Intrathymic B cells as Mediators of Autoimmune Type 1 Diabetes

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Abstract

B cells are implicated in the pathogenesis of type 1 diabetes (T1D), a serious autoimmune disease. It has been found that in nonobese diabetic (NOD) mice, a well described animal model of T1D, there is an enlarged thymic B cell population, not seen in non diabetic B6 mice, and such B cells may contribute to T1D development. The mechanisms by which B cells are populating the NOD thymus are unknown and delineating the mechanisms would offer new insights into abnormalities of the NOD thymus that may contribute to T1D.

This project investigated potential intrathymic B cell development of B cells in NOD mice, compared to B6 mice. Molecular biological approaches determined that transcripts of essential B cell development transcription factors were present in the NOD thymus. Flow cytometric studies established the presence of thymic pro and pre B cells, as well as thymic $CD19^{+}$ cells expressing RAG enzymes, indicating active rearrangement of the B cell receptor that is crucial for pro to pre B cell progression. Interestingly, comparative studies using B cell sufficient and deficient NOD mice revealed that mature B cells appear to play an important role in the enhancement of the thymic pro B cell population frequency. In contrast to pro B cells, the frequency of the earliest B cell progenitors in the NOD thymus was independent of mature B cells. B cell committed progenitors were decreased in the NOD thymus compared to the B6 thymus, suggesting that there may be an alternative B cell development pathway in the thymus compared to the bone marrow.

The data presented here provides a novel insight into the NOD thymus at the level of thymic B cells. Future studies to establish the definitive mechanisms for thymic B cell development, and their role in the T1D process will be informative.

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Declaration

I, Helen Davies, declare that this thesis is a presentation of original work and I am the sole author. This work has not previously been presented for an award at this, or any other, University. All sources are acknowledged as References.

Introduction

1.1. Diabetes - Facts and Figures

Diabetes mellitus is a condition affecting the body's ability to control blood sugar levels. According to statistics provided by Diabetes UK, diabetes affects approximately 3.9 million people in the UK, with many more sufferers, as yet, undiagnosed. The incidence of diabetes is rising rapidly, with the prevalence of diabetes in the UK now being more than double that of 1996 [1].

Diabetes results from either lack of, or resistance to, the hormone insulin. The pathogenesis of the disease depends on the type of diabetes in question. There are a few types of diabetes including gestational diabetes, seen in some pregnancies, type 1 diabetes, and type 2 diabetes [1]. Type 1 diabetes (T1D) is an autoimmune condition where the body's own immune system destroys insulin producing cells, resulting in a lack of insulin. Type 2 diabetes (T2D), on the other hand, is when the body becomes resistant to the action of insulin and insulin production is impaired. Both type 1 and type 2 diabetes result in a decreased ability to regulate blood glucose levels [2].

Insulin is produced in the pancreas by β cells in the Islets of Langerhans. It is a vitally important hormone involved with the maintenance of normal blood glucose levels, in particular stopping dangerous rises in blood glucose levels. Insulin's main actions are to stop the production of extra glucose when blood

glucose is already sufficiently high. It does this by keeping the brakes on mechanisms of glucose production such as glycogenolysis (breakdown of glycogen stores), lipolysis (breakdown of fats) and proteolysis (breakdown of protein). It also prevents the production of glucagon, the hormone which acts to raise glucose levels [3].

The dysregulation of blood glucose levels seen in diabetes is what causes the many complications seen in the disease. It is, therefore, crucial for the patient to carefully monitor and control blood glucose by diet and/or medication, depending on type and severity of the diabetes. Alongisde the characteristic diabetes symptoms of excessive thrist, drinking and urination, there are serious side effects both in the short and longer term. In the short term, extremes of blood glucose, either too high or too low, can lead to hyper- or hypoglycaemia which require fast treatment in order to prevent potentially lethal conditions such as diabetic ketoacidosis in T1D, or hyperosmolar hyperglcaemic state in T2D [1, 4]. In the longer term, prolonged mild hyperglycaemia caused by diabetes can cause micro- and macrovascular complications leading to blindness, neuropathy, kidney failure and a significantly increased risk of cardiovascular disease [4]. It is therefore vitally important for research into diabetes to continue, with the aim of finding a cure.

1.1.1. Type 1 Diabetes

This project is concerned only with type 1 diabetes (T1D). T1D is a serious autoimmune condition whereby the host's own immune system destroys β cells in the pancreatic Islets of Langerhans [5]. Destruction of β cells results in a lack of insulin and forces the patient to rely on self monitoring of blood glucose through regular finger prick blood testing and subsequent insulin administration. Until recently it was thought that T1D led to a total obliteration of β cells, however, it is now thought that very small numbers of β cells remain and are able to secrete insulin [6, 7]. Type 1 diabetes affects approximately 10% of all diabetes sufferers [1].

1.2. The Immune system

1.2.1. Role

The immune system evolved to protect animals from a variety of pathology-inducing agents such as bacteria, protozoa, viruses and fungi. The immune system is designed to recognise invading pathogens and eliminate them before they can cause harm to the host. The immune system consists of two interconnecting arms known as the innate immune system and the adaptive immune system. The innate system is the more primitive, non specific system whereas the adaptive immune system is highly specific and capable of recognising unique components on pathogens. The cells and processes involved in each arm of the immune system are different but are interconnected through cell-cell interactions and cytokine and chemokine secretion so that pathogens can be targetted in the most effective way [8–10].

1.2.2. The Innate Immune System

The innate immune system is characterised by being non specific, fast acting and incapable of forming immunological memory. The aim of the system is to prevent entry of pathogens and recognise, engulf and destroy pathogens which have managed to invade. Following this, pathogenic antigens are presented to cells of the adaptive immune system and inflammatory cytokines are produced to signal to other immune cells and boost the immune response [9].

The innate immune system has numerous ways of preventing pathogen entry, such as the skin and epithelial tight junctions providing physical barriers and enzymes, antimicrobial peptides and mucus to catch and kill pathogens trying to enter [9, 10].

However, should microorganisms gain entry to the body, there are innate immune cells which can take over. These include phagocytes (macrophages, dendritic cells (DCs) and neutrophils) and natural killer (NK) cells. To recognise

invading pathogens, cells of the innate immune system have a number of different receptors, both membrane bound and free in the cytosol. These pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), C-type Lectins, NACHT-leucine rich repeat receptors (NLRs) and Rig-like receptors (RLRs) are capable of recognising a variety of patterns expressed only by microbes, known as pathogen-associated molecular patterns (PAMPs) [11, 12]. Binding through receptors such as these transmits signals into the cells resulting in subsequent release of inflammatory cytokines, upregulation of major histocompatibility complex (MHC) Class II, upregulation of costimulatory molecules (vital for antigen presentation, see below), and upregulation of killing mechanisms following phagocytosis of pathogens. The ability to recognise these PAMPs gives the innate immune system to ability to distinguish self from non-self [11].

However, the immune system is not only there to protect from invading organisms. Damaged host cells also require removal by the immune system to prevent inflammation and potential autoreactive responses. In order to do this, it is thought that the innate immune system is also able to recognise damage-associated molecular patterns (DAMPs) on damaged host cells which can also bind to TLRs and ellicit an immune response [13, 14]

1.2.3. The Adaptive Immune System

The adaptive immune system is different from the innate immune system in that it is slower acting, highly specific and has the ability to form immunological memory [10]. The adaptive immune system is concerned with tailoring the immune response to a specific pathogen.

The adaptive immune system is composed of T and B lymphocytes, also known as T and B cells. These cells express receptors which are specific to a given antigen. Each T and B cell has a different antigen specificity, therefore, the adaptive immune system can recognise a huge number of antigens via B and T cell receptors [9].

However, B and T cells require activation before they can function. In order to

activate these cells, they must first encounter their cognate antigen (i.e, the antigen for which their receptor is specific) [10]. B cells are capable of recognising whole protein, whereas T cells require their antigen to be degraded into peptide fragments and presented to them by antigen presenting cells (APCs) [9]. Once activated, B and T cells are able to carry out their functions.

The functions of the adaptive immune cells vary. T cells have many different subsets with differing functions depending on the subset. Some are concerned with orchestrating the overall immune response (T helper cells) while others are designed to kill their target cells directly (killer T cells). These will be discussed in more detail in Section 1.3.3. B cells are capable of antigen presentation but their main function is antibody production. Antibodies are the secreted form of the B cell receptor and are designed to help with removal of the pathogen by phagocytosis or complement (a process consisting of a variety of different proteins aimed at the destruction of pathogens) and to help neutralise harmful toxins produced by microorganisms [15].

1.2.4. Receptor Rearrangement

As mentioned above, T and B cells have receptors on their surface that are specific to their cognate antigens. T cells have a T cell receptor (TcR) and B cells have a B cell receptor (BcR). Each B and T cell has a unique receptor which results in a massive repertoire of receptors, each capable of responding to a different antigen. Due to both types of receptor requiring a huge potential for diversity, the mechanisms which introduce this diversity during receptor development are largely the same for both receptor types.

There are two types of TcR, each consisting of two chains. The main one is an $\alpha\beta$ receptor which is most common, and the second is a $\gamma\delta$ receptor [9]. In the BcR, there are also two chains, known as the heavy and light chain [16]. The heavy chain is equivalent to either the β or δ chains, while the light chain is equivalent to the α or γ chains. The receptor chains consist of a variable and constant region. The variable region is the part which is capable of recognising

antigen [16].

The variable region is made up of V (variable), D (diversity, found only in heavy, β and δ chains) and J (joining) gene segments. Genomically encoded are multiple copies of different V, D and J genes and during rearrangement, one of each of these genes is selected at random and joined to produce different receptors [17]. This process is mediated by enzymes known as RAG (recombination activating genes) recombinases [10]. These enzymes recognise recombination signal sequences (RSS) found next to the coding regions of each V, D and J gene and bring the ends of the chosen genes near to each other to be joined [17, 18]. To join the segments, RAG proteins cleave at either end of the genes and leave hairpin loops of DNA. From here a nuclease nicks the loops leaving short sequences at the end of each gene to be joined. This allows the segments to join via non homologous end joining [19]. Further diversity is then added via the use of an enzyme called terminal deoxynucleotidyltransferase (TdT) which adds random base pairs between each segment [20]. Combined, all these mechanisms allow for the formation of approximately $10^{13\text{-}14}$ possible receptors [8, 20].

1.3. T cells

1.3.1. Development

T cells develop in the thymus. Since the thymus does not contain self-renewing thymocytes, the process of T cell development relies on the constant seeding of the thymus by progenitors from the bone marrow [21, 22]. However, the cell that is responsible for this seeding and acts as a T cell progenitor is not known. There are many candidates and these have been identified by looking at the surface markers that are required for a cell to be able to settle in the thymus and develop into a T cell.

Haematopoietic stem cells (HSCs) are the progenitors from which all blood cells derive. These cells are found in the bone marrow and, via the production of various progenitors, can produce megakaryocytes, erythrocytes, lymphocytes,

granulocytes, macrophages and NK cells. HSCs are characterised by their lineage $(lin)^-$, stem cells antigen-1 $(Sca\text{-}1)^{high}$, stem cell growth factor receptor $(c\text{-}kit)^{high}$ phenotype (so-called LSK cells). HSCs also lack surface fms-like tyrosine kinase 3 (Flt3) expression [23].

Following the HSC stage, multipotent progenitors (MPPs) form which have the capability of producing all types of blood cell, but lack the self-renewing ability of HSCs. These cells are LSK but also express $Flt3^{low}$ [23]. Interestingly, MPPs can express genes of multiple lineages [24] suggesting that this expression is due to priming for lineage committment, rather than commitment itself. It is thought that by expressing these genes, it maintains them in an accessible chromatin state, ready for commitment [23]. Evidence for this has been seen through changes in chromatin staus of lineage restricted genes during blood cell development [25].

Following MPP development, magakaryocytic and erythrocytic potential is much reduced and the so-called lymphoid primed multipotent progenitor (LMPP) arises. These cells are capable only of lymphocyte, macrophage, granulocyte and NK cell production [26]. LMPPs are characterised by being LSK but also $Flt3^{high}$.

Next, the interleukin-7 receptor-α (Il-7Rα) upregulates and Sca-1 and c-kit downregulate to form common lymphoid progenitors (CLP). These cells are capable only of B and T lymphocyte development and NK cell development [27] and have a $Sca\text{-}1^{low}$ $c\text{-}kit^{low}$ $Flt3^+$ $Il\text{-}7R\alpha^+$ phenotype. Flt3 is the receptor for Flt3 ligand (Flt3L) which is found in the bone marrow and acts as a growth promoter for CLPs. Flt3L also works synergistically with IL-7 to promote lymphocyte proliferation [28]. CLPs will be discussed further in Section 1.4.1.

There are many candidate thymic settling progenitors (TSPs). It appears that TSPs must express Flt3, which rules out HSCs as thymic seeding progenitors, [21], and also chemokine receptor (CCR) 9 or CCR7 or both [29]. CCR9 and CCR7 are chemokine receptors which are important for allowing thymic settling. While the expression of only one type of chemokine receptor is sufficient,

it is more efficient if both are expressed. These requirements for settling suggest that likely candidates for TSPs are CCR7+CCR9+ LMPPs and CLPs[21].

Following the thymic settling of precursors, T cells progress into the four step double negative phase (DN1-4), where they express neither cluster of differentiation (CD) molecules CD4 or CD8. During their time in this phase, particularly in DN3, the TcR begins to rearrange by the mechanisms outlined in Section 1.2.4 [30]. Following rearrangement, T cells are able to continue to the double positive (DP) stage where thy express both CD4 and CD8 molecules [31].

In the DP stage, T cells undergo their first round of education, known as positive selection. This is the process whereby all developing T cells are exposed to thymic cortical epithelial cells (cTECs) expressing MHC class I and MHC Class II. If the T cell doesn't recognise either MHC Class, or binds with a very high affinity, they receive a death signal and are deleted by apoptosis. However, for those T cells that bind to one of the MHC Classes with the correct affinity, they receive a survival signal and continue with their development. This process is to eliminate all those T cells that wouldn't be able to recognise self MHC presenting peptide and all those which may react dangerously to it [30, 32].

Assuming a T cell is positively selected, the type of T cell it will become ($CD4^+$ or $CD8^+$) depends on which MHC Class it recognised. For example, if a T cell recognised MHC Class II, it develops into a CD4 single positive ($CD4^+$) T cell or if it recognised MHC Class I, it becomes a CD8 single positive ($CD8^+$) T cell.

Following positive selection, T cells then move from the thymic cortex to the medullar where they are exposed to medullary thymic epithelial cells (mTECs) which mediate the process of negative selection [30]. This process is an important step in preventing autoreactivity as it is here that developing T cells are presented with self antigens. mTECs express autoimmune regulator (AIRE), an enzyme which is capable of producing many self antigens so that mTECs can present their peptides alongside MHC molecules to T cells to test the reactivity of the TcR to the host antigen [33]. The affinity with which T cells can recognise these self-antigens via their TcR is important for determining whether or

not a T cell is allowed to continue developing [34]. Cells which bind strongly to the self antigens are given a death signal as these T cells, upon completion of their development and migration out of the thymus into the periphery, could potentially recognise host antigen and attack the tissue. This is known as autoimmunity.

The aim of negative selection in the thymus is to only allow the release T cells that will not react to host antigens. Negative selection is, therefore, the first step in preventing autoimmunity by tolerising the T cell repertoire through removal of self-reative T cell clones [35] (T cell tolerance is discussed further in Section 1.3.5.)

1.3.2. Genes and Transcription factors driving T cell development

T cell development relies on expression of specific genes and transcription factors to drive development in the lineage. The first stage of T cell development is the development of early thymic progenitors (ETPs) from TSPs arriving in the thymus. There are a number of transcription factors that help with this process and, indeed, deficiency in any of these transcription factors can lead to a decrease in the ETP population without affecting their progenitors. These transcription factors include Notch1, T-cell factor 1 (TCF-1) and GATA-binding protein 3 (Gata3) [36–39].

Notch is a very important transcription factor for T cell commitment. The progenitors seeding the thymus arrive from the thymus and it is seems that some of these progenitors may retain B cell potential [40]. Notch expression is capable of suppressing B cell lineage commitment by suppression of B cell development transcription factors, such as paired box 5 (Pax5) (See Section 1.4.2) so that T cell development is promoted. It has been found that inactivation of Notch1 in bone marrow progenitors results in a block in T cell development and the formation of ectopic B cells in the thymus, suggesting it's expression is crucial for T cell development. Further evidence is shown by constitutive expression of Notch1 in the bone marrow causing T cells to develop abnormally in the bone

marrow [41]. These actions show that it is important for transcription factor balance to be maintained in order to support normal lymphocyte development in the correct place.

E2A is a protein encoding two transcription factors, E12 and E46. E2A proteins are also important in T cell development and their actions appears both upstream of Notch1 by affecting LMPPs [42], and downstream, helping to drive T cell transition from DN1 to DN2 [37]. As well as this, it appears to regulate the expression of Notch1 itself [42].

There are also other transcription factors involved in T cell development, such as RUNT-related transcription factor (Runx) and Bcl11b [37, 43]. Runx is important for progression from ETP to DN2 and DN2 to DN3, while Runx appears to be essential for absolute T lineage commitment in the late DN2 stage [37, 43]. Prior to absolute T cell commitment, DN2 cells retain DN, NK and macrophage potential [37].

1.3.3. Role

There are different types of T cells and they all have different functions as outlined below:

- $CD4^+$ Helper T lymphocytes - $CD4^+$ Helper T cells ($CD4^+$ T cells) are cells which are able to help other immune cells carry out their own functions. Helper T cells express CD4 which allows them to respond to peptide antigens held in MHC Class II on the surface of APCs. Activated $CD4^+$ T cells are able to help with $CD8^+$ cytotoxic T cell activation and B cell antibody production.

- $CD8^+$ Cytotoxic T lymphocytes - $CD8^+$ cytotoxic T cells (CTL) are produced through activation of $CD8^+$ T cells. Once activated, CTLs are capable of killing off their target cells in a highly specific manner.

- Regulatory T cell - There are many types of regulatory T cells (Tregs) which all act to suppress harmful immune responses in the periphery, either by

producing cytokines which inhibit harmful immune responses (such as IL-10), killing immune cells to prevent their harmful action, disruption of immune cell metabolism, or modulation of APC function/maturation [44].

1.3.4. T cell Activation

Antigen presentation is the process by which the innate immune system can signal to the adaptive immune system that an immune response is required. Professional APCs (macrophages, DCs and B cells (see Section 1.4) scavenge pathogens when they invade and internalise them. From here they breakdown the pathogen, then express it's antigen either on MHC Class II (to activate $CD4^+$ T cells), or MHC Class I (to activate $CD8^+$ T cells).

For T cells to become activated, they must be presented with their cognate antigen in the peptide/MHC complex on an APC. T cell activation requires three signals as follows [45, 46]:

1. Signal one - TcR must recognise the peptide/MHC complex on the APC
2. Signal two - Costimulatory molecules CD86 or CD80 on the APC must bind CD28 on the T cell. Costimulatory molecules are only expressed by APCs in inflammatory conditions
3. Signal three - Cytokines released by the APC influence the type of effector cell that the T cell will become. For example, $CD4^+$ helper T cells can become Th1, Th2 or Th17 effector cells, depending on signals from the APC and the type of immune response required [46]

$CD8^+$ T cells are activated differently to $CD4^+$ T cells, in that they will only respond to MHC Class I. This process requires a process known as cross-presentation by the APC. Normally, exogneous antigens (i.e. those from outside a cell) are processed and expressed on MHC Class II, whereas endogenous antigens (i.e. antigens from inside the cell, either damaged components or intracellular pathogens) are processed and displayed on MHC Class I. MHC Class

I is expressed on every nucleated cell and therefore provides a mechanism by which cells can signal that they are virally infected or cancerous.

However, for $CD8^+$ to become activated, they need to be presented with their antigen and costimulatory molecules which are only present on APCs. This requires a process known as cross presentation by the APC, whereby an exogenous antigen can be processed and subsequently presented on MHC Class I to activate $CD8^+$ T cells [47].

Following activation, $CD8^+$ T cells can become CTLs and kill off any cell expressing it's cognate antigen on MHC Class I. CTLs are therefore important cells for the clearance of viruses and can help with cancer prevention. CTLs kill off their target cells in a highly specific manner using perforins and granzymes [9].

1.3.5. Tolerance

Due to T cells having huge receptor diversity, there is potential for some TcR clones to be reactive to host antigens. Therefore, there are mechanisms in place to prevent the release or action of autoreactive T cells.

T cells begin their education and tolerisation process in the thymus, however, purging of autoreactive T cells in the thymus by negative selection, is not absolute. For example, not all self antigens can be expressed in the thymus therefore developing T cells cannot be tolerised to all self tissues. Another problem is that in negative selection, the avidity of binding to MHC/peptide complexes determines whether or not T cells are allowed to develop. The risk of making this process too stringent is that the receptor repertoire may be cut down too much and, therefore, the ability to fight pathogens may be reduced [35]. With these limitations in mind, it is necessary for there to be further regulatory mechanisms in place in the periphery.

