

Protective Regulatory T Cell Generation in Autoimmune Diabetes by DNA Covaccination with Islet Antigens and a Selective CTLA-4 Ligand

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DNA vaccination of autoimmune diabetes-prone NOD mice with unmodified target islet antigens, i.e., preproinsulin (PPIs) or glutamic acid decarboxylase 65 (GAD65), is poorly protective. However, in this study, we demonstrate protection against disease by covaccination with a mutant B7-1 molecule (B7-1wa) that binds the negative T cell regulator CTLA-4 (CD152), but not CD28. Codelivery of plasmids encoding a PPIs–GAD65 fusion construct and B7-1wa protected against both insulinitis and diabetes. *In vitro*, the T cells of covaccinated mice had negative responses to both insulin and GAD65, and this was restored by adding blocking antibodies to transforming growth factor β 1 (TGF- β 1), suggesting a role for this cytokine. Adoptive transfer experiments revealed that DNA vaccination generated protective CD4⁺ regulatory T cells (Tr) of either CD25⁺ or CD25⁻ phenotype. Furthermore, vaccinated mice had increased numbers of T cells with Tr-associated markers, such as CTLA-4, Foxp3, and membrane-bound TGF- β 1. Tr cells inhibited the responses of diabetogenic T cells to islet antigens, and depletion of T cells expressing membrane-bound TGF- β 1 abolished the suppressive effect. Thus, selective engagement of CTLA-4 during islet-antigen DNA vaccination induces Tr cells that protect against this autoimmune disease.

Key Words: CTLA-4, DNA vaccination, islet antigens, NOD mice, regulatory T cells

INTRODUCTION

Nonobese diabetic (NOD) mice are prone to develop autoimmune (type 1) diabetes mellitus (T1D), related to T helper 1 and cytotoxic T lymphocyte (CTL) reactivity to islet antigens [1–3]. There is increasing evidence that this results from a failure of regulatory T cell (Tr) activity [3–8]. Indeed, there are reports that transforming growth factor β 1 (TGF- β 1)-producing Tr cells protect against this disease, and their function declines with age [4–6]. We found that TGF- β 1 gene therapy prevents diabetes in NOD mice [9], and various protective approaches such as CD3 mAb therapy [8] act at least in part through induced Tr cells (iTr) that produce TGF- β 1. 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) [10–12].

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. One approach is DNA vaccination against target autoantigens (or their peptides), such as insulin or glutamic acid decarboxylase 65 (GAD65). This has been successful in some studies [13–17], but the results have been variable (reviewed in [1,18]) and, in most cases, the mechanisms of protection have not been clearly determined. Covaccination with islet antigen/Ig fusion and IL-4 genes has improved protection [19,20], presumably by inducing a T helper 2 (Th2) bias, but there is evidence that Th2 cells can be pathogenic [1], and to our knowledge other types of Tr cells have not been consistently generated by DNA vaccination.

In this study, we show that Tr cells can be generated by DNA vaccination when delivery of the antigen gene is combined with a CTLA-4 (CD152) ligand. This covaccination approach was strongly protective against T1D in NOD mice. CTLA-4 is a powerful negative regulator of T cells and, although there has been some controversy, appears essential for the activity of some Tr cells

[11,12,21]. Furthermore, recent studies reveal that CTLA-4 (along with PD-1) plays a critical role in the induction of T cell tolerance by dendritic cells [22]. No natural ligand discriminates between CTLA-4 and the positive costimulatory molecule CD28, since both molecules bind to B7-1 and B7-2 (expressed primarily by antigen-presenting cells), and no other ligands are known. To solve this problem, we used a mutated B7-1 molecule (B7-1wa), which has a single amino acid substitution and binds CTLA-4 but not CD28 [23–25]. Previously, we used preproinsulin I (PPIns) as a target molecule for DNA vaccination and covaccinated with B7-1wa, but this only partly protected against disease, and Tr cells were not identified [26]. Here, we employed a PPIns–GAD65 fusion (Ins-GAD) construct as the target antigen to introduce a larger number of autoantigenic target epitopes. DNA covaccination allows the linkage of antigen recognition by the T cell receptor with delivery of a negative regulatory signal. This consistently generated Tr cells that inhibited responses to insulin or GAD65 peptide and protected against spontaneous development of disease or adoptive transfer disease in NOD.scid mice. The Tr cells included CD4⁺ cells of both CD25⁺ and CD25⁻ phenotype and expressed markers associated with Tr function, i.e., CTLA-4, Foxp3, and membrane-associated TGF- β 1. This is the first study demonstrating induction of this type of Tr cell by DNA vaccination and provides a promising approach for vaccination against autoimmune diseases.

RESULTS

Reduced Incidence of Diabetes in VR-B7-1wa/VR-Ins-GAD-Covaccinated Mice

Untreated female NOD mice had an incidence of disease of 64% at 31 weeks of age and this was significantly reduced by VR-B7-1wa/VR-Ins-GAD covaccination (Fig. 1). We inoculated mice with plasmids, beginning at 4 weeks of age. The total amount of DNA was 50 μ g per mouse (see Materials and Methods). We repeated the vaccination four more times at 4-week intervals, and in each case we applied electroporation to increase gene transfer. Two vaccinations were less protective than five vaccinations (not shown). The incidence of diabetes was reduced modestly but significantly ($P < 0.05$) by inoculation with either a control blank plasmid (VRblank) or VR-B7-1wa. Inoculation with VR-Ins-GAD reduced diabetes more than VRblank and VR-B7-1wa, but this was not significant compared to VRblank and VR-B7-1wa, although significant versus untreated mice ($P < 0.05$). On the other hand, combined inoculation of VR-B7-1wa and VR-Ins-GAD reduced the incidence of disease to approximately 11.8%, and this was significantly lower than all other groups ($P < 0.01$). As in our previous studies [26], wild-type B7-1 was not protective in these experiments (Fig. 1).

