The Journal of Immunology

Th1 to Th2 Cytokine Shifts in Nonobese Diabetic Mice: Sometimes an Outcome, Rather Than the Cause, of Diabetes Resistance Elicited by Immunostimulation

David V. Serreze, Harold D. Chapman, Cristina M. Post, Ellis A. Johnson, Wilma L. Suarez-Pinzon and Alex Rabinovitch

J Immunol 2001; 166:1352-1359; ;
doi: 10.4049/jimmunol.166.2.1352
http://www.jimmunol.org/content/166/2/1352

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Th1 to Th2 Cytokine Shifts in Nonobese Diabetic Mice: Sometimes an Outcome, Rather Than the Cause, of Diabetes Resistance Elicited by Immunostimulation[1]

David V. Serreze,[2]* Harold D. Chapman,* Cristina M. Post,* Ellis A. Johnson,* Wilma L. Suarez-Pinzon,† and Alex Rabinovitch†

Numerous immunostimulatory protocols inhibit the development of T cell-mediated autoimmune insulin-dependent diabetes mellitus (IDDM) in the nonobese diabetic (NOD) mouse model. Many of these protocols, including treatment with the nonspecific immunostimulatory agents CFA or bacillus Calmette-Guérin (BCG) vaccine, have been reported to mediate protection by skewing the pattern of cytokines produced by pancreatic β-cell autoreactive T cells from a Th1 (IFN-γ) to a Th2 (IL-4 and IL-10) profile. However, most of these studies have documented associations between such cytokine shifts and disease protection rather than a cause/effect relationship. To partially address this issue we produced NOD mice genetically deficient in IFN-γ, IL-4, or IL-10. Elimination of any of these cytokines did not significantly alter the rate of spontaneous IDDM development. Additional experiments using these mice confirmed that CFA- or BCG-elicited diabetes protection is associated with a decreased IFN-γ to IL-4 mRNA ratio within T cell-infiltrated pancreatic islets, but this is a secondary consequence rather than the cause of disease resistance. Unexpectedly, we also found that the ability of BCG and, to a lesser extent, CFA to inhibit IDDM development in standard NOD mice is actually dependent upon the presence of the Th1 cytokine, IFN-γ. Collectively, our studies demonstrate that while Th1 and Th2 cytokine shifts may occur among β-cell autoreactive T cells of NOD mice protected from overt IDDM by various immunomodulatory therapies, it cannot automatically be assumed that this is the cause of their disease resistance. *The Journal of Immunology,* **2001, 166: 1352–1359.**

Insulin-dependent diabetes mellitus (IDDM)[3] in the nonobese diabetic (NOD) mouse model results from autoimmune destruction of pancreatic β-cells mediated by both $CD4^+$ and $CD8^+$ T cells (reviewed in Ref. 1). However, it has been widely reported that the pathogenic activity of β-cell autoreactive $CD4^+$ T cells in NOD mice can be inhibited if the predominant pattern of cytokines they produce is shifted from a Th1 (primarily IFN-γ) to a Th2 (primarily IL-4 and IL-10) profile. These conclusions are primarily based on reports that Th1 to Th2 cytokine shifts are often observed among β-cell-infiltrating T cells of NOD mice that are paradoxically protected from overt IDDM by many Ag-specific or nonspecific immunostimulation protocols (reviewed in Refs. 2–4). An explanation for such shifts may be provided by a report that at high stimulation levels, $CD4^+$ T cells can deviate from a Th1 to a Th2 cytokine production profile (5).