As such, peripheral tolerance mechanisms exist to regulate the autoreactive cells that have escaped central tolerance. These mechanisms include:

- Induction of anergy - T cells engage with their antigen on an APC but in the absence of costimulation. This stops the T cell becoming activated and renders it unresponsive to its antigen [48]
- Deletion [48]
- Suppression by regulatory T cells [48]

Autoimmune disease is believed to be linked to failures in tolerance mechanisms so that the production of autoreactive lymphocytes is not controlled properly.

1.4. B cells

B cells have two physiological roles, antigen presentation and antibody production. Whilst B cells are APCs, their main function is antibody production. Antibodies are an important part of the adaptive immune response which help with the neutralisation of toxins, phagocytosis of pathogens and destruction of bacteria and viruses. Early B cell development occurs in the bone marrow then immature B cells move to the spleen to mature.

B cells also play an important role in the pathogenesis of T1D. This will be discussed in more detail in Section 1.6

1.4.1. Development - Stem cells to maturity

B cell development is summarised in Figure 1.1.

B cell commitment

The bone marrow is the normal site of B cell development. As mention in Section 1.3.1, all blood cells, including lymphocytes are formed from Lin^{-} Sca^{high} c-kit^{high} HSCs found in the bone marrow (Figure 1.1, dark blue cell). HSCs then form Lin^{-} Sca^{high} c-kit^{high} Flt3^{low} MPPs which can form all types of blood cells but lack the self-renewal of HSCs (Figure 1.1, bright blue cell) [23]. From here,

Lin^- Sca^{high} c-kit^{high} $Flt3^{high}$ LMPPs form which are capable only of lymphocyte, macrophage, granulocyte and NK cell production (Figure 1.1, pale blue cell) [26]. CLPs are characterised by Sca-1^{low} c-kit^{low} $Flt3^+$ Il-7Rα^+ expression and are capable only of B and T cell and NK cell development (Figure 1.1, orange cell) [27].

However, further research into CLPs has revealed significant heterogenity within the population. Many different groups have used different methods, markers and reporter systems in order to try and identify a subpopulation of CLPs which is restricted to the B cell lineage. Some of these are outlined below:

- Mansson et al. [49] showed that, based on the expression of $\lambda 5$ (a B cell gene) and Rag1, the CLP compartment could be subdivided into cells that are able to develop into T, B and NK cells, cells that are able to develop into T and B cells, and those that are B cell restricted. Using $\lambda 5$ reporter mice crossed with Rag1 reporter mice, it was found that CLPs could be divided into three populations. $\lambda 5^-$Rag1^{low} cells which developed into T, B and NK cells, $L5^-$Rag1^{high} which developed into T and B cells with reduced NK cell potential, and $\lambda 5^+$Rag1^{high} cells which were B cell lineage restricted. They also found that the $\lambda 5^{high}$Rag1^{high} cells had increased expression of the surface marker Ly6D.

- Inlay et al. [50] on the other hand, used Ly6D expression as a marker for determining B cell lineage commitment. For their investigations they split CLPs into $Ly6D^+$ and $Ly6D^-$ fractions and then looked at their ability to produce T, B and NK cells. Interestingly, $Ly6D^-$ CLPs were able to produce all three types of progeny and were, therefore, termed all-lymphoid progenitors, ALPs. On the other hand, $Ly6D^+$ CLPs were almost totally B cell committed and, therefore, termed B cell-biased lymphoid progenitors, BLPs.

- Lastly, Zhang et al. [51] focused on the expression of Ly6D and Rag1 as markers to follow B cell commitment and differentiation. The CLP compartment was split based on Flt3 expression, Rag1 expression and Ly6D expression. It was found that those cells expressing Ly6D and Rag1 were the most

potent at producing B cells. Most of these cells also expressed Flt3. These B cell producing cells also had the highest levels of B cell gene transcripts (for example, Rag1, EBF, Pax5, see Section 1.4.2) indicating B cell lineage progression.

It therefore appears that within the CLP stage, there are subpopulations of cells which have leanings towards different lineages. It may be that the appearance of these different subpopulations is linked to the developmental stage of the CLP. For example, the more committed progenitors might be more developed CLPs compared to less committed progenitors. It appears that Rag1 and Ly6D are very important in determining B cell restricted progenitors and that it is reasonable to hypothesise that a committed B cell progenitor, B-cell biased progenitor (BLP), could have the phenotype of Sca-1^{low} c-kit^{low} $Flt3^{+}$ IL-$7R\alpha^{+}$ $Ly6D^{+}$ with evidence of previous Rag1 expression and robust levels of B cell development gene transcripts (Figure 1.1, red cell labelled 'BLP').

Following the development of B cell comitted progenitors, these cells then go on to express B220 (expressed on B cells and some plasmacytoid DCs), followed by CD19. Originally it was thought that B cells were only committed to the B cell lineage once B220 and CD19 were being expressed, however, there is now much evidence to suggest that this commitment step occurs prior to their expression [52].

Committed B cell development

Following commitment to the B cell lineage and the expression of CD19, B cells then progress through the following stages (Figure 1.1, light through to dark purple cells) [53]:

- Pro B cell - characterised as $CD19^{+}CD43^{+}IgM^{-}$
- Pre B cell - characterised as $CD19^{+}CD43^{-}IgM^{-}$
- Immature B cell - characterised as $CD19^{+}CD43^{-}IgM^{+}$

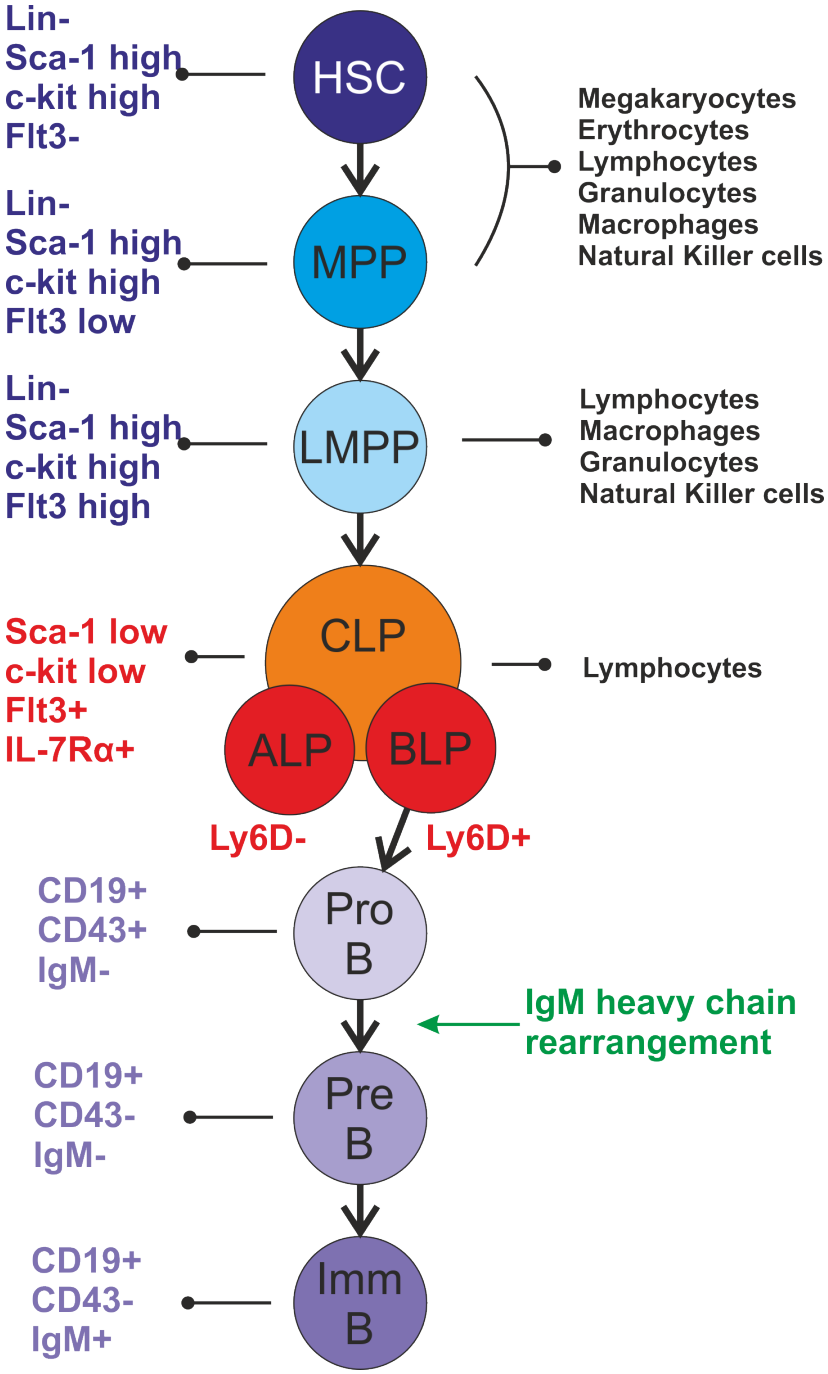


Figure 1.1: Schematic overview of B cell development in the bone marrow. Development of B cells from HSCs through the various progenitors is shown here. The surface markers associated with each developmental stage is shown on the left. On the right hand side, the haematopoietic cells which arise from each progenitor are listed. The point of IgM heavy chain rearrangement at the pro to pre B cell transition is shown in green. (Imm B = Immature B cell).

For progression from the pro B cell stage to the pre B cell stage, pro B cells must begin the rearrangement of their IgM heavy chain (see below).

Following the production of immature B cells in the bone marrow, they then leave the bone marrow and migrate to the spleen to mature. This is facilitated by the increase in IgM expression during development which allows immature B cells to become transitional B cells and migrate towards the centre of the bone marrow. From here they are carried by the central sinus, then venous circulation to the spleen [54].

B cell receptor development

As mentioned in Section 1.2.4, the BcR is the B cell's way of identifying pathogens in a very specific manner and there are many mechanisms in place in order to introduce high levels of diversity into the BcR repertoire.

The structure of a BcR consists of 2 heavy chains, which have a constant region and a variable region. Alongside these are two light chains, which also consist of a constant and variable region. Each light chain sits alongside the heavy chain with the two variable regions next to each other. Between them, the variable regions make up the antigen binding site [16].

In order to progress from the pro B to the pre B cell stage, the rearrangement of the IgM heavy chain must begin (Figure 1.1, green text/arrow). Once this has happened, it can be coupled to a surrogate light chain to form the pre-BcR which has the role of initiating light chain rearrangement so that a full IgM molecule can be produced [55]. The transition from pro B to pre B cells is also the first point of screening for autoreactivity [16].

The BcR may either be membrane bound or secreted in the form of antibody. Antibodies are produced by B cells that have differentiated into plasma cells and are secreted into the blood stream to help with pathogen toxin neutralisation, phagocytosis and complement activation [15]. The constant region gives the antibody its class, for example, IgM, IgD, IgE, IgA, IgG [16]. Each antibody

class has a different function, for example, IgE is important in allergic reactions and IgA is important for mucosal immunity.

1.4.2. Genes and Transcription Factors driving B cell development

The process of B cell development is reliant on the expression of particular genes and transcription factors.

Transcription factor PU.1 (PU.1) is an important transcription factor which requires repression in order to allow B cell development [56]. Without this repression, myeloid cell development is encouraged, therefore PU.1 is said to act in a dose dependent manner. PU.1 is regulated by growth factor independent 1 transcription repressor (Gfi-1), which is controlled by Ikaros family zinc finger protein 1 (Ikaros), a transcription factor known for it's importance in allowing B cell development [57, 58].

Lowered expression of PU.1 upregulates the expression of IL-7Rα, early B-cell factor 1 (EBF) and Pax5 which are all important factors for B cell development [59]. PU.1 acts on progenitors in order to aid production of CLPs [59].

Downstream from CLPs, factors such as E2A, EBF and Pax5 all become important in the commitment to, and progression within the B cell lineage [52]. E2A encodes two transcription factors, E12 and E46, both of which are important for B cell development [60], shown by the developmental block prior to the pro B cell stage in the absence of E2A [61]. E2A is also a requirement for the expression of EBF, which in turn activates genes crucial to BcR development, such as VPreB (a surrogate light chain) [23]. Alongside gene activation, Ebf-1 also activates the transcription factor forkhead box protein O1 (FOXO1) which is important for continued B cell development and Rag expression [62].

Pax5 is also an important transcription factor. Originally, it was believed to be the factor that coincided with B cell commitment, however, there is now evidence to suggest that B cell commitment occurs prior to Pax5 expression [23]. It is now thought that Pax5 is important for progression in the B cell

lineage, rather than commitment. Mature B cells made deficient in Pax5 have been shown to be able to dedifferentiate from the B cell lineage, suggesting that Pax5 is important in maintaining lineage restriction [63]. Pax5 is also important for the repression of the T cell lineage through repression of Notch1 [64].

Although not a transcription factor, the cytokine CXCL12 is important during B cell development in the bone marrow as it holds developing B cells in the bone marrow niche. It is produced by stromal cells and haematopoietic progenitor cells attach themselves to the processes extending from the CXCL12-producing cells and developing B cells attach themselves to the progenitor bodies [65].

1.4.3. Tolerance

Due to the huge receptor diversity produced by V(D)J recombination, it is inevitable that some combinations will produce receptors responsive to self antigens. Therefore, there are many mechanisms in place to help avoid the release and action of these potentially autoreactive clones.

Some methods of dealing with potentially autoreactive B cells are as follows:

- Deletion - B cells are exposed to self antigens in the bone marrow. If B cells are able to respond to self antigen, the maturation process of the B cell is halted and the B cell dies [66]
- Receptor editing - B cells can have another chance at rearranging their light chain in order to try and produce a different, non autoreactive recptor [67, 68]
- Receptor dilution - in rearranging an alternative light chain, the B cell expresses both the autoreactive and the non autoreactive receptors which downregulates the concentration of autoreactive receptor [67, 68]
- Induction of anergy - B cells become unreactive to their antigen [67]

These mechanisms are employed both in the bone marrow during development and following release into the periphery. However, once the B cells have left the

bone marrow, receptor editing is not an option.

1.5. The Nonobese Diabetic Mouse

The nonobese (NOD) mouse is a well described animal model of T1D, first discovered by Makino et al. [69] in 1980, where insulin producing β cells are believed to be destroyed by autoreactive T cells, predominantly $CD8^+$ CTL [70]. It is a model that is genetically predisposed to develop T1D by a well characterised pathogenesis, over a defined time course as outlined below and in Figure 1.2.

1. Sensitisation - Initial priming of T cells to islet antigen which takes place in the pancreatic lymph node (PLN)

2. Infiltration and insulitis - cells of the immune system move to the pancreatic islets and infiltrate the tissue. This is known as insulitis. For a while, the autoreactive cells' aggressive functions are suppressed by regulatory mechanisms and no damage occurs

3. β cell destruction - regulatory mechanisms become impaired and activ-

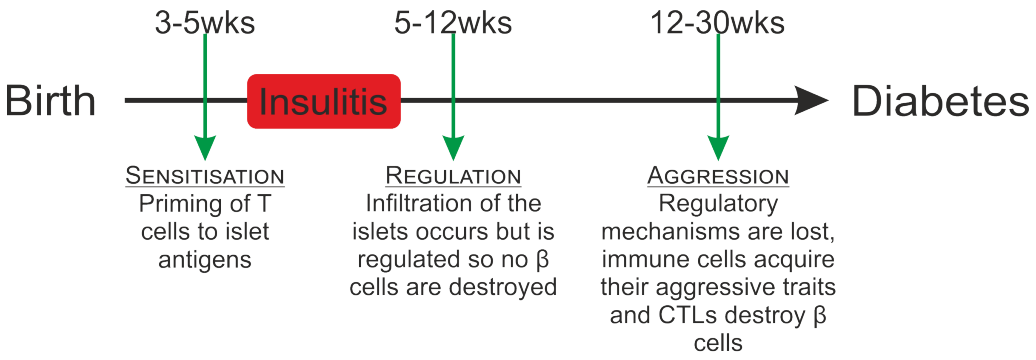


Figure 1.2: Schematic representation of the T1D process in NOD mice. At 3-4 weeks of age, autoreactive T cells become primed to islet antigen. Following this, immune cells move to the pancreatic islets, a process termed insulitis. Initially, regulatory mechanisms remain intact and β cells are preserved. However, regulatory mechanisms soon break down and by 12 weeks of age, CTL-mediated β cell destruction is well established and T1D is detectable in the NOD mouse colony.

ated $CD8^+$ T cells become cytotoxic T cells (CTLs) which begin to target and destroy β cells. β cells are believed to die by apoptosis [2].

It is believed that the NOD mouse is a relevant model for T1D research as the human disease is thought to follow a similar pathogenesis. It has been seen in donated human diabetic tissues that there is infiltration of islets, albeit reduced compared to that seen in the NOD mouse [7], and it is thought that β cell destruction is mediated by CTLs, similar to that seen in the NOD mouse [71].

Each of the stages of T1D development will be discussed further in the relevant sections below.

1.5.1. Initiation of T1D

It is thought that both the innate and adaptive immune system have roles to play in the initiation of T1D, through PRR binding, release of inflammatory cytokines and activation of T and B cells [72].

Autoantigens believed to be important in T1D are glutamic acid decarboxylase (GAD), insulin, islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) and insulinoma antigen-2 and 2β (IA-2 and IA-2β) [73, 74]. Under normal circumstances, these self antigens remain hidden from the immune system. However, in T1D, these autoantigens are exposed so that autoimmune cells can become primed towards them and mount an autoimmune attack. It is not known what triggers this exposure of autoantigens. However, genetic predisposition, such as T1D susceptible MHC, and environmental factors, such as infection [75], are thought to both be very important [76].

1.5.2. Infiltration and Insulitis

Following sensitisation to islet antigen, immune cells then begin to move into the islets to form an infiltrate. First only the edges of the islets are affected and this is known as peri-insulitis [77]. However, soon cells move into the islets too. Infiltrating cells include, but are not restricted to, DCs, macrophages, B cells

and T cells [78]. It is believed that insulitis is the point at which tolerance to β cell antigen is lost [77]. There is evidence to suggest that this pathogenesis is not limited only to the NOD mouse as pancreatic samples from diabetic humans also reveal infiltrated islets. Similar to the NOD mouse, this infiltrate contains $CD8^+$ T cells, which have potential to become CTLs, other lymphocytes and APCs [71].

1.5.3. β cell destruction

Following non-destructive insulitis, immune cells gain their aggressive traits and β cells begin to be destroyed. The destruction of β cells in NOD and human pancreatic islets is believed to be mediated by CTLs [71, 77, 78]. Only very few insulin-producing β cells survive [6, 7].

1.6. B cell involvement in T1D

It has been found that B cells have a critical role in diabetes pathogenesis and that without B cells, NOD mice are protected from the disease [79, 80]. However, the role which these cells play in T1D pathogenesis is not known. The main hypotheses relate to their two main physiological roles of antibody production and antigen presentation, along with cytokine secretion [81]. However, there is also potential that they are interefering with the process of T cell negative selection which could also contribute to the disease process.

These potential roles will be discussed in more detail below.

1.6.1. B cells are important in T1D

Investigations into the role of B cells in T1D have revealed that the presence of B cells is crucial for T1D onset.

Evidence for this came in 1996 when Serreze et al. [79] produced a mouse protected from diabetes by genetically depleting B cells from the NOD mouse. These mice retained normal T cell populations and all known diabetes suscept-

ibility genes, but lacked B cells, suggesting that the diabetes protection resulted from the lack of B cells. The resulting mouse was known as the NOD.Igμ^{null} mouse which had a functionally inactived Ig-μ chain (heavy chain) and, therefore, was unable to produce B cells. Further evidence came in 1997 when Noorchashm et al. [80] used anti Ig-μ antibody to deplete B cells from female NOD mice and compared the onset of insultis and blood glucose levels with control NOD mice which received whole rabbit IgG (which controlled for the process of antibody administration) and, therefore, retained their B cell populations. They found that insulitis and hyperglycaemia were not present in the Ig-μ treated mice, but were present in the controls. Interestingly, they also found that following removal of Ig-μ antibody, the B cell pool repopulated and insulitis returned, giving further evidence that insulitis and diabetes are linked to B cell presence.

More recently, the importance of B cells in T1D pathogenesis has been shown in both human diabetics and NOD mice through the effectiveness of B cell depletion treatments in the prevention/delay of T1D onset. CD20 is expressed on B cells and anti-CD20 monoclonal antibodies are able to bind to, and destroy B cells [82]. Administration of anti-CD20 antibody during the early onset of T1D, in both humans and mice, has an effect on slowing/preventing T1D progression. In man, it was found that Rituximab (an anti-CD20 antibody) treatment in newly diagnosed type 1 diabetics preserved β cell function for a year following treatment before the disease progressed again [83]. In mouse models, it has been shown that anti-CD20 antibody treatment can prevent or slow T1D progression depending on the time point at which it is administered [84].

As yet, there is no definitive answer as to the role of B cells in T1D. However, it is thought that it relates either to their antigen-presenting or autoantibody producing functions, or both.

