Plasmid Vaccination with Insulin B Chain Prevents Autoimmune Diabetes in Nonobese Diabetic Mice

Adrian Bot, Dan Smith, Simona Bot, Anna Hughes, Tom Wolfe, Lilin Wang, Catherine Woods and Matthias von Herrath

*J Immunol* 2001; 167:2950-2955; doi: 10.4049/jimmunol.167.5.2950
http://www.jimmunol.org/content/167/5/2950

**References**

This article cites 35 articles, 13 of which you can access for free at:
http://www.jimmunol.org/content/167/5/2950.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Plasmid Vaccination with Insulin B Chain Prevents Autoimmune Diabetes in Nonobese Diabetic Mice

Adrian Bot,* Dan Smith,* Simona Bot,* Anna Hughes,† Tom Wolfe,† Lilin Wang,* Catherine Woods,2* and Matthias von Herrath3†

The insulin B (InsB) chain bears major type 1 diabetes-associated epitopes of significance for disease in humans and nonobese diabetic (NOD) mice. Somatic expression of InsB chain initiated early in life by plasmid inoculation resulted in substantial protection of female NOD mice against disease. This was associated with a T2 shift in spleen, expansion of IL-4-producing and, to a lesser extent, of IFN-γ-secreting T cells in pancreatic lymph nodes, as well as intermolecular Th2 epitope spreading to glutamic acid decarboxylase determinants. A critical role of IL-4 for the Ag-specific protective effect triggered by plasmid administration was revealed in female IL-4−/− NOD mice that developed diabetes and higher Th1 responses. Coadministration of IL-4-expressing plasmid or extension of the vaccination schedule corrected the unfavorable response of male NOD mice to DNA vaccination with InsB chain. Thus, plasmid-mediated expression of the InsB chain early in diabetes-prone mice has the potential to prevent transition to full-blown disease depending on the presence of IL-4.


Insulin-dependent diabetes mellitus (IDDM) is an autoimmune disease of mixed, polygenic, and environmental etiology (1). The most frequently used models for IDDM are nonobese diabetic (NOD) mice that display spontaneous disease triggered by autoreactive CD4+ and CD8+ T cells (2). Major pathogenic roles are played by defective homeostasis of autoreactive T cells, possibly caused by leaky central negative selection (3), as well as by deficient function of APCs (4) with consequences for the T cell repertoire and production of regulatory cytokines (5).

Ag-based immune prophylaxis of IDDM has the potential to prevent progression to full-blown disease and avoid hormone replacement therapy. An ideal target for immune modulation would be an Ag common for humans and NOD mice, because the proof of concept can be addressed easier. Such an Ag is the insulin B (InsB) chain, recognized by T cells from DQ8+ humans predisposed to disease or with full-blown IDDM (6).

Ag-based therapeutic regimens may act via different mechanisms: 1) deletion/anergy of pathogenic T cells; 2) induction of regulatory cells that mediate bystander suppression; or 3) when initiated during the prediabetic stage, redirection of T cell precursors toward nonpathogenic cells. The significance of the Th2 shift noted in many cases of protection from IDDM is still under debate. For example, in the case of regimens inducing deletion/anergy of pathogenic cells, the resulting Th2-biased response may reflect just a higher tendency for Th1 cells for activation-induced cell death (7). Also, based on observations that protective treatments are associated with a Th (cytokine) switch toward Th2 (IL-4), Tr1 (IL-10), and/or Th3 (TGF-β) (8), it is not always clear whether such modifications are causal or secondary. Indeed, some previous studies documented protective effects of Th1 cells (9) and detrimental effects of Th2 cells (10) in IDDM. Intriguingly, the protective effect mediated by a plasmid expressing a dominant myelin basic protein epitope in an experimental autoimmune encephalomyelitis (EAE) model was dependent on the presence of T1-driving CpG motifs (11). This was in apparent discrepancy with a more recent report that suggested a critical role for IL-4 in the protection against IDDM conferred by a glutamic acid decarboxylase (GAD)-expressing plasmid (12). Thus, there likely are multiple mechanisms that can restore the normal immune homeostasis, depending on model, stage, as well as therapeutic strategy.