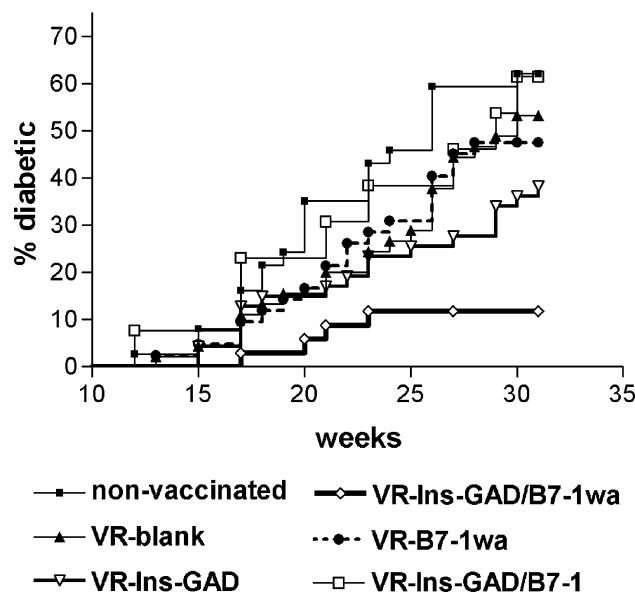


FIG. 1. Covaccination with VR-Ins-GAD and VR-B7-1wa protected against autoimmune diabetes more efficiently than each of the components separately. NOD mice were vaccinated by im DNA injection (with electroporation) at 4 weeks of age. The vaccination procedure was repeated four more times at 4-week intervals. Incidence of diabetes (% diabetic) in all groups is compared to the incidence in the blank-injected group, using log-rank statistics. $N = 32$ – 45 mice per group. Covaccination with VR-Ins-GAD and VR-B7-1wa was significantly different from all other groups ($P < 0.01$; see text). The data are representative of two independent experiments.

Insulinitis Scores

The administration of VR blank, VR-Ins-GAD, or VR-B7-1wa did not significantly improve insulinitis compared to untreated NOD mice (Table 1). However, covaccination with VR-Ins-GAD and VR-B7-1wa resulted in a remarkable amelioration of insulinitis versus all other groups ($P < 0.001$). These covaccinated mice had increased numbers of islets that either were normal (grade 0) or had only mild peri-insulinitis (grade 1) and a marked reduction in the number of islets with intraislet lymphocytic infiltration (true insulinitis; grade 3 or 4 lesions). When administered alone, VR-B7-1wa induced a modest reduction in the mean grade of insulinitis, which was significantly different from treatment with either VRblank or VR-Ins-GAD ($P < 0.01$), but not significantly different from untreated mice.

Adoptive Transfer of Disease and Identification of Tr Cells

In these experiments, we adoptively transferred diabetes by injecting (iv) 10^7 spleen cells from diabetic NOD mice into NOD.scid mice (Fig. 2). The transfer of spleen cells from diabetic mice resulted in the rapid induction of diabetes, but none of the NOD.scid mice receiving spleen cells from VR-B7-1wa/VR-Ins-GAD-covaccinated mice developed disease.

To identify putative Tr cells and their phenotype, we fractionated the spleen cells of VR-B7-1wa/VR-

TABLE 1: Intramuscular injection of VR-Ins-GAD and VR-B7-1wa protects NOD mice from autoimmune insulinitis

Vector treatment ^a	Mean grade of insulinitis	% of islets normal or grade 1 ^b	% of islets grade 3 or 4
Untreated	1.8	48	43
VR blank	2.1	37	51
VR-Ins-GAD	2.0	38	54
VR-B7-1wa	1.3 ^c	59	30
VR-Ins-GAD/ VR-B7-1wa	0.6 ^d	79	7

^a Female NOD mice (4 weeks old) were injected i.m. with 50 μ g (25 μ g in each leg) of vector DNA at day 0 and day 21 (two injections) with VR blank, VR-B7-1wa, VR-Ins-GAD, or VR-Ins-GAD and VR-B7-1wa vectors. Electroporation was applied as described under Materials and Methods.

^b Nondiabetic mice were killed at day 50 of the experiment, and the pancreas was removed for histological examination. Mean insulinitis grade is reported for each group of mice ($n = 20-24$); ≥ 100 islets were scored per group. Grade 0 islets are normal, and grade 1 have only a small peri-islet lymphocytic infiltrate (peri-insulinitis). Grade 2 islets have prominent peri-insulinitis. Grade 3 or 4 islets have true insulinitis, with lymphocytes clearly invading into the islets of Langerhans.

^c $P < 0.01$ versus VR blank and VR-Ins-GAD groups.

^d $P < 0.001$ versus all other groups.

Ins-GAD-covaccinated mice into three populations, i.e., CD4⁺CD25⁺ cells, CD4⁺CD25⁻ cells, and CD8⁺ cells. For the injections, we mixed 5×10^5 cells of either of the CD4⁺ subsets or 1.4×10^6 CD8⁺ cells with 10^7 diabetogenic cells. Adoptive transfer of these subpopulations with diabetogenic spleen cells revealed that the CD8⁺ T cells were not protective, while both CD4⁺ subpopulations were significantly, and almost equally, protective (Fig. 2). Thus, DNA vaccination generated regulatory T cells of both CD4⁺CD25⁺ and CD4⁺CD25⁻ phenotypes.

Increased Numbers of T Cells with a Regulatory Phenotype in Vaccinated Mice

Flow cytometry analysis revealed that VR-B7-1wa/VR-Ins-GAD-covaccinated mice had increased numbers of cells with regulatory phenotype, compared to nonvaccinated diabetic mice (Figs. 3A–3D and 4A–4D). Significantly more cells from the covaccinated mice coexpressed CD4/CD25 (Figs. 3A and 4A), CD4/Foxp3 (Figs. 3B and 4B), CD4/latency-associated peptide (LAP)-TGF- β (Figs. 3C and 4C), and CD4/CTLA-4 (Figs. 3D and 4D). In the VR-Ins-GAD/VR-B7-1wa-covaccinated mice there was also an increase in the number of T cells expressing neuropilin 1 (Nrp-1) or B7-1, and these cells overlapped extensively with T cells expressing other Tr-related markers (CD25, CTLA-4, membrane TGF- β) (data not shown).

