Microbiome and Cellular Players in Type 1 Diabetes: From Pathogenesis to Protection

Darshan Badal, Mahinder Paul, Neenu Jacob, and Naresh Sachdeva

Abstract Type-1 diabetes (T1D) is an autoimmune disease characterized by the loss of immune tolerance to the beta (β)-cells of the pancreas. In this disease, the islet infiltrating immune cells mainly comprising of autoreactive T cells target the β-cell associated antigens, such as preproinsulin (PPI) and in the process destroy β-cells, leading to insulin deficiency. Besides, genetically predisposing human leukocyte antigen (HLA) alleles, several environmental factors have been proposed in the initiation of T1D, as the disease develop years before the actual presentation of clinical symptoms. However, loss of tolerance to β-cells is the central event in the pathogenesis of T1D for which various cellular entities and cellular mechanisms have been implicated. This chapter provides a detailed review of involvement of these cells and mediators, right from the organogenesis of the pancreatic tissue till the destruction of the β-cells. Further, the chapter focuses on the role of various innate immune cells including, macrophages, monocytes, dendritic cells (DCs), neutrophils, natural killer (NK) cells, innate lymphoid cells (ILCs) and adaptive immune cells mainly different subsets of CD4+ and CD8+ T cells and B cells in causing β-cell damage with special focus on immune cells that infiltrate early in the pancreas during the disease process. Amongst the cellular mechanisms, factors such as endoplasmic reticulum (ER) stress and posttranslational modifications (PTM), neutrophil extracellular traps (NETosis), over-expression of major histocompatibility complex (MHC)-I, involvement of major chemokines and inflammatory cytokines have also been discussed. The latter half of the chapter discusses about various immunomodulatory cells, mainly regulatory T cells (Tregs) that are involved in the protection of β-cells and efforts to replace functional β-cells or prevent β-cell destruction. While the complete treatment of T1D is still far in sight, this chapter attempts to refresh the current knowledge on the pathogenesis of the disease from the perspective of cellular players, which might be helpful in exploring newer therapeutic approaches.

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Abbreviations

EPC	Endothelial progenitor cells
GZM	Granzyme
HSC	hematopoietic Stem cells
IDO	indoleamine 2,3-dioxygenase
IFN	Interferon-
IL-	Interleukin-
iNKT	Invariant NK T (iNKT) cells
mDC	Myeloid dendritic cell
MSC	Mesenchymal stem cell
MΦ	Macrophage
NK	Natural killer cell
NKT	Natural killer T cell
NO	Nitric oxide
pDC	Plasmacytoid dendritic cell
PFN	Perforin
PMN	Polymorphonuclear leukocytes (neutrophils)
PP	Perinatal period
Teff	Effector T cell
TNF	Tumour necrosis factor
Treg	Regulatory T cell
W	Weeks

1 Introduction

Type-1 diabetes (T1D) or autoimmune diabetes is one of the most common autoimmune diseases affecting more than 11,10,100 children and adoloscents worldwide (IDF 2019). The disease is characterized by the loss of immune tolerance to beta (β) cells associated antigens [1]. Because of an aberrant immunological response, the β-cells are attacked and destroyed by islet infiltrating immune cells mainly comprising of autoreactive T cells. Continuous β-cell destruction leads to insulin deficiency that results in impaired blood glucose metabolism and persistent hyperglycemia. Over time, the T1D patients become prone to micro- and macro-vascular complications like nephropathy, retinopathy, neuropathy, and cardiovascular diseases [2]. The primary risk factor for β-cell autoimmunity involves genetic factors i.e. individuals with either

human leukocyte antigen (HLA)-DR3-DQ2 or HLA-DR4-DQ8 haplotypes or both HLA class II alleles are at higher risk. Among the HLA class I alleles, HLA-A∗02 and HLA-B∗39 alleles further increase the risk in individuals possessing HLA class II DR3/4-DQ8 haplotype [3, 4]. However, development of clinical T1D typically requires a trigger from the environment as well, for which multiple factors have been implicated.

Till date, insulin replacement by exogenous insulin and oral anti-hyperglycemic drug remains the mainstay of T1D management. Although this approach is useful in preventing minor and early-onset complications, serious late-onset complications do pose a major challenge as they affect a large number of patients. Moreover, exogenous insulin therapy is never able to mimic physiological insulin responses leading to chaotic glucose profiles and life-threatening hypoglycemic episodes. Based upon the pathophysiology of diabetes, it appears that preserving insulin-secreting cells and stimulating their regeneration are the essential approaches for treating diabetes [2]. Since, the current management regimens are neither able to selectively eliminate diabetogenic immune cells nor able to protect the newly formed β-cells for the long term, therefore, there is a need to develop effective treatment against major autoimmune mechanisms involved in T1D [5]. This target can be achieved by abolishing the selective pathogenic reactivity of immune cells to β-cell auto antigens as well as preserving their full capacity to generate a normal immune response against foreign antigens. In addition to stopping the β-cell destruction process such a strategy would be able to restore immune balance in a safe and long-lasting fashion [6].

2 Role of Genetic Predisposition

T1D is a polygenic disorder with more than 40 different loci accounting for disease susceptibility. The HLA region located on chromosome 6 accounts for one-half of the genetic susceptibility [7]. HLA class II locus accounts for strongest association with T1D with DRB1∗04:01-DQB1∗03:02 and DRB1∗03:01-DQB1∗02:01 alleles conferring the greatest susceptibility. Their presence marks 55% chance for developing T1D [8]. On the other hand, some alleles such as, DRB1∗15:01 and DQA1∗01:02-DQB1∗06:02 are associated with disease resistance [9]. HLA class I locus also influences risk for T1D, mostly attributed to HLA-A and HLA-B genes. The susceptible alleles include HLA-B∗39, HLA-A∗02 and HLA-A∗24 while the protective HLA alleles are A∗11:01, A∗32:01, A∗66:01, B∗07:02, B∗44:03, B∗35:02, C∗16:01 and C∗04:01 [10]. The study conducted by Type 1 Diabetes Genetics Consortium (T1DGC), showed that HLA-B∗57:01 is significantly protective for T1D [11]. Similarly, a study conducted on African population found haplotype HLA DRB1∗03:02-DQA1∗04:01-DQB1∗04:02, has protection for T1D [12]. Various HLA alleles associated with susceptibility to T1D are listed in Table 1.

The other susceptibility loci include polymorphism in variable number tandem repeat (VNTR) in the promoter region of insulin gene [25]. A gain of function mutation in the protein tyrosine phosphatase, non-receptor type 22 (PTPN22) gene, which encodes for lymphoid protein tyrosine phosphatase (LYP) suppresses T-cell

Table 1 HLA susceptibility genes associated with risk of type-1 diabetes

S No:	HLA gene	Reference
1.	HLA DRB1∗04:01	[13]
2.	HLA B∗08:01	[14]
3.	HLA DRB1∗03 and DRB1∗04	[15]
4.	HLA DQA1∗05:01 and DQB1∗03:02	[16]
5.	HLA DQA1:03:01 and DQB1∗02:01	[17]
6.	HLA DPB1∗03:01 and DPB1∗02:02	[18–20]
7.	HLA A∗24	[21]
8.	HLA B∗39:06	[11, 22]
9.	HLA DRB1∗07:01-DQA1∗03:01-DQB1∗02:02	[17, 23, 24]
10.	HLA DRB1∗03-DQB1∗02:01, DQB1∗02/ DQA1∗03:01,DQB1∗03:02	[24]

receptor (TCR) signaling during thymic development, thereby allowing autoreactive T cells to escape negative selection [26]. A single nucleotide polymorphism (SNP) of the PTPN22 caused a A629T substitution in the biobreeding diabetes-prone (BBDP) rat. This resulted in 50% decrease in C-terminal Src kinase binding affinity which contributed to T cell hyper-responsiveness [27]. A study carried out in the cohort of Caucasian subjects showed increased frequency of PTPN22 C1858T polymorphism in diabetic patients [28]. A49G polymorphism has also been detected in the cytotoxic T lymphocyte associated protein (CTLA)-4 which causes a change in the primary amino acid sequence of CTLA-4 thereby reducing its surface expression on T cells [29]. Studies show that SNP CT60A/G in the CTLA-4 gene marks as a susceptibility factor for T1D [30]. A meta-analysis study involving 2238 participants from Chinese population showed a significant relationship between CTLA4 + 49A/G gene polymorphism and T1D [31]. Another gene, interferon-induced helicase 1 (IFIH1) codes for an IFN induced helicase that recognizes dsRNA from picornavirus, thus serving as a sensor for viral infection. Coxsackievirus, which is proposed to be a causative agent for T1D pathogenesis, belongs to *Picornaviridae* family. Polymorphisms in the IFIH1 gene have shown its enhanced gene expression in peripheral blood mononuclear cells in patients with T1D [32]. Studies also confirm the association of the polymorphism in IFIH1 locus with susceptibility to T1D [33] Fig. 1.

Symbol	Description
	Antibodies
	Interferon-α
	Cytokines
	Chemokines
	Granzyme
	Perforin

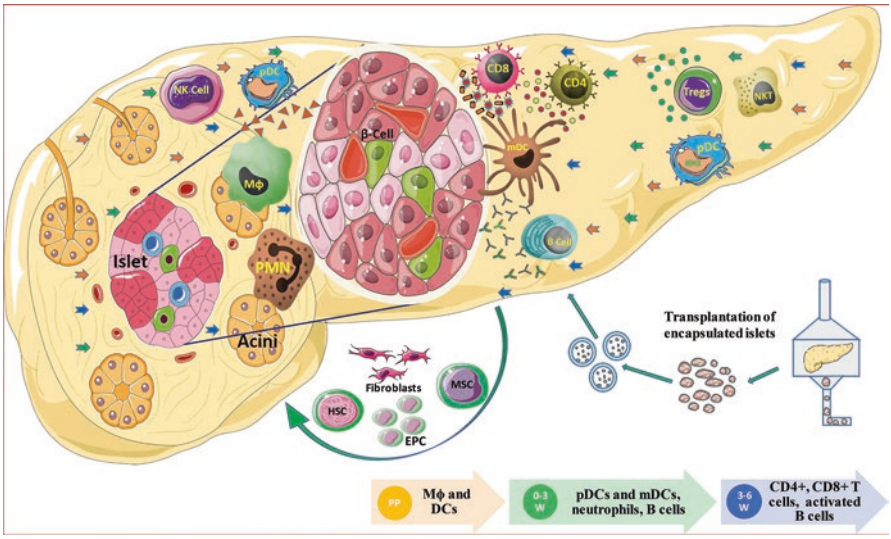

Fig. 1 Initiation of type 1 diabetes (T1D) is marked by the infiltration of innate and adaptive immune cells in pancreatic islets. Infiltrating antigen presenting cells including macrophages and myeloid dendritic cells (mDCs) capture and process β-cell antigens released following initial damage caused by inflammation, apoptosis, ER stress, viral infections or other environmental stimuli. Beta-cell destruction is primarily initiated by CD4+ T cells that recognize β-cell associated-antigens and produce IL-2 and interferon-γ (IFNγ) to activate CD8+ T cells. Cytotoxic CD8+ T cells mainly mediate the destruction of β-cells by releasing perforins and granzymes. Natural killer (NK) cells contribute to β-cell killing via release of IFNγ, granzymes and perforin. Activated macrophages can also cause β-cell death through secretion of tumour necrosis factor (TNF), IL-1β and nitric oxide. B cells present in and around the islets can present β-cell antigens to diabetogenic T cells and secrete auto-antibodies. pDCs infiltrate islets at early stages of T1D and are shown to produce IFN-α and augment Th1 responses. Neutrophils are also among the earliest islet infiltrating cells that are thought to play a role in pathogenesis through NETosis. Cells limiting β-cell damage include Tregs that inhibit effector T cells and inflammatory mDCs via various mechanisms. Indoleamine 2,3-dioxygenase (IDO) producing tolerogenic pDCs check the proliferation of effector T cells by limiting the amount of IL-2 in the milieu and by expanding Tregs. Invariant NK T (iNKT) cells can promote recruitment of tolerogenic DCs and pDCs. In the β-cell replacement cellular therapies, besides whole pancreas transplantation, islet transplantation is a safe and promising approach. Attempts are underway to encapsulate isolated islets with semi-permeable membranes or co-infuse them with accessory cells, such as endothelial progenitor cells (EPCs) or fibroblasts. Hematopoietic stem cells (HSCs) have been tried in β-cell regeneration and, MSCs due to their immunosuppressive nature are also being tried preserve the β-cell mass

3 Contribution of Environmental Factors

T1D develops years before the actual presentation of clinical symptoms [34, 35]. George S. Eisenberth in 1986, proposed a model, which suggests a steady progression in β-cell killing by autoreactive T cells that results in 80–90% of β-cell death [36]. Some of the extensive studies such as, The Environmental Determinants of Diabetes in the Young (TEDDY) [37], The Diabetes Auto Immunity Study in the

Young (DAISY) [38] and TrialNet [39], have been commenced to identify the prospective environmental triggers and biomarkers for T1D.

