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REVIEW ARTICLE

Multiple Immune Pathways to Type 1 Diabetes Mellitus: Lessons Learned from Human Clinical Trials and Animal Models of Disease

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ABSTRACT

Type 1 diabetes mellitus results from progressive autoimmune attack of the endocrine pancreas. Immune cells infiltrate the pancreatic islets and focus their attack on beta cells causing loss of insulin production. Progressive insulin loss leads to lifelong insulin replacement therapy and comorbidities. The ultimate clinical goal in type 1 diabetes is to restore and preserve beta cell function thus alleviating the need for exogenous insulin replacement. A secondary goal is to prevent complications that result from chronic inflammation including cardiovascular disease, kidney disease, neuropathy, and retinopathy. These goals are neither exclusive nor interdependent. The best clinical approach will target immune cells, although beta cell replacement in addition to immune targets might lead to a cure. At present, clinical trials have involved antigen specific therapies to attempt tolerance induction through depletion of pathogenic effector cells and/or generation of regulatory T cells; infusion of autologous Tregs to control the pathogenic inflammation; monoclonal antibodies that target total T cells, total B cells, or inflammatory cytokines; small molecule drugs; and targeting T cell co-stimulation. Moreover, newly developed pluripotent beta cell clusters with immune privilege achieved through CRISPR technology appear to restore insulin secretion and avoid immune surveillance. These approaches have not yet achieved the clinical goal of halting or reversing loss of C-peptide, a marker for beta cell function, or sustained long-term reduction of daily blood glucose and insulin requirements. Some therapies like Teplizumab (humanized anti-CD3 monoclonal antibody) have slowed loss of C-peptide and it appears that several study subjects have had long-term positive responses. The totality of clinical trials points to heterogeneity within those individuals labelled as "type 1 diabetes" making a single target approach unlikely to be successful. This review considers recent and current immune modulatory drugs in T1DM clinical trials. While none have yet been fully successful, valuable information about how to better approach this serious disease is emerging. The information from clinical trials further points to the possibility that rather than being a single disease, 'type 1 diabetes' may better be described as a family of diseases where different cellular mechanisms reach the same clinical outcome, loss of insulin production.

Introduction

Type 1 diabetes mellitus (T1DM) is a progressive autoimmune disease and even after decades of scientific pursuit the etiology is still unclear[1]. Likely contributors include both genetic and environmental factors[2]. What has become clear is the complexity of immune dysfunction involving adaptive and innate immune cells that orchestrate a cycle of chronic inflammation leading to beta-cell dysfunction and loss. Immune dysfunction potentially results in comorbidities suffered through the duration of the disease and can predispose to more autoimmune diseases. Early symptoms are non-descript including fatigue, polydipsia, polyuria, and blurry vision, thus diagnosis may not occur for months to years after symptoms onset. Persistence of symptoms prompts glucose tolerance testing or random glucose measurement that can display hyperglycemia prior to or at the onset of disease. Utilizing a genetic approach, incidence should be predictable. A major problem however is that current disease incidence trends drastically exceed expectations. In the United States, physician diagnosed T1DM in subjects aged 0 - 19 had an incidence rate of 1.48 per 1000 in 2001 that increased to 1.93 per 1000 by 2009 in all ethnic groups[3]. Adjusted for completeness of ascertainment, there was a 21.1% increase over 8 years that cannot be explained by genetics alone[3]. While substantial incidence increases are reported in first world countries, the world-wide incidence trend is likewise increasing at an alarming rate of 3 to 4%[4]. Epidemiologic patterns have been broken down by demographic, geographic, biologic, cultural and other factors to learn the natural history of T1DM but have provided little insight on disease causation[5]. There are no satisfactory explanations for the disease incidence increases, but it is suggested that viral infections play a role[5]. While type 1 diabetes has classically been considered a childhood disease, i.e., juvenile diabetes, in 2021 nearly half of all diagnosed new onset T1DM cases are adults (loosely defined as age >20 - 50 years)[6].

In addition to clinical concerns, diabetes results in heavy financial burdens. In 2017, the American Diabetes Association (ADA) estimated that $327 billion was spent on diabetes care with $237 billion accounting for direct medical costs. Current clinical approaches rely on disease management with only one recent therapeutic preventive option on the open market. There are pipeline drugs aimed at modulating the immune contributors in various phases of clinical trials, yet those trials that report data have been lackluster. The need to understand etiology, potential disease contributors, disease pathology, and ultimate risk is paramount. Through animal models of T1DM we have gained an understanding of the disease, but there are limitations to these models and knowledge deficits that have hindered therapeutic drug development for human T1DM.

1.0, PATHOGENESIS OF TYPE 1 DIABETES: HETEROGENEITY OF THE DISEASE.

The pancreatic islet is composed of five different cell types, alpha, beta, delta, gamma and epsilon[7]. Beta cells form a sizable portion of the islet and produce insulin[7]. Alpha cells produce glucagon, PP also called gamma cells produce pancreatic polypeptide, delta cells produce somatostatin and epsilon cells produce ghrelin, the hunger hormone. During

diabetogenesis, peripheral immune cells including T cells, B cells, and innate cells including macrophages and neutrophils, invade the islet establishing insulitis[8]. While the entire islet can become fully infiltrated, beta cells alone are the target of immune-mediated destruction. In mice this process occurs uniformly at 15 - 22 weeks of age[9,10]. In human subjects the amount of time likely takes months to years[11]. Age of onset can range from young, less than 5 years, up to greater than 50 years; the average age of onset is 17 yrs[6,11].

Attempts to find biomarkers led to the discovery of autoantibodies (AAbs) that were found in human subjects and later in NOD mice, the established research model for T1DM[10,12]. This approach was done in other autoimmune diseases as well. In rheumatoid arthritis models, serum from sick mice transferred disease to healthy recipients, proving antibody pathogenesis in that disease[13]. Serum from diabetic mice however did not transfer disease, thus immunoglobulin (Ig) alone was not sufficient for disease onset. To further address the potential contribution of B cells to disease pathogenesis, B cell knockout (KO) mice were generated on the NOD background[14]. Here, insulitis and diabetes developed in only 28% of NOD B cell KO mice. Thus, while B cells contribute to disease, they alone cannot fully account for disease development. Alternately, cellular immunity proved to be the primary driver of T1DM, and this involved both $CD4^+$, $CD8^+$T-cells, and cytokine interleukin-2 (IL-2)[15,16]. Further exploration led to the development of diabetogenic T cell clones in NOD mice. The first described clone, BDC2.5, a $CD4^+$ T cell, was isolated from spleens of diabetic NOD mice; BDC without the need of other cell types was able to transfer T1DM to young, NOD-scid (NOD mice with no T or B cells; severe combined immunodeficiency) recipients[17]. Other $CD4^+$ T helper clones were generated that could successfully transfer T1DM to NOD-scid recipients[18,19]. This breakthrough proved that T cells alone generated under chronic inflammatory conditions, transfer disease.

1.1, T CELL CLONES:

T cells clones, including the early described BDC2.5 express a single T-cell receptor (TCR). Over time the TCR sequences of various clones were determined[17,20]. Even though TCR sequences were found, the recognizable antigen(s) remained unknown. This was later resolved for the BDC2.5 clone. Initially, small peptide mimotopes were developed and eventually the target antigen was identified as chromogranin-A[21]. The early diabetogenic T cell clones were derived from $CD4^+$ helper T cells and those clones alone were successful at diabetes disease transfer. This implied that $CD8^+$ cells were not needed for disease transfer in the NOD mouse model[22]. Further complicating the issue, $CD8^+$ T cell clones from young NOD mice could transfer diabetes rapidly to irradiated NOD recipients[23]. Transfer of a specific $CD8^+$ T clone accelerated diabetes onset in NOD recipients[24]. A peptide mimotope that could elicit proliferation, cytokine secretion, differentiation, and cytotoxicity of a diabetogenic H-2K(d)-restricted CD8(+) T cell specificity (NY8.3) was developed[25]. Stimulation of splenic $CD8^+$ T cells with that mimotope led to preferential expansion of T cells bearing an endogenously derived TCR-alpha chain identical to the one

used by 8.3-TCR-alpha clones[25]. While the results involving $CD4^+$ and $CD8^+$ clones were initially contradictory, the controversy became resolved. Their differential effects highlight the heterogeneity of type 1 diabetes immunology even in the highly genetically restricted NOD mouse model.

