anergy is still induced in 125Tg immature B cells to effectively censor self-reactivity. We hypothesize that the IgM down-regulation seen in 125Tg immature B cells cultured with insulin may similarly diminish the amount of positive signaling generated, which may in part account for the reduced Ca^{2+} mobilization observed. Culture with 500 ng/mL insulin results in a modest reduction of surface IgM (Fig. 2-3A), yet results in more substantial impairment in Ca^{2+} mobilization (Fig. 2-5, D-E). Thus, even a modest reduction in surface IgM expression may correlate with biologically relevant functional outcomes, and highlights the exquisite sensitivity that immature B cells possess to detect cognate Ag. Additionally, ionomycin responsiveness, which bypasses the BCR, is also impaired (Fig. 2-5G), supporting that functional impairment involves changes in the way cells manage intracellular Ca^{2+} increases, and is not solely due to reduced Ag engagement of surface BCR.

Intracellular Ca^{2+} increases up-regulate calcineurin activity, which dephosphorylates NFAT to promote its nuclear translocation to induce proliferation in

mature lymphocytes (67). Calcineurin inhibition results in decreased 125Tg immature B cell proliferation to anti-CD40 (Fig. 2-6). We therefore propose a model in which immature 125Tg B cells chronically engaging insulin enter an impaired state by down-regulating the BCR. This limits the magnitude of intracellular Ca^{2+} increases derived from tonic BCR signaling, to diminish calcineurin activity. The magnitude of cell proliferation to anti-CD40 stimulation is therefore impaired, due to this reduction in tonic signaling. This may be achieved by reducing nuclear translocation of NFAT, a well-known target of calcineurin (67), which occurs in mature, anergic 125Tg B cells (2). Alternatively, calcineurin has also been shown to dephosphorylate cell cycle proteins, such as CDK4 (10), and calcineurin inhibition inactivates CDK2 in late G1 or S phase due to p27 accumulation (82), thus highlighting another potential method of cell cycle control.

BCR downregulation and impaired Ca^{2+} mobilization are observed after 3 h insulin exposure (Fig. 2-7, A-C), and immature B cells simultaneously exposed to insulin and anti-CD40 show impaired proliferative responses (Fig. 2-7D). Any transcriptional changes required to suppress proliferation appear to be able to occur concomitantly with those that support proliferation to result in a dominant anti-proliferative effect. These findings raise the possibility that the anergic state observed does not require substantial prior genetic reprogramming. Ca^{2+} is required for cell cycle progression (82); thus it seems likely that the impaired Ca^{2+} mobilization observed is linked to the downstream reduction in proliferative responses. Immature anti-insulin B cell anergy is reversible upon Ag withdrawal, as BCR surface expression, Ca^{2+} mobilization, and proliferation to anti-CD40 stimulation return to levels observed for naïve cells (Fig. 2-8, A-D), which is

also consistent with the highly sensitive and responsive qualities of intracellular Ca^{2+} mobilization.

The anti-insulin Ig Tg model (125Tg) enables the study of how tolerance is initiated and maintained for physiologically relevant proteins such as hormones which have low-valency interactions with the BCR, and highlights a different form of anergy induction from what is observed in other models of tolerance. *In vitro* culture with IL-7 generates naïve immature B cells not accessible *in vivo*. The affinity of the 125Tg BCR for human insulin (used in *in vitro* assays due to high purity) is 3×10^8 M^{-1} (172), which contrasts with the more than 100-fold higher affinity of IgHELMD4Tg for HEL (5×10^{10}, (11)), the only other low-valency model of B cell anergy. Comparable IgM surface down-regulation is observed following culture with beef insulin (Fig. 2-3B) which has a lower affinity (3×10^6 M^{-1}) than rodent insulin (8×10^6 M^{-1}) (172). If the reduction in positive signaling from surface BCR is responsible for the tolerance phenotype observed in immature 125Tg B cells cultured with human insulin, this suggests lower affinity species of insulin will also elicit anergy. The concentration of insulin present *in vitro* (50 – 5×10^4 ng/mL) is higher than the circulating concentration of insulin *in vivo* (1 ng/mL), which can increase 5-10 fold based on physiologic demand (56). However, BCR internalization of insulin *in vitro* reduces the concentration of Ag available throughout the culture duration, whereas insulin homeostasis *in vivo* is tightly regulated. The initial insulin concentration present is well below the concentration at which insulin forms dimers (100 μg/mL) (17). Immature B cells show a decreased threshold for activation through the BCR compared with mature B cells (reviewed in 85), thus it seems plausible that endogenous levels of rodent insulin, which are sufficient to occupy virtually all of

the surface BCR and induce anergy in mature 125Tg B cells *in vivo* (160), would be capable of inducing anergy in immature B cells as well. As little as 50 ng/mL insulin can mobilize Ca^{2+} in immature B cells (not shown), consistent with highly sensitive biological responses occurring with low concentrations of Ag.

Insulin is a key autoAg in T1D (206), and its availability in the bloodstream fluctuates hourly with physiological responses to increases in blood glucose (56), unlike other autoAgs which may be present at more constant levels. This may therefore represent an important consideration in understanding fully how tolerance is maintained for protein hormones, as this oscillation may allow a brief window for some autoreactive cells to escape into the periphery. The functionality of tolerance at multiple checkpoints is thus likely to be critical for autoimmune disease prevention, particularly with respect to this category of autoAgs. Developing a better understanding of how cells can become functionally silenced may aid in the identification of novel therapeutic targets for the treatment of autoimmune disease and graft rejection. The site and stage of tolerance induction is important in the consideration of designing novel therapeutics, and these studies highlight an important developmental stage for potential therapeutic intervention in autoimmune disease.

CHAPTER III

INSULIN INDUCES CENTRAL TOLERANCE IN A DEVELOPING POLYCLONAL B CELL REPERTOIRE

Abstract

Loss of immune tolerance in the B cell compartment accompanies many autoimmune disorders, including T1D. Peripheral tolerance for insulin is maintained by anergy, however regulation by central tolerance mechanisms has not been assessed. We find that B cells harboring an anti-insulin IgH Tg (VH125Tg) paired with targeted IgL Tgs show evidence of receptor editing *in vivo*. Insulin-binding B cells develop in bone marrow, but not in spleen polyclonal repertoires of VH125Tg C57BL/6 non-autoimmune disease-prone mice. In contrast, T1D-prone VH125Tg NOD mice generate anti-insulin B cells in the spleen, which are increased compared with the bone marrow. VH125Tg NOD bone marrow B cells cultured *in vitro* in the absence of Ag generate an increased percentage of insulin-specific B cells compared with VH125Tg C57BL/6 cells. B cells possessing VH125 (compared with non-insulin-specific VH281) paired with endogenous IgLs (VH125Tg and VH281Tg, respectively) show increased rag-2 transcript levels, consistent with increased receptor editing. These data suggest that insulin elicits central tolerance in developing insulin-specific B cells present in a polyclonal repertoire *in vivo*, which is dysregulated in the T1D-prone NOD strain. This is compounded by the enhanced potential of the NOD IgL repertoire to generate an autoreactive BCR. Multifactorial differences may therefore contribute to the development and allowance of insulin-specific B cells in the periphery of NOD mice.

Introduction

Breaches in B cell tolerance precede the development of many autoimmune diseases. Autoreactive B cells can directly mediate tissue damage when they differentiate and produce pathogenic autoAbs, such as anti-DNA Abs in SLE, or play an indirect role, as suggested for T1D, where B cells capture and present key self Ag to autoaggressive T cells (174). The mechanisms that normally remove autoreactivity include anergy, receptor editing, and deletion (61, 64, 128, 130, 152, 187). The bulk of studies describing the role of tolerance mechanisms in B cell development were made possible by Ig Tg models, as tracking the fate and function of Ag-specific B cells is facilitated in a restricted repertoire. Using this approach, we have shown that anti-insulin 125Tg B cells in the peripheral repertoire are anergic (160). In addition, the anti-insulin VH transgene (VH125Tg) paired with endogenous IgLs generates a detectable population of peripheral B cells that bind insulin in autoimmune-prone NOD mice; however similar B cells are not found in the periphery of C57BL/6 mice (78, 197).

The failure to detect insulin-binding B cells in VH125Tg C57BL/6 mice (197) raises the possibility that central tolerance may effectively cull these specificities in the non-autoimmune strain, either through receptor editing, deletion, or altered peripheral survival associated with anergy. Additionally, it has been previously shown that polymorphisms exist in NOD IgLs which are conserved among other autoimmune-prone strains and differ from non-autoimmune strains (197). It therefore seems plausible that the IgL repertoire of the NOD strain that pairs with VH125Tg may be better suited to generate an insulin-binding BCR. Whether central tolerance is elicited for insulin, which is impaired in NOD mice, and/or whether enhanced generation of insulin-binding B cells

is responsible for the presence of these cells in the periphery of NOD mice has not been addressed.

We find that B cells harboring anti-insulin VH125Tg paired with multiple targeted IgLs undergo receptor editing and replace the targeted IgL. Evidence for receptor editing in a polyclonal repertoire is also provided in C57BL/6 mice; rag-2, required for receptor editing, is increased in VH125Tg compared to non-insulin-binding VH281Tg animals, suggesting that an insulin-specific IgH might stimulate receptor editing. Further, insulin-binding B cells are detected in the developing, but not peripheral repertoire of VH125Tg C57BL/6 mice *in vivo*. This contrasts with T1D-prone VH125Tg NOD mice, which positively select for insulin-specific B cells in the periphery from the developing repertoire. An increased frequency of insulin-binding B cells develop *in vitro* in the absence of Ag in VH125Tg NOD mice. These data suggest that the IgL repertoire of NOD mice is more permissive for the generation of insulin-binding B cells when VH125 is present, and that flaws in central tolerance may seed autoreactive specificities in the repertoire of the autoimmune strain that are normally removed early in development.

Materials and Methods

Animals

The VH125Tg (anti-insulin), VH281Tg (non-insulin specific), and 125Tg (insulin-binding) mice used in this study harbor non-targeted Tgs on C57BL/6 or NOD backgrounds as described previously (81, 160, 186). IgHELMD4Tg mice that harbor anti-HEL BCRs (24) were purchased from The Jackson Laboratory. Previously

described Vκ1ki (Vκ1-110-Jκ1), Vκ4ki (Vκ4-81/Jκ4), and Vκ8ki (Vκ8-19/Jκ5) mice were kindly provided by Dr. Martin Weigert (University of Chicago) (34, 150). Vκ1ki, Vκ4ki, or Vκ8ki mice were intercrossed with VH125Tg animals to generate VH125Tg/Vκ1ki, VH125Tg/Vκ4ki, or VH125Tg/Vκ8ki mice, respectively. All data are derived from lines that have been backcrossed >20 generations to C57BL/6 or NOD, and which are hemizygous for all Tgs indicated. All mice were housed under specific pathogen-free (C57BL/6) or sterile housing (NOD) conditions, and all studies were approved by the Institutional Animal Care and Use Committee of Vanderbilt University.

Flow Cytometry

Flow cytometry analysis was performed using a FACSCalibur flow cytometer or LSRII (BD Biosciences). Ab reagents (BD Pharmingen): B220 (6B2), IgM^a (DS-1), IgM^b (AF6-78), and CD43 (S7). Insulin (Sigma) was biotinylated at pH 8.0 in bicine buffer using biotin N-hydroxysuccinimide ester (Sigma) and detected with streptavidin (BD Pharmingen). WinMDI 2.8 software (Dr. J. Trotter, Scripps Institute, San Diego, CA) was used for analysis.

Determination of Vκ Usage

Spleen cells from 6-10 individual animals were positively selected with IgM^a-biotin (BD Pharmingen, DS-1) and streptavidin magnetic beads (Miltenyi) through autoMACS sorting (Miltenyi) to >95% IgM^{a+} cell purity. RNA was isolated using the Tri Reagent manufacturer's instructions (Molecular Research Center). First strand cDNA was generated from total RNA using Superscript II RT (Invitrogen) and 0.67 μg oligo-dT

primer (Amersham Biosciences) in a standard protocol. Vκ sequences were amplified from first strand cDNA using the following primers: murine Cκ primer - 5'GGA TAC AGT TGG TGC AGC ATC and murine VκA - 5'ATT GTK MTS ACM CAR TCT CCA, where K=G or T, M=A or C, S=C or G, R=A or G. Vκ sequences were amplified using AmpliTAQ DNA Polymerase (2U/reaction) (Applied Biosystems), 200 nM dNTP, 1.25 mM $MgCl_2$, 13.35 mM Cκ primer and VκA primer; 94°C/1 min, 42°C/1 min, and 72°C/1 min for 40 cycles. PCR product was ligated into pGEM-T Easy Vector System I plasmid (Promega). Clones were sequenced using an Applied Biosystems 3730xl DNA Analyzer (Vanderbilt-Ingram Cancer Center, Nashville, TN). Vκ gene segment sequence alignments were assigned using the ImMunoGeneTics (IMGT) database (www.imgt.cines.fr:8104/). Individual clones are each derived from independent pools of RNA.

Real-Time Polymerase Chain Reaction (PCR)

RNA was prepared from freshly isolated bone marrow from VH125Tg or VH281Tg C57BL/6 mice using Tri Reagent according to the manufacturer's instructions (Molecular Research Center). First strand cDNA was generated as for Vκ usage analysis (above). Resultant cDNA template was used in real-time PCR with the following primers to amplify rag-2 and cd19 (55°C annealing temperature): rag-2 forward (FWD): 5'CACATCCACAAGCAGGAAGTACAC, rag-2 reverse (REV): 5'GGTTCAGGGACATCTCCTACTAAG, cd19 FWD: 5'GTGGCCAAAGCGTCCATC, cd19 REV: 5'GAGAGCACATTCCCGTAC, rag-2 transcript levels were normalized to cd19.

Cell Isolation and Culture

Bone marrow was eluted from femurs, tibias, and humeri of 4-6 week old animals with HBSS (Invitrogen) + 10% FBS (HyClone). RBCs were lysed and cells were stained for flow cytometry analysis or resuspended at 2 x 10^6 cells/mL in DMEM + 10% FBS + L-glutamine + HEPES + MEM sodium pyruvate + gentamycin + 2 x 10^{-5} M 2-ME (Invitrogen) + 20 % J558L supernatant-derived IL-7 or 15 ng/mL human rIL-7 (Peprotech) and cultured for 5 d in a 37 ^oC CO_2 incubator (IL-7 expressing J558L cell line kindly provided by John Cambier, National Jewish Medical and Research Center, Denver, CO). To remove IL-7, 5 d cultures were washed with HBSS + 10% FBS and were resuspended at 2 x 10^6 cells/mL in culture media without IL-7 and grown for an additional 2 d, at which point cells were harvested and stained for flow cytometry analysis. Spleens were harvested and RBCs were lysed using Tris-NH_4Cl. Cells were resuspended and stained for flow cytometry.

Statistical Analyses

Two-tailed, two sample, unequal variance t tests were used as indicated to determine statistical significance.

Results

Transgene-associated developmental acceleration through the IgM^- stage is not observed in VH125Tg/Vκki B cells

Non-Ig-targeted BCR Tg models (e.g. 125Tg) do not permit the study of how receptor editing physiologically proceeds, as this process is directed at the IgL loci. To

directly test whether B cells harboring anti-insulin VH125 undergo receptor editing, VH125Tg mice were intercrossed with mice harboring Igκ-targeted Vκ transgenes to generate three lines, VH125Tg/Vκ1ki, VH125Tg/Vκ4ki, and VH125Tg/Vκ8ki. Vκ1ki, Vκ4ki, and Vκ8ki Tgs are useful in this regard because their ability to undergo receptor editing when paired with an anti-DNA IgH has been well examined in elegant studies on anti-DNA specific BCRs (150, 199). In addition, a prior study in NOD found that VH125 can pair with closely related Vκ1-110 (Vκ1ki) and Vκ4-81 (Vκ4ki) genes (197). For ease, Tg models utilized are described in Table 3-1.

