

CD44 Gene Vaccination for Insulin-Dependent Diabetes Mellitus in Non-Obese Diabetic Mice

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Abstract

Background: Standard CD44 and its alternatively spliced variants were found to be associated with the metastatic potential of tumor cells and with cell migration of autoimmune inflammatory cells, including cells involved in experimental insulin-dependent diabetes mellitus.

Objectives: To investigate whether induction of anti-CD44 immune reactivity, through cDNA vaccination, could attenuate IDDM in a transfer model of NOD mice.

Methods: Our vaccination technique involved the insertion of CD44s or CD44v cDNA into a silicone tube filled with a 2.5 cm long segment of hydroxylated-polyvinyl acetate wound dressing sponge (forming a virtual lymph node) which was implanted under the skin of male NOD recipients reconstituted with diabetogenic spleen cells of female NOD donors. The VLN were implanted 20 days before and 3 days after cell transfer.

Results: In contrast to control groups of recipient mice, recipients vaccinated with VLN loaded with CD44v or CD44s cDNAs developed resistance to IDDM almost to the same extent. Our results suggest that the gene vaccination effect was mediated by anti-CD44 antibody rather than by cellular immunity. Histopathological examinations revealed a significant protection of pancreatic islets in the DNA-vaccinated recipients, whereas the islets of control recipients of diabetogenic cells were almost totally destroyed.

Conclusions: These findings may open new opportunities for IDDM therapy in the future.

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CD44 is a single-chain glycoprotein molecule containing a conserved N-terminal extracellular domain in which the ligand binding site is included, a non-conserved membrane proximal region in which the variable region is inserted, a conserved transmembrane-spanning domain, and a conserved cytoplasmic tail linked to the cytoskeleton by an adaptor protein. The structural nature of CD44 is related to alternative splicing of 10 (mouse) or 9 (human) variant exons inserted in different combinations between two constant regions, containing five or four constant

exons [for review, see 1-4]. Theoretically, over 800 CD44 variants can be generated by differential utilization of the variant exons [5], including several dozen isoforms that have already been detected [2]. For example, splicing and insertion of exons v3 to v10 between the two constant exons generate the molecule CD44v3-v10, which is preferentially expressed on normal keratinocytes [6] and synovial fluid cells from patients with rheumatoid arthritis [7]. A comparison of the transcript sequence of CD44v3-v10 derived from these two cell types revealed that the CD44 variant of synovial fluid cells from 33 of 43 patients with RA and psoriatic arthritis includes an extra trinucleotide, CAG, which is transcribed from the end of the intron flanking exon v5. This CD44 variant was designated CD44vRA, whereas CD44v3-v10 is the "wild type" molecule [7]. Direct splicing from constant exon 5 to constant exon 16, which skips all the variant exons, generates the shortest and most common isoform-standard CD44 (CD44s), which is preferentially expressed on hematopoietic cells [2].

The variability in CD44 structure is further increased due to N- and O-glycosylation as well as to the attachment of glycosaminoglycans (e.g., heparan sulphate, chondroitin sulphate) [1-4]. The GAG heparan is used for binding heparan sulphate-binding growth factors to the CD44 molecule [1]. For example, CD44vRA presents, in an exclusive orientation, fibroblast growth factor-2 to pro-inflammatory synovium cells of RA patients bearing the cognate FGF receptor-1. This allows enhanced proliferation of FGF-2-stimulated cells, thus aggravating the inflammatory cascade in these patients [7]. The multi-structural nature of CD44 is presumably associated with its multi-functionality, which includes cell-cell and cell-matrix interactions; support of cell migration; presentation of growth factors (as mentioned above), cytokines, chemokines and enzymes to other cells or to the surrounding tissues; as well as signal delivery from the cell surface to its interior, leading to programmed cell death or to cell survival and proliferation [reviewed in 1]. The rich isoform repertoire of CD44 may explain its ability to bind a considerable number of different counter-molecules (or ligands), some of which are presumably

