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Diabetes tolerogenic vaccines targeting antigen-specific inflammation

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Tolerance controls the magnitude of inflammation, and balance between beneficial and harmful effects of inflammation is crucial for organ function and survival. Inadequate tolerance leads to various inflammatory diseases. Antigen specific tolerance is ideal for inflammation control as alternative anti-inflammatory interventions are non-specific and consequently increase the risk of infection and tumorigenesis. With inherent antigen specificity, tolerogenic vaccines are potentially ideal for control of inflammation. Although the concept of tolerogenic vaccines is still in its infancy, tolerogenic mucosal vaccines and specific immuno-therapies have long been proven effective in pioneering examples. Now a body of evidence supporting the concept of tolerogenic vaccines has also accumulated. Here we comment on recent successes of the tolerogenic vaccine concept, present new evidence with a type 1 diabetes vaccine as an example and draw conclusions on the advantages and potential for inflammatory disease control at the bedside.

Keywords: antigen specific inhibition, autoimmune disease treatment, co-immunization, immuno-suppressors, immune suppression, inflammation, tolerance, suppressive vaccine, tolerogenic vaccine, type 1 diabetes

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Introduction

Tolerance controls the magnitude of inflammation, and balance between beneficial and harmful effects of inflammation is crucial for an inflamed organ's function and an individual's survival.[1,2] Inadequate tolerance leads to various inflammatory diseases, including those with proven autoimmune basis such as type 1 diabetes (T1D),[3,4] allergic diseases including asthma,[4-6] inflammatory cardiac disease such as atherosclerosis[7] and newly identified examples in neuro-degenerative Alzheimer Disease[8-10] and cancer.[11-13] Thus, the need to cure or control chronic and pathogenic inflammation can be urgent if not desperate, and vaccines to induce tolerogenic responses are the top priority for this task (**Fig. 1A**).

Antigen specific tolerance is ideal for inflammation control.[14] Classical interventions suppress inflammation through depletion (mono-clonal antibody[15-17]) of key parts of the immune system, or universally suppress the whole immune system (cyclophosphamide[18] or steroid immuno-suppressants[19]). With these interventions, pathogenic inflammation is surely in some degree brought under control but at the cost of impaired or retarded normal immune responses, a situation which increases the risks of infection and tumorigenesis (**Fig. 1B**). Therefore, the development of specific interventions that target only the pathogenic antigen(s) and suppress only the problem-causing immune responses could be an ideal strategy that circumvents the risks while efficiently delivering safer tolerance.

Indeed, with their inherent antigen specificity, tolerogenic vaccines have long been reasoned to be an ideal means of controlling inflammation (**Fig. 1C**). Since the first discovery of the live smallpox vaccine, vaccination approaches have evolved to contain a spectrum of immunogens ranging from live or dead whole pathogens, viral vectors, subunit proteins, to DNA or RNA molecules encoding antigens.[20-22] However, most vaccines are intended to enhance host immune responses rather than to suppress them. Only recently has the use of vaccines that induce tolerogenic immunity to suppress

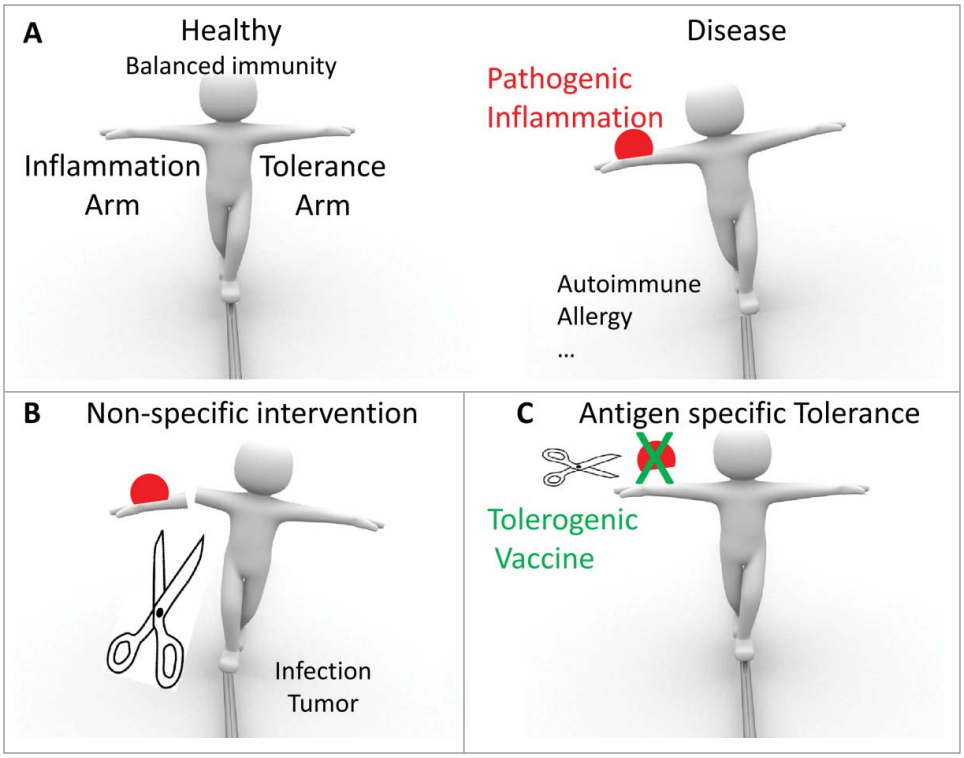


Figure 1. Tolerogenic vaccines are proposed to induce antigen specific control of pathogenic inflammation. (**A**) Balance between inflammation and tolerance guarantees immune homeostasis and individual survival. (**B**) Pathogenic inflammation leads to diseases such as autoimmunity, allergy, etc. (**C**) Non-specific intervention impairs protective immune responses and increases risks of infection or tumorigenesis. (**D**) Tolerogenic vaccine, as antigen specific intervention, may restore balance and provide a cure.