The pre-B cell compartment of mice that harbor Ig Tgs is greatly reduced in the absence of autoAg compared with WT mice; however this compartment is restored if the autoAg is present, in concert with receptor editing induction (135, 145). To identify whether a population of pre-B cells is present in VH125Tg animals harboring targeted Igκs, freshly isolated bone marrow was analyzed using flow cytometry. Figure 3-1 shows pro/pre ($B220^{+}$, IgM^{-}), immature ($B220^{mid}$, IgM^{+}), and mature recirculating ($B220^{high}$, IgM^{+}) B cell populations in these lines. The distribution of these compartments in VH125Tg/Vκki Tgs (Fig. 3-1, B-D) are compared to 125Tg (Fig. 3-1E) or IgHELMD4Tg (Fig. 3-1F) (anti-insulin or anti-HEL, respectively). As expected, 125Tg B and IgHELMD4Tg, which possess non-Ig-targeted Tgs, show accelerated development to the immature B cell stage (Fig. 3-1, E-F). In contrast, all three VH125Tg/Vκki targeted Tg lines (Fig. 3-1, B-D) show a four-fold increase in the IgM^{-} compartment when compared to 125Tg or IgHELMD4Tg B cells (Fig. 3-1, E-F). The distribution of B cell development in each VH125Tg/Vκki line more closely resembles VH125Tg, in which endogenous light chains must undergo rearrangement (Fig. 3-1A).

Table 3-1. Genotype Nomenclature for Heavy and Light Chain Transgene(s) Present

Genotype	Heavy Chain Transgene	Light Chain Transgene
WT	None - endogenous	None - endogenous
VH125Tg	Non-targeted, anti-insulin	None - endogenous
VH281Tg	Non-targeted, non-insulin-specific	None - endogenous
VH125Tg/Vκ1ki	Non-targeted, anti-insulin	Targeted, anti-DNA
VH125Tg/Vκ4ki	Non-targeted, anti-insulin	Targeted, anti-DNA
VH125Tg/Vκ8ki	Non-targeted, anti-insulin	Targeted, anti-DNA
125Tg	Non-targeted, anti-insulin	Non-targeted, anti-insulin
IgHELMD4Tg	Non-targeted, anti-HEL	Non-targeted, anti-HEL

HEL: Hen egg lysozyme

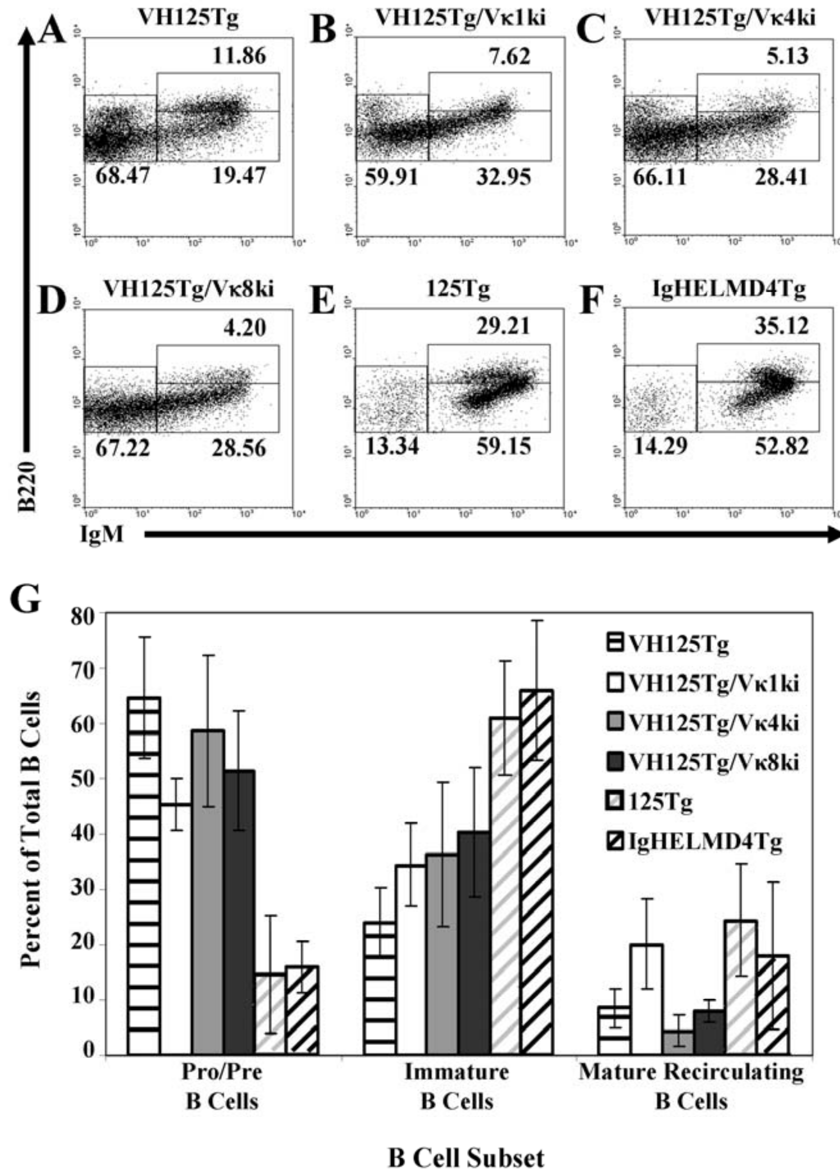

Figure 3-1. **VH125Tg B cells harboring Igκ-targeted Igκ transgenes are retained in the IgM$^-$ compartment while non-targeted transgenes are accelerated through this stage.** Flow cytometric analysis was used to determine B220 and IgM expression on freshly isolated bone marrow from VH transgenic mice with an endogenous Igκ locus (VH125Tg), a targeted Igκ transgene (VH125Tg/Vκ1ki, VH125Tg/Vκ4ki, and VH125Tg/Vκ8ki), or a non-targeted Igκ transgene randomly integrated into the genome (125Tg and IgHELMD4Tg). Bone marrow B cell developmental stages are: pro/pre (B220$^+$ IgM$^-$), immature (B220^{mid} IgM$^+$), mature recirculating (B220^{high} IgM$^+$). (A-F) Representative plots are shown for: VH125Tg (A), VH125Tg/Vκ1ki (B), VH125Tg/Vκ4ki (C), and VH125Tg/Vκ8ki (D), 125Tg (E), and IgHELMD4Tg (F), n = 8-19 animals, percentages are indicated. (G) Bars represent the average percentage of total B220$^+$ cells of the indicated developmental stage, n=8-19 animals ± SEM.

The increased size of the IgM^- compartment is consistent with the hypothesis that B cells harboring targeted Vκ initiate further IgL rearrangement to undergo receptor editing, rather than rapidly transiting to the immature compartment as occurs in B cells harboring non-targeted Vκ Tgs.

A pre-B cell compartment is well-maintained in targeted Vκ transgenic (VH125Tg/Vκki Tg) mice

The presence of a substantial population of IgM^- precursors in Igκ-targeted Vκ mice could represent either an increase in pro- or pre-B cells. To distinguish these possibilities, freshly isolated bone marrow was analyzed by flow cytometry to identify pro-B cells ($B220^+$ IgM^-, $CD43^+$) and pre-B cells ($B220^+$ IgM^-, $CD43^-$) (Fig. 3-2, A-B). Control comparisons were made between non-targeted Ig Vκ Tg mice (125Tg and IgHELMD4Tg), in which IgL rearrangement is suppressed, and mice lacking Vκ Tgs (VH125Tg), in which all B cells must initiate endogenous IgL rearrangement to exit the pre-B cell stage. B cells which possess Igκ-targeted Vκ Tgs (VH125Tg/Vκ(1, 4, or 8)ki) maintain an increased percentage of pre-B cells (Fig. 3-2, D-F) compared to non-Igκ-targeted Vκ Tg B cell models (IgHELMD4Tg and 125Tg, Fig. 3-2, G-H), and instead resemble VH125Tg mice (Fig. 3-2C). As expected, non-Igκ-targeted Vκ Tg mice (Fig. 3-2, G-H) had a clear decrease in the pre-B cell compartment compared to VH125Tg mice (Fig. 3-2C). These data are consistent with a developmental delay in B cells from VH125Tg/Vκ(1, 4, or 8)ki mice that would be consistent with receptor editing, and suggest that anti-insulin VH125 can support this process.

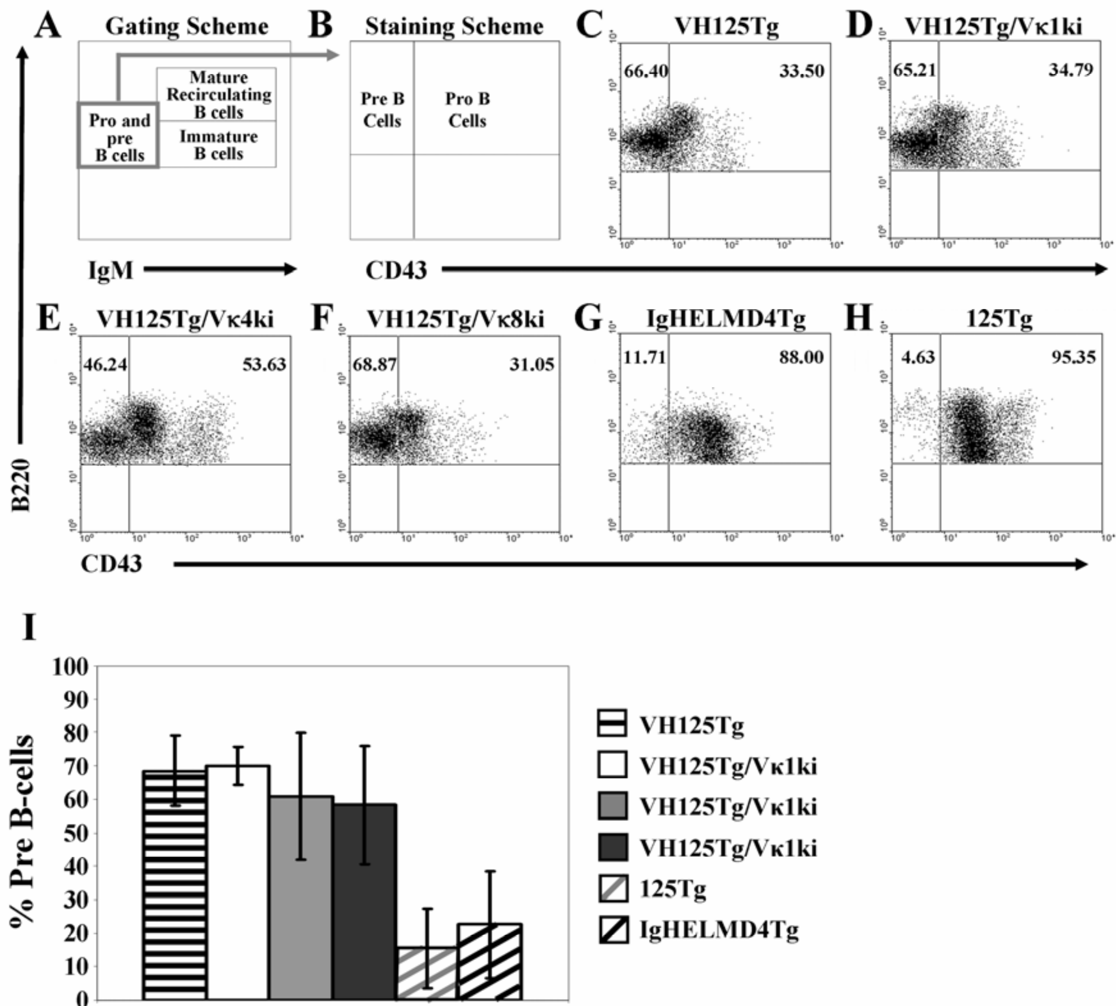

Figure 3-2. Pre-B cells are increased when VH125Tg is paired with Igκ-targeted Igκ transgenes compared with non-targeted Igκ transgenes. Flow cytometry analysis shows B220, IgM, and CD43 expression for freshly isolated bone marrow cells. CD43 expression in B220^{+} IgM^{-} cells identifies pro (CD43^{+}) and pre (CD43^{-}) B cells (A-B). (C-H) Representative scatter plots for a rearranged VH transgene paired with endogenous Igκs (VH125Tg, D), targeted Igκ transgenes (VH125Tg/Vκ1ki, D, VH125Tg/Vκ4ki, E, or VH125Tg/Vκ8ki, F), or a non-targeted Igκ transgene (125Tg, G, and IgHELMD4Tg, H). (I) Bars show the average percentage of pre-B cells (in B220^{+} IgM^{-} gated cells) of 8-14 animals ± SEM.

IgL replacement occurs in B cells possessing an anti-insulin heavy chain

To determine if the increased pre-B cell compartment observed in VH125/Vκki animals coincides with receptor editing, Vκ usage was assessed in a Vκ cDNA library amplified from spleen B cells isolated from VH125/Vκki mice. DNA sequencing of independent clones revealed that all three Igκ-targeted Vκ strains showed evidence of Vκ replacement: approximately one third of isolates from VH125Tg/Vκ1ki and VH125Tg/Vκ8ki B cells, or two thirds of isolates from VH125Tg/Vκ4ki B cells replaced the Tg-encoded Vκ (Table 3-2). Vκ usage for each of the three genotypes is shown in Table 3-3 through 3-5. None of the three targeted Vκ transgenic lines showed a detectable percentage of $Ig\lambda^+$ cells in the spleen, suggesting that editing to the Igλ locus is not occurring with any measurable frequency (not shown). These data are consistent with a correlation between an increased pre-B cell compartment and induction of receptor editing in VH125Tg B cells harboring Igκ-targeted Vκ Tgs.