IDDM = insulin-dependent diabetes mellitus

CD44s = standard CD44

CD44v = CD44 variants

VLN = virtual lymph node

RA = rheumatoid arthritis

GAG = glycosaminoglycan

FGF = fibroblast growth factor

yet to be discovered. The list of CD44 ligands is continuously expanding and so far includes hyaluronic acid, which is the principal ligand, as well as collagen, fibronectin, fibrinogen, laminin, chondroitin sulphate, mucosal vascular adhesion, serglycin/gp600, osteopontin, the major histocompatibility complex class II invariant chain (Ii), L-selectin, E-selectin and galectin-8 (the last was discovered in our laboratory) [8].

Since cell surface CD44 is implicated in cell migration and delivery of apoptotic signals, we hypothesized that diseases regulated by these functions should be sensitive to treatment by the enzyme hyaluronidase and/or anti-CD44 monoclonal antibodies. Indeed, we found that injection of the enzyme hyaluronidase and/or anti-CD44 monoclonal antibodies reduced the pathological activities in animal models of collagen-induced arthritis [9], type I diabetes and lymph node invasion by malignant lymphoma [10]. The mechanism of action was attributed either to the enzyme or antibody ability to avoid cell migration (i.e., the recruitment of the metastatic cancer cells or destructive inflammatory cells into the relevant tissues) [9,10], or the antibody capacity to deliver apoptotic signals in these cells [9].

Although the amelioration effect of vaccination-induced antibody (or cell-mediated immunity) is slower than passive immunization with antibody, its ability – apart from its effector functions – to generate immunological memory provides a significant advantage: the second experience with the same pathogen or self-antigen results in ultimate defensive or protective responses even when they occur a long time after the first experience with the same agent. This is especially important in endemic regions of infection and in chronic diseases, which can be predicted before the appearance of symptoms. Insulin-dependent diabetes mellitus is one of many examples in which the vaccination strategy should be experimentally examined. Focusing on IDDM, we already have some tools to predict disease onset, while several others are now under investigation, some of which will soon be clinically available.

Insulin-dependent diabetes mellitus is the result of autoimmune destruction of the insulin-producing beta cells found in the pancreatic islets of Langerhans. The disease process is characterized by a progressive infiltration of lymphocytes and monocytes into the islets (insulinitis), culminating in massive destruction of the β cells [11-13]. Studies in the non-obese diabetic mouse, a spontaneous murine model for IDDM, have demonstrated that CD4⁺ and CD8⁺ T cells are the primary mediators of β cell destruction [for review, see 14]. Furthermore, temporal analyses indicate that only a few β cell autoantigens such as glutamic acid, decarboxylase 65 (GAD65) and insulin are targeted in the early stages of disease development by CD4⁺ T cells [15,16].

As injection of anti-CD44 monoclonal antibodies reduced the diabetogenic activity in the transfer model of NOD mice [17], we decided to examine the ability of CD44 cDNA vaccination to generate anti-CD44 immunity in non-diabetic male NOD recipients of inflammatory splenocytes derived from diabetic female NOD donors. Indeed, we previously found that our method (see below) of cDNA vaccination produces protective anti-CD44 antibody (manuscript submitted) rather than a cell-mediated immune response.

