

DNA vaccines that induce regulatory T cells and protect against autoimmune diabetes

Review Article

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Abbreviations: antigen presenting cells, (APCs); blank vector, (VR); cyclophosphamide, (CYP); cytotoxic T lymphocyte, (CTL); dendritic cells, (DCs); glutamic acid decarboxylase 65, (GAD65); heat shock protein 60, (HSP60); immunostimulatory sequences, (ISS); induced Tr cells, (iTr); interferon γ , (IFN γ); interleukin 12, (IL-12); islet-specific glucose-6-phosphatase catalytic subunit-related protein, (IGRP); lymphocytic choriomeningitis virus nucleoprotein, (LCMV-NP); natural Tr cells, (nTr); PPIIns-GAD65, (Ins-GAD); preproinsulin, (PPIIns); regulatory T cells, (Tr); Th type 1, (Th1); Th type 2, (Th2); T-helper, (Th); Toll-like receptor 9, (TLR9); transforming growth factor beta-1, (TGF- β 1); tumor-associated antigens, (TAAs); type 1 diabetes, (T1D)

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Summary

DNA vaccination against autoantigens can protect against autoimmune diseases. In autoimmune (type 1) diabetes-prone NOD mice, or transgenic models of type 1 diabetes, disease has been ameliorated by delivery of plasmids encoding islet-cell antigens such insulin (especially the B chain), glutamic acid decarboxylase 65 (GAD65), or islet-antigen/IgG-Fc fusion constructs. Frequently, beneficial effects have only been noted when these genes were codelivered with another gene encoding an immunoregulatory molecule. Thus, we found that DNA vaccination with unmodified preproinsulin (PPIIns) or glutamic acid decarboxylase 65 (GAD65), was not effective. However, we were able to design protective vaccines by introducing cDNA encoding a mutant B7-1 molecule (B7-1wa) that selectively binds the negative T-cell regulator CTLA-4 (but not the positive stimulator CD28). In this case, the best target antigen was a PPIIns-GAD65 (Ins-GAD) fusion protein. We found that covaccination with Ins-GAD/B7-1wa generated CD4⁺ regulatory T cells (Tr) that could transfer resistance in adoptive transfer experiments. These T cells expressed markers frequently associated with Tr cells (including CD25 and CTLA-4) and acted, at least in part, by producing TGF- β 1. We conclude that DNA vaccination based on recognition of a self antigen and coligation of CTLA-4 induces the generation of protective Tr cells, and this is a novel approach which is relevant to autoimmune disease therapy.

I. Introduction

Many forms of immunotherapy are being studied with the objective of preventing or attenuating autoimmune diseases, but gene transfer approaches are particularly promising. Genetic (or nucleic-acid-based) vaccination involves the administration of a gene that encodes the target antigen, which is then synthesized in vivo, unlike classical vaccination which depends on the direct delivery of the antigenic molecule (Prud'homme et al, 2001; Calarota and Weiner, 2004; Rodriguez, 2004). The gene can be delivered as DNA or RNA, and carried by a variety of viral and non-viral vectors. However, most investigators have performed DNA vaccination by simple injection of naked plasmid DNA into skeletal muscle or other tissues. The efficiency of gene transfer, as well as

the potency of the DNA vaccine, are greatly enhanced by electroporation applied locally after DNA injection (Dupuis et al, 2000; Widera et al, 2000). The antigen gene (as cDNA) can be delivered on the same or a separate vector with cDNA encoding an immunomodulatory molecule(s) (DNA covaccination) (Prud'homme et al, 2001; Calarota and Weiner, 2004; Rodriguez, 2004), with the goal of either enhancing, deviating, or depressing responses.

As reviewed below, DNA vaccination strategies have been employed to ameliorate autoimmune insulin-dependent diabetes mellitus (type 1 diabetes [T1D]). This approach may appear counterintuitive, as DNA vaccines usually promote rather than depress immunity, primarily because plasmid DNA carries unmethylated CpG-

containing immunostimulatory sequences (ISS) (Krieg, 2002; Klinman, 2004). The ISS bind to Toll-like receptor 9 (TLR9) expressed by dendritic cells (DCs) and some other cells, stimulating inflammatory cytokine production and initiating an innate immune response (Krieg, 2002; Klinman, 2004). Evidently, any successful vaccine against autoimmunity has to block or overcome this stimulatory element.