host immune responses gained research momentum following the discovery of T_{reg} cells and their suppressive functions. Although the tolerogenic vaccine concept is still in its infancy, mucosal tolerance induced by vaccination[23] and by specific immunotherapy (SIT) for allergy desensitization[24] were long ago proven effective in successful pioneering studies.

Now a body of evidence is accumulating under the concept of tolerogenic vaccination. Here we provide a perspective and new data regarding a tolerogenic vaccine against autoimmune diabetes. In view of this constraint of content, readers interested in mucosal induced tolerance or allergen-specific immuno-tolerance (SIT) should read reviews by L Mayer,[25] O Pabst,[26] Valenta,[24] Mark Larché[27] and Ciprandi.[28]

Taming inflammation

Reflecting its importance, inflammation is normally meticulously controlled in body. However, genetic changes, environmental pollutants, infections, aging or unidentified factors could alter such tight control and lead to disease. Therefore, interventions targeting the armory of disease-causing inflammatory cells and molecules have been proposed and tested. As listed in **Table 1**, non-specific interventions that are routinely used include depletion of cell populations by cyclophosphamide,[18] depletion of cell subset by antibodies(anti-CD3 mAb for T cell depletion[29] or anti-CD20 mAb for B cell depletion[30,31]), depletion of inflammatory cytokines by antibody,[32,33] universal tolerance induction by immunosuppressant drugs (dexamethasone, FK506, rapamycin or cyclosporin A),[34-36] entrapment of all leukocyte into lymph nodes away from inflammatory tissues (FTY720),[37,38] etc. As already pointed out above, these approaches run into the risk of increasing the chance of infection and tumorigenesis(**Fig. 1B**). A better approach to avoid these problems would be to use the induction of antigen specific tolerance against the bad or unregulated inflammatory reactions. Such an approach has recently undergone rapid development.

Induction of antigen specific tolerance by vaccination

In contrast to a vaccine augmenting immune responses against a pathogen, a tolerogenic vaccine is aimed at inducing tolerogenic responses to suppress or impair immunity against antigens, which in most cases are auto-antigens or allergens.[39] Besides the mucosal tolerance and SIT, more attractive approaches to achieve tolerogenic responses and induce antigen specific suppression have emerged in recent years[14,27,40-44] (**Fig. 2**).

Among these novel approaches, we have discovered 2 vaccine technologies to induce antigen specific tolerogenic responses. One of which is "co-immunized vaccine" (protein antigen plus its cognate DNA),[45-47] the other one is "suppressed immunization vaccine" (protein antigen plus immunosuppressant)[48](**Fig. 2**). The co-immunized vaccine was discovered by accident when we compared a prime-boost protocol of DNA-protein with a formulation of mixed DNA and protein vaccines for the ease of application. To our surprise, such co-immunized DNA and protein regimens induced impaired cellular immune responses.[45] This co-immunized vaccine can be applied to different antigens as long as the DNA encoded antigen is the same as the co-delivered protein. We observed that the co-immunized vaccine could induce $CD4^+CD25\text{-}Foxp3^+$ T_{reg} cells in animals, including mice, cats, dogs and monkeys, and the suppression of activation of T effector cells was specific for the same antigen used in the co-immunized vaccine. This suppression could translate into tolerogenic responses in vivo and into effective protection/treatment of animals with inflammatory diseases including autoimmune diseases, (diabetes,[48,49] auto-immune ovary disease[50]), asthma,[51] allergic dermatitis[52]) and Alzheimer disease.[47] Taking

Table 1. Non-specific interventions of inflammation

Intervention	Target	Example	Application			
			Disease	Aprv	Spec	Risk
Depletion or Blocking	Cell subset	Muromonab-CD3	T1D, RA, MS, SLE, Crohn's disease	1986	—	+
		Rituximab		1997		
	Molecule	Omalizumab	Asthma	2003	—	+
		Etanercept	RA, AS, psoriasis	1998		
Suppression	Proliferation	Cyclophosphamide	RA, MS, SLE	1959	—	+
	Signaling	Glucocorticoids (DEX, etc.)	RA, MS, SLE, asthma	1988	—	+
Quarantine	Infiltration	Fingolimod(FTY720)	MS	2010	—	+