Our cDNA vaccination technique involves the insertion of standard CD44 (CD44s) or CD44 variant (CD44v) into a silicon tube filled with a 2.5 cm long segment of hydroxylated-polyvinyl acetate wound-dressing sponge. This device was loaded with plasmid containing a cytomegalovirus promoter and human CD44s or CD44v cDNA and then implanted under the mouse skin. Under such circumstances we induced in the silicon tube a local subcutaneous inflammatory reaction similar to that found in lymph nodes. Therefore the cDNA-loaded silicon tube was designated a virtual lymph node. We suggest that antigen-presenting cells penetrate into the VLN and internalize the CD44 cDNA, which is then expressed on their cell surface, thereby inducing an immune response. We used human CD44 cDNA, rather than mouse CD44 cDNA, to break the self-tolerance to CD44 in the mouse, thereby allowing production of anti-mouse CD44 antibodies (see Discussion). Implantation of VLN loaded with CD44s or CD44v cDNA plasmids under the mouse skin generated resistance to experimental allergic encephalomyelitis in SJL/J mice [18] and to mammary tumor progression in BALB/c mice (manuscript submitted). In either case, the CD44v cDNA plasmid generated stronger disease resistance than the CD44s cDNA plasmid. This is hardly surprising, since it is well known that cancer cells and inflammatory cells predominantly express CD44 variants rather than standard CD44 [1-4]. Possibly the variant isoforms have a biological advantage over the standard isoform, as the former supports better cell migration [19]. Equipped with this information we tested the ability of VLN loaded with CD44s or CD44v cDNAs to induce resistance to IDDM in the NOD mouse cell transfer model. Indeed, the CD44s and CD44v plasmids induced resistance to type I diabetes, yet the immunogenic potential of both vaccines was almost identical.

Materials and Methods

Mice

Male and female NOD mice obtained from the Jackson Laboratory were maintained in a specific pathogen-free animal facility at 21°C and supplied with acidified water (pH 2.7) and mouse chow *ad libitum*. The experimental protocol was approved by the institute's Animal Ethic Committee.

Construction of human CD44v3-v10 and CD44s expression plasmids for DNA vaccination

To clone human CD44v3-v10, total RNA was isolated from human keratinocytes (a gift from Dr. Ben Basat, Hadassah University Hospital). RNA was separated with the aid of a commercial kit (Promega, Madison, WI, USA). CD44v3-v10 cDNA was prepared by reverse transcriptase-polymerase chain reaction (PTC-100™ Programmable Thermal Controller, MJ Research, Watertown, MA), using primers representing the constant coding regions Ex1-sense, 5'-GAATTCGCCGCCACCATGGACAAGTTTTGGTGG 3'; Ex20 antisense, 5'-TCTAGATTACACCCCAATCTTCATG 3'.

PCR product size was confirmed by agarose gel electrophoresis, sequencing (ABI PRISM 310, Perkin-Elmer, Wellesley, MA)

PCR = polymerase chain reaction

or PstI digestion (New England BioLabs, Beverly, MA). The PCR product was excised from the gel, purified and subcloned into the pGEM vector (Promega). Positive clones were selected by white/blue screening. Plasmids were purified using a commercial kit (Promega), subjected to EcoRI/XbaI-double digestion and cloned into the pcDNA3.1 vector (Invitrogen, Paisley, UK) in which the gene product was expressed. For cloning of human CD44s, RNA was isolated from the HeLa cervical cancer cell line (obtained from ATCC), according to the above-described protocol. The absence of mutations was confirmed by sequencing. Plasmids lacking inserts (pcDNA3.1) served as the control.

Virtual lymph node

The virtual lymph node was developed by VLN LLC, Tarrytown, NY, as a novel medical tubular device mimicking the structure and function of a lymph node. Immune cells T and B lymphocytes as well as antigen-presenting cells gravitate to the inflamed site and accumulate inside the porous matrix of the VLN. The VLN is prepared from a 2.5 cm length of SILASTIC[®] silicone tubing (Dow Corning, NY) with an inner diameter of 0.15 cm and an outer diameter of 0.2 cm, fitted with a 2.5 cm long segment of hydroxylated-polyvinyl acetate wound-dressing sponge. The VLN is then immersed in a container of phosphate-buffered saline and autoclaved. Recipient NOD mice were anesthetized with Avertin. Two virtual lymph nodes per mouse were inserted under the skin through a 5 cm dorsal midline incision 20 days before and 3 days after cell transfer.