II. Immune mechanisms in DNA vaccination

A. The antigen presenting cell

Studies with bone marrow chimeras reveal that after delivery (intramuscular [i.m.] or other) of an antigen-encoding vector the expressed antigen is presented by bone marrow-derived antigen presenting cells (APCs) (Iwasaki et al, 1997), which are most likely DCs. It is still not totally clear how APCs acquire the antigen, but two mechanisms have been postulated. There could be direct transfection of APCs by plasmid, or uptake of protein antigen from other transfected cells (cross presentation). Cross presentation can lead to enhanced immunity (cross priming) or depressed immunity (cross tolerance). Both mechanisms of antigen uptake have been demonstrated (Ulmer et al, 1996; Casares et al, 1997; Fu et al, 1997; Chattergoon et al, 1998; Corr et al, 1999); however, there is evidence that cross-presentation is the most important pathway (Fu et al, 1997; Corr et al, 1999). For instance, it is possible to induce strong immunity against hepatitis B surface antigen by DNA vaccination with a plasmid carrying a muscle-specific desmin promoter (Kwissa et al, 2000), where antigen production is thought to be limited to myocytes. In addition, the uptake of DNA by mononuclear cells is associated with extensive DNA degradation which may impair the direct presentation pathway (Kwissa et al, 2000).

B. T-helper (Th) deviation and regulation of cytotoxic T lymphocyte (CTL) activity by DNA vaccination

In mice, immunity can be enhanced by coinjecting cytokine expression plasmids (or use of bicistronic plasmids), and can be deviated to a Th type 1 (Th1) or Th type 2 (Th2) response as reported by several investigators in infectious disease and tumor immunity models (Kim et al, 1998; Song et al, 2000a, b; Prud'homme et al, 2001; Scheerlinck, 2001; Calarota and Weiner, 2004; Rodriguez, 2004). For example, we found that i.m. injections of a plasmid encoding carcinoembryonic antigen (CEA) elicited both humoral and cellular immune responses, but only delayed the growth of transplanted syngeneic CEA⁺ tumor cells (Song et al, 2000a, b). Coinjection of the CEA vector with a vector encoding either interferon (IFN) or interleukin 12 (IL-12; bicistronic p35/p40) promoted a Th1 response, anti-CEA CTL activity and resulted in up to 80% tumor-free survival following a challenge. In contrast, coinjection of CEA and IL-4 vectors generated a Th2 response, and a decline in both CTL activity and resistance to a tumor challenge. Several cytokines are effective costimulatory adjuvants, boosting humoral as

well as Th- and CTL-mediated responses (Prud'homme et al, 2001; Scheerlinck, 2001; Calarota and Weiner, 2004; Rodriguez, 2004). Moreover, coinjection of either B7-1, B7-2 or CD40L genes, on the same or separate plasmid(s) as the antigen gene, can markedly improve the effectiveness of DNA vaccines (Rodriguez, 2004).

C. Breaking immune tolerance by DNA vaccination

DNA vaccination can break tolerance to self or transgenic neo-self antigens (Davis et al, 1997; Weber et al, 1998; Amici et al, 2000; Costagliola et al, 2000; Hawkins et al, 2000; Djilali-Saiah et al, 2002), and this is usually facilitated by covaccination with an inflammatory cytokine gene such as *IL-12*. Indeed, DNA vaccination has been used as a means of inducing autoimmunity against the liver or the thyroid gland for experimental purposes (Costagliola et al, 2000; Djilali-Saiah et al, 2002). A common maneuver promoting autoimmunity in DNA vaccination involves replacing a gene for a self antigen with one encoding a closely related xenogeneic antigen. Almost all tumor-associated antigens (TAAs) are nonmutated self molecules, and the induction of any immune response to these antigens involves breaking natural immune tolerance, which can result in autoimmune disease. As an example, melanocyte differentiation antigens are potential target TAAs for specific melanoma immunotherapy. DNA immunization against xenogeneic melanocytic antigens (e.g., gp100 and tyrosinase-related protein-1) has been exploited to break tolerance and generate anti-tumour immunity (Weber et al, 1998; Hawkins et al, 2000), and this is associated with various degrees of depigmentation (vitiligo) due to autoimmunity against melanocytes. On the other hand, as outlined below, some DNA vaccines have protected against autoimmunity.