Here, we investigated whether plasmid vaccination of NOD mice with an InsB-expressing vector would result in protection from IDDM. We reasoned that initiation of vaccination during the neonatal window may result in either anergy/deletion of specific autoreactive T cells in context of a poor expression of costimulatory molecules (13), or in induction of nonpathogenic/regulatory cells of Th2 profile (14). We found that plasmid vaccination of female NOD mice redirected the profile of autoreactive T cells and protected from IDDM. The Th2-associated cytokine IL-4 was critical for the protection, rather than being just a secondary outcome. Furthermore, coadministration of IL-4-expressing plasmid or extension of the vaccination schedule corrected the lack of protection of male NOD mice by plasmid vaccination.

Materials and Methods

Plasmids and reagents

The plasmid expressing the InsB chain (pInsB) was described previously (15) and the pGAD plasmid was a generous gift of Dr. D. Kaufman (University of California, Los Angeles, CA). As control, we used pCMV plasmid devoid of Ag open reading frame. The plasmids were produced in Escherichia coli and purified using EndoFree purification kits (Quagen, Valencia, CA).

We used the following custom-synthesized peptides: GAD-65 Aε22 restricted dominant epitopes 78–97 KPCNCPKGDVNYAFLHATDL.

Copyright © 2001 by The American Association of Immunologists
Mice and immunization

NOD/LtJ breeder mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed in specific pathogen-free conditions. The rate of diabetes in the colony was 75–90% (females at 30 wk of age). The IL-4-deficient NOD mice were obtained using a speed-congenic approach described previously (16). The naive IL-4−/− female mice developed diabetes similar to IL-4-competent NOD female mice (16, 17).

T cell assay

The analysis of T cell responsiveness was conducted by ELISPOT using responder cells isolated from spleen and pancreatic lymph nodes (PLNs). The ELISPOT plates (Millipore, Molsheim, France) were coated with anti-cytokine Abs, blocked, and the responder cells were incubated (range from 3 to 5×10^5/ml) in HL-1 medium, with or without stimulator cells (2×10^5/ml) and 20 μg/ml of peptides (InsB 9–23, 15–23, or a mixture of GAD peptides). In the case of splenocytes, we used an initial 5-day stimulation with peptides, followed by a 3-day stimulation with rIL-2 (10 U/ml) and a 3-day stimulation with mitomycin-treated feeder cells plus peptides. In the case of PLN cells, we used a 5-day stimulation protocol in the presence of peptide-pulsed feeder cells and 10 U/ml of rIL-2 during the last 48 h. Alternatively, we used 72-h polyclonal stimulation with a mixture of anti-CD3 + anti-CD28 mAbs (5×10^5/ml). The reaction was developed using detecting Abs (2 μg/ml) followed by streptavidin-HRP and 3-amin-9-ethylcarbazole substrate (Sigma, St. Louis, MO). The data were acquired using an automated system (Navitar, Rochester, NY) with ImagePro-Plus software (Media Cybernetics, Silver Spring, MD).

The cytokine production by islet-infiltrating cells was measured after overnight incubation of islets, using ELISA kits (BioSource International, Camarillo, CA and R&D Systems, Minneapolis, MN). For the assay, the islets were obtained by digestion with collagenase (Sigma) and Ficoll gradient centrifugation.

Statistical analysis

The comparison among disease profiles in the context of various treatment regimens was conducted by log-rank analysis. Values of p < 0.05 of the log-rank test were considered statistically significant, relative to the effect on kinetics of disease.

Results

Effect of plasmid vaccination of female NOD mice with InsB chain on development of IDDM

Because the InsB chain is a major potential target for Ag-based immune prophylaxis of IDDM, we tested whether immunization with plnBs initiated at day 7 after birth and repeated during the preclinical stage (wk 4 and 8) affected the kinetics of disease in NOD mice. As shown in Fig. 1A, plnBs delayed the development and suppressed the rate of IDDM in female NOD mice (p of log-rank test p > 0.05; Fig. 1A). The exclusion of the priming step on day 7 precluded suppression of IDDM by inoculation of plnBs (data not shown).