Multiple environmental triggers can result in autoimmunity. Viral infection has long been considered as a predisposing factor leading to T1D due to the discordance in monozygotic twins [40]. Many papers suggest enteroviruses (EV) especially coxsackievirus B (CVB) as the prime viral candidate for the precipitation of T1D. Serum antibodies against coxsackieviruses have been found in recent onset patients with T1D versus healthy controls [41]. CVB4 strain isolated from the pancreas of a deceased diabetic child, after passaging through murine cells, was found to induce diabetes after inoculation in mice [42]. After examination of pancreatic autopsy sample in patients with T1D, CVB3 RNA was detected in the islets but not in the exocrine tissue [43]. Recently this was validated by evidence of CVB5 particles exclusively in the endocrine cells but not in the exocrine cells of T1D primary human pancreatic cells [44]. A possible explanation for this difference is the higher basal and induced expression of signal transducer and activator of transcription (STAT)-1 regulated genes in alpha cells thus being able to clear viral infection more efficiently than β-cells [45]. There are mainly three pathways by which EVs have been proposed to kill β-cells, direct cytolysis of infected β-cells, local virus-induced inflammation, and molecular mimicry. A direct cytolytic effect of EVswas supported by the finding that EV can infect human β-cells *in vitro* [46, 47] and has been discovered in the islets at onset of T1D [43, 48]. Infection of β-cells, or other cells in close association to the islets, induces an inflammatory milieu [49, 50] that can be directly toxic to the islets [51, 52] or attract immune cells to the site of infection [53, 54]. The molecular mimicry that results due to the sequence homology between the EV protein 2C and the islet autoantigen glutamic acid decarboxylase (GAD)65 also results in β-cell killing [55]. The Diabetes Virus Detection study (DiViD) is the first study to examine the presence of virus in pancreatic tissue of T1D. The study was conducted on six type 1 diabetic patients, the findings of which revealed the presence of EV in pancreatic islets at the time of diagnosis [56]. Rotavirus infection has also been associated with progression of diabetes in children. Studies have shown that infection of non-obese diabetic (NOD) mice with rotavirus accelerated diabetes onset, which was evidenced by infection in the regional lymph nodes [57]. Apart from rotavirus, cytomegalovirus [58], parvovirus [59] and encephalomyocarditis virus [60] have also been found to be contributing factors for T1D.

Other environmental factors suspected to be involved in T1D is early exposure to cow's milk. The albumin in the milk cross reacts to islet cell autoantigen (ICA)-1 (p69), which is a β-cell surface protein [61]. Recent studies using hydrolyzed casein diet showed promising results in lowering T1D. Administering NOD mice with anti-diabetogenic casein hydrolyzed diet reduced the incidence of T1D. This result was corroborated with reduced levels of reactive oxygen and nitrogen species in the epithelial cells and distal intestine [62]. A study was conducted in Finland on infants with first-degree relatives with T1D. They received either hydrolyzed or conventional formula during first 4–6 months of their life. It was observed that the infants receiving hydrolyzed formula developed less autoantibodies than their counterparts [63]. However, this effect on islet autoimmunity was not confirmed in a larger phase

3 Trial to Reduce IDDM in the Genetically at Risk (TRIGR) study [64]. The DAISY study showed that increased intake of cow's milk in children with low/moderate HLA-DR genotype increases the risk of developing islet autoimmunity and further progression to T1D [65]. Another protein gluten, which is a storage protein present in several grains such as wheat, rye and barley, has also been implicated in T1D development. Gluten peptides are incompletely digested and reach the intestinal mucosa, where they are partly resistant to enzymatic degradation resulting in continuous exposure of the protein to the intestinal immune system [66]. Some of the gluten peptides, of which gliadin is most extensively studied are known to be immunogenic in nature. Increased reactivity of peripheral blood T cells to wheat gluten has been seen in T1D especially in celiac disease and reports have shown production of proinflammatory cytokines resulting from T cell activation [67, 68]. The use of animal models such as NOD mice has been able to provide a better understanding on the effect of dietary gluten on T1D progression. The occurrence of diabetes was reduced in offspring of NOD mice, which was supplemented with gluten-free diet during pregnancy [69]. Studies have also shown that gluten-free diet increased the percentages of $CD11c^{+}$ dendritic cells (DCs) in NOD mice spleen, thus providing a new insight into the stimulatory effect of gluten-free diet on innate immune cells [70]. A pilot study carried out to assess the beneficial effects of gluten-free diet on newly diagnosed children with T1D, showed better outcomes on haemoglobin A1c (HbA1c) and insulin dose-adjusted A1c (IDAA1c) levels [71]. Studies also showed that gluten-free diet resulted in reduction in HbA1c level from 7.8% to 5.8–6.0% without insulin therapy in a subject with T1D. Even after 16 months of diagnosis the fasting blood glucose was maintained at 4.1 mmol/l [72].

Vitamin D plays a crucial role in immune modulation and thus could impact the early onset and disease progression of T1D. A nationwide Diabetes Incidence Study in Sweden (DISS) diagnosed low levels of plasma vitamin D concentration in T1D subjects, suggesting its role in disease development [73]. Supplementation of 1, 25-dihydroxyvitamin D3 [1, 25(OH) 2D3] (an active form of vitamin D) in NOD mice promoted the generation of tolerogenic mature DCs that suppressed the activation of auto reactive T cells [74]. An *in vitro* treatment of T cells from T1D subjects as well as healthy subjects with TX527, a less calcemic analog of bioactive vitamin D, promoted the induction of $CD4^{+}CD25^{high}CD127^{low}$ Tregs [75, 76]. A Cross sectional study on Caucasian children and adolescents with T1D demonstrated a high prevalence of low levels of 25-hydroxyvitamin D [77]. Low concentrations of vitamin D during pregnancy time have also been implicated in the development of T1D in their offspring [78]. A genome wide association study discovered the expression of vitamin D binding protein (VDBP) on the alpha cells of pancreatic islets. The VDBP antibodies were detected in T1D subjects which suggest that they acquired auto-antigenicity during diabetic progression and hence could be a potential T1D biomarker [79]. Although many studies have shown reduced vitamin D levels in T1D subjects, there are few studies showing contradictory results as well. A study on Finnish and Estonian children participating in the DIABIMMUNE and Type 1 Diabetes Prediction and Prevention (DIPP) studies showed no correlation of plasma 25-hydroxyvitamin D [25(OH)D] concentrations with subjects positive or negative

for β-cell autoantibodies [80]. T1D prediction and prevention study carried out in Finland showed no variation in the circulating 25(OH)D concentrations between cases and control groups [81].

At present the incidence of T1D is increasing in developed countries highlighting the influence of infections in disease protection. Infections may help in disease protection by skewing the response towards Th2, ameliorating the Th1 response [82]. Improved sanitation and infection control has hampered the immunoregulatory mechanism of our body. Strachan et al. proposed the hygiene hypothesis in 1989 that explained the rise of allergic conditions [83]. Recently an extension of this hypothesis suggested, greater access to antibiotics and vaccination and improved hygiene increased the susceptibility to autoimmune disease [84]. Studies in NOD mice show an inverse relationship between microbial exposure and incidence of diabetes [85]. NOD mice infected with live attenuated *Salmonella typhimurium* showed reduced incidence of T1D [86]. Helminth infection has shown to modulate inflammatory responses in NOD mice. Infection of *Heligmosomoides polygyrus* (helminth parasite) to NOD mice at 5 weeks of age reduced the incidence of T1D. There was marked reduction in pancreatic insulitis and the expression of IL-4, IL-10 and IL-13 as well as the frequencies of CD4+ Tregs were elevated in mesenteric lymph nodes (MLN) and pancreatic lymph nodes (PLN) in helminth infected mice [87]. Helminth infection has also been shown to prevent diabetes in NOD mice by inducing non Tregs that produce IL-10 independent of STAT 6 signaling [88]. Recently a combinatorial therapy with helminth antigen and proinsulin prevented the onset of diabetes in NOD mice. This protective effect was associated with increased frequency of Tregs within the PLNs [89].

3.1 Obesity

Obesity is a disease which is caused by excess accumulation of body fat leading to predisposition to various cardiovascular and inflammatory diseases in an individual. Several factors influence the incidence of obesity, which includes a lack of physical activity, age pattern and various socioeconomic factors [90].

3.2 Obesity and T1D

The epidemic of obesity is increasing throughout the world and is now also prevalent among young adults with T1D. Until recently, the role of obesity in the development of T1D has not been a focus of active research but the field is picking up the pace recently. A study by Liu et al. (2010) observed that youth with T1D are more prone to be obese than their peers without T1D [91]. A time trend, of which was provided by 18 years' follow-up study, which observed 47% increase in the prevalence of overweight whereas seven-fold increase in the prevalence of obesity [92].

The risk for development of T1D is increased by obesity and may occur at an earlier age among obese individuals with a predisposition as shown by a recent mendelian randomization study that found association between 23 SNPs and childhood onset T1D [93]. Higher bodyweight, obesity and insulin resistance increases the risk of T1D development even though no longitudinal studies have simultaneously assessed their association during preclinical diabetes [94]. There could be a crucial link formed by inflammatory cytokine and adipokines between obesity and T1D. Obese patients have been shown to have high levels of IL-17, IL-23 and leptin, similarly the higher production of IL-17 is observed during the early stages of T1D [95, 96]. Several studies have shown that adipokines like leptin and resistin could play a role in the development of T1D as resistin, decreases beta cell viability and has increased levels in T1D patients [97, 98]. Similarly, in murine models leptin has shown to destruct beta cells through its proinflammatory effects [99]. Pancreatic adipocytes derived proinflammatory cytokines have a direct cytotoxic effects on pancreatic islets, additionally they also aid infiltration of Th1 and Th17 cells thereby inducing persistent inflammation in islets by increase chemokine ligand (CCL) 5 expression [100]. Obesity increases the risk for comorbidities like metabolic syndrome, along with macro- and microvascular diseases among individuals with T1D, collectively speaking, prevention of obesity may slow down the development of T1D and might also prevents the late complications in T1D [101].

3.3 Gut Microbiota

The gut microbiota is a complex community of microbes belonging to at least nine divisions of Bacteria and one division of Archaea, which may vary for each individual but mostly dominated by four phyla of bacteria like *Firmicutes, Bacteroidetes, Actinobacteria* and *Proteobacteria*, whereas archaea domain is dominated by *Methanobrevibacter smithii*, a methanogen that consumes hydrogen [102–104]. Most of them reside in large intestine which is home to estimated 10^{11} bacteria per gram of intestinal matter and plays an important role in various physiological functions such as helping in digestion and metabolism, absorption of nutrients, synthesis of several vitamins and inhibiting the growth of pathogenic microorganisms.

3.4 Gut Microbiota and Obesity

Studies in recent years especially those on germ free animals and transplant of microbiota have shed light on the influence of gut microbiota on human health and diseases and more importantly on metabolic disorders like obesity [102]. Many factors are known to affect composition of gut microbiome which can be linked to obesity like diet, genetic variations, use of antibiotics [105–107]. The initial evidence of link between obesity and gut microbiota was provided by Wostmann et al.

(1983) by their experiments on germ free animals, demonstrating that these mice require 30% more calories for sustaining their body mass than their conventional counterparts [108]. Several studies have shown increased bacteria of *Firmicutes* phyla over *Bacreroidetes* phyla, this is believed to have an association with enhanced low-grade inflammation and increased absorption of energy from food [109, 110]. The gut microbiota also plays an important role in the metabolism via the production of short chain fatty acids (SCFA) like acetate, propionate and butyrate. Several studies have shown the beneficial effects of SFCA on insulin resistance and glucose tolerance and obesity induced by diet etc. [111–113].

3.5 Gut Microbiota and T1D

The human gut microbiome has density which is highest in nature and it outnumbers his own cell number by100:1 [114]. The perfect storm for the development of T1D has been hypothesised which includes the trio factors such as an aberrant intestinal microbiota, a "leaky" intestinal mucosal barrier, and an altered intestinal immune responsiveness [115–117]. Recently gut microbial dysbiosis has been proposed as the main factor contributing to the pathogenesis of T1D. The DIPP study carried out in Finland provided a first line of evidence showing gut microbial alterations in T1D subjects with lower abundance of *Firmicutes* and increased abundance of phylum *Bacteroidetes* [118]. T1D subjects with proper glycemic control and good physical fitness displayed gut microbial profile comparable to that of matched subjects without diabetes. *Faecalibacterium* sp., *Roseburia* sp. and *Bacteroides* were the most abundant microbial species in the study cohort [119]. Studies were carried out to assess the gut microbiota in Infants from Finland and Estonia who are at risk for developing T1D. Significant alterations in the gut microbiota with shifts in both microbial phylogenetic and metabolic pathways were observed. Also increased intestinal inflammation characterized by high levels of human β-defensin 2 (hBD2) (an antimicrobial product induced by colonic epithelial during inflammation) have been observed in the study cohorts [120]. A case control study carried out in Caucasian children with T1D showed a significant difference in *Firmicutes* to *Bacteroidetes* ratio as well as difference in the number of *Bifidobacterium, Lactobacillus* and *Clostridium.* These differences correlated with glycemic level in the group with diabetes [121]. A study conducted on the comparison of fecal microbiota of Mexican children with T1D with that of controls, reported high levels of *Prevotella* in controls while *Bacteroides* dominated T1D subjects. These results were attributed to the dietary intake, where *Bacteroides* were associated with high protein and fat diet while *Prevotella* is associated with carbohydrate rich diet. The role of *Bacteroides* in thinning of the mucin layer in intestinal epithelial cells (IEC) thereby causing increased gut permeability and inflammation also supports its role in T1D development. Studies have shown a low abundance of lactate producing as well as butyrate producing species in children with β-cell specific autoimmunity. These include *Bifidobacterium adolescentis*, *Roseburia faecis* (a member of

Clostridium cluster XIVa), and *Fecalibacterium prausnitzii* (a member of Clostridium cluster IV) [122]. Diet rich in plant polysaccharide and low in fat as well as animal proteins has been found to favor the development of tolerogenic commensal bacteria. This has been proved in a comparative study between African and European children. The African children's diet comprised mainly of fiber and plant while the European children were fed on a high fat western diet. The fecal microbiota of African children consisted mainly of *Actinobacteria, Prevotella* and *Xylanibacter*, and more SCFA while the European children's microbiota comprised of *Proteobacteria* [123].