III. DNA vaccination against autoimmune diabetes

A. Antigenic targets in NOD mice

NOD mice develop T1D, which is more frequent in females. Insulin-producing cells are destroyed in an autoimmune process characterized by infiltration of islets of Langerhans by macrophages and islet-cell antigen reactive, autoaggressive T cells (Serreze and Leiter, 2001; Adorini et al, 2002; Rabinovitch and Suarez-Pinzon, 2003). The islet inflammatory process is termed insulinitis. The disease can be adoptively transferred with T lymphocytes, provided both CD4⁺ and CD8⁺ cells are included, and diabetogenic T-cell clones reactive to various islet antigens have been isolated. The development of diabetes is greatly accelerated by administering cyclophosphamide (CYP), which induces a burst of IFN production (possibly by depleting regulatory T cells), and accentuates the autoimmune reaction.

To design a DNA vaccine against T1D it is important to select an appropriate target antigen, but this is complicated by the fact that several antigens have been identified. Islet-cell antigens include proinsulin/insulin, glutamic acid decarboxylase isoforms 65 and 67 (GAD65, GAD67), IA-2 (or related tyrosine phosphatases), islet-

specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), p69, heat shock protein 60 (HSP60), carboxypeptidase H, gangliosides and others (Simone et al, 1999; Bach, 2001; Bach and Chatenoud, 2001; Chase et al, 2004; Fourneau et al, 2004; Yang and Santamaria, 2004; Nakayama et al, 2005). GAD isoforms and insulin are the best studied antigens for vaccination (Bach, 2001; Nakayama et al, 2005; Prud'homme et al, 2005), but the relative importance of the various islet antigens has not been clear.

B. Plasmid inoculation in NOD mice

Several DNA vaccination approaches have been

protective in diabetes-prone mice (**Table 1**). One group was able to prevent diabetes by injection of a blank vector alone (Quintana et al, 2000), but other authors have not reported this effect. However, as outlined in **Table 1**, protection has been noted following DNA vaccination to HSP60 (Quintana et al, 2002), insulin B chain (Coon et al, 1999; Bot et al, 2001; Urbanek-Ruiz et al, 2001) and GAD65 (Balasa et al, 2001; Weaver et al, 2001; Jun et al, 2002). Some of the mechanisms proposed by the investigators to account for the beneficial effect are included in this table, but most often Th2 deviation has been implicated.

Table 1. DNA vaccination against autoimmune diabetes

Model of diabetes	DNA Vaccine (+a Covaccine)	Disease Severity	Putative Mechanism of protection	Reference
TG	Ins B	^a	Th2 deviation	Coon et al, 1999
RIP-LCMV ^b				
TG	PPIIns,			Karges et al, 2002
RIP-B7.1 ^c	PIns, Ins GAD65			Karges et al, 2002
NOD	Empty		Th2 deviation	Quintana et al, 2000
	HSP60		Th2 deviation	Quintana et al, 2000
NOD/CYP	HSP60		Th2 deviation	Quintana et al, 2002
NOD	Ins B9-23		IFN	Urbanek-Ruiz et al, 2001
NOD	Ins B9-23 (\pm IL-4)		Th2 deviation	Bot et al, 2001
NOD	InsA-IgG-Fc (\pm IL-4)			Weaver et al, 2001
	InsB-IgG-Fc (\pm IL-4)			Weaver et al, 2001
NOD	PPIIns			Prud'homme et al, 2002
	PPIIns (+ ^a B7-1wa) (CTLA-4 ligand)		T-cell anergy	Prud'homme et al, 2002
NOD	GAD65			Balasa et al, 2001; Glinka et al, 2003; Gauvrit et al, 2005
NOD	rVV-GAD65		Th2 deviation	Jun et al, 2002
	spGAD		IL-10 , TGF 1	Glinka et al, 2003
NOD	(secreted)			
	PPIIns/GAD65 fusion + B7-1wa (CTLA-4 ligand)		Tr cells (TGF 1)	(Glinka and Prud'homme, submitted)
NOD	GAD55		Th2 deviation	Filippova et al, 2001
NOD/CYP	(secreted)			
NOD	GAD65-IgG-Fc ^d			Tisch et al, 2001; Weaver et al, 2001
	GAD65-IgG-Fc ^d (+IL-4)		Th2 deviation	Tisch et al, 2001; Weaver et al, 2001
NOD (pro-islet transplant)	GAD65-IgG-Fc ^d (+ IL-4/IL10)		Tr cells (IL-4 , IL-10)	Seifarth et al, 2003

