The Role of B Lymphocytes in Type 1 Diabetes

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Abstract

While autoreactive T cells are known to induce beta cell death in type 1 diabetes (T1D), self-reactive B cells also play an important role in the pathogenesis of T1D. Studies have shown that individuals living with T1D have an increased frequency of self-reactive B cells that escape from the bone marrow and populate peripheral organs, become activated and participate in disease. These failed tolerance mechanisms may be attributed to genetic risk alleles that are associated with the development of T1D. Once in the periphery, these self-reactive B cells act as important antigen-presenting cells to autoreactive T cells and produce autoantibodies that are used to predict individuals at-risk for or diagnosed with T1D. Here we discuss the evidence that B cells are important in the pathogenesis of T1D, how these cells escape normal tolerance mechanisms, their role in disease progression, and how targeting these cells and/or monitoring them as biomarkers for response to therapy will be of clinical benefit.

Introduction

Autoimmunity generally ensues in the setting of genetic susceptibility, occurring in response to an environmental trigger, leading to a loss of central and peripheral immune tolerance. Type 1 diabetes has been predominantly considered an autoimmune condition in which T cells play a prominent pathogenic role in destruction of the insulin-producing beta cells of the pancreas, but B cells and other antigen presenting cells are also required in a complex network for autoimmunity to occur. The pathogenic process occurs over many years, with autoantibodies, being one of the earliest markers of this disease. It is relatively difficult to study aspects of the pathogenesis of T1D, as the target organ – the beta cells of the pancreatic islets of Langerhans, are inaccessible, lying deep within the human body abdominal cavity. Our insights into many aspects of the pathogenesis have been signposted by the Non Obese Diabetic (NOD) mouse model, which has remarkably similar features to some of the human clinical aspects of disease, including both high genetic susceptibility with the Major Histocompatibility Complex contributing a large proportion of risk, as well as influence of the environment in development of diabetes, although the actual factors are different.

In this chapter, the focus on the role of B cells in diabetes development will be discussed, with insights gained from the NOD mouse model highlighted alongside the observations that these have led to in humans. The many B cell functions will be explored, with consideration of the various ways in which B cells may contribute to the autoimmunity in T1D, including antigen presentation and diversification of the immune response, cytokine production, autoantibody production, development of follicular dendritic cells, and alteration in immune regulation.

What is the evidence that B cells are involved in the pathogenic processes leading to T1D? NOD mice that have a targeted IgM gene deletion (μMT−/−) resulting in B cell developmental arrest (Serreze et al. 1996; Akashi et al. 1997; Wong et al. 1998), or B cells depleted by the administration of antibodies at a very early age (Noorchashm et al. 1997) have a very low incidence of autoimmune diabetes. This has been even more strongly emphasized by the depletion of B cells in NOD mice with agents that have included anti-CD20 (Hu et al. 2007; Xiu et al. 2008), toxin Calicheamicin conjugated to anti-CD22 (Fiorina et al. 2008), neutralization of the B cell growth factor BAFF (Zekavat et al. 2008), and BCMA-huFc fusion protein (Marino et al. 2009) to more specifically deplete follicular and marginal zone B cells, which have all resulted in protection against the development of autoimmune diabetes. Importantly, studies in recently diagnosed individuals with T1D demonstrated that depletion of B cells using anti-CD20 (Rituximab) preserved beta cell function (i.e. decreased rate of c-peptide loss) and reduced exogenous insulin needs up to one year following treatment (Pescovitz et al. 2009). However, these benefits were no longer seen by the end of the second year, once B cell numbers had been restored to normal levels following the initial course of treatment (Pescovitz et al. 2014). Thus, these lines of evidence provide strong support for the proposition that autoreactive B cells play an important role in the etiology and/or progression of T1D and that targeting B cells may have therapeutic potential. However, there are practical considerations in the choice of the therapy that could be used (see below).

Breakdown of B cell tolerance in T1D

Previous studies have shown that as many as 70% of B cells generated in the bone marrow are autoreactive (Wardemann et al. 2003). In healthy individuals these

self-reactive B cells are normally tolerized (silenced) by one of three mechanisms: 1) receptor editing, 2) clonal deletion, or 3) anergy (Fig. 1a). Central tolerance in the bone marrow encompasses both receptor editing and clonal deletion, whereas peripheral tolerance includes anergy. B cells that bind self-antigen with high avidity in the bone marrow undergo receptor editing, in which strong B cell receptor (BCR) signals induce rearrangement of the antigen receptor light chain genes, silencing one allele and expressing a second. If the new antigen receptor lacks self-reactivity, the B cell can continue development and populate the periphery as a naïve B cell capable of responding to pathogenic insults (Halverson et al. 2004; Meffre and Wardemann 2008). For many B cells this process is successful, but when it is not, death by clonal deletion/apoptosis occurs. If the BCR has a moderate avidity for self-antigen, the B cell can exit the bone marrow and enter the periphery but is suppressed via anergy (Bluestone et al. 2010; Jeker et al. 2012). Anergic B cells are characterized by an inability to become activated, proliferate, and differentiate into antibody secreting cells (Gauld et al. 2005; Gauld et al. 2006; Merrell et al. 2006; Cambier et al. 2007; Duty et al. 2009). Chronic stimulation by self-antigen (signal 1) in the absence of T cell help (signal 2) is critical for induction and maintenance of B cell anergy. Anergic B cells downregulate their BCR, particularly surface IgM, and increase activation of negative regulatory signaling molecules, such as PTEN and SHIP-1 (O'Neill et al. 2011; Getahun et al. 2016; Getahun et al. 2017). Importantly, anergy is reversible if the autoantigen dissociates from the BCR or the autoreactive B cell receives help from a cognate T cell.

A breakdown in the tolerance mechanisms discussed above likely contributes to development of T1D. Menard and colleagues found that self-reactive B cells, as defined by binding of their antibody to permeabilized Hep-2 cells, are increased among

the new emigrant/transitional and mature naïve B cells in individuals with T1D (Table 1), suggesting impairment of both central (receptor editing or clonal deletion) and peripheral (anergy) B cell tolerance (Menard et al. 2011). Importantly, these autoreactive B cells were found to be polyreactive, binding also to LPS and insulin. Furthermore, the frequency of recombining sequence (RS) rearrangements in lambda positive B cells, which is a surrogate measure of receptor editing, is decreased in T1D subjects compared to healthy control individuals (Panigrahi et al. 2008). Taken together, these results demonstrate T1D subjects exhibit a breakdown in central tolerance mechanisms, which likely allows escape of autoreactive B cells into the periphery.