VH125Tg/Vκ(1, 4 or 8)ki mice do not generate insulin-binding B cells

A substantial proportion of VH125Tg/Vκ(1, 4, or 8)ki B cells retained the Igκ-targeted Vκ (Table 3-2); therefore, if pairing of these Vκ with VH125 produces an insulin-binding specificity, insulin-binding B cells should be readily detectable, and might explain the substantial editing observed. To detect these B cells in the spleen, B220, IgM, and insulin-binding expression was assessed in freshly isolated VH125Tg/Vκ1ki, VH125Tg/Vκ4ki, and VH125Tg/Vκ8ki cells using flow cytometry. As shown in Figure 3-3, compared with insulin-binding 125Tg B cells, VH125Tg/Vκ1ki, VH125Tg/Vκ4ki, and VH125Tg/Vκ8ki B cells do not measurably bind insulin, even

Table 3-2. Frequency of Replacement of a Targeted Light Chain Paired with VH125

Heavy Chain Transgene	Light Chain Transgene	% Replacement of the Targeted Light Chain Allele
VH125Tg	Vκ1ki (Vκ1-110, Jκ1)	30
VH125Tg	Vκ4ki (Vκ4-81, Jκ4)	65
VH125Tg	Vκ8ki (Vκ8-91, Jκ5)	40

Percentages are based on 20 independent clones for each genotype

Table 3-3. Vκ/Jκ Usage in VH125Tg/Vκ1ki Spleen B Cells		
Clone #	**Clone Name**	**Vκ, Jκ Utilized**
1	1-1A2	Vκ1-110, Jκ1
2	1-1B	Vκ1-110, Jκ1
3	1-2C	Vκ1-110, Jκ1
4	1-3AS	Vκ1-110, Jκ1
5	1-3-2A	Vκ1-110, Jκ1
6	1-3-4A	Vκ1-110, Jκ1
7	1-3-5A	Vκ1-110, Jκ1
8	1-4A	Vκ1-110, Jκ1
9	1-5A	Vκ1-110, Jκ1
10	1-5BS	Vκ1-110, Jκ1
11	1-5C	Vκ1-110, Jκ1
12	1-5D	Vκ1-110, Jκ1
13	1-6BS	Vκ1-110, Jκ1
14	1-6CS	Vκ1-110, Jκ1
15	1-1C	Vκ9-120, Jκ2
16	1-2A	Vκ14-111, Jκ5
17	1-2B	Vκ14-100, Jκ2
18	1-3BS	Vκ9-120, Jκ5
19	1-3-6A	Vκ4-57, Jκ4
20	1-4D	Vκ4-61, Jκ5

Clones which replaced the targeted allele are shaded in gray, all clones are derived from independent pools of B cells

Table 3-4. Vκ/Jκ Usage in VH125Tg/Vκ4ki Spleen B Cells

Clone #	Clone Name	Vκ, Jκ Utilized
1	4-1A	Vκ4-81, Jκ4
2	4-3A	Vκ4-81, Jκ4
3	4-5AS	Vκ4-81, Jκ4
4	4-7CS	Vκ4-81, Jκ4
5	4-8A	Vκ4-81, Jκ4
6	4-9A	Vκ4-81, Jκ4
7	4-9B2	Vκ4-81, Jκ4
8	4-1B	Vκ19-93, Jκ1
9	4-2A	Vκ1-135, Jκ4
10	4-2BS	Vκ4-91, Jκ2
11	4-3B	Vκ17-127, Jκ2
12	4-4AS	Vκ1-135, Jκ1
13	4-4BS	Vκ1-110, Jκ2
14	4-5B	Vκ1-117, Jκ1
15	4-6A	Vκ4-91, Jκ1
16	4-6B	Vκ1-110, Jκ2
17	4-7A	Vκ4-57, Jκ4
18	4-7B	Vκ4-80, Jκ2
19	4-8B	Vκ4-72, Jκ1
20	4-9C3	Vκ5-39, Jκ5

Clones which replaced the targeted allele are shaded in gray, all clones are derived from independent pools of B cells

Table 3-5. Vκ/Jκ Usage in VH125Tg/Vκ8ki Spleen B Cells

Clone #	Clone Name	Vκ, Jκ Utilized
1	8-1A	Vκ8-19, Jκ5
2	8-3AS	Vκ8-19, Jκ5
3	8-3B	Vκ8-19, Jκ5
4	8-4BS	Vκ8-19, Jκ5
5	8-6AS	Vκ8-19, Jκ5
6	8-7AS	Vκ8-19, Jκ5
7	8-7B	Vκ8-19, Jκ5
8	8-7C	Vκ8-19, Jκ5
9	8-8AS	Vκ8-19, Jκ5
10	8-8BS	Vκ8-19, Jκ5
11	8-9A	Vκ8-19, Jκ5Jκ2
12	8-9B	Vκ8-19, Jκ5
13	8-1BS	Vκ4-68, Jκ2
14	8-2A	Vκ4-74, Jκ2
15	8-2B	Vκ9-120, Jκ2
16	8-2-1B	Vκ4-57, Jκ2
17	8-4A	Vκ1-110, Jκ2
18	8-5A2	Vκ14-130, Jκ1
19	8-5B2	Vκ1-110, Jκ2
20	8-6BS	Vκ14-111, Jκ4

Clones which replaced the targeted allele are shaded in gray, all clones are derived from independent pools of B cells

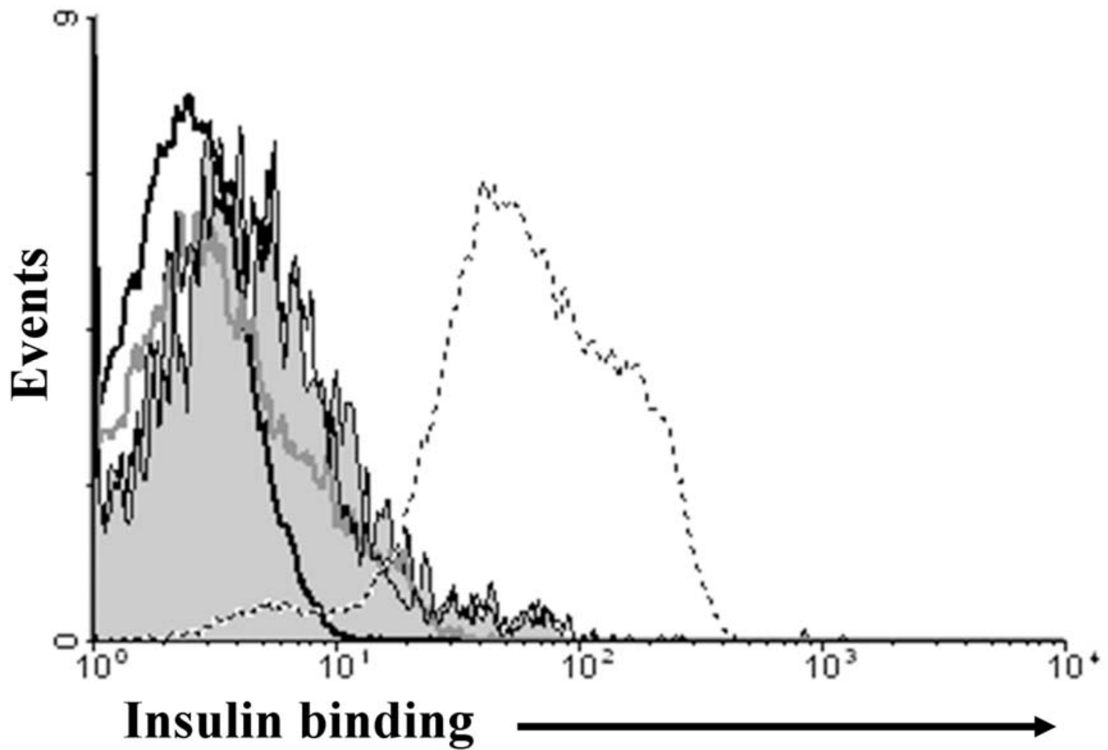

Figure 3-3. **Igκ-targeted Igκ transgenes do not generate insulin-binding BCRs when paired with an anti-insulin IgH (VH125).** Flow cytometry was used to measure insulin binding in freshly isolated B220$^+$ IgM$^+$ spleen cells from VH125Tg (shaded, negative control), VH125Tg/Vκ1ki (thick black line), VH125Tg/Vκ4ki (thick dark grey line), VH125Tg/Vκ8ki (thin black line), or 125Tg (dashed line, positive control) upon staining with 5 μg/mL biotinylated insulin. Data represent ≥ 6 animals.

when incubated with high concentrations of insulin (5 μg/mL; detection of insulin-binding of 125Tg B cells is readily achieved with 0.05 μg/mL insulin). Insulin binding mediated by the Igκ-targeted Igκs is essentially negative and resembles VH125Tg animals, in which insulin-binding cells are not detected in the periphery. An insulin-binding population was also not detected in bone marrow B cells, and measurable levels of insulin-binding Ab were not found in serum enzyme-linked immunosorbent assay (ELISA) (not shown). This suggests that strong insulin reactivity is not driving the observed receptor editing.

Insulin-specific B cells are removed from the peripheral repertoire of C57BL/6, but not T1D-prone NOD VH125Tg mice

Although VH125Tg B cells possessing the Igκ-targeted Igκs show evidence of receptor editing, the question of whether insulin could drive this process was not resolved. A population of insulin-binding B cells is found in the spleens of VH125Tg NOD mice (78, 197), but is not detectable in the spleens of C57BL/6 mice (197). As the anti-insulin IgH present is identical, C57BL/6 animals should also possess the potential to generate insulin-binding B cells. Flow cytometry was therefore used to identify whether insulin-binding B cells were present among developing bone marrow B cells in the C57BL/6 strain, which may not have completed receptor editing-mediated replacement of an insulin-reactive BCR. To avoid studying B cells with non-specific BCR:Ag interactions, insulin-binding was plotted against IgM^a, as a linear relationship is expected between Ag-binding and BCR expression, and cells were incubated with 10-fold excess unlabeled insulin competitor. VH281Tg mice were used as a negative control, since

VH281 differs from VH125 by two amino acids which are necessary to confer insulin-binding specificity (186).

In NOD mice, insulin-specific B cells were detected in the bone marrow (Fig. 3-4 E) and spleens (Fig. 3-4M) of VH125Tg, but not VH281Tg mice (Fig. 3-4, G and O). In C57BL/6 mice, a small population of insulin-specific B cells was also sometimes detected in the bone marrow of VH125Tg (Fig. 3-4A), but not VH281Tg mice (Fig. 3-4C). Importantly, this population was not observed in the spleens (Fig. 3-4, I and K). A statistically significant increase of insulin-specific B cells was present in the bone marrow of VH125Tg NOD compared with C57BL/6 mice (Fig. 3-4Q). Interestingly, this percentage was significantly increased further in the spleen of VH125Tg NOD mice, as compared with the bone marrow (Fig. 3-4Q). These findings suggest that anti-insulin VH125 can pair with endogenous IgLs to produce a small population of insulin-specific B cells, which are observable in the developing polyclonal repertoires in bone marrow from both C57BL/6 and NOD mice. This population of insulin-specific B cells is found in the periphery of NOD, but not C57BL/6 VH125Tg mice, which suggests that while tolerance mechanisms effectively prevent insulin-specific B cells from reaching the periphery on the C57BL/6 background, such mechanisms fail to censor them on the NOD background. In contrast, the NOD strain selects for insulin-specific B cells, as shown by the increased percentage present in the spleen compared with bone marrow. Defects in censoring anti-insulin B cells may therefore exist in the T1D-prone NOD strain.

An increased frequency of insulin-specific B cells develop *in vitro* in the absence of antigen from VH125Tg NOD bone marrow, compared with VH125Tg C57BL/6 mice

An increased percentage of insulin-specific immature B cells were present in VH125Tg bone marrow from NOD, compared with C57BL/6 mice. This suggests either that the NOD IgL repertoire is more permissive for developing anti-insulin BCR when paired with VH125, or that central tolerance was more rapidly and effectively removing such specificities from the repertoire of C57BL/6 mice. To identify whether NOD VH125Tg mice spontaneously generate a higher frequency of insulin-specific B cells, bone marrow cells were cultured *in vitro* using a well-established IL-7-driven culture system (16, 42, 161, 163) in the absence of exogenous insulin. In this system (depicted Fig. 2-2A), freshly isolated bone marrow is grown with IL-7 for 5 d to enhance pro-B cell proliferation while B cells that have differentiated beyond this IL-7-dependent stage die, leaving only Ag-naïve B cells which would not have been censored by endogenous insulin (122). A population of insulin-specific B cells is observable in bone marrow cultured from VH125 C57BL/6 mice (Fig. 3-5A), which is significantly increased in VH125 NOD animals (Fig. 3-5C). This suggests that NOD developing B cells are more permissive for the generation of insulin-reactive BCRs.

rag-2 is elevated in anti-insulin VH125Tg-restricted, polyclonal B cells

Increased rag expression is known to correlate with receptor editing (187), thus to further probe whether receptor editing is evoked in VH125Tg C57BL/6 animals, real-time PCR was used to quantify relative rag-2 transcript levels in freshly isolated VH125Tg bone marrow cells. VH281 differs from VH125 by two amino acids necessary

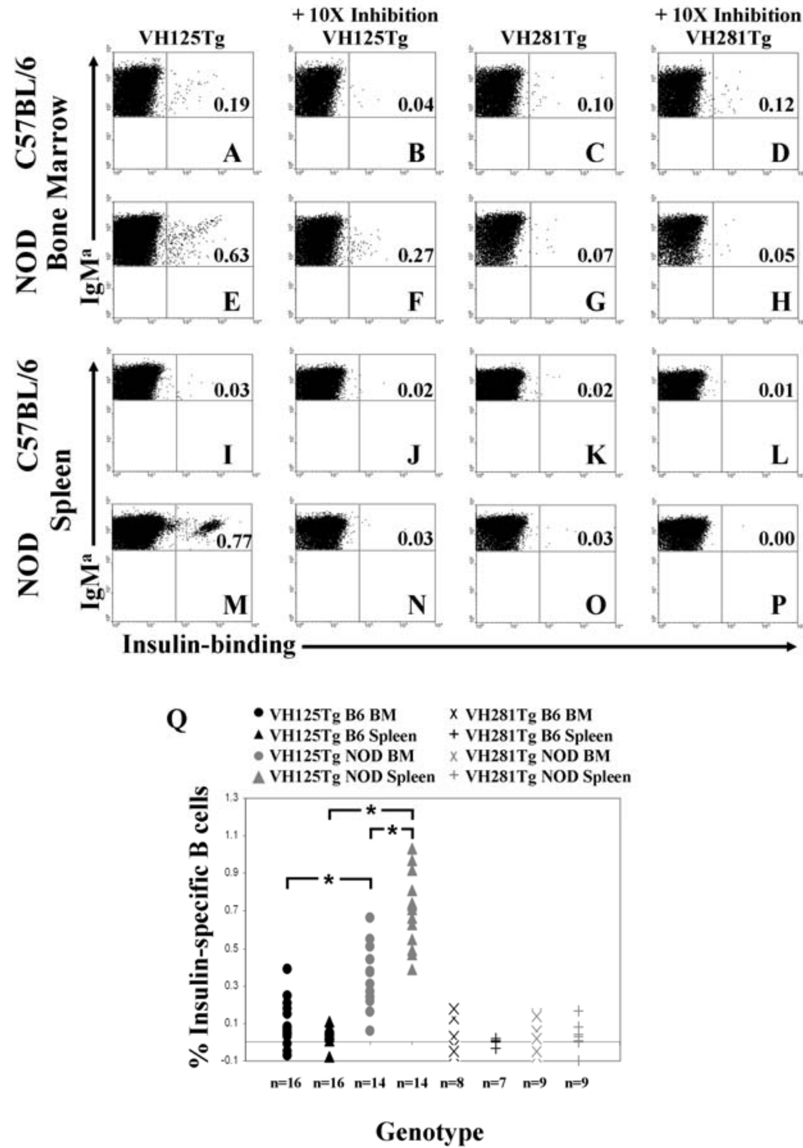

Figure 3-4. **VH125 NOD mice develop an increased frequency of insulin-specific B cells in the bone marrow, which accumulate in the spleen *in vivo*.** Freshly isolated bone marrow (A-H) or spleen (I-P) cells from C57BL/6 or NOD VH transgenic (anti-insulin VH125 or non-insulin-binding VH281) are incubated with anti-B220, anti-IgM, and biotinylated insulin/streptavidin to detect insulin-binding B cells using flow cytometry. Bone marrow scatter plots are gated on $B220^{mid}$ IgM^{a+} $7AAD^{-}$ events to show immature B cells, spleen plots are gated on $B220^{+}$ IgM^{a+} $7AAD^{-}$ lymphocytes to show total B cells, plots are representative of ≥ 7 mice. (Q) Using the gating scheme listed above, the percentage of positive cells observed following incubation with biotinylated insulin and 10X excess unlabeled insulin competitor was subtracted from cells incubated in the absence of competitor (e.g. Panel A – Panel B). Results for individual mice are plotted, C57BL/6 are shown in black, NOD are shown in grey; VH125 bone marrow (●, ●), VH125 spleen (▲, ▲), VH281Tg bone marrow (**X**, X), and VH281Tg spleen (+, +) are shown. * $p < 0.0001$, as calculated by a two tailed t test.