While the induction of IDDM resistance in NOD mice by many immunostimulatory protocols has been associated with Th1 to Th2 cytokine shifts among β-cell autoreactive T cells, such alterations have not been demonstrated to be the true cause of disease prevention. One frequently overlooked consideration that may complicate interpretation of these associational studies are reports that Th1 are more prone than Th2 cells to activation-induced cell death (AICD) (6, 7). NOD APCs are characterized by a series of genetically controlled T cell activation defects (8–12). The stimulation threshold required to induce T cell deletion by AICD is higher than that needed to trigger their immunological effector functions (13–15). Thus, the impaired stimulatory capacity of NOD APC could preferentially inhibit their ability to induce AICD-mediated deletion of autoreactive T cells without fully abrogating their ability to trigger immunological effector functions. Correcting an impaired ability of APC to trigger AICD-mediated deletion of β-cell autoreactive T cells might provide another explanation for how various immunostimulatory agents inhibit IDDM development in NOD mice. Furthermore, if they are indeed characterized by differential sensitivity to AICD, this deletional process might preferentially spare β-cell autoreactive T cells, producing Th2 rather than Th1 cytokines. Such an "unmasking event" might be misinterpreted as a Th1 to Th2 deviation among β-cell-infiltrating T cells of NOD mice protected from overt IDDM by various immunostimulatory treatments.

Several other factors also call into question whether the pathogenicity of β-cell autoreactive $CD4^+$ T cells in NOD mice can be strictly compartmentalized on the basis of currently defined Th1 and Th2 cytokine production profiles. These include reports of Th1 and Th2 clonotypic T cells isolated from NOD mice that, contrary to expectations, had a respective ability to inhibit or promote IDDM development (16–18). Furthermore, it was recently reported that prior skewing in vitro to either a Th1 or a Th2 cytokine production profile did not alter the ability of an NOD-derived β-cell autoreactive $CD4^+$ T cell clone to passively transfer IDDM

*The Jackson Laboratory, Bar Harbor, ME 04609; and †Department of Medicine, University of Alberta, Edmonton, Canada

Received for publication October 27, 1999. Accepted for publication October 19, 2000.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by National Institutes of Health Grants DK51090, DK46266, and AI41469 and Cancer Center Support Grant CA34196 as well as by grants from the Juvenile Diabetes Foundation International. A.R. is supported by a Medical Scientist Award from the Alberta Heritage Foundation for Medical Research.

[2] Address correspondence and reprint requests to Dr. David V. Serreze, The Jackson Laboratory, 600 Main Street, Bar Harbor, ME 04609. E-mail address: dvs@jax.org

[3] Abbreviations used in this paper: IDDM, insulin-dependent diabetes mellitus; NOD, nonobese diabetic; AICD, activation-induced cell death; BCG, bacillus Calmette-Guérin.

0022-1767/01/$02.00

(19). Adding additional confusion to the potentially pathogenic role of Th1 cells are reports that IDDM development in NOD mice does not require IFN-γ signaling (20–22). Similarly, there is confusion regarding the potentially protective role of Th2 cytokines. Systemic administration of either IL-4 or IL-10 can inhibit IDDM development in NOD mice (23–25). However, IDDM development is respectively inhibited or accelerated in NOD mice transgenically expressing IL-4 or IL-10 in pancreatic β-cells (26, 27).

We reasoned that better insights into some of the uncertainties described above could be gained through analyses of NOD stocks made genetically deficient in IFN-γ, IL-4, or IL-10. Thus, in the current study we used such stocks to determine whether various cytokines associated with Th1 and Th2 responses play an obligatory role in promoting or blocking IDDM development in NOD mice. These stocks were also used to determine whether treatment with CFA or bacillus Calmette-Guérin (BCG) vaccine, two nonspecific immunostimulatory protocols proposed to block IDDM development in standard NOD mice by eliciting Th1 to Th2 cytokine shifts among β-cell autoreactive T cells (reviewed in Ref. 2), actually do so. We report that in some cases Th1 to Th2 cytokine shifts among β-cell autoreactive T cells of NOD mice rendered IDDM resistant by immunostimulation are merely a secondary outcome of protection actually mediated through different mechanisms.