The role of gut microbiota in T1D diabetes progression has been reported in animal models as well. The absence of Myeloid differentiation primary response gene 88 (Myd88), an essential signal transducer in toll like receptor (TLR) signaling in NOD mice protected it from diabetes development [124]. But the protection against diabetes was abrogated in Myd88$^{-/-}$ mice, when it was transferred to germ free environment, however under specific pathogen free conditions (SPF) NOD Myd88$^{-/-}$ mice were protected from T1D [125]. The oral administration of broad spectrum antibiotics such as streptomycin, colistin and ampicillin) or vancomycin alone from the time of conception until adulthood resulted in increased diabetes incidence in male NOD mice [126]. Also NOD mice receiving either continuous low-dose antibiotic or pulsed therapeutic antibiotics (PAT) early in life had higher incidence of T1D as well as gut microbial alterations [127].These data indicates that antibiotic treatment as well as germ free environment disrupts the commensal microbial population that plays a major role in disease protection. Lower abundances of *Lactobacillus* and *Bifidobacterium* have been observed in BBDP as compared with healthy diabetes-resistant BioBreeding (BB) rats [128].

The gastrointestinal tract is lined by intestinal epithelial cells that act as a protective barrier against harmful antigens as well as helps in nutrient absorption. In BioBreeding rats an increased intestinal permeability was observed at an early age. This was correlated with decreased expression of tight junction protein claudin [129]. An alteration in intestinal barrier function was observed in non-celiac T1D which was associated with mucosal ultra-structural alterations [130]. Dietary microbial toxins have been shown to promote T1D by damaging beta cells thereby releasing autoantigens. Injection of bafilomycin A1 extracted from Streptomyces into mice resulted in impaired glucose tolerance and, reduced islet size and relative beta cell mass [131]. A study carried out by Bosi et al. (2006) showed significant increase in intestinal permeability in subjects with T1D compared to healthy individuals [132].

In recent years there has been a drastic change in the dietary habits of individuals due to increased consumption of processed food which are rich in carbohydrates and fats. Hence the recommended intake of dietary fibers which is 30 g daily has been reduced to one half [133]. The fluids in the gastrointestinal tract cannot digest the dietary fibers; hence they are broken down by gut microbiota into metabolites such as SCFA. A study comparing intestinal microbial composition of T1D subjects positive for at least two autoantibodies revealed low abundance of bifidobacteria and butyrate-producing species [134]. The fecal transfer from male to female NOD

mice conferred diabetes protection in female with an associated increase in butyrate producing bacteria [135, 136]. These SCFA exerts anti-inflammatory effects by producing immunosuppressive cytokines and Immunoglobulin A [137]). The SCFA especially butyrate stimulated the colonic mucus secretion in rats [138], in addition butyrate accelerated the assembly of tight junction proteins as well as increased the AMP-activated protein kinase (AMPK) activity in Caco-2 cell monolayer model [139]. In addition, SCFA can maintain immune homeostasis by modulating inflammatory responses. Butyrate and propionate suppressed the expression of lipopolysaccharide (LPS)-induced cytokines such as IL-16 and IL-12p40 [140]. Another *in vitro* study demonstrated that butyrate stimulated the DCs to express immunosuppressive enzymes such as indoleamine 2,3-dioxygenase 1 (IDO1) and aldehyde dehydrogenase 1A2 (Aldh1A2), which enabled the conversion of naïve T cells into FoxP3+ Tregs and eventually suppressed its conversion into IFNγ+ T cells [141]. Consumption of dietary fiber enhanced SCFA production in the small intestines, which induced the expression of the vitamin A-converting enzyme Aldehyde dehydrogenase 1 (RALDH1) on CD103+ tDCs in MLN. This in turn, promoted the differentiation of FoxP3+ Tregs from naive T cells [142]. Intraperitoneal administration of butyrate to NOD mice increased the pancreatic cathelicidin-related antimicrobial peptide (CRAMP) production by the beta cells. CRAMP exerts immunoregulatory effects on pancreatic macrophages and cDCs thereby maintaining immune homeostasis in pancreas via induction of Tregs. The induction of CRAMP by SCFA was mediated through G protein-coupled receptors (GPR) 43 and GPR41 expressed on beta cells [143]. Feeding NOD mice with acetate and butyrate releasing diet provided complete protection against T1D. Interestingly these two diets had their respective mode of action such as markedly decreasing autoreactive T cells in the lymph nodes as well as boosting the number and function of Tregs [144].

It is a universally accepted that providing new born with human milk protect them from infections. Human milk has the unique composition of proteins, fats, carbohydrates, vitamins and minerals as well as essential fatty acids, enzymes, hormones and many other biologically active compounds that provide health benefits [145]. Early life introduction of human milk oligosaccharides provides an interesting strategy for T1D prevention. Two population based cohort study from Norway and Denmark supports the contention that prolonging the breast feeding for more than 12 months reduced the risk for T1D [146]. There are only a few reports available on the effect of Human milk oligosaccharide (HMOS) on T1D. In breast fed infants these complex oligosaccharides can influence the composition of intestinal microbiota with abundance of *Bifidobacterium* [147]. It has been shown that *Bifidobacterium infantis* and B*ifidobacterium bifidum* grow well on HMOS as it is their only carbohydrate source [148, 149]. The HMOS grown bifidobacteria can maintain gut integrity by reducing occluding relocalization and inducing the expression of cell membrane glycoprotein. They also cause higher expression of anti-inflammatory cytokines such as IL-10 in Caco-2 cells [150]. A recent study showed the immune-modulatory potential of non-digestible short chain galacto- and long chain fructo-oligosaccharides (scGOS/lcFOS) on human monocyte derived dendritic cells (MoDC). These scGOS/lcFOS mimicked the HMOS and promoted MoDC to release IL-10 *in vitro* [151].

It is said that the PLNs as well as the MLNs drain the pancreatic tissue. There is an immunologic connection between the gut associated lymphoid tissue (GALT) and the pancreatic islets since orally administered antigens are able to activate T cell responses in the PLNs [152]. Also the T cells activated in the gastrointestinal tract migrate to islets that express mucosal vascular addressin cell adhesion molecule-1 (MAdCAM-1) [153]. In NOD mice infection with *Citrobacterium rodentum* which disrupts intestinal epithelial barrier has been found to accelerate the development of diabetes and the administration of this antigen via gastric route was found in the PLN and MLN of infected NOD mice [154]. These data suggest that enteral antigens and immune responses arising in GALT may be able to target islet beta cells for destruction.

Whether Th17 cells plays a role in pathogenesis or provides protection from T1D remains a controversial issue. A study conducted by Martin et al. (2009) on NOD mice show increased expression IL-17A or IL-7F in islets that correlated with development of insulitis [155]. Further the deficiency of IL-17 in NOD mice reduced the severity of insulitis and delayed the onset of diabetes [156]. The gut microbial modulations profoundly influence the balance between Th17 cells and Tregs that may influence intestinal immunity. A study by Ivanov et al. (2008) found that specific commensal microbiota such as Cytophaga-Flavobacter-Bacteroidetes (CFB) bacteria was required for the Th17 differentiation in Lamina Propria (LP) and the absence of these bacteria was accompanied by increased $Foxp3^{+}$ Tregs in the LP [157]. Later colonization of segmented filamentous bacteria (SFB) in the small intestine of LP in mice has been found to be potent inducers of Th17 cells [158]. Although many studies are in favor of the pathogenic role of Th17 cells in T1D, some studies also show the protective effect Th17 cells in T1D when gut microbiota is manipulated. Feeding of BBDP rats with *Lactobacillus johnsonii* strain N6.2 (LjN6.2) from Bio-Breeding diabetes-resistant rat conferred diabetes resistance to BBDP. This was correlated with TH17 cell bias within the MLNs [159, 160]. The SFB colonization in NOD female mice showed only 20% incidence in diabetes development, while those without SFB colonization had 80% incidence by 30 weeks of age. The Th17 cells in SFB positive mice correlated with SFB levels in feaces. Indeed these Th17 cells are assumed to be Foxp3+/RORũFE;t + IL-17-producing T regulatory cells that migrate to the site of inflammation and protect NOD mice from diabetes [161].

4 Development of Pancreas and Beta-Cells

Since the pathogenesis of T1D involves destruction and regeneration of the islets, it is important to have some knowledge about various cells and cellular factors involved in the ontogeny of the pancreas. The pancreatic development starts when the embryonic foregut gives rise to surrounding mesenchymal tissue by endodermal budding [162]. The intricate interactions between mesenchyme-epithelium tissues give rise to branching of pancreatic ducts and differentiation, whereas morphogenesis results in the growth of the acini and pancreatic islets. Other organ systems, particularly the circulatory and nervous systems strongly influence pancreas development [163]; signals like vascular endothelial growth factor (VEGF) are provided by blood vessels, resulting in the induction of pancreas organogenesis [164].

4.1 Beta-Cell Development in Mouse

Mouse pancreas development has been studied in much more detail and can be operationally divided in three major time periods: first, is a primary transition of embryonic day (E) (E9.5 to E12.5), second is a secondary transition (E12.5 to birth), and third and the final one is postnatal period from birth to weaning, which in mouse also coincides with adolescence onset. During the first phase, the development of pancreas initiates with endoderm thickening, followed by proliferation of the pancreatic progenitors cells at E9.5, and the evagination of dorsal and ventral pancreatic bud around E9.75 [165–167]. The pancreatic endocrine progenitor cells expressing neurogenin 3 (*Ngn3*) differentiate into β-cells [168]. Additionally, expression of several transcription factors [167] (Table 2) are required for the formation of a functional glucose-sensing and insulin-secreting β-cells [169–171]. After initial differentiation, maximum fetal β-cells remain functionally immature till late gestation period [172–174]. Beta-cells can be considered mature when they are capable of sensing physiological signals like glucose and secrete appropriate levels of insulin to match them. After birth, the β-cells of new-born mice rapidly mature to confront the new host energy sources and requirements [28]. A recent study by Sasson et al. (2016), suggested that pericytes plays an important role in the islet niche, and directly influence the maturity and functionality of β-cells. When the pericytes were depleted from the islets it resulted in the reduction of insulin content and expression. The pericyte devoid islets had impaired glucose-stimulated insulin secretion, along with a reduced expression of β-cell function and reduced levels of the MafA and Pdx1 transcription factors [175].

4.2 Role of Immune Cells during Pancreas and Beta-Cell Development

Immune cells are present in the pancreatic islets during the neonatal periods in both mice and humans, but their role during the development of pancreas and β-cells was not given much focus. There is hardly any literature on whether there is a link between the early presence of immune cells during β-cell development and pathogenesis of T1D.

4.2.1 NOD Mice Neonates

The presence of macrophages is a well-recognized component of adult pancreas in rodents, although their presence in the neonatal and fetal pancreases are not well understood. Large number of several types of macrophages especially the mature BM8+ scavenger macrophages were found to be localizing around periphery of blood vessels, ducts, nerves and islets, and also scattered in the septa and exocrine tissue in

Table 2 Factors involved in beta-cell development and maturation

Associated gene-expression changes	
Factors increased	References
Ldha	[176–179]
Npy	[179–183]
Mmp-2, Spd	[184]
Ck-19	[179, 184]
Factors decreased	References
Ins2	[185, 186]
Glut2	[186, 187]
Gck, Glp1r, Pcsk 1/3	[186]
Oxidative metabolism genes (Pyruvate carboxylase, mitochondrial shuttles, etc.)	[187]
Transcriptional regulators	
NeuroD1	[179]
MafA	[186, 188–190]
MafB	[190, 191]
Islet1	[192]
Ngn3	[193, 194]
Nkx2.2	[195, 196]
Pdx1	[197–200]
Vhl	[201, 202]
Other factors	
αvβ3 and αvβ5 integrin	[203]

pancreas of NOD control and NOD/*SCID* mice [204–207]. At the time of birth, BM8+ and ER-MP23+ macrophages, and CD11c + DCs were more abundant in the pancreas of NOD/*SCID* and NOD than C57BL/6, DBA/2 and BALB/c controls, which is suggestive of ongoing abnormal events in islet milieu [206]. Few weeks after birth, the number of macrophage progressively decline in all mouse strains till weaning and rebound subsequently only in NOD and NOD/*SCID* strains with diabetic background [206]. DC precursors like ER-MP581, Ly6C^{hi} and Ly6C^{low} were present in fetal pancreases of prediabetic NOD and control mice. Ly6C^{hi} and Ly6C^{low} DC precursors were capable of developing into CD11c + MHCII+ CD86+ DCs capable of processing DQ-OVA antigen. Additionally, ER-MP581 cells in the embryonic and pre-diabetic NOD pancreas had a higher proliferation capacity than controls [208].

Additionally, during the tissue remodeling in pancreas, apoptosis of β-cells peaks around 2 weeks of age and is significantly increased in NOD neonates as compared with controls. Although apoptosis is considered a non-immune response generating process, but certain studies have indicated that apoptotic cells can preferentially activate DCs capable of activating autoreactive T cells by presenting auto-antigens on their surface blebs and have also been shown to induce autoantibodies formation. In NOD and transgenic NOD mice, the immune cell infiltration into pancreatic islets appears around 15 days of age and coincides with neonatal β-cell apoptosis with

accelerated onset of autoimmune diabetes [209]. The NOD mice younger than 15 days of age do not develop diabetes even after the transfer of functional T-cells from adult BDC 2.5 TCR transgenic mice to 10-day-old NOD recipients, the possible reason may be the lack of autoantigens or absence of antigen presenting cells (APC) [210].