If central tolerance mechanisms fail, B cells that enter the periphery should undergo a state of anergy or unresponsiveness. However, studies indicate that individuals with T1D have an impaired ability to maintain self-reactive B cells via anergy. Analyzing the frequency of total anergic B cells (termed B_{ND}) versus insulin-binding anergic B cells along a continuum of diabetes development, it was found that autoantibody positive first-degree relatives and recently (< 1 year) diagnosed individuals with T1D have a significant decrease in total and insulin-reactive anergic B cells in their peripheral blood compared to healthy controls and individuals living with long standing diabetes (Table 1) (Smith et al. 2015; Habib et al. 2019). Interestingly, some autoantibody negative first-degree relatives display a similar loss of insulin-reactive anergic B cells in their peripheral blood, suggesting that loss of anergy may precede activation and differentiation of these cells into autoantibody- secreting cells. Recent studies have identified a subset of B_{ND} cells, termed $B_{ND}2$, which expresses increased markers of activation, including the T cell co-stimulatory molecules CD80 and CD86, and are functionally no longer anergic in T1D donors (Table 1). Importantly,

insulin-binding $B_{ND}2$ cells were increased in the peripheral blood and pancreatic lymph nodes of young-onset T1D donors, suggesting activation of previously anergic autoreactive B cells occurs at an increased frequency in individuals with T1D (Fig. 1b). Given that insulin-binding $B_{ND}2$ cells have increased surface expression of CD80 and CD86, it is tempting to speculate that previously islet-specific anergic B cells may participate in pathogenic responses (Stensland et al. 2023).

Association of genetic risk alleles with B cells in T1D

It has long been known that Human Leukocyte Antigen (HLA) alleles play a major role in susceptibility to T1D, but there are many other contributory genetic loci, with currently more than 90 gene regions identified by Genome Wide Association Studies (GWAS) (Redondo et al. 2023). Fine mapping to genetic loci that are associated with development of T1D indicates that these susceptibility loci are shared with other autoimmune conditions and involve genes associated with immune cell function. These include loci that influence B and T cell responses, immunoregulatory cell activity, as well as some that play a role in innate immunity.

Given that development of T1D is driven in part by genetic risk alleles, it seems likely that these factors could mediate their effects by promoting loss of central and peripheral B cell tolerance. The T1D risk allele most affecting odds ratio for disease development is HLA class II. DR4-DQ8 followed by DR3-DQ2 confer the greatest risk (Erlich et al. 2008; Concannon et al. 2009). CD4 T cells recognizing self-peptides in the context of DR4-DQ8 could evoke loss of B cell tolerance. In line with this, loss of anergic insulin-binding B cells and acquisition of $B_{ND}2$ cells is associated with carriers of the DR4-DQ8 haplotype (Smith et al. 2015; Stensland et al. 2023). The genetic polymorphism conferring the second highest risk is in the VNTR region of the insulin

(*INS*) gene (Concannon et al. 2009). This polymorphism is thought to increase the number of insulin-specific T cells in the periphery due to impaired T cell tolerance induction in the thymus (Pugliese et al. 1997). Hence, an increase in insulin-specific T cells would promote activation of insulin-reactive B cells, driving them to participate in disease. Indeed, studies have found that loss of anergic insulin-binding B cells is associated with insulin allotypes, suggesting T cells are likely driving loss of B cell anergy (Smith et al. 2018b).

In addition, impaired B cell tolerance is associated with polymorphisms in the genes encoding the phosphatases, *PTPN22* and *PTPN2,* both of which are expressed in B and T cells and involved in regulation of B and T cell receptor signaling (Cerosaletti and Buckner 2012). Mutations in *PTPN22,* which encodes the lymphoid tyrosine phosphatase, Lyp, confer the third highest contributor to T1D risk, after HLA and the *INS* genes (Concannon et al. 2009). Individuals who express the R620W variant of PTPN22 have reduced signaling through the B cell receptor (BCR), and this is suggested to increase the release of autoreactive B cells into the periphery (Rieck et al. 2007). The R620W variant also predicts that, in those individuals who become positive for insulin autoantibodies, these insulin autoantibodies will appear first (Steck et al. 2014). This variant, which is also found in other autoimmune conditions, increases the frequency of autoreactive and polyreactive B cells in the peripheral blood that have recently emigrated from the bone marrow (Menard et al. 2011). Targeted ectopic expression of the risk allele in B cells *in vivo* leads to autoimmunity (Dai et al. 2013).

The *PTPN2* gene encodes another protein tyrosine phosphatase that has been shown to have a range of functions, including negative regulation of JAK/STAT signaling (Simoncic et al. 2002) and T cell receptor signaling (Wiede et al. 2011). A

study in which PTPN2 was deleted in the hematopoietic compartment of adult mice demonstrated that these mice developed autoimmunity characterized by an increase in the number of B cells, including germinal center B cells, as well as anti-nuclear autoantibody production (Wiede et al. 2017). Studies in the Smith lab have shown that B cell-specific deletion of PTPN2 in C57BL/6 mice leads to activation of B cells, a hyperresponsive phenotype, and autoantibody production (Alexander and Smith, unpublished). Thus, polymorphisms in genes whose products function as negative regulators of BCR signaling may confer T1D risk by impairing central and peripheral B cell tolerance. Other T1D associated allelic variants of genes expressed in B cells, such as BACH2 and SH2B3, may also in time be proven to impair B cell tolerance.

While no genetic risk alleles are known to exist for PTEN, a negative regulator of the PI3-kinase pathway, it has been shown that total B cells from individuals with new onset T1D exhibit decreased expression of PTEN compared to control subjects (Smith et al. 2019). Defects in regulation of the PI3-kinase pathway (i.e. gain-of-function (GOF) mutations) can lead to increased infections, cancer, and autoimmunity (Fruman et al. 2017; Michalovich and Nejentsev 2018). Hence one might speculate that decreased expression of a negative regulator, such as PTEN, in all B cells would alter signaling thresholds, allowing rogue activation of autoreactive B cells. Further studies are needed to support this idea.

B cells and autoantibodies

Autoantibodies recognizing antigens expressed in the islets were one of the earliest indications of T1D having an autoimmune basis – with antibodies to insulin (Palmer et al. 1983), glutamic acid decarboxylase (GAD) (Baekkeskov et al. 1990), tyrosine phosphatase like protein I-A2 (Payton et al. 1995), Zinc transporter 8 (ZnT8) (Wenzlau et al. 2007) and most recently tetrapanin-7 (McLaughlin et al. 2016).