Materials and Methods

Mice

NOD/Lt mice are maintained at The Jackson Laboratory (Bar Harbor, ME) by brother-sister mating. Currently, IDDM develops in 90% of female and 63% of male NOD/Lt mice by 1 year of age. Stocks of NOD mice homozygous for *IFN-γ*, *IL4*, or *IL10* alleles functionally disrupted by homologous recombination (28–30) were generated by our previously described speed congenic approach (31). Using this approach, we produced sixth backcross generation (N7) male and female heterozygous carriers of the *IFN-γ*^{null} (formal designation *Ifng*^{tm1Ts}) or *IL4*^{null} (formal designation *Il4*^{tm1Cgn}) alleles that were also fixed to homozygosity for genetic linkage markers delineating all previously identified diabetes susceptibility (*Idd*) loci of NOD origin. Male and female heterozygous carriers of the *IL10*^{null} (official designation *Il10*^{tm1Cgn}) allele that were fixed to homozygosity for these NOD-derived *Idd* loci became available at the N8 backcross generation. The appropriate N7 or N8 progenitors were intercrossed to produce lines of NOD mice homozygous for the *IFN-γ*^{null}, *IL4*^{null}, or *IL10*^{null} allele, which were maintained by brother-sister matings. All mice were maintained under specific pathogen-free conditions and allowed free access to food (National Institutes of Health diet 31A, Ralston Purina, Richmond, IN) and acidified drinking water.

PCR typing of disrupted cytokine genes

Segregants homozygous or heterozygous for the *IFN-γ*^{null} allele were identified using a four-primer-based PCR assay. The four primers were: no. 126, 5′-AGAAGTAAGTGGAAGGGCCCAGAAG-3′; no. 127, 5′-AGGGAAACTGGGAGAGGAGAAATAT-3′; no. 128, 5′-TCAGCGCAGGGGCGCCCGGTTCTTT-3′; and no. 129, 5′-ATCGACAAGACCGGCTTCCATCCGA-3′. Primer pairs 126 and 127 amplify a 220-bp product from the wild-type *IFN-γ* allele, while the 128 and 129 primer pairs amplify a 375-bp product from the *IFN-γ*^{null} allele. Segregants homozygous or heterozygous for the *IL4*^{null} allele were identified using the previously described three-primer-based PCR assay (32). Segregants homozygous or heterozygous for the *IL10*^{null} allele were also identified using a three-primer-based assay. The three primers were: no. 86, 5′-GTGGGTGCAGTTATTGTCTTCCCG-3′; no. 87, 5′-GCCTTCAGTATAAAAGGGGGACC-3′; and no. 88, 5′-CCTGCGTGCAATCCATCTTG-3′. Primer pairs 86 and 87 amplify a 200-bp product from the wild-type *IL10* allele, while the 86 and 88 primer pairs amplify a 400-bp product from the *IL10*^{null} allele.

Assessment of diabetes and insulitis development

The indicated mice were monitored for development of glycosuria with Ames Diastix (supplied by Miles, Elkhart, IN). Glycosuric values of ≥3 were considered diagnostic of diabetes onset. Pancreases from mice assessed for insulitis development were fixed in Bouin's solution and sectioned at three nonoverlapping levels. Granulated β-cells were stained with aldehyde fuchsin, and leukocytes were stained with a hematoxylin and eosin counterstain. Islets (at least 20/mouse) were individually scored as follows: 0, no lesions; 1, peri-insular leukocytic aggregates, usually periductal infiltrates; 2, <25% islet destruction; 3, >25% islet destruction; and 4, complete islet destruction. An insulitis score for each mouse was obtained by dividing the total score for each pancreas by the number of islets examined. Data are presented as the mean insulitis score ± SEM for the indicated experimental group.

Treatment of mice with nonspecific immunostimulatory agents

Four-week-old NOD, NOD.*IL4*^{null}, NOD.*IL10*^{null}, and NOD.*IFN-γ*^{null} female mice were injected in a hind foot pad with 1.0 mg of heat-killed BCG vaccine (Connaught, Willowsdale, Canada) or 50 μl of CFA (Sigma, St. Louis, MO). Previous studies have demonstrated that it is the mycobacterial components of CFA or BCG that elicit IDDM-resistant NOD mice, because no palliative effects are observed following IFA treatment (32, 33). Controls consisted of females similarly injected with 50 μl of saline. Four mice from each group were sacrificed at 8 wk of age for comparison of intraislet cytokine mRNA levels as described below. All other mice in each group were monitored for IDDM and insulitis development.