4.2.2 Human Neonates

There are very few reports on the infiltration of immune cells in humans especially during neonatal and fetal period. Infiltration of lymphocytes was observed parallel to the two successive waves of β-cell apoptosis/islet degeneration during the pancreatic development as reported in an early study of human pancreas [204, 206]. Another study by Jasen et al. (1993) showed the presence of large focal lymphocyte infiltrates, containing primarily T cells in capsule and connective tissue of septa of fetal and neonatal human pancreas. Abundant endothelial venule-like structures, macrophages and DCs were also observed in periphery of fetal islets [211]. Presence of lymphocytes and expression of MHC class II antigens were also confirmed in pancreas of human fetuses [212]. Collectively, these reports suggest that presence of lymphocytes, macrophages and DCs during developmental periods is an essential part of the pancreatic milieu, which requires special attention in understanding T1D pathogenesis. These cells have also been shown to play a role during the development of limb, nervous system, retina, kidney, gut and thymus in rodents, during various stages of organogenesis, such as angiogenesis/vasculogenesis, neurogenesis/perinatal nerve degeneration and epithelial branching. Macrophages, in particular, are well-recognized for their role during tissue remodeling, phagocytosis during embryogenesis and their interaction with apoptotic cells during developmental periods and are also known to secrete numerous factors, including, growth factors, cytokines, and extracellular matrix proteins [213] (Table 3).

In fact, the mesenchymal compartment of every organ throughout embryogenesis is populated by macrophages, where they support tissue regeneration and organogenesis by regulating remodeling of the extracellular microenvironment. Mussar et al. (2014), shed some light on their specific role in islet development by describing that M2 macrophages regulate cell cycle progression and migration of pancreatic progenitors cells by modulating adhesion receptor, neural cell adhesion molecule (NCAM) and transcription factor, paired box protein (PAX6) in the epithelium [214]. Further, the role of macrophages was also observed in β-cell proliferation following injury, where their depletion blocked connective tissue growth factor (CTGF) mediated β-cell proliferation [215].

5 Loss of Self-Tolerance

Immune tolerance is a state of unresponsiveness to antigens that can elicit an immune response. There are mainly two types of immune tolerance, central and peripheral tolerance. Central tolerance is generated at sites of lymphocyte development, such as thymus and bone marrow for T and B cells respectively. This helps to

Table 3 Growth and differentiation factors produced by macrophages involved in islet development

Factors	Synthesized by macrophages
Mesenchyme and extracellular matrix	
Activin A	+
β-Cellulin	–
Fibronectin	+
Follistatin	–
Laminin	?
Matrix metalloproteases (MMPs)	+
Cytokines and growth factors	
Epidermal growth factor (EGF)	+
Fibroblast growth factor (FGF)	+
Hepatocyte growth factor (HGF)	+
Insulin growth factors	+
Interferon-γ (IFN-γ)	+
Interleukin-6 (IL-6)	+
Keratinocyte growth factor (KGF)	–
Nerve growth factor (NGF)	+
Transforming growth factor-α or -β (TGF-α or -β)	+
Tumor necrosis factor-α or -β (TNF-α or -β)	+
Vascular endothelial growth factor (VEGF)	+

distinguish self and non-self-antigens, whereas peripheral tolerance is generated at sites of antigen recognition and processing mainly in the lymph nodes. This helps prevent over reactivity to environmental triggers such as gut microbes and allergens. Failure of central and peripheral tolerance can lead to development and expansion of effector T cells, which eventually lead to progression of autoimmunity. T1D ensues as a result of breakdown of this tolerance, which leads to commencement and progressive destruction of insulin producing β-cells. Self-reactive T cells are eliminated in the thymus by negative selection process. The thymic medulla express the transcription factor, autoimmune regulator (AIRE), which controls the transcription of broad array of organ-specific genes, including preproinsulin, thereby creating an immunological umbra in the thymus [216, 217], thereby eliminating autoreactive T cells. Yet many autoreactive T cells escape this immune regulation in the thymus. This partial clearance of autoreactive T cells in the thymus could be attributed to lower HLA binding affinity of self-peptide epitopes [218], low avidity of the TCR recognizing self-epitopes presented on the HLA molecules, and variances in post-transcriptional [219, 220] and post-translational expression regulation in peripheral tissue versus thymus [221]. The autoreactive CD8+ T cell tolerance is achieved by immunological ignorance, if the avidity of self-peptide presentation in the draining lymph node is low or by anergy or death mediated by high expression of Bim, a pro-apoptotic protein [222]. The breakdown of tolerance depends on the phenotypic and functional characteristics of DC that is whether DCs promote tolerance or present antigens in an immunological manner. Also, the avidity of interac-

tion between autoreactive TCRs and their respective cognate antigens presented by DCs must reach a certain threshold to trigger activation of autoreactive CD8 + T cells in PLNs [223]. Peripheral tolerance is also maintained by recognition of self-antigens on APCs other than DCs. Stromal cells present tissue-specific antigens in lymph nodes in association with AIRE [224, 225]. Mutations in genes encoding AIRE and PTPN22 have been involved in T1D [226, 227]. A gain-of function mutation in PTPN22 gene results in lower T-cell activation and IL-2 production [26] resulting in compromised immunoregulation by Tregs.

There is ambiguity regarding the factors involved in loss of β-cell tolerance, but it is evident that β-cells are themselves responsible for their demise rather than being an innocent victim of autoimmune attack [228]. Viral infection or ER stress provokes an immune response in β-cells leading to activation of immune system. Infiltration of leukocytes (insulitis) towards islets is preceded by hyper-expression of MHC I, IFN-α, and CXCL10, that attracts immune cells expressing CXCR3 towards the islets [229–231]. The NOD mice develop autoimmunity with overt hyperglycemia (where 70% of the β-cell have been destroyed) by around 3–5 months of age much later than the actual development of insulitis, which begins at 3 weeks of age. This delayed disease onset and occurrence of β-cell destruction has been evidenced from a study where, adoptive transfer of pathogenic polyclonal CD4+ and CD8+ T cells from the spleen of diabetic NOD mice to syngeneic immune deficient recipients resulted in diabetes incidence in these mice [232–234]. It is still unclear whether a single antigen or a repertoire of antigens is responsible for autoimmunity. Also it is unknown which candidate antigen is responsible for pathogenic auto-reactivity or bystander islet autoimmunity [235, 236]. There is still an enigma on why loss of tolerance to certain antigens expressed in islets and other tissues lead to tissue specific pathogenesis. Nonetheless, breakdown of this tolerance leads to activation and recruitment of T lymphocytes, which have an important involvement in the disease process.

5.1 Endoplasmic Reticulum (ER) Stress and Post-Translational Modifications (PTM)

During the initiation and progression of insulitis, immune cells move towards the pancreatic islets after sensing inflammation, although the factors causing this initial inflammation and infiltration are not well defined. Beta-cells are predisposed towards ER stress due to their secretory nature and rapid turnover of insulin molecules. Inflammation causes ER stress in β-cells which they try to resolve by activating unfolded protein response (UPR) pathways, but if ER stress remains prolonged and unresolved, the UPR switches from a pro-adaptive to pro-apoptotic outcome leading to the death of β-cells [237]. Several studies have suggested link between disruption of ER homeostasis and β-cell dysfunction and diabetes, as misfolded insulin was shown to induce diabetes in both mouse mod-

els and humans [238, 239]. Also, mutations in genes critical for ER function results in β-cell failure and diabetes onset both in experimental models and humans [240–242].

ER stress and dysfunction also leads to abnormal protein folding and post-translational modifications (PTM), affecting protein function and may give rise to "neo-antigens" with increased immunogenicity [243]. Coxsackie viral infection is also linked to ER stress and PTM via disruption of ER membrane and release of Ca2+ from the ER into the cytosol [244, 245]. The risk of developing T1D increases considerably with increase in number of target auto-antigens, which can happen via PTM. PTM includes phosphorylation, citrullination, acetylation, carbamylation, amidation, and oxidation [246]. Once the β-cell ER stress increases, it leads to the release of β-cell related neo-antigens which are processed and then presented by APCs to T cells in draining lymph nodes leading to the increased infiltration of auto-reactive T cells. Beta-cells under ER stress may secrete cytokines and chemokine's that attracts immune cells to islets [247]. With increase in immune infiltration into the islets the ER stress also increases progressively [248]. Increased ER stress could lead to rise in cytosolic Ca2+ that enhances the activity of tissue transglutaminase 2 (Tgase2) and Peptidylarginine deiminases (PAD) enzymes. PTM by the Ca2+ dependent enzymes Tgase2 (deamidation) or PAD (deimidation) increases the immunogenicity of several β-cell proteins [246] (Table 4). Recent study by Marre et al. (2016) demonstrated that ER stress increases immunogenicity in the human β-cells. Induction of ER stress by thapsigargin in human islets and insulinomas increases the recognition of deamidated GAD65 by 135–360 fold by human T cells and increased activation of the PTM enzyme Tgase2 was found to accompany this increase in immunogenicity [249].

Table 4 Post-translational modifications (PTM) in beta-cell associated antigens occurring during endoplasmic reticulum (ER) stress

Autoantigen	PTM	References
Phogrin	Deamidation	[250]
Proinsulin	Oxidation	[219]
CHGA (WE14)	Crosslinking/ Isospeptide bond	[251, 252]
Preproinsulin	Deamidation	[250]
ICA69	Deamidation	[250]
ZnT8	Deamidation	[250]
IA-2	Deamidation	[250]
IGRP	Deamidation	[250]
GAD65	Citrullination	[253]
	Deamidation	[250, 253]
GRP78	Citrullination	[254]

CHGA, Chromogranin A; GRP78, Glucose regulated protein 78; GAD65, glutamic acid decarboxylase 65; IA-2, insulinoma antigen-2; ICA69, islet cell autoantigens; IGRP, islet-specific glucose-6-phosphatase catalytic subunit related protein; ZnT8, zinc transporter-8

5.2 *Role of Chemokines, Cytokines and Cell Signaling Pathways*

In T1D, disease onset is preceded by leukocyte infiltration to the pancreatic islets suggesting the role of chemokines expressed in the pancreatic islets in disease pathogenesis. Pancreas produce numerous chemokines such as CXCL10, CCL5, CXCL9 and CCL2 [255, 256] implicating the recruitment of pathogenic [257] or Treg [258] cells into the pancreatic islets. Studies also indicate that chemokine receptor (CCR)7 and its ligands are important for T cell recruitment to pancreatic islets. During insulitis, β-cells secrete chemokines such as CXCL10 and CXCL9, which act as driving forces for the accumulation of cytotoxic T cells expressing CXCR3 [256]. Genes encoding chemokines, mainly CXCL10 and also CXCL9 and CXCL11 are the response genes in pancreatic β-cells that are elevated in inflammatory conditions. The circulatory levels of these chemokines are also elevated in NOD mice [259]. Islets obtained from 4 weeks old NOD/*SCID* mice showed the basal expression of several chemokine ligands. CXCL10 was predominantly expressed followed by CCL22, CCL21, CCL3, CCL17 and CCL2 [260]. Gene expression analyses detected the presence of mRNA for CCR7 as well as its ligands CCL19 and CCL21 in inflamed islets but not in uninflamed islets of NOD mice, suggesting their role in disease pathogenesis [261]. In a population-based registry of children diagnosed with T1D from 1997 to 2005, the levels of five inflammatory chemokines (CCL2, CCL3, CCL4, CCL5 and CXCL8) were analyzed from the serum samples. The levels of CCL2, CCL3, CCL4 and CXCL8 varied based on seasonal variations with higher levels during summer period. The study also showed an inverse relationship of CCL4 chemokine with age [262]. Expression of CCL2 by β-cells, recruits monocytes and macrophages thereby causing insulitis and islet cell destruction [263]. CCL2 has also been shown to attract the tolerogenic CD11c + CD11b + DC (DCs) to pancreatic islets, thereby reducing diabetes incidence in NOD mice [264]. Pancreatic islets release CXCR1/2 ligands such as CXCL1 and CXCL8 in response to inflammation [265] and the circulatory levels of these ligands are elevated in humans and mouse models of T1D reflecting an anti-islet autoimmune activity [266]. Neutrophils are the primary leukocytes expressing CXCR2 and the depletion of neutrophils in combination with CXCR1/2 inhibitors efficiently prevented diabetes in NOD mice [267].

During early islet inflammation, proinflammatory cytokines are released by a small number of early infiltrating immune cells, including, IL-1β, TNF-α, and IFN-γ. IL-1β and/or TNF-α plus IFN-γ induce β-cell apoptosis via the activation of β-cell gene networks under the control of the transcription factors nuclear factor-κB (NF-κB) and STAT(STAT-1), attracting the DCs and other immune cells to pancreatic islets [268]. NF-κB activation leads to production of nitric oxide and chemokines and depletion of ER calcium [269]. The execution of β-cell death then occurs through activation of mitogen-activated protein (MAP) kinases, via triggering of ER stress and by the release of mitochondrial death signals [268, 270]. Upon further activation, more mediators like Fas/FasL, perforin/granzyme, and pro-inflammatory cytokines come into play to produce their deleterious effects on β-cells secreted by islet invading immune cells [271].

