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Genetic vaccination for re-establishing T-cell tolerance in type 1 diabetes

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Key words: immunoregulation, gene transfer, autoimmunity, plasmid DNA, viral vectors

Abbreviations: AAV, adeno-associated virus; Ad, adenovirus; APC, antigen presenting cells; AAT, α1-antitrypsin; AS-ODN, antisense oligonucleotides; DC, dendritic cell; ds, double stranded; GAD65, glutamic acid decarboxylase 65; GFP, green fluorescent protein; HO-1, heme oxygenase-1; Treg, immunoregulatory T cells; i.m., intramuscular; i.p., intraperitoneal; i.v., intravenous; LCMV, lymphocytic choriomeningitis virus; mIP, mouse insulin promoter; NM, nonmitogenic; NOD, nonobese diabetic; PLN, pancreatic lymph nodes; pDNA, plasmid DNA; ss, single stranded; STZ, streptozotocin; T1D, type 1 diabetes

Type 1 diabetes (T1D) is a T cell-mediated autoimmune disease resulting in the destruction of the insulin-secreting β cells. Currently, there is no established clinical approach to effectively suppress long-term the diabetogenic response. Genetic-based vaccination offers a general strategy to reestablish β cell-specific tolerance within the T cell compartment. The transfer of genes encoding β cell autoantigens, anti-inflammatory cytokines and/or immunomodulatory proteins has proven to be effective at preventing and suppressing the diabetogenic response in animal models of T1D. The current review will discuss genetic approaches to prevent and treat T1D with an emphasis on plasmid DNA- and adeno-associated virus-based vaccines.

Introduction

Type 1 diabetes (T1D) is characterized by the autoimmune-mediated destruction of the insulin producing β cells residing in the pancreatic islets of Langerhans.[1-4] The disease process is viewed as a chronic inflammatory response of the islets, typically progressing over a number of years until the functional mass of β cells is insufficient to meet the body's insulin needs. It is well established from studies carried out in spontaneous rodent models of T1D, such as the nonobese diabetic (NOD) mouse, that the primary mediators of β cell destruction are $CD4^+$ and $CD8^+$ T cells.[5-7] Indirect evidence for a role for T cells in human T1D is provided by detection of increased β cell-specific $CD4^+$ and $CD8^+$ T cells in peripheral blood lymphocytes of at risk and/or diabetic individuals, and the presence of T cell infiltrates in the islets of pancreatic specimens from diabetic cadavers.[8-11] The breakdown of β cell-specific tolerance is complex, involving both genetic and environmental factors, which contribute to dysregulation of mechanisms promoting T cell tolerance.[12-14]

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The latter is marked by increased development of type 1 $CD4^+$ and $CD8^+$ effector T cells characterized by the secretion of pro-inflammatory cytokines such as IFNγ and TNFα.[15] The apparent skewed differentiation of naïve β cell-specific T cells towards pathogenic type 1 effectors correlates with reduced numbers and/or function of immunoregulatory T cells (Treg), and/or reduced sensitivity of established type 1 T effectors to Treg-mediated regulation.[11,16-21] A number of subsets of Treg have been identified which are defined by the: (1) type of cytokine(s) secreted, (2) effector function(s) employed to regulate an immune response and (3) overall potency.[22]

To date most immunotherapies have focused on reestablishing the functional balance between pathogenic type 1 T effectors and Treg to prevent and/or treat T1D. In the clinic the most promising results have been achieved with non-mitogenic (NM) anti-CD3 antibodies administered to recent onset diabetic patients. β cell mass is maintained in these patients; however, protection is relatively short-lived and is associated with transient depletion of T cells which may lead to recurrent viral infections.[23-25] Other strategies of immunotherapy have been tested in experimental models and the clinic. Antigen-specific immunotherapies have proven to be effective at preventing overt diabetes in NOD mice and transgenic models of T1D, but clinical findings have largely been disappointing with only recent studies providing cause for optimism.[26-36] This approach is appealing since administration of β cell antigens or peptides under various conditions can be used to selectively manipulate β cell-specific T cell reactivity, with minimal if any effect on the "normal" function of the immune system. Depending on the protocol, administration of β cell antigen may lead to: (1) T cell deletion or induction of a state of unresponsiveness (e.g., anergy), and/or (2) differentiation and expansion of Treg. Clonal anergy or deletion induced by high dose soluble antigen for instance, is exquisitely specific for those T cells recognizing the injected antigen.[37] However, at late stages of disease progression when pathogenic $CD4^+$ and $CD8^+$ T cells recognize multiple autoantigens and epitopes, anergy/deletion of a select pool of T cell clones is typically ineffective.[37,38] Accordingly, promoting Treg differentiation and/or expansion has generally been the preferred outcome.[38-41] Once