We next assessed the cytokine profile of T cells from PLN of treated mice and controls. Mere incubation of T cells from plnB-vaccinated mice with mitomycin-treated syngeneic APCs resulted in substantial generation of spot-forming colonies (SFC) (Fig. 1, B and C). The total number of IFN-γ- and, particularly, IL-4-producing SFC was higher in PLN from mice treated with plnBs, as opposed to controls. In addition, polyclonal stimulation of PLN T cells from plnB-immunized mice had a limited additional effect on the number of IL-4 relative to IFN-γ SFC (Fig. 1, B and C), suggesting that most of the IL-4-producing T cells in the PLN from protected mice are active. However, whereas the total number of polyclonally stimulated IL-4 SFC was significantly higher (2 orders of magnitude) in plnB-immunized mice, the number of IFN-γ SFC was in the same range compared with that of naive controls. Treatment with pGAD failed to similarly modify the number of SFC in the PLN (Fig. 1, B and C), resembling the administration of mock plasmid (data not shown).

Impact of vaccination with plnB on the cytokine profile of Ag-specific T cells and islet-infiltrating lymphocytes

We next assessed whether plasmid vaccination persistently modified the cytokine profile of InsB and GAD-specific T cells in PLN and spleen. Short-term incubation of PLN T cells from 30-wk-old naive mice with peptide-pulsed APC did not result in substantial generation of IL-4 and IFN-γ SFC (Fig. 2, A and B). However, using a similar protocol of stimulation, the number of both IFN-γ- and IL-4-producing InsB-specific colonies was greatly enhanced in plnB-vaccinated NOD mice that were free of disease by wk 30. Immunization with pGAD failed to reproduce a similar expansion in the pool of PLN SFC (Fig. 2, A and B), although it increased to a certain extent the reactivity to both InsB and GAD.

In sharp contrast to the observed increase in both IL-4 and IFN-γ in PLNs from plnB-immunized NOD mice, there was a clear shift to T2 immunity in the spleen. We compared the cytokine profiles of SFC generated ex vivo by stimulation with dominant InsB or major GAD epitopes. As shown in Fig. 2D, the mice vaccinated with plnBs and protected against IDDM displayed enhanced frequency of IL-4-producing SFC generated with either GAD or InsB peptides. In addition, the frequency of IFN-γ-producing SFC generated with InsB peptide was lower than that of...
In addition, there was a trend for decreased IFN-γ cytokine production in the expansion of IL-4-producing SFC (Fig. 2D). In contrast, pGAD vaccination did not prevent disease in naive NOD mice (Fig. 2C). In contrast, pGAD vaccination did not result in the expansion of IL-4-producing SFC (Fig. 2D). Conversely, we measured a substantial increase in the frequency and total number of IFN-γ-producing SFC obtained with InsB and GAD peptides in pGAD-immunized NOD mice (Fig. 2C). Collectively, the data generated using splenocytes show opposite effects of plasmid vaccination with InsB chain and GAD-65: Th2-biased profile in the first case and a pattern dominated by T1 immunity in the latter. Interindividual epitope spreading was noted in both pInsB- and pGAD-immunized mice (Fig. 2, C and D).

The effect of plnsB vaccination on the activity of islet-infiltrating T cells was assessed by measuring in vitro cytokine production. Despite its ability to prevent IDDM, plasmid vaccination did not preclude peripheral infiltration of the islets (data not shown). Compared with age-matched naive mice or mice injected with mock plasmid, the islet-infiltrating cells from 12-wk-old plnsB-vaccinated mice produced significantly less IL-1β, but more IL-4 (Fig. 3). In addition, there was a trend for decreased IFN-γ and increased TGF-β production by cells from plnsB-vaccinated mice.

The cytokine profile of islet-infiltrating cells from plnsB-treated mice was somewhat similar to that of naive NOD mice that remained free of disease by the age of 30 wk (Fig. 3). No detectable IL-10 production was measured (data not shown), arguing against the induction of T1 cells by plasmid vaccination.

Because potential induction of anti-insulin Abs may be used to monitor the response to treatment or anticipate side effects, we measured the induction of specific Abs subsequent to plnsB vaccination. No significant enhancement was noted, in contrast to control animals immunized with InsB peptide formulated in IFA (E. Liu, data not shown). This may have been due to more limited exposure of B cells to Ag, subsequent to plasmid vaccination.