5.3 Infiltration of Immune Cells during Early Stages of T1D

Early infiltration of immune cells in the pancreatic islets always precedes inflammation and onset of autoimmunity in both NOD mice and humans. The islets are normally encapsulated by a layer of peri-islet basement membrane and an interstitial matrix and this layer must be breached by the infiltrating immune cells to cause any β-cell damage [272]. At the same time, the islets are highly vascular in nature, providing abundant cell adhesion molecules for T cell interactions [272]. Pancreatic infiltration predominated by monocytes and B-lymphocytes indicates an early expression of autoimmune phenomena in NOD mice [273]. Infiltrating mononuclear cells consists of CD4 + T cells, CD8+ T cells, B cells, and macrophages, out of which CD8 + T cells being predominant followed by macrophages both in NOD mice and humans [274, 275]. Novel techniques like two photon and intravital microscopy gave much more clear and detailed insight of the islet infiltrates and their phenotype. T cell trafficking studies by Coppieters et al. (2010, 2012) gave us a much better insight of some of the happenings during onset of experimental T1D. According to these studies CD8+ T cells enters pancreatic islets by extravasation through post capillary venules in a random-walk fashion and they move freely in and out of the islets with no time-lag at the islet–exocrine interface [276–278].

The islets seem to be exposed to both antigen-specific and non-antigen-specific T cells, with both cell trafficking to and from the pancreas similarly [279]. One recent study by Lindsay et al. (2015) suggested that these cells halted and mostly interacted with APCs during early stages of disease [280]. These studies also suggest that some other signals in addition to chemokines and cytokines may be involved in the recruitment of T cells to the islets as many of the T cells found at islets of both mouse models and humans are non-islet antigen-specific [278]. A recent study of population dynamics of islet-infiltrating cells by Magnuson et al. (2016) found out that insulitic lesion is open to constant cell influx and turnover, predominated by B and T cells along with CD11b + c + myeloid cells. They have also shown that Tregs exist in peripheral lymph nodes but their migration towards the pancreas is slow and sluggish, which might be the reason for their decreasing proportion in islets as T1D progresses [281]. Innate immune cells, like plasmacytoid DC (pDCs) have also been implicated in initial progression of islet inflammation, especially in NOD mice, as early as 2 weeks of age [282].

6 Cellular Players and Pathological Mechanisms Involved in Beta-Cell Destruction

6.1 Innate Immune Cells

The innate immune system is the first line of defense that provides prompt response following infection or injury. The primary mediators of innate response are circulating factors and cells of non-lymphoid lineage like DCs, monocytes/macrophages, neutrophils and other rare lymphocytes. It recognizes threats by using cell surface,

intra-cellular and secreted, pattern recognition receptors (PPRs), like TLRs, nucleotide binding oligomerization domain (NOD)like receptors and RIG-I like receptors [283].

6.1.1 Dendritic Cells (DCs)

DCs are APCs with functions extending to both innate and adaptive immunity. They play a crucial role during infections and in maintaining immune tolerance to self-tissues and commensal microorganisms [284]. DCs can be divided into two main subtypes: myeloid DCs (mDCs) and pDCs.

6.1.2 Myeloid DCs (mDCs)

mDCs are CD11c + and can be further divided into two major types according to their migratory and tissues localization properties namely, migratory mDCs and lymphoid tissue-resident mDCs. Migratory mDCs are immature and sample antigens in peripheral tissues and subsequently migrate to local lymph nodes via the afferent lymphatics and develop into mature or semi-mature mDCs [285, 286]. Semi-mature mDCs are thought to induce tolerance whereas mature mDCs primarily induce immunity and have a high expression of co-stimulatory molecules and MHC II [287]. DCs found in lymphoid organs like lymph nodes are called lymphoid tissue-resident mDCs and they play a major role in priming CD4+ and CD8+ T cells.

The role of DCs in T1D is well studied; their peri-islet accumulation can be seen in NOD mice as early as 4 weeks of age and was concomitant with the influx of lymphocytes. Earlier studies found yield, function and phenotype of DCs from subjects at risk of developing T1D to be impaired. Lower yield of DCs from adherent peripheral blood mononuclear cells along with reduced expression of CD1a and co-stimulatory molecules like CD80 and CD86 was observed in T1D relatives compared to controls. Additionally, abridged stimulation potential of DCs for autologous CD4+ T cells from relatives of T1D subjects and some recently diagnosed subjects was observed [288]. Saxena et al. (2007) have shown that, the ablation of CD11b + CD11c + DCs leads to the loss of T cell activation, insulitis, and diabetes mediated by CD4+ T cells, and the same was restored when the cells were added back [289]. Decreased numbers of mDCs and pDCs with, a reduced CCR2 expression in recent-onset T1D were also observed. This abnormality of DCs in T1D may have an effect on the initiation and intensity of auto-immune responses, due to the important role that CCR2 plays in DC chemotaxis and differentiation of Th1 subsets [290]. A recent study described that DCs can also guide islet autoimmunity via processing and presentation of restricted autoantigens in a unique and a highly immuno-dominant form by the high-risk HLA-DR [291]. It has also been demonstrated that human BDCA1+ DCs from pancreas-draining lymph nodes and blood effectively engulf β-cells and induce interferon (IFN)-α/β responses and have suppressed Th2 cytokines [292].

6.1.3 Plasmacytoid DCs (pDC)

The ability of pDCs to secrete copious amounts of IFN-α upon viral encounter has defined their role as front runners of virus induced adaptive immune responses [293]. pDCs once activated through TLR7 and TLR9 stimulation by CpG nucleotides containing DNA, start releasing large amounts of IFN-α [294, 295]. pDCs can also play important role as APCs and the uptake and presentation of antigen to CD4+ T cells or CD8+ T by human pDCs enhances when stimulated in the presence of antigen-specific immunoglobulins [296, 297].

The role of pDCs in autoimmune diabetes has been proposed by several studies. Increased production of IFN-α and pDCs were detected in autoimmune diabetes patients at diagnosis, along with high expression of IFN-α induced genes in prediabetic children [298–300]. One of the reasons for the infiltration of pDCs in islets during the initiation of autoimmune diabetes, could be the release of self-nucleic acids (genomic DNA, mitochondrial DNA, RNA etc.) by dying β-cells. As pDCs and monocytes can capture β-cell specific nucleic acids during normal scavenging process akin to other autoimmune diseases like systemic lupus erythematosus (SLE) and psoriasis, these cells might get activated to a pro-inflammatory phenotype [301–303]. In the islets of NOD mice accumulation of pDCs were observed as early as 2 weeks of age, where they get activated via TLR 9 by self-DNA-cathelicidin-related antimicrobial peptide (CRAMP) complexes, leading to the production of IFN-α and induction of autoimmune diabetes. Their role in the initiation of autoimmune diabetes was also confirmed by depletion treatments [282]. T1D subjects both at risk and newly diagnosed were found to have increased pDCs compared to controls. Increased IFN-α production in T1D subjects by PBMCs upon stimulation with influenza viruses was observed that correlated positively with pDC numbers. Additionally by *in vitro* studies authors also demonstrated that IFN-α produced by pDCs augments Th1 responses, as a greater proportion of IFN-γ-producing CD4+ T cells from T1D subjects were observed [304]. A potential role of TLR9 induced IFN-alpha in T1D development can be deduced, as CpG 2216 induced IFN-α production by pDCs was found to be highest in T1D relatives even though lower pDCs numbers were observed both in T1D patients and their relatives [305]. A disease-promoting role of E2–2 dependent pDCs was recently described during autoimmune diabetes in the NOD mice. After knocking out E2–2, abridged recruitment of pDCs was observed in pancreatic islets along with decreased CpG1585 induced IFN-α production that markedly reduced diabetes incidence [306].

A tolerogenic role of pDCs has also been suggested by some studies, Welzen-Coppens et al. (2013) reported the accumulation of pDCs and lymphocytes in pancreas of NOD mice 10 weeks onwards. These pDCs expressed Indoleamine-pyrrole 2,3-dioxygenase (IDO) and were found to be responsible for reduced insulitis and slow disease development [307]. In another study, ablation of DCs from NOD mice lead to accelerated insulitis, marked by the loss of pDC and localized loss of IDO, which was restored on the return of pDCs to the depleted mice [289].

6.1.4 Monocytes and Macrophages

In addition to diabetogenic T cells and B cells, several studies suggest a role for monocytes/macrophages in autoimmune mediated β-cell destruction. In a study, passively transferred diabetogenic T cells failed to induce diabetes following depletion of monocytes. Additionally, activated macrophages are also known to kill β-cells directly *in vitro* [308, 309]. Convincing evidence was provided by Martin et al. (2008) using multiple transgenic mouse models, that monocytes can induce diabetes by destroying β-cells even in the absence of functionally mature T and B cells, following their recruitment to pancreatic islets under the transgenic expression of chemokine CCL2 in β-cells [263]. Apart from their direct effect, macrophages also help in the recruitment of other cells to islets by producing chemokines CXCL1 and CXCL2, which recruit CXCR2-expressing neutrophils from the blood. This recruitment of neutrophils is important for the induction of diabetes as its blockade at early age by CXCR2 antagonist diminishes T cell responses and development of the disease [310, 311].

6.1.5 Neutrophils

Neutrophils are also part of the list of innate immune cells involved during the initial phases of T1D as their numbers are decreased in the peripheral circulation of recently diagnosed T1D subjects which may be attributed to their increased infiltration in the pancreas [312]. Additionally, neutrophil extracellular traps (apoptosis of neutrophils resulting in the release of DNA complexes or NETosis) and associated serum biomarkers like neutrophil elastase (NE) or proteinase 3 (PR3) are increased in recently diagnosed T1D subjects compared to controls [313]. Although a new study by Qin et al. (2016) contradicts the previous study and has shown that, NETosis-associated serum biomarkers, NE and PR3 are decreased in T1D subjects in association with the reduced neutrophil count [314].

6.1.6 Natural Killer (NK) Cells

NK cells are granular lymphocytes that lack B or T cell receptors and recognize their target cells via presence or absence of specific cell surface receptors like MHC molecules. They are cytotoxic in nature and destroy their target cells by exocytosis of perforin and granzyme, and are also known to secrete IFN-γ and TNF-α [315]. Some early studies suggested role of NK cells in TID by showing that NK cells are involved in destruction of islet cells in BB rat and NOD mice [315]. The mechanism of β-cell killing was further explored by Gur et al. (2010), where they identified that presence of ligand to NKp46 or NCR1 on β-cells is responsible for activation of NK cell receptor which leads to their degranulation and onset of diabetes in NOD mice [316]. Tregs are capable of regulating NK cells in islets by limiting amounts of IL-2 [317]. In humans altered frequency and phenotype of NK

cells has been observed by many studies, the first of those observing slight reduction in blood NK cells at the time of onset with very high secretion of IFN-γ [318]. NK cells from T1D children were found to be reduced in number with reduced responses to IL-2 and IL-15; finally defects in activating NK cell receptor, NKG2D were also observed [319]. A recent study by Duangchan et al. (2016), showed that NK cell subsets in long standing T1D are skewed towards more activated or less regulatory phenotype [320].

6.1.7 Natural Killer T (NKT) Cells

NKT cells are unconventional T cells that act as a link between innate and adaptive immune systems. Their best-known subset invariant-NKT (iNKT) cell expresses semi-invariant TCR, Vα14-Jα18 and Vα24-Jα18 in mice and humans respectively, and recognizes glycolipid ligands, presented by highly conserved CD1d molecule. In a recent study, they have been postulated to play regulatory role during T1D through various mechanisms [321]. Absence or abnormalities in their frequency and function relates to the acceleration of autoimmunity and diabetes, whereas their increased frequency or function prevents β-cell autoimmunity in both NOD mice and humans [322–325]. Studies on iNKT cells in NOD mice associates T1D protection with a Th2 shift in the effector T cell responses that involves IL-4 and IL-10, along with their ability to induce tolerogenic DCs that generates Tregs in PLNs [326–329]. **S**tudies in humans have shown decreased IL-4 production by iNKT cells sourced from the PLNs and peripheral blood [330]. Additionally, defective Th2 cytokine production and Th1 bias by iNKT cells was also observed by another study [331]. A recent study by Usero et al. (2016) found that iNKT cell suppression of effector T cells is defective in T1D patients. The mechanism involved was cell contact independent and IL13 was described to exert the suppressive effect [332]. Collectively these studies support the notion that exploring iNKT cell alteration in T1D could open a new path in T1D intervention.

6.1.8 Innate Lymphoid Cells (ILCs)

Innate lymphoid cells (ILCs) belong to a family of developmentally related cells that lack specific antigen receptors but can promptly mount an immune response on microbes by producing copious amounts of an array of effector cytokines. They have functions in tissue remodeling, lymphoid organogenesis, inflammation and antimicrobial immunity predominantly at mucosal barrier surfaces [333]. The family of ILCs comprises of three subsets, named as group 1, 2 or 3 ILCs, on the basis of common of surface markers, transcription factors and cytokines produced. Group 1 ILCs (ILC1s) constitutively express T-bet, secrete cytokines like IFN-γ and TNF and respond to IL-12. Group 2 ILCs (ILC2s) have high expression of GATA3, secrete IL-4, IL-5, IL-9, IL-13 and respond to IL-25, IL-33 and TSLP, Group 3 ILCs (ILC3s) expresses RORγt, secrete IL-17 and/or IL-22 and respond to IL-1β, IL-6

and IL-23 [334]. There is scant information on their role in T1D. NOD mice have an increased frequency of type 3 ILCs along with decreased frequency of type 1 ILCs in the MLN at all stages of disease and in the PLNs at 8 weeks of age [335]. A novel CD25+ ILC population in the pancreas is also been identified, but more studies are required to ascertain its role if any in T1D [336].