established, Treg can traffick to the islets and draining pancreatic lymph nodes (PLN) and through secretion of cytokines regulate β cell autoimmunity independent of the antigen-specificity of the pathogenic effector T cells.[38-42] Nevertheless, the efficacy of antigen-based immunotherapy generally wanes at late pre-clinical and clinical stages of intervention partly reflecting the increased numbers of pathogenic type 1 T effectors, and the need for a sufficiently large frequency of Treg.[40,42-44] Administration of cytokines to promote differentiation and/or expansion of different subsets of Treg has also proven to be effective in preventing overt diabetes in NOD mice. For instance, ongoing β cell autoimmunity is suppressed in NOD mice treated with recombinant IL-4 and IL-10 and the subsequent induction of IL-4 and IL-10 secreting Treg, respectively.[45,46] In addition, diabetes is prevented in NOD mice receiving IL-2-antibody complexes which promotes expansion of highly potent Treg expressing the transcription factor FoxP3 (FoxP3$^+$Treg).[47,48] However, the pleiotropic effects of cytokines administered systemically are an important concern, especially if the cytokines need to be administered long-term to maintain protection.

Genetic vaccines offer a strategy to enhance the efficacy of antigens, cytokines and other immunomodulatory proteins used to reestablish T cell self-tolerance. Transfer of genes obviates the need to express, purify and store recombinant proteins. Furthermore, genetic vaccination enables greater flexibility in manipulating the nature of a T cell response, in addition to directly modifying in vivo the "tolerogenicity" of the target tissue (e.g., β cells). We will review the most studied approaches of genetic vaccination used to prevent and/or suppress β cell autoimmunity, in addition to highlighting the respective strengths and weaknesses of these strategies.

Clinial Scenarios for Immunotherapy in T1D

There are three general clinical scenarios in which immunotherapy can be applied to suppress β cell autoimmunity and reestablish tolerance within the T cell compartment.[41,43,49-51] Firstly, immunotherapy can be used to prevent the onset of clinical diabetes in at risk individuals. These individuals are identified by detection of autoantibodies specific for various islet and β cell antigens in serum, in addition to altered insulin responses upon glucose challenge. Secondly, immunotherapy can be applied for the purpose of rescuing residual β cell mass in recent onset and long-term diabetic subjects. At the time of clinical diagnosis a sufficient amount of functional β cell mass persists so that remission of diabetes may be induced if islet inflammation is suppressed.[50] Furthermore, indirect evidence suggests that protection of even minimal β cell mass in chronic diabetic patients can result in more efficient glycemic control.[52] Finally, immunotherapy can be applied in the context of β cell replacement in chronic diabetic individuals. Recently islet transplantation has proven to be a feasible strategy to provide a "cure" for chronic diabetic patients.[51] However, long-term survival of islet grafts depends on persistent tolerance within the pool of β cell-specific T cells. Similarly, efforts to promote β cell regeneration/expansion in vivo are only possible with suppression of the diabetogenic response.

Importantly, the efficacy of a given immunotherapy to suppress β cell autoimmunity is dictated by the number of pathogenic type 1 effector T cells present, and the overall proinflammatory *milieu* that is established in the islets at the time of intervention. In this regard the most stringent conditions are expected at late preclinical and clinical stages of disease progression.

Genetic Vaccination to Manipulate β Cell-Specific T-Cell Reactivity

To date the use of genetic vaccines to suppress β cell autoimmunity has been studied largely in NOD mice and murine transgenic models of T1D; only recently has this approach been assessed in the clinic. In general, two strategies of genetic vaccination have been studied in depth; namely plasmid DNA (pDNA)- and viral vector-based vaccines (**Tables 1 and 2**). Recently, a third genetic approach entailing the use of antisense oligonucleotides (AS-ODN) has also proven to be effective for manipulating β cell autoimmunity.