**IL-4 is required for protection from IDDM conferred by plnsB vaccination**

Despite the fact that bystander suppression was documented in various protocols of peptide or protein-based immune therapy (18), use of plasmid expression vectors for a similar purpose (11, 12) is intriguing because unmethylated CpG motifs on bacterial DNA are strong Th1 inducers (19). Various mechanisms were previously proposed to explain the efficacy of plasmid vaccination in a transgenic model of autoimmune diabetes (15) and models of EAE (11, 20), ranging from bystander suppression, to anergy or Th1 cytokine-mediated protection.

Because the association of increased IL-4 production and protection from IDDM supports but does not demonstrate a causal role for IL-4, we sought to address this question by using recently described IL-4−/− × NOD mice (16). If expansion of IL-4-producing T cells is just an epiphenomenon, then plnsB should protect the IL-4-defective NOD mice against IDDM.

Cultures of splenocytes from naive IL-4−/− NOD mice, with or without polyclonal stimulation, confirmed the lack of IL-4 production and showed an increased frequency of IFN-γ-producing SFC (Fig. 4, A and B). This was expected due to the role of IL-4 as negative regulator of T1 immunity (21). RT-PCR analysis showed that sterile IL-4 transcripts were still produced in IL-4−/− mice (data not shown), indicating that the generation of Th2-like cells associated with specific chromatin remodeling and opening of specific loci (22) was not completely prevented.

In contrast to IL-4-competent mice, the deficient mice were not protected from IDDM by plnsB vaccination (Fig. 4C; p < 0.05). Furthermore, we showed that the number of IFN-γ SFC obtained by stimulation of splenocytes with InsB and GAD peptides was increased in the IL-4−/− mice, as compared with IL-4-competent mice that developed IDDM (Fig. 4D) or were protected from disease (Fig. 2D). In contrast to the plnsB-vaccinated NOD mice protected from IDDM, the diabetic mice showed significantly decreased numbers of IL-4 but not IFN-γ-producing SFC in PLN (Figs. 2 and 4). Thus, vaccination with plnsB of IL-4-deficient NOD mice failed to prevent IDDM and to substantially change the T cell profile in spleen and PLN.

**Coadministration of IL-4 or expansion of the vaccination schedule ameliorates the response of male NOD mice to InsB-expressing plasmid**

Because the male NOD mice develop disease with a lower rate and delayed kinetics compared with females, we assessed whether plnsB vaccination had an impact on disease in males. Interestingly, in contrast to female NOD mice, the vaccination of males failed to

---

**DNA VACCINE WITH InsB CHAIN PREVENTS DIABETES**

**FIGURE 2.** Effect of plasmid vaccination with InsB chain on the cytokine profiles of autoantigen-specific T cells from PLN and spleen. A and B, Cytokine profiles of Ag-specific T cells from PLN of naive, pInsB- or pGAD-vaccinated 30-wk-old female NOD mice were measured by ELISPOT subsequent to stimulation with peptides and feeder cells according to Materials and Methods. Data were expressed as total number of SFC/PLN of one mouse (means ± SEM of 4 mice/group; results are representative of two independent experiments). C and D, Cytokine profile of peptide-specific T cells from spleen, measured in 30-wk-old vaccinated mice and controls. Results were expressed as means of SFC frequency/mouse + SEM, normalized to 10⁶ responder cells (n = 4/group).

**FIGURE 3.** Cytokine profile of islet-infiltrating lymphocytes from vaccinated and control mice. A, Cytokine production, measured by ELISA, of infiltrating lymphocytes from naive prediabetic (12-wk-old) and 30-wk-old normoglycemic female NOD mice. B, Cytokine production of infiltrating lymphocytes from 12-wk-old mice vaccinated with plnsB or injected with control plasmid (pCMV). The results in A and B were expressed as means ± SEM of cytokine concentration for individual cultures (total islets per pancreas were cultured in 2 ml; n = 5 pancreata/group).
significantly influence the kinetics of IDDM (Fig. 5A; p of log-rank test 0.05) or to shift the cytokine profile toward T2 immunity (data not shown). We reasoned that this failure of plnsB vaccination in males may be due to either one (or both) of the following factors: 1) an ineffective exposure (timewise) of insulin-specific T cells to self-Ag expressed by plasmid; or 2) a defect of male NOD mice to mount T2 immunity to InsB.