6.1.9 Mucosal Associated Invariant T (MAIT) Cells

Mucosal associated invariant T (MAIT) cells are innate like T cells in peripheral blood of humans and abundantly found in intestinal mucosa that display both innate and effector like functions to confer protection against microbial activity and infection. These cells express an invariant α-chain (TRAV1–2-TRAJ33/12/20 in humans and TRAV1-TRAJ33 in mice) coupled with a limited repertoire of β-chains, imparting them with the ability to recognize precursors of riboflavin of bacterial origin (vitamin-B related antigens), presented by the MHC-I related protein MR1 [337]. Recently, Rouxel et al. (2017), have suggested an important role of MAIT cells in the development of T1D. Firstly, they discovered that in recent onset T1D children, the frequency of circulating MAIT cells is significantly lower and the phenotype of these cells was also different in the recent onset T1D children, than their age matched controls [338]. In the recent onset T1D children, the MAIT cells had higher expression of activation marker, CD25 and exhaustion marker, programmed death-1 (PD-1), but lower expression of tissue homing chemokine receptor, CCR6 and adhesion molecule CD56. Additionally, upon stimulation the MAIT cells derived from these children showed lower expression of IFN-γ, but higher expression of TNF-α, IL-4 and granzyme-B, upon stimulation with PMA/ionomycin. The authors further showed that in an inflammatory milieu, as expected during islet inflammation, these cells secrete high levels of granzyme-B, in response to increased upregulation of MR1 by the pancreatic β cells, implicating their role in direct participation in β cell killing. In NOD mice as well progression to diabetes is associated with decreased production of IL-17A and IL-22 from MAIT cells in the ileum and an accumulation of IFNγ- and granzyme-B (GzB) –producing MAIT cells in the pancreatic islets. Compared to humans (6%) the frequency of MAIT cells is lower in NOD mice (0.1%) in peripheral circulation, however, such cells can be traced in pancreas or peripheral blood by using MR1 tetramers loaded with the riboflavin derivative 5-OP-RU [339, 340].

6.2 Adaptive Immune Cells

6.2.1 T Cells

T1D results from the destruction of insulin-producing pancreatic β-cells mainly by T cells recognizing the self-islet associated antigens. Best studied antigens include preproinsulin [341], GAD65 [342], insulinoma antigen-2 (IA-2) [343], ICA [344],

heat shock protein (HSP) [345], islet-specific glucose-6-phosphatase catalytic subunit related protein (IGRP) [346], imogen-38 [347], zinc transporter-8 (ZnT8) [348], pancreatic duodenal homeobox factor 1 (PDX1) [349], chromogranin A (CHGA) [350] and islet amyloid polypeptide (IAPP) [351]. However, CD4+ T cells recognizing post translational modified peptides [246, 249] and hybrid insulin peptide also have been detected in NOD mice and T1D subjects [352]. Recently, Delong et al. (2016) reported that CD4+ T cells recognizing epitopes formed by covalent cross-linking of proinsulin peptides to other peptides present in β-cell secretory granules such as CHGA and IAPP can be detected in islets of T1D subjects [352].

6.2.2 CD4+ Helper T (Th) Cells and Subsets

The autoreactive CD4+ T cell is likely at the heart of this disease, as an orchestrator of the immune attack on β cells. Loss of CD4+ T cell tolerance to β-cell associated antigens is a key step involved in pathogenesis of T1D (221). CD4+ T-cells are activated upon interaction with APCs presenting β-cell autoantigens mainly in PLNs followed by a formation of specialized junction called immunological synapse at the T-cell interface [353]. Recognition of antigen by CD4+ T cells can lead to activation or anergy/death depending upon the co-stimulatory molecule involved in process. Signaling through CD28, TNF family members, CD154 (CD40L) and OX40 leads to activation of CD4+ T cells whereas CTLA4 and PD-1 inhibit T cell activation [354, 355]. Following activation, CD4 + T cells (Th1) cells secrete IL-2, which activates CD8+ T cells. At late stages of disease, autoreactive T cells become resistant to suppression by Tregs that may also have diminished regulatory capacity, ultimately leading to complete β-cell destruction [356]. It has been reported that CD4 + T cells specific for β-cell auto-antigens present more proinflammatory phenotype and secret IFN-γ and IL-17[357].

6.2.3 Th17 Cells

Several line of evidences from animal and human studies indicate that Th17 cells are involved in pathogenesis of T1D which were previously thought to be mediated by only Th1 cells [358]. Role of Th17 cells in β-cell destruction is now being explored in T1D subjects. Deficiency of IL-17 in NOD mice delayed the onset of diabetes [156]. Inhibition of Th17 cells using anti-IL-25 or anti-IL-17 decreased GAD65 autoantibody levels, increased the frequency of Tregs, significantly suppressed development of diabetes in 90% of treated animals [359, 360]. IL-23, regulator of IL-17, promotes development of diabetes in sub-diabetogenic doses of streptozotocin treatment by expansion of Th17 cells and IFN- γ production in male C57BL/6 mice [361]. Moreover, deficiency of IL-17A ameliorates streptozotocin-induced diabetes [362]. Adoptive transfer of islet associated antigen-specific Th17 cells induced diabetes in immunodeficient mice [363, 364]. Studies have reported that PLNs of T1D subjects possess increased population of Th17 cells [365].

Furthermore, increased population of IL-17 secreting T cells were observed in new onset T1D children [366]. Interestingly, circulating memory CD4+ T cells from T1D subjects showed increased IL-17 secretion and expression of IL-17, IL-22 and retinoic acid-related orphan receptor C isoform 2 (RORC2) *ex vivo,* indicating activation of IL-17 pathway *in vivo* [96]. Upon *in vitro* stimulation with β-cell autoantigens including proinsulin, insulinoma-associated protein, and GAD65 peptides, the circulating CD4+ T cells from T1D subjects have been shown to produce IL-17 [367]. These observations clearly indicate a Th17 biased response in T1D patients.

6.2.4 Th40 Cells and TCR Revision

A central paradigm of immunology holds that once T cells exit the thymus, TCR molecules do not undergo alteration. To the contrary, several laboratories have shown that peripheral T cells re-express recombination activating genes 1 (RAG1) and RAG2 proteins and subsequently alter TCR expression [368–371]. Th40 cells are subsets of Th cells defined by expression of CD40 and capable of undergoing TCR revisions [372–376], a process by which T cells can alter expression of TCR even in the periphery by inducing RAG1 and RAG2 [374–376]. Th40 cells have been shown to become highly pathogenic in autoimmune disease models [372–376]. CD40 acts as a co-stimulatory molecule on T cells, which upon engagement induces RAG1/RAG2 TCR recombination machinery via interaction with Ku proteins, DNA polymerases and helicases leading to alteration of TCR expression [374–378]. Alterations in the expression of TCR-α [73, 104] and TCR-β [370, 379, 380] in long-standing peripheral T cells occurs following the induction of RAGs [369, 374, 381]. Th40 cell numbers in spleen and peripheral lymph nodes of young NOD mice are equivalent to non-autoimmune mice, but in PLNs, Th40 cell numbers are expanded significantly as early as 3 weeks of age [375]. Pathogenicity of Th40 cells is demonstrated by their ability to transfer T1D to NOD/SCID recipients [373, 375–377]. Th40 cells are stimulated in the PLNs and are then recruited to infiltrate islets. Since Th40 cells are capable of TCR revision, the odds of increasing autoreactive T cells on site would be increased dramatically. Th40 cells are capable of producing IL-17 [377, 382, 383] and IFN- γ to drive diabetogenesis.

6.2.5 CD8+ Cytotoxic T Cells

Infiltrating CD8+ T cells recognize epitopes presented with MHC-I molecules on the surface of β-cells and destroy them. During this period there is hyperexpression of MHC-I molecules on the surface of the β-cells, allowing enhanced epitope presentation to the infiltrating CD8+ T cells [384]. Among the major epitopes recognized by the autoreactive CD8+ T cells, preproinsulin derived epitopes are the primary ones to be recognized by the CD8+ T cells, during the progression of the disease [385].

These autoreactive CD8+ T cells kill target cells mainly by releasing cytotoxic granules or interaction with TNF family-related death receptors. Cytotoxic degranulation involves release of perforin, which facilitates the entry of co-released gran-

zymes with serine protease activity into cells and thus results in rapid cell death. Fas ligand (FasL) is the best-characterized TNF family-related death receptor, binding to Fas expressed on the target cell surface and initiating a series of intracellular pathways resulting in apoptosis. It has been well established in T1D that CD8+ T cell mediated killing of β-cells predominantly use cytotoxic degranulation pathway [386, 387]. This period is also marked by a change in the phenotype of autoreactive CD8+ T cells, whereby there is a shift towards the effector phenotype and an increase in the proliferative potential [388]. Destruction of β-cells results in shedding of other islet associated antigens and presentation of these antigens leads to infiltration of pancreatic islets by diverse population of T cells (predominantly tissue specific), by a process called epitope spreading [389]. Rate of progression of β-cell destruction may vary, depending upon frequency, proliferative and pathogenic potential of CD8+ T cells [388]. Beta-cell associated antigen-specific CD8+ T cells have been characterized and shown to express memory cells markers [390]. Therefore, targeting memory T cells in T1D subjects to preserve residual β-cell mass seems plausible [391].

6.3 B Cells

B cells play an additional key role in the pathogenesis of T1D, yet their functions are less explored. B cells produce autoantibodies against insulin, GAD-65, IA-2, and ZnT8 which are commonly used as biomarkers in predicting disease onset [392], besides routine clinical diagnosis of autoimmunity in diabetes. Although they produce antibodies, these are not thought to be pathogenic, rather their islet antigen presenting capabilities appear to be critical in disease pathogenesis [393]. To explore their role in antigen presentation, a transgenic NOD mouse was generated which could not secrete immunoglobulin but present the antigen. This resulted in increased insulitis and development of diabetes in NOD mice [394]. Early therapy, with either anti-CD20 or anti-B cell activating factor (BAFF) mAb, before the onset of insulitis merely delayed disease progression in NOD mice [395, 396]. A recently identified subtype of B cells, immunosuppressive B cells, also known as B regulatory cells (Bregs) are CD1d^{high}, CD5+ and produce IL-10 [397]. Studies have shown that expansion of Bregs by tolerogenic DCs, subsequently reversed new-onset T1D in NOD mice [398].

6.4 Pathological Mechanisms Underlying Beta Cell Death in T1D

Heterogeneous population of immune cells infiltrates pancreatic islets during the progression of the disease. However, T cells comprise the major proportion of the cells causing damage to β-cells [399]. Following antigenic recognition in lymph nodes, naïve T cells expressing self-reactive TCRs become activated, proliferate and

differentiate into various subsets: central memory T cells and effector memory T cells and effector T cells. Effector T cells invade pancreatic islets and destroy β-cells. Central memory T cells persist in lymph nodes, exhibit high sensitivity to antigenic stimulation, are less dependent on co stimulation and are able to differentiate into IFN-γ producing effector cells. Effector memory T cells can home to inflamed tissue; express high levels of perforin and mount rapid effector responses [400]. Effector T cells are short lived, while long term survival of central memory and effector memory T cell subsets pose major hurdle for immunotherapeutic approaches [401]. On the other hand, CD4+ T cells also participate in activation of CD8+ T cells and B cells. Due to loss of self-tolerance to β-cell associated antigens, β-cells are targeted by immune cells by various effector mechanisms including, (1) Granzymes and perforin pathway (2) Fas-FasL pathway (3) Cytokine mediated death (4) Production of reactive oxygen species. Granzyme and perforin mediated apoptosis is the principle pathway used by CD8+ T cells to kill β cells [386]. In the presence of Ca^{2+} ions, perforin monomers inserted in membrane polymerize to form a cylindrical pore of 5–20 nm through the membrane, which assist the entry of granzymes to cytoplasm. Granzymes activate the caspase cascade resulting in apoptosis of β-cells. Pretreatment of preproinsulin specific CD8+ T cells clones with concanamycin A, which results in perforin degradation, significantly reduce the β-cell death *in vitro* [386]. Quite surprisingly, a recent report by Mollah et al. (2017), have demonstrated that Granzyme A, normally considered as a pro-apoptotic mediator of cell mediated cytotoxicity, may be associated with protection to T1D. In their finding, the authors demonstrated that Granzyme-A knock out NOD mice progressed towards diabetes much faster, implicating its role in maintenance of peripheral tolerance [402].

TNF receptor superfamily member Fas is expressed on the surface of β-cells. Islet infiltrating autoreactive T cells can also activate the caspase dependent pathways of β-cell death by binding of FasL expressed by them. Disruption of Fas-FasL signalling using targeted overexpression of a dominant negative form of Fas-associated death domain adaptor protein in pancreatic β-cells significantly delays the onset of diabetes in NOD mice, implicating a role for Fas in the early stages of autoimmune β-cell destruction [403].

Pro-inflammatory cytokines such as type II interferons including, IFNγ, IL-1 β, TNFα also induce β-cell death [404]. IFNγ is mainly secreted by Th1 subset of CD4+ T cells. Binding of IFNγ to their receptor activates the JAK STAT signaling pathway, which induces β-cell death via regulating the expression of FAS, inducible nitric oxide synthase (iNOS) and caspases. In the absence of STAT 1, major downstream transcription factor of IFNγ signaling, IFNγ mediated destruction of β-cells is disrupted in NOD mice [405]. Apart from the role of IFN- γ in pathogenesis of disease, recent study by John P et al. (2017) also reported that IFN- γ can also limits the activation of diabetogenic CD8+ T cells implicating its role in induction of tolerance [406]. Type 1 interferons, IFNα and IFNβ, also provide signals responsible for accelerating the β-cell death. Type 1 interferons regulate the effector functions and augment the cytotoxity of CD8+ T cells by rapid phosphorylation of STAT4 and induction of Granzyme B. Additionally, studies revealed that overexpression of IFN α in pancreatic β-cells of non-diabetes-prone mice regulate the onset of diabetes in

mice with severe insulitis, while expression of IFNβ in islets of NOD mice accelerated autoimmunity [407].