In humans, the presence of autoantibodies to GAD, IA-2 and insulin have been used to predict future development of T1D (Ziegler et al. 2013). Indeed, T1D is now

staged; pre-diabetes or Stage 1 and Stage 2, is recognized as the presence of 2 or more diabetes autoantibodies without dysglycemia (Stage 1) or with dysglycemia (Stage 2), as proposed by Insel and colleagues and adopted for screening programs (Insel et al. 2015). Although these autoantibodies are now recognized as very important biomarkers for the future development of T1D and for diagnosing an individual with T1D, current dogma suggests they are not pathogenic.

There are multiple lines of evidence from the NOD mouse that suggest that autoantibodies are not necessary for the development of autoimmune diabetes, although some studies have suggested a modulating role. The direct transfer of human serum antibodies into SCID mice (Petersen et al. 1993) or NOD mouse serum into NOD mice (Serreze et al. 1998) does not induce diabetes, nor is transfer of antibodies through milk important (Washburn et al. 2007). However, when B cell-sufficient offspring are born to B cell-deficient mice, the incidence of diabetes is reduced, implying that maternally transmitted antibodies may be important in mice (Greeley et al. 2002). Furthermore, through embryo transfer experiments, NOD offspring born to non-diabetes susceptible mothers developed insulitis but had reduced diabetes (Kagohashi et al. 2005). Additionally, mice that have B cells that express surface antibody but are lacking in soluble antibody production are able to develop diabetes, albeit at a much lower rate (Wong et al. 2004). In humans it is interesting that offspring of mothers who have diabetes autoantibodies are not at increased risk of future development of T1D (Koczwara et al. 2004) and there is a greater risk of development of diabetes in offspring where fathers have diabetes compared with mothers (Warram et al. 1984). Indeed, in a subset of individuals (HLA DR3+ but DR4/DQ8-), the presence of autoantibodies appeared to be protective (Koczwara et al. 2004). These

studies indicate that soluble antibodies likely do not play a major role in causing diabetes, but is this lack of pathogenic effect absolute?

There are a number of observations that suggest that autoantibodies may impact disease pathogenesis. Autoantibodies can play a pathogenic role through FcR-mediated antigen-antibody uptake and activation by dendritic cells and macrophages that then present antigen to self-reactive T cells. It has been demonstrated that FcR-deficient NOD mice are protected from diabetes and insulitis is alleviated (Inoue et al. 2007). Moreover, secretion of anti-islet autoantibodies act in an FcR-mediated fashion to enhance the expansion of islet-reactive CD4 T cells in mice (Silva et al. 2011). Studies in humans indicate a tight correlation with the number of autoantibody specificities present and progression to diabetes. Importantly, of all the possible autoantibodies that an individual can develop, it has only been shown for anti-insulin antibodies that higher titer levels correlate with disease progression (Steck et al. 2011; Steck et al. 2016), suggesting a pathogenic role for anti-insulin antibodies in T1D. Hence, the current dogma that autoantibodies are likely not pathogenic remains uncertain. Nevertheless, other aspects of B cell function, such as antigen presentation to T cells, may be more important.

B cells as antigen-presenting cells

Much evidence suggests that of the many functions of B cells, their ability to present antigen to T cells, is of considerable importance (Serreze et al. 1998; Silveira et al. 2002; Marino et al. 2012) (See Figure 2). B cells are the only antigen-specific APCs that recognize antigen via the more than 10^5 B cell receptors on their cell surface, making them very potent and efficient at processing and presenting self-antigen to cognate autoreactive $CD4^+$ (Kendall et al. 2013; Pearson et al. 2020) and

CD8$^+$ T cells (Marino et al. 2012; Boldison et al. 2020). In the NOD mouse model, if B cells are prevented from presenting antigen via either class I or class II, diabetes development is reduced, demonstrating the importance of B cells to present antigen to both CD4$^+$ and CD8$^+$ T cells (Noorchashm et al. 1999; Marino et al. 2012). In addition, BCR specificity is particularly important as NOD mice that have a reduced antigen-specific BCR repertoire also develop a reduced incidence of diabetes (Silveira et al. 2002; Wong et al. 2004). Conversely, accelerated autoimmune diabetes occurs in NOD mice that express an anti-insulin heavy chain gene (VH125Tg.NOD), rendering ~1-2% of peripheral B cells insulin-binding (Hulbert et al. 2001; Kendall et al. 2013). The same increased rate and penetrance of diabetes development is seen in the V_H125^{SD}.NOD mouse, in which the VH125 gene is directly targeted into the IgH locus, enabling class-switch recombination to occur (Felton et al. 2018). In both models, the heavy chain is fixed but can pair with a variety of endogenous light chains. Thus, most peripheral B cells are non-insulin-reactive, but the 1-2% of B cells that are insulin-reactive are sufficient to drive accelerated diabetes development. These anti-insulin B cells from the VH125.NOD mouse models can act as antigen-presenting cells to insulin-reactive T cells (Kendall et al. 2013; Felton et al. 2018; Boldison et al. 2019). Reciprocal effects of insulin-specific CD4$^+$ T cells on insulin-reactive B cells have been studied in transgenic mice in which increased levels of both T and B insulin-reactive cells are expressed. The pathogenic CD4$^+$ T cells, 8F10, recognizing insulin amino acids B12-20, when expressed as a transgene in the NOD mouse do not cause disease, but accelerate diabetes when the T cell transgene is on the NOD RAG1$^{-/-}$ genetic background (Mohan et al. 2013). When the 8F10 transgenic NOD mouse was crossed with the V_H125^{SD}.NOD mouse, which develops accelerated disease, diabetes incidence was considerably reduced. Interestingly, however, when naïve 8F10 CD4$^+$