Application of pDNA vaccination to induce β cell-specific T cell tolerance. pDNA vaccines have been mostly studied for infectious diseases and cancer, with more recent efforts focusing on autoimmunity.[53-55] Intramuscular (i.m.) injection of soluble or "naked" pDNA results in significant levels of protein expression of the encoded transgene that may persist for 6 weeks or longer.[53,56] pDNA vaccines are considered to be safe, in that pDNA fail to integrate into the host genome, exhibit limited immunogenicity, and are well tolerated in the clinic.[53,56] From a production standpoint, pDNA are readily manufactured and stored.[57] However, the in vivo transfection frequency of pDNA is low, and different cell types are transfected which may lead to varying levels of transgene expression.[53-56] Different strategies of delivery have been used to increase the efficiency of pDNA transfection. Transfection is markedly enhanced via "gene gun" vaccination for instance, which involves bombardment of the epidermis of the skin with pDNA-coated gold particles.[58-60] pDNA complexed with cationic polymers or liposomes or the use of electroporation have also been used to increase transfection efficiency.[61,62] Nevertheless, pDNA-induced antibody or T cell responses specific for foreign or tumor antigens have generally been weak and/or transient in human subjects.[53,54] The latter, however, may in fact be beneficial for preventing and treating autoimmunity, where exacerbating an ongoing pathogenic response must be avoided.

Distinct approaches of pDNA vaccination have been used to immunoregulate β cell autoimmunity (**Table 1**). For instance, i.m. injection of pDNA encoding CCL4 or CXCL10 to young NOD mice results in the induction of neutralizing antibodies specific for the respective chemokines.[63,64] Consequently, T cell trafficking to the islets is blocked and the development of diabetes prevented. This approach, however, is limited by the lack of specificity for the autoimmune response.

Induction of β cell-specific Treg differentiation and/or expansion has typically entailed the use of pDNA encoding anti-inflammatory cytokines, β cell autoantigens or the combination of both. Delivery of a short course of pDNA encoding IL-4 or

Table 1. Approaches of pDNA vaccination for reestablishing β cell tolerance

Approach	Transgene	Efficacy at blocking β cell autoimmunity		
		Preclinical		Clinical
		Early	Late	
Chemokine Neutralization	CCL4	+[64]		
	CXCL10	+[63]		
Cytokine-induced Treg	IL-4	+[65]		
	IL-10	+[66]		
	IL-4 + IL-10	+[128]		
	TGFβ1		+[70]	
Cytokine Neutralization	IFNγR-Ig	+[71]		
β cell autoantigen-induced Treg	Intracellular GAD65	+[83,129,130]	+[129]	
	Secreted GAD65	+[83]		
	GAD65IgFc		+[60]	
	Insulin B chain	+[73,74]		
	Proinsulin		+[75]	
	HSP60		+[77]	
Combined pDNA vaccination	Intracellular GAD65 + IL-4		+[80]	
	GAD65IgFc + IL-4		+[67]	
	GAD65IgFc + IL-10		+[68]	+[69]
	GAD65IgFc + IL-4 + IL-10		+[68]	+[69]
	GAD65-Proinsulin fusion + mCD80	+[81]		
	Proinsulin + mCD80	+[131]		
	Secreted GAD65 + BAX		+[132]	
	GAD65 (intracellular) + NMαCD3			+[125]
	Proinsulin + αCD40L	+[76]		

IL-10 to NOD mice early in the diabetogenic response, results in a transient increase in systemic levels of the respective cytokines and prevention of overt diabetes.[65,66] These cytokines influence both the differentiation of type 1 and Treg effectors, and block the activation/maturation of antigen presenting cells (APC) such as dendritic cells (DC) and macrophages. However, when administered at later preclinical stages of the diabetogenic response, the efficacy of IL-4 and IL-10 encoding pDNA is reduced.[67-69] In this instance, islet infiltration is unaffected and diabetes continues to develop in the treated NOD mice. Failure to suppress β cell autoimmunity under increasingly stringent conditions partly reflects inadequate cytokine levels established in the relevant target tissues, namely the islets and draining PLN. The relative immunoregulatory potency of IL-4 and IL-10 may also be a key factor. For example, overt diabetes is prevented in NOD mice at a late preclinical stage of T1D following i.m. injection of pDNA encoding TGFβ1.[70] However, protection is dependent on repeated pDNA injections raising the concern that elevated levels of systemic TGFβ1 long-term may impair normal immune function. An alternative cytokine-based strategy has been to neutralize a given proinflammatory cytokine by administration of pDNA encoding the corresponding soluble receptor. Vaccination with pDNA expressing a soluble fusion molecule consisting of the IFNγ receptor (IFNγR) and IgG-Fc domain prevents islet infiltration and autoimmune diabetes induced by multiple low dose injections of streptozotocin (STZ) in NOD mice.[71] However, systemic and persistent expression of the IFNγR-Ig fusion molecule may again impair protective type 1 T cell-mediated immunity specific for pathogens.