In another mechanism, signaling through IL-1β leads to activation of NF-κβ in rodent and human islet cells. Translocation of NF-κβ to nucleus induces the β-cell death. Prevention of NF-kβ activation by an inhibitory B (I B) "super-repressor" protects pancreatic cells against cytokine-induced apoptosis. It has been demonstrated that overexpression of NF-κβ super-repressor in rodents protect pancreatic β-cells against cytokine-induced apoptosis [404] and transgenic mice expressing an NF-κβ super-repressor are resistant against experimental diabetes induced by multiple low-doses streptozotocin [408].

TNF-α causes destruction of β-cells by activation of NF-kβ and extrinsic pathway of apoptosis. An important role for TNF-α in β-cell killing was demonstrated in TNF-R1 null mutant NOD mice, which fail to develop spontaneous diabetes [399]. Moreover, treatment of NOD mice with anti-TNF-α antibodies also prevents diabetes development implicating the role of TNF-α in β-cells destruction [409]. Reactive oxygen species e.g. nitric oxide induce β-cell death by causing DNA damage and in turn activation of p53 in a concentration dependent manner. However, reactive oxygen species seems to have a less relevant role for cytokine-induced β-cell death in humans and mice. Blocking of iNOS does not prevent cytokines induced β-cell death [410] while islets obtained from an iNOS knockout mouse are only partially protected against death induced by IL-1β and IFN- γ [411, 412].

7 Protection of Beta-Cells

Targeting immune cells that are associated with β-cell destruction remains the mainstay of most of the approaches in protecting β-cells. Initial attempts to target the immune cells were more generalized, had limited success and were associated with risks of infection. With time, as the information about the cells and factors involved in the disease process became clearer, targeted approaches have been pursued. However, till date, none of the treatment approaches has been able to achieve the goal of selective elimination of immune cells causing β-cell damage, without any compromise on the general immune responses.

7.1 Immunosuppressive Agents

It has been proven in combined outcomes of several trials that blocking T cell function in T1D leads to β-cell preservation by the use of immune-suppressive agents such as cyclosporine (CsA) and azathioprine. Although the continuous CsA treatment in patients with new-onset T1D can eliminate the need for exogenous insulin for some duration, continuous treatment and chronic CsA therapy to maintain remission has been found to be associated with toxic effects in the kidneys leading to decline in the

enthusiasm for its use in T1D patients [413]. Another promising drug, rapamycin (sirolimus) inhibits the critical mammalian target of rapamycin (mTOR) pathway which is involved in cell growth, proliferation, motility, and survival [414]. Rapamycin monotherapy has also been found to increase in serum C-peptide and a reduction in exogenous insulin requirement in patients with long-term T1D [415]. However, rapamycin in combination with IL-2 has also been shown to impair β-cell function [5, 416].

7.2 Monoclonal Antibodies (mAbs)

Among several newer immunotherapies developed in the recent past, selecting mAbs against different immune cell receptors appeared as another promising approach [5]. In an attempt to replace the use of immunosuppressive drugs globally, several agents like anti-CD3 mAb (teplizumab/otelixizumab), anti-CD20 mAb (rituximab), and CTLA-4-Ig (abatacept) directed at the co-inhibitory receptors have been evaluated in new onset T1D patients [417].

7.3 Anti-CD3 mAbs

In contrast to pharmacological immunosuppression treatment, anti-CD3 therapy transiently depletes T cells and exerts long-lasting immune regulatory effects [413]. Administration of anti-CD3 mAbs has shown substantial benefits in recently diagnosed T1D patients in the initial clinical stages. Another report revealed that this therapy particularly teplizumab and otelixizumab can help in preserving the β-cell function for more than 2 years in patients [418–420]. Otelixizumab treatment preserved insulin production for more than 3 years depending on patient age and baseline residual β-cell mass. Moreover, preservation of residual β-cell function was observed following brief teplizumab treatment as long as 5 years in a small group of patients [421]. Therefore, it seems that a short treatment course with Anti-CD3 mAbs may eliminate the need for chronic treatment by triggering lasting tolerance. However, the targeted permanent arrest of the C-peptide decline rate could not be achieved as observed in a series of immune modulation trials in new-onset T1D. Hence, it is to be evaluated whether further optimization of therapeutic antibody concentration and timing of treatment would be able to provide better outcomes or not [413]. Furthermore, the risks of T cell depletion in predisposing individuals to infectious diseases must also be evaluated.

7.4 Anti-CD20 mAb (rituximab)

Being APCs, B cells play a crucial role in the pathogenesis of T1D as these cells themselves are involved in infiltrating the pancreatic islets, presenting autoantigens to T cells and secreting autoantibodies. Therefore, anti-human (h) CD20 mAb were

used to delay or revert diabetes by depleting B cells in transgenic NOD mouse having human CD20 receptors on their B cells with positive outcomes [5, 395]. Rituximab has also been used in Phase II clinical trials. The study showed an initial improvement in T1D by promoting C-peptide levels, reducing HbA1c levels and reducing insulin dose, although this protective effect was short lived. However, continued B cell depletion and associated adverse events as well as the risk of lowering systemic immunity limit the utility of anti-hCD20 mAbs [417, 422].

7.5 CTLA-4-Ig (abatacept)

Besides the main antigen-driven signal, co-stimulatory signals are required to keep immune T cells fully activated. In humans, the susceptibility of T1D has an association with CTLA-4 locus and its immunopathogenesis is linked with T-cell autoimmunity. Therefore, modulating this co-stimulatory signal is another promising strategy in treating T1D. The target can be achieved by using abatacept, which has been observed to modulate co-stimulation and prevent full T-cell activation, as an estimated 9.6 months delay in C-peptide reduction had been achieved with continued administration of abatacept. Despite this, a continued parallel deterioration of β-cell mass as well as function was also observed, inhibiting its further use [423].

7.6 Antithymocyte Globulin (ATG)

ATG is an effective immune-depleting agent and a rabbit polyclonal gamma immunoglobulin (IgG) which is active against thymocytes of human. It is specific for various receptors presented on T cells as well as other immune cells. Short-term ATG therapy in recent onset T1D patients preserved residual C-peptide production and lowered the requirement of insulin but could not induce long-lasting remission [424].

7.7 Low Doses of Interleukin-2 (IL-2)

IL-2 also called a T cell growth factor secreted by T cells itself, can stimulate both effector T cells and Tregs in a dose dependent manner. IL-2 activates primarily STAT5 in Tregs, whereas IL-2 also induces the MAP kinases and phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) pathways in effector T cells [425, 426]. Due to higher expression of IL-2 receptor, Tregs require less IL-2/Il-2R signaling [427]. It has also been reported that IL-2 mediated signaling is dispensable for effector T cells but not for Tregs [428]. Defects in IL-2 mediated signaling have been reported in T1D [429–431]. High dose of IL-2 is associated with many severe side effects [428, 432]. Besides side effects, high dose of IL-2 also carries risk of expansion of

effector T cells that mediate autoimmunity [428]. These key points permit the development of targeted Tregs therapy using low-dose IL-2 administration. First trial with low dose IL-2 (0.33–1 MIU/day) reported that it is well tolerated in the T1D subjects with mild side effects [433]. The minimal doses that are required for the purpose are not fully known and are being investigated in an ongoing dose-finding trial in recently diagnosed T1D children (NCT01862120).

7.8 Phytotherapeutic Approaches

As discussed earlier, prevention of the degeneration of β-cells and stimulation of endogenous islets regeneration are currently the essential approaches for the treatment of T1D. Among several antidiabetic plants investigated so far, a small fraction has been shown to pose pancreatic β-cell protection and/or regenerative properties as well (2). *Allium sativum* [434], *Azadirachta indica* [435, 436], berberine [437], *Crocus sativus* [438], *Gymnema sylvestre* [439], *Juglans regia* [440, 441], *Momordica charantia* [442] and *Nigella sativa* [443–445] have been reported to possess β-cell regenerative property [446]. Many of these agents and their extracts have also been shown to reduce insulin resistance. Hence, their consumption may help in reducing insulin dependence in diabetic patients.

8 Cell Based Treatments

As T1D is caused by functional loss in pancreatic β-cells, replacing them with functional β-cells from various sources provides a new hope for treating T1D. For this purpose, whole-pancreas transplantation, initiated in 1966 is a widely accepted therapeutic modality as evidenced by the fact that several thousand pancreatic transplants have been performed until now. Normal HbA1c levels achieved using this strategy allow long-term insulin independence over 2 years after transplant. However, pancreas transplantation is a surgical procedure that involves high risk of systemic infection that requires lifelong immunosuppression in the recipients. In order to overcome these complications, pancreatic islet cell transplantation has been introduced to replace whole organ transplantation due to new research efforts which presents as a better procedure requiring lesser invasive procedure [447]. However, the procedure requires harvesting the islet cells, preferably from the brain-dead donors and mostly requires two or three donors to achieve insulin independence. Also, to protect the transplanted islets from host's anti-donor HLA and anti-islet responses, various immune-isolation strategies, such as encapsulation in semi permeable matrices are also being explored. Further, in view of the limited availability of pancreas donors, xenografts from other sources like pig islets, have also been considered and pursued further for research.

8.1 Stem Cell-Based Therapies

Stem cells have become an important therapeutic entity due to their inherent regenerative, differentiation capacities as well as their immunomodulatory potential. While the regenerative and differentiation potential can be utilized to avail a supply of glucose-responsive insulin-producing cells for transplantation, the immunomodulatory properties of multipotent mesenchymal stromal cells and hematopoietic stem cells (HSCs) can be used to seize cell damage, preserve the remaining cell mass, promote the regeneration of endogenous cells as well as prevent graft rejection [448]. In view of these regenerative and immunomodulatory characteristics, a variety of stem cells from different sources including, embryonic, bone marrow-derived HSCs and bone marrow-derived MSCs, umbilical cord blood-derived MSCs, adipose tissue-derived MSCs (ADSCs) and pancreas-derived multipotent precursor cells as well as pancreatic cell progenitors have been tested and various studies have provided promising outcomes for the treatment of T1D as follows:

8.1.1 Mesenchymal Stem Cells (MSCs)

Mesenchymal stem cells (MSCs) are multipotent progenitor cells that were originally identified in the bone marrow. MSCs can also be isolated from cord blood, peripheral blood, fallopian tube, fetal liver and lungs. In preclinical T1D studies [449–451], MSCs have been shown to induce and expand Tregs thereby suppressing the immune responses. MSCs can also induce immature IL-10-secreting DCs *in vitro*, thus they potentially interrupt the priming and amplification capacity of autoreactive T cells involved in tissue inflammation. These DCs can assist in the inhibition of inflammatory T cell responses to islet antigens and promoting the anti-inflammatory, regulatory responses exerted by MSCs [452]. Being non-immunogenic in nature, MSCs can also provide protection after allogeneic transplantation and hence they are more attractive for cell based therapies [453]. In spite of the source, whether bone marrow [454] or adipose tissue [455] used for their aspiration, MSCs have been proven to be well-tolerated in T1D patients. Moreover, MSCs have also been documented to improve T1D parameters such as C-peptide preservation [455].

8.1.2 Hematopoietic Stem Cells (HSCs)

In contrast to MSCs, hematopoietic stem cells (HSCs) are found in stem cell niches such as bone marrow, which are situated in the entire body or in umbilical cord blood. HSCs are comprised with the ability to initiate and promote neovascularization rather than an effective differentiation and therefore their prime use is to treat immune-related disorders [456]. Voltarelli et al. have reported increase

in β-cell function, prolonged independence from exogenous insulin in 80% of the patients after high-dose immunosuppression and autologous transplantation of hematopoietic bone marrow-derived stem cells with acceptable toxicity in newly diagnosed T1D patients [457]. Further, in another study by Couri et al. (2009) autologous nonmyeloablative HSCs transplanted in patients with newly diagnosed T1D resulted in significant increase in C-peptide levels and insulin independence in most of the patients with good glycemic control [458]. In another study by Li et al. (2012), it has been reported that autologous HSCs transplantation helps in modulating lymphocytes and preserving β-cell function in Chinese patients with new onset of T1D and diabetic ketoacidosis [459].

8.2 Regulatory T Cells (Tregs) Based Therapies

The discovery that CD4+ Tregs play indispensable role in maintaining self-tolerance [460, 461] has led to the prospect of these cells in cell based treatments to restore tolerance and treat autoimmune diseases such as T1D. These Tregs are CD4 + CD25 + Foxp3+ and suppress the proliferation of autoreactive T cells by producing cytokines, cytolysis, deprivation of cytokines and contact-induced cell modulation [462]. Two types of Tregs are engaged in maintaining the tolerance, natural Tregs (nTregs) and induced Tregs (iTregs). nTregs develop from thymic TCR high affinity T cells selection whereas iTregs are peripherally generated FoxP3+ T cells under immunogenic stimulation [463]. Both Treg subsets express CD25, FoxP3, GITR (glucocorticoid-induced TNF receptor) and CTLA-4 but nTregs exhibit a higher expression of programmed cell death-1 (PD-1), neuropillin 1(Nrp-1) and Helios compared with iTregs [464]. There are many evidences, which show that Tregs have the potential to prevent destruction of pancreatic islets, thereby protecting from T1D. Hence, strategies to increase Treg cell numbers and/or function are being explored as potential therapeutic approaches in treating T1D. In fact most of the antigenic/immunosuppressive treatment approaches to reverse diabetes in NOD mice worked via induction of Tregs or proliferation of Tregs [465–467]. Trials on therapy of T1D subjects with Tregs have indeed shown to prolong survival of pancreatic islets [468].