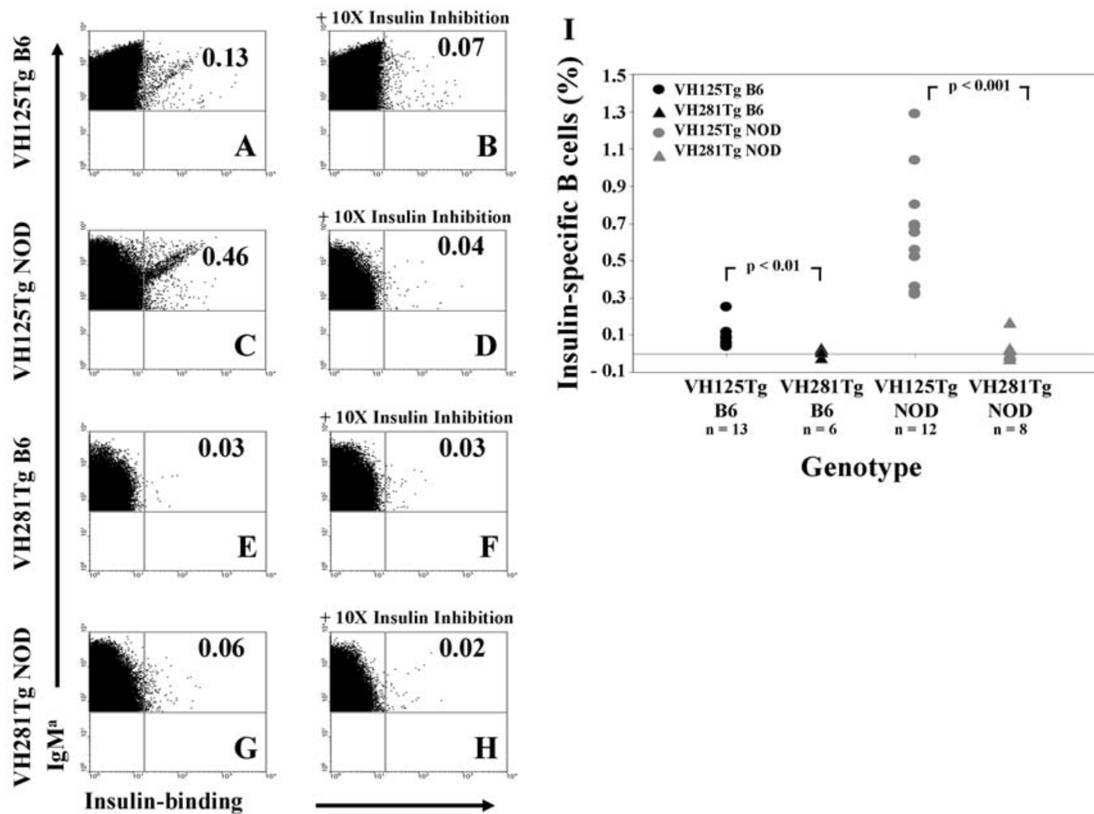

Figure 3-5. VH125Tg NOD mice develop an increased percentage of anti-insulin B cells *in vitro* in the absence of antigen compared with VH125Tg C57BL/6 mice. Bone marrow B cells from C57BL/6 or NOD VH transgenic animals (anti-insulin VH125 or non-insulin-binding VH281) were cultured with IL-7 for 5 d to enrich for Ag-naïve B cells. IL-7 was withdrawn, and cells were cultured in the absence of exogenous insulin for an additional 2 d. Insulin-specific bone marrow B cells were identified using flow cytometry as in Fig. 4. (A-H) Plots are representative of ≥ 6 mice. (I) Results for individual mice are plotted, C57BL/6 are shown in black, NOD are shown in grey; VH125Tg (●, ●) and VH281Tg (▲, ▲) are shown. * $p < 0.01$, ** $p < 0.001$, as calculated by a two-tailed t test.

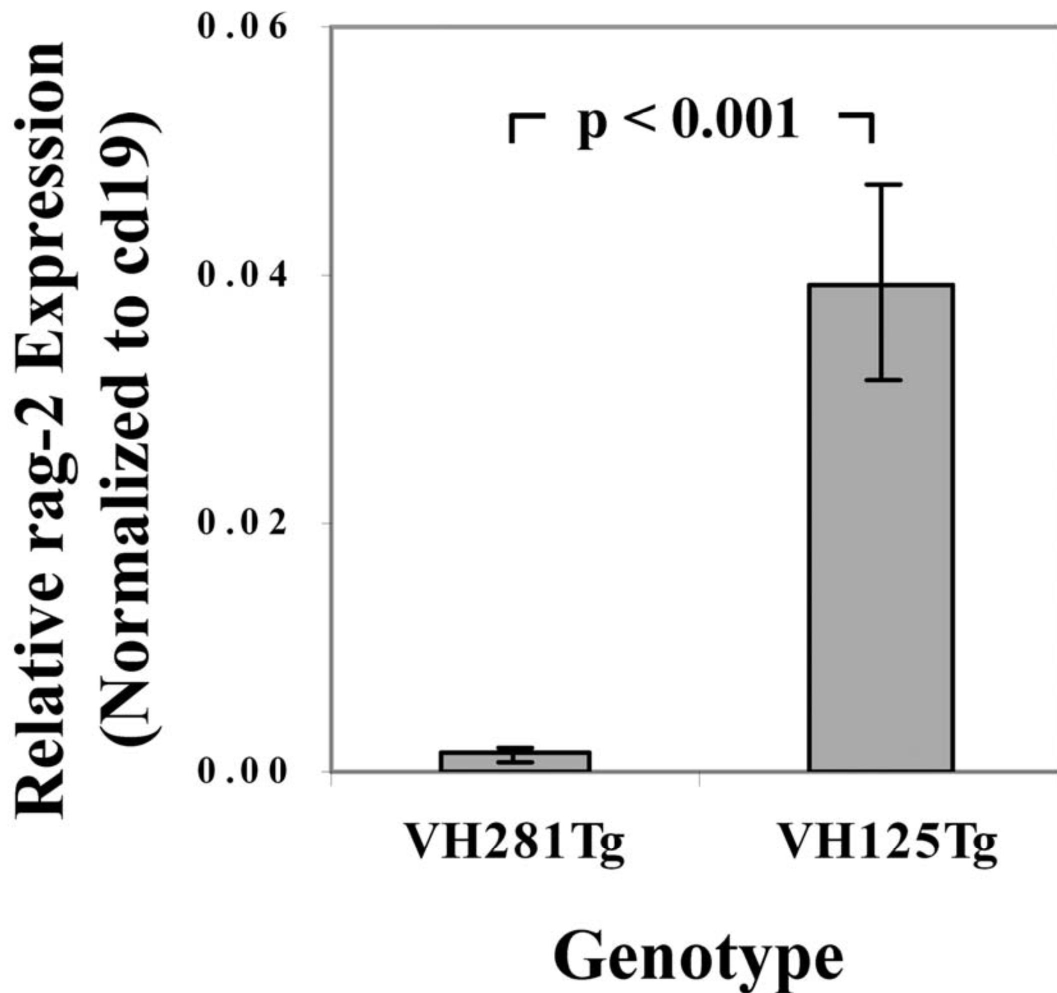

Figure 3-6. **Anti-insulin VH125Tg mice show increased rag-2 transcript levels.** RNA was harvested from freshly isolated C57BL/6 bone marrow from mice that possess either functionally rearranged anti-insulin (VH125Tg) or insulin-unreactive (VH281Tg) VH transgenes. Real-time PCR was used to measure relative rag-2 transcript levels in cDNA (normalized to CD19). Bar graphs represent the average relative rag-2, error bars represent SEM, n = 6-9 animals.

for insulin binding when the IgH is paired with an anti-insulin Igκ (186); thus high structural similarity and similar pairing efficiency with endogenous IgLs is expected, and the expression of rag stimulated by "normal" IgL rearrangement should be similar. IgH Tg allelic exclusion is well maintained in these models (160), therefore IgL rearrangement events should exclusively account for rag expression observed (52). As shown in Figure 3-6, mice possessing anti-insulin VH125 show a 28-fold increase of relative rag-2 expression compared to control VH281Tg B cells. Expression of rag-2 was normalized to cd19 expression, a B cell lineage-specific marker. These data suggest that anti-insulin VH125 may up-regulate rag, an enzyme critically involved in receptor editing, to elicit central tolerance through IgL replacement *in vivo*. Taken together, these data show that B cells harboring an anti-insulin IgH replace Igκ-targeted Vκs, and suggest that the small protein hormone, insulin, can elicit receptor editing of insulin-reactive B cells in a polyclonal repertoire.

Discussion

Strong BCR engagement, such as that produced by multivalent or high-affinity interactions, is capable of stimulating receptor editing (34, 68, 76, 207), however the extent to which moderate affinity interactions which do not elicit a high degree of BCR crosslinking can drive this process *in vivo* is unknown. To address the lower threshold of interactions necessary for receptor editing induction, the interaction of B cells harboring an anti-insulin IgH paired with restricted or polyclonal IgLs were studied. Mice harboring anti-insulin VH125 paired with several different Igκ-targeted Vκs show indirect (Fig. 3-1 and 3-2) and direct evidence (Table 3-2) of Igκ-targeted Vκ

replacement in peripheral B cells. Anti-insulin B cells can be observed in freshly isolated bone marrow of VH125Tg C57BL/6 mice, and a discrete population is clearly observable following culture in the absence of insulin *in vitro*, which is enhanced in VH125Tg NOD mice, suggesting that NOD developing B cells are more permissive for generation of insulin-binding BCRs. In contrast with C57BL/6 mice, in which insulin-binding B cells are not found in the spleen, such autoreactive cells are present in the spleens of VH125Tg NOD mice at an increased frequency compared with the bone marrow. This suggests that in contrast with C57BL/6 mice, in which tolerance prevents insulin-binding B cells from reaching or surviving in the periphery, such cells appear to be positively, rather than negatively selected on the NOD background. Additional studies using anti-insulin VH125Tg C57BL/6 B cells possessing a polyclonal IgL repertoire show increased rag-2 expression compared with non-insulin-specific (VH281Tg) controls, which is consistent with increased receptor editing, perhaps to censor any anti-insulin B cells that develop. Together, these findings extend the importance of central tolerance in maintaining B cell tolerance by suggesting that a small protein hormone, such as insulin, can drive this process.

Polyclonal IgL usage in VH125Tg animals complicates direct detection of IgL replacement events. This problem was circumvented by generating VH125Tg/Vκki lines that possess Igκ-targeted Vκ Tgs, in which a known Vκ is dominantly expressed, but is susceptible to receptor editing-mediated replacement. The presence of a pre-B cell compartment in such BCR Tg mice is associated with receptor editing induction due to autoreactivity, and is not found in an autoAg-free model (145). The finding that the pre-B cell compartment of VH125/Vκ(1, 4, or 8)ki mice is 4-5 times greater than in mice

possessing non-Igκ-targeted Vκ Tgs (IgHELMD4Tg or 125Tg) is therefore consistent with a receptor editing-induced developmental delay (Fig. 3-1 and 3-2). Alternatively, chromatin remodeling events required for Igκ locus accessibility might also account for the delayed developmental progression, as non-targeted Igκ genes may not require these events for expression. However, replacement of 30-60% of the targeted Igκ in peripheral B cells (Table 3-2) provides direct evidence that receptor editing is active and likely contributing to the enhanced pre-B cell compartment observed.

As all three VH125Tg/Vκki lines showed evidence of receptor editing, we hypothesized that VH125 confers insulin specificity to the BCRs. However, failure to detect insulin-binding B cells in any of these lines (Fig. 3-3) suggests that either the interaction with insulin, albeit physiologically relevant, is not possible to detect with these methods, that an unidentified autoAg is being engaged, or that the editing observed is Ag-independent. Because such possibilities are difficult to rule out in the VH125/Vκki models, we also studied Ag-specific interactions with insulin in VH125 animals which possess a polyclonal (endogenous) IgL repertoire. Insulin-binding B cells are not detected in the periphery of VH125Tg C57BL/6 animals (197), which suggests either that VH125 does not pair with the endogenous IgL repertoire to generate insulin-binding B cells, or that insulin-reactive B cells are censored during development in the bone marrow. *In vitro* culture in the absence of insulin should alleviate negative selection through central tolerance to circulating insulin, and thus give an accurate view of the frequency of insulin-binding B cells that develop from the endogenous IgL repertoire. Insulin-specific B cells are observed in VH125Tg C57BL/6 bone marrow that is freshly isolated or cultured in the absence of insulin *in vitro*, and this frequency is significantly

increased among VH125Tg NOD B cells (Fig. 3-4 and 3-5). These findings suggest that while insulin-binding B cells are present in the developing repertoires of both strains, the IgL repertoire of NOD mice may be preferentially suited to pair with VH125 and generate insulin-binding B cells. The observation that multiple polymorphisms exist in IgLs of the autoimmune-prone NOD strain (197) may therefore explain the enhanced ability to generate insulin-reactive B cells.

Insulin-specific B cells are not found in the spleens of C57BL/6 mice (Fig. 3-4 and (197)), suggesting that although they are present in the developing repertoire, they are effectively removed from the peripheral repertoire. The mechanism(s) of tolerance that are operating to remove insulin-binding B cells from the periphery of C57BL/6 mice appear to be faulty in NOD mice, as insulin-specific B cells fail to be removed from the spleen (Fig. 3-4). Furthermore, the frequency of insulin-binding B cells present in the developing VH125Tg NOD repertoire significantly increases in the peripheral repertoire, suggesting that inappropriate selection of the insulin-reactive B cells is occurring in the periphery (Fig. 3-4). It does not seem likely that the culling of insulin-reactive specificities observed is due to deletion, as B cells with an insulin-restricted BCR (125Tg) which retain the autoreactive BCR do not show evidence of deletion, even following culture with up to 50 μg/mL insulin *in vitro* (Fig. 2-3), but rather undergo anergy *in vitro* (Chapter II), and following exposure to circulating insulin *in vivo* (160). It is however plausible that insulin-reactive B cells encountering endogenous insulin are removed from the repertoire through receptor editing.

The hypothesis that the lack of insulin-binding B cells observed in the periphery of VH125Tg C57BL/6 animals is due to receptor editing is supported by the finding that

anti-insulin VH125Tg B cells show almost 30-fold more rag-2 expression than B cells that express a non-insulin-binding variety of the same IgH (VH281) (Fig. 3-6). As a functionally rearranged IgH transgene suppresses rag expression required for IgH rearrangement (120, 182), the rag expression observed likely derives from ongoing IgL rearrangement. This expression may result either from primary rearrangement of endogenous IgLs, or secondary rearrangement induced by receptor editing (61, 152, 187). A wide variety of Igκ genes are reported to pair with VH125 and related VH9 genes, so a general problem with chain pairing is not likely (197). Insulin-specific B cells make up a small fraction of the VH125Tg repertoire, however it is possible that additional, unappreciated autoreactive specificities are also generated in this model, which are partially responsible for the elevated rag-2 expression observed.