Delivery of pDNA encoding β cell autoantigens has proven to be effective at selectively blocking β cell autoimmunity. pDNA encoding insulin B chain, proinsulin, glutamic acid decarboxylase 65 (GAD65) and heat shock protein 60 (HSP60) suppress autoimmunity at various stages of disease progression in NOD mice.[67-69,72-77] A number of factors, however, impact the efficacy of pDNA-mediated β cell specific tolerance. The context of β cell autoantigen expression is a key parameter determining overall efficacy. Induction of Treg by pDNA encoding antigens that are intracellularly expressed is dependent on direct transfection of APC and/or cross-presentation by professional APC of antigen derived from transfected cells, such as myocytes in the case of i.m. injection of pDNA.[53] Consequently the number of professional APC such as DC which process and present the corresponding epitopes to T cells may be low, thereby limiting the induction of a sufficient pool of β cell-specific Treg. Increased doses and repeated injections of pDNA may enhance efficacy.[75] An alternative approach has been to engineer β cell autoantigens that are secreted.[67,78,79] The frequency of GAD65-specific Th2 cells and subsequent diabetes prevention are markedly increased in NOD mice injected i.m. with pDNA encoding a secreted GAD65-IgFc

Table 2. Viral-based vectors primarily used in the treatment of Type 1 diabetes

Viral vector	Approach	Ex vivo islet transduction	i.p./i.v./i.m. delivery	In vivo pancreas transduction
			Transgene	
Adenovirus				
	Antigen:	N.D.	proinsulin[133]	N.D.
	Immune modulating cytokines:	TGFβ[134,135]	TGFβ[135,136]	N.D.
		TNFα[137]		
		IL-12p40[137,138]		
		IL-4[139]	IL-4[140]	
		IL-10[139]		
	Pro-inflammatory modulators:	Indoleamine 2,3-dioxygenase[141]	N.D.	N.D.
		IL-1Rα[142-144]		
		vascular endothelial growth factor[142,144]		
		hepatocyte growth factor[143]		
		human Fas ligand[145,146]		
		insulin-like growth factor 1[147]		
	Co-stimulatory blockade:	CTLA-4[134]	N.D.	N.D.
		CTLA-4Ig[134,137]		
rAAV				
	Antigen:	N.D.	GAD[95,96]	N.D.
		N.D.	preproinsulin[97]	N.D.
	Immune modulating cytokines:	IL-4[148]	IL-4[98]	IL-4[116]
		IL-10[100]	IL-10[98-101,149,150]	IL-10[116]
	Pro-inflammatory modulators:	N.D.	HO-1[103]	glucagon-like peptide 1[117]
			AAT[102]	IκB[116]
	Co-stimulatory blockade:	N.D.	N.D.	N.D.
Lentivirus				
	Antigen:	N.D.	proinsulin[151]	N.D.
	Immune modulating cytokines:	IL-4[152]	N.D.	N.D.
		TGFβ[134]		
	Pro-inflammatory modulators:	thioredoxin[153]	N.D.	N.D.
		IL-1Rα[154]		
		cFLIP[155]		
	Co-stimulatory blockade:	CTLA-4Ig[134]	N.D.	N.D.
		CTLA-4[134]		

N. D., Not Determined.

fusion molecule compared to native GAD65 expressed intracellularly.[67] Secretion of a β cell autoantigen by pDNA transfected cells ensures widespread distribution in vivo, and in turn an increased frequency of APC that endocytose, process and present the corresponding epitopes.