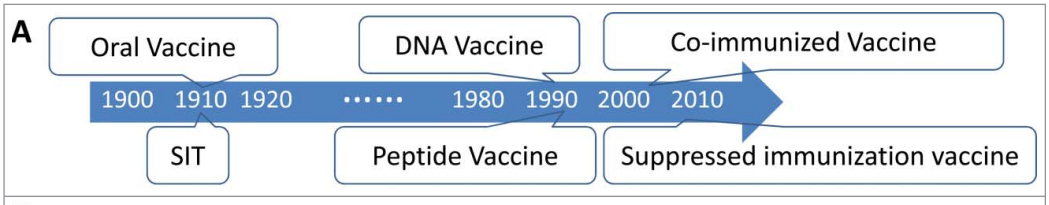
Abbreviations: Aprv: Approved by USA FDA or European Union. Spec: antigen specificity. Risk: risk of infection or tumorgensis. Muromonab-CD3: anti-CD3 (T cell) monoclonal antibody. Rituximab: anti-CD20 (B cell) monoclonal antibody. T1D: type 1 diabetes mellitus. RA: rheumatoid arthritis. MS: multiple sclerosis. SLE: systemic lupus erythematosus.Omalizumab: anti-IgEmAb. Etanercept: TNFR2 ECD–Fc (IgG1) fusion protein. AS: Ankylosing spondylitis. DEX: dexamethasone.

autoimmune diabetes for example:1) co-immunized vaccine (insulin plus DNA encoding proinsulin) prevented type 1 diabetes (T1D) progress from hyperglycemia in NOD mice with a 93.8% efficacy for 36[49] and 52 weeks (unpublished data). Controlled inflammation in pancreas and suppressed T cell response in spleen were observed in the co-immunized vaccine group, consistent with the proposed mechanism; 2) "suppressed immunization vaccine" of antigen + immunosuppressant (insulin dominant epitope B:9–23 plus dexamethasone, Dex) prevented T1D in NOD mice and protected 10/12 mice from hyperglycemia for 35 weeks (83.3% efficacy). Tolerance establishment was verified under auto-antigen re-challenge.[48] We report here further studies undertaken to clarify the mechanisms involved.

Results and Discussion

Expanding the concept of treatment for diabetes

We tested if the suppressed immunization vaccine could also induce tolerogenic responses when used to treat newly diabetic animals. When NOD mice showed signs of diabetes at week 12, they were treated with the suppressed immunization vaccine (recombinant insulin and dexamethasone) at days 1, 4 and 7. Their hyperglycemia was controlled for at least 1 month at 83.3% (5/6) efficacy (**Fig. 3A**). The apparent tolerogenic response was antigen-specific since irrelevant antigen suppressed immunization vaccines did not show any benefit for such amelioration (not shown). To confirm this induced tolerogenic response, T_{reg} cells were analyzed. They were found to be up-regulated significantly



B

Tolerogenic Vac	Td	Vaccine	Target Disease	Aprv	Trial	Spec	Ref
Oral Tolerogenic Vaccine	1910s	Allergen	Food Allergy	+	>1000 on MS, RA, T1D	+	39
SIT---Specific Immunotherapy	1910s	Allergen	Asthma, allergic rhinititis	+	>400 on allergy, T1D	+	40,41
Synthetic peptide vaccine	1990s	Epitope peptide	Allergy, autoimmune	-	~10 on allergy, MS, RA, T1D	+	23,42
DNA vaccine	1990s	plasmid	Allergy, autoimmune	-	~5 on allergic asthma, conjunctivitis	+	10,43
Co-immunized Vaccine	2005	Antigen +DNA	Allergy autoimmune	-	2 on allergic dermatitis, AD	+	44-46
Suppressed immunization vaccine	2008	Antigen +Ist	Allergy autoimmune	-	1 on T1D	+	47

Figure 2. A history of tolerogenic vaccines as specific interventions for inflammatory diseases. (**A**) Timeline of vaccine discovery. (**B**) Current state of tolerogenic vaccines. SIT: specific immunotherapy. TolerogenicVac: tolerogenic vaccine. Td: Time of discovery. Trial: clinical trials from USA, Europe and China. Ist: Immunosuppressant. Aprv: Approved by USA FDA or European Union. Spec: antigen specificity. RA: rheumatoid arthritis. MS: multiple sclerosis. T1D: type 1 diabetes. AD: Alzheimer Disease.

in draining lymph nodes after the suppressed immunization (**Fig. 3B**). The main function of T_{reg} cells is to suppress T cell mediated inflammation and in the case of diabetes, the disease is a result of attack on islet cells by auto-reactive T cells, most of which are insulin specific cytotoxic $CD8^+$ T cells. We therefore assessed the activities of cytotoxic $CD8^+$ T cells reacting against islet cells (**Fig. 3C**), insulin 10–18 epitope (**Fig. 3D**) or preproinsulin 15–24 (**Fig. 3E**). The level of specific cytotoxic $CD8^+$ T cell activity was suppressed substantially after the suppressed immunization vaccine (**Figs. 3C–D**), although suppression of activity against preproinsulin 15–24 epitope did not reach statistical significance. This data suggested not only that induced T_{reg} cells inhibited diabetes by suppressing auto-reactive cytotoxic $CD8^+$ T cells but also that the epitope specificity of the target $CD8^+$ cells influenced the degree of susceptibility to inhibition by the T_{reg}. Overall, the degree of suppression by the induced T_{reg} cells was similar to that seen in mice treated with anti-CD8 antibodies. The induced tolerogenic responses were antigen-specific since irrelevant antigen suppressed vaccines did not show any benefit for such amelioration.

With its inherent advantage of an antigen specific tolerogenic response, tolerogenic vaccination has shown proof-of-concept in these animal studies and become an attractive approach for translational research and human trials.