Experimental design

Diabetic (confirmed by glucosuria; see below) female donor NOD mice aged 15–20 weeks were killed, and suspensions of spleen leukocytes were washed twice in RPMI 1640 medium containing 10% fetal calf serum (Beit HaEmek, Israel). A quantity of 25×10^6 leukocytes was injected intravenously into each irradiated male recipient mouse aged 8–10 weeks. The recipient mice were anesthetized and the site of the inserted VLN was shielded with lead to avoid exposure to the irradiation. The mice were conditioned by total body irradiation with a single dose of 650 cGy delivered by a linear accelerator (Varian 6X) at a source to skin distance of 80 cm and a dose rate of 170 cGy/min.

Different groups of recipient mice were implanted with virtual lymph nodes loaded with CD44s or CD44v cDNA plasmids as well as empty plasmid or PBS (control recipients) 20 days before and 3 days after cell transfer [18]. To monitor the development of diabetes, the percentage of disease-free animals (glucosuria negative) was recorded over time. Each experiment was repeated at least twice. The statistical significance of the findings was determined by the GraphPad Prism according to the Logrank test.

Assessment of diabetes by glucose determination

Diabetes was monitored by testing urinary glucose with a Teststrip (Medi-Test, Combi 9, Macherey-Nagel, Düren, Germany) twice weekly and was considered positive after the appearance of glucosuria in at least two determinations. The intraperitoneal

glucose tolerance test was performed as follows: blood was drawn from the paraorbital plexus at 0 and 60 minutes after an i.p. injection of glucose (1 g/kg body weight). Plasma glucose levels were determined (as glucose mmol/L) with a Glucose Analyzer 2 (Beckman Instruments). A glucose level above 15 mmol/L at the 60 min time point was considered a positive IPGTT.

Histopathology

Pancreatic tissue was fixed in 4% buffered formalin and embedded in paraffin, and 5 µm sections were stained with hematoxylin and eosin. Islet sections from each pancreas were screened and scored by an uninformed observer according to the following criteria: 0 = no cell infiltration, 1 = periinsulinitis and cell infiltration involving up to 20% of islet area, 2 = cell-infiltration involving up to 50% of islet area, 3 = cell infiltration involving up to 75% of islet area, and 4 = cell infiltration involving 90–100% of islet area.

Results

VLN loaded with CD44 cDNA plasmids induce IDDM resistance

Diabetogenic female NOD donor cells were intravenously infused into irradiated non-diabetic male NOD recipients. VLN containing CD44v3-v10 (CD44v) cDNA or standard CD44 (CD44s) cDNA were inserted 20 days before and 3 days after the cell transfer [18]. Control recipients were inserted at the same times with VLN lacking insert (pcDNA3.1) (empty vector) or administered PBS. The percent of diabetes-free recipients was monitored by glucose detection in the urine as indicated in Materials and Methods. All recipient mice that were administered PBS were diabetic within 5 weeks, while 83% of the recipients given VLN loaded with empty vector were diabetic within 11 weeks, indicating a certain non-specific effect mediated by VLN containing empty vector. In contrast, recipients implanted with VLN loaded with CD44s cDNA or CD44v cDNA showed resistance to diabetes development, i.e., 86% and 75% of these male NOD recipients were respectively free of diabetes 11 weeks after the cell transfer [Figure 1]. Since the difference between these two last groups was statistically insignificant, we concluded that they generate mostly anti-standard CD44 immunity, leading to the development of IDDM resistance. Therefore, in most cases, our analysis considered the CD44s and the CD44v-vaccinated groups as a single group, designated a CD44 cDNA-vaccinated mouse group. Note that the cDNA-vaccinated recipients whose VLN implantation site was not protected during irradiation by lead developed IDDM (results not shown).

All mice were killed 12 weeks after cell transfer. Ten diabetes-free recipient NOD mice that were implanted with VLNs loaded with either CD44s cDNA or CD44v cDNAs were subjected to IPGTT before killing. Six of these mice cleared the glucose challenge normally (i.e., they were IPGTT negative), indicating lack of latent diabetes.