1.2, DISEASE TRANSFER WITH PERIPHERAL MONONUCLEAR CELLS:

Early attempts to transfer disease using whole T cell populations, isolated from diabetic NOD spleens, showed that T cells from diabetic donors transferred disease more readily than T cells from pre-diabetic donors[26]. Unfractionated T cells (having both $CD4^+$ and $CD8^+$) from diabetic donors transferred diabetes with 100% of recipients becoming diabetic after 40 weeks[26]. Fractionated $CD4^+$ cells caused diabetes in 87% of recipients while fractionated $CD8^+$ cells did not cause diabetes[26]. A separate study showed that $CD4^+$ splenic T cells isolated from either pre-diabetic or diabetic donor mice transferred diabetes equally well[27]. In that study, purified $CD8^+$ cells were unable to transfer diabetes[27]. If, however, only a small addition of $CD4^+$ T cells were added, disease onset was rapid; suggesting that $CD8^+$ T cells do cause diabetes but require some level of $CD4^+$ help. In these disease transfer models cell transfer numbers were typically 1 x 10^7 or greater and disease kinetics ranged from 4 to 10 weeks in some experiments[27] and up to 40 weeks in others[26].

A unique subset of $CD4^+$ helper T cells called Th40 cells was identified because they express the CD40 receptor that has typically been associated only with antigen presenting cells (APC). Th40 cells produce IFNγ (Th1 cytokine), IL-17 and IL-22 (Th17 cytokines)[28,29,30]. Within the helper T cell category, Th40 cells were defined as $CD3^+$ $CD4^+$ $TCR\alpha\beta^{low}$, $CD40^+$[31]. Th40 cells proved to be pathogenic in the NOD mouse model[31]. In non-autoimmune mouse strains, Th40 cells constitute between 5 - 15% of the helper T cell compartment. In NOD mice, prior to insulitis, Th40 cell numbers in the periphery are low, at 5%, while numbers in pancreatic lymph nodes are as high as 60%[28]. As insulitis expands in pre-diabetic NOD mice a concurrent expansion of Th40 cell numbers is detected in the periphery[31]. Th40 cells migrate to and infiltrate islets[28]. Once in the islet, Th40 cells interact with resident macrophages and dendritic cells to further perpetuate insulitis (cf. Fig. 1). When the BDC2.5 T cell clone was analyzed, it was found to be CD40-positive[30,32]. BDC2.4, a T cell clone generated from the spleen of the same diabetic NOD mice that gave BDC2.5, did not transfer diabetes[33] and was found to be CD40-negative[30]. These findings helped to define CD40 as a pathogenic T cell biomarker in T1DM. If CD40 mediated signals were ablated early, less than 9 weeks of age, in NOD mice, disease onset was prevented, and so if CD40 mediated signals were ablated after 9-weeks of age, disease onset occurred normally[31].

Figure 1

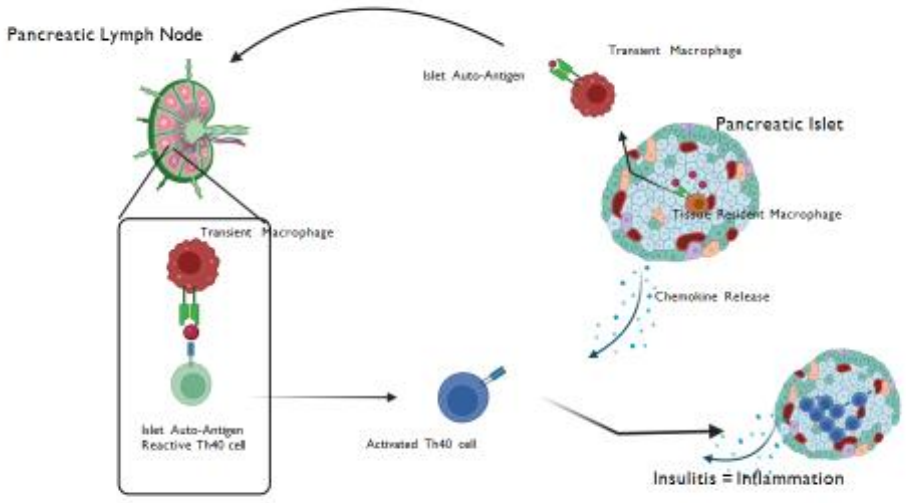

Figure 1: *T cell activation and infiltration to islets*. Beta cell Autoantigens (β-CAA) are generated in the islet. Tissue resident macrophages take up and process the antigens, then migrate to pancreatic lymph nodes. The lymph node has T cells including Th40 cells and when a T cell with a responsive TCR encounters the Macrophage/β-CAA the T cell activates. Chemokine release by the islets attracts activated Th40 cells to infiltrate the islet. During insulitis, insulin production ceases.

Unlike entire splenic T cell preparations, isolated Th40 cells transferred diabetes at 10-times lower (1 x 10^6) cell numbers[31]. Diabetogenic T cell clones needed much larger cell numbers (2 x 10^7) in disease transfer experiments. Of note, Th40 cells represent a varied TCR repertoire. Given the difference in numbers of cells needed for disease transfer between canonical T-cell clones and Th40 cells, a wider TCR repertoire clearly promotes disease more readily than a single antigen repertoire. The success of T cell clones, having a single antigen specificity, in diabetes pathogenesis suggested that a single target antigen for diabetes treatment could be successful. In human trials, this has unfortunately not proven to be the case. Single antigen approaches with insulin peptide or GAD65 (glutamic acid decarboxylase 65 Kd) protein have uniformly failed to prevent T1DM[34]. The fact that each of these differing T cell types are diabetogenic further shows the heterogeneity of human T1DM.

2.0, MOUSE MODELS AND HUMAN DISEASE: NON-OBESE DIABETIC MICE:

Non-obese Diabetic (NOD) mice spontaneously develop diabetes over time in a predictable fashion[20], but there is a female sex bias. In a typical colony, female disease incidence is approximately 80% and male incidence 30% by 40 weeks of age. The autoimmune nature of the disease showed that $CD4^+$ T cells attack islets and establish insulitis[26,27,28]. While the exact nature of beta-cell demise is still debated, several fates exist. These outcomes include beta cell death

through apoptosis, necrosis, or necroptosis. Moreover, the inflammatory microenvironment inhibits glucose-stimulated insulin secretion or injures cellular machinery necessary for insulin production, and both may occur simultaneously[35]. In NOD mice, once 80% to 90% of islets are infiltrated, serum insulin levels serum decreased enough for development of hyperglycemia. In some human T1DM postmortem cases, insulitis is not detected at all and in many cases, insulitis was detected only if examined less than 1 year post diagnosis[36]. Improved techniques over time reported more insulitis, but levels were substantially lower than in NOD mice, ranging between 20 and 30% of human islets[37]. The meaning of the differences in insulitis between mice and humans is not clear.

Further concerns about the differences between mouse models of disease and human T1DM arose from autopsy studies of human subjects who died with or from T1DM. A seminal study published by Foulis and colleagues on 119 pancreata collected from autopsy specimens revealed that only 50% of islets had areas of insulitis, while only 23% of insulin containing islets demonstrated insulitis[38]. Furthermore, the histological appearance of insulitis was vastly different from that seen in NOD mice which typically shows aggressive T cell infiltration into the islet and peri-insulitis prior to disease onset[39]. This observation was reconfirmed by analysis of human pancreas specimens obtained by the Network for Pancreatic Organ Donors with Diabetes (nPOD)[40]. It is noteworthy that two patients in the Foulis cohort had diabetes for less than 2 weeks and one patient had diabetes diagnosed at 18 months. In each case, normal islets without insulitis were found in diabetic human subjects. These children had monogenetic forms of diabetes, for example mutation in the sulfonylurea receptor leading to a "type1-like" form of clinical diabetes.