Translational research for tolerogenic vaccines

Before a move into human trials, there are 2 issues of concern in translating a candidate tolerogenic vaccine from lab into bedside: safety and efficacy.

In considering safety, it is evident that caution is needed when new formulations are used to develop tolerogenic vaccines since there is no history of such applications being tested in humans, For this reason, drugs such as dexamethasone, rapamycin or cyclosporin A that are already established for use in humans

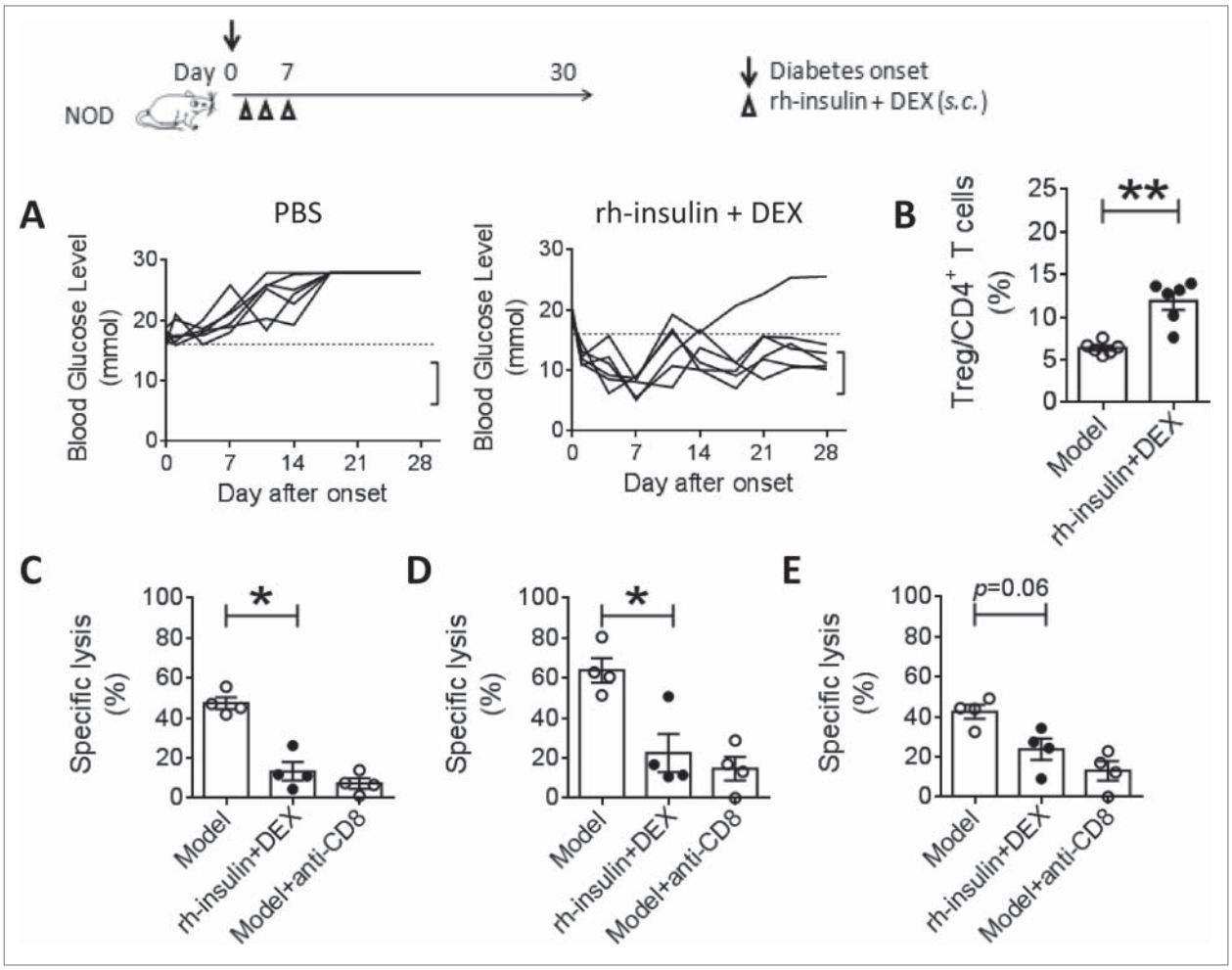


Figure 3. Combination vaccination controlled new-onset diabetes and cytotoxic inflammation in NOD mice. New-onset diabetic NOD mice were treated with rh-insulin plus DEX (10 μg+10 μg) at days 1, 4 and 7. (**A**) Blood glucose was measured at intervals for 28 d Dashed line indicates the diabetes threshold (16.7 mmol/L). Square bracket indicates the normal glucose range of healthy animals (5~12 mmol/L). (**B**) Percentage of regulatory T cells among draining lymph node cells. n = 6. On day 14, in vivo cytolytic assay (CTL) was performed against islet cell (**C**), insulin 10–18 epitope (**D**) or preproinsulin 15–24 (**E**). Unvaccinated mice were used as CTL control. Anti-CD8 mAb was *i.p.* injected to block CTL in unvaccinated mice as positive control. n = 4. Data represent 3 independent experiments.