1.6.2. Antibody production

Autoantibodies are present in T1D and have in fact been useful in identifying candidate islet autoantigens [74]. However, the role that they play in the pathogenesis of T1D is not known.

To assess the potential role of autoantibodies, autoantibodies from diabetic NOD mice were transferred to NOD.Igμ^{null} mice to see if diabetes susceptibility could be transferred. However, there was no change in diabetes protection so the mice remained disease free [85]. This gives the impression that, while autoantibodies may be important in the disease, they are not sufficient to cause disease onset and are not likely to be the primary role of B cells in the T1D pathogenesis.

Further to this, the role of maternal autoantibodies in NOD offspring has been investigated. Greeley et al. [86] has shown that the removal of insulin autoantibodies from mothers of NOD offspring prevented T1D in offspring. However, others, such as Koczwara et al. [87] have shown that maternal autoantibodies have very little effect on the T1D progression in the offspring in NOD mice. This shows that there is conflicting evidence for the role of autoantibodies in T1D. The lack of clear cut evidence implicating autoantibodies as a major player in T1D pathogenesis suggests that their production is unlikely to be the only role of the B cell in T1D.

To assess the relative importance of autoantibody production versus antigen presentation by B cells in T1D pathogenesis, Wong et al. [88] compared NOD.μMT mice (mice that are unable to rearrange their IgM heavy chain and, therefore, unable to progress beyond the pro B cell stage [89]) with transgenic mice that were unable to secrete antibodies but still displayed a normal BcR. They found that the transgenic mice had an increased incidence of T1D compared to the NOD.μMT controls, suggesting that B cells drive T1D pathogenesis via an antibody independent mechanism [88].

1.6.3. Antigen presentation

Following the finding that autoantibodies are not likely to be the principle mechanism of action for B cells in T1D pathogenesis, Serreze et al. [85] looked into the antigen presenting capacity of B cells. In particular, their ability to present GAD and ellicit T cell responses was investigated. It was noted that B cells were critical for the initial priming of T cells to GAD, shown by the lack of spontaneous T cell response to it in NOD.Igμ^{null} mice compared to control NODs. However, it seemed that following initial priming, other APCs were sufficient to present to T cells on restimulation with GAD antigen. This could explain why transfer of T cells from NOD mice into other mice lacking B cells such as NOD/SCID mice (which have no T or B cells and are protected from T1D) can cause disease, as APCs other than B cells in the recipient mice would be sufficient to reactivate the transferred T cells [90].

B cells acting as APCs to activate autoreactive T cells was further investigated by determining the effect of rendering B cells deficient of MHC Class II. In this study, I-A ^{g7} (the MHC Class II expressed in NOD mice [91]) expression was deficient in B cells but normal on other non-B cell APCs [92]. These mice were resistent to T1D, despite the presence of normal, non-B cell, APCs. This suggests that it is a process relating to MHC Class II and, therefore, antigen presentation, that B cells are required for.

B cell specificity to self antigen is important in T1D pathogenesis. Evidence for this comes from the finding that T1D onset can be accelerated by modifying the BcR to be more reactive to insulin. In this study by Hulbert et al. [93], B cells were manipulated so that 1-3% of the B cell population were insulin specific. Controls were also modified using similar transgenes but these gave limited insulin binding capabilities. It was seen that the mice with insulin specific B cells developed T1D at a much faster rate than controls, suggesting that antigen specifity of B cells is an important determinant of T1D onset.

In terms of activating T cells, it is thought that $CD8^+$ T cells are the main culprits

for β cell destruction in T1D. As mentioned in Section 1.3.4, to activate $CD8^+$ T cells, APCs have to present their cognate antigen on MHC Class I via the process of cross-presentation. Whilst this process is usually carried out by DCs, there is evidence that B cells can cross present islet antigen to $CD8^+$ T cells in the PLN. This was shown by a decrease in activated $CD8^+$ T cell population both in B cell deficient mice and in mice whose B cells have been rendered MHC Class I deficient [94]. This gives the impression that B cells could be playing an important role in activating $CD8^+$ in the islets, allowing them to become CTLs which could destroy β cells.

Further evidence for the activation of T cells by B cells is that NOD B cells that have infiltrated the islets in T1D appear to have increased levels of costimulatory molecules, B7-1 and B7-2 [95]. This could suggest that they have an increased ability to present to, and activate, T cells which could aid the progression of the diabetic process in the islets.

The evidence to date suggests that presentation of self antigen to autoreactive T cells is the principle action of B cells in the pathogenesis of T1D.

1.7. Thymic B cells

It is normal for small numbers of B cells to be present in the thymus of non diabetic humans and mice [96–98]. However, in the NOD mouse, this population of B cells is dramatically increased [85, 99]. It is not known how B cells come to populate the thymus, it is thought to either be as a result of migration to the thymus or development from progenitors within the thymus. Development is more likely due to the fact that some thymic progenitors do have B cell potential [40] and developing pro and pre B cells are seen in the thymus [96].

The increase in B cells is intriguing as it appears to be age-related and correlates with the progression of the disease in the NOD mouse. This could suggest that either thymic B cells are involved in driving the disease progression, or it may be that they are a result of disease progression.

This increase in thymic B cells is not entirely unique to T1D in NOD mice, it is also a phenomenon seen in other autoimmune diseases such as myasthenia gravis [100, 101] and, therefore, unravelling the origin and function of thymic B cells in the NOD mouse, may have benefits beyond those in the field of T1D.

The function of thymic B cells is not known. It has been hypothesised that B cells within the thymus may play a role in the negative selection of developing T cells. Evidence of this has come from the discovery that thymic B cells position themselves next to mTECs [102], are able to express AIRE [103] and that they are able to delete T cell clones from the T cell repertoire by displaying self anitgens [104]. However, these models utilise non-autoimmune prone mice that have been manipulated to develop autoimmunity. It therefore remains to be seen whether these hypotheses hold true in an autoimmune-prone model, such as the NOD mouse.

1.8. Project Hypotheses and Aims

Figure 1.3 outlines the current understanding of B cell development in the bone marrow, T cell development in the thymus and potential intrathymic B cell development.

The importance of establishing the source of thymic B cells stems from the increasing evidence that thymic B cells can manipulate central thymic tolerance, and the finding of abnormally large populations of thymic B cells in disease-prone NOD mice. This project aims to understand more fully the potential pathway of intrathymic B cell development.

It is thought that B cells found in the thymus of NOD mice appear there due to intrathymic development. Assuming this is the case, it is likely that these B cells will be arising from undifferentiated progenitor cells which migrate to the thymus with retained B cell potential. This forms the first project-driving hypothesis that, within the thymus of NOD mice, it should be possible to see all stages of B cell development from the very earliest committed B cell progenitor

(the BLP), through pro, pre and immature B cell stages to mature B cells, if development is occurring there. As well as seeing the different developmental stages of B cells in the thymus, it is also hypothesised that there should be evidence of B cell development milestones, in particular, BcR rearrangement, within the thymic environment. Further to this, it is expected that findings in young NOD mice will be different to older NOD mice, due to the strong age-related nature of T1D disease progression. In particular, it is expected that the intrathymic B cell development potential in the NOD mouse will change with age.

In order to test the hypotheses outlined above, these are the main questions that this project aims to investigate:

- Are early B cell progenitors and developing B cells present in the thymus of NOD mice?
- Is the NOD thymus able to support B cell receptor rearrangement on developing thymic B cells and, if so, are these events age-related?

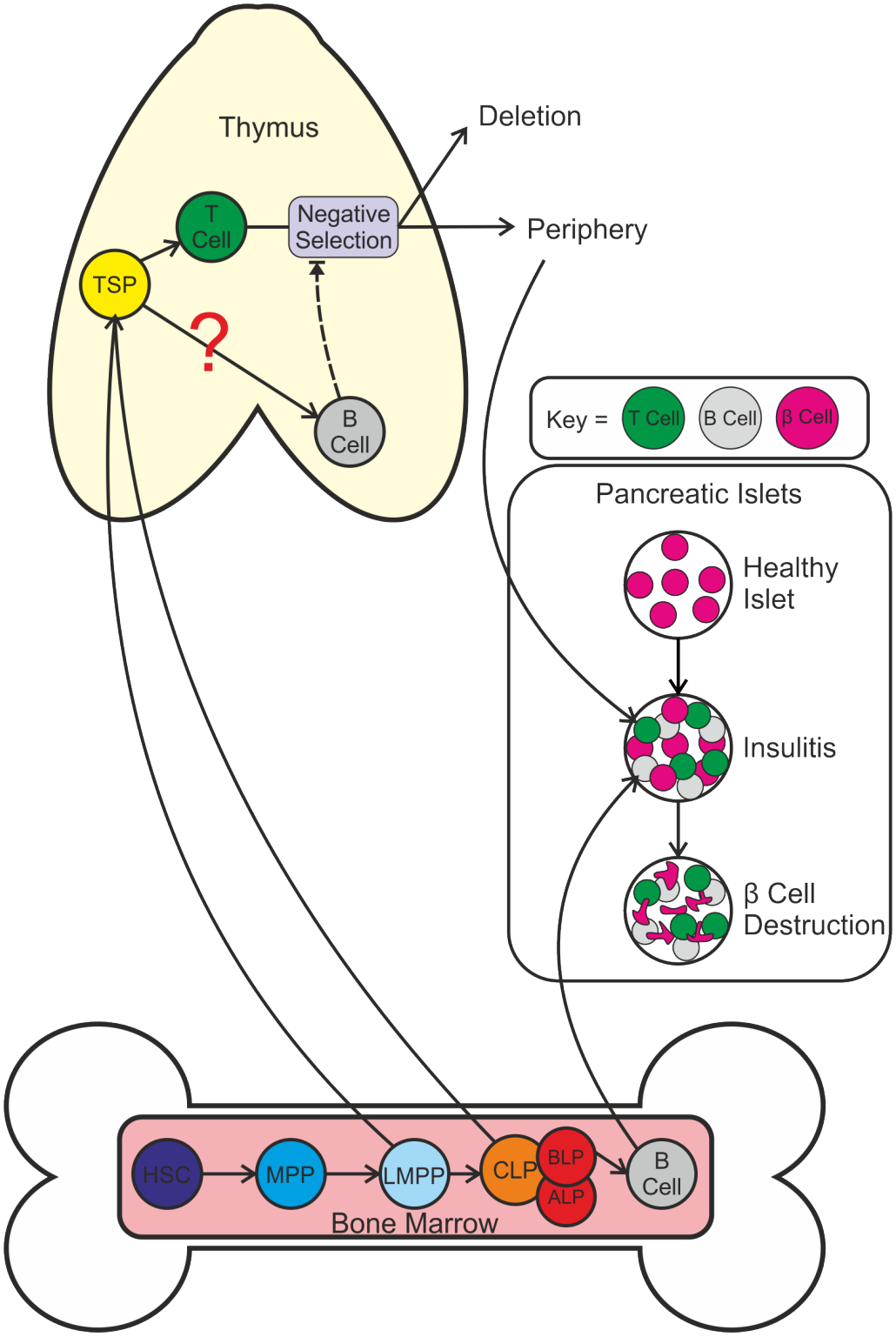


Figure 1.3: B cells develop in bone marrow, T cells develop in thymus. In seeding of the thymus with TSPs destined for the T cell fate, some progenitors may retain B cell potential. These cells may be differentiating into B cells in the thymus, resulting in a population of thymic B cells. T cells go through negative selection in the thymus as the first stage of tolerisation. Those which survive this process are released in the periphery. In the case of T1D, healthy islets become infiltrated with immune cells including T and B cells (potentially including some of thymic origin) and subsequently, insulin-producing β cells are destroyed by CTLs. It may be that thymic B cells are contributing to T cell negative selection in some way (black dashed arrow).

Materials and Methods

2.1. Mice

NOD, NOD.μMT$^{-/-}$, NOD-RAG2p-GFP, NOD.μMT$^{-/-}$.RAG2p-GFP and C57BL/6 (B6) mice were used.

NOD.μMT$^{-/-}$ (hereafter referred to as NOD KO) mice are deficient in B cells and have been described elsewhere [79]. They have a mutation in their IgM heavy chain which prevents heavy chain rearrangement and subsequent progression from the pro B cell stage to the pre B cell stage. This block in development results in an inability to produce mature B cells and antibody secreting plasma cells.

RAG2p-GFP reporter mice on a FVB background have also been described elsewhere [105]. RAG2p-GFP reporter mice express green fluorescent protein (GFP) under the control of the RAG promoter so that when RAG is expressed, so is GFP. When RAG transcription is deactivated, so is GFP transcription. However, the protein decays over time with a half life of approximately 56 hours [106], meaning that GFP^{+} cells can be seen even after RAG deactivation.

For use in this project, FVB-RAG2p-GFP mice were backcrossed 12 generations (N12) to the NOD mouse to produce NOD-RAG2p-GFP reporter mice (hereafter referred to as NOD-RAG-GFP mice).

NOD.μMT$^{-/-}$-RAG2p-GFP reporter mice (hereafter referred to as NOD KO-RAG-GFP mice) were also used in this project. For this, N12 NOD-RAG2p-GFP mice were crossed with NOD.μMT$^{-/-}$ mice. Heterozygous progeny were then crossed back to NOD.μMT$^{-/-}$ mice. Mouse genotypes were determined by flow cytometric analysis of blood samples to assess IgM and/or GFP presence.

All mice were housed in specific-pathogen-free barrier conditions at York University Biological Services Facility. All experimental procedures were conducted under U.K. Government Home Office guidelines. For mouse studies, investigations were carried out double or single blind, as stated in results, following ARRIVE guidelines [107]

2.2. Cell preparation

2.2.1. Preparation from frozen tissue

Frozen immune cells housed at -80 °C in the Green Lab biobank, were quickly defrosted by incubation in a waterbath at 37 °C. Defrosted cells were immediately resuspended in 5 mL phosphate buffered saline (PBS) supplemented with 0.5% bovine serum albumin (BSA) (hereafter referred to as 0.5% PBS/BSA) and transferred to 15 mL tubes. Cells were then centrifuged for 3 minutes at 290 relative centrifugal force (rcf), supernatant discarded and the washing step was repeated once more to ensure complete removal of the solution the cells had been frozen in. The cells were then resuspended in 2 mL 0.5% PBS/BSA and put in incubator at 37 °C, 5% CO_2 for 2 hours to enable re-expression of cell-surface molecules that had been downregulated due to the freezing process. Cells then centrifuged as before, supernatant discarded and cells resuspended in a volume appropriate for the subsequent procedure they were to be utilised in.

2.2.2. Preparation from fresh tissue

Freshly prepared cells were isolated from the thymus, spleen, inguinal lymph nodes, pancreatic lymph nodes and bone marrow. For preparation of bone marrow cells, the femur and tibia were isolated and tissue gently removed using a razor, leaving clean bone. The cells were isolated by flushing of femur and tibia of mouse with 1 x PBS. For thymi and spleens, harvested tissues were homogenised using a seive and 2 mL syringe plunger. Lymph nodes were isolated and placed in a petri dish containing 5 mL PBS. The cells were liberated by grinding the tissue between two frosted glass slides. The cell suspensions were centrifuged for 3 minutes at 290 rcf to pellet cells then the supernatant was discarded. Where appropriate, red blood cells were lysed by resuspending cells in 1 mL of water for 6 seconds, followed by 1 mL 2 x PBS to neutralise toxicity of the water. The cells were then centrifuged as before, supernatant discarded and the cells were resuspended as appropriate for subsequent procedure.

2.3. Flow cytometry

Single cell suspensions were prepared as in Section 2.2 and the cells were resuspended in 1 mL of 1 x PBS. 100 μL of cell suspension (equvalent to 10^6 to 10^7 cells) was then transferred to an appropriate vessel. The cells were incubated with 1:300 dilution of anti-CD16/32 antibody and incubated for at least 10 minutes at 4 °C. This was carried out to block Fc receptors to avoid non specific binding of analytic antibodies used in the subsequent staining. The cells were then stained using appropriate fluorescently labelled antibodies and incubated for 25 minutes at 4 °C in the dark. Antibodies (from eBioscience or BD Pharmingen) were used at 1:300 dilution, apart from anti-CD4 and anti-CD8 antibodies which were used at 1:800 dilution. The cells were washed in 3 mL 1 x PBS and centrifuged for 3 minutes at 290 rcf to remove unbound antibodies. The supernatant was discarded then the cells were resuspended in 500 μL of 1 x PBS.

Table 2.1: Antibody clones and suppliers

Antibody	Clone	Supplier
CD4	RM4-5; GK1.5	eBioscience, BioXcell
CD8	53-6.7; 56.3.7	eBioscience, BioXcell
CD19	eBio1D3	eBioscience
CD43	eBioR2/60	eBioscience
IgM	11/41	eBioscience
Sca-1	D7	eBioscience
C-kit	2B8	eBioscience
Flt3	A2F10	eBioscience
Il-7Rα	A7R34	eBioscience
Ly6D	49-H4	eBioscience
CD16/32	93	eBioscience
CD25	PC61	eBioscience
CD44	IM7	eBioscience
CD11c	N418	eBioscience
B220	RA3-6B2	eBioscience
TcRβ	H57-597	eBioscience
Streptavidin	-	BD Pharmingen

If biotin-conjugated antibodies were used, the dilution, incubation and washing steps were conducted as above. Subsequently, fluorescent-labelled streptavidin was added at 1:800 dilution in 1 x PBS and incubated for 20 mins at 4 °C. Unbound streptavidin was removed by washing with 3 mL of 1 x PBS as above and the cells were resuspended in 500 μL of 1 x PBS.

All antibodies used in this study were assessed and optimised for use against isotype controls.

Data was acquired on a Dako CyAn™Flow Cytometer using Summit software and analysed using FlowJo 7.6.1 software.

Antibody clones and suppliers are shown in Table 2.1.

2.4. Magnetic cell sorting

2.4.1. Miltenyi beads and columns

The cells were prepared as in Section 2.2. For a cell lineage depletion, lineage cell depletion kit (Miltenyi Biotech) was used. For separation of samples into $CD19^+$ and $CD19^-$ fractions, CD19 microbeads (Miltenyi Biotech) were used. For both lineage depletion and CD19 separation, Miltenyi LS columns were used. There were two deviations from the manufacturers protocol. Firstly, the addition of an Fc receptor-blocking step prior to the addition of the beads using anti-CD16/32 antibody at 1:300 dilution. Secondly, the buffer used was 0.5% PBS/BSA, rather than the buffer suggested in the protocol. Purity of the appropriate cells was verified by flow cytometric analysis. The cells were resuspended in a volume of buffer appropriate for the procedure that they were to be used in.

2.4.2. Qiagen BioMag goat anti-rat IgG beads

Cells were prepared as in Section 2.2 and resuspended in 500 μL of 0.5% PBS/BSA. A 1:300 dilution of anti-CD16/32 antibody was added and samples were incubated at 4 °C for 10 minutes to block Fc receptors. The cells were then incubated with the appropriate purified antibodies depending on which cell populations were to be depleted. Antibodies were added at appropriate concentrations and incubated for 20 minutes at 4 °C. 1 mL of BioMag goat anti-rat IgG beads (Qiagen, 1mg/mL) were transferred to a 15 mL tube and 10 mL PBS was added. The solution was placed on a magnet and left for approximately 10 minutes to allow the beads to adhere. A pasteur pipette was used to remove the supernatant and the beads were resuspended in 500 μL of PBS. The beads were put back on the magnet and allowed to adhere again before removing supernatant as before. A final wash in 500 μL PBS performed then beads finally resuspended in 500 μL PBS.

The cells were prepared by washing in 3 mL PBS and centrifuging for 10

minutes at 290 rcf. The cell pellet was then resuspended with the 500 μL solution of beads and incubated for 15 minutes at 4 °C.

The samples were then placed on a magnet and beads allowed to adhere. Supernatant containing unbound cells were removed with clean pasteur pipette, kept, and put back on magnet to remove residual beads. The cellular supernatant was isolated as before and centrifuged for 3 minutes at 290 rcf. The cells were then resuspended in 500 μL 1 x PBS for subsequent flow cytometric analysis.

2.5. RNA preparation

Cells were prepared as in Section 2.2, then RNA extracted using RNeasy mini kits (Qiagen) following the maunufacturers protocol. A DNAse step was included where deoxyribonuclease I (Sigma) was added to the column membrane for 10 minutes at room temperature. The addition of a DNAse step was to prevent the binding of primers to residual genomic DNA. RNA quality was assessed on the NanoDrop (ThermoScientific NanoDrop 2000), with the aim of producing RNA with 260/280 and 260/230 values of near to, or above, 2.0 which indicates a good purity of RNA. RNA was resuspended in RNAse-free water and stored at -80 °C.