We propose a new hypothesis that T1DM, rather than being a singular defined disease, is a group of insulin deficient conditions with a common theme of irreversible beta-cell damage. This group of diseases certainly includes "autoimmune" beta cell destruction, but also recognizes new insights into malfunctions of insulin secretory machinery, genetic low beta cell mass, environmental factors that disrupt beta cell function, and beta cell de-differentiation. Mechanisms of disease development have been further proposed once islet infiltrations were phenotypically described. From decades of research, a predicted disease development model has emerged. Damage to beta cells could be caused by oxidative stress from resident macrophages, cell induced damage/ dysfunction from cytokines[41], and cytotoxic T-cells (CD8 bearing cells) that release perforin and granzyme B (Fig. 2)[41]. Some reports suggest that beta cells are damaged by Fas/ FasL interactions (Fig. 2)[41]. Other mechanisms of beta-cell death have been described and several of these discoveries have led to therapeutic human clinical trials in patients with newly diagnosed T1DM including a human anti-IL-1 beta antibody called Canakinumab and human interleukin-1 receptor antagonist called Anakinra, unfortunately without success[42]. According to one strongly supported hypothesis, resident macrophages or dendritic cells take up beta-cell antigens after cellular injury occurs and migrate to pancreatic lymph nodes[43] (cf. Fig. 1). If auto aggressive TCR bearing cells are present, they

may interact with the migrant antigen presenting cells and beta cell auto-antigen. Attempts to therapeutically regulate these disparate diabetes mechanisms must consider the differences. Therefore, a single drug approach that targets a specific mechanism may be beneficial in one cohort of patients but not others. Targeting a pathway that intersects multiple mechanisms or combinational therapies over different patient cohorts will be needed.

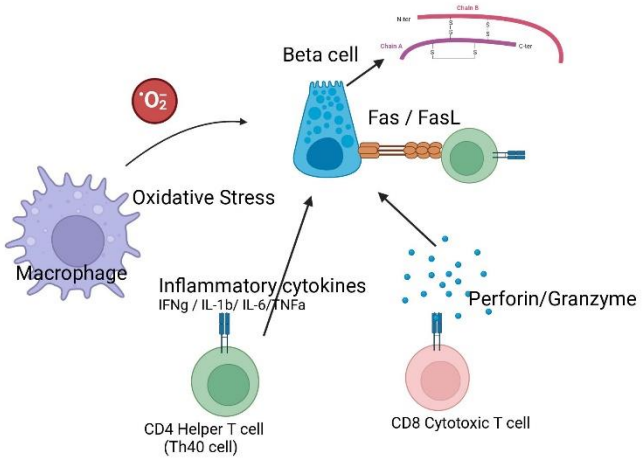

Figure 2: Mechanisms of beta cell death. Beta cells exposed to oxidative stress from tissue resident macrophages; exposed to inflammatory cytokines produced by helper T cells including Th40 cells; exposed to granzyme and perforin from CD8+ T cells; or exposed to Fas L from activated T cells; can undergo cell death.

3.0, OTHER ANIMAL MODELS OF HUMAN TYPE 1 DIABETES:

Animal models have been crucial to the understanding of T1DM pathogenesis. In well controlled laboratory conditions, rodent models of T1DM were developed through cross breeding experiments. For research models, mice have predominated because they are less expensive to keep, easier to manipulate experimentally, and there are more reagents including mouse specific monoclonal antibodies for experimentation and cell phenotyping. Mice have also predominated for genetic manipulation although congenic rat strains have been developed.

3.1, Rat models:

While particularly useful for basic immunology, the NOD model has not been particularly useful for drug development. Alternate models were looked for, and data generated in rat models have correctly predicted the outcome of several human diabetes prevention trials. Notably, the failure of nicotinamide and of low dose parenteral and oral insulin therapies were predicted in rat diabetes models[44]. The best-known rat diabetes model is the Diabetes-prone BioBreeding (DP-BB) rat. An outbred strain spontaneously developed diabetes and was named the BB/Wor rat[45]. Other spontaneous disease models were developed including the LETL, the Komeda

diabetes prone, and the IDDM Lewis (LEW.1AR1-iddm) rat[44,46]. In all rat models, distinctive genetic mutations have been identified[44]. In these models, like in NOD mice, insulitis leading to beta cell damage occurs[47,48]. Unlike NOD mice, the rat models do not have sex bias[44]. BBDP/Wor rats become lymphopenic with a noted reduction in $Art2^+$ cells, a rat Treg cell type[47], suggesting the importance for sustained Tregs in disease prevention. The insulitis seen in the BB/Wor rat was described as more "human-like" in comparison to NOD mice, with no peri-insulitis, varied and lower levels of insulitis, and a Th1 cell predominance[44,48,49]. These models have been well described in detailed review articles[44,46].

3.2 Canine diabetes:

Canine diabetes mellitus is a common endocrine disorder in companion dogs (pets) with a prevalence ranging from 0.26 to 1.33% accounting for ~150,000 to 200,000 diabetic dogs in the U.S. alone[50]. Canine diabetes involves persistent hyperglycemia and insulin deficiency with massive beta cell loss. The symptoms and clinical consequences including comorbidities are much like human diabetes[51]. While canine diabetes is completely insulin dependent, calling it immune-mediated diabetes has been controversial[51,52,53]. Human T1DM etiology involves demonstrable immune cell infiltrations that attack beta cells, but in dogs that etiology seems less clear[54]. It has been reported that autoantibodies to insulin, GAD-65 and/or canine islet antigen 2 are detected in some but not all diabetic dogs[52]. It also has been reported that reduced beta cell numbers and notable insulitis occur in diabetic dogs[50,55]. This finding has led to the term islet hypoplasia in canine diabetes[56]. Dogs are a larger animal model and companion dogs are treated in a veterinary clinical setting and live under non-laboratory conditions. Canine diabetes has been proposed as a valuable model for better understanding diabetes heterogeneity. However, there are specific limitations to drug testing in this model. In fact, for testing in companion animals an Investigational New Animal Drug (INAD) certification must be obtained from the Center for Veterinary Medicine (CVM) of the FDA. While an INAD application is less rigorous than its human counterpart IND, the CVM still needs safety, toxicology, PK and PD studies. This model with its disease heterogeneity could be highly beneficial for future human drug development and testing.

4.0 HUMAN TYPE 1 DIABETES CLINICAL TRIALS:

The primary goal of phase 1 clinical trials is to obtain safety/tolerability data for a new drug but in some cases, a phase 1 trial can generate efficacy data. In addition, a phase 1 trial finds pharmacokinetics (PK, how long does the drug stay in the body) and pharmacodynamics (PD, where does the drug go and where is it metabolized for clearance). Phase 2 trials are directed towards efficacy. In all cases, the FDA needs data reporting. During the clinical trial, the sponsor decides which outcome measures will be reported. In Phase 1 trials, data often include injection site reactions, infusion reactions, blood chemistry panels and complete blood counts (CBC). Severe adverse events (SAE) and adverse events (AE) to the drug are recorded and the range of these vary, changes in leukocytes, infection rates, adverse physiologic responses, symptom

variations etc. While the sponsor defines what constitutes an SAE versus AE, the FDA must approve those definitions.

The primary goal of a T1DM drug is restoration of beta cell function, thus negating the requirement for exogenous insulin. Because internal insulin production generates the release of C- peptide, measuring this analyte in peripheral blood shows beta cell health. Assaying for C- peptide typically involves administering a mixed meal tolerance test (MMTT) or oral glucose tolerance test (OGTT). After the MMTT/OGTT, blood is collected every 15 or 30 minutes for up to 4 hours. A concentration curve is generated, and the Area-Under-Curve (AUC) is calculated. The ideal outcome for a new type 1 diabetes drug would be consistent and sustained increase in C-peptide. The method in which AUC is reported lies with the sponsor. Direct measurement with or without a baseline, or deviation from baseline may be reported. Also, percent changes from baseline or as a direct comparison between drug treated and placebo may be reported. The inclusion of placebo data is at sponsor's discretion. Other important outcomes include daily glucose changes and daily insulin dose changes. Glycated hemoglobin A1c (HbA1c) is considered a marker for disease management and study subjects typically have A1c greater than 7%. Normal A1c is less than 5.7%, prediabetes is from 5.7 - 6.4%, and 6.5% and greater is defined as diabetes.