VLN loaded with CD44 cDNA plasmids attenuate insulinitis

The degree of insulinitis in the pancreatic islets was histopathologically analyzed and scored by a non-informed pathologist [Table 1]. The number of islets in representative tissues with

identical size was lower in mock-vaccinated recipients [14] than in CD44 cDNA-vaccinated mice [38], implying islet protection effect by the gene vaccination. Scoring the degree of islet insulinitis, the invasion of inflammatory cells into islets of mock-vaccinated recipients was more intensive (average score 3.9 ± 0.5) than into CD44 cDNA-vaccinated recipients (average score 1.4 ± 0.5). While high insulinitis and minimal to mild perinsulinitis was detected in the mock-vaccinated recipient NOD mice, the reverse was observed in the CD44 cDNA-vaccinated recipients. The morphological appearance of intact islets in the VLN CD44-implanted recipients appears normal. In contrast, atrophic and infiltrated islets were detected in the control recipients [Figure 2].

CD44 cDNA vaccination is not mediated by cellular immunity

We previously found that CD44 cDNA vaccination induces protective anti-CD44 antibodies (manuscript

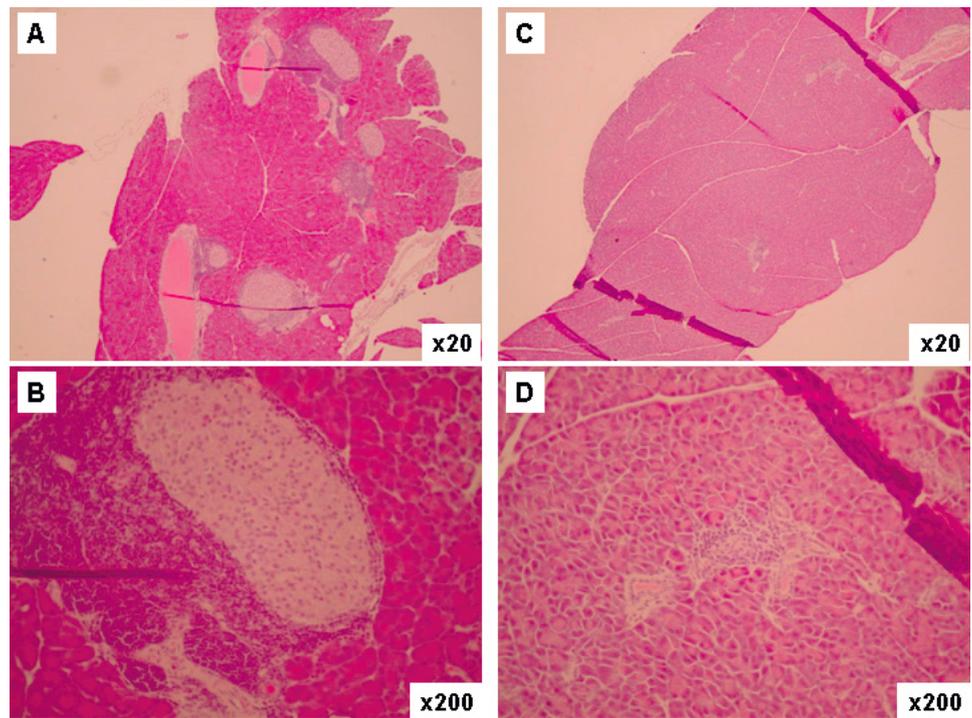


Figure 2. Histological analysis of representative pancreatic islets derived from recipients implanted with VLN containing CD44 cDNA [A and B] or from control recipients [C and D]. Pancreatic tissues from implanted recipients were fixed in 4% formalin and embedded in paraffin, and 5 µm sections were stained with hematoxylin and eosin. The pancreatic endocrine islets of the control recipients are atrophic and infiltrated with mononuclear cells [C and D] whereas mice implanted with VLN containing CD44 cDNA displayed a focal infiltrate at the periphery only [A and B]. Note that the number of islets in the DNA-vaccinated mice is much higher than in the control mice.