2.6. Reverse transcription of RNA to cDNA

RNA was reverse transcribed to cDNA using Superscript II reverse transcriptase (Invitrogen, 2000 U/μL). RNA was taken and heated at 55 °C for 10 minutes before being placed on ice. 7 μL of first buffer (4 μL 5 x 1st strand buffer (Invitrogen), 2 μL 10mM dNTP mix (Thermo Scientific), 1 μL OligodT 0.5 μM (Invitrogen)) was added to 10 μL of RNA and the solution was placed in RNAse-free PCR strips. Amount of RNA was normalised across samples using data from NanoDrop in order to try and keep quantities of cDNA constant across samples. The volume was then made up to 10 μL by the addition of RNAse-free water. The samples were then incubated at 65 °C for 5 minutes to uncoil RNA, then cooled quickly on ice. Three μL of a second buffer (1 μL 0.1M DTT, 1 μL RNAse

out, 1 μL Superscript II (all Invitrogen)) was then added to each sample and the solution incubated at 65 °C for 45-60 minutes to make cDNA. cDNA was stored at -20 °C until needed.

2.7. Primer design

Specific primers for polymerase chain reaction (PCR) to look for B cell development genes were designed using sequences derived from the National Center for Biotechnology Information (NCBI) nucleotide database [108] then looking for suitable primers for these genes using Primer3 (www.primer3.ut.ee) [109]. The primers were then put through a primer blast [110] to check for specificity of the primer pair for the desired gene. Designed primers (obtained from Sigma Genosys) were then tested to find their optimum annealing temperature by carrying out a temperature gradient PCR from 52-62 °C.

Primers are as follows:

- E2A. Forward TTG ACC CTA GCC GGA CAT AC. Reverse TGC CAA CAC TGG TGT CTC TC. Expected product size: 150bp. Optimum annealing temperature: 61.8 °C.
- EBF. Forward CAG TTC TGC AAA GGG ACA CC. Reverse CAA TGT CGG CAG CTC TCT TC. Expected product size:226 bp. Optimum annealing temperature: 59.4 °C.
- Pax5. Forward AAC TTG CCC ATC AAG GTG TC. Reverse CTG ATC TCC CAG GCA AAC AT. Expected product size: 217bp. Optimum annealing temperature 61.3 °C.
- VPreB. Forward CGA TAT CCC ACC TCG CTT CT. Reverse CCG AGC AAA GCA AAC TCT GT. Expected product size: 238 bp. Optimum annealing temperature: 59.4 °C.
- CXCL12. Forward GCT CTG CAT CAG TGA CGG TA. Reverse TAA TTT CGG GTC AAT GCA CA. Expected product size: 184 bp. Optimum anneal-

ing temperature: 60.5 °C.

2.8. Polymerase chain reaction

Non quantitative polymerase chain reaction (PCR) was carried out to look for the genes of interest using the primers outlined above. PCR samples consisted of 1 μL cDNA, 1.125 μL forward primer, 1.125 μL reverse primer, 2.5 μL 25 mM $MgCl_2$ (Applied Biosystems), 2.5 μL 25 mM Amplitaq360 buffer (Applied Biosystems), 15.5 μL dH_2O, 0.125 μL 250 U AmpliTaq 360 DNA Polymerase (Applied Biosystems). cDNA was amplified following 35 cycles of 1 minute at 94 °C, 1 minute at the appropriate annealing temperature for the primer set, 1 minute at 72 °C. A final elongation step of 10 minutes at 72 °C was performed once at the end of the 35 cycles. Amplified cDNA was subsequently analysed by gel electrophoresis.

2.9. Gel electrophoresis

Gel electrophoresis of PCR products were carried out using 3% agarose gels (Lonza SeaKem LE Agarose) made in 1 x Tris acetate-EDTA (TAE) buffer (Sigma) supplemented with 1 μL of 10 mg/mL ethidium bromide (Sigma) per 25 mL TAE buffer. 5 μL of 6x loading dye (Thermo Scientific) were added to each 25 μL PCR product sample. Total volume of 30 μL was then transferred to wells in the gel, held in a gel rig containing 1 x TAE buffer. 100 base pair (bp) ladder also put in a well of the gel to show size of bands seen in the gel. Gels were run at constant 70 Volts until bands had separated sufficiently and imaged using SynGene software.

2.10. Statistical Analyses

All graphs were drawn using GraphPad Prism and statistical analyses (One way ANOVA, Tukey's tests and Mann-Whitney tests) were performed using built-in functions within the software.

Results

3.1. B cells can develop intrathymically

3.1.1. B cell numbers in the NOD mouse thymus increase with age

As mentioned previously in Section 1.7, it is normal for a small population of B cells, about 0.1-0.5% of thymocytes, to be present in the thymus of both humans and mice [102]. However, it has been noted by our lab (Varian and Green, unpublished observations) and others [99], that this population is significantly increased in NOD mice, compared to non diabetes prone control mice, such as B6 mice. It is thought that these cells may have a role in the process of T cell negative selection and, whilst some literature suggests this may be a beneficial role [102, 104], it may be that in autoimmunity-prone mice, they are potentially impeding central tolerance of T cells. For this reason, it is important to establish when and why increased thymic B cells occur.

It is not clear whether the increased frequency of thymic B cells in NOD mice compared to non-NOD mice is age-related; that is, whether or not the increased population of thymic B cells correlates with a specific stage of the T1D process. To confirm and extend our previous observations of the increase in thymic B cells in NOD mice compared to control B6 mice, a time-course, flow cytometric study was conducted. The time points for analysis were chosen to correlate with different stages of the disease process in NOD mice (see Figure 1.2). As

shown in Figure 3.1, as NOD mice age and progress along the T1D development pathway, the frequency (Figure 3.1 A) and absolute numbers (Figure 3.1 B) of mature thymic B cells increases significantly in comparison to control B6 mice (Varian and Green, unpublished observations).

Thus, at 4 weeks - the time point in the disease pathway corresponding to priming of the autoreactive T cell repertoire to islet antigens occurs - no differences were seen in either frequencies Figure 3.1 A or absolute numbers Figure 3.1 B of mature IgM^+ B cells in the two strains of mice. However, by 9 weeks of age, when insulitis is well established and initial CTL activity to β cells is active, there is a significant increase in both the frequency and absolute numbers of thymic B cells in NOD mice with respect to control B6 mice. Furthermore, at 12 weeks of age - a time when β cell destruction is extensive and initial T1D development is detectable in our colony - this disparity in thymic B cell frequencies between the two strains of mice is even more prevalent.

Due to the interesting observation in the thymus, the bone marrow of NOD mice was also investigated to determine if there is a similar age-related increase in B cell frequencies in comparison to control B6 mice. To assess this possibility, a time-course, flow cytometric study was carried out to look at the frequencies of B cells in the bone marrow of NOD and B6 mice at the same time points as the thymus time-course study. As shown in Figure 3.1 C, there is no significant difference between the frequency of B cells in the NOD and B6 bone marrow at any age. Furthermore, no significant difference in B cell frequencies were seen in either spleen or lymph nodes of NOD and B6 mice (Data not shown). This would suggest that the increase in B cells is specific to the thymus, not to the mouse as a whole.

Finally, to assess whether this increase in thymic B cells impacted on the development of the main T cell subtypes in the thymus, the frequencies of DN, DP and SP T cells in NOD and B6 thymi were compared using flow cytometry. Analysis of this data, shown in Figure 3.2, revealed that there was no significant difference between NOD and B6 developing T cell populations at any age.

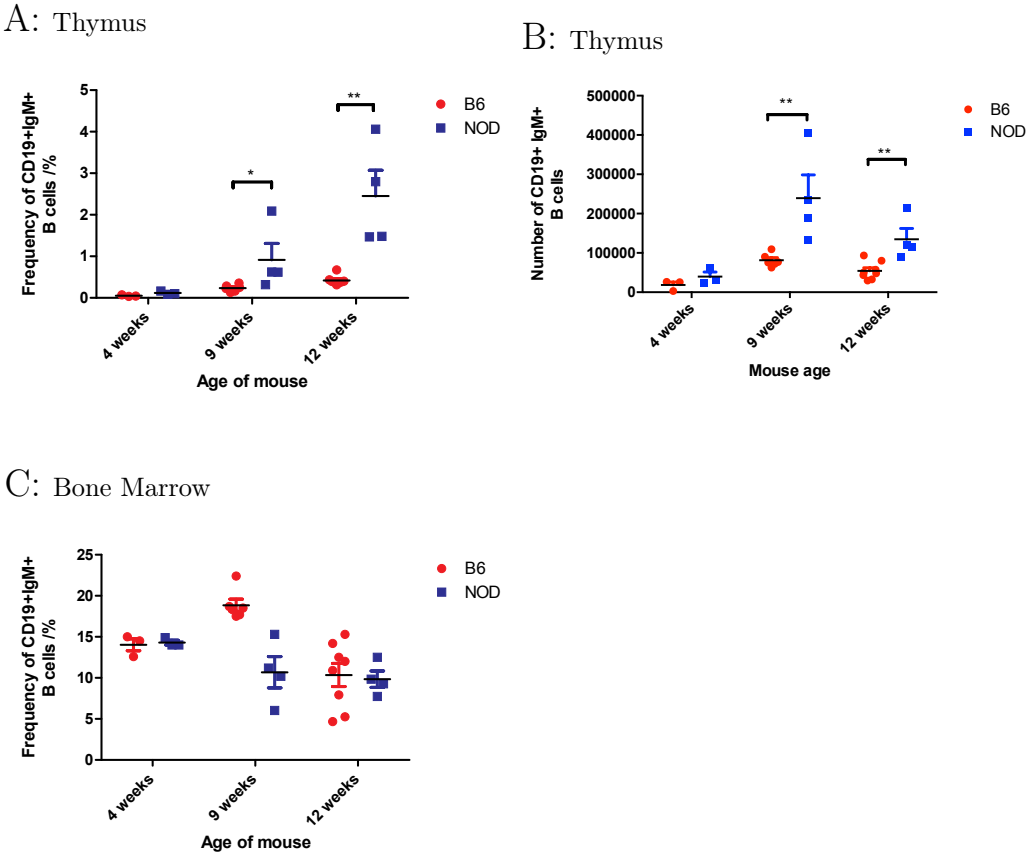


Figure 3.1: B cell numbers in the NOD mouse thymus increase with age. Cells were isolated from the thymus and bone marrow of NOD (n=3-4) and control B6 (n=3-6) mice at the ages stated. The cells were incubated with anti-CD19 and -IgM antibodies and the frequencies (A, C) and absolute numbers (B) of $IgM^{+}CD19^{+}$ mature B cells in a live, single cell gate, was determined by flow cytometry. Statistical significance in B cell populations between NOD and B6 mice was determined using the Mann Whitney non-parametric test. The Mann Whitney test was used to compare the difference in mature B cell frequency at each age. Differences between NOD and B6 were statistically significant at both 9 and 12 weeks of age (P=0.0190 and P=0.0040, respectively). The difference at 4 weeks is not statistically significant. Black line shows the mean and error bars show standard error of the mean. Acknowledgements to Jennifer Varian for provision of raw data.

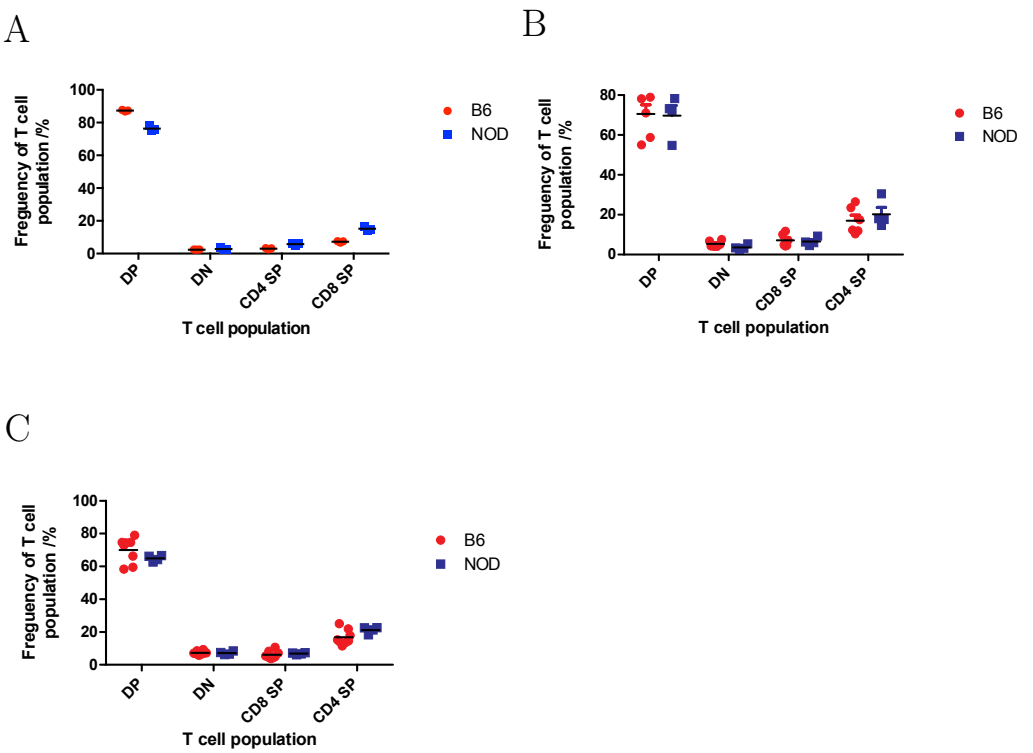


Figure 3.2: The frequency of major T cell populations in the thymus of the NOD mouse are the same as those seen in B6 control mice. Cells were isolated from the thymus of NOD and B6 mice at the stated ages. Cells were subsequently incubated with anti-CD4 and -CD8 antibodies for flow cytometric analysis. T cell subsets were identified within a live, single cell gate. A shows 4 weeks of age (NOD n=3, B6 n=3), B shows 9 weeks of age (NOD n=3, B6 n=6) and C shows 12 weeks of age (NOD n=4, B6 n=8). Black line shows the mean and error bars show standard error of the mean. Acknowledgements to Jennifer Varian for provision of raw data.

3.1.2. The NOD thymus contains B cell development transcription factors

If B cells are increasing within the thymus, this begs the question as to whether the NOD thymus is providing an environment conducive to B cell development. To try and answer the question of whether B cells are developing within the thymus, rather than migrating there, primers were designed that were specific for B cell development factors. If B cell development is occurring in the NOD thymus, it would be expected that these development factors would be seen in the there as they are a requirement for successful B cell development (see

Section 1.4.2). Genes such as E2A, EBF, Pax5, VPreB and CXCL12 were chosen as they are important for lineage commitment and/or lineage progession and stabilisation (see Section 1.4.2). E2A is important for the formation of pro B cells and is a requirement for the expression of EBF [23]. EBF is then important for activating genes and transcription factors crucial for BcR rearrangement, such as VPreB and FOXO1 [62]. Pax5 is important for the progression of progenitors within the B cell lineage and for the repression of transcription factors driving development of other lineages, for example, Notch1 for T cell development [23, 64]. CXCL12 is a cytokine responsible for holding B cell progenitors in the bone marrow niche during development [65].

Non-quantitative PCR was performed to compare whether or not B cell development genes and transcription factors are present in the thymus of 11-12 week old NOD mice. The bone marrow was included as a control tissue since this is the normal site of B cell development. All transcription factors would be expected to be detectable in the bone marrow and this was the case, as shown in Figure 3.3. Interestingly, all the genes, besides CXCL12 (Figure 3.3 E), were also present in the NOD thymus too. This reinforces the idea that the thymic environment is potentially able to support and/or promote B cell development. The lack of CXCL12 in the thymus is not a surprising result as it is a cytokine responsible for holding developing B cells in the bone marrow niche. Therefore, it may be more bone marrow specific rather than B cell specific. This raises the question as to what chemokine is holding developing B cells in the thymic niche to enable completion of their development if it isn't CXCL12.

It would be beneficial to carry out quantitative PCR to determine relative expression levels between the bone marrow and thymus of NOD mice, as non quantitiative PCR can only indicate presence of a transcription factor. It would also be useful to compare B6 control mice and NOD mice to see if the thymic environments differed between the diabetes susceptible and non diabetic control mice. Unfortunately, due to time constraints it was not possible to carry out quantitative PCR for this project.

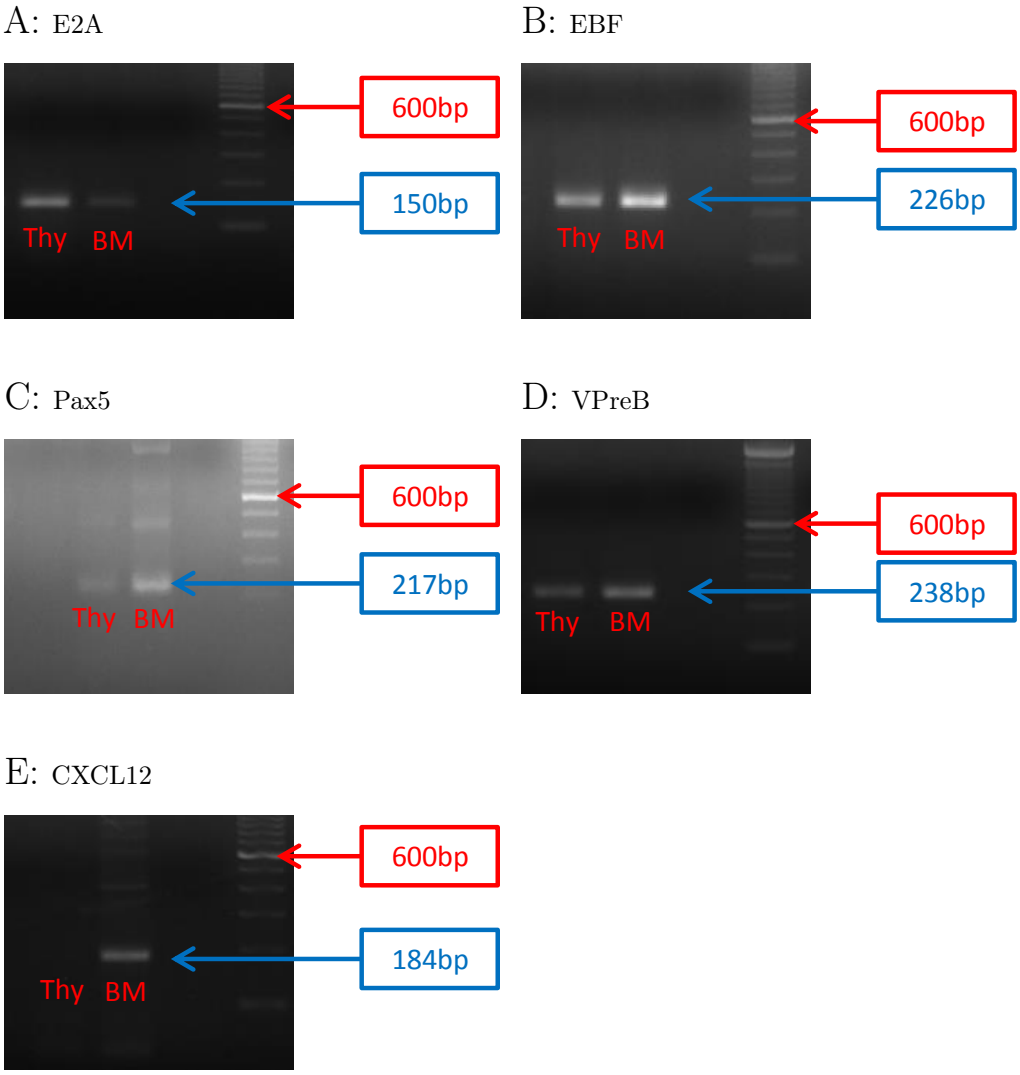


Figure 3.3: Non quantitative PCR reveals B cell development transcription factors are present in the NOD thymus. RNA was prepared and reverse transcribed to cDNA. Non-quantitative PCR was performed using primers specific for B cell development transcription factors and genes. Gel electrophoresis was carried out to detect PCR product. Gels were run in 3% agarose in 1 x TAE buffer. Gels were supplemented with 1 μL ethidium bromide per 25 μL of 1 x TAE buffer. Gels were run at constant 70V. Lanes from left to right; Thymus, Bone marrow, 100 bp marker. 600bp mark is indicated by the red arrow and box. The expected product size is shown by the blue arrow and box. A: E2A; B: EBF; C: Pax5; D: VPreB; E: CXCL12. Mice analysed were 12 weeks old.

3.1.3. There are pro and pre B cells present in the NOD thymus

The mechanism by which B cell frequencies/numbers increase in the NOD thymus is unknown. Two potential hypotheses to account for the increase are development from progenitors entering the thymus which have B cell potential [40, 96], or migration of B cells from the bone marrow. The current consensus is that B cells are developing within the thymus and the data above would support the hypothesis that the NOD mouse thymus may support B cell development.

To further investigate whether B cells are developing within the thymus, rather than migrating there, we must first consider the different developmental stages of the B cell (see Section 1.4.1 and Figure 1.1). The first of these stages is the $CD19^{+}CD43^{+}IgM^{-}$ pro B cell stage. Subsequently, the IgM heavy chain is rearranged and coupled to a surrogate light chain (such as VPreB), forming the preBCR. The sucessful formation of a preBCR allows progression to the $CD19^{+}CD43^{-}IgM^{-}$ pre B cell stage. Next, the light chain rearranges resulting in a full IgM molecule and the B cell can be released from the bone marrow to migrate to the spleen to finish its development. An indicator of B cell development within the thymus of the NOD mouse would be if these pro and pre B cells are present in the thymus.