Characterization of Tr Cells *in Vitro* and Putative Mechanism of Action

To characterize further the phenotype of Tr cells in VR-Ins-GAD/VR-B7-1wa-covaccinated mice, we fractionated the spleen cells of these mice into various subpopulations and examined the ability of these subpopulations to suppress the responses of T cells of diabetic mice to islet antigens *in vitro*. These experiments confirmed that the

suppressor (Tr) activity resided in the CD4⁺ population (Fig. 5). Indeed, CD8⁺ T cells enhanced responses and had no apparent regulatory activity. Thus, the *in vitro* experiments were in accord with the *in vivo* adoptive transfer experiments.

To analyze the potential contribution of IL-10 and TGF- β 1 as a mechanism of suppression, we added blocking antibodies against these cytokines in assays consisting of spleen cells of VR-Ins-GAD/VR-B7-1wa-vaccinated mice and islet cell antigens (Fig. 6A). While anti-IL-10 antibodies had no effect, we found that anti-TGF- β 1 antibodies restored the ability of the unfractionated cells of covaccinated mice to respond to islet antigens. This supports the view that TGF- β 1 is a key suppressor cytokine in this assay; however, islet-antigen stimulation did not reveal significantly increased concentration of this cytokine in culture supernatants (not shown). An alternative possibility is that TGF- β 1 is primarily cell bound, as suggested by the cell depletion experiments described below.

We separated CD4⁺ cells into various subsets to estimate their suppressive capacity upon addition to the antigen-stimulated diabetogenic cells. Surprisingly, depletion of cells coexpressing CD4 and CD25 did not reduce the suppressive activity of the T cells of VR-Ins-GAD/VR-B7-1wa-covaccinated mice (Fig. 6B). However, this might

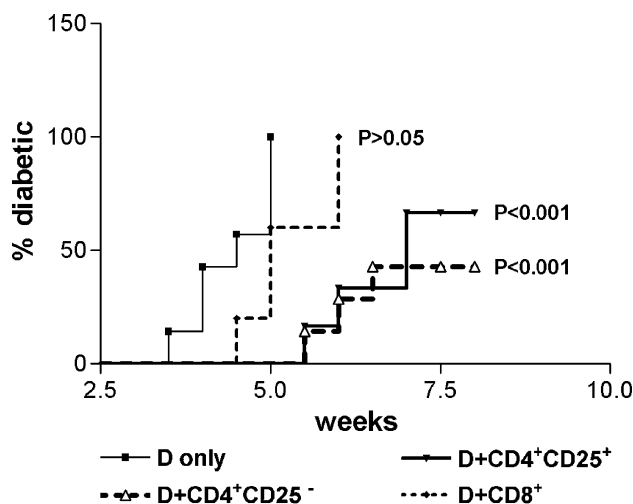


FIG. 2. Prevention of disease by adoptive transfer. Adoptive transfer of splenic T cells from the Ins-GAD/B7-1wa-covaccinated mice prevented autoimmune disease induced by transfer of diabetogenic spleen cells (D) into NOD.scid mice. Spleen cells from covaccinated mice (V) were separated into CD4⁺ and CD8⁺ fractions by negative sorting. CD4⁺ cells were further sorted into CD25⁺ and CD25⁻ subpopulations. Prior to adoptive transfer, diabetogenic spleen cells were mixed with either CD4⁺CD25⁺ or CD4⁺CD25⁻ cells at a ratio of 20:1 (D:V) and with CD8⁺ cells at a ratio of 7:1 (D:V). Each scid mouse ($N = 7-8$ /group) received 10^7 diabetogenic cells in a single injection. The incidence of diabetes is compared to that in the group that received only diabetogenic cells, using log-rank statistics, and P values are indicated on the curves. Both CD4⁺CD25⁺ and CD4⁺CD25⁻ cells were protective. Unfractionated CD4⁺ cells were also protective (not shown).