a.+, with covaccine; \pm , with or without covaccine; , increased; , decreased; , unchanged.

b. TG RIP-LCMV, transgenic mice with islet-restricted expression of lymphocytic choriomeningitis virus nucleoprotein under the control of the rat insulin promoter (RIP).

c. TG RIP-B7, transgenic mice with islet-restricted expression of B7.1 under the control of the rat insulin promoter (RIP).

d. GAD65-IgG-Fc, a construct of GAD65 peptides fused to an IgG-Fc segment.

C. DNA vaccination against insulin

DNA vaccination against insulin or its precursor

forms has either aggravated disease (Karges et al, 2002), or had little effect (Prud'homme et al, 2002), in NOD mice or transgenic models of T1D. On the other hand, vaccination against the B-chain only (especially the 9-23 peptide) has been more successful. Mice expressing lymphocytic choriomeningitis virus nucleoprotein (LCMV-NP) as a transgene in their cells develop T1D only after LCMV infection. Injection of plasmid DNA encoding the insulin B in these mice reduced the incidence of disease by 50% (Coon et al, 1999). This was associated with the induction of regulatory CD4 lymphocytes that reacted with the insulin B chain, secreted IL-4, and reduced the activity of LCMV-NP-autoreactive CTLs. Urbanek-Ruiz et al, (2001) vaccinated NOD mice against the B9-23 residue and observed delayed onset and a lower incidence of disease but, surprisingly, insulinitis and T-cell reactivity to insulin were not diminished. Nonetheless, there was decreased production of IFN γ by pancreatic lymph nodes in response to insulin, suggesting immune deviation. In similar experiments, Bot et al, (2001) also reported protection against diabetes, associated with expansion of IL-4-producing and, to a lesser extent, of IFN γ -secreting T cells in pancreatic lymph nodes, as well as intermolecular Th2 epitope spreading to GAD determinants. IL-4-null NOD mice still developed diabetes, implying a role for this cytokine. Unlike the females, male NOD mice did not respond favorably, but this was corrected by adding an IL-4-expressing plasmid or extension of the vaccination schedule. Taken together, these results suggest that a Th2 deviation accounts at least in part for the beneficial effect of DNA vaccination. Weaver et al, (2001) obtained contrasting results, but they used B-chain/IgG-Fc constructs which may have different immune properties.

D. DNA vaccination against GAD65

DNA vaccination against GAD65 with plasmid or viral vectors has been reported by some authors to protect against diabetes in NOD mice. GAD65 plasmid inoculation (2001) moderately ameliorated insulinitis, although T-cell responses to this antigen were not markedly altered and regulatory T cells were not generated. B7/CD28 costimulation with bicistronic GAD65/B7 plasmids abrogated the beneficial effect. A GAD65 vaccinia vector (Jun et al, 2002) shifted the autoimmune response to a Th2 type and, furthermore, splenocytes from treated NOD mice prevented the adoptive transfer of diabetes in NOD.scid recipients, revealing a role for regulatory cells.