Administration of pDNA encoding a β cell autoantigen alone has proven to be insufficient at suppressing islet inflammation at later stages of disease progression; in fact β cell autoimmunity may be exacerbated.[67,72] Accordingly, one approach has been to co-administer pDNA encoding antigen and anti-inflammatory cytokines to "shape" the nature of the T cell response.[67-69,80] Intramuscular injection of pDNA encoding GAD65-IgFc and IL-10 suppresses β cell autoimmunity and protects syngeneic islet grafts in diabetic NOD recipients.[68,69] In this case co-injection of pDNA encoding IL-10 enhances differentiation of GAD65-specific IL-10-secreting and FoxP3-expressing Treg, and may also potentiate protection by downregulating pathogenic effector T cells and islet resident APC.[68,69] Co-injection of pDNA encoding a modified CD80 molecule that binds to CTLA-4-only (mCD80) has also been used to enhance β cell-specific Treg reactivity and prevent diabetes in young NOD mice.[81] The site and mode of vaccination also influences the nature of the T cell response elicited by the pDNA encoded β cell autoantigen. For instance, i.m. injection of pDNA results in preferential induction of type 1 effector T cells which can exacerbate β cell autoimmunity in NOD mice.[72] Differentiation of type 1 T effectors is

partly attributed to CpG motifs found in the vector backbone that bind Toll-like receptor 9 and promote a proinflammatory response.[53,56,57] On the other hand, delivery of pDNA to the epidermis via gene gun results in preferential induction of IL-4-secreting Th2 cells independent of CpG motifs.[58-60] Currently it is unclear why Th2 cell differentiation is selectively promoted but may be due to the tolerogenic properties of epidermal Langerhans cells.[82] Similarly, the tolerogenic nature of mucosal tissues can be exploited to manipulate T cell reactivity by delivering pDNA via intranasal or oral routes.[76,83] In this way induction/expansion of Treg versus pathogenic type 1 T effectors can be further enhanced.

Promising results have been obtained in a recent phase I/II randomized, dose escalation trial in which diabetic patients receive weekly i.m. injections of pDNA encoding full-length human proinsulin.[84] Notably, β cell function as determined by insulin C-peptide levels is maintained over a 12 month period in patients vaccinated with the pDNA encoding proinsulin, leading to improved glycemic control compared to subjects receiving the placebo control. The proinsulin encoding pDNA vaccine is well tolerated and efficacy correlates with reduced anti-insulin antibody titers.[84]

Viral vector-based vaccination: the use of recombinant adeno-associated virus vectors to induce T cell tolerance. The majority of studies using viral vector-based vaccines have focused on immunity to infectious pathogens and tumor antigens, although this approach has been employed for prevention and treatment of T1D experimentally (**Table 2**).[85] The key advantage of this approach relative to pDNA vaccination is that viral vector-based vaccines typically transduce cells with greater efficiency in vivo. This can result in more robust expression levels of the encoded transgene, and a broader range of tissues (e.g., islets) that can be targeted in vivo. On the other hand, vector toxicity to transduced tissue and vector-specific immunity are key concerns. For instance, the efficacy of replication-defective adenovirus (Ad) vectors is reduced by pre-existing immunity to capsid proteins used to package the recombinants, which in turn affects levels and persistence of transgene expression and limits repeated injection of the recombinant.[86] In this regard, recombinant adeno-associated virus (rAAV) vectors have garnered a great deal of interest as an efficient and safe gene transfer platform.

rAAV vectors are highly amenable for gene delivery for a number of reasons. rAAV vectors transduce both dividing and nondividing cells, and exhibit broad tissue tropism with minimal toxicity that leads to long-term transgene expression in vivo without significant immunogenicity.[87,88] Furthermore, the risk of genomic insertion and insertional mutagenesis is minimal since rAAV persists as nonintegrating circular monomers or concatemers in the nucleus.[89] Clinical studies using rAAV-mediated gene transfer to complement genetic disorders have generated promising results.[90] Moreover, improved methods to engineer and produce packaged rAAV coupled with the availability of multiple serotypes to manipulate the immunogenicity of the recombinants enhance clinical application of rAAV-based gene transfer.[87,88] The development of double-stranded (ds) rAAV vectors has further improved the approach. Upon delivery, traditional single-stranded (ss) rAAV vectors become transcriptionally active upon conversion to a double stranded DNA template, which results in a slow onset of transgene expression.[91] The use of dsAAV vectors eliminates this rate limiting step to accelerate the onset and increase the level of transgene expression.[92-94] Consequently, lower doses of dsAAV versus ssAAV can be delivered to achieve sufficient levels of transgene expression.