4.1: Antigen Specific Therapy:

The development of T cell clones, each with a single antigen specificity, suggested that single antigens could be used to tolerize patients. Directed tolerance mechanisms are those that anergize and/or deplete pathogenic effector T cells. Passive tolerance involves generating/expanding natural or adaptive regulatory T cells (Tregs). Understanding how tolerance works originated from tolerance induction to food and external allergens. In those trials, long-term, escalating low-dose administration of an allergen tolerized patients to that allergen[57]. For example, continuous, escalating low-dose exposure to pollen or bee venom over time creates tolerance through complex alterations in T and B cell repertoires that need to delicately balance both effector and regulatory compartments. The concept of tolerance induction has been reviewed recently[57]. If autoimmune contributors behave similarly to allergy mediators, then the antigen specific therapy approach could be beneficial, but will not be a stand-alone therapy. T1DM autoantigens include various forms of insulin, glutamic acid decarboxylase and others described recently[58,59,60,61].

4.1.1 Insulin Specific Therapy:

An early AST approach in T1DM was insulin perhaps because it is produced exclusively by beta cells, and because in NOD mice mutating one amino acid in the insulin $B_{9\text{-}23}$ epitope prevents diabetes[62]. Oral insulin administration was developed based on data in NOD mice. Mice were administered porcine insulin or human B chain insulin orally. If administered earlier than 9 weeks of age, diabetes onset was delayed, but if administered later than 9 weeks diabetes developed normally[63]. Disappointingly, insulin administered orally to pre-diabetic human subjects has not yet been successful (*ClinicalTrials.gov* and references[34,60]). The most recent trial, starting in 2015 and posting

data in 2020, administered oral insulin and followed changes in levels of GAD autoantibodies but no significant changes were detected. In 2018 an adjuvanted antigen (insulin B chain) trial began, although no results have been reported. Trials involving subcutaneous injections and nasal administration have not been successful; insulin AST trials have been reviewed previously[34].

4.1.2 Glutamic Acid Decarboxylase specific therapy:

Glutamic acid decarboxylase (GAD) is an enzyme that catalyzes the conversion of glutamate, an excitatory neurotransmitter, to gamma-aminobutyric acid (GABA) an inhibitory neurotransmitter[65]. GAD was identified as a diabetes antigen in T1DM in 1987[66] and since then has been a topic of extensive research. It is not understood why a non-beta cell specific antigen (GAD) would become a major part of the humoral immune response in T1DM. Moreover, cellular immunity to GAD65 auto-antigen has been debated too. In one study, the frequency of positive $CD4^+$ T cells in subjects with T1DM was higher when compared to non-diabetic controls, but this was not statistically significant[67]. Further clinical studies have not showed a significant benefit in T1DM prevention with GAD antigen[68,69]. At least 2 GAD trials including a direct injection of protein in lymph nodes were conducted with no significant improvements in C-peptide AUC, insulin reduction, reduced glucose *etc.*, being reported (reference[69] and *ClinicalTrials.gov).*

4.1.3 Blocking Human Leukocyte Antigens:

Antigens and autoantigens are presented by professional antigen presenting cells (APC) including B cells, macrophages, and dendritic cells through classic MHC class II (HLA-DR, DP, DQ and others) molecules[70,71]. High risk Human leukocyte antigen (HLA) alleles for T1DM have been determined and blocking HLA antigen presentation is a potential option for controlling autoimmune responses. The drug methyldopa is a hypertension drug that binds HLA-DQ8 molecules on antigen presenting cells[72]. Because HLA-DQ8 is associated with T1DM development, it was postulated that engaging DQ8 with methyldopa would potentially thwart autoreactive TCR bearing T cells and thereby delay or prevent type 1 diabetes. A clinical trial was conducted and after 12 weeks of treatment, an ex vivo reduction in T cell response to DQ8 was reported in treated subjects. T cells from those subjects were isolated and exposed to a chimeric DQ8 source; there was no in vivo efficacy study. A slight improvement in C-peptide AUC was noted comparing pre-treatment levels to post treatment levels, and reduction in HbA1c was reported. However, there was no comparison to placebo and thus these outcomes cannot be interpreted appropriately. A phase 1b multiple ascending dose trial (IMT-002) was completed in August 2021, although data has not yet been posted.

Summary: Single antigen specific therapy approaches have not proven successful thus far. A likely problem is that a multiple antigen approach will be necessary. T cells isolated from islets of mice or humans have a varied TCR repertoire. It also is probable that not all T1DM associated autoantigens have been discovered and moreover a multiple autoantigen trial has not yet been approved. While DQ8 clearly is highly associated with

T1DM, it is not the only high-risk HLA allele detected in T1DM. It is possible that other HLA molecules present autoantigens and therefore circumvent the blocking of a single HLA molecule. While T cell involvement in insulitis is clear, targeting T cells for tolerance may not be sufficient to control diabetes. A broader approach that includes adaptive and innate immune cells may be needed.

4.2 Regulatory T cells.

Suppressor T cells were first suggested by Steve Miller and Henry Claman[73]. The idea of a specific suppressive cell type remained controversial until Foxp3$^+$, CD25$^+$, CD4$^+$ T cells were described[74,75]. Extensive work over the years led to better understanding of suppressor mechanisms and ultimately regulatory T cells (Tregs) were defined. Tregs were described as CD4$^+$ cells that express the high affinity IL-2 receptor alpha chain CD25 and the transcription factor Foxp3[75]. Foxp3 functions to down regulate multiple inflammatory genes while up regulating non-inflammatory/regulatory genes[76,77]. Tregs that develop in the thymus are considered natural Tregs (nTregs), but Tregs can be induced (iTreg) in the peripheral lymph nodes. Both function through bystander mechanisms. Tregs express TGFβ and interact through the TGF receptors I or II on target cells[78] or can secrete regulatory cytokines including IL-4, IL-10, IL-12, type 1 interferons, and TGFβ to affect bystander cells. Most of the work defining Tregs was done in mice. In human subjects, defining Tregs has been more difficult. Low expression of the IL-7 receptor, CD127, on CD4$^+$CD25$^+$Foxp3$^+$ cells was proposed to further differentiate human Tregs[79,80,81,82]. A phase I safety and dose finding study was performed using polyclonal CD4$^+$CD127^{lo}CD25$^+$ Tregs in subjects with established type 1 diabetes[81].

4.2.1 Polyclonal Regulatory T Cells:

Early experiments in allergic responses compared low dose single antigen administration with high antigen dose[83]. Low dose antigen induced immune activation followed by tolerance, while high dose antigen induced tolerance alone. However, while both low and high dose antigen provided tolerance, isolation of immune cells from the host only transferred tolerance with the low dose approach[84]. It was eventually found that low dose antigen created Tregs, and high dose resulted in pathogenic T effector cell depletion. De novo induction of Tregs requires a strong agonistic ligand for the TCR under sub-immunogenic conditions, i.e., low antigen dose that targets T cells with high affinity T cell receptors[85]. Higher antigen doses activate the P13k-AKT-mTOR pathway that inhibits Treg development[86]. Initial attempts to capitalize on Tregs therapeutically focused on an autologous polyclonal approach[81].

Thus far, there are six clinical trials listed for use of polyclonal Tregs in T1DM and two of those have posted results. In one study, polyclonal T regs (CD4$^+$CD25$^+$CD127$^{low/-}$) were extracted from subjects, expanded by treating with CD3 and CD28 on immobilized magnetic beads in the presence of recombinant IL-2 and autologously infused. Initially, new onset T1DM, young adults were selected, and subjects were watched over 2 years. Cohorts of five subjects were treated with four ascending concentrations of Tregs, 0.05 x 10^8, 0.4 x 10^8, 3.2 x 10^8, and 26 x 10^8. In all cohorts mild and moderate adverse

events (AE) were recorded with the least number in cohort (0.05 x 10^8). Six grade 3 Severe Adverse Events (SAE) were recorded in the 3rd cohort and two grade 4 life-threatening adverse events were noted in subjects at the highest dose (*ClinicalTrials.gov*). Efficacy data was obtained as per study protocol. C-peptide levels were measured at 26 and 52 weeks after treatment began (cf. Fig. 3). The lower concentrations of Tregs stabilized C-peptide AUC (cf. Fig. 3) over the trial while higher concentrations showed substantial loss. An improvement in daily insulin use was seen with the 0.4 x 10^8 dose of Tregs. In the second clinical trial with reported results there were 110 subjects divided into high dose, low dose, and placebo cohorts. At 52 and 104 weeks there were no improvements in C-peptide loss, HbA1c levels increased in all cohorts and insulin use increased in all cohorts. The data were taken directly from the clinical trial report (*ClinicalTrials.gov*).