In contrast, we found that i.m. DNA vaccination against wild-type GAD65 (full length protein) was not protective in NOD mice (Glinka et al, 2003), and others have reported detrimental effects (Gauvrit et al, 2005). The discrepancy between studies might be due to differences in vectors or methods. At any rate, in our hands there was little evidence that the procedure was modifying T-cell immunity to GAD65. This protein is cytosolic and it lacks a signal for the secretory pathway. We hypothesized that it might not be efficiently picked up and presented by APCs. We constructed an expression plasmid encoding a chimeric GAD65-derived molecule

(spGAD) with a signal peptide originating from IL-4, a secretable protein, to facilitate the release of this antigen. Immunization with an spGAD plasmid had a protective effect against diabetes, reduced insulinitis and altered the response of lymphocytes. Immunization resulted in increased production of IL-10 and TGF- β 1 by GAD65 peptide-stimulated lymphocytes *in vitro*. Moreover, spGAD plasmid-immunized mice had high serum IL-10 levels and low serum IFN γ levels compared to other groups, suggesting a systemic effect. These alterations in regulatory cytokine production were apparent both early and late after the treatment was initiated, and persisted for months after the last of multiple DNA injections. Another group has reported similar findings in CYP-accelerated NOD-mouse diabetes, with a secreted GAD55 DNA vaccine (Filippova et al, 2001). Our results do not point to classical Th2 deviation as a mechanism of disease resistance, but are more consistent with the generation of regulatory T cells that produce IL-10 and/or TGF- β 1.

Similar changes in the serum IFN γ /IL-10 ratio in NOD mice had been observed previously, and it seems likely that the ratio of these two cytokines is a relevant factor, perhaps more important than the exact level of each cytokine. We previously reported that neutralization of IFN γ by a gene therapy approach was beneficial (Chang and Prud'homme, 1999; Prud'homme and Chang, 1999). On the other hand, IL-10 has many immunosuppressive and anti-inflammatory effects that could block autoimmunity (Hawrylowicz and O'Garra, 2005). Indeed, IL-10 reduces MHC and B7-2 expression on APCs, induces T-cell anergy, promotes the differentiation of regulatory T cells, and increases T-cell apoptosis. Other investigators have reported that systemic delivery of IL-10 (Pennline et al, 1994), IL-10-Ig fusion protein (Zheng et al, 1997), or IL-10-producing islet-reactive T-cell clones (Moritani et al, 1996) protects NOD mice against diabetes. Taken together, these results suggest that disease is likely to progress when mice have a high IFN γ /IL-10 ratio, but not when they have a low ratio. Thus, successful immunotherapies and/or vaccines should aim to produce a low ratio. Based on our findings, increased TGF- β 1 production could also be an important factor. Indeed, we reported that delivery of this strongly suppressive cytokine by i.m. gene transfer prevents diabetes in NOD mice (Piccirillo et al, 1998).

E. Modification of the GAD65 response by fusion with IgG-Fc and coinjection of IL-4

GAD65 immunization can be improved by fusing this molecule with IgG-Fc, as demonstrated by Tisch et al, (2001). Their GAD65 fragment contained three peptide epitopes (amino acid residues 206–220, 217–236, and 290–309), which are recognized by CD4⁺ T-cell clones derived from NOD mice. I.m. injection of plasmid DNA coexpressing GAD65-IgG-Fc and IL-4 protected NOD mice from diabetes, whether they were treated at early or late preclinical stages of disease. The response was antigen-specific, inasmuch as plasmid DNA encoding hen egg lysozyme-IgG-Fc and IL-4 was ineffective. This beneficial effect appears related to GAD65-specific regulatory Th2 cells, which are induced by the addition of

IL-4 cDNA. It can be presumed that IL-4 negates, at least in part, the Th1-inducing effects of ISS carried by plasmid.

Consistent with a beneficial effect of IL-4, Tisch et al, (2001) found that GAD65-specific Th1 cell reactivity was significantly enhanced in animals immunized with plasmid DNA encoding GAD65-IgG-Fc without IL-4. They also observed that IL-4-null mice were not protected by their DNA vaccine, confirming an important role for this cytokine. Importantly, DNA vaccination begun late, when NOD mice already have insulinitis, reduced the incidence of diabetes markedly. Thus, the vaccine did not have to be administered before the onset of autoimmunity to be effective. As in some other reports, vaccination against unmodified GAD65, with or without IL-4, was not successful (Tisch et al, 2001), suggesting that the IgG-Fc segment plays an important part in tolerance induction. It seems plausible, in view of other results with secreted GAD, that secretion of the IgG-Fc construct is a key feature, but the Fc segment could also be acting by other mechanisms, perhaps involving Fc receptors.