Insulin is a critical autoAg in T1D (206), and represents an important class of self Ags for which preservation of tolerance is critical for human health. NOD mice which lack B cells, or possess B cells restricted to irrelevant Ag specificities are protected from spontaneous development of T1D (3, 78, 173, 179), however, the presence of anti-insulin IgH (VH125Tg) or IgH and IgL Tgs (125Tg) promote disease development (78), emphasizing the key role of insulin as an autoAg. Notably, although insulin-reactive B cells in the periphery are anergic, this form of tolerance is insufficient to prevent autoimmune disease in the NOD strain (78, 160), suggesting additional tolerance checkpoints may be required to prevent disease. NOD B cells specific for HEL fail to be rendered anergic or undergo partial deletion in response to soluble HEL engagement, consistent with tolerance defects (178). We extend these studies by implicating tolerance defects in VH125Tg NOD mice which fail to remove insulin-specific B cells from the

peripheral repertoire, through inappropriate survival and/or inefficient receptor editing. The percentage of anti-insulin B cells present in VH125Tg NOD spleen exceeds the percentage of cells that are generated in the bone marrow (Fig. 3-4), suggesting that in addition to a breakdown in adequate elimination of insulin-reactive B cells through receptor editing, defective peripheral selection of the autoreactive B cells may also compound disease generation in the NOD model.

Further confirmation and characterization of how insulin stimulates receptor editing will be difficult using the current Tg models available. The percentage of insulin-binding B cells in the repertoire of VH125Tg mice is very low, and is likely rapidly censored through receptor editing *in vivo* during development. Use of the 125Tg model, in which the majority of B cells bind insulin, is also problematic in the study of receptor editing, because the Igκ is randomly integrated into the genome and is thus not subject to regulatory control aimed at the Igκ locus. These cells may initiate "frustrated" editing attempts which are overridden by continued expression and signaling of the Igκ Tg, and thus lack appreciable developmental delay at the IgM$^-$ stage of B cell development or altered BCR specificity. Consistent with this notion, 125Tg B cells which do not require rag expression for successful BCR expression and developmental progression express increased rag-2 transcript levels compared with VH281Tg animals, which must up-regulate rag to successfully rearrange an IgL (not shown). Definitive study of insulin-induced receptor editing would thus be greatly facilitated by the generation of an IgL-targeted anti-insulin Tg model.

CHAPTER IV

ANTI-INSULIN B CELLS UNDERGO RECEPTOR EDITING IN THE PRESENCE OF CIRCULATING INSULIN *IN VIVO*

Abstract

Central tolerance is critically important in censoring autoreactive B cell specificities that arise during B cell development. One such mechanism, receptor editing, can edit the BCR of B cells that receive strong signals from highly crosslinking Ags, however the minimum BCR engagement threshold required to induce this process has not been sufficiently explored. Insulin, a key T1D autoAg, can be used as a model autoAg to probe this question, as it is present at a low concentration and does not interact with the 125Tg BCR with high affinity or avidity. We therefore developed the Igκ-targeted model, Vκ125ki, to address whether endogenous insulin could stimulate receptor editing. Whereas ~80% of VH125/Vκ125ki B cells retain the insulin-binding specificity in the bone marrow *in vivo*, this percentage is diminished to 40% in the T1 B cell compartment in the spleen, suggesting that ~60% of B cells exported from the bone marrow have replaced the anti-insulin IgL. A similar percentage of follicular and marginal zone B cells show evidence of receptor editing based on loss of insulin-binding, which was confirmed by sequence analysis of Vκ/Jκ usage. An increased percentage of anti-insulin IgL replacement was observed in B cell clones harboring Vκ125ki paired with VH125 compared with non-insulin-binding VH281, confirming that Ag specificity contributed to the receptor editing observed. This is the first demonstration that a small protein hormone, such as insulin, can promote receptor editing and highlights the exquisite sensitivity of this pivotal central tolerance mechanism.

Introduction

Autoimmune diseases develop as a result of faulty maintenance of immune cell tolerance. Tolerance in the B cell compartment is actively maintained throughout B cell development via receptor editing, deletion, and anergy. As 55-75% of newly formed B cells in humans are estimated to be autoreactive, receptor editing represents a critical salvage pathway in B cell development (193). Additionally, only 10-20% of immature B cells formed in the bone marrow survive the maturation process long enough to emigrate from the bone marrow, and only 3% survive as mature B cells in the periphery (5, 57, 69, 164). Together, these data suggest that the majority of newly formed B cells are autoreactive. The repertoire of specificities is altered between immature and mature B cell populations, suggesting that selection is occurring between this transition (66, 153, 168). Multiple checkpoints exist that serve to remove autoreactive specificities generated in the bone marrow; however these checkpoints are dysregulated in patients with SLE, thus permitting escape of autoreactive cells into the periphery (167, 205). These findings suggest that central tolerance defects may promote the development of human autoimmune disease (167, 205), and highlight the importance of understanding how B cell tolerance is maintained for key autoAgs.

If signaling emanating from BCR surface expression is absent, due to faulty pairing of the IgL with the IgH (38, 54), or if the signal is too strong, due to engagement with Ag, receptor editing may be initiated (61, 152, 187). This results in developmental retention of the B cell and prolonged activity of recombinational machinery to promote continued IgL rearrangement (31, 187). Ig Tgs restrict the B cell repertoire and facilitate the study of Ag-specific interactions, and have been used to show that receptor editing

occurs upon engagement of the BCR with membrane-bound Ags, such as membrane-bound HEL (76, 189), MHC class I (93, 187), DNA (34, 61), and type IV collagen (207). This form of tolerance is also stimulated by soluble HEL exposure (76, 189), for which the BCR has a high affinity (5 x 10^{10} M^{-1}) (11). The lower limits of the threshold for receptor editing induction *in vivo* remain to be defined; thus the key T1D autoAg, insulin, will be used to further probe this question.

Anti-insulin IgH and IgL pair together in the 125Tg model to produce a B cell repertoire in which > 95% of the B cells bind insulin (160). We have demonstrated that these B cells bind circulating insulin *in vivo* and thus allow the study of a physiologically relevant BCR:Ag interaction (160). Classically, hormones are expected to induce clonal ignorance, as they are soluble proteins present at low concentrations. Surprisingly, peripheral 125Tg B cells are not clonally ignorant, but rather are rendered anergic *in vivo* (160), despite the fact that circulating insulin is present at very low concentrations (up to 1-5 ng/mL post-prandially) (56), insulin is a small, soluble protein that does not appreciably cross-link the BCR, and the affinity of the interaction is modest (1 x 10^7 M^{-1}) (172). This model has been also been used to demonstrate a role for anergy in maintaining tolerance to insulin in the developing B cell compartment (Chapter II).

We show that VH125/Vκ125ki B cells undergo receptor editing *in vivo*, based on loss of the BCR insulin-binding specificity, as well as sequence analysis confirmation of replacement of the Igκ-targeted Vκ125 (Vκ125ki). This does not appear to be due to inefficient expression of Vκ125ki, as ~80% of B cells present in the bone marrow bind insulin. Whereas VH125/Vκ125ki B cells bind insulin, VH281/Vκ125ki B cells do not, thus allowing the contribution of Ag specificity to receptor editing to be assessed.

VH125/Vκ125ki spleen B cells show an increased frequency of Vκ125ki replacement compared with VH281/Vκ125ki spleen B cells, suggesting B cells capable of binding circulating insulin undergo a higher extent of receptor editing that is likely Ag-dependent.

Materials and Methods

Generation of Vκ125ki Targeting Vector

The pSVG-125Vκ vector used to generate Vκ125 Tg mice (160) was digested with EcoRI and SacII to liberate the Vκ125/Jκ5 fragment which was subcloned into the pBluescript SK(-) vector (Stratagene) and was verified through sequencing at the Vanderbilt Sequencing Core. Vκ125/Jκ5 was then amplified from this vector using the primers NotI FWD 5' AAGCGGCCGCAGGGTCTGTCGAT and SalI REV 5' AAAGTCGACGGATGAGTCTCCTTCTC. The 1.6 kb Vκ125 fragment was subsequently cloned into NotI and SalI sites in the pVKR3-83-2NEO targeting vector, which was kindly provided by Roberta Pelanda, National Jewish Medical and Research Center, Denver, CO, and the pVKR-Vκ125ki targeting construct was verified through sequencing. pVKR3-832neo differs slightly from pVKR3-83neo used to generate the 3-83κi model (144), in that no downstream Jκ segments exist on the targeted allele.

Generation of Igκ-Targeted Anti-insulin Vκ125ki Mice

The targeting vector was linearized through digestion with ClaI. 200 μg of the linearized DNA was electroporated into 3.5 x 10^7 TL1 embryonic stem (ES) cells using the Bio-Rad Gene Pulser II system set at 0.800kV and the capacitance at 3.0μF. Cells

were selected with 200 µg/mL G418 and 2 µM Gancyclovir. Double-resistant colonies were screened by Southern blot. The 1.6 kb EcoRI probe (144) kindly provided by Roberta Pelanda was hybridized to blots of SacI-digested ES cell clone DNA to identify endogenous (5.5 kb) and targeted (6.3 kb) allele bands. Positive clones (33 of 458 double resistant clones screened) were subsequently verified through PCR using FWD 5' TCTGCAAATGTCTGATGAGT and REV 5' CTCGTGCTTTACGGTATCGC primers. Two clones were injected into blastocysts which were transplanted into pseudopregnant females, who gave birth to two lines of chimera progeny. All of this work was performed with the help of the Vanderbilt Transgenic Mouse/Embryonic Stem Cell Shared Resource. Chimeric males were intercrossed with C57BL/6 females and brown-coated progeny were screened through PCR (FWD #194 5' GGACGTAAACTCCTCTTCA and REV #195 5' CCGCTCTAGACTCGAG; Fwd #182 5' TCTGCAAATGTCTGATGAGT and REV #183 5' CTCGTGCTTTACGGTATCGC) and Southern blot analysis to confirm the presence of the targeted allele. PCR was also used to confirm that the intact Vκ125/Jκ5 was present in targeted mice (FWD #69 5' AATGGATTTTCAGGTGCAGAT and REV #88 5' GCTCCAGCTTGGTCCCAGCA). Six chimeric founder males were backcrossed to WT C57BL/6 females to generate mice carrying the targeted Vκ125 allele. The six independent lines of progeny were all found to contain the targeted allele, based on PCR and Southern blot analysis.

Animals

Vκ125ki mice were intercrossed with VH125Tg C57BL/6 females (78) to generate VH125/Vκ125ki offspring. Progeny from all six founder lines exhibited similar

percentages of “edited” non-insulin-binding B cells as assessed by flow cytometry, which remained consistent at various stages of backcrossing. One line was subsequently used for continued backcrossing and experiments shown. 125Tg mice which harbor functionally rearranged anti-insulin IgH and IgL Tgs are previously described, and have been backcrossed > 20 generations to the C57BL/6 background (78, 81). C57BL/6 mice were purchased from Taconic Farms, IghelMD4 (HELTg) C57BL/6 mice were purchased from The Jackson Laboratory. All mice were housed under specific pathogen-free conditions, and all studies were approved by the institutional use and animal care committee of Vanderbilt University.

Flow Cytometry and Antibodies

Flow cytometric analysis was performed using a FACSCalibur flow cytometer or LSRII (BD Biosciences). Ab reagents (BD Pharmingen): B220 (6B2), IgM^a (DS-1), IgM^b (AF6-78), and CD43 (S7). Insulin (Sigma) was biotinylated at pH 8.0 in bicine buffer using biotin N-hydroxysuccinimide ester (Sigma) and detected with streptavidin (BD Pharmingen). WinMDI 2.8 software (Dr. J. Trotter, Scripps Institute, San Diego, CA) was used for analysis.

Determination of Vκ Usage

Spleen cells from 6-10 individual animals were positively selected with IgM^a-biotin (BD Pharmingen, DS-1) and streptavidin magnetic beads (Miltenyi) through autoMACS sorting (Miltenyi) to >95% IgM^{a+} cell purity. RNA was isolated using the Tri Reagent manufacturer’s instructions (Molecular Research Center). First strand cDNA

was generated from total RNA using Superscript II RT (Invitrogen) and 0.67 μg oligo-dT primer (Amersham Biosciences) in a standard protocol. Vκ sequences were amplified from first strand cDNA using the following primers: Cκ primer - 5'GGA TAC AGT TGG TGC AGC ATC and VκA - 5'ATT GTK MTS ACM CAR TCT CCA, where K=G or T, M=A or C, S=C or G, R=A or G. Vκ sequences were amplified using AmpliTAQ DNA Polymerase (2U/reaction) (Applied Biosystems), 200 nM dNTP, 1.25 mM $MgCl_2$, 13.35 mM Cκ and VκA primers; 94°C/1 min, 42°C/1 min, and 72°C/1 min for 40 cycles. PCR product was ligated into pGEM-T Easy Vector System I plasmid (Promega). Clones were sequenced by the VU Sequencing Core. Vκ gene segment sequence alignments were assigned using the ImMunoGeneTics database (www.imgt.cines.fr:8104/). Individual clones are each derived from independent pools of RNA.

Results

A functionally rearranged anti-insulin Igκ transgene is targeted to the Igκ locus to produce a novel model to assess receptor editing in insulin-specific B cells

We hypothesized that 125Tg B cells may initiate "frustrated" receptor editing attempts, such that receptor editing initiates, but fails to remove and replace the autoreactive anti-insulin Igκ Tg, which is not integrated at the Igκ locus. To directly assay for receptor editing, we developed a model in which anti-insulin Vκ125, was targeted to the Igκ locus, as outlined in the targeting schematic (Fig. 4-1A). As described in Methods, Vκ125/Jκ5 (which encodes the anti-insulin IgL of 125Tg mice) was cloned into the pVKRneo2 targeting vector, a derivative of that used to generate the 3-83ki line (144), kindly provided by Roberta Pelanda. Electroporated ES cells were screened with

PCR and Southern blotting (Fig. 4-1B) and positive clones were injected into blastocysts. Progeny from resulting chimeras were also screened using PCR and Southern blotting and targeting of the anti-insulin Igκ was verified in six independent lines of mice (Fig. 4-1, C-E). One line was ultimately chosen for extensive backcrossing to the C57BL/6 background.