rAAV vectors have been applied in multiple ways to block β cell autoimmunity in NOD mice and other models of T1D (**Table 2**). Intramuscular injection of rAAV1 or rAAV2 vectors and systemic expression of β cell autoantigens (e.g., proinsulin, GAD65),[95-97] and cytokines (e.g., IL-10),[98-100] suppresses ongoing β cell autoimmunity at both early and late preclinical stages, and prevents overt diabetes in NOD mice via induction of Treg. Furthermore, i.m. delivery of a rAAV vector expressing IL-10 protects syngeneic islet grafts implanted into diabetic NOD recipients, demonstrating that the approach is robust even at clinical stages of T1D.[101] Established β cell autoimmunity in NOD mice is also suppressed by i.m. delivery of rAAV encoding anti-inflammatory molecules such as human α1-antitrypsin (AAT)[102] a serine protease inhibitor and heme oxygenase-1 (HO-1)[103] a stress-response enzyme that catalyzes the degradation of heme to free iron, carbon monoxide and biliverdin. Here protection is mediated primarily due to the effects of AAT and HO-1 on innate effector cells. Depending on the dose and transgene, the encoded proteins by a given rAAV can be detected several weeks post-injection.[95-103] Whether sustained expression of high systemic levels of these proteins compromises normal immune function, however, has not been assessed. Inducible promoters to regulate transgene expression can be used to address this potential concern. Intramuscular injection of rAAV encoding an AAT transgene driven by a tetracycline/doxycycline inducible promoter results in increased AAT expression and suppression of collagen-induced arthritis when mice are fed doxycycline containing chow.[104] Notably, the level and length of time of gene expression can be effectively manipulated with an inducible promoter so that tolerance can be established and maintained in a safe manner.

A major feature of rAAV-based vaccination is the ability to directly modify the tolerogenicity of β cells in vivo in a cell-specific manner. In this way, possible complications associated with systemic expression of an immunoregulatory molecule are obviated. In addition, direct expression of a given protein in the islets may more readily establish immunotherapeutic levels that otherwise are not attained via a systemic route. The latter may also reduce the required dose of rAAV thereby minimizing the possibility of inducing immunity to the recombinant. Studies have shown employing rAAV encoding green fluorescent protein (GFP) that the efficiency of in vivo transduction of pancreatic tissue is influenced by the serotype of the capsid proteins used for packaging, and the route of rAAV delivery. For instance, rAAV8 vector is highly efficient at transducing murine β cells and acinar cells of the exocrine pancreas when administered via i.v. or intraperitoneal (i.p.) routes, whereas rAAV6 vector is the preferred choice for pancreatic intraductal infusion.[105] Importantly, rAAV transduction has no effect on β cell function.[105-108] It is

noteworthy that studies of viral capsid protein structure and the corresponding receptors have led to the engineering of tissue-specific capsids. Random peptide ligand libraries have been used to generate AAV capsid proteins specific for tissues previously resistant to rAAV infection.[110-112] Furthermore, pseudotyped rAAV have been established in which relevant amino acid sequences from different capsid proteins are swapped to create a tissue-specific chimeric recombinant.[113-115] These strategies may lead to the future development of capsid proteins that promote efficient, "β cell-only" transduction by rAAV vectors.