Akashi et al. [96] has shown pro and pre B cells in the thymus of B6 mice. It is unclear whether similar populations occur in NOD mice, therefore, to determine this, a comparative flow cytometric study was carried out using NOD and B6 mouse thymi. As a control, the populations of pro and pre B cells in the bone marrow were also assessed, as this is the normal site of B cell development.

As shown in Figure 3.4 B, pro and pre B cells can be seen in the thymus of NOD and B6 mice. The presence of pro and pre B cells suggests that these stages of B cell development may be supported by the thymus. As expected, the bone marrow (Figure 3.4 C) also shows populations of pro and pre B cells which appear similar between mouse strains. The gating strategy for revealing pro and pre B cells in the bone marrow and thymi of NOD and B6 mice is shown in Figure 3.4

A. The positions of the gates were decided by using the bone marrow as a tissue control where clear populations of $CD19^+$, IgM^- cells could be seen. These gates were then applied to the thymus. The divide between $CD43^+$ and $CD43^-$ was also determined by looking at bone marrow samples. Subsequent experiments could also include isotype controls to show true negative populations for each marker.

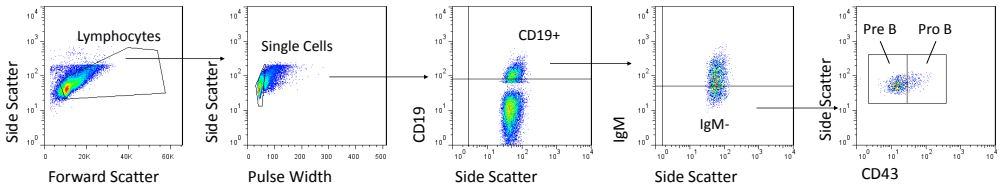
3.1.4. Thymic B cell development may be dependent on the presence of a mature B cell

The presence of pro and pre B cells in the thymus suggests that thymic B cell development may occur in a similar manner to that occurring in the bone marrow.

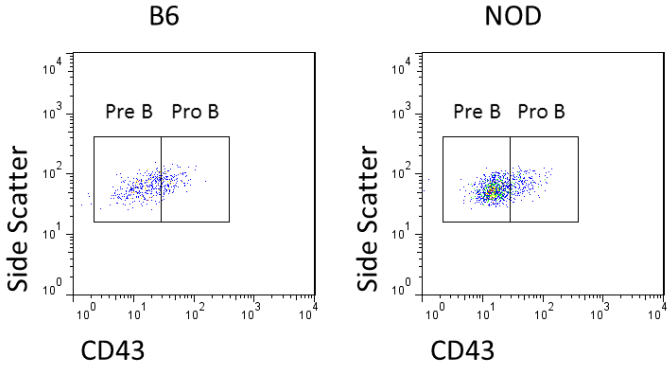
In the bone marrow, progression from the pro B cell stage to the pre B cell stage requires rearrangement of the IgM heavy chains. Ablation of IgM heavy chain rearrangement results in blockade of pro to pre B cell stage, and as a consequence an accumulation of pro B cells. To assess whether similar pro to pre B cell transition is governed by IgM heavy chain rearrangment in the thymus, comparative flow cytometric analysis of NOD mice with NOD KO mice (that are unable to rearrange the IgM heavy chain, Section 2.1) was performed.

Comparative, flow cytometric studies of NOD and NOD KO bone marrow, as expected, revealed that in NOD KO mice, B cell development was blocked at the $CD19^+CD43^+IgM^-$ pro B cell stage (Figure 3.5 B). It was, therefore, expected that in the NOD KO thymus there would also be a population of pro B cells, similar to that seen in the thymus of NOD mice. However, this does not appear to be the case. The gating quadrants shown in Figure 3.5 B were placed as shown by looking at the picture in the NOD bone marrow which shows populations of pro, pre and immature/mature B cells. These gates were then applied to the NOD KO bone marrow and showed that the bone marrow showed only pro B cells and lacked pre and immature/mature B cells. This gave the impression that the gating was correct as literature relating to this mouse model states that the B cell is unable to rearrange a IgM heavy chain Serreze et al. [79] and

A



B: Thymus



C: Bone marrow

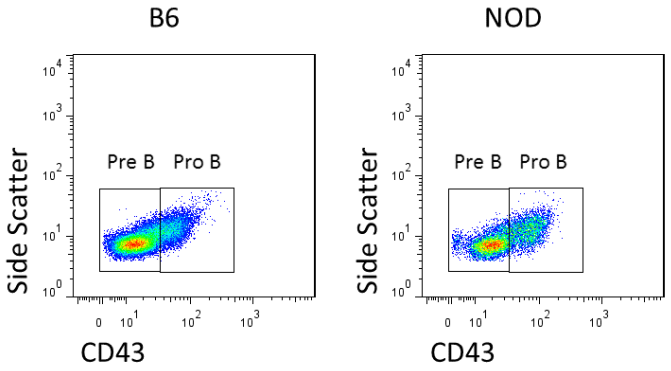


Figure 3.4: Pro and pre B cells are found in the thymus (B) and bone marrow (C) of both NOD (n=3) and B6 (n=4) mice aged 6-9 weeks. Single cell suspensions were prepared and incubated with anti-CD19, -IgM and -CD43 antibodies for flow cytometric analysis. Pro ($CD19^{+}IgM^{-}CD43^{+}$) and pre ($CD19^{+}IgM^{-}CD43^{-}$) B cells within a live, $CD19^{+}$, IgM^{-}, single cell gate were identified by flow cytometry. The gating strategy for both thymic and bone marrow samples is shown in A by a representative thymic sample. For the thymus, the cells were acquired on a $CD19^{+}$ gate to enrich for B cells.

therefore development should be stopped at the pro B cell stage. A method to further validate the positioning of the gates would be to include isotype controls in subsequent experiments to show true negative populations for each marker.

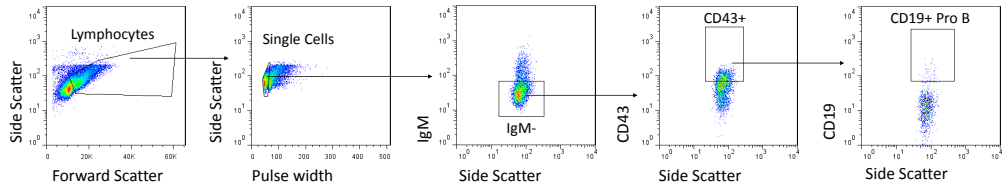
When analysing this data, it was important to establish a gating system whereby the two different strains could be compared fairly. NOD KO mice have no pre B cells and no IgM^+ cells. Therefore, previous gating to look at pro and pre B cells ($CD19^+$ then analysed on IgM and CD43 expression) would be skewed in the NOD KO as 100% of the cells would be pro B cells - it would not be possible to tell whether or not the population was increased or decreased with respect to the other strains. With this in mind, IgM^+ cells were gated out, as were $CD43^-$. This left only IgM^-CD43^+ cells, of which any that are $CD19^+$ should be pro B cells. This means that the frequencies of pro B cells can be compared fairly by the frequency of $CD19^+$ cells present within the IgM^-CD43^+ population. The frequencies of pro B cells in both strains of mice are shown in Figure 3.5 C.

As shown in Figure 3.5 D, the frequency of pro B cells in the NOD KO mouse thymus is significantly decreased in comparison to the frequency of pro B cells in the NOD mouse thymus. This is an interesting finding as it suggests that the enhancement of pro B cells in the thymus may be dependent on the presence of a mature B cell.

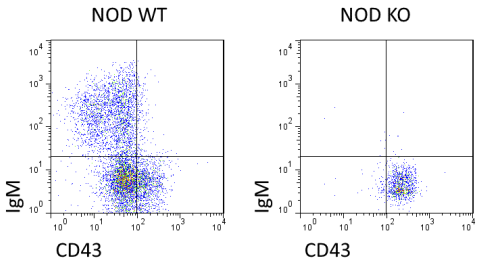
3.1.5. Mature NOD thymic B cells are killed following transfer into NOD KO recipients

The finding that the frequency of pro B cells was decreased in NOD KO mice with respect to NOD mice (Figure 3.5), suggests that mature B cell presence may influence thymic B cell development. However, it is not known whether these potential effects are due to peripheral or thymic resident B cells. In order to investigate the role of thymic B cells in general, and in particular, determine whether or not they have an effect on the pro B cell population seen in the thymus a suitable experimental approach would need to be decided upon first. A potential approach would be to transfer mature thymic B cells from NOD

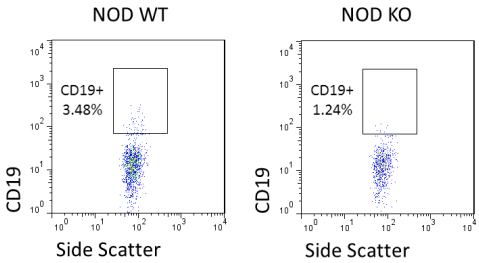
A



B



C



D

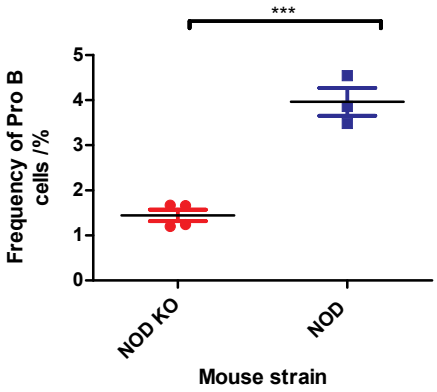


Figure 3.5: NOD mice have an increased frequency of thymic pro B cells compared to NOD KO mice. Single cell suspensions were prepared from 6-8 week old NOD (n=3) and NOD KO (n=4) mouse thymi and subsequently incubated with appropriate analytic fluorescent-labelled antibody for flowcytometric analysis. B shows a representative FACS plot of NOD and NOD KO bone marrow gated on $CD19^{+}$, live, single cells. Thymic samples were gated on acquisition for $CD19^{+}$ cells. $CD19^{+}$ pro B cells were identified in the thymus of NOD and NOD KO mice (C) from $CD43^{+}IgM^{+}$ cells in a live, single cell gate; the gating strategy for which is shown in A. D shows the frequencies of pro B cells in NOD and NOD KO thymi. Statistical significance was determined using an unpaired T test. P=0.0004 ($P<0.05$ is deemed significant). The black line shows the average frquency and the error bars represent the standard error of the mean. Data shown are from one experiment that has been repeated 5 times. A total of 32 mice were examined. Experiment was carried out single blind to avoid bias.

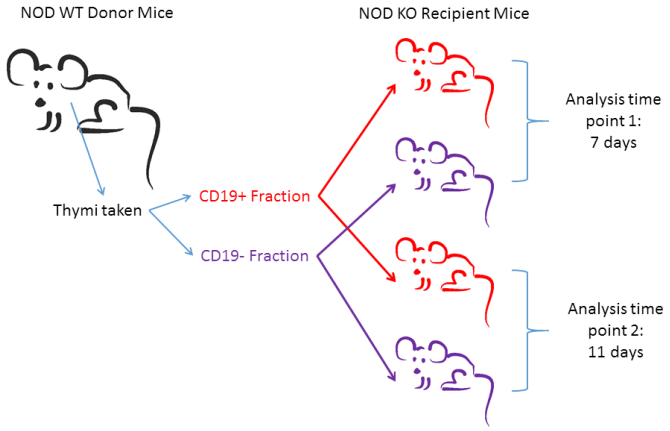
donors into NOD KO recipients. A similar approach has been tried by Serreze et al. [85] which resulted in CTL destruction of the transferred B cells, suggesting that this approach may not work. However, Serreze et al. [85] transferred splenic B cells rather than thymic B cells and since the properties of thymic B cells are not well understood, it is not clear whether thymic B cells would be similarly susceptible to death in NOD KO mice.

To investigate this, a pilot study was performed whereby thymic cells, including thymic B cells, were isolated from NOD mice and transferred into NOD KO mice, to see whether they could survive in the NOD KO recipients.

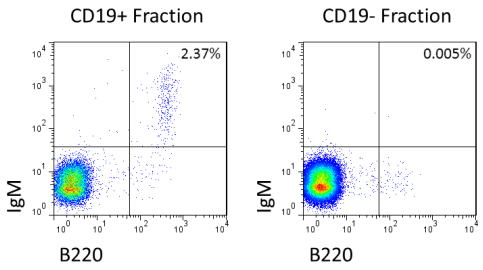
Due to the preliminary nature of the transfer experiment, very small numbers of mice were used. Ten week old female donor NOD and recipient NOD KO mice were used. At 10 weeks of age, numbers of thymic B cells are high in the NOD thymus enabling sufficient numbers for isolation and transfer. The experimental plan for the transfer experiment is outlined in Figure 3.6 A. Thymocytes were prepared from two donor mice and $CD19^+$ and $CD19^-$ populations separated using magnetic bead assisted cell sorting. This approach yielded a thymocyte fraction enriched for $CD19^+$ cells ($CD19^+$ fraction) and a thymocyte fraction largely devoid of $CD19^+$ cells ($CD19^-$ fraction). The purity of the two cellular fractions in terms of B cell presence/absence was determined by flow cytometry (see Figure 3.6 B).

The donor populations were transferred by intravenous injection into the recipient mice, then recipient tissues were analysed by flow cytometry at the stated time points. As shown in Figure 3.7, flow cytometric analysis revealed that mature B cells ($CD19^+IgM^+$) were only present in the recipient mice at the 7 day post transfer time point. The inability to detect mature B cells at the later, 11 day timepoint, may reflect cytotoxic killing of these cells in NOD KO mice [85]. At either time point, no mature B cells were detectable in the bone marrow or any lymph node analysed (Data not shown). In contrast, both the spleen Figure 3.7 A and thymus Figure 3.7 B of recipient NOD KO mice contained mature B cells at the 7 day time point.

A



B



C

Fraction	Total cell count	% B cells	Number of B cells /recipient
CD19+	133,333	2.7	1295
CD19-	3.67 x 10^7	0.005	710

Figure 3.6: Experimental setup and donor cell populations for transfer experiments. A shows a diagram of the experimental setup. B shows the efficiencies of the MACS separations carried out. Mature IgM^+B220^+ B cells were identified within a live, single cell gate for both the $CD19^+$ and $CD19^-$ fractions. C shows the total thymic cell counts of the donor mice along with the frequencies of mature B cells. Also shown is the number of B cells transferred into the recipient mice for each fraction.

Although B cells are seen in the thymus and spleen of both recipients at the 7 day time point, it seems that there is a higher frequency of $CD19^+IgM^+$ cells in the recipients that received the $CD19^+$ cell containing donor cells. This finding is not surprising as this recipient received more mature B cells in the transferred cells.

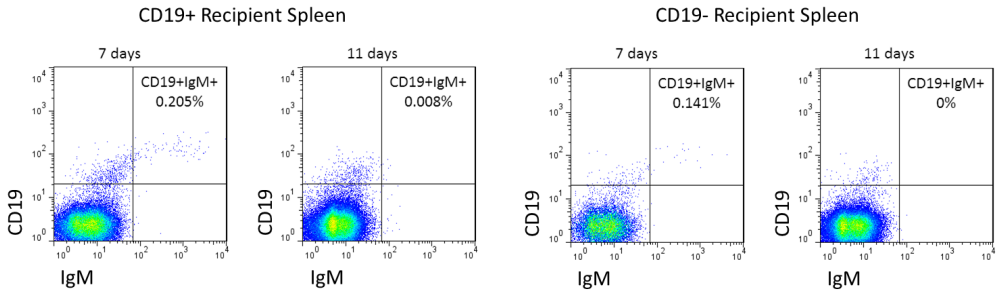
In the $CD19^-$ recipients, there are also mature B cells present in the spleen and thymus at the 7 day time point. The population in the spleen could reflect contaminating B cells which were transferred. It is unlikely that these B cells seen have developed from $CD19^-$ progenitors due to B cells not normally developing in the spleen. However, while the population of B cells in the thymus could also be due to contaminating transferred B cells, it may be that within the thymus, there is the potential for $CD19^-$ B cell progenitors to develop into mature B cells there.

The aim of the experiment was to look at the lifespan of B cells following transfer into recipient NOD KO mice, to see if this would be a viable method for investigating the effect of mature B cells on thymic pro B cell frequency. However, it has shown that this methodology is not appropriate for these investigations due to the disappearance of transferred cells between 7 and 11 days post transfer. Future studies in which recipient NOD KO mice are first sublethally irradiated and subsequently transfused with bone marrow from NOD KO mice supplemented with mature thymic B cells [85], may offer a more successful approach. By using this approach, it should mean that endogenous T cells are developing in the presence of B cells and should, therefore, become tolerised to them. This, in turn, should prevent any CTL-mediated B cell destruction occurring during transfer and allow the effects of thymic B cells on thymic pro B cell populations to be investigated.

3.1.6. The NOD thymus can support BcR rearrangement

Initial rearrangement of the BcR takes place at the pro to pre B cell transition phase, and the finding that pre B cells are present in the NOD thymus suggests

A: Spleen



B: Thymus

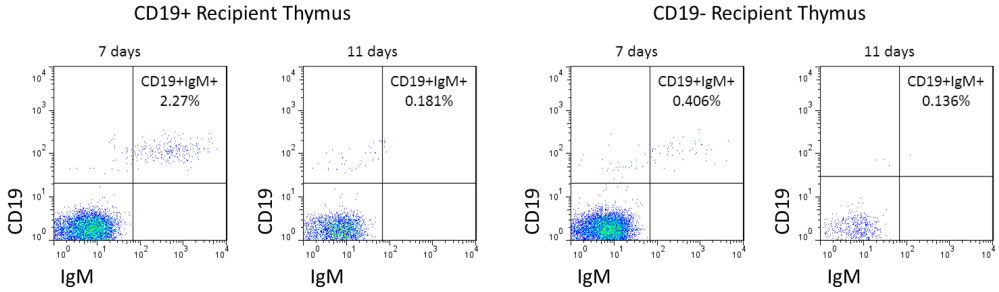


Figure 3.7: Donor B cells are found in the spleen and thymus of recipient mice at 4 days post transplant. Cells were isolated from the recipient bone marrow, thymus, spleen, PLN and axillary lymph node and incubated with anti-CD19 and -IgM antibodies for flow cytometric analysis. Thymic samples were gated on acquisition for $CD19^{+}$ cells. For analysis, mature $CD19^{+}IgM^{+}$ B cells were identified within a live, single cell gate. A shows the spleen and B shows the thymus from both recipients and both time points, as stated. Spleen, PLN and axillary lymph node data not shown. n=1 for each type of recipient. The experiment was carried out double blind to avoid bias during analysis.

that the thymus can support early rearrangments of the BcR. To confirm this, NOD-RAG-GFP reporter mice were utilised to look for RAG expression by developing thymic B cells. If RAG is being expressed by $CD19^{+}$ cells in the thymus, this gives more definitive evidence that B cells are developing in the thymus, rather than just relying on seeing cells with the phenotypes of pro and pre B cells.

NOD-RAG-GFP reporter mice express GFP under the control of the RAG promoter, therefore, during receptor rearrangement when RAG is being transcribed, GFP is also produced and can act as a marker for RAG activity. Further to this, while transcription of RAG and GFP cease simultaneously, GFP degrades slowly over time with a half life of approximately 56 hours in vivo [106]. This means that the intensity of GFP signal seen on flow cytometry correlates with how recently RAG/GFP transcription was active. For example, as shown in Figure 3.8 B, the GFP^{high} population in the bone marrow represents the developing B cells which are actively rearranging their receptor. The GFP^{low} population shows the cells that have recently rearranged their receptor and subsequently deactivated RAG/GFP transcription, therefore, the GFP signal is decreased. The GFP^{-} population represents cells which rearranged their receptor more than 56 hours previously.

Murine strains of RAG-GFP reporter mice have been invaluable at documenting the differing frequencies of developing, newly developed and long term resident thymic T regulatory cells depending on co-stimulatory signals [111]. Thus, NOD-RAG-GFP mice enabled analysis not only of BcR rearrangment in developing B cell populations, but also the proportion of developing, newly developed and long term resident thymic B cells.

To investigate whether the thymus may be able to support BcR rearrangement, depending on the stage of the disease, cells of the B cell lineage were identified by expression of CD19 then these cells were interrogated for their GFP expression. As shown in Figure 3.8 A, there are $CD19^{+}GFP^{high}$ cells within the thymus of NOD mice at 4, 7 and 11 weeks of age. This finding confirms that

the NOD thymus supports rearrangment of the NOD BcR, as no $CD19^{+}GFP^{+}$ cells would be detectable if it did not. Further to this, as shown in Figure D, interestingly, it appears that the frequency of $CD19^{+}GFP^{+}$ cells decreases as mice progress through the T1D process. In particular, the frequency of $CD19^{+}GFP^{low}$ cells decreases significantly between 4 and 11 weeks of age. Conversely, the frequency of the $CD19^{+}GFP^{-}$ increases significantly between 4 and 7 weeks of age. The significant decrease in $CD19^{+}GFP^{low}$ cells in the NOD thymus could show that there is a decreased frequency of newly developed B cells in the thymus of older NOD mice. This could be due to a decrease in B cell development, or increased emigration of newly developed B cells. This is an interesting result as it could suggest that the support of early BcR rearrangement in the thymus is limited to younger mice.