Figure 3

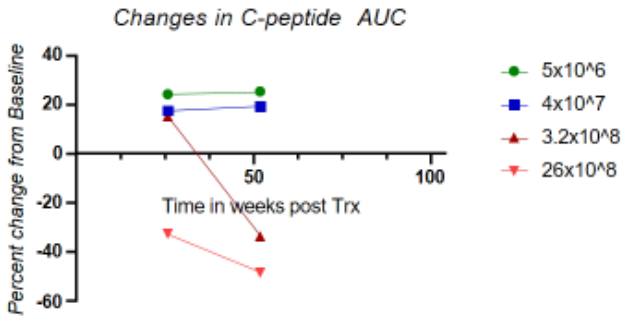

Figure 3: Polyclonal Tregs administered during clinical trial and effect on C-peptide area-under-curve. The curves were generated from the posted tabular data at the ClinicalTrials.gov website; the application identifier: NCT 01210664, "*T1DM Immunotherapy using CD4+CD127lo-/CD25+ Polyclonal Tregs (Treg)*". First Posted Sept 28, 2010; Results posted July 11, 2018. Sponsor: *University of California San Francisco*; Collaborators: *JDRF and National Institute of Allergy and Infectious Diseases*. Over a one-year period, the lower Treg doses, 0.05 x 10^8 and 0.4 x 10^8 preserved c – peptide percent change from baseline; the 3.2 x 10^8 Treg dose preserved c – peptide levels through 26 weeks with a sharp decline at 52 weeks. The 26 x 10^8 Treg dose saw sharp decrease at 26 weeks that progressed through 52 weeks.

4.2.2 *Low dose Interleukin-2:*

Tregs are interleukin-2 (IL-2) dependent[87,88] and low dose IL-2 promotes Treg development in vivo[89]. Treg expansion in vivo using recombinant IL-2 was tried. A clinical trial using recombinant interleukin-2 (rIL-2) was begun in 2014 and was completed in May 2016. In earlier studies, Tregs were well tolerated but undetectable 3 months later. With added administration of low dose IL-2 trying to sustain Treg numbers for longer, Tregs were expanded[90]. Unexpectedly

activated NK cells, mucosal associated invariant T cells, and clonal $CD8^+$ cells were also increased. In all cases, Tregs alone, or Tregs + low dose IL-2, there were still decreases in C-peptide, increases in HbA1c, and insulin use over 104 weeks[90].

4.2.3 Antigen Specific Regulatory T Cells:
At present there are no antigen specific Treg clinical trials listed for T1DM. A drug pipeline includes the development of Chimeric-Antigen-Receptor T cells (CAR T cells) with antigen specificity for beta cell autoantigens[91]. CAR-T cells are engineered to express a specific T cell receptor that exclusively targets the desired antigen. The rationale is that high affinity Tregs, in this case targeting known T1DM associated antigens, would be more effective in supplying a defense for the beta cell. CAR-T cells show promising clinical outcomes in cancer by targeting and eliminating tumor cells through enhancing tumor targeting effector T cells. The CAR-Treg translation to autoimmune disease may be more difficult. Currently there is no mechanism to eliminate CAR T cells once they have supplied the necessary defense. One could postulate an effective cell suicide mechanism to eliminate CAR-T reg cells when functions are completed, but in autoimmune disease their persistence may be beneficial, potentially providing long-term bystander tolerance. This "yin-yang" of immune system balance is a confounding factor that needs scientific clarity. Moreover, the single antigen approach described for AST raises concern that multiple antigen specific Tregs would be necessary to control autoimmunity.

Summary: The ascending Treg dose trial was informative and suggests that a too drastic disruption in Treg to non-Treg balance is problematic. Increasing Treg numbers without affecting pathogenic effector T cell numbers did not reestablish homeostasis. When it was found that polyclonal Tregs lasted only 3 months, a low dose IL-2 approach was performed. That approach sustained Tregs longer, but also significantly increased NK and $CD8^+$ cells causing concerns[90]. Another concern is that Tregs can convert from regulatory to effector status. In murine studies, Tregs develop stochastically from precursor cells that are either $CD25^{low}$ or $Foxp3^{low}$ and carry self-antigen reactive TCR molecules[92,93]. During natural development, CD25 and Foxp3 expression on the nascent Treg increases. Foxp3 expansion coincides with a pro-apoptotic T cell phenotype. When Foxp3 levels subside, the cells are less susceptible to apoptosis thus increasing the risk of auto-aggressiveness[93]. A further complication is that inflammatory conditions reduce CD25 and Foxp3 levels on Tregs[94,95]. These cells have been termed "exTreg", which produce both IL-17 and IFNγ, inflammatory cytokines[92,93]. Thus, Tregs in a highly inflammatory milieu have divergent fates: apoptosis or loss of Foxp3 expression ("ex-Treg"). As said, the loss of Foxp3 leads to increased production of IL-17 and IFNγ further contributing to inflammatory conditions. CD40 engagement was shown to reduce Foxp3 levels that results in increased numbers of pathogenic effector cells and those cells became highly pathogenic in a diabetes model[94]. The tremendous efforts over decades to develop a clear understanding of Tregs has become murkier. Most of the work has been done in mice and understanding, even defining human Tregs, has been more

difficult. Present understanding is that human Tregs are highly heterogenous. Treg persistence could be beneficial so long as they keep regulatory status.

Another concern is that rather than dysfunction in Tregs, pathogenic effector cells in T1DM are dysfunctional compared to regulation[96]. It was shown in a murine model that pathogenic effector T cells escape typical regulatory mechanisms including directed exposure to TGF, IL-10 and CTLA-4[97]. Furthermore, dysregulation of effector function was dependent upon CD40 and CD40 is a major player in driving chronic inflammation[98]. If the concern of pathogenic effector cell dysregulation in human disease holds, then the Treg option whether polyclonal or AST directed has limited success potential. Much work must be done to better understand Tregs and their potential functions.

4.3 Targeting Cytokines:

Cytokines are produced by both adaptive and innate immune cells. Directly upstream of multiple inflammatory cytokines is the CD40-CD154 pathway. CD154 is expressed on activated T cells with maximal expression occurring at 12-18 hours post activation[98], on platelets[99], on APC[100], and on some tissue specific cell types including astrocytes[101] in the central nervous system. CD40 is expressed on APC including B cells and macrophages that produce inflammatory cytokines and is expressed on a subset of effector T cells[102]. During an infection, antigen is processed and presented by innate immune cells that activate T cells. T cell activation leads to localized increases in CD154[103,104]. Interferon-gamma (IFNγ) production increases CD40 expression[97,104]. In a CD154 rich milieu, CD40 expressing cells produce inflammatory cytokines. As antigen becomes less available, CD154 concentration recedes, and CD40-mediated cytokines are reduced[105]. CD40 mediated pro-inflammatory cytokines include IL-1β, IL-6, IL-12, IL-17A, IL-18, IL-21, IL-22, IL-23, and TNFα, that have been detected at elevated levels in serum/plasma of T1DM subjects well past diagnosis. Many of these cytokines are found at the lesion of diabetic islets in test models. Clinical trials using monoclonal antibody approaches in T1DM have been undertaken to try to modulate pathogenesis derived from specific inflammatory cytokines.

Canakinumab/IL-1β: Conducted 2009 – 2020 with no difference in C-peptide AUC over 12 months. SAE and AE were elevated above placebo.