Currently since rAAV serotypes that efficiently transduce β cells also transduce other tissues, it is necessary to engineer rAAV vectors with an appropriate promoter to target transgene expression in a tissue-specific manner. Here, the use of an insulin II promoter (IP) has proven to be highly effective for tightly-regulated and stable β cell-specific expression of rAAV encoded transgenes.[105,116] Evidence that T1D can be manipulated by targeting β cells in vivo is provided by a study in which dsAAV8 recombinants encoding IL-4 and IL-10 transgenes driven by a mouse IP (mIP) were administered i.p. to young NOD mice.[116] Diabetes is prevented in NOD mice receiving dsAAV8-mIP-IL4, which correlates with reduced islet infiltration and an increase in FoxP3$^+$Treg in the periphery. Interestingly, no effect on β cell autoimmunity is detected in dsAAV8-mIP-IL10-treated NOD mice. These observations suggest that local versus systemic expression of a cytokine can have markedly different effects on β cell autoimmunity. For instance, in contrast to β cell-specific expression, rAAV-driven systemic expression of IL-10 but not IL-4 protects NOD mice from diabetes.[98] Importantly, the above study provides proof-of-principle that rAAV can be used to modulate the tolerogenicity of β cells in vivo. Whether this strategy is sufficiently robust under more stringent conditions (e.g., late preclinical or clinical stages of T1D) still needs to be determined. In addition, the efficacy of other anti-inflammatory cytokines and/or immunomodulatory molecules need to be tested. It is noteworthy that i.p. injection of dsAAV8 carrying a mIP-driven transgene encoding glucagon-like peptide-1 blocks autoimmune diabetes induced by STZ in BALB/c mice.[117]

Finally, rAAV vector-based vaccination may also be applied for genetically modifying islet grafts ex vivo for the purpose of inducing transplantation tolerance. Feasibility for this general approach has been provided by numerous studies using Ad vectors (**Table 2**). Genes encoding cytokines, anti-inflammatory and anti-apoptotic proteins and molecules to block T cell co-stimulation (e.g., CTLA-4Ig) have been successfully used to increase islet graft survival.[118] Although enhanced, islet graft survival is nevertheless transient due in part to low transduction efficiencies and the immunogenicity of Ad vectors. Accordingly, dsAAV vectors are well suited for modifying the tolerogenicity of islet grafts due to limited immunogenicity and rapid transgene expression. dsAAV packaged in serotypes 2, 6 and 8 efficiently transduce human islets without impairing β cell function.[109]

Application of antisense therapy to induce T cell tolerance. Transfer of genes encoding autoantigens and immunomodulatory proteins has been the predominate approach to genetically manipulate autoimmunity in general and T1D specifically. An alternative strategy, however, is to block expression of relevant genes by targeting RNA. A number of different approaches including ribozymes, DNAzymes, aptamers and AS-ODN have been used to mediate "antisense therapy".[119,120] Of these approaches, the use of AS-ODN is arguably the most direct therapeutic strategy and multiple clinical trials testing AS-ODN in for example hematology, oncology and neuromuscular diseases are ongoing.[120] AS-ODN are single-stranded DNA molecules designed to specifically hybridize to the complementary RNA. Upon binding, AS-ODN block the function of mRNA by altering splicing events, inhibiting protein translation by influencing ribosome assembly, and/or eliciting endogenous RNase H enzymes.[120]

AS-ODN have been successfully used to modify the stimulatory capacity of DC either ex vivo or in vivo and in this way suppress β cell autoimmunity in NOD mice. DC uptake of AS-ODN specific for CD40, CD80 and CD86 blocks expression of these co-stimulatory molecules and establishes a robust tolerogenic phenotype.[121] Diabetes is prevented in NOD mice following a single injection of bone marrow-derived DC treated with AS-ODN, and protection correlates with an increase in Treg.[121] Currently, a phase I clinical trial is underway to test the safety of ex vivo expanded, autologous DC treated with AS-ODN and injected into diabetic patients.[122] The potential of antisense therapy to treat T1D has been further demonstrated in a study examining the efficacy of microspheres containing CD40, CD80 and CD86 AS-ODN injected into recent onset diabetic NOD mice.[122] Here the microspheres were delivered at a site anatomically proximal to the PLN in an attempt to enhance targeting of the relevant pool of DC. The AS-ODN induced an increased frequency of FoxP3$^+$Treg and diabetes was in fact reversed in some NOD mice.[122] Although the efficiency of in vivo uptake of AS-ODN by DC and selective targeting of tissue-specific DC are key issues that still need to be resolved, the above findings provide evidence that this strategy can be effective even under the most stringent of treatment conditions.