T cells were co-transferred with V_H125^{SD}.NOD B cells into RAG-/- mice, diabetes was accelerated compared to naive 8F10 $CD4^+$ T cells alone or with non-transgenic B cells (Wan et al. 2016). In the presence of the 8F10 T cells, the frequency of germinal centres (GC) in pancreatic lymph nodes were considerably increased, and these GC were also increased in the mesenteric, inguinal, and axillary lymph nodes, with concomitant high production of insulin autoantibodies, which were class-switched. These GC responses were also found when 8F10 and V_H125^{SD} were co-transferred into the RAG-/- mice (Wan et al. 2016). Thus, the insulin-specific $CD4^+$ T cells increased the auto-antibody production from the insulin-reactive B cells, which in turn were also able to activate the antigen-specific $CD4^+$ T cells. In a different model, the regulatory insulin-specific $CD4^+$ T cells 2H6 (Du et al. 2006) which play a suppressive/regulatory role on recognition of insulin B9-23, when crossed with VH125 BCR transgenic mice to generate 2H6VH125 double transgenic mice, also develop reduced spontaneous diabetes (Pearson et al. 2020). The regulation promoted by the 2H6 cells, which produce TGFβ, reduced the expression of MHC class II and costimulatory molecules on the VH125 B cells, and reduced antigen presentation. Reciprocally, the 2H6 cells also demonstrated weaker proliferation when activated by the VH125 B cells but the presence of the B cells from the VH125 BCR did not affect the regulatory phenotype of the 2H6 $CD4^+$ T cells (Pearson et al. 2020). Furthermore, the expression of these 2H6 regulatory T cells reduced the germinal centres seen in the pancreatic lymph nodes of the VH125 BCR transgenic mice. Thus, the antigen-specific $CD4^+$ T cells will modulate the pathogenic antigen-specific B cells, by altering GC responses and concomitant autoantibody responses, dependent on their phenotype. Reciprocally, the antigen-specific B cells also reinforce the phenotypes of the $CD4^+$ T cells.

Due to the inherent difficulties of demonstrating directly that islet-reactive B cells are presenting antigen to cognate islet-reactive T cells in Stage 1, 2 or 3 T1D individuals, most evidence that B cells act as important antigen-presenting cells to T cells has come from studies in mice. Nevertheless, studies of the pancreas from donors with T1D (discussed in more detail below) has demonstrated a strong correlation with the number and proximity of B cells found in inflamed islets with $CD8^+$ T cells, suggesting B cells may be acting as antigen-presenting cells to $CD8^+$ T cells in the pancreas (Willcox et al. 2009). Future studies are needed to expand upon these findings to determine the role of B cells more conclusively in the pancreatic islets.

Regulatory B cells in T1D

As we have discussed above it is well-established that B cells are associated with a pathogenic role in disease; however, it is important to note that under specific circumstances and environments, B cells can exert regulatory effects. Regulatory B cells (Bregs) in T1D have recently been extensively reviewed (Ben Nasr et al. 2021; Boldison and Wong 2021) and so in this chapter we will only provide a brief overview, highlighting some recent observations. Many regulatory B cell subsets suppress inflammation via the production of IL-10. However, unlike regulatory T cells, there are no definitive markers of regulatory B cells and therefore, without assessing IL-10 (or other anti-inflammatory cytokines such as TGFβ and IL-35), it is difficult to define a regulatory B cell. However, with increased multi-parameter flow cytometric capabilities, many distinct subsets of regulatory B cells, distinguished by the expression of a selection of immune markers, have been identified. These regulatory B cell populations suppress inflammatory responses from T cells, DCs and monocytes in both mice and humans, although it should be noted that in permissive environments,

most B cells can differentiate into Bregs (Rosser and Mauri 2015). In T1D, there is evidence for both numerical and functional defects in specific Breg populations; however, some disparity exists between studies (which is extensively discussed in (Boldison and Wong 2021)), likely due to the use of different markers, lack of IL-10 assessment or different donor cohorts. More recent studies have reported a decrease in $CD25^{hi}$ Bregs, a population high in IL-10 and TGFβ production (Kessel et al. 2012), in T1D donors compared to healthy controls (Zhang et al. 2022). Tompa and Faresjo characterized Bregs in children with T1D (and/or celiac disease) and demonstrated a decrease in memory Bregs ($CD24^{hi}CD27^{+}$) but an increase in $CD5^{+}$ Bregs (Table 1) (Tompa and Faresjo 2024). Additional studies will be required to fully elucidate the complex network of regulatory B cell subsets and allow understanding of the relationship between the changes we observe and different donor demographics.

Recent experiments in NOD mice have shown that a unique $CD103^{+}$ B cell population with immunosuppressive properties are expanded in the NLRP6-deficient mouse and can protect from diabetes development (Pearson et al. 2023). $CD103^{+}$ Bregs produced IL-10 and TGFβ, reduced antigen-specific CD4 T cell responses, and were controlled by the presence of NLRP6. This work supports the notion that in some environments B cells can play a vital role in maintaining immunity. Furthermore, antigen-specific engineered B cells have the capacity to protect from autoimmune diabetes induced by both insulin-reactive $CD8^{+}$ T cells and antigen-specific $CD4^{+}$ T cells (Chen et al. 2023). These studies suggest that B cells could be manipulated to enhance their regulatory capacity to suppress autoimmunity. Additional research is required to fully elucidate the importance of Bregs in the development of diabetes and whether we can harness their regulatory capacity.

B cells in the pancreatic tissue

So far, many of the studies evaluating B lymphocytes in the pancreas have used immunohistochemical techniques on fixed tissue collected post-mortem from individuals diagnosed with T1D. However, early studies on pancreatic biopsy specimens from donors with newly-diagnosed T1D observed the presence of B cells in inflamed pancreatic islets (Itoh et al. 1993). Pancreatic samples from individuals with a type 1 diabetes diagnosis are still relatively rare, and samples from donors who have had a recent diagnosis or are at risk of developing diabetes are rarer still (Leete 2023). Therefore, much of the research on B cell phenotype and function within the pancreatic tissue has relied on T1D mouse models. ~~These models, such as the NOD mouse, can serve as a valuable tool for studying infiltrating B lymphocytes and allow strategic signposting into clinical studies.~~ In the NOD mouse, B-1a cells, which are innate-like B cells that mainly reside in the peritoneal cavity, can be detected as early as 2 weeks of age in the pancreas, and these cells can activate plasmacytoid DCs via dsDNA-specific IgG immune complexes resulting in IFNα production and the initiation of diabetes (Table 1) (Diana et al. 2013). Depletion of B-1a cells in the NOD model can inhibit the diabetogenic T cell response and protects the mice from the development of disease (Kendall et al. 2004; Ryan et al. 2010; Diana et al. 2013). During the development of diabetes in the NOD model, B-1a cells are replaced with a more follicular B cell phenotype in the pancreas, which is characterized by the expression of IgD, the upregulation of CD138 (Ryan et al. 2010; Serreze et al. 2011; Boldison et al. 2019) and an increase in $CD138^{+}CD44^{hi}$ plasmablasts (Table 1) (Boldison et al. 2021; Ling et al. 2022). However, in human T1D pancreatic tissue, very few $CD138^{+}$ or $Ki67^{+}$ B cells are observed (Arif et al. 2014), indicating that, so far, in the donors assessed, the presence of blasting or plasmablast-like B cells are rare.