Tocilizumab/IL-6 receptor blocker: Conducted 2014 – 2020 with no improvement or slowing of C-peptide loss over 2 years.

Daclizumab/CD25 (high affinity IL-2 receptor): Conducted 2004-2020. Daclizumab in combination with mycophenolate showed no difference in C-peptide AUC over 12 months in subjects with new onset T1DM.

Secukinumab/IL-17A: Conducted 2014 – 2016 with no data; study stopped by sponsor.

Ustekinumab/IL-23 Conducted 2017 – 2017: No data reported.

Efalizumab/CD11a: Proposed in 2008 and withdrawn in 2014: No data posted.

Etanercept/TNFα: Conducted 2015 -2021 in Sweden. Dosing was daily by subcutaneous injection once per week for 90 days; the doses included Vitamin D. There was a measurable decrease in GAD 65 antibody but no change

in C-peptide or other T1DM clinical parameters. The drug currently is being evaluated primarily in islet transplantation.

Golimumab/TNFα: A phase 1b posted in 2017: No data posted.

Summary: Targeting individual cytokines has yet to be a practical therapy for T1DM. As major players in driving inflammation the approach is sound. Multiple inflammatory cytokines are upregulated during diabetes development and throughout the duration of the disease[106]. A likely issue is redundancy of action of inflammatory cytokines, thus targeting IL-1β without targeting TNFα, IL-17A, IL-23 or IL-6 etc., may be insufficient. There also have been unintended consequences from targeting of individual cytokines. Etanercept a monoclonal antibody that targets TNFα for example increases tumor development risk. A more ideal strategy would be to regulate multiple inflammatory cytokines without eliminating any single cytokine. In other words, reestablishing homeostasis is the key.

4.4 Targeting Effector Cells:

Monoclonal antibodies (mAbs) as therapeutic agents have advanced considerably. Monoclonals recognize a cell specific molecule, bind to it, and target the cell for antibody mediated cell death. Once the antibody attaches, complement binds to the Fc part of the antibody and the cell is deleted. An alternate mechanism of mAb action is to create molecular interference disrupting receptor-ligand interactions, which does not necessarily involve cell death. For autoimmunity, the ideal target is an inflammatory pathway molecule. A logical approach was to target T cells or B cells in T1DM. Initially subjects with established disease were recruited to clinical trials but because considerable damage to the islet occurs prior to clinical diagnosis, new onset T1DM subjects became ideal clinical trial candidates. Minimizing immune damage as early as possible would seem to be important. Recent trials have been able to identify and target 'at-risk' subjects for therapeutic intervention trials as well.

4.4.1 Alefacept, anti-CD2:

CD2 is a cell adhesion molecule found on the surface of T cells and natural killer (NKT) T cells; it interacts with LFA-3 and acts as a T cell costimulatory molecule[107]. In 2009 a phase 2 trial enrolled 49 T1DM participants. Subjects received weekly intramuscular (IM) injections for 12 weeks, followed by a 12 week off period and then a second 12-week round of injections[108]. The outcome was modestly positive. Drug recipients saw a measurable increase in C-peptide following a mixed meal tolerance test (MMTT) at 52 weeks that declined by 104 weeks (Fig. 4). Insulin use trended toward lower in drug recipients compared to placebo, but the difference did not achieve statistical significance. One SAE occurred and multiple AE's including leukopenia, lymphadenopathy, and lymphopenia were reported. Common symptoms included nausea, vomiting and diarrhea consistent with cytokine release syndrome (CRS).

Summary: Overall, increased infections indicative of immune suppression was reported. The trial was stopped because the drug manufacturer discontinued production. The approach of overall T cell targeting suggested improvements; however, this approach was insufficient.

Figure 4

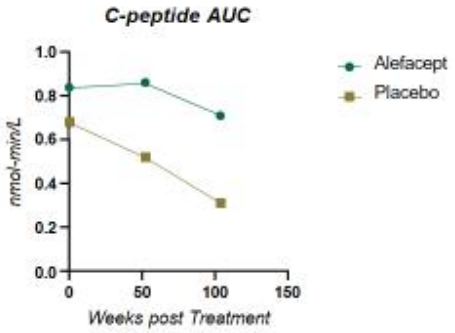

Figure 4: Alefacept Effects on C - peptide. The curves were generated from reported tabular data at ClinicalTrials.gov website; the application identifier: NCT00965458 *"Inducing Remission in Type 1 Diabetes with Alefacept (T1DMAL)"*. First posted: August 25, 2009; Results posted: January 7, 2015. Sponsor: *National Institute of Allergy and Infectious Disease;* Collaborator: *Immune Tolerance Network; Juvenile Diabetes Research Foundation; National Institute of Diabetes and Digestive and Kidney Diseases.* Study Terminated July 6, 2017.The baseline level was set to zero and at 3, 6, 12, 18 and 24 months, combined changes from baseline were reported in tabular form, this was converted here to graph curves. There were noted differences in c – peptide AUC between placebo and drug at baseline. Both curves trended down; Alafacept treated subjects had stable c – peptide at 52 weeks that decreased by 102 weeks. At each time point Alefacept treated subjects were significantly higher than placebo.

4.4.2 Rituximab/Ocrelizumab, anti-CD20:

CD20 is a calcium channel originally described on B cell subsets excluding pro-B and plasma cells[109]. It was later discovered that a subset of $CD3^+$ T cells also express CD20[110,111,112] and Th40 cells express CD20[113]. Rituximab was developed as a human/mouse chimeric monoclonal antibody to treat chronic lymphocytic leukemia, non-Hodgkin's lymphoma, and mantle cell leukemia[114]. A pathophysiologic role for B cells in diabetes was suggested when it was shown that genetic or physical depletion of B cells in NOD mice arrested diabetes development at pre-insulitis[115]. Later work showed that anti-CD20 treatment in NOD mice reversed established hyperglycemia in one third of treated mice[116]. A clinical trial to evaluate Rituximab in T1DM subjects was sponsored by the National Institute of Diabetes, and Digestive and Kidney diseases (NIDDK) looking at the effects over a one-year period. When compared directly to placebo, Rituximab slowed C-peptide loss as analyzed in a mixed meal tolerance test; after 1 year; C-peptide levels were 0.580 pmol/ml blood compared to placebo at 0.429 pmol/ml (Data taken from the Clinical Trial report, *ClinicalTrials.gov*). The rate of decline was similar between drug and placebo for 8.2 months but then shifted to favor drug treated subjects; however, this improvement disappeared at 30 months[117]. There were severe and adverse events including lymphopenia, and cardiac disorders in twelve out of 57 recipients. Common complaints were nausea, diarrhea, and

vomiting, all of which were attributed to CRS[118,119]. Another study was planned for 2019 but enrollment was suspended.

Summary: Rituximab recipients showed a decreased antibody response to neoantigens and significantly lower titers after recall immunization with diphtheria and tetanus toxoid[120]. As B cell numbers recovered, immune responses returned toward normal[121]. The original intent was that peripheral B cell depletion would eliminate autoreactive B cells, however, autoreactive B cells returned to pre-treatment levels with removal of drug[121]. The consensus was that while Rituximab treatment showed temporary improvement in C-peptide loss, after 30 months that protection disappeared. Thus, *"Rituximab delays the fall in C-peptide but does not appear to fundamentally alter the underlying pathophysiology of the disease"*[122]. Unlike NOD mice, human B cell depletion did not alter disease pathophysiology.

4.4.3 Abatacept/CTLA-4-Ig:

T cells receive activation signal one through T cell receptor (TCR) interaction with an antigen and MHC Class II molecule (major histocompatibility complex also called human leukocyte antigen HLA on human cells) on antigen presenting cells[123]. Activation to cytokine production requires a second costimulatory signal. The first well described T cell costimulatory molecule was CD28 along with its family members CTLA-4 and ICOS[124]. Other T cell costimulatory molecules include CD2 and CD40[107,125,126,127]. The ligands for CD28, ICOS and CTLA-4 were identified as B7-1 (CD80), B7-2 (CD86), B7h (CD275), PD-L1 (CD274), PD-L2 (CD273), B7-H3 (CD276), and B7x (B7-H4 or B7S1)[128]. Abatacept, rather than being a monoclonal antibody, is the CTLA-4 protein engineered onto an immunoglobulin (Ig) backbone, creating CTLA4-Ig[129]. Abatacept was designed for use in juvenile idiopathic arthritis, psoriatic arthritis, and rheumatoid arthritis. The approach was evaluated in a mouse diabetes model using the BDC2.5.NOD TCR transgenic mouse strain where CTLA4-Ig prevented diabetes onset[130].