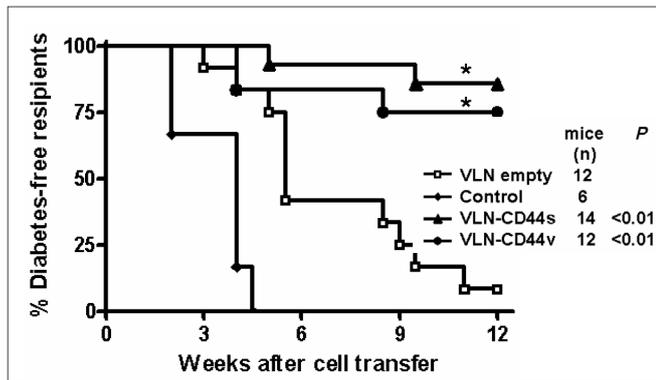


Figure 1. Vaccination with VLN loaded with CD44 cDNA ameliorates IDDM in transfer model of NOD mice. Spleen cells from diabetic NOD female mice were intravenously infused into non-diabetic NOD male recipients. VLN containing CD44v3-10 (CD44v) cDNA or standard CD44 (CD44s) cDNA were inserted into the recipients 20 days before and 3 days after the cell transfer. Control recipients were transplanted at the same times with VLN lacking insert (pcDNA3.1) (empty VLN) or administered PBS. The percent of diabetes-free recipients was monitored by glucose detection in the urine. The graph shows integrated data from two independent but similar experiments. The Logrank statistical analysis was used to compare the empty VLN group with all the other groups.

PBS = phosphate-buffered saline
IPGTT = intraperitoneal glucose tolerance test

Table 1. Insulinitis inhibition by VLN loaded with CD44*

Mouse no.	Treatment	No. of scored islets	Perinsulinitis	Average score per islet
1	Control	4	Minimal	4
2		1	Minimal	2
3		6	Mild	4
4		3	Minimal	4
Total		14		
Mean 3.9 ± 0.5				
5	VLN loaded with CD44 cDNA	11	Marked	1
6		11	Moderate	1
7		9	Mild	2
8		7	Moderate to Marked	2
Total		38**		
Mean $1.4 \pm 0.5^*$				

* Male NOD recipients loaded with VLN containing CD44 cDNA or control recipients were subjected to histopathological analysis. Insulinitis was graded on a scale of 0 to 4, 12 weeks after cell transfer as described in Material and Methods. At this time, the anti-diabetogenic effect of the VLN loaded with CD44 cDNA was clearly observed by the glucose test. The number of scored islets per mouse and the average insulinitis score per mouse, as well as the total score and mean \pm SD for each group, are shown.

** $P < 0.01$ by t-test.

submitted). To rule out the possibility that cell-mediated immunity is simultaneously developed, we mixed 25×10^6 splenocytes from diabetes-free CD44 cDNA-vaccinated recipients with 25×10^6 diabetogenic donor cells and infused them i.v. into four irradiated recipients. In parallel, we mixed, under identical conditions, naïve normal cells with diabetogenic donor cells and injected them into six recipients. All mice displayed IDDM within 24 weeks with no significant difference in the disease development between the groups (results not shown), suggesting that antibody response rather than cell-mediated immune response is generated after CD44 cDNA vaccination.

Discussion

VLNs loaded with CD44v3-v10 (CD44v) cDNA or standard CD44 (CD44s) cDNA induced resistance to insulinitis and IDDM in male NOD recipients reconstituted with female diabetogenic donor cells. No difference was detected in the vaccination capacity of the two constructs, suggesting that anti-CD44s immune response is generated after vaccination. Interestingly, virtual lymph nodes loaded with the same CD44v cDNA identical to the one reported here induced stronger resistance to EAE development in SJL/J mice [18] and to mammary tumor progression in BALB/c mice (manuscript submitted) than VLN loaded with the corresponding CD44s cDNA. We suggest, therefore, that the predominant target protein in the EAE and tumor models (manuscript submitted) is CD44v, whereas in the IDDM transfer model it is CD44s. Different mechanisms of inflammation and/or differences in the mouse genetic background may explain this discrepancy.