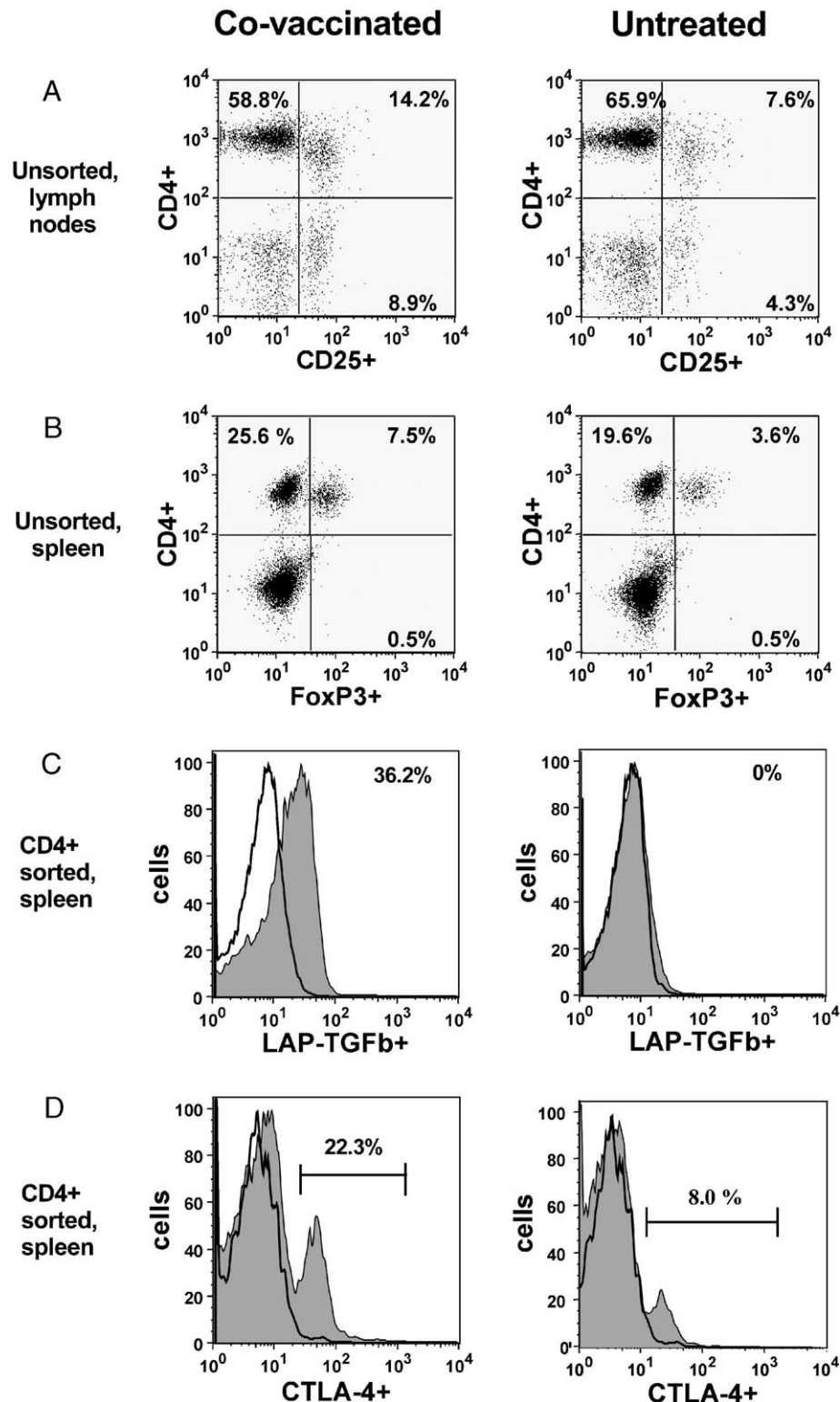


FIG. 3. Tr cell phenotypes in vaccinated NOD mice. The spleen or lymph node cells from an Ins-GAD/B7-1wa-covaccinated mouse (left column) or from an untreated mouse (right column) were analyzed by multicolor flow cytometry. (A) Coexpression of CD4 and CD25 in mesenteric lymph node cells (unsorted prior to staining). (B) Coexpression of CD4 and Foxp3 in spleen cells. (C) Expression of LAP-TGF- β in CD4⁺ sorted spleen cells (negative magnetic sorting). (D) Expression of CTLA-4 in CD4⁺ sorted spleen cells (negative magnetic sorting). The percentages of the positive cells are indicated in relevant sections of the histograms. In C and D, the histograms of isotype controls are denoted by unshaded areas and those of LAB-TGF- β and CTLA-4 by shaded areas.

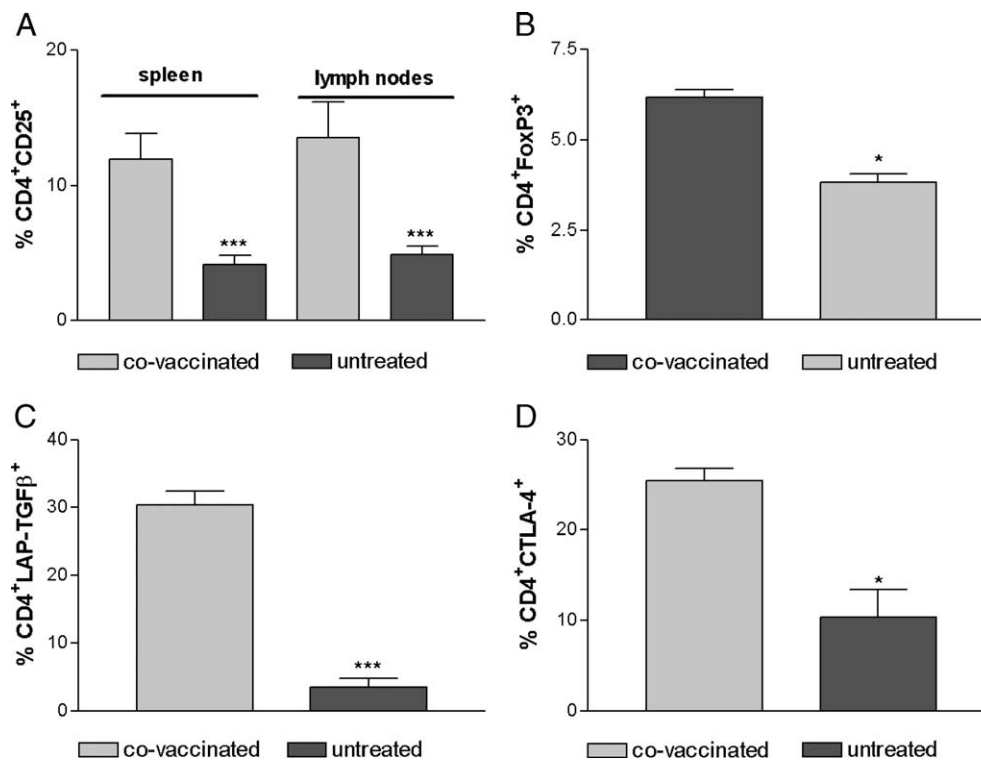


FIG. 4. Tr cell phenotypes in vaccinated and untreated NOD mice. Spleen and mesenteric lymph node cells were stained for multicolor flow cytometry analysis as in Fig. 3. (A) Percentage of cells coexpressing CD4 and CD25 in spleen and lymph nodes. (B) Percentage of cells coexpressing CD4 and Foxp3 in spleen cells. (C) Percentage of sorted splenic CD4⁺ cells expressing LAP-TGF-β. (D) Percentage of sorted splenic CD4⁺ cells expressing CTLA-4. The data represent the means of 3 to 6 independent experiments. The difference between covaccinated and untreated mice was statistically significant: * $P < 0.05$; *** $P < 0.001$.

have resulted in enrichment of a CD25⁻ population of Tr cells. Importantly, depletion of membrane-bound LAP-TGF-β⁺ T cells totally removed Tr activity, suggesting again that TGF-β was involved in suppression. Depletion of cells coexpressing CD4 and B7-1, or CD4 and Nrp-1, had a significant but lesser effect on reducing Tr activity, perhaps because these cells overlap with those expressing TGF-β.

Antigen Specificity of Suppression *in Vitro*

T cells from VR-Ins-GAD/VR-B7-1wa-covaccinated mice suppressed proliferation of diabetogenic T cells induced by insulin and GAD peptides, but not the proliferation of T cells of chicken ovalbumin (OVA) DNA-activated mice stimulated by OVA (Table 2). In contrast, the T cells from VR-OVA-vaccinated mice were not suppressive of T cells stimulated by either insulin or GAD peptides. These data suggest that the Tr cells of VR-Ins-GAD/VR-B7-1wa-covaccinated mice are specific for islet antigens and presumably exert their activity only after stimulation by these antigens.