Quantitation of intraislet Th1 and Th2 cytokine mRNA levels

T cell-infiltrated pancreatic islets were isolated as previously described (34) and subsequently pooled from four NOD, NOD.*IL4*^{null}, or NOD.*IL10*^{null} female mice that had been treated 4 wk earlier with BCG or CFA. Pools of pancreatic islets were also isolated from two separate groups of four NOD, NOD.*IL4*^{null}, or NOD.*IL10*^{null} female mice treated 4 wk earlier with saline. Comparison of these separate samples from two independent groups of saline-treated controls allowed for an assessment of potential intragroup variation. Levels of IFN-γ, IL-4, IL-10, and cyclophilin mRNA transcripts within the islets were assessed as previously described in radiolabeled semiquantitative RT-PCR assays (35). In addition, TGF-β mRNA levels were assessed using the primer pair 5′-TTGGTATCCAGGGCTCTCC-3′ and 5′-TGAGTGGCTGTCTTTTGACG-3′. The intensity of each radiolabeled signal was measured with a Fujix BAS imaging system (Fuji, Tokyo, Japan) and expressed as phosphor-stimulated luminescence units. Each cytokine signal was normalized as a percentage of the cyclophilin signal for the same sample. All samples compared were amplified in the same PCR run to avoid interassay variation.

AICD rates among NOD CD4$^+$ T cells functionally activated in a Th1 or Th2 cytokine environment

NOD CD4$^+$ T cells were purified from splenic leukocytes using a streptavidin-conjugated magnetic bead system (Miltenyi Biotec, Auburn, CA) to deplete CD8$^+$ T cells, macrophages/granulocytes, and B lymphocytes that had been prestained with biotinylated Abs directed against lineage-specific markers. CD8$^+$ T cells and the macrophage/granulocyte populations were, respectively, depleted with the mAbs 53-6.72 and M1/70, while B lymphocytes were removed with a goat polyclonal antiserum specific for mouse Ig molecules (Sigma). The purified CD4$^+$ T cells were suspended at a concentration of 5×10^6/ml in the previously described culture medium (36). These were seeded into tissue culture dishes that had been precoated as previously described (37) with 3.125 μg/ml of the mAb 145-2C11 (PharMingen, San Diego, CA) capable of activating T cells by binding the CD3 component of the TCR. To drive the anti-CD3-stimulated CD4$^+$ T cells into a Th1 or Th2 mode, the medium was further supplemented with either 50 U/ml rat recombinant IFN-γ (supplied by P. van de Meide, Rijswijk, The Netherlands) combined with 5.0 μg/ml of the murine IL-4 neutralizing mAb 11B11 (PharMingen), or 500 U/ml murine rIL-4 (BioSource, Camarillo, CA) combined with 10.0 μg/ml of the murine IFN-γ neutralizing mAb XMG1.2 (PharMingen). After incubation at 37°C for the indicated period of time, the proportion of CD4$^+$ T cells that had been driven into apoptotically mediated AICD upon anti-CD3 stimulation under Th1 or Th2 cytokine conditions was assessed by FACS analysis (FACScan, Becton Dickinson, San Jose, CA). Apoptotic cells were identified by positive TUNEL staining using a fluorescein-based in situ cell death detection kit (Roche, Indianapolis, IN). To determine the cytokine secretion profile of the surviving CD4$^+$ T cells, they were washed free of exogenous cytokines, resuspended at a concentration of 5×10^6/ml in medium, and subsequently restimulated for 24 h in tissue culture dishes precoated with 3.125 μg/ml of the CD3-specific mAb. Following this secondary stimulation, the culture supernatants were assessed with commercially available ELISA kits for IFN-γ, IL-4, and IL-10 concentrations (PharMingen).