Studies from the VH125 BCR transgenic mouse demonstrate specific recruitment of insulin-reactive B cells to both the PLN and pancreas (Smith et al. 2018a; Boldison et al. 2019). Importantly, these insulin-reactive B cells in the target tissue and draining lymph node have increased expression of CD86, a marker of B cell activation and an important co-stimulatory molecule necessary for T cell activation (Henry et al. 2012; Smith et al. 2018a). Studies from mice have now been translated to humans. Recently, it was shown that insulin-reactive B cells are also found in increased frequencies in the PLN of donors with T1D compared to non-diabetic controls (Stensland et al. 2023).

Gene studies in NOD mice, comparing $CD19^+$ B cell populations in the pancreas and the pancreatic lymph nodes (PLN), when the pancreas has extensive insulitis, have demonstrated that B cells are transcriptionally different to the PLN, with induction of an innate immune signature characterized by IFNα–related genes i.e. *Irf7* and *Tlr7* alongside proinflammatory cytokine-related genes such as *Il6* and *Il1b* (Table 1) (Boldison et al. 2023). In this same study, TLR7 protein expression in $CD20^+$ B cells in the NOD mouse pancreas, was possibly induced by the presence of IFNα and could play a role in promoting damage through cytokine production (Boldison et al. 2023). Moreover, deletion of TLR7 in the NOD mouse model suppresses diabetes development (Debreceni et al. 2020; Huang et al. 2021), specifically by altering the functional responses of B cells and inhibiting cytotoxic $CD8^+$ T cell activation (Huang et al. 2021). It is not yet known if $CD20^+$ B cells in human T1D pancreatic tissue adopt an IFN-signature, but it is well-established that IFNα is expressed by the beta cells of individuals with T1D (Foulis et al. 1987) and IFN-associated genes are overexpressed in the islets of new-onset T1D donors (Lundberg et al. 2016). Currently, little phenotyping has been performed on $CD20^+$ B cells found in human T1D pancreatic

tissue, and therefore it is still unknown if specific subsets of B cells are recruited to the tissue, how early they are found in the pancreas during the development of T1D or if they are altered within the inflamed pancreatic environment. However, a new body of evidence is now accumulating indicating that B cells may play a crucial role in approaches to new therapeutic strategies, which will be discussed below.

Evidence for B cell-targeted treatment stratification.

Several ground-breaking studies have recently proposed that there are distinct immune phenotypes or endotypes in T1D, with age of diagnosis being the major factor. Seminal work by Willcox et al. identified $CD20^+$ B cells in the pancreas of recent-onset donors, with the most abundant frequencies observed at the later stages of beta cell destruction. These B cells were strongly associated with the presence of $CD8^+$ T cells (Willcox et al. 2009), suggesting B cell presentation of antigen and B cell-CD8 T cell crosstalk (See Figure 2). Further immunohistological analysis in this T1D donor cohort (Exeter Archival Diabetes Biobank (EADB)) noted that recent-onset donors, diagnosed at a young age, had significantly larger numbers of $CD20^+$ B cells as part of their insulitic pancreas profile. Donors who were diagnosed at older ages had fewer immune cells, and very few $CD20^+$ B cells were present in the pancreas (Arif et al. 2014). The follow-up in depth study by Leete and colleagues on a larger number of donors from the EADB, DiViD (Diabetes Virus Detection study) and nPOD (Network for Pancreatic Organ Donors with Diabetes) confirmed the earlier observations (Leete et al. 2016). In this follow-up study, using the age of diagnosis and the ratio of $CD20^+$ B cells to $CD4^+$ T cells present in inflamed pancreatic islets, it was evident that donors diagnosed with T1D when <7yrs of age had a high $CD20^+$ B cell:$CD4^+$ T cell ratio, compared to donors diagnosed >13yrs of age and above, and this was correlated with

fewer insulin-containing islets. Furthermore, a detailed study using imaging mass cytometry reported that 1 of 4 recent-onset T1D donors who were diagnosed at a young age displayed a prominent $CD20^+$ B cell profile in the pancreas (Damond et al. 2019). Recent gene expression studies were performed in a select number of donors from the EADB cohort (diagnosed young with an increased frequency of $CD20^+$ B cells [T1DE1] and diagnosed >7 years of age and with fewer $CD20^+$ B cells [T1DE2]). These studies demonstrated a number of genes overexpressed in T1DE1 donors associated with lymphocyte regulation, including *IKZF3* (Torabi et al. 2023) which is involved in B cell differentiation and activation (Schmitt et al. 2002). Other gene expression studies using whole blood RNA sequencing from new-onset T1D donors revealed fast-progressors, characterized by a rapid loss of C-peptide, is predicted by age of diagnosis and associated with a B cell gene signature (Linsley et al. 2019b).

B cell targeted therapeutic strategies

In NOD mice, a number of therapies targeting B cells were shown to protect the mice from developing autoimmune diabetes, both when given before overt disease, or even after diabetes had developed as discussed earlier (Hu et al. 2007; Xiu et al. 2008). In humans, the early studies using a single course of Rituximab (anti-CD20) (Fig. 3a) showed that the treatment clearly depleted B cells for the first six months following treatment, but B cell numbers returned to normal levels by 12-18 months post therapy. As mentioned above, individuals with T1D who were treated with Rituximab showed reduced requirement for insulin and preserved C-peptide one year following treatment, which was not sustained at the two-year follow-up visit. Hence, like many other therapies given at the time of overt Stage 3 type 1 diabetes, the Rituximab trial showed B cell depletion after sufficient beta cell mass has been

462 destroyed to require exogenous insulin administration does not have a lasting effect,
463 and therefore, may have been too little, too late (Pescovitz et al. 2009; Pescovitz et al.
464 2014). One year following treatment, the study showed a reduction of IgM antibodies,
465 which may take longer than 1 year to return to pre-treatment levels, with a
466 corresponding reduction in the B cell response to new antigens during this time.
467 However, IgG responses were maintained. In addition, the ability of B cells to respond
468 to a previously encountered antigen (recall response), as well as new antigens, was
469 restored once the B cells recovered, with naive B cells recovering more rapidly than
470 memory B cells (Pescovitz et al. 2011). Following treatment with Rituximab, the newly-
471 generated B cells included just as many autoreactive cells as at the baseline visit
472 (Linsley et al. 2019a) (Chamberlain et al. 2016), which implies that there was not a
473 fundamental change in the mechanisms that prevent the generation and/or release of
474 autoreactive B cells from the bone marrow. Therefore, to be effective in the long term,
475 further treatment would be required.