Receptor editing occurs in VH125/Vκ125ki B cells exposed to circulating insulin *in vivo*

To directly assay for replacement of the Igκ-targeted anti-insulin Igκ, Vκ125ki mice were crossed with VH125Tg mice, which harbor a non-Ig-targeted anti-insulin IgH, to generate VH125/Vκ125ki mice. As > 95% of 125Tg B cells bind insulin (which possess the non-Igκ-targeted version of the Igκ) (160), a similarly high percentage of VH125/Vκ125ki B cells should bind insulin if the targeted Vκ125ki transgene is dominantly expressed and receptor editing is not induced. Expression of B220, IgM^a, Igκ, and insulin-binding specificity was therefore assessed in freshly isolated bone marrow and spleen cells using flow cytometry. As shown in Fig. 4-2A, ~70% of the immature (IgM^{a+}) bone marrow B cells bind insulin. The ~30% of IgM^{a+} immature B cells that do not bind insulin could represent cells which have undergone receptor editing, or cells in which dominant expression of Vκ125ki did not occur. The percentage of non-insulin-binding B cells in the spleen is increased to ~50% (Fig. 4-2C), suggesting that the insulin-binding BCR is being negatively selected during B cell maturation, perhaps through receptor editing. The percentage of non-insulin-binding B cells was also

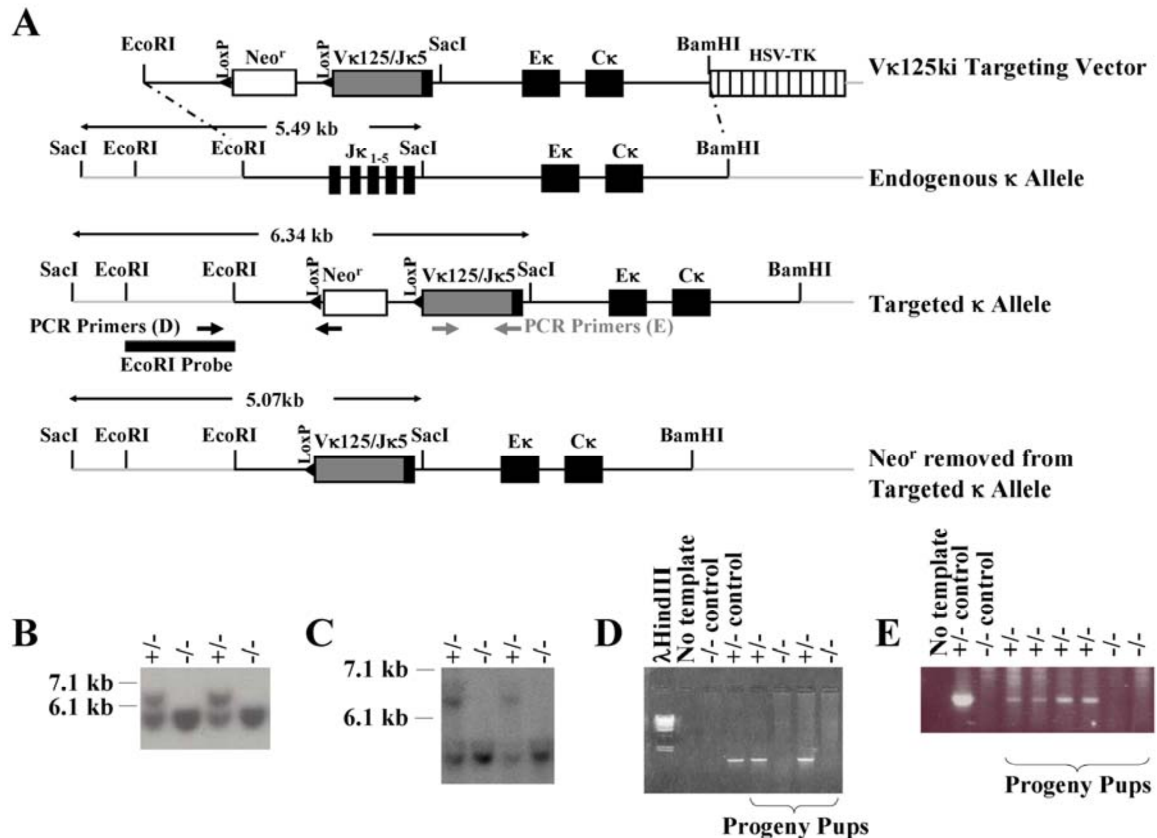

Figure 4-1. Generation of an Igκ-targeted anti-insulin Igκ transgenic model, Vκ125ki. (A) Targeting vector schematic. (B) ES Clones were digested with SacI and Southern blots were performed using the EcoRI probe to identify presence of endogenous (5.5 kb) or targeted (6.3 kb) alleles. (C-D) Tail DNA of chimeric founder progeny was screened for the presence of the targeted allele by (C) Southern blot analysis as in (B), or (D) PCR analysis of amplified fragments using primers to detect the properly targeted allele (FWD #182 and REV #183 primers ; FWD #194 and REV #195 primers) or (E) Vκ125 (FWD #69 and REV #88 primers). Six independent founder lines produced progeny which harbored the targeted allele, as assessed by both Southern blot and PCR analysis (not shown).

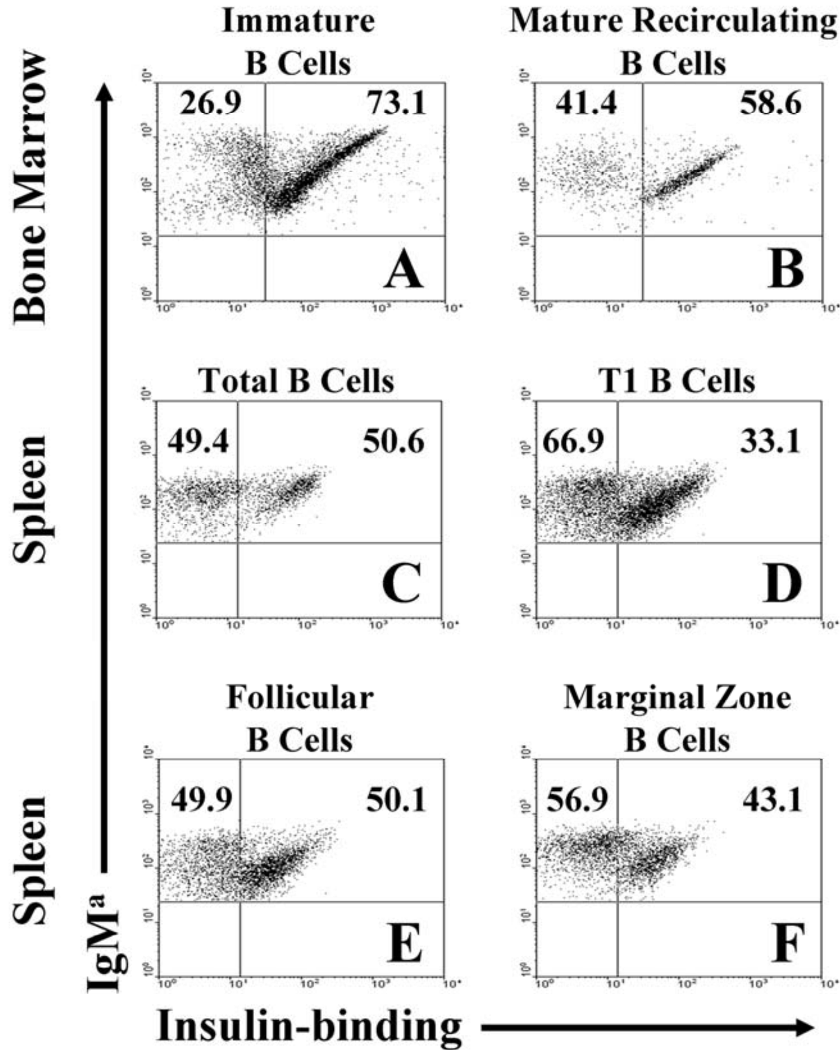

Figure 4-2. VH125/Vκ125ki mice show evidence of receptor editing in the presence of endogenous circulating insulin *in vivo.* Vκ125ki mice were intercrossed with C57BL/6 VH125Tg mice to produce VH125/Vκ125ki mice. (A-B) Expression of B220, IgM^{a}, Igκ, and insulin-binding were assessed in freshly isolated bone marrow cells using flow cytometry. The average percentage ± SD of >10 mice of non-insulin binding "edited" B cells was 26.9 ± 8.4 in immature B cells (B220^{mid}, IgM^{a+} Igκ^{+}, A), and 38.4 ± 7.6 in mature recirculating B cells (B220^{high}, IgM^{a+} Igκ^{+}, B). (C-F) Expression of B220, IgM^{a}, Igκ, CD21, CD23, and insulin-binding were assessed in freshly isolated spleen cells using flow cytometry. The average percentage ± SD of non-insulin binding "edited" B cells was 48.3 ± 11.5 in total B cells (lymphocyte-gated, B220^{+}, IgM^{a+} Igκ^{+}, C), 61.5 ± 11.5 in T1 B cells (lymphocyte-gated, B220^{+}, IgM^{a+} Igκ^{+}, CD21^{lo}, CD23^{lo}, D) 49.3 ± 12.6 in follicular B cells (lymphocyte-gated, B220^{+}, IgM^{a+} Igκ^{+}, CD21^{lo}, CD23^{high}, E), and 64.5 ± 13.4 in marginal zone B cells (lymphocyte-gated, B220^{+}, IgM^{a+} Igκ^{+}, CD21^{high}, CD23^{lo}, F). Similar percentages of non-insulin binding B cells were observed in VH125/Vκ125ki independent lines of mice derived from 6 founders (not shown). Averages of >10 mice are shown for each plot.

increased in the mature recirculating B cells present in the bone marrow (Fig. 4-2B), although to a lesser extent than in the spleen.

B cells undergoing receptor editing spend an extended period of time in the editing-competent compartment (31), thus we hypothesize that the lower percentage of edited B cells present in the bone marrow is due to rapid emigration of the edited cells to the periphery, following successful expression of a non-autoreactive BCR. To confirm this hypothesis, CD21 and CD23 expression was additionally assessed in spleen cells to allow identification of T1, follicular, and marginal zone B cells. T1 B cells are recent bone marrow emigrants, and mature into T2 and mature B cells within 48 hours (106). ~60% of T1 cells did not bind insulin, consistent with the hypothesis that extensive editing occurs in the bone marrow (Fig. 4-2D). Edited (non-insulin-binding) B cells make up ~50% of the follicular B cell compartment (Fig. 4-2E), and this frequency is elevated to ~65% in the marginal zone (Fig. 4-2F). The percentages of non-insulin-binding B cells were consistent among VH125/Vκ125ki mice from multiple independent founder lines and at multiple backcross generations, suggesting that the mixed genetic background of the mice was not influencing the extent of receptor editing. $Ig\lambda^{+}$ B cells were not appreciably detected in the bone marrow or spleen of VH125/Vκ125ki mice, suggesting that Vκ125ki expression was predominantly replaced by the endogenous Igκ allele, and not by switching to the Igλ locus. These findings suggest that a small protein hormone, such as insulin, present at low concentrations *in vivo* is capable of inducing receptor editing.

VH125/Vκ125ki B cells show enhanced Vκ125ki replacement over non-insulin-binding VH281/Vκ125ki B cells

Insulin-binding is not detectable in VH281Tg/Vκ125Tg B cells (186), thus a similar phenotype is predicted for VH281/Vκ125ki B cells. As assessed by flow cytometry, VH281/Vκ125ki B cells do not bind insulin as expected (Fig. 4-3). We therefore hypothesized that insulin encounter would not induce substantial Vκ125ki replacement in VH281/Vκ125ki B cells *in vivo*, as they do not appear to bind this Ag. To confirm that the receptor editing observed in VH125/Vκ125ki B cells correlates with the insulin-reactive specificity of the BCR, Vκ/Jκ usage was assessed in non-insulin binding VH281/Vκ125ki and insulin-binding VH125/Vκ125ki B cells freshly isolated from spleens. IgM^{a+} cells were positively selected using MACS sorting to purify $VH125Tg^{+}$ cells. Flow cytometry analysis was used to confirm that >95% of sorted cells were $IgMa^{+}$. As depicted in Table 4-1, VH125/Vκ125ki B cells show a higher percentage (68%) of clones which utilize endogenous Vκs, compared with VH281/Vκ125ki B cells (40%), indicating that a higher percentage of B cells retain the targeted allele when it is paired with a non-insulin-specific IgH. Individual Vκ/Jκ usage from 20 independent clones is depicted in Table 4-2 and 4-3. These data are consistent with receptor editing enhancement by an anti-insulin specificity of the BCR.

Discussion

The contribution of receptor editing in maintaining tolerance for insulin has previously not been investigated, due to limitations of available anti-insulin BCR Tg models. We therefore developed a novel model in which the anti-insulin Vκ (Vκ125)

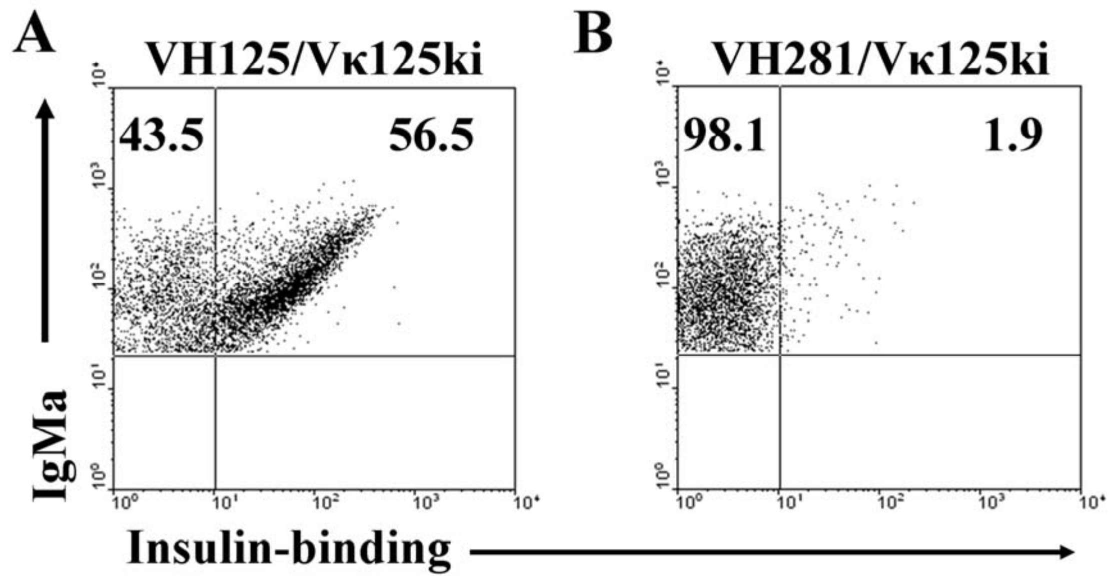

Figure 4-3. VH281/Vκ125ki B cells do not detectably bind insulin. B220, IgM[a], and insulin-binding expression were assessed on freshly isolated bone marrow cells using flow cytometry. The average ± standard deviation of three mice is as follows: (A) VH125/Vκ125ki, 49.2 ± 4.8, (B) VH281/Vκ125ki, 98.1 ± 0.1.

Table 4-1. Frequency of Replacement of a Targeted Anti-Insulin Light Chain (Vκ125ki) Paired with VH125 or VH281

Heavy Chain Transgene	Insulin-binding BCR Specificity?	% Replacement of the Targeted Light Chain Allele
VH125Tg	yes	68
VH281Tg	no	40

Percentages are based on ≥ 20 independent clones for each genotype

Table 4-2. Vκ/Jκ Usage in VH281/Vκ125ki Spleen B Cells

Clone #	Clone Name	Vκ, Jκ Utilized
1	14	Vκ4-74, Jκ5
2	16A	Vκ4-74, Jκ5
3	16D	Vκ4-74, Jκ5
4	16L	Vκ4-74, Jκ5
5	16N	Vκ4-74, Jκ5
6	20A	Vκ4-74, Jκ5
7	20C	Vκ4-74, Jκ5
8	20D	Vκ4-74, Jκ5
9	20G	Vκ4-74, Jκ5
10	20H	Vκ4-74, Jκ5
11	20I	Vκ4-74, Jκ5
12	20J	Vκ4-74, Jκ5
13	15A	Vκ17-121, Jκ5
14	16B	Vκ17-121, Jκ5
15	16C	Vκ4-57, Jκ4
16	16J	Vκ9-120, Jκ2
17	16K	Vκ4-59, Jκ5
18	16M	Vκ14-100, Jκ4
19	20B	Vκ9-124, Jκ4
20	20L	Vκ16-104, Jκ2 or 5

Clones which replaced the targeted allele are shaded in gray, all clones are derived from independent pools of B cells

Table 4-3. Vκ/Jκ Usage in VH125/Vκ125ki Spleen B Cells

Clone #	Clone Name	Vκ, Jκ Utilized
1	5G1	Vκ4-74, Jκ5
2	5J2	Vκ4-74, Jκ5
3	12C1	Vκ4-74, Jκ5
4	12E1	Vκ4-74, Jκ5
5	12H1	Vκ4-74, Jκ5
6	12J1	Vκ4-74, Jκ5
7	94M4	Vκ4-74, Jκ5
8	5A4	Vκ4-63, Jκ4
9	5C1	Vκ4-59, Jκ5
10	5H4	Vκ9-120, Jκ2
11	5L1	Vκ16-104, Jκ2
12	12B1	Vκ5-39, Jκ5
13	12D4	Vκ5-39, Jκ5
14	12F1	Vκ4-68, Jκ4
15	12I4	Vκ5-39, Jκ5
16	12L1	Vκ4-79, Jκ2
17	26A1	Vκ4-68, Jκ1
18	26B1	Vκ4-91, Jκ2
19	26C1	Vκ4-81, Jκ5
20	26D1	Vκ4-80, Jκ4
21	26E1	Vκ14-111, Jκ2
22	26F2	Vκ4-50, Jκ1

Clones which replaced the targeted allele are shaded in gray, all clones are derived from independent pools of B cells

was targeted to the Igκ locus, and is thus subject to physiologic regulation of the locus, such as secondary rearrangement and replacement events. The bone marrow of VH125/Vκ125ki mice consists of ~30% non-insulin-binding B cells, which is elevated to ~50% in the spleen. The T1 B cell stage is highly transient and consists of recent bone marrow emigrants (106). Sixty percent of T1 B cells are non-insulin-binding, suggesting that positive selection in the periphery is not responsible for the frequency of non-insulin-binding B cells observed, but rather that receptor editing during B cell development is occurring. These data suggest that the Vκ125ki transgene is dominantly expressed, and is replaced in a substantial fraction of peripheral B cells that have encountered physiologic levels of circulating insulin *in vivo*. A small protein hormone, such as insulin, is therefore capable of eliciting receptor editing *in vivo* as a means for maintaining B cell tolerance for a key autoAg in T1D.