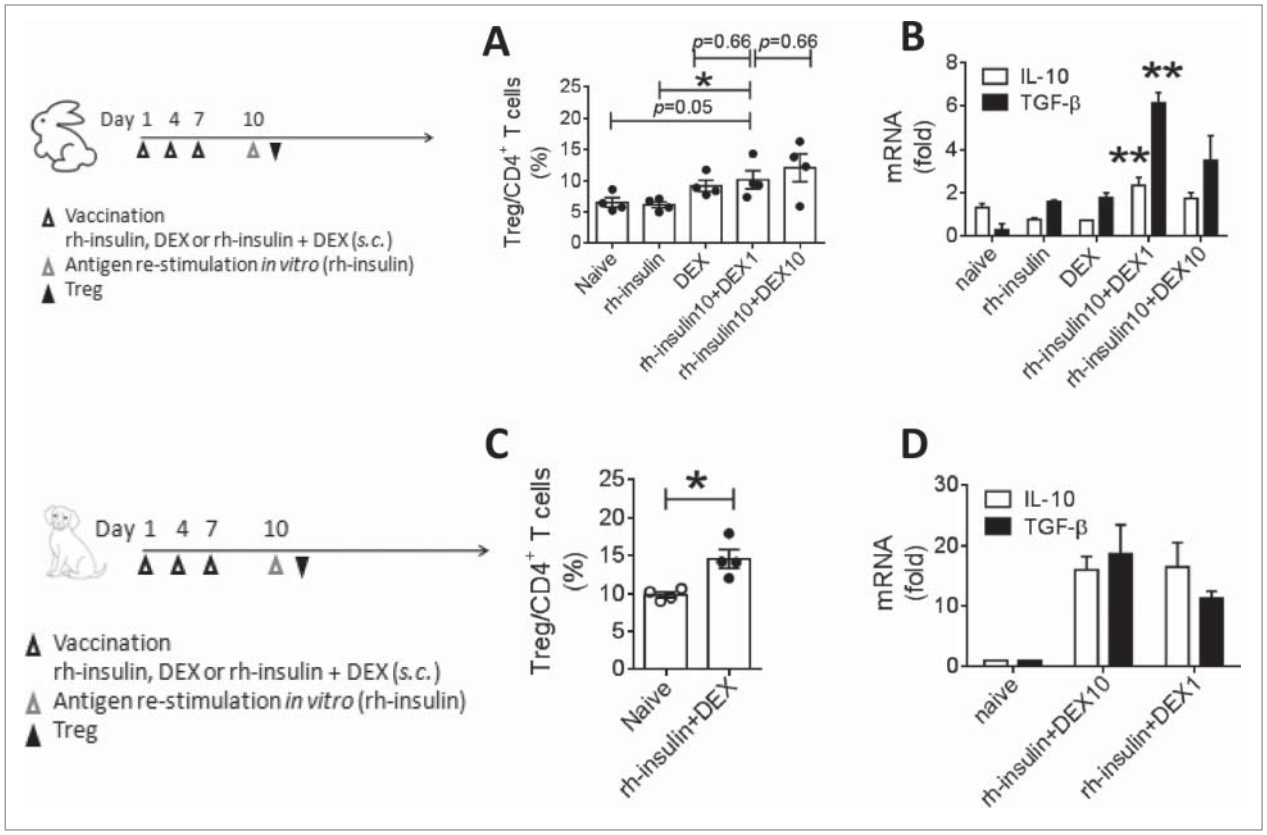


Figure 4. Combination vaccination induced immune suppression in rabbit and dog. Rabbits and dogs were vaccinated at day 1, 4 and 7. At day 10, T_{reg}s were analyzed in draining LNs of rabbits (**A**) or dogs (**C**). After in vitro restimulation with rh-insulin for 24 h, IL-10/TGF-β mRNA was analyzed in LN cells of rabbits (**B**) or dogs (**D**). n = 4. Data represent 3 independent experiments.

should be considered the first choice for administration with the antigen since their history of adverse effects is well documented.

For efficacy, evidence from studies in larger animals should be obtained before test in humans. To this end, we tested the T1D tolerogenic suppressed immunization vaccines in rabbits and dogs, assessing tolerogenic responses in addition to safety. As shown in **Figure 4**, the levels of T_{reg} cells, IL-10 and TGF-β were increased when immunized with the suppressed immunization vaccine compared with other groups in rabbits and dogs. The tolerogenic responses are apparently dose-dependent response. When this suppressed immunization vaccine regimen was incubated with fresh isolated PBMC of T1D or healthy individuals in vitro, we observed that co-stimulation molecule CD40 was down-regulated (**Fig. 5A**) and IL-10 was upregulated (**Fig. 5B**) in $CD1a^+$ dendritic cells compared with incubations of other regimens. The results suggest that the tolerogenic suppressed immunization vaccine could induce tolerogenic responses against auto-reactive responses in larger animals, and maybe in humans as well. In the safety evaluations, we did not observe notable signs of hypoglycemia or behavior abnormalities in the vaccinated large animals (**Table 2**).

Taking the results together, tolerogenic vaccination appears to be a promising strategy for treating T1D and should be considered for moving to clinical tests if other requirements are met.