DISCUSSION

A major goal in the therapy of autoimmune diseases is to restore tolerance to self antigens. Many approaches have

been investigated to achieve this, although clinical applications have thus far been limited. One avenue involves coupling T cell recognition of autoantigens with the delivery of signals that turn T cells off or induce their differentiation to a nonpathogenic type. It is not easy to accomplish this by conventional antigen therapy, but DNA vaccination provides this opportunity. Previous studies in diabetes-prone NOD mice, or in models of experimental autoimmune encephalomyelitis, have suggested that DNA vaccination against target autoantigens was protective [13–17]. However, in other studies very similar protocols aggravated these diseases, for reasons that were not clear [1,18,34,35]. 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 [19,20]. 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. In this study we investigated a different approach, in which 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. Further-

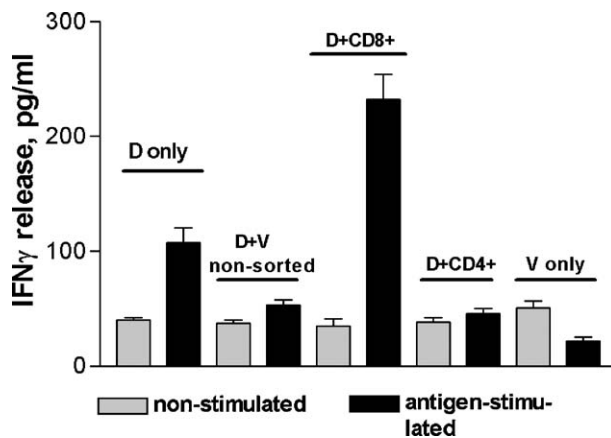


FIG. 5. *In vitro* suppression is produced by CD4⁺ Tr cells. Unsorted and CD4⁺ sorted spleen cells from Ins-GAD/B7-1wa-covaccinated mice (V, unsorted spleen cells) suppress antigen-stimulated release of IFN- γ by diabetogenic cells (D), while CD8⁺ cells from the covaccinated mice have no suppressive activity. The cells were stimulated with a mixture of insulin and GAD peptides (50 μ g/ml each) for 72 h before harvesting. The bars represent the means \pm SEM. IFN- γ release by diabetogenic antigen-stimulated cells alone or mixed with CD8⁺ cells was significantly different from that of unstimulated cells or diabetogenic cells mixed with CD4⁺ cells ($P < 0.05$). The data are representative of four independent experiments.

more, it is expressed by both iTr and nTr cells and may be essential for the activity of either type or regulatory cells [7,10–12].

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 [36]. 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. Studies by others [23,24] and us [25] have confirmed the inability of this molecule to engage CD28. In a previous study [26] 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. However, the protective effect was limited, and disease incidence was reduced by only approximately 50%. In other experiments, we found that B7-1wa did not improve the effects of DNA vaccination against GAD65 (unpublished observations). In the case of PPIs/B7-1wa vaccination, the mechanism of protection 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, albeit a very important one [37]. GAD65 (and the GAD67 isoform), IA-2, and other target antigens have been identified. 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 [38].

Although there has been some controversy about the relative importance of various islet autoantigens, there is evidence that both insulin and GAD65 are major antigens [1,15,17,37,38]. Therefore, we designed Ins-GAD, consist-

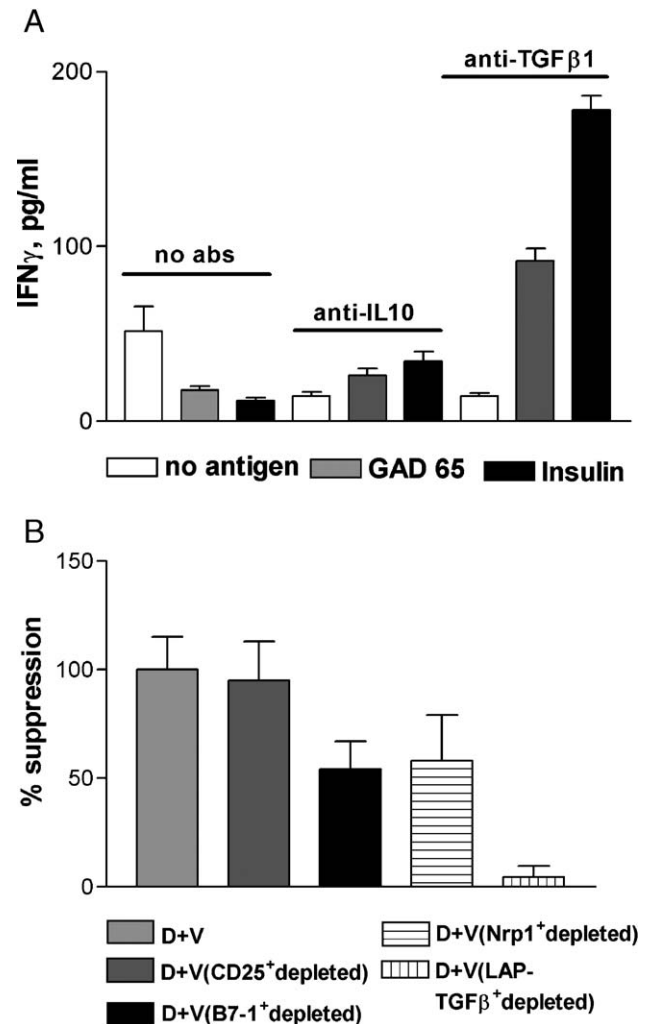


FIG. 6. Markers and cytokine production of Tr cells suppressing the response *in vitro* against islet antigens. (A) Spleen cells from Ins-GAD/B7-1wa-covaccinated mice stimulated separately with either insulin or GAD65 peptides recover responsiveness to islet antigens (IFN- γ release) upon neutralization of TGF- β , but not IL-10, with specific antibodies. The difference between the antigen-induced cytokine release in the absence and the presence of anti-TGF- β antibodies was statistically significant ($P < 0.01$). (B) Antigen-stimulated (insulin/GAD65 peptides mixture) proliferation of diabetogenic cells (D) was suppressed *in vitro* by CD4⁺ T cells from the covaccinated mice (V). CD4⁺ cells depleted of either the B7.1⁺ or the Nrp-1⁺ subpopulation of cells had moderately reduced suppressive activity. Depletion of LAP-TGF- β ⁺ almost totally abolished suppression. Percentage suppression was calculated as described under Materials and Methods. Spleen cells were treated with diabetogenic antigens, insulin and GAD65, separately (A) or in a mixture (B), at 50 μ g/ml each for 72 h before harvest. Proliferation assays were performed and the conditioned medium was tested for IFN- γ . The data, means \pm SEM, are representative of a minimum of three independent experiments.