We have shown previously that CD44 cDNA vaccination of mice generates anti-CD44 antibodies, which reduce the CD44-dependent pathological activity (manuscript submitted). Furthermore, in a different study we proved that vaccination with this construct induced protection against pathology, which was independent of cell-mediated immunity [18]. This conclusion is supported by the finding reported in the present article. Spleen cells from IDDM-free recipient mice vaccinated with VLN loaded with CD44 cDNA could not reduce the ability of diabetogenic donor cells to transfer IDDM to naïve recipients when mixed together. Collectively, our results demonstrate that VLN vaccination with CD44 constructs induced antibody-mediated protective activity, with very restricted, if at all, cell-mediated immunity.

Although the cDNA vaccination approach has not been developed beyond the experimental stage, its future potential as a leading public health vaccine is widely discussed. This method makes use of a gene encoding an antigen instead of the antigen itself [20]. It provides several advantages over conventional "proteinic" vaccination: DNA vaccine is cheap and easy to produce, is stable under diverse physical conditions, does not require purification processes, and lacks the toxicity of protein vaccines. The antigen is presented to the immune system in a structure and conformation closer or identical to the native protein, including post-translational modifications. DNA vaccination mimics the effect of vaccination with live attenuated pathogen since it can

induce not only a humoral response but also a T cell-mediated response. It induces a stronger and possibly longer lasting immunity.

In our research we used human constructs of CD44 to break the self-tolerance to this protein. Indeed, virtual lymph nodes containing mouse CD44 cDNA rather than human CD44 cDNA were unable to induce resistance to the mammary tumor growth (unpublished data). This observation is hardly surprising since the mouse is tolerant to its own CD44. Therefore, we used human CD44 to break the tolerance. The CD44 of mouse and man share some epitopes, while diverging in others, allowing breaking the tolerance to self CD44 in the mouse by human CD44. The non-homologous epitopes of human CD44 are immunogenic in the mouse, allowing activation of helper T cells that recognize these epitopes. Linked recognition is then generated between helper T cells recognizing the foreign epitopes of human CD44 and B cells recognizing the shared human-mouse epitopes of the same molecule, resulting in breaking of tolerance and synthesis of anti-mouse CD44 antibodies. This concept is well established and is used to explain breaking of tolerance to self-antigens, leading to autoimmunity.

The concept of gene vaccination, rather than protein vaccination, to establish autoimmunity against cancer, to induce resistance to viral infection or to ameliorate autoimmune pathological activities has been successfully challenged in animal models [21-29]. Focusing on IDDM, intramuscular injection of cDNA encoding either GAD65 alone (to make the inflammatory cells more susceptible to apoptosis) [21] or GAD65-IgGfC and IL-4 (to shift the immune system toward Th2 anti-inflammatory response) [22] prevented IDDM in NOD mice. Pro-apoptotic gene vaccination, which reduces IDDM activity, can also be accomplished by cDNA coding the pro-apoptotic protein BAX into plasmid DNA of secreted GAD55. This DNA vaccination effect is mediated by regulatory T cells [23]. Amelioration of IDDM in NOD mice was also obtained by vaccination with DNA encoding membrane-bound preproinsulin and a chimeric gene vector encoding mutant B7.1/CD40 fusion protein. The mutant B7.1 product binds exclusively to CTLA4 on antigen-presenting cells, resulting in the delivery of a tolerogenic signal to insulin [24]. These findings and the results reported in this communication may open new opportunities for IDDM therapy in the future.

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EAE = experimental allergic encephalomyelitis

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