3.1.7. Early B cell progenitors are present in the thymus of NOD and B6 mice

As shown in Figure 3.1 A, there is an age-related, significant increase in thymic B cells in NOD mice compared to B6 control mice that do not develop T1D. The age-related increase correlates with the onset of insulitis in NOD mice and increased activity of autoreactive T cells. Given this differences between NOD and B6 mice, and the evidence that B cell development is supported in the thymus, the question arose as to whether thymic seeding progenitor cells with B cell potential are present within the NOD thymus at higher frequencies than control B6 mice.

To investigate this, it was important to first of all decide on the specific progenitor that would be looked for. Although the B cell commitment pathway remains undefined, as mentioned in Section 1.4.1, it appears that a so-called BLP may be the best described B cell progenitor. In which case, the cells of interest would be Sca-1^{low} c-kit^{low} $Flt3^{+}$ IL-$7Ra^{+}$ $Ly6D^{+}$ [49–51] (See Figure 1.1).

However, the matter was complicated further due to the fact research into B cell commitment and development is focussed on B cell development in the bone marrow. Research into the development of B cells in the thymus, therefore,

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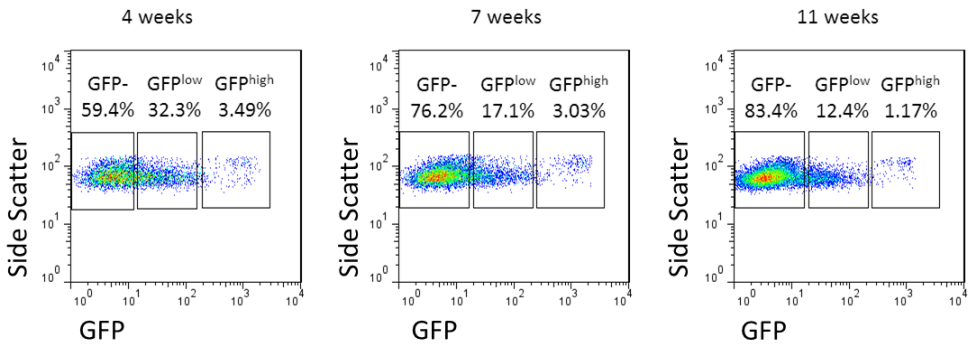

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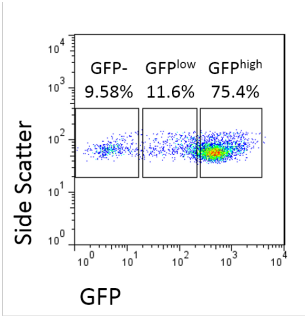

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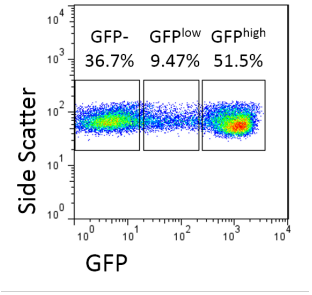

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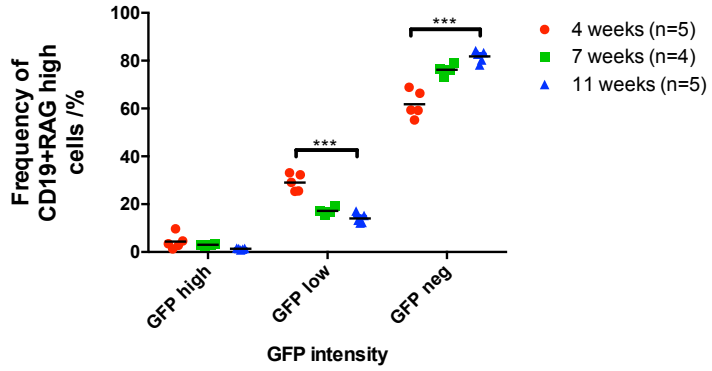


Figure 3.8: Some CD19^{+} cells in the NOD thymus express RAG. Cells from the bone marrow and thymus were isolated from NOD-RAG-GFP mice and incubated with anti-CD19 antibody for flow cytometric analysis. Thymic samples were gated on acquisition for CD19^{+} cells to enrich for B cells. For analysis, GFP^{high}, GFP^{low} and GFP^{-} CD19^{+} cells were identified within a live, single cell gate. This data is shown in A. Equivalent data is shown for the bone marrow in B. Gates were set for thymic RAG expression using the RAG expression levels in the total thymic sample shown in C. The difference in RAG expression at the different ages is shown in D. Statistical significance was determined using one-way ANOVA and post hoc Tukey's test. $P<0.0001$ ($P<0.05$ is deemed significant) for both GFP^{high} and GFP^{low} CD19^{+} cells. Black line represents average frequency. Data shown are representative of all 14 mice that were examined.

requires the assumption that the developmental pattern will match that of the bone marrow. While it is not known how well the developmental pattern in the thymus mirrors that of the bone marrow, it is a good place to start due to the wealth of literature relating the B cell development in the bone marrow.

The aim of the investigations was to determine whether or not the frequencies of BLPs in the NOD mouse were the same as that seen in the control B6 mice and in the NOD KO mice. This would give insight into two areas. Firstly, if NOD mice have an increased frequency of B cell progenitors in their thymus compared to B6 mice, this could potentially explain the increased number of B cells in the NOD thymus. And secondly the analysis could ask if BLPs are affected in the same way as pro B cells by the presence or absence of a mature B cell. This would be shown by a decreased frequency of BLPs in the NOD KO compared to the NOD.

For these studies, all mice used were 6-8 weeks of age, a time point that correlates with the onset of increased accumulation of thymic B cells in NOD mice, with respect to control B6 mice.

Due to the small size of progenitor populations in comparison to mature cell populations in the thymus, it was first necessary to deplete the thymus of the majority of mature cells before looking for progenitors. For depletion, magnetic-activated cell sorting (MACS) was used. There are different MACS-based approaches to enrich for progenitor cells. The decision of which approach to use was taken after investigating the purity and yield of lineage negative cells following separation using Miltenyi lineage-depletion kits or Qiagen BioMag goat anti-rat beads combined with our own anti-lineage antibody cocktail (see Section 2.4).

Firstly, Miltenyi columns and lineage depletion beads were tested to assess their efficiency. To start with, only one round of depletion was carried out but the depletion was not complete enough, therefore, two rounds of depletion were carried out. This improved the efficiency, however, the yield was very low meaning that very few cells were left to analyse (data not shown).

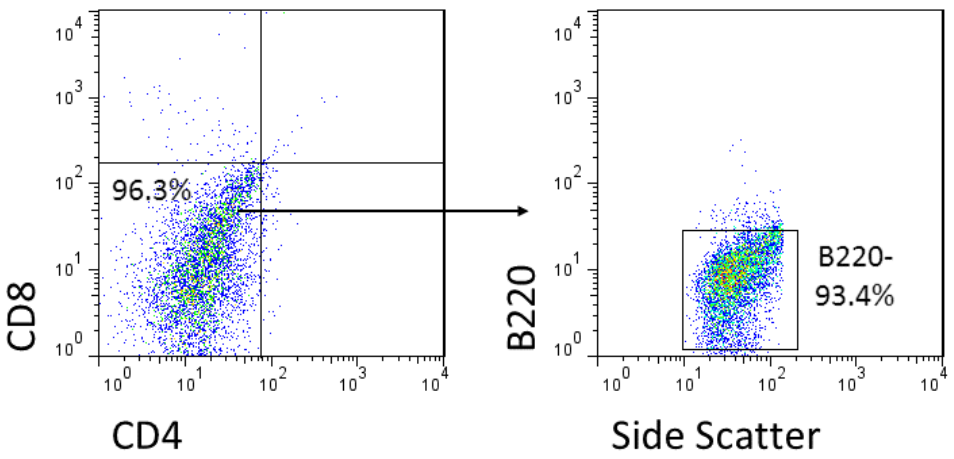


Figure 3.9: Flow cytometric analysis of lineage depletion using Qiagen beads. Thymic cells were isolated from NOD mice and incubated with purified anti-CD4, -CD8 and -CD19 antibodies. Mature T and B cells were then removed using Qiagen BioMag goat anti-rat beads and a magnet. Lineage depleted samples were then incubated with fluorescently labelled anti-CD4 and -CD8 antibodies, of a different clone to those used for depletion, along with anti-B220. Flow cytometric analysis was carried out to determine lineage depletion efficacy. $CD4^-$ $CD8^-$ $B220^-$ cells were identified as shown, within a live, single cell gate. Data shown is representative of the 7 mice examined.

Qiagen BioMag goat anti-rat beads were also tested as an alternative to the Miltenyi kits. As shown in Figure 3.9, the efficiency of this method was also very good and the yield was also much improved compared to the Miltenyi beads therefore this was the chosen method of depletion.

In order to assess the frequency of BLPs present in the thymus of NOD, NOD KO and B6 mice, it was first necessary to establish a suitable gating strategy. Firstly, data was gated to find the CLP population within the thymus. For this a lymphocyte and single cell gate was applied then data was gated to anaylse only cells which were Sca-1^{low}c-$kit^{low}$$Flt3^+$IL-$7R\alpha^+$. From this population, it is then possible to determine which of these cells are BLPs and, therefore, likely to be B cell committed, depending on their Ly6D expression. This gating pattern

is shown in Figure 3.10 A.

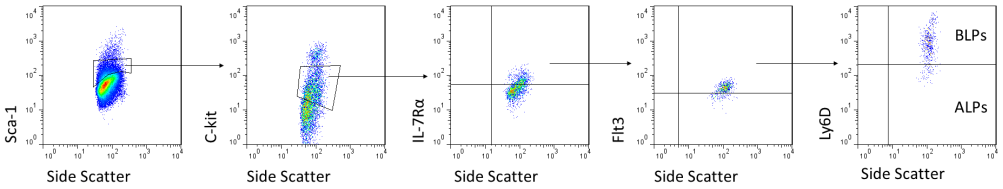
When examining the frequencies of BLPs within the thymic CLP population, it appeared that BLPs are present in the thymus of all strains of mice. However, interestingly, the frequency of BLPs in the thymic CLP population was significantly decreased in NOD and NOD KO mice compared to control B6 mice, as shown in Figure 3.10 B.

The presence of BLPs in the B6 thymus could suggest that they are the normal progenitor for the normal population of thymic B cells seen in nondiabetic animals. The significant difference in BLP frequencies between NOD mouse strains and control B6 mice may suggest that in NOD mice, thymic B cells originate from an alternative progentitor or developmental pathway.

Further to this, the presence of a mature B cell does not appear to have an impact on the frequency of BLPs in NOD mice of this age, as shown by the similarity in BLP frequency between NOD and NOD KO mouse thymi.

Finally, to confirm that BLP frequency was independent of mature B cell presence, a further group of NOD and NOD KO mice were assessed at 12-13 weeks of age, a time point when thymic B cell numbers peak in the NOD mouse. Unfortunately, no control B6 mice were available at this age to be included in the analysis. As shown in Figure 3.11, there is no difference in the frequency of $Ly6D^+$ BLPs within the CLP population. This may suggest that the action of a mature B cell to increase the pro B cell population in B cell sufficient mice, as opposed to B cell deficient mice, is after the BLP stage. However, the spread of data for NOD KO mice is much greater than for the NOD mice, therefore, further repeats would be beneficial.

A



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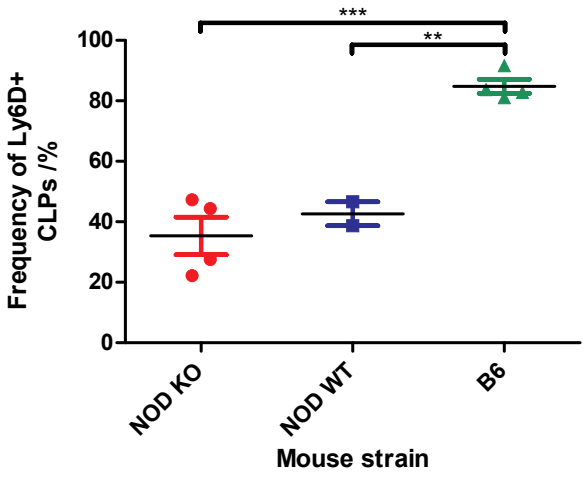


Figure 3.10: BLPs are significantly decreased in the NOD mouse thymus compared to the B6 mouse thymus. Thymic cells were isolated from 6-8 week old NOD (n=3), NOD KO (n=3) and B6 (n=4) and depleted of mature B and T cells by MACS using purified anti-CD4, -CD8 and -CD19 antibodies and Qiagen BioMag goat anti-rat beads. Samples were then incubated with fluorescently labelled analytic anti-CD4, -CD8 (of different clones to those used for lineage depletion), -Sca-1, -c-kit, -Flt3, -IL-7Rα and -Ly6D for flow cytometric analysis. For analysis, BLPs were identified as Ly6D$^+$ cells within a live, single cell, CD4$^-$, CD8$^-$, Sca-1^{int}, c-kit^{int}, Flt3$^+$, IL-7Rα^+ gate. This gating pattern is shown in A (live, single cell, CD4$^-$CD8$^+$ gating not shown). The difference in frequency of BLPs in the different strains of mouse is shown in B. Statistical significance was determined using one-way ANOVA (P=0.0003) and post hoc Tukey's test. Black line shows the mean and error bars show standard error of the mean. Data are representative of one experiment where 11 mice were examined. The experiment was carried out single blind to avoid bias during analysis.

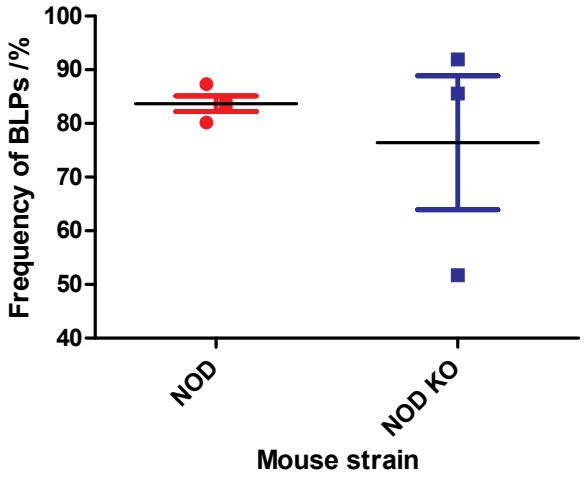


Figure 3.11: The frequency of thymic BLPs is the same in both NOD and NOD KO thymi. Thymic cells were obtained from NOD (n=5) and NOD KO (n=5) thymi. Preparation and staining was the same as samples in Figure 3.10 Statistical significance was assessed using the Mann Whitney test but the difference between the NOD and NOD KO thymic BLP frequency was found to be not significant. Black line shows the mean and error bars show standard error of the mean.

3.2. Some RAG+ cells in the thymus express B and T cell markers

B cells normally develop from progenitor BLPs in the bone marrow. Therefore, initially, BLPs were looked for in the NOD thymus to assess whether or not the normal B cell developmental pattern could be seen in the thymus. However, following the results suggesting that NOD thymi may have less thymic BLPs compared to the control B6 mice (Figure 3.10 B), an increase in early B cell progenitors is unlikely to account for the increase in thymic B cells in the NOD. This would suggest that there is an alternative pathway of B cell development in the thymus that is different to the pathway seen in the bone marrow.

3.2.1. A large proportion of $RAG^{+}CD19^{+}$ cells in the NOD thymus express CD4 and CD8

During the course of study, in-depth phenotyping of GFP^{+} cells in the thymus revealed a surprising finding that $CD19^{+}$ GFP^{+} cells expressed $CD4$ and CD8 T cell markers. It was decided to follow up this preliminary observation by

determining whether there may be a point where thymocytes are not yet fully lineage committed and are expressing markers of both T (CD4, CD8) and B (CD19) cells.

To investigate this, time course flow cytometric studies of the thymus, or control bone marrow, using 4 and 7 week old NOD-RAG-GFP mice were performed. Mice of 4 and 7 weeks of age were analysed and a representative FACS plot of a 4 week old NOD thymus and bone marrow are shown in Figure 3.12. Here, the difference between the thymus (Figure 3.12 A) and bone marrow (Figure 3.12 B) is shown. As shown in Figure 3.12 A, approximately 69.2% of thymic $GFP^{high}CD19^{+}$ cells also co-expressed CD4 and CD8 molecules. In contrast, only approximately 5.14% of $GFP^{-}CD19^{+}$ cells also co-expressed these T cell markers. This uniqueness for GFP^{high} $CD19^{+}$ cells to co-express CD4 and CD8 molecules was restricted to the thymus, as similar analysis of both the bone marrow and spleen revealed 0.016% (Figure 3.12 B) and 0.38% (Figure 3.12 C) of $GFP^{+}CD19^{+}$ cells co-express CD4 and CD8 molecules, respectively.

The lack of $RAG^{high}CD19^{+}CD4^{+}CD8^{+}$ cells in the bone marrow suggests it is unlikely that these cells are a normal part of B cell development (Figure 3.12 B). It is also unlikely that they are a normal part of T cell development as T cell commitment is thought to occur fully in the DN development stage, therefore when developing T cells are still $CD4^{-}CD8^{-}$.

This suggests that they are thymus specific and it may highlight another abnormality within the NOD thymus that may or may not be related to the increased population of thymic B cells. It could potentially suggest an alternative mechanism by which B cells are increased in the NOD thymus. It may be that a cell is beginning to commit to the T cell lineage, then at some point receiving a signal which is causing it to begin switching lineages and start expressing B cell markers. These cells could then potentially redifferentiate fully into B cells. This potential redifferentiation could explain the existence of both B and T cell markers on the cells, and account for the decreased frequency of conventional B cell markers in the NOD mouse thymus compared to the B6 control thymus.

However, further research would be required to explore this further.

These $RAG^{high}CD19^{+}CD4^{+}CD8^{+}$ cells are present in the thymus of NOD mice at both 4 and 7 weeks of age. As shown in Figure 3.12 E, the difference in frequency of these cells between the thymus and bone marrow is statistically significant at both ages. However, it was found that there was no significant difference in frequency of $RAG^{high}CD19^{+}CD4^{+}CD8^{+}$ cells in the thymus between 4 and 7 weeks of age (P=0.3429).

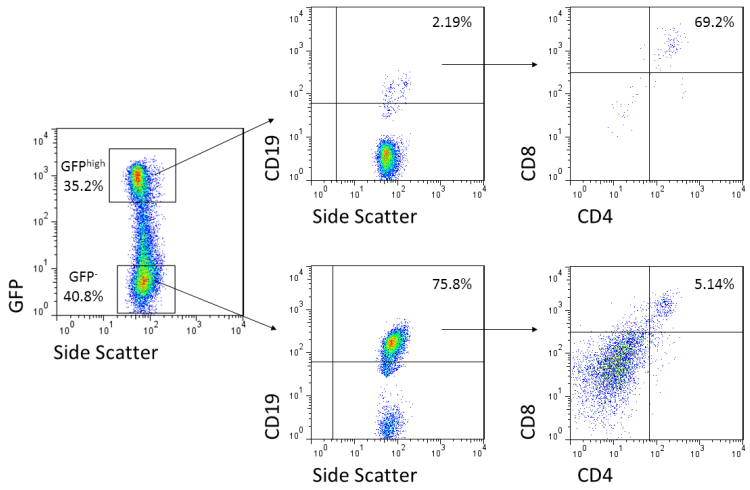
3.2.2. $RAG^{+}IgM^{+}TcR\beta^{+}$ cells are present in the NOD thymus

While $GFP^{+}CD19^{+}CD4^{+}CD8^{+}$ cells could potentially be lymphocytes transitioning from one lineage to another, this is not the only hypothesis relating to these cells. It may be that a developing T or B cell starts to receive signals which cause it to express markers of both the T and B cell lineage. If this is the case, it was wondered whether this cell would continue development and eventually co-express the receptors for both lineages. To test this hypothesis, $IgM^{+}TcR\beta^{+}$ cells were looked for in the NOD thymus.

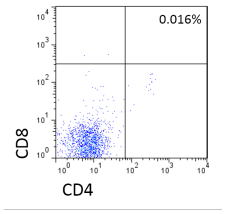
As shown in Figure 3.13, it appears that there is a small population (0.440%) of $RAG^{+}IgM^{+}TcR\beta^{+}$ in the thymi of NOD mice. Bone marrow preparations were included as a control and, as shown in Figure 3.13 B, there are no $IgM^{+}TcR\beta^{+}$ present there. The difference in frequency of $IgM^{+}TcR\beta^{+}$ cells between the bone marrow and thymus is statistically significant, as shown in Figure 3.13 C.