8.2.1 Polyclonal Versus Antigen-Specific Tregs

While considering therapy with Tregs, there are two available choices, polyclonal or antigen-specific (or epitope-specific) Tregs. Administration of polyclonal Tregs may be associated with significant off-target effects, including global immunosuppression that may compromise beneficial immune responses to infections and cancer cells. Therefore, the objective of research in recent times

has shifted to antigen-specific therapeutic approaches that can reverse the disease by selectively halting the harmful immune response without requiring lifelong immune suppression. Adoptive transfer studies suggest antigen-specificity is required by Tregs for trafficking and maintenance in inflammatory tissues such as the pancreas in T1D [389, 469]. Moreover, antigen-specific Tregs are much more potent in suppressing effector T cell responses, as demonstrated in a tumor rejection model, than polyclonal Tregs, which were only partially suppressive [470]. Another study has demonstrated that small number of *in vitro* expanded antigen-specific Tregs are sufficient to reverse T1D whereas large numbers of polyclonal Tregs are required to reverse the disease [471]. Antigen-specific Tregs have been reported to exhibit a much lower threshold for activation and may be activated by a broad range of loosely-defined analogs of their cognate antigen [472]. Besides, the site-specific mode of action, antigen-specific Tregs also have the ability to act as bystander suppressors locally in the organ under attack. It has also been shown in mice that antigen-specific Tregs treat autoimmunity without compromising antibacterial immune response [473]. However, isolation of sufficient number of antigen-specific Tregs is a major challenge, particularly when sampling is limited to peripheral blood. Moreover, success in inducing antigen-specific tolerance has been hampered by the inability to identify peptides triggering the diabetogenic versus the regulatory response. It has been established that islet-associated antigen-specific Tregs can be generated from CD4 + CD25- T cells. Alice et al. (2009) observed that GAD65 derived epitope specific Tregs suppress not only proliferation of GAD specific effector cells but also of tetanus toxoid (TT) specific effector cells when the GAD was present. Suppression was not observed when TT was present alone [474]. Therefore, these observations indicate that it might be possible to reverse autoimmune diabetes by small number of epitope-specific Tregs rather than having Tregs specific for all the diabetes associated antigens.

8.3 Dendritic Cells

Being the most specialized APCs, DCs have the ability to remove or inactivate diabetogenic T cells, convert them into Tregs or re-stimulate the preexisting Tregs [475]. Therefore, they have been chosen several times for immunomodulation in autoimmune diseases especially T1D. At present, phase 1 and phase 2 clinical trials are ongoing with the purpose to evaluate the safety and efficacy of this therapeutic strategy. Of these trials, phase 1 has been completed in one (NCT00445913), but study results have not yet been posted till date. This trial has included candidates of age ranging between 18–60 years with established diabetes. Another clinical trial (NCT02354911), which is in phase 2, is still ongoing and has included new onset T1D candidates aged between 12–35 years.

8.4 Cord Blood Derived Cells

Umbilical cord blood (UCB) is a rich source of Tregs [476, 477] besides other tolerogenic cells such as immature DC and MSCs, all of which have been shown to play key role in immune tolerance [478, 479]. UCB derived CD4 + CD25 + T cells have been shown to contain greater Foxp3 expression than their peripheral blood counterparts, suggesting the greater abundance of Tregs in UCB than peripheral blood [476]. Based upon preliminary observations, it has been found that autologous cord blood transfusion is helpful in slowing down the loss of endogenous insulin production and is a safe procedure in T1D children [480]. Further, it has also been documented that highly functional populations of Tregs are available in UCB and this increased Treg population may be available in the peripheral blood of subjects after more than 6 months of cord blood infusion as evidenced by mechanistic studies [480]. Autologous UCB transfusion in T1D pediatric patients has also been reported to be safe [481]. As the, collection and banking of UCB is becoming widespread all over the world, its utility as a source of therapeutic Tregs is expected to rise further.

8.5 Fibroblasts

Attempts to determine efficacy of stable IDO-expressing dermal fibroblasts in cellular therapy of autoimmune diabetes have been tried in NOD mice. IDO-expressing fibroblasts were found to significantly reduce islet infiltration by immune cells. Diabetes progression was reversed by inhibiting autoreactive CD8+ T cells and Th17 and through the induction of Tregs. Additionally, it was also observed that when IDO-expressing fibroblasts were cultured with islet β-cells they successfully reduced IL-1β levels and β-cell apoptosis [482].

9 Combinatorial Therapies

The accessory cells and biomaterials can provide a definite therapeutic benefit to save islets and their functional improvement. Currently, majority of the combinatorial approaches have been explored in islet transplantation, although, most of them are in experimental phases. The main goal is to recreate an islet friendly niche in a carrier or capsule to provide β-cell interactions within its native environment i.e. creating a microenvironment that includes accessory cells, proteins, as well as the local immunosuppression enclosed within a biocompatible material along with the islet cells. For the purpose, several accessary cells and therapies have been proposed and tested to achieve successful transplantation.

9.1 Cell Encapsulation

Cell encapsulation is a concept by which cells are encased within a biocompatible matrix. In this way a barrier against immune cells and cytotoxic molecules is created to prevent injury and hence avoid rejection while still allowing the active diffusion of essential molecules like oxygen, nutrients and hormones [483]. This way, other β-cell sources (e.g., xenogeneic islets and stem cell–derived β-cells) can also be used for clinical therapy [484]. In a previous report, vortex-induced silk hydrogels have been documented to provide a 3D environment for islets encapsulation *in vitro* thereby allowing the co-encapsulation of proteins found in extracellular matrix and secondary stromal cells to maintain function and viability of islet cells [485]. In a study by Borg et al. (2011) star-PEG-heparin cryogel scaffolds which are tunable in architecture, mechanical characteristics and biomolecular functionalization, and having the ability to load accessory cells, have been reported as highly promising supportive carriers for pancreatic islets in the context of transplantation in various alternate sites [484].

Although encapsulated islet transplantation has been supported in various animal model studies, the process has several limitations such as biocompatibility of encapsulation material, the damaging actions of cytokines, oxygen deficiency in implanted tissue at the transplantation sites and hindered secretion of insulin from capsules, which still remain to be solved [486]. The biggest of these problems is prevention of islet revascularization and oxygen transport to islets. This is associated with development of a hypoxic core within the islets that may result in reduced tissue function and ultimately, death. Therefore, several approaches to enhance microencapsulated islet survival and function have been proposed. For instance, incorporating a perfluorocarbon emulsion into alginate microcapsules to enhance oxygen permeability may help protect islets from hypoxia. Another approach is scattering the islets and allowing them to recluster into smaller size than the original islet. These smaller clusters are less likely to develop a necrotic core and they can function normally because of adequate oxygen supply and better cell-cell communication. Further, 10,000~20,000 IEQ/kg placed in a collagen matrix in stainless steel mesh tubes, with a polytetrafluoroethylene rod in the cassette have been successfully used in 11 T1D patients. This approach resulted in decrease in exogenous insulin requirements in more than 50% patients for up to 4 years [487]. Cadaveric human islets encapsulated in alginate microcapsules transplanted into T1D subject have also shown some beneficial effects [488]. However, fibrotic reactions still occur in alginate microcapsule leading to graft rejection.

9.2 Use of Accessory Cells

As it is known that islet transplantation is gradually becoming a popular diabetes therapeutic strategy, therefore, another emphasis of research is promoting angiogenesis and increasing blood vessels density around transplanted islets. In a recent study by Cao et al. (2016) the combination of allogeneic islet transplantation and

bone marrow mesenchymal stem cells (BM-MSCs) was pursued into NOD mice to investigate the effect of BM-MSCs in transplanted islet function and neovascularization. It was observed that BM-MSCs can migrate to transplanted islets along with promoting neovascularization. In addition, BM-MSCs enhanced immune tolerance of the allograft by improving lymphocytic chimerism of the donor [489]. The endothelium is also known to play an important role in the native islets function and revascularization process after islet transplantation. Endothelial progenitor cells (EPCs) are a population of rare circulating cells in the, cord blood, vessel walls, peripheral blood and bone marrow with the ability to adhere to endothelium at sites of hypoxia with subsequent differentiation into endothelial cells. EPCs/islet co-transplantation, have shown beneficial effects on islet transplantation in rodent models of diabetes [490, 491]. EPCs mediate their functions via direct differentiation into new vessels and pericytes, through secretion of paracrine factors (angiogenic and β-cell mitogenic) [492], via thrombospondin (Tsp)-1-mediated activation of TGF-β1, [493, 494] and through modulation of the expression of the β-cell gap junction protein connexin, a key element in coordinating β-cell function [491] resulting in enhanced insulin secretion.

The adoptive transfer of Tregs as accessory cells can be used to improve islet graft survival, as inflammatory immune response to alloantigens and recurrence of autoimmunity following islet transplantation are the major contributors to pancreatic islet transplant dysfunction. Experimental studies in murine models demonstrate that co-transfer of Tregs and islets can improve the graft survival [495]. Golab et al. (2014) have shown that, the anchoring of human *ex vivo* expanded Tregs to the surface of human pancreatic islets creates an immune barrier and decreased immunogenicity of the islets was shown *in vitro* [496] and the group is currently working on translating this work in animal models.

Alternatively, immune privilege can also be induced locally by accumulating immune-suppressive Tregs at the site of islet transplantation as done by Vågesjö et al. (2015), they co-transplanted islets with a plasmid encoding the chemokine CCL22 into the muscle of MHC-mismatched mice. Myocyte pCCL22 expression and secretion resulted in local accumulation of Tregs, which resulted in significantly fewer effector T-lymphocytes in close proximity to the islets, leading to delayed graft rejection [497]. However, data on human studies on efficacy of autologous Tregs in prevention of effector T cell mediated destruction of islets is very scarce. Several clinical trials have been completed or in process to evaluate different strategies of cell-based therapies in T1D patients some of which are summarized in Table 5.

10 Conclusions

The pathogenesis of T1D is a highly complex process involving various cellular entities and mechanisms, in addition to predisposing genetic factors and environmental triggers. While it is still unknown that how the central tolerance to β-cells is broken, the role of various immune cells infiltrating the pancreas at various stages

Table 5 Major clinical trials on cell-based therapies in type 1 diabetes

Study	Intervention	Phase	Status
NCT00873925	Transfusion of autologous umbilical cord blood plus vitamin D and omega 3 fatty acids to preserve β-cells function in children with recent onset type 1 diabetes	Phase 1	Completed (April 1, 2013)
NCT00468403	Islet transplantation in type I diabetes with LEA29Y (Belatacept) maintenance therapy (CIT-04)	Phase 2	Completed (march 9, 2016)
NCT01379729	Transplantation of encapsulated β-cells	Phase 2	Ongoing
NCT02763423	Umbilical cord mesenchymal stem cell	Phase 2	Ongoing
NCT00160732	Intraportal infusion of allogenic islet cells	Phase 1 & Phase 2	Ongoing
NCT01897688	Islet cell transplant	Phase 3	Ongoing
NCT00790257	Encapsulated human islets in a "Monolayer Cellular Device"	Phase 1	Completed (April 13, 2016)
NCT00708604	Islet after kidney transplantation (IAK)	Phase 1	Completed (July 2, 2014)
NCT02803905	Allogeneic islet cells transplanted into the Omentum	Phase 2	Ongoing
NCT00530686	Islet cell transplantation	Phase 1	Ongoing
NCT01630850	Islet transplantation in patients with "Brittle" type I diabetes		Ongoing
NCT00014911	Islet transplantation using the Edmonton protocol of steroid free immunosuppression	Phase 2	Completed (June 4, 2014)
NCT01210664	*Ex vivo* expanded human autologous polyclonal regulatory T cells	Phase 1	Ongoing
NCT00445913	Autologous dendritic cell therapy for type 1 diabetes suppression: A safety study	Phase 1	Completed (February12, 2016)
NCT02354911	Immunoregulatory dendritic cells	Phase 2	Ongoing
NCT02772679	Treg+IL-2	Phase 1	Ongoing

of disease process is getting clearer. Availability of latest technologies such as two photon and intravital microscopy, multicolor flowcytometry, single cell analysis and proteomics have thrown more light and provided more clearer and detailed insight of the islet infiltrates and their phenotype. Studies with animal models, mainly NOD mice and human subjects have provided abundant information and data about the mediators of the disease. Most of the studies have confirmed the role of T cells as principle mediators of β-cell damage, however, at the same time the role of previously unknown immune cells such as pDCs, NKT cells, ILCs is also coming into picture. The previously known CD4+ T and CD8+ T effector cells are now characterized in a better way and novel auto-antigens and modifications in antigens, such as PTM and peptide fusion have been identified. All this information has provided newer therapeutic targets and novel cellular modalities in targeting the disease. It is now becoming clear that antigen specific approaches, such as induction of PPI spe-

cific Tregs have better prospects in immunoprotection of β-cells, as compared to generalized approaches. Further, improvements in islet isolation and use of accessory cells in various clinical studies have provided momentum in strategies aimed at β-cell replacement or regeneration. Although, we are still far away from the ultimate goal i.e. complete treatment of T1D, recent developments have been quite encouraging and show better prospects for the future.

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