Mechanisms behind induction of antigen specific tolerance

Proper interpretation of immune regulation, particularly after the discovery of regulatory T cell (T_{reg}), requires an understanding of tolerance mechanisms.[53] In simple terms, T_{reg} is a subset of $CD4^+$ T cells that is characterized by expression of transcription factor Foxp3, and this subset helps to keep inflammation under control in healthy individuals. Deficiency of T_{reg} or Foxp3 leads to inflammatory reactions and multi-organ autoimmune diseases. Elegant reviews on T_{reg}'s origin, development and function have been published elsewhere.[54-56]

The use of T_{reg} cells as a tool to treat inflammation and autoimmune diseases has been proposed and well received in the scientific community. There are several approaches as induction of tolerogenic responses and their applications listed in **Table 3** and **Figure 6**. Two of them, the co-immunized vaccine[57] and suppressed immunization vaccine,[48] were discovered in our labs. Both of these approaches are aimed at inducing antigen specific T_{reg} cells to control inflammatory reactions and autoimmune diseases. We also discovered that the co-immunized vaccine regimens entered DC cells via a caveolae-dependent route that generated negative signaling, to shut down the IRANK-NF-kB pathway and CD40 expression, but turned on the expression of IL-10 and TGF-b. In this way, the DC became

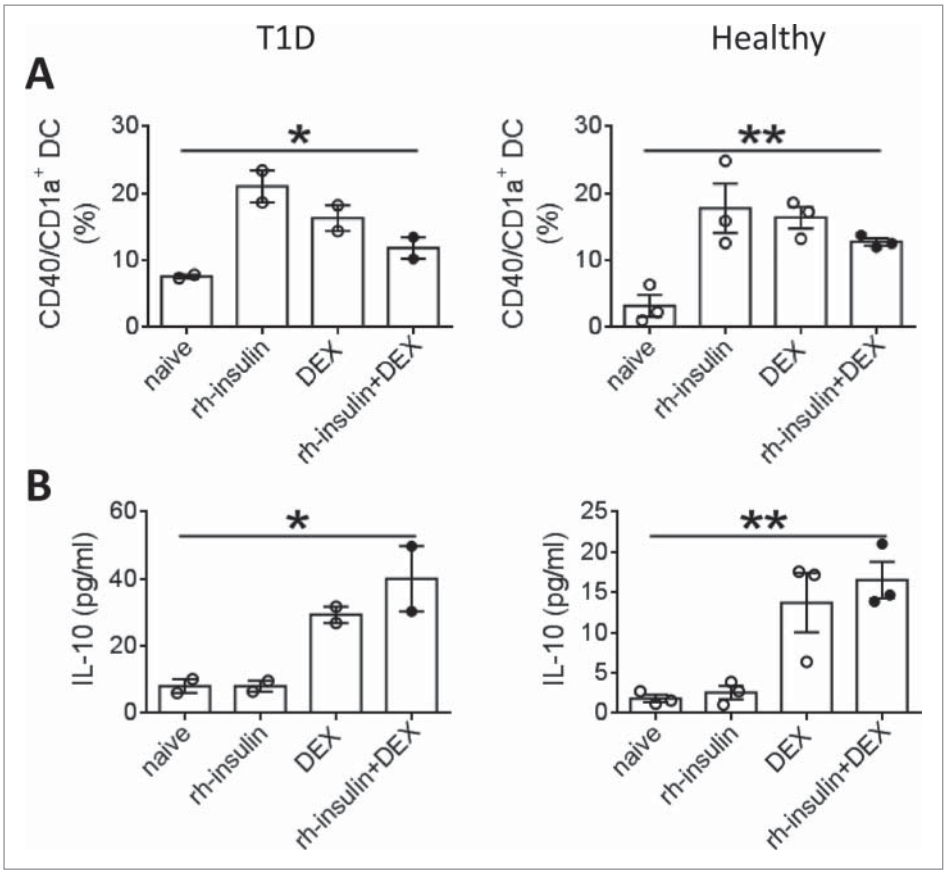


Figure 5. Suppressed immunization vaccine controlled human dendritic cell maturation. PBMC from 2 T1D patients (**A, C**) and 3 healthy donors (**B, D**) were cultured with rh-insulin, DEX or the combination for 3 d. Dendritic cell maturation (**A, B**) and IL-10 secretion in culture were analyzed (**C, D**).

$CD40^{low}IL\text{-}10^{+}$ regulatory dendritic cells (DC_{reg}) that could in turn cause naïve T cells to differentiate into inducible regulatory T cells (iT_{reg}).[58] Since this differentiation into iT_{reg} required MHC-II-TCR signaling, this production of iT_{reg} cells required antigen specific recognition and accordingly resulted in suppression of cognate antigen specific T cell activation and inflammation.

In another hand, the understanding of exact mechanism of tolerance induction by the suppressed immunization vaccine (e.g. protein+Dex) is improved. Dexamethasone preferentially induces apoptosis of dendritic cells (cDCs and pDCs),[59] effector $CD4^+$ T cells (T_{eff})[60] and B-2 cells;[61] whereas the $CD11c^{lo}CD40^{lo}CD86^{hi}MHCII^{lo}$ macrophages, $CD4^+Foxp3^+$ T_{reg} cells, and $IL\text{-}10^+$ B-1 cells are relatively protected.[59-61] As a result, Ag immunization in the presence of Dexamethasone preferentially expands Ag-specific T_{reg} cells and B-1 cells while blocking the recall responses of T_{eff} and B-2 cells, which leads to tolerance.[59,61]

Taking these new findings together, iT_{reg} induction by vaccination is an attractive and feasible approach toward human applications although its detailed mechanisms remain to be further investigated.