To address the first possibility, we injected male NOD mice four times (an additional injection at the age of 12 wk). That led to a significant suppression of IDDM (Fig. 5A; p of log-rank test A similar to coadministration of IL-4). Interestingly, this is reminiscent of a recently published study, that while confirming the inability of GAD-expressing plasmid to suppress IDDM in female NOD mice, showed that plasmid vaccination with an IgG-GAD chimeric construct together with IL-4 resulted in significant disease suppression (12). Profound effects of plasmid vaccination were noted in the PLN of protected males (Fig. 5B). Although administration of plnsB using an expanded schedule resulted in substantial increase in the pool of IL-4-producing T cells (4-fold) and IFN-γ-producing T cells (2-fold), coadministration of IL-4 and InsB-expressing plasmids resulted in diminution of the number of cytokine-producing T cells in PLN.

**Discussion**

A potential strategy to down-regulate autoimmune processes is ectopic expression of self-Ags that may restore normal immune homeostasis by directly affecting autoreactive T cells, or indirectly via generation of regulatory cells.

We showed that protection against disease by plasmid vaccination with the InsB chain of female NOD mice was associated with a substantial increase in the total number of cytokine-producing T cells in PLN (Figs. 1 and 2). The enhancement in the overall number of IL-4-producing T cells was not paralleled by a reduction of the IFN-γ-producing T cell pool in the PLN from protected animals. However, as revealed by the estimation of cytokine-producing cells in various conditions of in vitro stimulation, a large per-

![FIGURE 5. Plasmid vaccination with InsB chain of male NOD mice. A, Kinetics of IDDM in male NOD mice injected with control plasmid (pCMV), plnsB, plnsB + pIL-4 (three times), or using an expanded vaccination schedule (plnsB 4×). Mice injected with plnsB + pIL-4 or four times with plnsB displayed significantly reduced disease rate (p of log-rank test n/group between 8 and 20). B, Cytokine profile in PLN of recently diagnosed diabetic mice (diab) or 30-wk-old normoglycemic mice (ndiab), was assessed by ELISPOT analysis upon in vitro stimulation with anti-CD3 + anti-CD28 mAbs. The results were expressed as total number of SFC/PLN/mouse (means + SEM, n = 3–6/group).](http://www.jimmunol.org/)
centage of IL-4-producing cells were active in situ. Thus, the mere ex vivo incubation of PLN T cells with APC was able to render substantial IL-4 production, with no additional effect of polyclonal activation (Fig. 1C). In contrast, a substantial percentage of IFN-γ-committed T cells in PLN from mice vaccinated with pInsB were quiescent, because they required strong polyclonal activation to expand in vitro (Fig. 1B). Together, our data support a T2-biased profile in the PLN of pInsB-vaccinated female NOD mice protected against disease. Vaccination with GAD-expressing plasmid failed to trigger a similar expansion of T2 cells in PLN (Figs. 1C and 2B).

The effect of pInsB vaccination on the splenic T cell profile was more clear-cut (Fig. 2, C and D). Collectively, the data generated using splenocytes show opposite effects of plasmid vaccination with InsB chain and GAD-65: Th2-biased profile in the first case and a pattern dominated by T1 immunity in the latter. Intermolecular epitope spreading was noted in both pInsB- and pGAD-immunized mice. In addition, paralleling the pInsB-driven modification of the T cell profile in secondary lymphoid organs, there was a change in the cytokine pattern of islet-infiltrating cells, consisting in down-regulation of proinflammatory and up-regulation of suppressor cytokines (Fig. 3).

In our model, bacterial plasmid devoid of Ag-open reading frame lacked any effect on the kinetics of disease. In addition, underlining the Ag dependency, plasmids expressing different self-Ags displayed differential outcomes in terms of specific T cell responses.