The lower frequency (~30%) of non-insulin-binding B cells present in the bone marrow compared with the spleen (~50%) could be due to selection and accumulation of non-insulin-binding B cells in the periphery, or could reflect a reduced amount of time that non-insulin-binding, edited B cells spend in the bone marrow relative to their insulin-binding counterparts. The latter possibility seems more likely, as the enhanced percentage of edited B cells is observable in T1 B cells that have recently exited the bone marrow, and also because no accumulation of non-insulin-binding B cells is observed between the T1 and follicular or marginal zone compartments. Time spent in the T1 B cell stage is ~48h (106), so although it is possible that receptor editing may continue following emigration from the bone marrow, edited clones exiting the T1 stage do not appear to be further positively-selected in subsequent maturational stages.

Although the frequency of clones that retained the targeted allele was increased in VH125/Vκ125ki B cells compared with VH281/Vκ125ki B cells, 40% of VH281/Vκ125ki B cell clones showed evidence of IgL replacement based on sequence analysis of Vκ/Jκ transcripts. This could be explained either by imperfect Vκ125ki transgene expression dominance, or by low levels of autoreactivity present in the VH281/Vκ125ki BCR. Measurable insulin-binding is not detected by flow cytometry analysis of these B cells, but it is possible that undetectable levels of autoreactivity, either for insulin or unidentified autoAgs may be present which are sufficient to drive receptor editing. Such possibilities are not addressed in the current studies; regardless, this frequency is enhanced in VH125/Vκ125ki B cells that demonstrably bind insulin, suggesting that Ag specificity is playing a role in enhancing receptor editing incidence. The frequency of receptor editing observed based on clonal analysis of Vκ/Jκ usage (Table 1) is also consistent with the frequency of receptor editing as identified by non-insulin-binding B cells identified through flow cytometry.

SLE-susceptibility genes appear to dampen receptor editing of anti-DNA BCRs (105), so it is tempting to speculate that a similar defect may be present in the NOD background. Future studies will address whether differential degrees of receptor editing are observed in non-autoimmune (C57BL/6) versus autoimmune-prone (NOD) strains of mice carrying the anti-insulin VH125/Vκ125ki BCR.

Interaction of the high affinity ligand, soluble HEL, with anti-HEL B cells (5×10^{10} M^{-1} affinity, (11)) induces receptor editing *in vivo* (76), however the current studies present the first evidence to suggest that B cells recognizing cognate Ag with low avidity and affinity ($\sim 8 \times 10^{6}$ M^{-1}) (172) that is physiologically regulated are subject to receptor

editing. These studies thus suggest that the threshold for receptor editing is lower than previously appreciated, and highlight the exquisite sensitivity of developing B cells for Ag interaction. Receptor editing stimulation by a wide range of BCR:Ag interaction strength would be consistent with previous findings reporting that receptor editing occurs in a high percentage of developing B cells (34, 68, 76, 167, 193). The anti-insulin VH125/Vκ125ki model therefore establishes that B cell tolerance for a critical autoAg in T1D is regulated through receptor editing. Future studies addressing the role of this process in the disease state, as well as the malleability of receptor editing induction during the prodrome of disease may yield important methods of therapeutic intervention for autoimmune disease.

CHAPTER V

DISCUSSION AND FUTURE DIRECTIONS

B cells possessing self specificities are constantly being generated in the bone marrow, and studies indicate that the majority of newly generated B cells are autoreactive (193). These important cells of the immune system are therefore presented with the enormous challenge of defusing or removing potentially dangerous B cells from the repertoire to prevent autoimmune disease. Given the devastating effects of many human autoimmune diseases, it is not surprising that multiple levels of regulation exist to control B cell autoreactivity. These include central tolerance, which acts in the bone marrow to censor the developing B cell repertoire, and peripheral tolerance, which acts in the periphery as a second line of defense against autoreactive B cells that escape central tolerance. The work presented in this dissertation focuses on the role that central tolerance plays in regulating insulin-reactive, developing B cells, as it was unknown whether or how central tolerance was maintained for small protein hormones.

B Cell Anergy Is Induced by Non-Crosslinking Antigens

Several Ig Tg B cell models exist in which tolerance is manifested through anergy. The affinity and valency of BCR:Ag interactions range; limited models currently exist in which the Ag recognized by the Tg BCR is not a highly multivalent interaction. These include the anti-insulin Tg models generated in the Thomas lab, the anti-HEL Tg models (64, 76), and the anti-glucose-6-phosphate isomerase (GPI) model (77). Two major categories of Ig Tgs also exist: non-Ig-targeted Tgs, which are randomly integrated

into the genome, and Ig-targeted Tgs, which are integrated in the physiologic loci. The tolerance outcome observed in the various BCR Tg models depends on these factors.

Anti-HEL B cells recognize HEL with a high affinity (5 x 10^{10} M^{-1}), and both soluble (monovalent) and membrane-bound (multivalent) forms of the Ag are expressed by Tg mice (11, 64). The tolerance outcome differs between the two models; soluble HEL induces anergy, whereas membrane-bound HEL predominantly induces deletion in animals harboring non-Ig-targeted BCR Tgs (64). Ig-targeted anti-HEL Tg models also exist, and receptor editing is favored over deletion when membrane-bound HEL is encountered (76). Evidence of both anergy and receptor editing were observed if soluble HEL was present (76). Anergic anti-HEL B cells show chronic elevated intracellular Ca^{2+} oscillations, continuous translocation of NFAT to the nucleus, and activation of ERK (72).

The anti-GPI model encompasses a range of high to low affinity BCR interactions with autoAg (77). This diverse pool of autoreactive B cells show a range of tolerance outcomes, including anergy, receptor editing, and possibly clonal ignorance (77). Tolerance outcomes in response to non-crosslinking Ags can also be addressed using anti-insulin BCR Tg models. The original 125Tg model generated in our lab consists of non-Ig-targeted, randomly integrated anti-insulin IgH and Igκ genes, which were identified from BALB/c mice immunized with insulin (51, 160, 186). Spleen B cells harvested from these mice were found to be functionally silenced, as defined by many hallmarks of anergy. 125Tg spleen B cells show impaired or no Ca^{2+} mobilization following ionomycin, suboptimal anti-IgM, or insulin stimulation (2). This is likely due to enhanced SERCA pump activity to sequester Ca^{2+} in the ER, based on the finding that

treatment of 125Tg spleen B cells with thapsigargin, an inhibitor that poisons SERCA pumps which pump Ca^{2+} into the ER from the cytoplasm inhibitor, results in increased ER Ca^{2+} release when compared with WT spleen B cells (2, 109, 123). These cells show reduced IP_3 levels, as well as decreased basal NFATc1 and reduced NFATc1 nuclear translocation following stimulation when compared with WT spleen B cells (2). Downstream changes are also evident, as 125Tg spleen B cells show reduced proliferation following stimulation with anti-IgM, anti-CD40, and LPS (1). Development proceeds normally, and the marginal zone compartment is enhanced (1). Low levels of circulating, Tg-derived Abs are detectable, and little production of anti-insulin Ab occurs spontaneously or following T cell-dependent immunization (160).

Tg mice expressing non-Ig-targeted anti-HEL BCR and soluble HEL Tgs most closely mimic the 125Tg system, however, important differences exist. The concentration of circulating insulin is around 1 ng/mL, which can increase 5-10 fold post-prandially (56). The circulating concentration of soluble HEL is ~17 ng/mL (64), which does not fluctuate based on physiologic demand as insulin does (56). The affinity of the MD4 BCR for HEL is 5×10^{10} M^{-1} (11), which is significantly higher than that of the 125Tg BCR for rodent insulin, 8×10^{6} M^{-1} (172). Multiple differences exist in the cellular mechanism of functional silencing induced as well, as highlighted above. These differences, in addition to the key role that insulin plays as an autoAg in T1D (140), outline the importance of studying tolerance in the 125Tg BCR Tg model.

Insulin Elicits Anergy in Immature Anti-Insulin B Cells

Anergy is readily accepted as a form of tolerance that functions in the periphery

(64), however evidence to suggest a role during B cell development in the bone marrow was limited prior to these studies (13, 22). Furthermore, it was unknown whether a small protein hormone, such as insulin, could induce anergy in immature anti-insulin (125Tg) B cells; mature 125Tg B cells are rendered anergic *in vivo* (1, 2, 160), but the ontogeny of this process was undiscovered. Chapter II demonstrates that anergy plays a role in developing bone marrow B cells that encounter the non-crosslinking Ag, insulin. 125Tg bone marrow B cells cultured with human insulin *in vitro* show IgM down-regulation (Fig. 2-3), impaired intracellular Ca^{2+} mobilization in response to BCR-dependent and -independent stimulation (Fig. 2-5), as well as impaired proliferation to anti-CD40 (Fig. 2-4) when compared with naïve cells. Such changes appear to occur shortly following exposure to insulin (Fig. 2-7), and are reversible upon Ag withdrawal (Fig. 2-8). This suggests that anergy in developing B cells is dynamically regulated and maintained to promote a high degree of functional elasticity in the tolerant state observed in these cells.

The bulk of the studies presented in Chapter II were performed using human insulin, for which the 125Tg BCR has a ~100-fold higher affinity than rodent insulin (172). Technical difficulties exist that impair isolation of immature B cells exposed to insulin *in vivo*, therefore the question arises as to whether the *in vitro* findings translate to immature 125Tg B cells encountering endogenous rodent insulin. Availability of purified rodent insulin was experimentally limiting, however the affinity of the 125Tg BCR for beef and rodent insulin is highly similar, so IgM down-regulation following culture with beef insulin was also assessed (Fig. 2-3B). The ability of identical concentrations of beef insulin doses to promote comparable IgM down-regulation in 125Tg bone marrow B cells compared with human insulin suggests that even this lower affinity Ag can produce a

biologically important outcome. As other tolerance outcomes are stimulated by a lack of surface BCR signaling, such as receptor editing in response to soluble HEL (171), I anticipate that rodent insulin is capable of inducing a similar state of functional impairment in immature B cells *in vivo*, given its ability to reduce surface BCR levels. Further confirmation that beef insulin impairs other functional responses in a similar manner would support this hypothesis.

B Cells: Potential Type 1 Diabetes Therapeutic Target?

It is commonly thought that effective treatment of T1D will likely require a multifaceted therapeutic approach. Developing the ability to transplant islets would represent a key breakthrough, but autoimmune attack of transplanted islets must also be controlled to result in long-term insulin-independence. T cells directly mediate β cell destruction (reviewed in 159), however B cells are also necessary for T1D development in murine disease models (3, 173). B cell-targeted therapy has been successfully used to treat other autoimmune diseases (47, 74, 75, 147). Rituximab, a B cell targeted therapy, was approved for treatment of rheumatoid arthritis in 2006 (reviewed in 118), so it seems plausible that a better understanding of how B cell tolerance is dysregulated in the disease state may yield key insight into the design of future therapeutics. Trials are currently underway to assess Rituximab efficacy in the treatment of T1D.

To date, the anti-insulin BCR Tg models developed by our lab (VH125Tg and 125Tg) are the only BCR Tgs shown to be capable of promoting disease in the NOD mouse (1, 78); other non-islet-specific BCR Tgs actually protect against T1D generation

(78, 179). For these reasons, this dissertation sought to elucidate how central tolerance is induced in developing B cells reactive with a key T1D autoAg, insulin (140).

B Cell Tolerance Breaches Occur in Autoimmune Disease-Prone Mice

Receptor editing and deletion triggered by encounter of MHC class I or membrane-bound HEL in cognate B cells is intact in NOD mice, and production of circulating Ab is impaired in anti-HEL NOD B cells when soluble HEL is present (178). However, B cells recognizing soluble HEL show a more reversible anergy phenotype and impaired partial deletion when present on the NOD background, as evidenced by an increased ability of CD40 co-stimulation to rescue proliferation, suggesting that tolerance induction may differ in the autoimmune strain (178). NOD T1 B cells are also more resistant to apoptosis than non-autoimmune strains, also suggesting that normal tolerance checkpoints may be dysregulated (178).

Whereas non-insulin-specific VH281Tg is protective, VH125Tg and 125Tg mice develop T1D, suggesting that the presence of insulin-specific B cells is involved in the disease process (1, 78). Anti-insulin B cells are rendered anergic in the periphery of both C57BL/6 and NOD mice (1). Insulin-reactive B cells form the majority (>95 %) of the mature peripheral repertoire of both strains (1, 160), suggesting that deletion and/or receptor editing are failing to remove these self specificities from the repertoire. These findings imply that anergy induction is insufficient to protect against T1D generation in the NOD mouse, thus highlighting the importance of multiple functional arms of tolerance in preventing insulin autoimmunity (1). It therefore seems likely that anergy in 125Tg NOD mice may impair certain B cell functions, such as proliferation and plasma

cell differentiation, but may preserve other functions, such as Ag presentation. B cells likely contribute to T1D by presenting islet Ags to autoreactive T cells (174), thus if 125Tg NOD B cells retain the capacity to present Ags, this may explain the breach of immune tolerance and ultimately disease development that occurs, which depends on the insulin-specificity of the BCR (1, 78).

As previously discussed, the IgL of the 125Tg model is not targeted to the physiologic locus, thus it is not possible to conclude that receptor editing cannot promote tolerance to insulin using this model. Receptor editing could however function to censor insulin-binding B cells in the VH125Tg model, as endogenous IgLs encode the BCR specificity, and are thus subject to replacement. This model has the additional advantage of permitting the study of insulin-specific B cells in the context of a polyclonal repertoire, which should more closely mimic how this process occurs in a natural (endogenous) repertoire.

It was previously shown that insulin-binding B cells are present in the spleens of VH125Tg NOD, but not C57BL/6 mice (78, 197), however it was unknown whether this was due to differences in the ability to generate and/or censor these specificities. In Chapter III, I present findings indicating that VH125Tg C57BL/6 mice can generate insulin-specific B cells in the bone marrow, which are not present in the spleen *in vivo* (Fig. 3-4, A and I), suggesting that VH125 can pair with endogenous IgLs on the C57BL/6 background to produce an insulin-binding BCR. It is therefore likely that the absence of insulin-binding B cells observed in the periphery is due to induction of one or more B cell tolerance mechanisms.