We note that besides the $CD4^+$ T_{reg} cell, immuno-tolerance can be induced by other types of cells including CD8 T_{reg},[62,63] regulatory B cells (B_{reg}s)[64-66] and IL-10 producing B cells (B10 cells).[67] Since these cells can also participate in tolerance induction in inflammatory contexts, they too might potentially be drawn into application in tolerogenic vaccines against autoimmune related diseases.

Concluding remarks

In conclusion, the tolerogenic vaccine provides a feasible easy means to induce specific tolerogenic responses to reduce inflammation and has a great

Table 2. Safety assessments

		Hypoglycemic symptoms			Behavior abnormalities	
	Group	Vomit	Seizure	Shock/Death	Depression	Dysphoria
Rabbit	Naive	−	−	−	−	−
	rh-insulin	−	−	−	−	−
	DEX	−	−	−	−	−
	rh-insulin10+DEX1	−	−	−	−	−
	rh-insulin10+DEX10	−	−	−	−	−
	Overdose	+	+	+	+	+
Dog	Naive	−	−	−	−	−
	rh-insulin	−	−	−	−	−
	DEX	−	−	−	−	−
	rh-insulin10+DEX1	−	−	−	−	−
	rh-insulin10+DEX10	−	−	−	−	−
	Overdose	+	+	+	+	+

−: no signs during monitoring. +: shows signs. rh-insulin: recombinant human insulin. DEX: dexamethasone. rh-insulin10+DEX1: 10 μg rh-insulin mixed with 1 μg DEX. rh-insulin10+DEX10: 10 μg rh-insulin mixed with 10 μg DEX.

potential for clinical application against major inflammatory diseases, such as allergy, asthma, autoimmune diseases, atherosclerosis and Alzheimer disease. Accordingly, we propose here that efforts should be devoted to development of these novel vaccine strategies, expansion of the spectrum of diseases targeted and translation from bench to bedside. To achieve this the underlying mechanisms will require further exploration.

Table 3. Mechanism of induction of T_{reg}s by tolerogenic vaccines

Tolerogenic Vaccines	T_{reg} induction	T_{reg} specificity
Oral Tolerogenic Vaccine	34,35	36
SIT	37	38
Synthetic peptide vaccine	39	70
DNA vaccine	71	71
Co-immunization vaccine	26,27	28
Suppressed immunization vaccine	24	24

Material and Methods

Animals and samples

Foxp3-eGFP mice of Balb/c background, NOD mice, New Zealand White female rabbits and female Beagles dogs were purchased from VitalRiver (Beijing, China) and maintained in Specific-Pathogen-Free conditions. Peripheral blood was collected from healthy donors with informed consent. All animal experiments were reviewed and permitted by the Experimental Animal Ethics Committee of Shanghai Medical College. All experiments using human PBMC were reviewed and permitted by the Clinical Experiment Ethics Committee of Shanghai Medical College.

Vaccination, re-challenge, re-stimulation and culture

The rh-insulin was purchased from Eli Lily(Indianapolis, Indiana, USA) in 400IU/10 ml packages, and dose conversion was calculated as 1IU = 35 μg as instructed. Dexamethasone was purchased from Sigma Aldrich and dissolved first in 0.2 MNaOH then diluted in PBS to final concentration of 10 mM and the pH adjusted to 7.2–7.4. Suppressed immunization vaccines were prepared at doses suitable for the different animals: mouse, 10 μg insulin + 10 μg DEX; rabbit 10 μg insulin + 10 μg DEX or 10 μg insulin + 1 μg DEX; dog, 0.15IU/kg insulin + 10 μg/kg DEX or 0.15IU/kg insulin + 1 μg/kg DEX. At days 1,4 and 7, animals were s.c. injected with single or suppressed immunization vaccine in 100 μl (mouse and rabbit) or 200 μl (dog) PBS.

Vaccinated mice were given a challenge dose of rh-insulin, B9–23 epitope or ovalbumin(OVA) at day 10 and after 24 h, the draining lymph nodes(Inguinal LNs) were collected to test for T_{reg} responses. Vaccinated rabbits or dogs were sacrificed then draining lymph nodes were collected for in vitro re-stimulation. Single cell suspensions were cultured with rh-insulin 10 μg/ml for 24 h, then total RNA was extracted by Trizol and reverse transcribed into cDNA with oligo-dT as primer in 42°C for 1 h. PCR was performed to test expression of IL-10 and TGF-β. For human PBMC stimulation, PMBCs from patients or healthy donors were first induced into dendrtic cells with GMCSF and IL-4, then rh-insulin and/or DEX were added into cultures at a final concentration of 10 μg/ml each for 3 d CD40 expression on $CD1a^+$ cells was then analyzed by FACS, and IL-10 secretion into the supernatant was analyzed by FlexSet (BD Biosciences, San Jose, CA,USA). All cell culture was performed with DMEM with 5% FBS, in 5% CO_2 incubator at 37°C.