It may be that $RAG^{+}IgM^{+}TcR\beta^{+}$ cells are mature cells that develop from $RAG^{+}CD19^{+}CD4^{+}CD8^{+}$ cells seen in the thymus. However, the data presented cannot link the two populations. It may be that the two populations are totally separate from each other and represent two different populations found in the NOD thymus. More investigation into these cells in both diabetes-prone and diabetes-resistant mice is warranted.

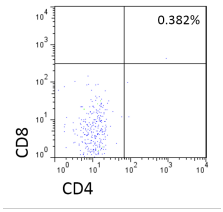
A: Thymus



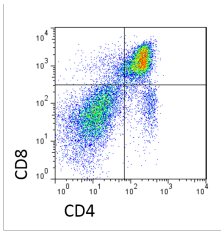
B: Bone marrow



C: Spleen



D: T cell gate



E

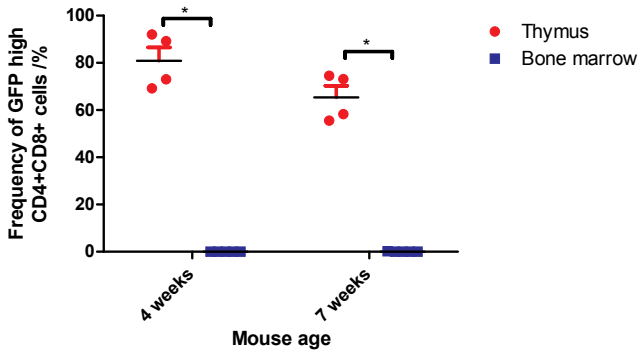
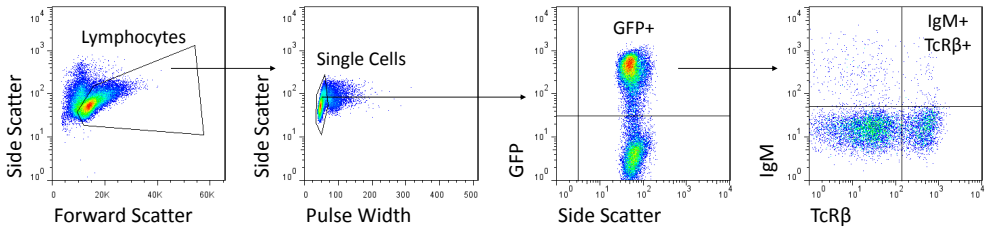


Figure 3.12: There are $GFP^+CD19^+CD4^+CD8^+$ cells present in the NOD thymus. Cells were isolated from the thymus, bone marrow and spleen of 4 (n=5) and 7 (n=4) week old NOD mice. Cells were incubated with anti-CD19, -CD4 and -CD8 antibodies for flow cytometric analysis. Thymic samples were gated on acquistion for $CD19^+$ cells. Firstly a live, single cell gate was applied (not shown) then the gating pattern shown in A was used to identify $CD4^+CD8^+CD19^+$ in GFP^+ and GFP^- populations. CD4 and CD8 gates were set using normal thymic T cell populations as a control, as shown in D A representative FACS plot of $GFP^+CD4^+CD8^+CD19^+$ cells in the NOD mouse BM (B) and spleen (C) is also shown following application of the gating shown in A. Frequencies of $GFP^+CD4^+CD8^+CD19^+$ in NOD mouse thymi and bone marrow at 4 and 7 weeks of age is shown in E. Statistical significance between frequencies in the thymus and bone marrow was determined using the Mann Whitney test. 4 weeks, P=0.0108; 7 weeks P=0.0151 ($P<0.05$ is deemed significant). Black line shows the mean and error bars show standard error of the mean. Data is representative of all 9 mice examined.

A



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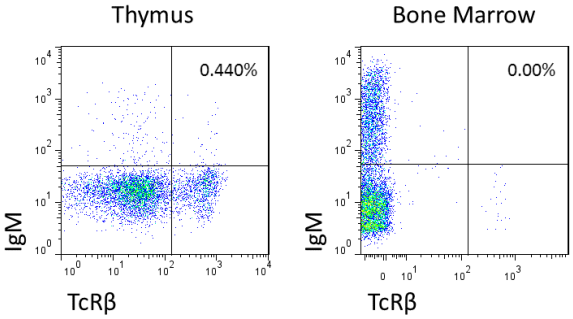

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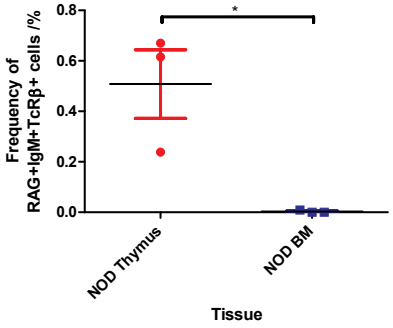

Figure 3.13: There is a small population of GFP$^+$IgM$^+$TcRβ^+ cells in the NOD thymus. Cells were isolated from the thymus and bone marrow of 11 week old NOD-RAG-GFP (n=3) incubated with anti-CD19, -IgM and -TcR antibodies and analysed by flow cytometry. Thymic samples were gated on acquistition for CD19$^+$ cells. For analysis, IgM$^+$TcRβ^+ cells were identified within a live, single cell, GFP$^+$ gate. Gating strategy shown in A. Representative FACS plots showing presence/absence of IgM$^+$TcRβ^+ for the NOD thymus and bone marrow are shown in B. C shows the frequencies of GFP$^+$IgM$^+$TcRβ^+ cells in the NOD thymus and bone marrow. Statistical significance in frequencies between the two tissues was determined using an unpaired T test and found to be significant, P=0.0206 (P<0.05 is deemed to be significant). Black line shows the average frequency and the error bars represent the standard error of the mean. Data shown are from one experiment which has been repeated twice. A total of 8 mice were examined. The experiment was carried out single blind to avoid bias.

Discussion

Type 1 diabetes is believed to be due, in part, to a breakdown in central tolerance of T cells in the thymus. Our studies, and others, have correlated a significant increase in thymic B cells in NOD mice, with respect to diabetes-resistant murine strains, with T1D progression to the β cell destruction phase. The origin and role of these B cells are not known, but they might possibly be involved in the breakdown of central tolerance. Due to the strong correlation between thymic B cell numbers and T1D susceptibility, it is important to establish how intrathymic abnormalities occur in NOD mice. This project aims to investigate more fully the potential intrathymic development of B cells.

Through this project, it has been confirmed that B cells are increased in the NOD mouse thymus with respect to B6 control mice, and that this increase is age-related. In terms of potential intrathymic development, it has been shown that the thymus contains B cell development transcription factors and, therefore, could be conducive to B cell development. Also, pro and pre B cells are present in the thymus of NOD mice, though the population frequency appears to be dependent on the presence of a mature B cell. Further to this, there is evidence of active B cell receptor rearrangement in the thymus, indicative of active B cell development. However, the progenitor from which thymic B cells are originating from is less clear. Whilst BLPs are present in both NOD and B6 mouse thymi, they are significantly decreased in the NOD mouse compared to B6 mouse suggesting an alternative progenitor for thymic B cells, at least in the

NOD mouse.

Also, further to these findings, there were some interesting results which suggested the presence of a cell in the thymus expressing markers of both T and B cells, and in fact a small population expressing both a B and T cell receptor. These could provide an alternative mechanism for B cell development through re-differentiation of T cells.

4.1. There is an age-related increase in thymic B cells in the NOD mouse

The results showing an age-related increase in thymic B cells in the NOD mouse compared to the B6 control mouse suggests there may be a correlation between thymic B cells and T1D progression. T1D onset occurs with initial priming of T cells between 3-5 weeks of age. At this point there is minimal responsiveness of T cells to T1D antigens and the population of B cells in the NOD thymus is not increased compared to control B6 mice. At 5 weeks of age, the islets start to become infiltrated with immune cells and by 9 weeks, CTL mediated β cell destruction is initiated and progresses to destroy the majority of β cells. In terms of thymic B cell numbers, B cells are significantly increased at 9 weeks of age in NOD mice compared to B6, and further increased by 12 weeks.

T cell development and education occurs within the thymus, in particular the first step of T cell tolerisation, negative selection. This is where autoreactive T cells are purged from the repertoire to prevent potential autoimmunity [112]. T1D is believed to be due to a breakdown in tolerance and, therefore, it may be that B cells found in the thymus of NOD mice may be contributing to this process. There is evidence that B cells are able to contribute to the process of negative selection, as shown by Frommer and Waisman [104] and Yamano et al. [103]. Yamano et al. [103] have demonstrated that B cells found in the thymus, but not the periphery, are able to express AIRE, similar to mTECS which classically mediate negative selection. Using AIRE-reporter mice, donor splenic B cells were transferred into recipient mice then the thymi of the recipient mice were analysed one week after transfer. It was observed that there were AIRE

expressing donor cells in the thymus of the recipient mice. This suggests that there may be something in the thymic environment which is causing B cells to express AIRE upon entering the thymus. MHC class II, also important for mediation of negative selection, was also upregulated on the immigrating thymic B cells. Meanwhile, Frommer and Waisman [104] utilised a mouse that specifically expressed myelin oligodendrocyte glycoprotein (MOG) on MHC class II of all B cells, including thymic B cells (B^{MOG} mice). These mice were then crossed with a transgenic mouse with has a T cell repertoire specific for MOG (2D2 mice). Interestingly, in the resulting mice (B^{MOG}/2D2 mice), the frequency of $CD4^+$ T cells was significantly reduced in the thymus and periphery, compared to 2D2 mouse controls. After ruling out the possibility of mTECs and other APCs in the thymus expressing the MOG antigen and, therefore, contributing to the negative selection of the MOG specific T cells, it was shown that the deletion of MOG specific T cells in the thymus was as a result of MOG expression on MHC Class II of B cells.

In the above studies, non-autoimmune prone mice were utilised in which the autoimmune process was inititated by inducing the inappropriate expression of host antigens and biasing the T cell repertoire to recognise these antigens. In these cases, the role of the B cells in the thymus appeared to be beneficial to the host in the tolerance induction in developing T cells. However, it is plausable that in autoimmune prone mice, such as the NOD mouse, the B cells may, in fact, be detrimental to the negative selection of T cells. It could be that thymic B cells are a driving force in the breakdown of negative selection, and that the increase in thymic B cells seen in the NOD thymus, compared to control B6 mice, is linked to an increasing breakdown in negative selection. On the other hand, as previous literature suggests that the negative selection provided by thymic B cells is beneficial in T cell education, it may be that increased thymic B cells are a consequence of the disease process, such that their development is triggered to help with an increasing population of developing autoreactive T cells.

Investigation into the role of thymic B cells was outside the remit of this project

but it remains a fundamental area that neccessitates further research in the future.

4.2. B cells are developing intrathymically

Thymic pro and pre B cell presence

The main hypotheses for the origins of thymic B cells are (a) intrathymic development from progenitors, or (b) migration of B cells from the bone marrow to the thymus. The general consensus is that B cells are developing within the thymus with very little contribution from the circulation. Experiments involving parabiotic congenic mice (where two congenic mice share the same blood system) showed that of the thymic B cells, the majority were of the endogenous phenotype, not the parabiotic partner. This gave the impression that the presence of thymic B cells have more to do with the signals within the thymus itself, rather than alteration in the circulation pattern of B cells [102]. Similarly, Akashi et al. [96] found that the majority of thymic B cells were of host origin. To investigate this, donor adult (Ly5.1 x Ly5.2)F1 mouse splenic B cells were injected into Ly5.1 recipient mice. The percentages of B cells of donor- and recipient-derived B cells were then evaluated in the thymus and periphery of the recipient mice. In the periphery, approximately 5-7% of B cells were of donor origin, compared to approximately 0.06% of thymic B cells of donor origin. This gives the impression that thymic B cells are mainly of host origin, rather than as a result of B cells entering the thymus from the circulation.

This project aimed to further investigate this intrathymic development and the evidence from the data shown agrees with the hypothesis of intrathymic B cell development.

Firstly, pro and pre B cells were investigated in the thymus of both NOD and B6 mice and shown to be present. This is similar to the findings of both Hashimoto et al. [113], who showed presence of pro B cells and a smaller population of pre B cells in the thymus of BALB/cJ mice, and Akashi et al. [96], who both

showed presence of pro and pre B cells in the thymus of B6 mice. It would seem, therefore, that the ability of B cells to develop in the thymus, at least to the pro B cell stage, is common for both diabetes-prone and diabetes-resistant strains of mice.

Hashimoto et al. [113] also showed an interesting finding that in BALB/cJ mice, there was an accumulation of pro B cells in the thymus that were inefficient at maturing to the pre B cell stage and beyond. Through foetal thymic organ culture (FTOC), it was hypothesised that the thymic environment renders B cell progenitors hyporesponsive to lymphopoietic signals, such as IL-7. This was shown by seeding lobes with B cell progenitors from the bone marrow, then harvesting the cells at both 2 and 7 days post seeding. Following harvest, cells were cultured on S17 bone marrow stromal cell lines with B cell permissive conditions and it was shown that only those harvested at the 2 day time point were able to produce B cells, whereas the 7 day time point cells were not. Il-7 is important for B cell development [114]. Hashimoto et al. [113] showed that B cell progenitors from FTOC showed a decreased response to IL-7 compared to the same B cell progenitors taken directly from the bone marrow. However, these data were acquired using in vitro approaches therefore it is not known whether this is applicable to the processes in vivo.

The presence of pro and pre B cells in the NOD thymus suggests that B cells may be developing there as they are two fundamental stages of normal B cell development (see Figure 1.1). The findings by Hashimoto et al. [113] could also suggest a potential mechanism by which B cells are increased in the NOD thymus compared to the control B6 mouse thymus, in that the NOD thymic environment may be less effective at dampening B cell progenitor responsed to lymphopoietic signals, such as IL-7.

Interestingly, it appears that the frequency of the thymic pro B cell population is dependent on whether or not mature B cells are present. That is, in NOD KO mice, the frequency of thymic pro B cells was significantly reduced compared to in both NOD and B6 mouse thymi. Some potential roles of a mature B cell

include the following:

- A mature B cell may be helping with the recruitment of B cell progenitors from the bone marrow to the thymus
- A mature B cell may provide a survival factor for B cell progenitors within the thymus so that they survive to be able to develop
- Mature B cell presence may kick start the process of B cell transcription factor expression within the thymus, so that the thymus can provide an environment conducive to B cell development
- A mature B cell may provide a survival factor for newly developed B cells so that once they have developed, they are able to survive.

A potential mechanism for investigating the role of mature B cells on the thymic pro B cell population would be to transfer mature B cells from a B cell sufficient NOD mouse into the NOD KO mouse to see if these donor B cells can have an effect on the thymic pro B cell population. However, a pilot study of this method was attempted and showed that the donor B cells did not survive to 11 days post transfer.

A similar approach to infuse mature B cells into NOD KO mice has also been attempted by Serreze et al. [85] where mature splenic B cells were transferred into NOD KO recipient mice. A similar finding was observed in that the B cells disappeared between 6 and 11 days post transfer. Serreze et al. [85] showed that this disappearance was due to CTL destruction of the donor B cells, presumably due to the lack of B cell presence during endogenous T cell development, therefore, the T cells would never have been tolerised to B cells. It is not known if CTL mediated B cell destruction was also occuring in the transfer utilising thymic B cells (rather than splenic), or whether it was a different mechanism causing their disappearance. However, given the fact that these B cells disappear following transfer, it suggests that this approach may not be appropriate for determining the effect of mature B cells on the thymic pro B cell population. For mature B cells to exert their effects on the thymic pro B cells, they may

need to be present for longer than the period they are detectable in the recipient NOD KO mice, therefore, an alternative approach must be explored.

Serreze et al. [85] continued investigations by irradiating recipient mice then transplanting NOD KO bone marrow supplemented with NOD B cells. This results in endogenous effector immune cells developing in the presence of B cells and should avoid the T cell destruction of donor B cells. This could, therefore, be a way forward to investigate the effect of B cells on the population of pro B cells in the NOD KO thymus. By adding purified mature B cells, it would be interesting to see if this was sufficient to increase pro B cell frequency in the KO thymus. Following this, it would also be of interest to try transplanting KO bone marrow supplemented with B6 B cells to see if it is a characteristic of a NOD B cell that can increase thymic pro B cells. However, transfer of B6 B cells into NOD mice would result in rejection therefore it would be necessary to use mice such as B6.H-2g7 ([115]) which express NOD MHC and would not be rejected. This then carries the caveat of the MHC being of NOD origin and may mean that the differential effects of a NOD B cell and a B6 B cell on the thymic pro B cell population may be hidden if their action has anything to do with MHC. Furthermore, it is not known whether the mature B cell needs to be of splenic or thymic origin, therefore, these experiments could also test this by separately transferring bone marrow supplemented with B cells from both tissues.

BcR rearrangement in the thymus

For pro B cells to progress to the pre B cell stage, the IgM heavy chain must be rearranged. Therefore, it was wondered whether the thymus is able to support this developmental step. To tackle this question, RAG expression within the thymic $CD19^+$ cells was assessed in NOD mice. It was seen that there are populations of $CD19^+RAG^{high}$, $CD19^+RAG^{low}$ and $CD19^+RAG^-$ cells in the NOD thymus. The RAG^{high} cells are very likely developing there within the thymus as this very bright signal was equivalent to that seen in actively developing T cells in the thymus and actively developing B cells in the bone marrow. This

gives the impression that RAG is being actively transcribed in $CD19^+$ cells in the thymus, indicating active BcR rearrangement and B cell development. In addition, if B cells were rearranging their receptors in the bone marrow and then migrating to the thymus, the GFP expression seen in the thymic B cells would be lower due to the kinetics of GFP expression. Evidence for this is seen when screening the blood of GFP^+ transgenic mice to see if they are indeed GFP^+. In this case, no GFP^{high} cells are seen in the blood of the mice suggesting that GFP expression is decreased following release from the bone marrow. Therefore, GFP^{high} B cell in the thymus is more likely to be due to active development there, rather than left over GFP from rearrangement in the bone marrow (E.A. Green, personal communication).

These RAG expressing $CD19^+$ cells were looked for in NOD-RAG-GFP mice of 4, 7 and 11 weeks of age and interestingly, the $CD19^+RAG^{high}$ population frequency didn't change, whereas the $CD19^+RAG^{low}$ frequency decreased significantly. This gives the impression that as mice age, there are less newly developed B cells in the thymus, potentially as a result of decreased development. This decrease suggests that the development of B cells within the thymus may be restricted to younger mice and that that intrathymic B cell development may be deactivated at a certain time point in the disease process. However, it may be that more newly developed B cells are leaving the thymus in older mice compared to younger ones so that the population of newly developed B cells within the thymus is decreased.

If the decrease in $CD19^+RAG^{low}$ cells is due to a decrease in development, this may be due to the fact that within the thymus there are multiple different niches harbouring different types of cells. For example, the proliferation of DN thymocytes depends on the availability of stromal niches within the thymus and this is capable, therefore, of regulating the thymus size [116]. It may be that within the thymus, there is a niche that is more capable of allowing B cell development than others. Further to this, it may be that this niche is more prevalent in mice of NOD background compared to non diabetic controls. If this is the case, down-regulation of B cell development may occur as a result of the thymic B cell niche

becoming full and competition may prevent the development of more B cells. Further to this, thymi undergo atrophy with age, therefore, a thymus which is decreasing in size would have less space available for cells, including B cells, to reside in, so it may be that with increasing age, B cell development is decreased. Thymic atrophy is believed to be accelerated in the NOD mouse. As shown by Ferreira et al. [117], thymic involution and loss of thymic architecture is seen by 9 months of age in the NOD mouse, whereas equivalent thymic degradation in the B6 mouse is not seen until 15 months of age.

On the other hand, if newly developed B cells are leaving the thymus and travelling elsewhere, it may be that the thymus is acting like the bone marrow, harbouring B cell development then allowing their release to mature in a different tissue, such as the spleen. In this regard, it would be interesting to know if thymic egressed B cells contribute to breakdown in peripheral tolerance of T cells to β cells.

Neither of these suggestions account for the overall picture of an increase in B cells in the NOD mouse thymus compared to the B6 mouse thymus. It does, however, suggest that the increased population of thymic B cells may not be directly linked to the rate of B cell development, but more to proliferation of developed B cells.

By understanding the kinetics of intrathymic B cell development, and the potential impact of thymic B cells on the normal functioning of the thymus, this could aid in the design of potential therapeutics. This understanding could elucidate a specific disease time point to target with a therapeutic agent, in order to have the greatest impact on limiting disease progression.

BLPs are present in the NOD thymus

As mentioned in Section 1.4.1, the commitment of progenitors to the B cell lineage is thought to occur at the Sca-1^{low}c-kit^{low}Flt3^{+}IL-7Rα^{+}Ly6D^{+} BLP stage (Figure 1.1, red cell labelled 'BLP'). Therefore, it was wondered whether these BLPs would be present in the NOD thymus and, if so, if they are increased in

the NOD mouse compared to the B6 mouse. When looking for potential TSPs, cells with the correct markers to allow homing were considered [21], therefore, it may be that marker expression in the bone marrow is abherrent on B cell progenitors, resulting in an excess of B cell precursors being able to migrate to the thymus alongside T cell progenitors. For example, due to their fundamental role in allowing thymic settling of progenitors, it would be of interest to see how the levels of Flt3, CCR7 and CCR9 expression compared between bone marrow BLPs and BLPs found in the thymus [21, 29]. It may be that some express excess levels of these markers which allow B cell progenitors to migrate to the thymus along with TSPs.