Based on the T cell profile data, we tested whether IL-4 was an essential cytokine participating in the protection conferred by pInsB plasmid. We demonstrated that IL-4-defective NOD mice could not be protected against diabetes by plasmid vaccination (Fig. 4). This result points toward IL-4 as a central mediator of pInsB-triggered protection from IDDM. This interpretation is also supported by the intermolecular spreading of Th2 immunity toward GAD determinants in pInsB-immunized mice (Fig. 2, C and D). Such a phenomenon may be of paramount importance in deactivating autoaggressive cells against multiple Ags (23). In addition, a bystander/regulatory effect may explain the modification noted in the profile of cytokines produced by islet-infiltrating lymphocytes (Fig. 3). Conversely, pGAD-immunized mice displayed Th1-biased immunity and epitope spreading toward the InsB determinant (Fig. 2). This was associated with lack of protection against disease and may be due to earlier recruitment of GAD determinants in the autoimmune process (24) resulting in refractoriness of GAD-specific T cells to modulation. The requirement for early priming with pInsB for the protective effect to occur supports the model that timing of Ag recruitment to the autoimmune process is an important parameter. It is a likely possibility that IL-4 produced by Th2 cells primed during neonatal window (25) may inhibit the expansion of Th1 and CTL (26) or modulate the activity of APC in the PLN and pancreas (27). This may explain the limited induction of autoaggressive T cells recognizing a dominant, MHC class I-restricted epitope on the InsB chain (28) in pInsB-vaccinated IL-4-competent but not IL-4-deficient NOD mice (Fig. 4E).

Previous studies showed that in contrast to adult vaccination, neonatal DNA immunization triggers both Th2 and Th1 cells (25) despite the Th1 driving effect of CpG motifs. Similar to studies addressing the mechanism of peptide-based immune modulation in IL-4-deficient (29) or STAT-6-defective mice (30), as well as to a recent study addressing the response to a GAD peptide-expressing plasmid (12), our findings underline a crucial role for the Th2-derived cytokine IL-4 in the protection from IDDM. However, our model likely involves different effector mechanisms than those described in a recent report demonstrating a requirement for Th1-inducing factors (11) and lack of bystander suppression (31) in the protection against EAE after vaccination with an myelin basic protein-expressing plasmid. Further emphasizing the pleiotropism of protective mechanisms, various immunostimulatory protocols of preventing diabetes in NOD mice depend to different extents on IL-4 (16, 32). Finally, the dependency of protection on IL-4 in this model is not concordant with a potential role for IL-10-producing cells (33).

A similar vaccination schedule with pInsB was not effective in the case of male NOD mice. However, extension of vaccination schedule or addition of IL-4-expressing plasmid to the inoculum provided significant protection against disease (Fig. 5). The outcome on the T cell profile was different as compared with that measured in pInsB-vaccinated female NOD mice. Thus, coadministration of IL-4 is not merely shifting the profile from T1 to T2 but has a more profound impact on the overall peripheral repertoire, consistent with an effect on APC (27). Because neonatal inoculation of plasmid is associated with substantially shorter persistence of transgene (34), the prolongation of vaccination schedule may optimize exposure of emerging self-reactive T cells to InsB chain in circumstances when the intrinsic disease kinetics is slower, as in the case of male NOD mice.

In summary, our study demonstrates the efficacy of InsB, an Ag common for DQ8 humans and NOD mice (6, 35), in the prophylaxis of IDDM conducted by plasmid inoculation. The current data extend our previous results from a transgenic model (15) to a spontaneous disease model involving multiple epitopes (NOD mice) and underline the importance of the following factors in the success of plasmid-based immune modulation: 1) the nature of Ag, 2) timing of vaccination, 3) regulatory cytokines, and 3) gender (in a broader context, the heterogeneity regarding disease-kinetics and pathogenesis). Because the 9-23 peptide of the InsB chain is a dominant epitope in humans at risk to develop IDDM (6), plasmid vaccination with this Ag may be of clinical interest. The addition of IL-4 may improve the efficacy of Ag-based immunotherapy with plasmid vectors. Finally, in contrast to Ags recruited earlier in the autoimmune process (i.e., GAD), immune prophylaxis with InsB chain may be safer and more effective.

Acknowledgments
We are grateful to Dennis McLaughlin (Alliance Pharmaceutical Corporation) for the excellent technical assistance with animal husbandry and Diana Frye (Scirpp Research Institute) for assistance with manuscript preparation.

References

DNA VACCINE WITH InsB CHAIN PREVENTS DIABETES