Interestingly, DNA vaccination has also been reported to be an effective strategy to mediate long-term protection of pro-islet grafts, produced by syngeneic neonatal pancreas implantation under the kidney capsule of NOD mice (Seifarth et al, 2003). These grafts are usually rejected by NOD mice through an autoimmune process. However, the conditions for protection of transplanted islets by DNA vaccination were more stringent than protection of native islets, in that IL-4 and IL-10 co-delivery was required to suppress destruction of grafted islets. Efficient protection of pro-islet grafts correlated with a marked reduction in GAD65-specific IFN reactivity and an increase in IL-10-secreting (regulatory) T cells.

IV. Immunoinhibitory DNA vaccines

There is increasing evidence that autoimmune diabetes in NOD mice results from a failure of Tr activity (Anderson and Thornton, 2004; Belghith et al, 2004; Bour-Jordan et al 2004; Gregg et al, 2004; Pop et al, 2005; You et al, 2005). Indeed, there are reports that transforming growth factor γ (TGF- γ)-producing Tr cells protect against this disease, and their function declines with age (Pop et al, 2005). We found that TGF- γ gene therapy prevents diabetes in NOD mice (Piccirillo et al, 1998), and various protective approaches such as CD3 mAb therapy (Anderson and Thornton, 2004; Belghith et al, 2004) act at least in part through induced Tr cells (iTr) that produce TGF- γ . Other types of Tr cells might also be implicated, particularly the CD4⁺CD25⁺ Tr cells that appear to act by direct cell contact, and are denoted natural Tr cells (nTr) (Piccirillo and Thornton, 2004; Sakaguchi, 2005).

Procedures such as CD3 mAb therapy may find clinical applications, but they lack specificity in that all T cells are targeted by the antibody. It would be desirable to design a vaccine that acts selectively on autoantigen-reactive T cells, and one approach is DNA vaccination against target autoantigens (or their peptides), such as insulin or glutamic acid decarboxylase 65 (GAD65). This can involve coupling T-cell recognition of autoantigens

with the delivery of signals which turn T cells off, or induce their differentiation to a nonpathogenic type. As noted above, covaccination with IL-4, or both IL-4 and IL-10, has been proposed as an improvement to this strategy and, indeed, promising results have been obtained. However, these cytokine-based approaches appear to act mostly by the induction of Th2-like cells, which are not devoid of pathogenic potential. These cells can mediate allergic-like tissue injury, and might be detrimental in humans. We have investigated a different approach, where the immunoinhibitory molecule CTLA-4 is used as a target molecule. Although the immunobiology of CTLA-4 is complex, and not fully understood, it is abundantly clear that this molecule plays a key role in tolerance (Prud'homme, 2004; Greenwald et al, 2005). Furthermore, it is expressed by both iTr and nTr cells, and may be essential for the activity of either type of regulatory cells (Paust and Cantor, 2005).

A limitation to the use of CTLA-4 as a therapeutic molecule in autoimmune diseases or transplantation has been the lack of a specific agonistic ligand. Antibodies against CTLA-4 usually have a blocking activity, and enhance immune responses as demonstrated most notably in cancer therapy experiments and clinical trials (Prud'homme, 2004). To overcome this limitation, we have employed a mutated B7-1 molecule, B7-1wa, which binds CTLA-4 but has lost the ability to bind CD28 (Guo et al, 1995; 1998). Studies by others (Guo et al, 1995; 1998) and us (Chakrabarti et al, 2005) have confirmed the inability of this molecule to engage CD28. Initially, we found that DNA covaccination of NOD mice with PPIs and B7-1wa abrogated T-cell reactivity to insulin and reduced the incidence of disease (Prud'homme et al, 2002). However, the protective effect was limited, and disease incidence was only reduced by approximately 50%. The mechanism of action was unclear, and regulatory T cells were not identified. We hypothesized that this was due to the fact that insulin is only one of several antigens that have been identified in this disease, although it is clearly a major one (Nakayama et al, 2005). GAD65 (and the GAD67 isoform), IA-2, and other target antigens have been identified (Bach and Chatenoud, 2001). It is notable that investigators have reported that immunity to GAD65 was one of the first autoimmune responses to occur in NOD mice, and that tolerance induction to this molecule was protective (Bach and Chatenoud, 2001). Although there has been some controversy about the relative importance of various islet autoantigens, it seems clear that both insulin and GAD65 are major antigens. Therefore, we designed Ins-GAD, consisting of full-length PPIs and GAD65, as a composite target antigen that would include many key epitopes.