Deletion of insulin-specific B cells seems unlikely, as culture with up to 50

μg/mL human insulin *in vitro* does not result in a reduction in cell recovery, based on cell counts and the percentage of B220$^+$ cells present (Fig. 2-3). Furthermore, as discussed in Chapter I, deletion typically occurs when receptor editing fails (68). Another possibility is that anergy is being induced in insulin-binding B cells of VH125Tg mice, resulting in either a severely reduced lifespan or an inability to compete for follicular niches. The T1 B cell compartment is formed by recent bone marrow emigrants (5, 139); thus autoreactive T1 B cell survival is not influenced by follicular exclusion by competition with non-autoreactive cells. The similar percentage of insulin-binding B cells present in the T1 versus follicular and marginal zone B cell compartments of VH125/Vk125ki mice, which are capable of editing the Igκ-targeted anti-insulin Igκ, suggests that insulin-binding B cells do not exhibit a survival disadvantage in the periphery, even when competition from non-insulin-binding B cells exists (Fig. 4-2). It therefore does not seem likely that defective survival of anti-insulin B cells would account for the lack of insulin-binding B cells in the spleen of VH125Tg mice.

The final potential tolerance outcome, receptor editing, is the mechanism that seems most probable. Unpublished preliminary observations suggest that insulin-binding B cells present in the bone marrow are absent at the T1 stage of B cell development in VH125Tg C57BL/6 mice, thus it seems unlikely that the absence of insulin-binding B cells in the spleen could be due to differences in cell turnover or follicular exclusion. Additionally, Chapter IV formally demonstrates that circulating insulin *in vivo* can elicit receptor editing in anti-insulin VH125/Vκ125ki B cells (Fig. 4-2), thus supporting the likelihood that receptor editing may also function to censor anti-insulin B cells in a polyclonal repertoire.

Although it is difficult to identify which mechanism(s) of tolerance may be responsible for removing insulin-reactive B cells from the periphery of VH125Tg C57BL/6 mice, it is clear that one or more of the forms of tolerance that are functioning on the C57BL/6 background is/are dysregulated on the NOD background, which permits the development of insulin-specific B cells in the periphery of these mice. Moreover, rather than removing insulin-specific B cells from the mature repertoire, VH125Tg NOD mice appear to positively-select for these cells, as the percentage is significantly increased in the spleen compared with the bone marrow (Fig. 3-4Q). A significantly increased ability of VH125Tg NOD mice to generate insulin-binding B cells (compared with VH125Tg C57BL/6 mice) may further enhance the peripheral population of insulin-binding B cells, and thus likelihood of T1D development in NOD mice (Fig. 3-5Q). These findings are summarized in a model (Fig. 5-1).

A Novel BCR Transgenic Model (VH125/Vκ125ki) Shows Evidence of Receptor Editing in the Presence of Circulating Insulin *in vivo*

As detailed previously, the existing 125Tg model presents major limitations for the study of receptor editing, as novel rearrangements at the IgL loci will not inhibit expression of the Vκ125 Tg integrated in a non-physiologic (non-Igκ or -Igλ) locus. Chapter IV describes the generation of a new model, in which Vκ125 is targeted to the Igκ locus. Expression of the Igκ-targeted Vκ125ki Tg should thus be subject to normal regulatory mechanisms aimed at the Igκ locus. These animals are intercrossed with VH125Tg mice to generate VH125/Vκ125ki mice, which should develop insulin-binding B cells that are subject to receptor editing. Additional Jκ segments are not present on the targeted allele (Fig. 4-1), thus non-insulin-binding B cells that express the Tg IgH

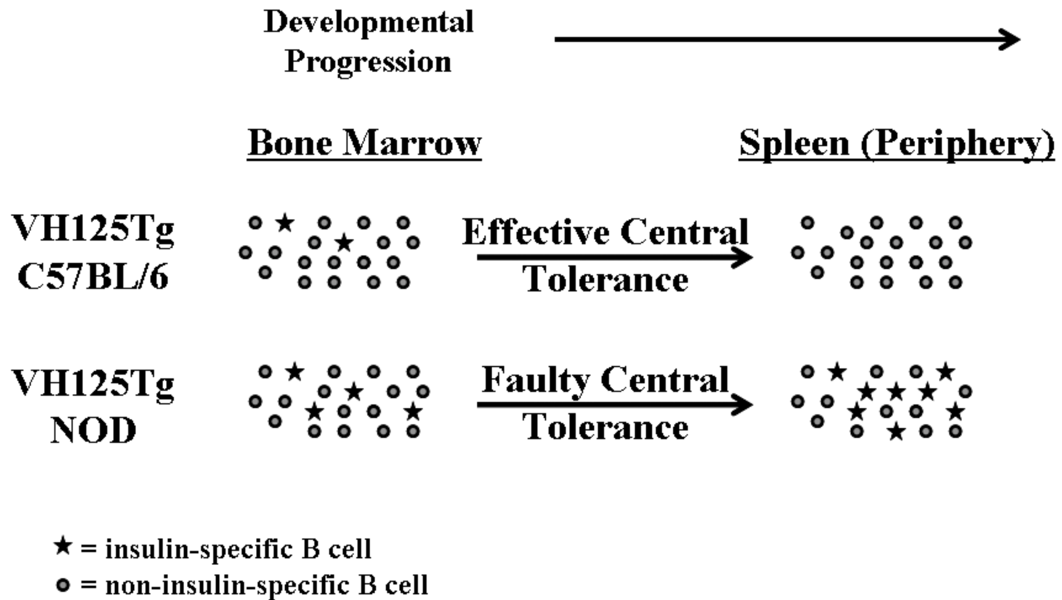

Figure 5-1. NOD mice show faulty central tolerance and fail to remove insulin-specific B cells from the peripheral repertoire. Insulin-specific B cells (represented by black stars) develop in the bone marrow of both VH125Tg C57BL/6 and NOD mice amidst a polyclonal repertoire (non-insulin-specific B cells indicated by grey circles). The number of stars shown represent relative percentages present in the various compartments (actual percentages of the total repertoire are much lower). These autoreactive B cells are absent in the periphery of C57BL/6 mice, suggesting that one or more mechanisms of tolerance are functioning to censor these cells. This contrasts with NOD mice, which permit selective accumulation of insulin-specific cells in the periphery, which indicates a breakdown in one or more tolerance mechanisms is responsible. The elevated percentage of insulin-specific B cells in the bone marrow of NOD mice suggests a bias towards generating insulin-reactive B cells in the developing repertoire. Multiple factors therefore contribute to the generation of anti-insulin B cells in the periphery of VH125Tg NOD mice.

(confirmed by IgM^a expression) must express an IgL that rearranged on the other Igκ allele or either of the Igλ loci. Insulin-binding specificity of the BCR therefore provides a direct readout of B cell receptor editing in VH125/Vκ125ki mice.

Chapter IV shows that circulating insulin results in Vκ125ki replacement in ~50% of peripheral VH125/Vκ125ki B cells. The low percentage (~30%) of B cells which have replaced the insulin-binding specificity in the bone marrow is increased to ~60% in the T1 B cell compartment, suggesting that expression of Vκ125ki is likely dominant over endogenous IgLs, and that once immature B cells successfully replace the insulin-binding BCR, they rapidly leave the bone marrow (Fig. 4-2). I hypothesize that the ~30% of non-insulin-binding B cells present in the bone marrow are cells which have undergone receptor editing, rather than those which never expressed Vκ125ki, although future experiments will be required to prove this.

Assessment of Vκ/Jκ usage showed that a higher percentage (68%) of VH125/Vκ125ki B cells replaced the Igκ-targeted Vκ125 Tg, compared with 40% of VH281/Vκ125ki B cells (Table 4-1). VH281 differs from VH125 by two amino acids which are responsible for insulin-binding (186), thus VH281/Vκ125ki B cells are not predicted to bind insulin. These findings therefore suggest that the increased receptor editing observed in VH125/Vκ125ki B cells is due to insulin reactivity of the BCR, as it is unlikely that pairing efficiency differences exist among the two IgHs, given their high degree of structural similarity. It also confirms that poor expression of the Vκ125ki Tg does not solely account for receptor editing, as both strains should exhibit the same efficiency of Tg expression dominance. Interestingly, VH281/Vκ125ki B cells do show an appreciable, albeit lower level of replacement of the Igκ-targeted Vκ125ki Tg. One

possible explanation is that Vκ125ki isn't dominantly expressed, however this seems unlikely, given that 70% of IgM^{a+} B cells in the bone marrow bind insulin, and thus likely express the targeted allele, at least initially (Fig. 4-2). Another explanation is that VH281/Vκ125ki B cells are also autoreactive, perhaps due to undetectable, yet physiologically relevant interactions with insulin, or with another unidentified autoAg *in vivo*. The data presented in this thesis do not confirm or deny these possibilities, but regardless of the "background" receptor editing in VH281/Vκ125ki B cells, receptor editing is enhanced in insulin-specific VH125/Vκ125ki B cells, supporting the conclusion that circulating insulin can induce receptor editing.

Interestingly, VH281/Vκ125ki, VH125Tg/Vκ1ki, and VH125Tg/Vκ8ki B cells all show a lower extent of receptor editing (30-40%), whereas VH125Tg/Vκ4ki and VH125/Vκ125ki B cells show a similarly higher percentage of clones that have replaced the targeted allele (Table 3-2 and 4-1). It is therefore tempting to speculate that Vκ4ki and Vκ125ki may both confer a higher degree of autoreactivity to the BCR when paired with VH125. In another study, mice possessing only the targeted Vκ4ki showed evidence of receptor editing, suggesting that Vκ4ki may be predisposed to interact with IgHs to produce an autoreactive BCR (150). As they are both Vκ4 family members (Vκ4-81 and Vκ4-74, respectively), perhaps there are structural similarities among the targeted Igκs (Vκ4ki and Vκ125ki) that contribute to this phenotype. Further characterization would be required to assess whether this explanation is correct.

In summary, insulin is capable of eliciting multiple forms of tolerance in developing anti-insulin B cells following insulin exposure, including anergy and receptor editing. Replacement of the anti-insulin BCR specificity is not observed in the 125Tg

model, thus tolerance for insulin is principally manifested through anergy in this model. When the anti-insulin specificity of the BCR stems from either endogenously rearranged light chains (VH125Tg) or an Igκ-targeted anti-insulin IgL (VH125Tg/Vκ125ki), receptor editing-mediated replacement of the autoreactive IgL becomes possible. Accordingly, replacement of the anti-insulin IgL is observed in VH125Tg/Vκ125ki peripheral B cells, suggesting that tolerance for insulin can also be maintained through receptor editing. These findings are summarized in Fig. 5-2.

Future Studies

The development of the VH125/Vκ125ki BCR Tg model opens the door to a diverse array of new questions. Approximately half of the mature B cell repertoire have replaced the targeted Igκ through receptor editing, however the remaining half of the B cells retain the insulin-binding specificity (Fig. 4-2). Allelic inclusion can "dilute out" an autoreactive BCR on the surface of the cell, and has been shown to actually facilitate autoimmunity by preserving the autoreactive B cell in the repertoire in a functional state (100). It is unknown whether allelic inclusion occurs in VH125/Vκ125ki B cells, which may thus relax the tolerance stringency imposed and allow enhanced production of circulating insulin Ab. It will therefore be important to assess whether allelic inclusion is occurring, and whether insulin-binding VH125/Vκ125ki B cells show evidence of anergy.

Another important question which has not been addressed is whether danger signals, such as TLR engagement, alter receptor editing. In general, bacteremia or

BCR Interaction Strength

	Original Antigen Specificity	Light Chain Transgene	Clonal Ignorance	Anergy	Receptor Editing	Clonal Deletion
125Tg	Insulin-Restricted	Non-targeted	No	Yes	No	No
VH125Tg	Insulin/Polyclonal	Polyclonal	No	?	?	Unlikely
VH125Tg/Vκ125ki	Insulin-Restricted	Targeted	No	?	Yes	No
			Retention of Original Specificity in Repertoire		Removal of Original Specificity from Repertoire	

Figure 5-2. B Cell Tolerance Outcomes Observed in Anti-Insulin Transgenic Mice. Decreased BCR interaction strength is classically recognized to permit retention of the autoreactive BCR specificity, whereas stronger interactions result in removal of the specificity from the repertoire. Both anergy and receptor editing function to censor insulin-reactive immature B cells.

viremia is associated with a substantial infection, thus it seems logical that pathogen-specific bone marrow B cells should be preserved, rather than removed from the repertoire. In support of this, LPS, a TLR4 agonist, drives immature B cell proliferation (121). RAG proteins are negatively regulated by cell cycle progression (102, 104), therefore I hypothesize that LPS stimulation of immature B cells will suppress receptor editing induced by cognate Ag engagement by inducing cell cycle which negatively regulates RAG expression, thus protecting pathogen-specific B cells from tolerance induction.

Chapter III shows that insulin-binding B cells, present in the developing repertoires of both VH125Tg C57BL/6 and VH125Tg NOD mice, are only present in the periphery of VH125Tg NOD mice. This prompts the hypothesis that the efficiency of receptor editing on the C57BL/6 background may be more effectively removing these specificities, thus the Vκ125ki line is additionally being backcrossed to the NOD background to answer this question. Although some evidence exists to suggest that tolerance defects are present in NOD mice (178), these mice will allow us to probe whether receptor editing for monovalent proteins is altered in T1D-prone mice, which has not been addressed. Differential receptor editing might be due to altered signaling threshold sensitivity of NOD versus C57BL/6 immature B cells, or perhaps in the kinetic window in which receptor editing must be achieved prior to bone marrow export. Peripheral absence (or accumulation) of insulin-reactive B cells might also be explained by efficiency differences of bone marrow B cell to home to the spleen among the two strains. Future studies will therefore aim to identify and explain any insulin tolerance defects present in NOD B cells.

Summary

Small protein Ags which do not strongly engage the BCR were classically assumed to induce clonal ignorance, due to the expectation that they would fall below the biological threshold of BCR detection. However, mature anti-insulin B cells show evidence of anergy in the periphery, indicating that tolerance is maintained for this critical T1D autoAg (1, 2, 160). The role that central tolerance plays in preventing insulin autoreactivity has been explored throughout this body of work, and I find that multiple avenues of central tolerance can be employed to keep insulin-specific B cells in check, including anergy and receptor editing. Limited data exist regarding the role of central tolerance for developing B cells recognizing monovalent Ags. HEL-specific B cells show signs of anergy in the periphery, however these studies are the first to describe anergy in immature B cells recognizing monovalent Ag. Receptor editing has also been described for IgHELMD4Tg B cells, however our model uses an Ag that represents a much lower affinity interaction; the affinity of 125Tgki for insulin *in vivo* is 10000 times lower than that of MD4Tg B cells for HEL (11, 172). The work presented in this dissertation challenges the dogma of the field and shows that small protein hormones can elicit active tolerance maintenance in immature B cells, and thus redefines the "antigen-sensitivity rheostat" of developing B cells.

APPENDIX A

LIST OF PUBLICATIONS

I. **Henry, R.A.** and Thomas JW. Insulin Induces Central Tolerance in a Developing Polyclonal B Cell Repertoire. *In Preparation*.

II. **Henry, R.A.,** Acevedo-Suárez CA, and Thomas JW. 2009. Functional Silencing Is Initiated and Maintained in Immature Anti-Insulin B Cells. *J. Immunol*. 182(6): 3432-3439.
PMID: 19265121

III. Antar, A.A.R., Konopka, J.L., Campbell, J.A., **Henry, R.A.**, Perdigoto, A.L., Carter, B.D., Pozzi, A., Abel, T.W., and Dermody, T.S. 2009. Junctional Adhesion Molecule-A Is Required for Hematogenous Dissemination of Reovirus. *Cell Host & Microbe* 5(1):59-71.
PMID: 19154988

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