An Abatacept human clinical trial for T1DM with sponsors NIDDK and collaborators including NIAID (National Institute of Allergy and Infectious Diseases), NICHD (National Institute of Childhood and Hereditary Diseases), JDRF (Juvenile Diabetes Research Foundation) and ADA (American Diabetes Association) was started in February 2008 and completed in May 2012. The trial involved 112 participants in a randomized triple blind (sponsor, test subjects, principal investigator) study. Doses of 10 mg/kg were given every other week for the first two doses then every 28 days for the next twenty-seven doses (a 2-year dosing strategy). The most common adverse event was pulmonary infections. Another trial from NIDDK was proposed but suspended by sponsor (NIDDK) in 2021. The data from the trial were reported in *The Lancet*[131].

Abatacept treatment improved C-peptide levels over placebo when compared to baseline. Importantly, both placebo and Abatacept showed a drop in C-peptide at 3 months, and both maintained a downward trend; placebo had a sharper negative slope through 6 months compared to Abatacept (also a negative slope) with both continuing to decline through the 2-year study period[132]. If difference from baseline is considered, placebo saw a sharp drop at 3 months with a recovery at 6 months that continued to uptrend through

18 months followed by a decrease at 24 months. Abatacept treated subjects also saw a drop at 3 months, but less severe than placebo. From 3 to 6 months C-peptide levels increased but decreased again at 12 months. At 18 months, levels increased slightly then decreased again at 24 months. At 24 months, the C-peptide level in subjects treated with Abatacept was higher than placebo, but still had decreased significantly from baseline. Insulin use and HbA1c levels were improved in Abatacept recipients compared to placebo, but both trended upwards[131], which is the opposite of desired effect. Abatacept treated subjects saw a decline in central memory $CD4^+$ helper T cells, with no effect on $CD8^+$ cells[132]. Studies show that a decrease in $CD4^+$ central memory cells positively correlate with slower C-peptide decline[133].

Summary: Abatacept treatment improved clinical outcomes initially when compared directly to placebo treated subjects; however, this improved status was temporary. Over a 2-year period C-peptide levels steadily trended downward while insulin use and HbA1c levels trended upwards. The conclusion is that Abatacept slows but does not cease or reverse T1DM progression. By interacting with the B7 molecules, Abatacept necessarily ablates CD28 stimulation, a mechanism for IL-2 induction. When CD28 knockout mice were bred onto the NOD background, those mice developed rapid extensive beta-cell destruction and diabetes[134]. $CD28^{-/-}$ or $B7^{-/-}$ mice experience a dramatic reduction in Tregs[134,135]. Also, because pathogenic effector cell regulation can occur through CTLA-4 expression[97,102] on the effector cell itself, blocking the B7s reduces pathogenic effector cell self-regulation.

4.4.4 Teplizumab, anti-CD3:

CD3 is the signal transduction component for the TCR complex associated with both $CD4^+$ T helper cells and $CD8^+$ cytotoxic T cells. When the TCRαβ is engaged, the strength of that signal is conveyed through the CD3 complex. CD3 is composed of two epsilon (ε) chains, one that associates with a delta (δ) chain and the other that associates with a gamma (γ) chain, and two zeta (ζ) chains. Epsilon, delta, and gamma chains have extracellular motifs, while the zeta chains are intracellular only. The delta and gamma chains have one intracellular signaling motif each and the zeta chains have three signal transduction motifs each[136]. Antibodies to the human epsilon chain were generated in mice. Mechanism of action studies showed that the anti-CD3ε antibody inhibited cytotoxic activity of T cells[137]. The antibody was quickly moved to clinical trials for kidney and other organ transplants[138]. The anti-CD3ε was further assessed in multiple sclerosis[139]. In those early trials, life-threatening cytokine release syndrome were reported[140]. To address these clinical concerns, a mouse anti-CD3ε antibody was developed, and those studies showed that the Fc receptor non-specifically bound to monocytes or macrophages that contributed to the CRS. CRS could be prevented by mutating sections of the Fc receptors[141]. This improved antibody was assessed in the NOD autoimmune disease models. Neonatal injection of the modified anti-CD3ε prevented diabetes onset[142]. Later experiments showed that low dose administration of anti-CD3ε to new onset diabetic NOD mice induced diabetes remission[143].

These modifications were done to the human anti-CD3ε but added concern that the murine

origin of anti-(human) CD3ε would lead to anti-antibody responses in human subjects led to the creation of a chimeric antibody. The F(ab')2 region of anti-CD3ε was genetically grafted onto a human Fc backbone[144]. Because of the serious complication issues previously seen, and because the antibody targets overall T cells, concerns about dosing remained. Clinical trials were set up to prove efficacious and safe doses. A clinical trial was begun in 2005 and completed in 2017 using T1DM subjects. The study was a 14-day course of ascending doses beginning at 51 ug/m^2 increasing up to 826 ug/m^2 at day fourteen then repeating the 14-day course 1 year later. At study termination there were ten severe adverse events out of fifty-two participants in the drug cohort and one out of twenty-five participants in the placebo cohort. There were 52/52 adverse events in drug and 23/25 AE in placebo. When all participants were evaluated, there was a distinct difference between placebo and drug cohorts in terms of C-peptide AUC[145]. In drug treated subjects at 6 months there was little decline in C-peptide compared to placebo. From 6 through 24 months drug treated subjects maintained increased C-peptide levels compared to placebo; however, the slope of the lines was the same and showed downward trajectory. HbA1c levels and daily insulin use were lower, but not yet significant[145]. An interesting finding was that the Teplizumab treated group could be sorted into 'responders' and 'non-responders.' Examining C-peptide, the "non-responder" designates were slightly but not significantly better than placebo, while the "responder" group was significantly higher than placebo and non-responders throughout 24 months[146]. At 18 to 24 months post treatment a downward trend began. Longitudinal study, up to 7 years, reported that in the "responder" group C-peptide loss remained reduced compared to placebo and non-responders[146]. This has been the most promising T1DM drug outcome to date.

In 2009 an intervention study was performed in designated "pre-T1DM" subjects. Seventy-six autoantibody positive, pre-T1DM, subjects aged 8 - 45 were recruited and administered the 14-day consecutive dosing regimen and monitored for diabetes onset. The rate of diabetes per 100-participant-years in drug treated was forty-three in drug treated subjects compared to seventy-two in the placebo group *(ClinicalTrials.gov)*. This study showed enough potential that Teplizumab received FDA approval in 2023 under the brand name "Tzield". There were severe adverse and adverse events with the majority associated with immune suppression related.