Concluding Remarks

Preclinical studies provide evidence indicating that genetic vaccination in general and specifically pDNA- and AAV vector-based vaccines and AS-ODN are effective at reestablishing β cell-specific T cell tolerance. Each of these strategies has key strengths. pDNA vaccination offers a relatively facile and safe strategy to express proteins and modulate β cell autoimmunity systemically. In addition the nature of the T cell response can be readily manipulated by co-delivery of pDNA encoding antigen, cytokines and/or anti-inflammatory modulators. The recent phase I/II trial studying administration of proinsulin encoding pDNA to diabetic patients[84] and encouraging findings from a phase II trial in which multiple sclerosis patients were treated with myelin basic protein expressing pDNA,[123] provide evidence suggesting that pDNA vaccination may indeed be effective to manipulate T cell-mediated autoimmunity in the clinic. rAAV vector-based vaccination on the other hand offers an approach to directly modify and enhance the tolerogenicity of β cells in vivo

and ex vivo. dsAAV vectors can be used to express immunoregulatory proteins specifically in β cells in vivo by choosing: (1) the appropriate route of delivery, (2) capsid proteins that preferentially transduce islets and (3) promoters which selectively drive transgene expression in β cells. Anti-sense therapy via AS-ODN provides a strategy to alter the phenotype and effector function of APC and possibly T cells in the periphery (e.g., PLN). Whether a given strategy of genetic vaccination alone is sufficient to establish long-term protection in patients, especially at late preclinical and clinical stages of T1D is a question that still needs to be addressed. However, genetic vaccination may also prove to be effective in the context of a combinatorial immunotherapy. In this regard, two potential scenarios can be envisioned.

In the first scenario, different genetic vaccine strategies are combined, similar to heterologous prime-boost vaccination protocols that exploit the properties of distinct vaccines to induce immunity to pathogens.[124] One possible approach for example is to induce β cell-specific Treg via autoantigen-encoding pDNA and quench the inflammatory *milieu* of the islets with dsAAV encoding an anti-inflammatory molecule(s) (e.g., IL-4, AAT, HO-1). This combination may reduce the stringency needed to suppress β cell autoimmunity at later preclinical or clinical stages of T1D in terms of the number and/or type of pDNA-induced β cell-specific Treg, and/or the efficiency of islet transduction and the level of transgene expression by the rAAV vector. Similarly, a more robust β cell-specific Treg response may be elicited by inducing tolerogenic DC in vivo via encapsulated AS-ODN targeting co-stimulatory molecule expression coupled with pDNA- or rAAV vector-encoded β cell autoantigen.

In the second scenario, genetic vaccination can be paired with other "nongenetic-based" strategies of immunotherapy. A recent study assessed the efficacy of i.m. injected pDNA encoding GAD65 combined with i.v. injected NM anti-CD3 antibody in a transgenic model of T1D in which the lymphocytic choriomeningitis virus (LCMV) glycoprotein is a neo-autoantigen expressed by β cells.[125] Diabetes is induced in this model by LCMV infection. Notably, the combination immunotherapy reverses diabetes in a greater frequency of recent onset mice relative to either approach alone. This synergy correlates with an increased GAD65-specific Treg response induced by the pDNA-GAD65 and NM anti-CD3 antibody combined treatment. The NM anti-CD3 antibody establishes conditions permissive for GAD65-specific Treg differentiation/expansion by in part depleting pathogenic type 1 T effectors and reducing the overall proinflammatory *milieu* in the islets and PLN.[126,127] These findings demonstrate the potential potency of a combinatorial immunotherapy, and establish rationale for combining genetic vaccines with approaches based on administration of other antibodies (e.g., anti-CD20) and/or immunomodulatory proteins (e.g., vitamin D).

In conclusion, genetic vaccination can be used to manipulate the diabetogenic response either systemically, and/or by directly modifying the tolerogenicity of β cells. The inherent flexibility of the approach provides immense potential for clinical application either as a stand alone or combinatorial immunotherapy. Continued preclinical and clinical studies, however, are needed to meet this potential. How treatment parameters (e.g., dose, route of administration) and antigen-specificity of pDNA vaccination influence β cell-specific T cell reactivity at various stages of disease progression for instance, need to be assessed in patients. In addition further preclinical development of dsAAV vectors to improve selective targeting of β cells in vivo, and identifying the most effective immunoregulatory proteins that suppress inflammation in the islets is required. Finally, improved in vivo targeting of and uptake by specific cell types (e.g., DC) is needed to enhance the clinical application of AS-ODN microspheres.

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