TABLE 2: Antigen specificity of CD4⁺CD25⁺ Tr suppression

Effector T cells	Ins-GAD/B7wa CD4 ⁺ CD25 ⁺ Tr cells ^a		OVA CD4 ⁺ CD25 ⁺ Tr cells ^b	
	1 × 10 ⁴ cells	3 × 10 ⁴ cells	1 × 10 ⁴ cells	3 × 10 ⁴ cells
Diabetic NOD+islet antigens ^c	22.3 ± 6.5% ^d	63.73 ± 5.0% ^d	0% ^d	0% ^d
OVA-vaccinated NOD + OVA ^c	0% ^d	0% ^d	Not done	Not done

^a Tr cells obtained from Ins-GAD/B7-1wa-covaccinated NOD mice.

^b Tr cells obtained from OVA DNA-vaccinated mice.

^c The effector (responder) cells were either spleen cells of diabetic NOD mice stimulated with insulin and GAD peptides or spleen cells from NOD mice DNA-vaccinated with OVA and stimulated with OVA protein.

^d The results represent the percentage of suppression (mean ± S E M) of antigen-stimulated proliferation calculated as described under Materials and Methods. In all cases where 0% suppression is reported, proliferation was increased by adding CD4⁺CD25⁺ rather than suppressed. OD readings: spleen cells from NOD diabetic mice + islet antigens = 0.670 ± 0.033; T cells from OVA-vaccinated mice + OVA = 0.720 ± 0.045. Unstimulated cells generated OD < 0.200. Three experiments yielded similar results.

ing of full-length PPIs and GAD65, as a composite target antigen that would include many key epitopes. As mentioned previously, vaccination against either PPIs or wild-type GAD65 did not protect but, as shown in this study, Ins-GAD vaccination alone has a moderate protective effect. Furthermore, as noted above, vaccination combining GAD65 and B7-1wa did not improve results over GAD65 alone, suggesting that Ins-GAD has additional properties. Therefore, our results are not supportive of the notion that vaccination against a smaller peptide would be preferable, but we have not studied this question systematically, and we make no claim that Ins-GAD is necessarily the optimal antigen.

Compared to mice that were not treated, inoculation with either blank vector or VR-Ins-GAD did not improve insulinitis scores and caused only a modest reduction in the incidence of diabetes. This might be related to plasmid-related CpG stimulation of DCs or other cells through Toll-like receptor 9 [39], but we have not investigated this question. Injection of the B7-1wa plasmid alone was no more effective than VR alone at preventing diabetes, although it caused a modest improvement in insulinitis scores. In sharp contrast, coinjection of VR-Ins-GAD and VR-B7-1wa caused a significant reduction of disease versus all other groups. Thus, the incidence of diabetes in this group was only 11.8% compared to 64% in unmanipulated 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 VR-Ins-GAD/VR-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⁺ cells exerted no beneficial effect. This could mean that both

CD25⁺ and CD25⁻ exert regulatory functions, 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 [40]. Notably, Foxp3-expressing Tr cells of the CD25⁻ phenotype have been found to convert to a CD25⁺ phenotype after homeostatic expansion of these cells in lymphopenic hosts. Surface expression of CD25 on Tr cells appears to be labile, and it can be lost and regained [40].

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⁺, CD4⁺Foxp3, and CD4⁺CTLA-4⁺ phenotypes in VR-Ins-GAD/VR-B7-1wa-covaccinated mice, compared to mice of other groups. Furthermore, there were significantly increased numbers of CD4⁺ T cells expressing membrane-bound LAP-TGF-β, which has been previously linked to Tr function. Nrp-1 [32,33] and B7-1 were also increased in expression but their significance is less certain. Of all these markers, Foxp3 is the one that has shown the most specificity for Tr cells, and its expression was significantly elevated in the covaccinated mice. In NOD mice receiving VR, VR-Ins-GAD, or VR-B7-1wa alone the numbers of these cells were not significantly altered compared to untreated mice (not shown).

We performed *in vitro* assays to characterize the Tr cells further. CD8⁺ T cells had no regulatory activity, but rather increased responses to GAD65 peptides and insulin antigenic stimulation. In contrast, CD4⁺ T cells completely abolished this response. To address the relative importance of T cells bearing various putative Tr markers, we selectively depleted subpopulations of cells expressing a single marker from the entire CD4⁺ population. Deletion of cells expressing CD25 did not diminish suppression, suggesting that a large proportion of Tr cell were CD25⁻. As noted above, this result must be interpreted with caution, because we cannot exclude the possibility that CD25⁻ T cells reexpressed this marker in culture. In fact, a role for CD25 seems likely since addition of anti-CD25 mAb reduced suppression (not shown). Importantly, depletion of LAP-TGF-β had the greatest effect and completely abolished suppression.

Depletion of cells expressing either B7-1 or Nrp-1 reduced the suppressive effect partially, and we speculate this is due to the considerable overlap of these populations with LAP-TGF- β -expressing cells. Thus, LAP-TGF- β^+ cells appear to be the dominant Tr population induced by Ins-GAD/B7-1wa covaccination. Interestingly, LAP-TGF- β^+ T cells were found in almost equal numbers in the CD4⁺CD25⁺ and CD4⁺CD25⁻ populations (data not shown). There has been controversy about the significance of membrane-bound LAP-TGF- β , but there is increasing evidence that this is a key feature of Tr cells, whether natural or induced [6,41–47]. Taken together, our results indicate that our vaccination procedure generates induced Tr cells bearing LAP-TGF- β .