4.4.5 Otelixizumab: Other versions of anti-CD3ε were created including otelixizumab, a chimeric anti-CD3ε antibody that was altered to remove glycosylation sites in the Fc domain with the intention of limiting ability of the antibody to bind complement or Fc receptors[147]. The heavy chains are humanized γ1 from rat and the light chains are chimeric human/rat λ. Like Teplizumab, Otelixizumab was administered as an infusion, over 8 or 14 days and in some trials the fourteen consecutive day dose was repeated a year later. The trial in 2007 was stopped due to severe adverse events and several adverse events. This occurred because of a misstep in the protocol that neglected to include a dosing filter and allowed much too high dosing. From 2010 through 2017 a Phase 3

DEFEND (Durable-response therapy Evaluation for Early onset or New onset type 1 Diabetes; Sponsors: GlaxoSmithKline and Juvenile Diabetes Research Foundation) trial involving 179 participants receiving an 8-day dose schedule was performed. The trial was stopped however, due to ineffective dosing as per ClinicalTrials.gov statement from sponsor. This study was prior to the dosing standards study. Given the concerns around efficacious dosing, a multi-center dose ascending trial was performed in thirty subjects[148]. Cohort one was placebo receiving saline solution by IV infusion. Cohort two received 1.5 mg Otelixizumab infused over 30 mins for six consecutive days totaling 9 mg. The same strategy for cohort three except 3 mg daily totaling 18 mg and cohort four received 4.5 mg daily totaling 27 mg. Data were reported after 24 months. When C-peptide AUC was measured the best outcome was in cohort two, the 9 mg group. New onset T1DM subjects were used and all cohorts including placebo saw an increase in C-peptide at 3 months post treatment; Cohort 1 had the highest increase in C-peptide (Fig. 5). Cohort 2 (9 mg), delayed return to baseline levels through 18 months but fell below baseline at 24 months (Fig. 5). The fastest loss in C-peptide did not occur in the placebo group but occurred in the 18 mg treatment group. Cohort 4 (27 mg) performed second best, but subjects experienced a greater number of severe adverse events including life-threatening events. Data was taken from the clinical trial report (*ClinicalTrials.gov*). Using a computerized pharmacokinetic and pharmacodynamic modeling analysis that simulates interplay between drug administration, molecular target engagement and down modulation, maximum target engagement was determined to be 18 and 27 mg of otelixizumab[149]. As of 2022 more clinical trials are planned or recruiting. The teplizumab and otelixizumab data proved safer dosing levels with possible good outcomes.

Figure 5

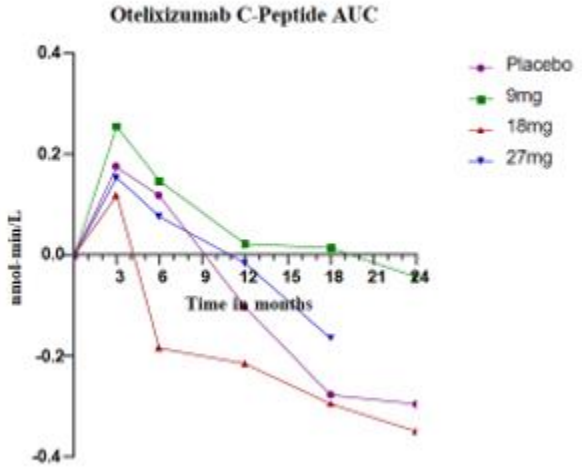

Figure 5: Otelixizumab Effects on C - peptide. The curves were generated from reported tabular data at ClinicalTrials.gov website; the application identifier: NCT02000817 *"Investigation of Otelixizumab in New Onset Type 1 Diabetes Mellitus Patients"*. First posted: December 4, 2013; Results posted: June 24, 2019. Sponsor: *GlaxoSmithKline* Collaborator: *Parexel*. The baseline level was set to zero and at 3, 6, 12, 18 and 24 months, combined changes from baseline were reported in tabular form, this was converted here to graph curves. At 3 months all cohorts (results averaged by the Sponsor), including placebo saw increases in c – peptide AUC, the 9 mg Otelixizumab recipients saw the greatest increase. All cohorts at 6 months saw decline, the 18 mg Otelixizumab cohort was the only cohort to drop below baseline at 6 months. In each of the treatment cohorts a steady decline in c-peptide was seen. At 12 and 18 months the 9 mg cohort was the only cohort above baseline and at 24 months all cohorts were below baseline.

Summary: In clinical trials thus far, for both teplizumab and otelixizumab adverse and severe adverse events included degrees of immune suppression and resulting complications. High doses caused life-threatening events and low doses were ineffective. Achieving a sweet-spot dose is essential for therapeutic efficacy and thus dosing standard trials were necessary. Teplizumab has achieved this standard leading to FDA approval while Otelizumab has not. The potential for slowing loss of C-peptide with Teplizumab through 7 years is promising and is the first successful immunomodulatory agent for new onset human T1DM.

4.4 Beta Cell Replacement with Immune Privilege:

Islet transplantation became an exciting new possibility for late stage T1DM in 2000 with the publication of data by Shapiro and Lackey showing insulin free survival for up to 15 months in a small Canadian patient cohort using a glucocorticoid-free immunosuppressive regimen[150]. After two decades, islet transplantation did not prove to be as robust as initially hoped with 8% insulin-independence at 20 years in a larger cohort of 201 T1DM subjects[151]. In addition to a relative lack of viable pancreatic islets for transplantation, human islet transplantation requires significant immunosuppression. However, these ground-breaking studies opened the possibility of transplanting beta-cells (with unlimited supply) to reverse T1DM. Indeed, Shapiro and colleagues in collaboration with Viacyte have embarked on clinical trials using human pluripotent stem cells (PEC-01) which have ability to differentiate in vivo to insulin producing cell clusters but still require immunosuppression [Clinicaltrials.gov: NCT03163511]. Encouraging preliminary data has already been published showing engraftment of this pluripotent stem-cell derived pancreatic endodermal cell mass [152,153]. The goal of unlimited islet cells for transplantation is not quite ready for widespread application, but this research is moving forward quickly.

In addition, beyond traditional encapsulation methods to overcome immune rejection of transplanted pluripotent islet cells, new CRISPR-Cas 9 (gene editing) tools have been deployed to remove or add critical cell surface molecules that attract T cells, NK cells, and APCs. Indeed, stem cell derived islets have been engineered to eliminate Class I and Class II MHC molecules which attract cytotoxic T cells and effector T cells, respectively. Moreover, overexpression of PD-L1 further inhibits T cell responses. These exciting research endeavors open new avenues for T1DM subjects that might yield cell transplantation without immune suppression[154].

5.0. Conclusions:

The approaches to develop therapeutics in T1DM have focused on immune modulation, given the autoimmune nature of the disease. Cell contributors to pathogenesis were discovered using mouse and rat disease models. Those models each showed distinct advantages and disadvantages. Human clinical trials have focused on total T cells, total B cells, T cell co-stimulation pathways, individual inflammatory cytokines, Treg infusions and antigen specific therapy. These approaches, while encouraging, have yet to be fully successful. Many treatments slowed C-peptide loss for one year, and a few others,

Abatacept, Teplizumab, and Otelixizumab, slowed loss for up to 2 years. The most encouraging meta-analysis of the Teplizumab trial showed a cohort of subjects with slowed C-peptide loss up to 7 years. An important revelation in the anti-CD3 trials was the observation that T1DM subjects could be differentiated into 'responder' and 'non-responder' subgroups. The responder groups slowed C-peptide loss significantly better and longer than any other group. Understanding what differentiates responders from non-responders is important. Collectively these clinical trials point to the overall heterogeneity of T1DM. Consideration of this finding is crucial for advancing any treatment opportunity. In addition, cell replacement therapy with immune privilege appears to be moving forward after 25-30 years of attempted islet transplantation.

Every individual immune system has its own unique immune fingerprint. That is, an individual's immune experiences over time created a reservoir of memory T and B cells unique to that person. This unique reservoir influences eventual immunologic outcomes including response to various immune modulating drugs. Considering the prospect that subjects in a clinical trial will respond uniquely to the drug should be considered as shown by the meta-analysis of the Teplizumab study. Furthermore, attempts to differentiate responders and non-responders will be important. Thus, added research efforts must be undertaken.

It is time to recognize that T1DM is a heterogeneous disease. This understanding creates novel approach opportunities. It is likely that a combination drug approach that targets adaptive immune cells but also targets innate cells will be needed. It is likely that different drug combinations will be needed for distinct cohorts of T1DM patients. Regardless of the drugs, the ideal approach will modulate but not ablate immune responses. Current approaches are trending in that direction.

Conflict of Interest Statement:

Dr. Wagner is the founding scientific officer for Op-T-Mune, a company in the early-stage development of peptide-based therapy to prevent type 1 diabetes. Dr. Bleich was a consultant for Provention Bio, Inc., a company that was recently acquired by Sanofi Aventis for humanized anti-CD3 monoclonal antibody, teplizumab, to treat new onset type 1 diabetes. This drug received recent FDA approval.

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Contributions:

Drs. Wagner and Bleich contributed equally to this manuscript in conceptualizing, researching, writing, and editing.

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