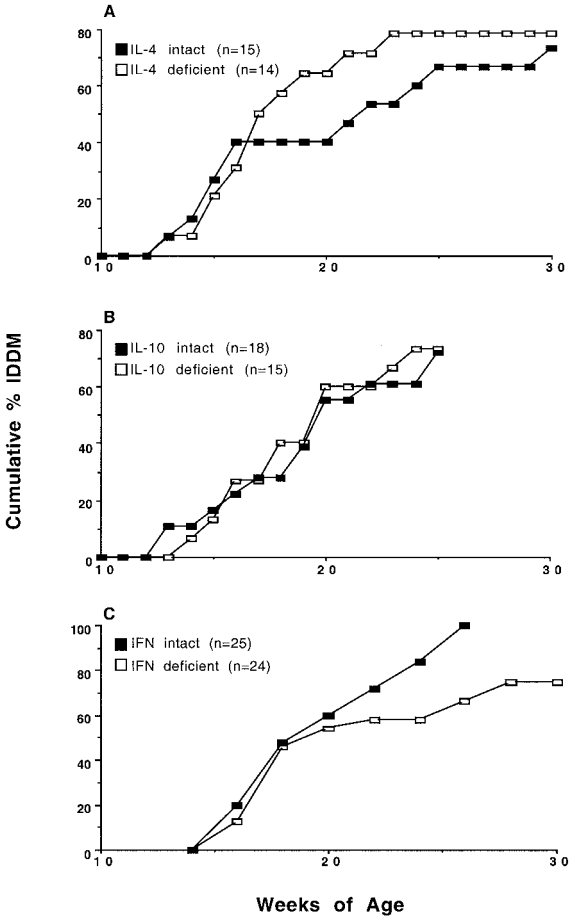

FIGURE 1. IDDM development is not significantly modulated in NOD mice made genetically deficient in IL-4 (*A*), IL-10 (*B*), or IFN-γ (*C*). Heterozygous carriers of the inactivated cytokine genes that were also fixed to homozygosity for markers of NOD alleles at all previously identified *Idd* loci were intercrossed at the N7 ($IL4^{null}$ and $IFN\text{-}\gamma^{null}$) or N8 ($IL10^{null}$) backcross generation. Data represent the rate of IDDM development in female intercross progeny homozygous for the $IL4^{null}$, $IL10^{null}$, and $IFN\text{-}\gamma^{null}$ alleles compared with those capable of producing these cytokines (pooled null/+ and +/+ segregants).

Results

Genetic ablation of IFN-γ, IL-4, or IL-10 does not alter IDDM development in NOD mice

If Th1 and Th2 cytokines, respectively, promote or inhibit IDDM development, it might be expected that disease onset would be accelerated in NOD mice made genetically deficient in IL-4 or IL-10. Conversely, IDDM development could conceivably be impaired in NOD mice made genetically deficient in IFN-γ. To address these possibilities, heterozygous carriers of the inactivated cytokine alleles that were also fixed to homozygosity for genetic linkage markers delineating all previously identified *Idd* loci of NOD origin were identified at either the N7 ($IL4^{null}$ and $IFN\text{-}\gamma^{null}$) or N8 ($IL10^{null}$) backcross generation and intercrossed. It was then determined whether the rate or frequency of IDDM development in female intercross progeny that were homozygous for the $IL4^{null}$, $IL10^{null}$, or $IFN\text{-}\gamma^{null}$ alleles differed from those segregants capable of producing these cytokines (pooled null/+ and +/+ genotypes). Similar to results reported by another group (38), we found no differences in the frequency or rate of IDDM development in IL-4-deficient or intact NOD females (Fig. 1*A*). IDDM development was also unaltered in IL-10-deficient NOD female mice (Fig. 1*B*). As reported by another group (20), there was only a slight retardation in IDDM development in NOD.$IFN\text{-}\gamma^{null}$ females (Fig. 1*C*). Collectively, these results demonstrated that the rate of IDDM development in NOD mice cannot be prevented by solely eliminating their naturally produced levels of the Th1 cytokine IFN-γ or accelerated by eliminating the Th2 cytokines IL-4 and IL-10.