Another question is whether cytokine production mediates the suppressive effect of the T cells of DNA-vaccinated mice. Analysis of the supernatants of mixed cultures of Tr and effector cells revealed decreasing levels of IFN- γ as Tr cell numbers were increased, but in all cases there were only very low levels of IL-10 and TGF- β 1. Nevertheless, antibodies against TGF- β 1, but not IL-10, antagonized the suppressive activity of the Tr cells. Because levels of TGF- β were low in culture medium, we hypothesize that membrane-bound TGF- β is neutralized and that this is sufficient to block negative regulatory effects.

The antigen specificity of the Tr cells is of major interest. Many of our experiments were performed with combined insulin and GAD65 peptide stimulation, because this improved the magnitude of the responses (which are otherwise low) and facilitated the analyses. However, in Fig. 6A we show the results of spleen cells of DNA-vaccinated mice (VR-Ins-GAD + VR-B7-1wa) stimulated with either GAD65 or insulin. In that case, no cytokine production is noted with either antigen, unless TGF- β 1 is blocked with a monoclonal antibody. Since TGF- β 1 appears to be the main suppressor molecule in our assays, this reveals that the regulatory T cells can suppress responses stimulated with either GAD65 or insulin. Moreover, the results of Table 2 show that the Tr cells were completely ineffective at suppressing the responses of OVA-reactive T cells against OVA. From this we conclude that the Tr cells appear to have antigen specificity. However, these analyses have limitations, and further studies are warranted to analyze specificity.

As a caveat, we must also consider the possibility that vaccination induces IFN- γ -producing CD8⁺ (CTLs). This might appear to be the case from the results presented in Fig. 5, in which addition of purified CD8⁺ T cells from vaccinated mice increased IFN- γ production when added to unsorted diabetogenic NOD spleen cells. However, this assay was designed to detect suppression and not to analyze CTL function, and these results do not really provide any information on the number of islet-reactive CTLs generated by the vaccine. This is because depletion of CD4⁺ regulatory T cells should result in an increase in

IFN- γ production by residual CTLs. Since the CD8⁺ cells did not appear to be suppressive, their addition to unsorted diabetogenic T cells should result in considerably more interferon production, and it does. Whether DNA vaccination generates effector cells in our model is obviously relevant, but would require a new study directed at that question. Furthermore, the interpretation of any *in vitro* study based on antigen stimulation would require appropriate depletion of regulatory T cells. However, it is difficult to establish that all Tr cells have been depleted, and it might be technically problematic to quantitate the number of islet-reactive CTLs.

An important question raised by our study is how CTLA-4 engagement during the process of DNA vaccination encourages the generation of Tr cells. Although CD28 and IL-2 have been recognized as essential factors in the generation of these cells [7,11], there have been few studies suggesting that CTLA-4 is required. However, Vasu *et al.* [48,49] have shown that targeted coengagement of CTLA-4 and cellular or tissue antigens with bispecific antibodies induces Tr cells with properties similar to those in our current study. This activity of CTLA-4 may be explained by the recent studies of Zheng *et al.* [50], who found that negative CTLA-4 costimulation is necessary for generating adaptive CD4⁺CD25⁺ Tr cells. TGF- β could not induce these cells from CTLA-4 null mice to express normal levels of FoxP3 or to develop Tr activity. Time-course studies suggested that CTLA-4 ligation of CD80 shortly after T cell activation enables TGF- β to induce CD4⁺CD25⁻ cells to express FoxP3 and develop suppressor activity.

In conclusion, 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 numbers of T cells bearing markers associated with Tr function. 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 Tr cells. This approach is promising and could find wide applicability in the prevention of autoimmune diseases. We did not note any adverse effects; however, vaccination against a self antigen(s) is not without risk, and animal experiments cannot answer all questions related to safety. These issues can be resolved only in clinical trials.

MATERIALS AND METHODS

Mice. Female NOD mice were purchased from Taconic Farms (Germantown, NY, USA). They were housed under specific pathogen-free conditions and fed *ad libitum* with a regular chow, i.e., Charles River No. 5075 irradiated chow. All procedures on animals were approved by the St. Michael's Hospital (Toronto) Animal Care Committee, in accordance with the guidelines of the Canadian Council for Animal Care.

Diagnosis of diabetes. The blood sugar levels of the mice were monitored weekly with Advantage Comfort strips and with an AccuSoft Advantage monitor (Roche Diagnostics, Laval, QC, Canada). Diabetes was diagnosed when the blood glucose level exceeded 15.0 mmol/L on two consecutive readings.

DNA vaccine preparation. Wild-type murine GAD65 cDNA, provided in the Bluescript KS(+) vector, was a generous gift from Dr. H. McDewitt (Stanford University, Stanford, CA, USA). Full-length PPIs I, generated as previously described [26] but without a stop codon, and full-length GAD65 were fused by overlapping PCR, performed as we have described [27,28]. This produced a cDNA segment in the order 5'-Ins-GAD65-3', denoted Ins-GAD, which was inserted into compatible restriction sites of the VR1255 expression plasmid [29] (Vical, Inc., San Diego, CA, USA), from which the original cDNA luciferase segment had been deleted. The resulting plasmid is denoted VR-Ins-GAD. Transient transfection of COS-7 cell with VR-Ins-GAD resulted in the secretion of an Ins-GAD hybrid molecule in culture medium, as determined by immunoblotting performed as we have described [17]. Similarly, im injection of this vector, with electroporation, resulted in the production of Ins-GAD by local muscle cells, as determined by both immunoblotting and radioimmunoassay of extracted proteins (not shown), which were obtained from transfected muscle as we have previously described [28]. The empty VR1255-derived plasmid (denoted VR) was used as a control in these experiments. We have previously described the properties of VR-B7-1wa [25,26], which encodes a B7-1 molecule with a single tryptophan to alanine substitution at position 88 [23,24]. Similarly, full-length sequence of chicken OVA was introduced into the same blank plasmid, and this construct is denoted VR-OVA. These VR1255-derived plasmids have human cytomegalovirus (CMV) immediate-early enhancer/promoter elements, CMV intron A, and a rabbit β -globin terminator sequence [29].