DNA vaccination against Ins-GAD caused only a minor reduction in the incidence of diabetes, which was not significant when compared to the injection of the blank vector (VR) alone (Glinka and Prud'homme, manuscript submitted). Interestingly, injection of VR alone also reduced the incidence of disease modestly, for reasons that are unclear. This might be due to plasmid-related CpG stimulation of DCs or other cells through toll-like receptor 9 (TLR9), but we have not investigated this question.

Injection of the B7-1wa plasmid alone was no more effective than VR alone. In sharp contrast, coinjection of the VR-Ins-GAD and VR-B7-1wa plasmids caused a significant reduction of disease versus all other groups. Thus, the incidence of diabetes in these mice was <12%, compared to > 62% in untreated mice. This is a result superior to all our previous vaccination studies with islet antigens in this disease model.

We examined the response of lymphocytes from vaccinated mice both *in vitro* and *in vivo*. Adoptive transfer of T cells from vaccinated mice, injected with or without diabetogenic lymphocytes obtained from diabetic mice, revealed that the T cells of vaccinated mice could not transfer disease in NOD.scid mice, and could significantly delay disease induced by the diabetogenic lymphocytes. Thus, the T cells of Ins-GAD/B7-1wa vaccinated mice exerted a regulatory effect *in vivo*. We further fractionated the protective T cells into CD4⁺CD25⁺, CD4⁺CD25⁻ and CD8⁺ subpopulations and repeated the experiments. We found that both CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells were protective, whereas CD8⁺ cell exerted no beneficial effect. This could mean that both CD25⁺ and CD25⁻ exert regulatory function, but this must be interpreted with caution in light of recent studies showing that CD25⁻ can convert to CD25⁺ after adoptive transfer in congenic or lymphopenic recipients (Zelenay et al, 2005).

We also performed flow cytometry analysis to identify changes in T-cell numbers of various phenotypes in the vaccinated mice. We identified a significant increase in the numbers of cells with CD4⁺CD25⁺CTLA-4⁺B7-1⁺ phenotype in Ins-GAD vaccinated mice, compared to diabetic control mice. Furthermore, there were increased numbers of CD4⁺ T cells expressing both neuropilin-1 (Nrp1) and membrane-bound TGF- β , which are two markers that have been previously linked to Tr function (Bruder et al, 2004; Di Giacinto et al, 2005). We also found by quantitative PCR that Foxp3 expression was increased in treated mice and, consistent with this, there is evidence that Nrp1 expression correlates with Foxp3 expression (Bruder et al, 2004). Thus, DNA vaccination induced increases in populations of T cells that have been found in other studies to exert suppressor function.

These results did not establish, however, whether there were multiple populations of Tr cells, or one population expressing all these markers. We performed *in vitro* assays to further characterize the Tr cells. Latency associated peptide (LAP)-TGF- β cells appear to be the dominant Tr population induced by Ins-GAD/B7-1wa covaccination. However, as determined by flow cytometry, these cells overlapped considerably with Nrp1⁺ and B7-1⁺ cells. In summary, our preliminary studies suggest that Tr cells are heterogeneous in their marker expression, with a dominant population expressing membrane-bound LAP-TGF- β . Antibodies against TGF- β 1, but not IL-10 antagonized the suppressive activity of the Tr cells.