476 However, as a pan-B cell depletion therapy, there are elements of
477 immunosuppression which may limit the use of Rituximab, especially if it would require
478 further courses of administration. Since Rituximab is an early generation chimeric
479 anti-B cell antibody, the likelihood of anti-chimeric antibodies developing is increased.
480 The possibility of using second generation humanized anti-B cell antibodies may
481 therefore be a useful strategy. Thus, whilst targeting B cells using Rituximab had
482 obvious C-peptide preserving effects, there is clearly scope for improving on the
483 current outcomes. For example, initiating treatment early in the course of disease,
484 such as in Stage 1 when autoantibodies are first detected, may be superior. In addition,
485 future studies are needed to help identify which individuals are likely to be a responder
486 versus a non-responder. For example, it was found that a subgroup of individuals

treated with Rituximab had an increase in T cell proliferative response to antigens, including islet autoantigens, earlier after treatment, suggesting that these individuals may have more potential for further islet beta cell damage, and are less likely to respond favorably to Rituximab treatment (Linsley et al. 2019a). It may also be important to identify and target other cell types that treatment with Rituximab has uncovered. These include T follicular helper cells ($CD4^+CXCR5^+ICOS^+$ T cells) that are increased in individuals with T1D, and which were shown to decrease with Rituximab treatment (Xu et al. 2013). It is also worth noting other B cell-targeted therapies, such as a BAFF blockade, which may be more effective at mediating T1D protection (Wang et al. 2017) and circumvents the possibility that the CD20 molecule is downregulated on B cells upon entry into the pancreas (Serreze et al. 2011). Similarly, recent studies using CD19-targeting chimeric antigen receptor (CAR) T cells to deplete B cells in various autoimmune conditions, such as SLE, idiopathic inflammatory myositis, and systemic sclerosis have demonstrated they are safe and effective, and therefore, may warrant testing in the treatment or prevention of T1D (Mackensen et al. 2022; Muller et al. 2024).

CTLA4Ig (Abatacept), which blocks costimulatory molecules, CD80 and CD86, that are expressed on antigen-presenting cells, such as B cells, has been transiently effective when given to individuals at the time of overt clinical presentation (Orban et al. 2011), although not when administered earlier in Stage 1 (Fig. 3b) (Russell et al. 2023). Further analysis of the effects of Abatacept suggest that individuals who respond less well to treatment had an increase in the number of B cells at baseline, and an increase in gene expression of alternative costimulatory ligands *ICOSLG* (interacting with ICOS) and *CD40* (interacting with CD154), which are both strongly expressed on B cells. These findings suggest that these B cells may preferentially use

512 other ligands when CD80 and CD86 are blocked (Linsley et al. 2019b), making this
513 treatment less effective.

514 Given the potential for immunosuppression that general depletion of B cells
515 may cause, a recent study in NOD mice showed that targeting insulin-specific B cells
516 may be effective in reducing autoimmune diabetes. Alleva and colleagues used a
517 metabolically inactive recombinant Fc fusion protein, AKS-107, comprising the human
518 insulin A and B chains linked to human IgG1 Fc fragment, which binds to and depletes
519 insulin-specific B cells (Fig. 3c). They demonstrated that treatment in prediabetic
520 VH125Tg.NOD mice, as well as WT NOD mice, reduced the development of diabetes
521 (Alleva et al. 2024). Recent work has further indicated the importance of insulin-
522 specific B cells in human type 1 diabetes, particularly in young-onset T1D (Stensland
523 et al. 2023). Other antigen-specific B cell targeted therapies have been tested in the
524 NOD mouse and have shown promising results (Henry et al. 2012; Leon et al. 2019;
525 Zhang et al. 2019). It would potentially be an interesting type of reagent to trial in
526 humans at risk of Type 1 diabetes, perhaps together with another agent.

527 Collectively, there is now increasing evidence that B cell targeted therapy will
528 be most effective in patients that develop T1D at a young age. Indeed, in pediatric T1D
529 patients combined therapy of Rituximab and autologous Tregs was superior to Treg
530 monotherapy alone (Zielinski et al. 2022). In addition, in the early Rituximab trial the
531 participants who were youngest in age tended to respond better than those who were
532 older at onset (Pescovitz et al. 2009). Furthermore, it may also be true of other
533 demographics, aside from age, that is associated with a B cell-immune phenotype that
534 we have not yet explored. For more effective B cell targeted therapies, it will be
535 necessary to understand who would benefit most from a B cell intervention, and which
536 B cell intervention strategy is likely to be the most successful. Lastly, combination

537 therapies will likely be needed to provide the most robust targeting of the immune
538 system to prevent ultimate progression to T1D.
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B cell subset	*Phenotype*	*Mechanism(s) of action*	*Change in tissue*	*References*
Human T1D				
New emigrant/transitional	Poly/autoreactive CD19+ CD27- IgMhi CD24hi	Unknown	↑ in blood	Menard et al. 2011
Mature naïve	Poly/autoreactive CD19+ CD27- IgM+ IgD+	Unknown	↑ in blood	Menard et al. 2011
Anergic (B_{ND})	CD19+ CD27- IgM-/lo IgD+ +/- Insulin-binding	Tolerized / unresponsive self-reactive	↓ in blood	Smith et al. 2015, Habib et al. 2019
Activated previously anergic ($B_{ND}2$)	Insulin-binding CD19+ CD27- IgM- IgD+ CD21- CXCR5-	Unknown	↑ in blood in young-onset (≤10 yrs old)	Stensland et al. 2023
Breg	CD25hi Breg	Inhibit T cell and APC responses	↓ in blood	Zhang et al. 2022
	Memory Breg (CD24hi CD27+)	Inhibit T cell and APC responses	↓ in blood	Tompa and Faresjo 2024
	CD5+ Breg	Inhibit T cell and APC responses	↑ in blood	Tompa and Faresjo 2024
NOD mouse				
B1a	CD5+	Innate-like; can activated pDCs	↑ in pancreas at 2 weeks of age	Diana et al. 2013
Plasmablasts	CD138+ CD44hi	Antibody-secreting cells	↑ in pancreas when diabetic	Boldison et al. 2021, Ling et al. 2022
Follicular B cell	IgD+ CD138+	Unknown	↑ in pancreas when diabetic	Ryan et al. 2010, Serreze et al. 2011, Boldison et al. 2019

Inflammatory B cells (via gene expression)	↑ expression of IFNα-related and pro-inflammatory genes	Unknown	↑ in the pancreatic lymph node	Boldison et al. 2023
TLR7+ B cells	TLR7+ B cells	Unknown	↑ in the pancreas	Boldison et al. 2023

542 **Table 1**. B cell subsets that have been shown to be altered in human T1D and NOD mouse.