Inhibition of IDDM in NOD mice by CFA or BCG treatment does not require IL-4 or IL-10 induction

Given that their β-cell autoreactive $CD4^+$ T cells normally fail to produce significant levels of IL-4 or IL-10 (reviewed in Ref. 3 and 4), it is probably not surprising that IDDM development is not enhanced in NOD females made genetically deficient in these Th2 cytokines. In contrast, there have been many reports that IDDM can be inhibited in NOD mice if a normally absent Th2 response is induced among β-cell autoreactive $CD4^+$ T cells (reviewed in Refs. 2–4). Such an up-regulation of Th2 cytokine production by β-cell autoreactive $CD4^+$ T cells has been proposed to be an essential component of IDDM suppression mediated by the nonspecific immunostimulatory agents CFA and BCG (reviewed in Ref. 2). However, as shown in Fig. 2, treatment with either BCG or CFA effectively inhibited IDDM development in NOD.$IL4^{null}$ female mice (0 vs 70% in controls by 20 wk of age). Similarly, the

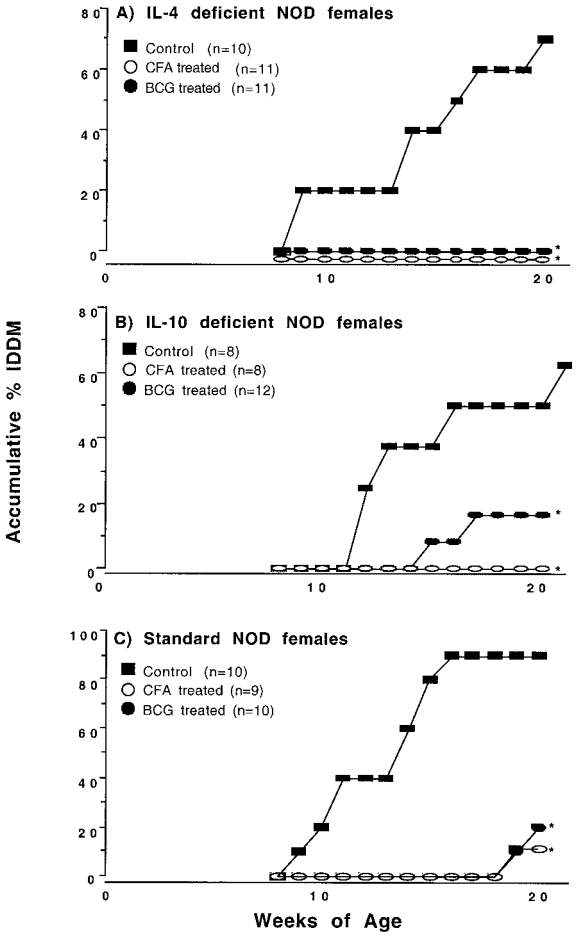

FIGURE 2. Inhibition of IDDM development in NOD mice by CFA or BCG treatment does not require induction of the cytokines IL-4 and IL-10. NOD.$IL4^{null}$ (*A*), NOD.$IL10^{null}$ (*B*), or standard NOD females (*C*) were injected in a rear foot pad at 4 wk of age with CFA, BCG, or saline and subsequently monitored for IDDM development. ∗, IDDM incidence significantly less ($p < 0.05$, by Kaplan Meier life table analysis) than in saline-treated controls.