A role for cell-membrane TGF- β -expressing Tr cells has been proposed in other models of autoimmunity. In the T-cell transfer model of colitis, Di Giacinto et al, 2005 reported that CD4⁺CD25⁻ T cells bearing LAP on their

membrane suppressed CD4⁺CD45RB^{high}-induced colitis by a TGF- β -dependent mechanism. A population of CD4⁺ T cells, both CD25⁺ and CD25⁻, could be stained with a goat anti-LAP polyclonal antibody without being stimulated. Almost all these LAP⁺ cells were also positive for thrombospondin, which can convert latent TGF- β to the active form. *In vivo*, the regulatory function of CD25⁻LAP⁺ cells was blocked by anti-TGF- β mAb. This study substantiates the hypothesis that Tr cells bearing LAP-TGF- β on their membrane regulate autoimmunity. Moreover, our current study demonstrates that Tr cells with this phenotype can be generated by DNA vaccination. It has not been clearly established whether the Tr cells synthesize the LAP-TGF- β or acquire it from other cells and, conceivably, both mechanisms could coexist.

In summary, DNA covaccination with Ins-GAD and B7-1wa plasmids induced tolerance to islet antigens, and protected NOD mice against autoimmune diabetes. This protection could be transferred to NOD.scid mice with CD4⁺ T cells obtained from immunized NOD mice. Furthermore, the vaccinated mice had increased number of T cells bearing markers associated with Tr function. In particular, CD4⁺Nrp1⁺LAP-TGF- β T cells were increased in numbers and exerted suppressor activity *in vitro*. DNA covaccination of this type is a novel way of combining T-cell autoantigen recognition with signals that inhibit T cells and/or induce their differentiation to a Tr cell phenotype.

V. Concluding remarks and future prospects

DNA vaccination against autoimmune diseases is a new approach that has shown both promise and limitations. Thus far, it has been unclear which vector elements or factors determine a favorable outcome. This has led investigator to test vaccines that incorporate genes encoding immunomodulatory elements, particularly cytokines. This approach has produced positive results, and might lead to safer vaccines. However, there are significant interspecies differences in the response to DNA vaccines, and clinical studies must be conducted with caution.

To design a safe and effective vaccine for humans, several variables must be taken into consideration. This includes factors such as antigen modification (e.g., secretion and construction of Ig fusion products), the level and length of vector expression, the effects of electroporation when it is applied, the choice of target tissues and the method of DNA delivery. The role of various DC subpopulations and CpG stimulation must also be taken into consideration. CpG motifs could play a detrimental role by inducing the production of IL-6 which antagonizes the activity of CD4⁺CD25⁺ regulatory T cells (Pasare and Medzhitov, 2003). Therefore, DNA vaccines might be improved if IL-6 or other cytokines that negatively affect regulatory T cells were somehow neutralized. There is a concern that vaccination to prevent autoimmunity might result in dangerous anaphylactic reactions (McDevitt, 2004). This is indeed worrisome, and suggests that vaccines must be carefully designed to avoid

inducing Th2 cells, which are linked to this type of hypersensitivity.

There are important differences in the response of human and mouse DCs to CpG elements that will undoubtedly affect the response to DNA vaccines. Another consideration is that transfection efficiency, at least in muscle, is lower in large mammals than in rodents, and methods of DNA delivery will have to be adapted accordingly. Nevertheless, several recent studies reveal that DNA vaccination is feasible in humans, dogs, pigs, and other large species. These studies are almost all designed to test immunostimulatory vaccines, but there are no obvious reasons to believe that inhibitory vaccines cannot be produced. Thus far, DNA vaccines have had an impressive safety profile. Indeed, there are good reasons to be optimistic about the future in this area. A major advantage of DNA vaccines over other immunotherapeutic methods is their remarkable simplicity. This approach is straightforward and inexpensive, and when it is sufficiently developed could be easily applied in the clinic by any physician.

Acknowledgements

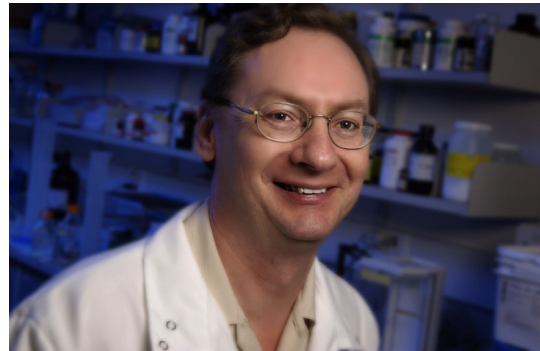
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