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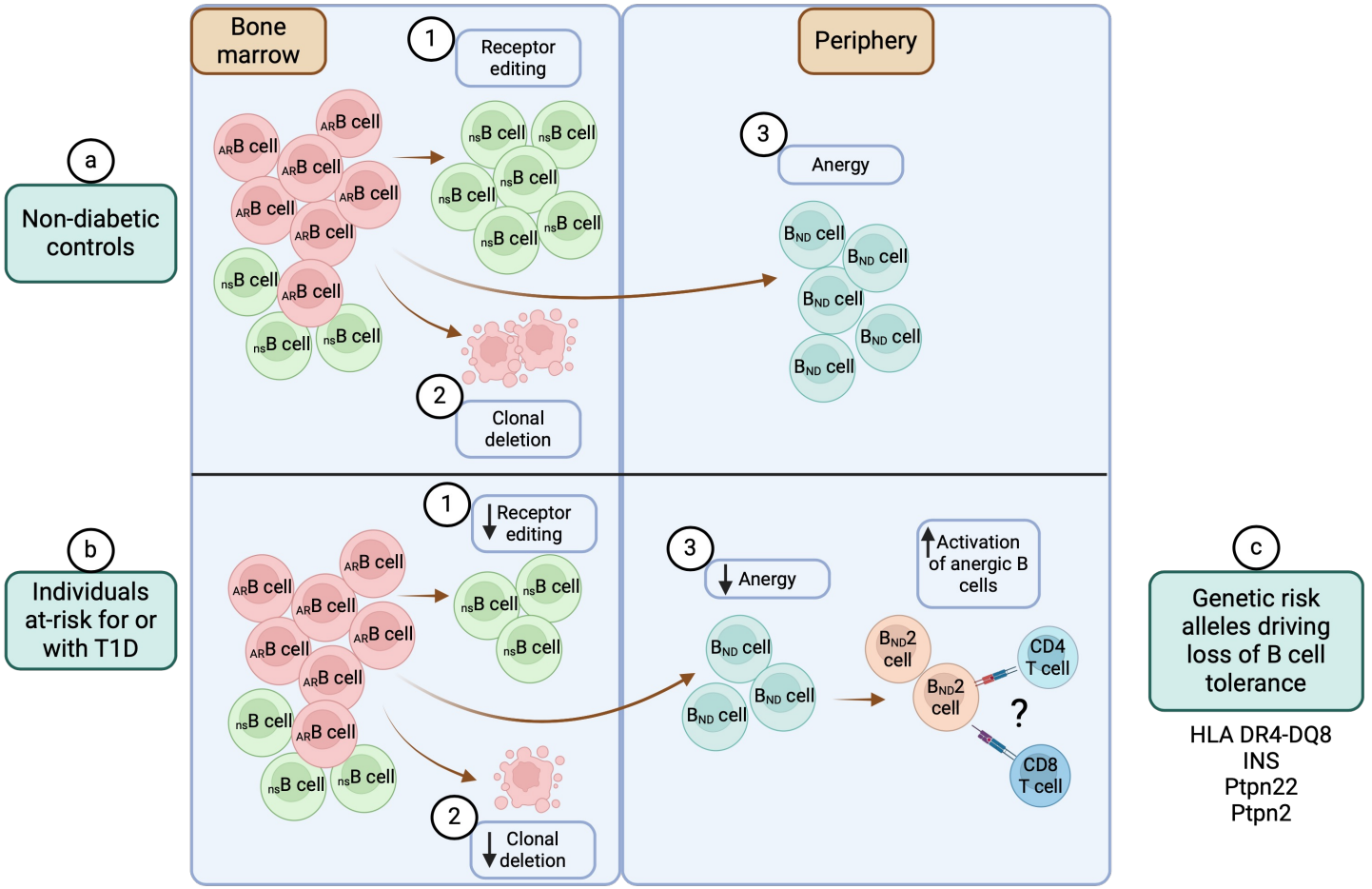

Figure 1. Failed B lymphocyte tolerance mechanisms in T1D. (a) Central tolerance occurs in the bone marrow and includes receptor editing and clonal deletion, while peripheral tolerance occurs in the periphery and includes anergy. It has been shown that ~70% of B cells made in the bone marrow are autoreactive ($_{AR}$B cell). In non-diabetic individuals, $_{AR}$B cells with high affinity for self-antigen will undergo 1) receptor editing. If these cells fail to edit their B cell receptor (BCR) to a non-autoreactive BCR, they will then undergo 2) clonal deletion/apoptosis. $_{AR}$B cells with low-moderate affinity can escape into the periphery, where they will undergo B cell anergy, becoming a B_{ND} cell. Together these B cell tolerance mechanisms help prevent development of autoimmunity. (b) In individuals at-risk for or with T1D, it has been shown that $_{AR}$B cells fail to undergo proper silencing by receptor editing, clonal deletion, and anergy. Autoreactive B cells that escape into the periphery become activated, becoming $B_{ND}2$ cells, and likely interact with cognate $CD4^+$ and $CD8^+$ T cells to help drive development of T1D. (c) Loss of these B cell tolerance mechanisms in T1D are associated with high-risk genetic risk alleles, including expression of the HLA DR4-DQ8 haplotype and polymorphisms in INS, Ptpn22, and Ptpn2.