Type 1 diabetes model

Young female NOD mice were monitored for hyperglycemia every 2 d Mice with 2 consecutive elevations of blood glucose level (>16.7 mmol/L) were defined as new-onset diabetes (Day 0) and then

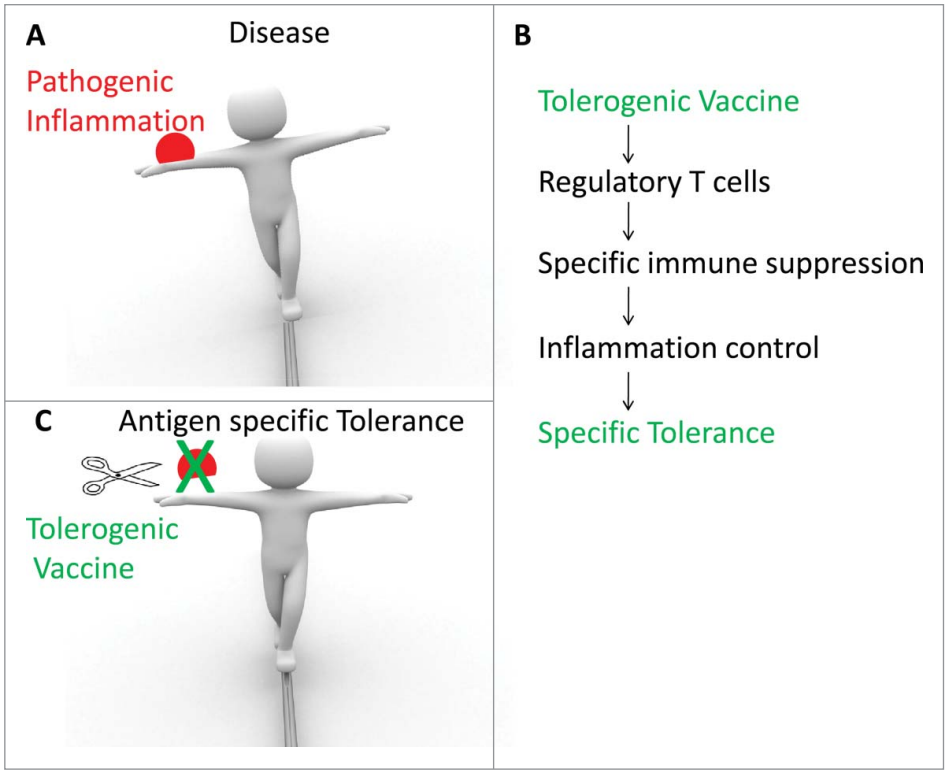


Figure 6. T_{reg} as a mechanism for tolerogenic vaccines. Specific tolerance targeting pathogenic inflammation (**A**) can be induced by tolerogenic vaccine through various mechanisms including T_{reg} (**B**), and in turn ameliorates disease progression (**C**).

treated with vaccine at days 1, 4 and 7. Blood glucose was monitored until day 28.

Flow cytometry

To detect murine T_{reg}, single cell suspensions were prepared then stained with anti-CD4-APC andanti-CD25-PE. Foxp3 was labeled with endogenous GFP or was intracellular-stained with anti-Foxp3-FITC mAb. For rabbit or dog, surface staining of CD4-FITC(clone KEN-4 or clone YKIX302.9) was followed by intracellular staining of Foxp3-APC(clone FJK-16s) with T_{reg} staining kit (eBioscience, San Diego, CA, USA). For PBMC maturation markers, 72 h after in vitro re-stimulation, cells were washed with PBS and stained with CD1a-FITC, CD40-PE/CD80-PE/CD83-PE/CD86-PE. All antibodies were checked for species specificity and ordered from eBioscience. Data were collected with BD Calibur(BD Biosciences) and analyzed with Flowjo (Treestar, USA).

In vivo CTL

New-onset diabetic NOD mice were treated with suppressed immunization vaccine at days 1, 4 and 7. At day 14, target cells were prepared either with islet cells from naive NOD mice, or with naive NOD splenocytes incubated with 500 μg/ml peptide epitope (insulin B10–18 epitope or preproinsulin 15–24 epitope[68,69]). Control cells were prepared with naive NOD splenocytes without epitope. Target cells and control cells were labeled with eFluor670 (10 μM for target cells, 2 μM for control cells), mixed at 1:1 ratio, then *i.v.* transferred (2×10^6 totalcells/mouse) into vaccinated diabetic mice. After 12 h, spleens of recipient mice were collected to assess specific cytolysis. Percentage of specific cytolysis was calculated as (Control cell-Target cell)/Control cell×100%. Purified anti-CD8 mAb (100 μg) was *i.p.* injected at days11, 12 and 13 into new-onset diabetic NOD mice as a depletion control for CTL suppression.

Evaluation of hypoglycemic symptom

Each NOD mouse was closely observed for hypoglycemic signs (vomit, seizure, shock or death) and behavior abnormalities (depression or dysphoria) for 4 h after vaccination and then once a day between injections. The incidence of these signs was recorded as either positive or negative. Overdose of rh-insulin (500 μg per rabbit or 0.3IU/kg for dog) was used as hypoglycemia positive control, and animals experiencing hypoglycemic shock were injected *i.v.* with saline containing 10 g glucose to rescue.

Statistics

Two-tailed Mann-Whitney test (**Figs. 3B–E**, **4A and C**) or 1-way ANOVA test (**Figs. 4B and 5**) was used to calculate significance of data.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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