It was interesting when investigating the presence of BLPs that the frequency of BLPs in the Sca-1^{low}c-$kit^{low}$$Flt3^{+}$IL-$7R\alpha^{+}$ population in the thymus of the NOD mouse, was significantly decreased compared to the B6. This was surprising due to the increased population of B cells seen in the NOD thymus compared to that of the B6.

However, before any assumptions can be made on this data, it is first necessary to consider the model of B cell development from BLPs. For example:

- Are BLPs the sole progenitor for B cell development? There is evidence to suggest that this is a progenitor that is restricted to the B cell lineage [50], however, it is not clear if this is the only progenitor capable of differentiating into a B cell.

- Is the B cell developmental pathway the same in the thymus as the bone marrow? The B cell development pathway in the bone marrow has been investigated extensively [23], however, the pathway in the thymus is much less well understood, therefore, whether the bone marrow development pathway can be applied to the thymus is not known.

In order to help determine the answers to the questions above, it would be necessary to carry out further experiments. To determine whether B cells can develop from thymic BLPs, it would be interesting to culture BLPs on thymic

stromal cell lines, such as OP9-DL1 cell lines (Notch ligand-expressing, bone marrow derived cell line) which can, to an extent, simulate the thymic environment [118]. It would, therefore, be useful to see if BLPs are able to develop into B cells on cell lines such as these. It would also be of use to confirm whether the BLPs identified in the thymus in this project are able to differentiate into B cells under the same conditions as those described by Inlay et al. [50] when characterising the bone marrow-derived BLP. For this, liquid culture, supplemented with stem cell factor (SCF), Flt3L and IL-7, was used and differentiation of BLPs into B cells was seen. By replicating these conditions and culturing thymus-derived BLPs, it could suggest whether or not they are able to produce B cells in the way that bone marrow-derived BLPs can.

It is unlikely that B cell development from BLPs is restricted only to the bone marrow, shown by the presence of BLPs in the B6 thymus. These may be the origin of the normal, small population of B cells in the B6 thymus. However, the NOD thymus has a significantly smaller frequency of BLPs in the thymus compared to the B6. Despite this, NOD mice still have increased thymic B cells suggesting that there are different mechanisms in the NOD mouse which increase thymic B cells that are not present in the B6 mouse.

Interestingly, when comparing NOD and NOD KO thymic BLPs, there was no difference observed in the population frequency. This means that if a mature B cell is having an effect on the pro B cell population, it's action must be after the formation of the BLP, and prior to the formation of the pro B cell. For this reason, it would be of interest to investigate stages between these two cells, to see whether they are affected by the presence/absence of mature B cells and to try and pinpoint the time point when mature B cells have their effect.

Do thymic T cells transition to B cells?

To understand another potential mechanism for increased thymic B cells, the finding of $RAG^+CD19^+CD4^+CD8^+$ cells in the thymus needs to be considered. It is worth noting that the numbers of mice investigated was relatively small and,

therefore, requires further repetitions, along with inclusion of B6 control mice for comparison. However, assuming that this population is a true, distinct cell population in the thymus of NOD mice, there are a few potential hypotheses relating to these cells. One in particular is of interest for the increased thymic B cells seen in the NOD mouse. These cells expressing markers of both T and B cells suggest that there may be an intrathymic, post-CLP stage when developing T and B cells express dual T/B cell markers. However, if this is the case, it is unlikely that this stage is a normal part of B cell development in the bone marrow due to the lack of $RAG^+CD19^+CD4^+CD8^+$ there.

Thymic progenitors are normally committed to the T cell lineage at the DN stage, that is, prior to $CD4^+$ and $CD8^+$ expression. It is, therefore, worth considering whether $RAG^+CD19^+CD4^+CD8^+$ cells represent developing T cells which are late to commit to the T cell lineage and are retaining some characteristics of B cells (CD19). On the other hand, they may be cells developing with characteristics of both T and B cells. This may be possible due to the finding of a small population of cells expressing both a TcR and BcR ($IgM^+TcR\beta^+$) in the thymus that may be the mature cell for which $RAG^+CD19^+CD4^+CD8^+$ cells are the progenitor.

Another possibility which could then contribute to the B cell population in the thymus, is that these cells are the midpoint of a T cell transitioning to a B cell (or vice versa, although this would not account for increasing thymic B cells). This could account for the decreased BLPs in the NOD compared to B6, as B cells developing from T cells would not develop originally from BLPs and could, therefore, provide an alternative 'progenitor' to a mature B cell.

To investigate the potential for T cell to B cell (or vice versa) transition, it would be of use to look at the relative levels of B and T cell transcription factors Pax5 and Notch1 in the thymus of NOD mice compared to the thymus of B6 mice.

Maintaining the correct balance of transcription factors is very important for development of lymphocytes. For example, as mentioned in Section 1.4.2, Pax5 expression is important for repressing T cell lineage commitment through the

repression of Notch1 [64]. This was shown by expressing Pax5 in all HSCs and MPPs and it was found that the constitutive expression of Pax5 led to an increase in B cell development, and a simultaneous decrease in T cell development due to Pax5 dependent repression of Notch1. Further to this, deletion of Notch1 led to a loss of developing T cells and an increase in thymic B cells [119]. As shown by Feyerabend et al. [119], they found that when Notch1 was deleted from progenitor T cells, this was associated with an increase in thymic B cells, some of which arose from the progenitor T cells switching fate from T to B cell lineage. However, unexpectedly, the majority of these B cells arose from Notch1 sufficient progenitors and, therefore, became B cells as a result of a cell-extrinsic factors acting due to the deletion of Notch1 in progenitor T cells.

That said, Notch1 is not the only important transcription factor that needs to be kept in balance. As mentioned in Section 1.3.2, Gata3 is an important transcription factor for T cell development which requires repression by EBF for B cell development to occur [120]. Banerjee et al. [120] showed, through the use of quantitative PCR, that EBF is capable of significantly reducing Gata3 transcription. This was done by transfecting $ebf^{-/-}$ progenitor cells prior to the pro B cell stage, with EBF and/or Pax5. Following induction of Pax5 expression, Notch1 transcripts were found to be consistently decreased and EBF expression reduced Gata3 transcript abundance. This suggests that EBF is also able, to an extent, to control the T/B cell fate decision and, therefore, any abnormality in expression levels may affect the populations of B and T cells in the thymus. In terms of BLPs identified in this project, when Banerjee et al. [120] looked into the levels of Gata3 transcripts in these cells they found a robust decrease compared to that seen in all-lymphoid progenitors (ALPs). This coupled with the lack of T cell lineage cells arising from BLPs suggests that Gata3 may be downregulated when T cell potential is lost.

Cobaleda et al. [63] have shown that B cells, in the correct conditions, may be able to de-differentiate back to progenitors, and then differentiate into T cells. To show this, Pax5 was deleted from mature B cells from $Ly5.2^{+}$ donor mice and the B cells were then transferred into recipient $Ly5.1^{+}$ mice. It was found that

8 weeks after transfer, some of the donor mature B cells had dedifferentiated back to the pro B cell stage, shown by their Ly5.2$^+$ pro B cell phenotype. Also, the recipient mice (that were T cell deficient) showed reconstitution of T cell development in the thymus 8 weeks following transfer of Pax5 deleted B cells.

These data, combined, show the importance of transcription factor balance. It may be that in the thymus of NOD mice, an imbalance in transcription factor expression causes the increased B cell population. Given the information on transcription factor balance importance above, it would also be beneficial to compare the relative levels of Notch1 and Pax5, and Gata3 and EBF seen in the thymus of NOD and B6 control mice. This could indicate any imbalance, unique to the NOD mouse, which could be accounting for the increased thymic B cell population seen in the NOD thymus. Unfortunately, due to time constraints, quantitative PCR studies to assess the transcription factors above could not be conducted.

In the future, to investigate the potential transitioning of a T cell to a B cell (or vice versa) in the thymus, RAG$^+$CD19$^+$CD4$^+$CD8$^+$ cells could be isolated and their final developmental fate determined following incubation with either a stromal cell line (such as OP9-DL1), or more relevantly, a reaggregated thymic organ culture (RTOC). In this latter approach, developing thymocytes in ex-vivo generated foetal thymic organ cultures (FTOCs) would be killed, leaving the thymic stromal network. This stromal network could then be seeded with RAG$^+$CD19$^+$CD4$^+$CD8$^+$ cells and their end fate monitored. For example, their potential progression to IgM$^+$TcRβ^+ cells, mature B cells or mature T cells could be assessed. On the other hand, these further investigations could show that these RAG$^+$CD19$^+$CD4$^+$CD8$^+$ cells form a distinct population that is unrelated to other populations seen in the thymus.

4.3. Conclusions and Future Directions

This project aimed to more fully understand potential intrathymic B cell development in the NOD mouse model of T1D. These mice show a significant

increase in thymic B cells when compared to non-diabetes prone mice, such as the B6 mice used for controls in this project.

There were two main aims for this project. Firstly, the presence of B cell progenitors (at the point of B cell commitment and beyond) within the thymus was investigated to give an indication as to whether B cell development in the thymic environment is likely. Secondly, it was wondered whether or not the thymic environment is able to support the initial IgM heavy chain rearrangement process that is crucial for early B cell development at the pro to pre B cell transition stage.

Literature to date has suggested that intrathymic B cell development is a likely process and the data presented in the project supports this hypothesis on a number of levels.

Due to the literature available on the development of B cells in the bone marrow, it was hypothesised that the development of B cells within the thymus would follow a similar pattern. Investigation into this revealed that, in a similar fashion to the bone marrow, the thymus holds populations of pro and pre B cells, indicative of B cell development there. Further to this, there are $CD19^{+}$ cells in the thymus that, via the use of a RAG-GFP reporter mouse, show evidence of RAG expression. These findings together suggest that the thymic environment is able to support development of B cells from the pro to pre B cell stage. Interestingly, it appeared that the development of B cells may be age-related, shown by a decrease in newly developed B cells in the thymus of older NOD mice.

BLP presence in the thymus was also investigated due to evidence that this is the first progenitor committed to the B cell lineage. Interestingly, while these were present in the NOD thymus, their frequency was much reduced in the NOD mouse thymus, in comparison to the B6 control mouse thymus. This findings gives the impression that the increase in thymic B cells seen in the NOD mouse, compared to the control B6 mouse, is unrelated to the BLP population, and may indicate an alternative progenitor/mechanism for thymic B cell development.

A hypothesis for a potential alternative developmental pathway arose upon the finding of $RAG^+CD19^+CD4^+CD8^+$ cells in the NOD thymus. It was postulated that this population may represent the midpoint of a T cell transitioning to a B cell and, therefore, present a mechanism by which B cells could be produced without the BLP progenitor. With this in mind, this could explain why thymic BLPs are decreased in NOD mice compared to B6 control mice, even though thymic B cell numbers are increased in the NOD mouse thymus.

These processes occurring in the NOD mouse are summarised in Figure 4.1. Alongside these potential, abnormal processes, the normal pattern of B cell development in the bone marrow, and the pathogenic process occurring in the islets are shown in the schematic. Of particular interest is the fact that TSPs (yellow cell) may be retaining B cell potential and differentiating, via the BLP stage and RAG expression into mature B cells (blue arrows). There is also potential for the existence of cells with characteristics of both B and T cells (green and grey cell), which may or may not be the result of a redifferentiating T or B cell (purple arrows). The contribution of thymic B cells to the normal functioning of the thymus is not known, however, it is hypothesised that they may be contributing to T cell negative selection (black, dashed arrow). It is also not known how thymic B cells are contributing to the overall disease pathogenesis, however, it may be that they are moving from the thymus to the islets and contributing to insulitis.

By understanding the likely intrathymic development of thymic B cells in the NOD mouse, in the future, it may be possible to manipulate this process to impact on the disease progression. With this in mind, the field of thymic B cells may be able to contribute to the discovery and development of new therapeutics for T1D.

Specific future directions have been outlined within the discussion section of this project. However, in more general terms, the future direction of this project should be concerned with aiming to characterise the B cell progenitor responsible for production of thymic B cells. This would indicate whether or not the

increased thymic B cell numbers in NOD mice, compared to control B6 mice, is due to an abnormality allowing excess B cell development in the thymus, or whether it is a lack of regulatory mechanisms relating to their survival. For example, if the NOD mouse possesses an abnormality which allows seeding of the thymus with progenitors which have retained B cell potential and preferentially differentiate into B cells, this could account for the increase. On the other hand, it may be that B cell development in the NOD mouse is normal, but there is an abnormality in the NOD mouse which allows thymic B cells to survive and/or proliferate, where they would not be allowed to in control mice.

It would also be of importance to understand the role of the thymic B cell, in order to know how it functions in T1D. It would be useful to know if it has a role in driving disease progression, or whether it is more concerned with helping with the purging of autoreactive T cells. It would therefore be an obvious progression to look into the functioning of thymic B cells and how their actions have an effect on the T1D pathogenesis. By linking the development and functioning of thymic B cells, it may be that there is an optimum time point for intervening in the thymic B cell development process in order to have a beneficial effect on the disease.

The deepening understanding of the field of thymic B cells has far reaching consequences, not just in the field of T1D, but in other autoimmune diseases where increased thymic B cells are also seen. This highlights the relevance of the research and the importance of its continuation in the future to bring therapies, as yet undiscovered, one step closer.

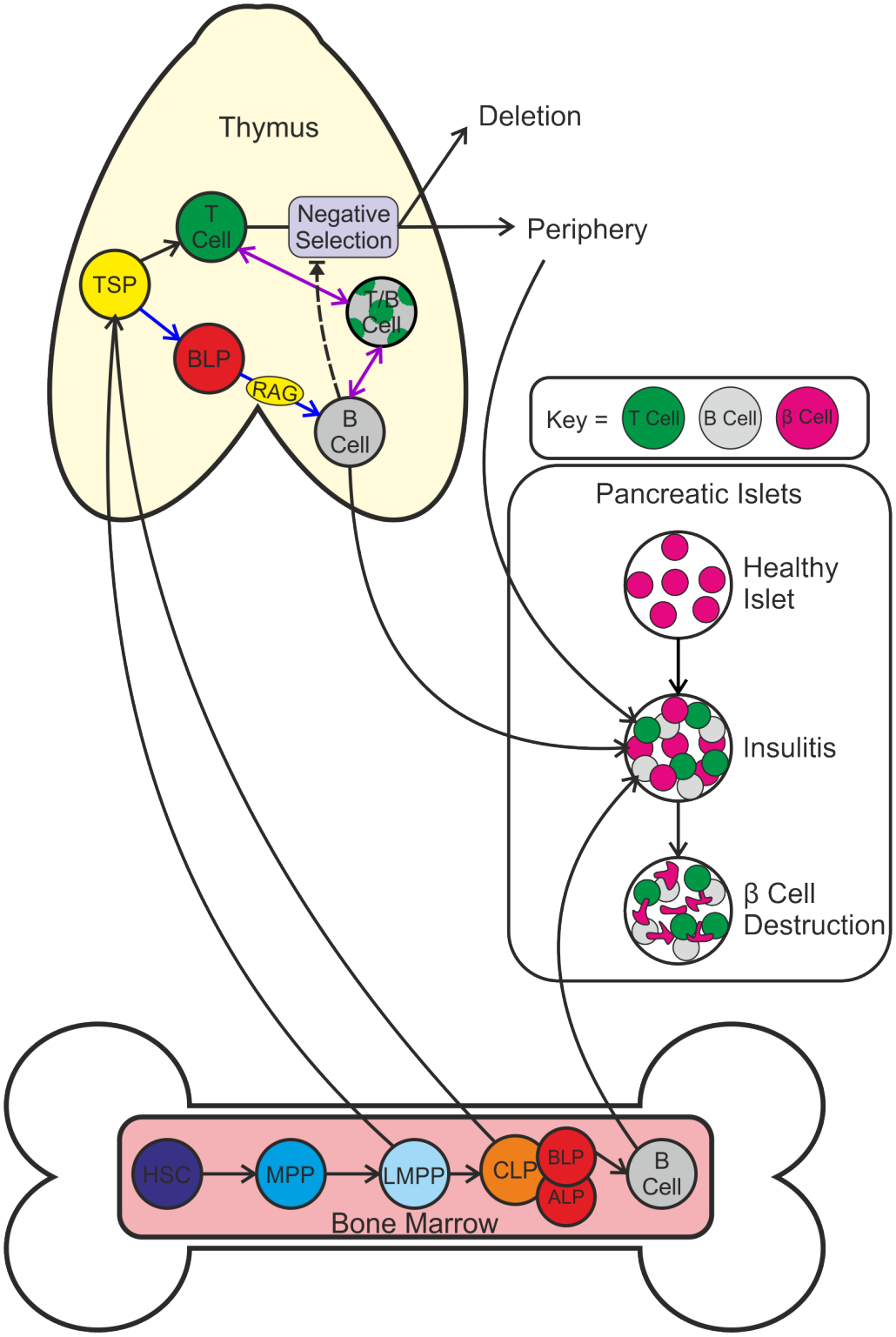

Figure 4.1: Diagrammatical summary of the project findings. B cells develop in the bone marrow. T cells develop from thymic settling progenitors believed to be Flt3^{+}CCR7/9^{+} LMPPs or CLPs which migrate to the thymus. Here they develop into T cells and pass through negative selection, for which the outcome is either clonal deletion or release into the periphery. B cells may also be developing in the thymus, either following the same pathway as seen in the bone marrow (bone marrow), or from T cells developing into B cells (red arrows). There also appears to be evidence of a cell expressing both T and B cell markers which may arise from a B or T cell that has been triggered to display both markers (purple arrows), or may be the mid point of a transitioning T or B cell. Thymic B cells may contribute to insulitis.

Abbreviations

AIRE Autoimmune Regulator

ALP All lymphoid progenitor

APC Antigen presenting cell

ARRIVE Animal Research: Reporting of In Vivo Experiments

B6 C57BL/6

Bcl11b B cell lymphoma/leukaemia protein 11B

BcR B cell receptor

BLP B cell biased lymphoid progenitor

bp base pair

BSA Bovine serum albumin

CCR7 Chemokine receptor 7

CCR9 Chemokine receptor 9

CD Cluster Differentiation

cDNA Complementary DNA

c-kit Stem cell growth factor receptor

CLP Common lymphoid progenitor

cTEC Cortical thymic epithelial cell

CTL Cytotoxic T lymphocyte

CXCL12 C-X-C motif chemokine 12

DAMP Damage-associated molecular pattern

DC Dendritic cell

DN Double negative

DNA Deoxyribonucleic acid

dNTP Deoxynucleotide triphosphates

DP Double positive

DTT Dithiothreitol

E2A Transcription factor 3

EBF Early B cell factor

ETP Early thymic progenitor

Fc Fraction crystallisable

Flt3 Fms-like tyrosine kinase 3

Flt3L Fms-like tyrosine kinase 3 ligand

FOXO1 Forkhead box protein O1

GAD Glutamic acid decarboxylase

Gata3 GATA-binding protein 3

Gfi-1 Growth factor independent 1 transcription repressor

GFP Green fluorescent protein

HSC Haematopoietic stem cell

IA-2 and 2β Insulinoma antigen 2 and 2β

Ig Immunglobulin

IGRP islet-specific glucose-6-phosphate catalytic subunit-related protein

Ikaros Ikaros family zinc finger protein 1

IL Interleukin

IL-7Rα Interleukin 7 receptor α

KO Knock out

Lin Lineage

LMPP Lymphoid primed multipotent progenitor

Ly6D Lymphocyte antigen 6 complex, locus D

MgCl2 Magnesium chloride

MHC Major histocompatibility complex

MPP Multipotent progenitor

mTEC Medullary thymic epithelial cell

NCBI National Centre for Biotechnology Information

NK cell Natural killer cell

NLR NACHT-leucine rich repeat receptors

NOD Nonobese diabetic

PAMP Pathogen-associated molecular pattern

Pax5 Paired box 5

PBS Phosphate buffered saline

PCR Polymerase chain reaction

PLN Pancreatic lymph node

PRR Pattern recognition receptor

PU.1 Transcription factor PU.1

RAG Recombination activating genes

rcf Relative centrifugal force

RLR Rig-like receptors

RNA Ribonucleic acid

RSS Recombination signal sequences

Runx RUNT-related transcription factor

Sca-1 Stem cells antigen-1

SCID Severe combined immunodeficiency

SP Single positive

T1D Type 1 diabetes

T2D Type 2 diabetes

TAE Tris acetate-EDTA

TCF-1 T cell factor 1

TcR T cell receptor

TcRβ T cell receptor subunit β

TdT Terminal deoxynucleotidyltransferase

TLR Toll-like receptor

Treg Regulatory T cell

TSP Thymic settling progenitor

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