DNA vaccination. Intramuscular DNA injections and *in vivo* electroporation were performed as we have previously described [26]. Briefly, anesthetized mice were injected with 25 μ g of DNA in each tibialis anterior muscle (total of 50 μ g DNA per mouse). In all cases, two plasmids were coinjected, and they were premixed in a ratio of 1:1 for a total of 25 μ g DNA per muscle. The amount of DNA, when necessary, was adjusted with blank plasmid, such that all mice received the same amount of DNA. Immediately after the injection of DNA, local electroporation was performed by applying eight pulses at 200 V/cm and 20 ms duration with an Electro Square Porator Model ECM830, using a caliper-type electrode (Genetronics, Inc., San Diego, CA, USA) applied to the overlying skin coated with conductive gel.

Histological grading of insulinitis. To grade insulinitis, pancreata were fixed in 10% buffered formaldehyde, and histological slides were stained with hematoxylin and eosin. Grading of the islets was performed as follows: 0, no evidence of infiltrate; 1, peri-insular infiltrate less than a quarter of the islet circumference; 2, peri-insular infiltrate greater than a quarter of the islet circumference; 3, intraislet lymphocytic infiltration with good preservation of islet architecture; 4, severe insulinitis with numerous intraislet inflammatory cells and a loss of normal architecture or a small residual islet.

In vitro spleen cell stimulation, proliferation, and cytokine assays. Spleen cell suspensions from individual DNA-immunized mice, after lysis of the red blood cells, were plated in serum-free chemically defined medium AIM V (Invitrogen Canada, Burlington, ON, Canada), supplemented with 0.05 mM 2-mercaptoethanol. Antigens were added to the culture medium at a final concentration of 0.05 mg/ml. An equimolar mixture of three GAD65 synthetic peptides (Sheldon Biotechnology Centre, McGill University, Montreal, QC, Canada) was prepared. These peptides were TYEIA PVFVLLLEYVTLKMKREIIGWPGGSGD (amino acids 206–236), AALGIGTDSVILIKCDEIRGK (amino acids 290–309), and VPPSLRTLEDNEERMSRLSKVAPVIKARMEYGTG (amino acids 509–543). These peptide fragments cover most of the antigen determinants recognized by T cells, as summarized in [19]. Bovine insulin was purchased from Sigma Chemicals (St. Louis, MO, USA). Endotoxin-free bovine serum albumin was from Calbiochem (San Diego, CA, USA). Endotoxin-free OVA

was purified from fresh chicken eggs as described in [30] and added to cultures at a concentration of 0.05 mg/ml. In preliminary tests, this OVA preparation did not affect proliferation of naive T cells.

Proliferation was determined with the MTT assay as described [31], based on the accumulation of formazan (the product of MTT reduction). In brief, MTT solution was added to the cells in 96-well plates after 72 h of antigen stimulation in a final concentration of 1 mg/ml. The assay was performed in quadruplicates. After 4 h, the reaction was stopped with isopropanol acidified with 0.04 M HCl. Antigen-stimulated proliferation was measured as the increase in optical density (OD) at 540 nm induced by addition of antigen to the cells. In mixed cell assays in which suppression was observed, the percentage suppression of antigen-stimulated proliferative responses was calculated as follows: percentage suppression = $[1 - (\text{OD of Te} + \text{Tr})/(\text{OD of Te})] \times 100$, where Te represents effector/responder T cells and Tr represents regulatory/suppressor T cells.

For cytokine secretion assays, the cells were plated into 96-well plates, 5×10^5 cells per well. After 72 h in culture, the supernatants were pooled and measured in single aliquots for the evaluation of IL-10, IFN- γ , and TGF- β 1 release. TGF- β 1 was assayed after acidic activation of the conditioned medium (10 μ l of 1 N HCl per 100 μ l of the medium). ELISA kits from BD Biosciences (Mississauga, ON, Canada) were used to determine cytokine levels, as per the manufacturer's instructions. Monoclonal antibodies against mouse IL-10 and human TGF- β 1 (also recognizing mouse cytokine) were from R&D Systems (Minneapolis, MN, USA). Neutralizing levels of the antibodies were established in previous experiments.

Flow cytometry analysis. FITC- or PE-labeled rat anti-mouse CD4, CD25, CD86 (B7.1), CD152 (CTLA-4), and isotype-matched IgG were from BD Pharmingen (Mississauga, ON, Canada). Mouse anti-Foxp3 mAb was obtained from eBioscience (San Diego, CA, USA). Unlabeled and biotinylated mouse anti-human/mouse LAP-TGF- β 1 were from R&D Systems. Rabbit anti-rat/mouse Nrp-1 polyclonal IgG was purchased from Oncogene (through EMD, La Jolla, CA, USA). Anti-LAP-TGF- β 1 and mouse isotype IgG were labeled with Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA) according to the manufacturer's protocol. Anti-Nrp-1 IgG and rabbit normal IgG were labeled with Alexa Fluor 647.

Separation of cell subpopulations and regulatory T cell assays. CD4⁺ or CD8⁺ cells were purified from the total spleen cell suspension (depleted of red blood cells), by negative magnetic sorting using Mouse CD4⁺ or CD8⁺ EasySep kits, respectively, from StemCell Technology, Inc. (Toronto, ON, Canada) as per the manufacturer's instructions. The purity assessed by flow cytometry was 92–95.7%. CD4⁺ cells were further separated into CD25⁺ and CD25⁻ subsets using the Mouse Biotin Selection EasySep kit from the same company and biotinylated rat anti-mouse CD25 antibody (BD Pharmingen) according to the manufacturer's instruction with minor modifications. Similarly, biotinylated rat anti-mouse B7.1 (BD Pharmingen), biotinylated mouse anti-human/mouse LAP-TGF- β , and rabbit anti-rat/mouse Nrp-1 plus biotinylated goat anti-rabbit IgG as second antibody (BD Pharmingen) were used with the same positive selection kit as above to deplete total CD4⁺ lymphocytes of the B7.1⁺, Nrp-1⁺, or LAP-TGF- β cells. The separation was performed according to the manufacturer's instruction without modification.

Statistical analysis. Statistical analyses were performed with the GraphPad Prism 3.0 program (GraphPad Software, Inc., San Diego, CA, USA). The incidence of diabetes was plotted using the Kaplan-Meier method and statistical comparisons made with the log-rank test. The significance of differences in insulinitis scores was determined with the χ^2 test. The differences between groups in the *in vitro* proliferation and cytokine release assays were determined by analysis of variance. $P < 0.05$ was considered significant.

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