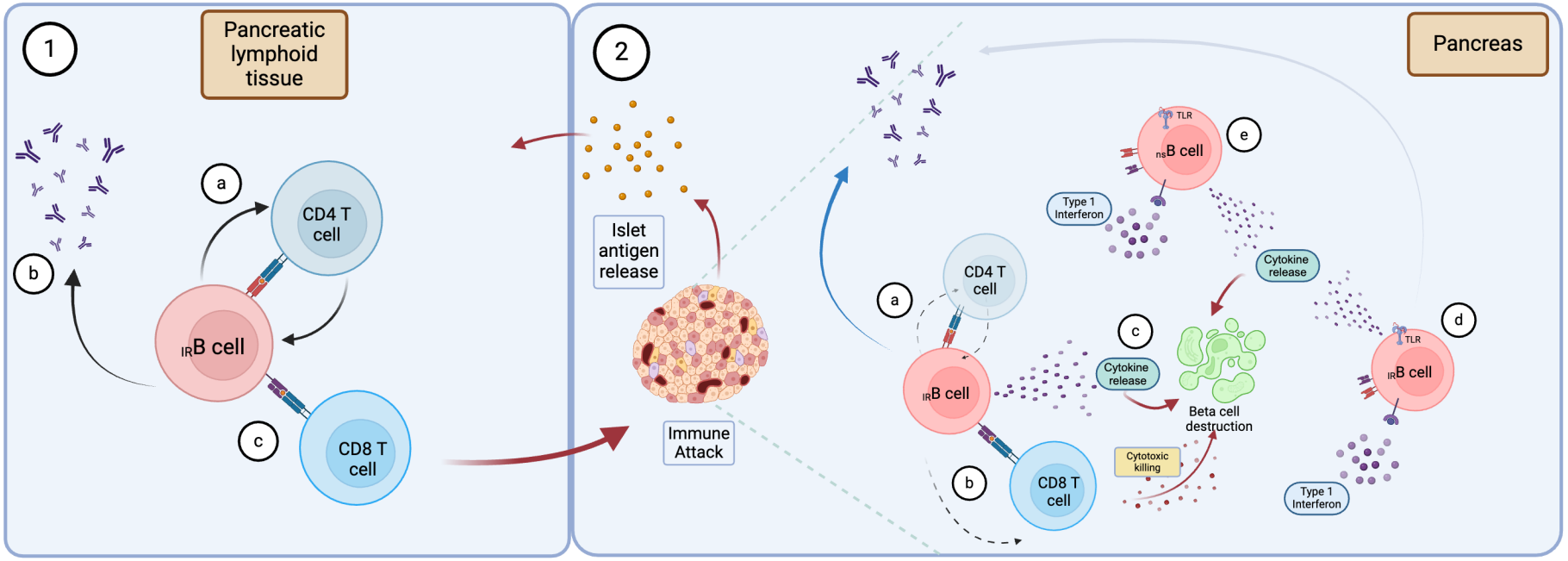

Figure 2. B lymphocyte involvement in T1D. 1. In the peripheral lymphoid tissue islet reactive B cells ($_{IR}$B cells) process and present islet antigens to CD4 T cells (a) and receive CD4 T cell help to produce islet autoantibodies (b), which are used as a biomarker in T1D. $_{IR}$B cells will also cross present antigen to CD8 T cells leading to activation and islet immune attack. 2. In the pancreatic tissue it is still unknown which B cells are present during beta cell destruction, and how they perpetuate beta cell demise. We have suggested several roles which may occur in the tissue during T1D: a) $_{IR}$B cells present islet antigens to CD4 T cells. b) $_{IR}$B cells cross present islet antigens to CD8 T cells which may lead to further cytotoxic CD8 T cell activation and B cells to (c) release pro-inflammatory cytokines due to the altered environment and received signals. d) $_{IR}$B cells may undergo activation without T cell help, activated by cytokines such as IFN**α** or an innate signal through the Toll-like Receptor (TLR) pathways leading to cytokine release and possible antibody production. (e) Non-specific B cells ($_{ns}$B cells) could be part of the pancreatic immune repertoire and undergo similar activation discussed in (d). As B cells are more prominent in the islet tissue in individuals with young onset diabetes, this scheme of events would be more likely to be operative in those individuals.

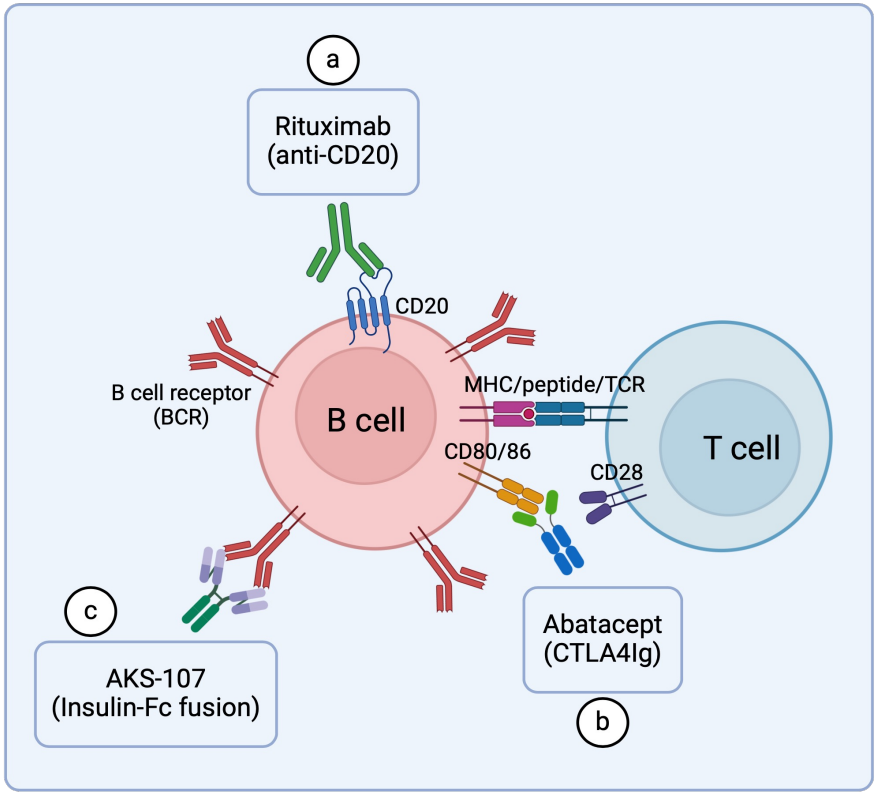

Figure 3. Examples of B cell targeted therapies. A variety of B cell targeted therapies have been used to delay or treat human T1D and autoimmune diabetes in the NOD mouse. These include (a) Rituximab, which targets CD20 expressed on B lymphocytes, (b) Abatacept, which blocks the interaction of CD80/86 and CD28, preventing stimulation of T cells, and (c) antigen-specific therapies, such as AKS-107, which is a fusion protein comprising insulin and the human IgG1 Fc region, that selectively binds to and depletes insulin-reactive B cells.