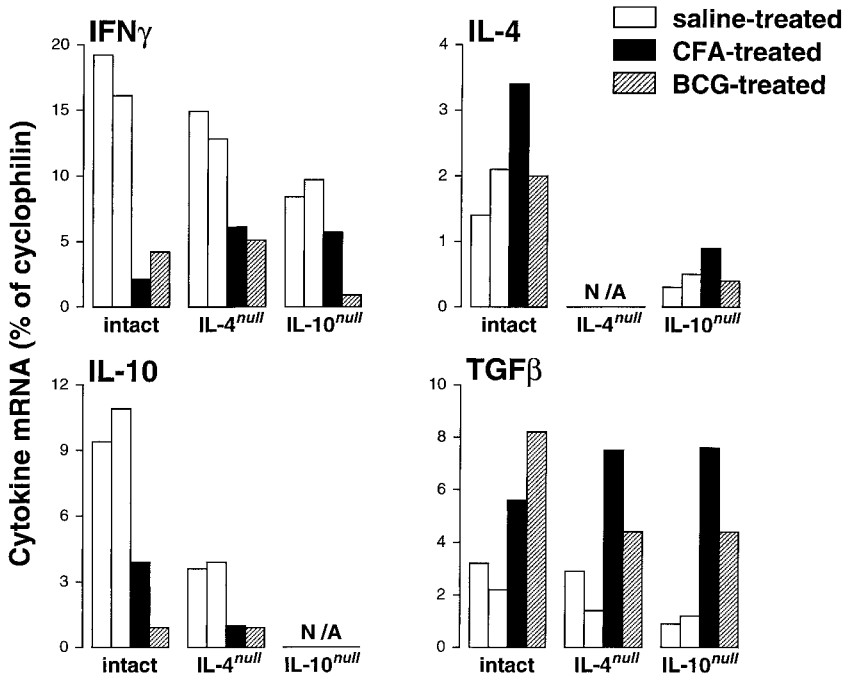

FIGURE 3. Intraislet TGF-β and IFN-γ mRNA levels are, respectively, increased and decreased in CFA- or BCG-protected NOD, NOD.*IL4*^{null}, and NOD.*IL10*^{null} mice. RNA was isolated from pooled pancreatic islets of four NOD, NOD.*IL4*^{null}, or NOD.*IL10*^{null} female mice that had been treated 4 wk earlier with CFA or BCG. RNA was also extracted from pools of pancreatic islets isolated from two separate groups of four NOD, NOD.*IL4*^{null}, or NOD.*IL10*^{null} female mice treated 4 wk earlier with saline. Samples were assessed for levels of IFN-γ, IL-4, IL-10, TGF-β, and cyclophilin mRNA transcripts in semiquantitative RT-PCR assays. Cytokine mRNA levels are depicted as percentages of cyclophilin mRNA for the same sample.

extent of IDDM development by 20 wk of age in NOD.*IL10*^{null} females treated with BCG (16.7%) or CFA (0%) was significantly less than that in saline-treated controls (62.5%). As expected, BCG or CFA treatment also effectively inhibited IDDM development in standard NOD female mice (10–20% incidence in treated mice vs 90% in controls). These results indicated that neither IL-4 or IL-10 induction by itself is required to mediate the IDDM-protective effects of CFA or BCG treatment.

*Intraislet TGF-β and IFN-γ mRNA levels are respectively increased and decreased in CFA- or BCG-protected NOD, NOD.*IL4^{null}*, and NOD.*IL10^{null} *mice*

The studies described above demonstrated that the mechanism by which CFA or BCG treatment inhibits IDDM development does not entail an absolute requirement for IL-4 or IL-10 induction. However, it is possible that following CFA or BCG treatment, β-cell autoreactive CD4$^+$ T cells unable to produce one of these Th2 cytokines instead up-regulate production of the other in a reciprocal compensatory fashion that is then responsible for the inhibition of IDDM development. This possibility was addressed by semiquantitative RT-PCR analyses of cytokine mRNA levels in pancreatic islets from control and CFA- or BCG-treated NOD, NOD.*IL4*^{null}, and NOD.*IL10*^{null} mice. It should be noted that the quantities of RNA that can be isolated from islets of individual mice is not sufficient to conduct RT-PCR analyses. Thus, to control for intragroup variation, RNA was extracted from the pooled islets of four mice in each treatment group. The validity and reproducibility of this approach are illustrated by the fact that closely matched cytokine mRNA levels were found in pools of pancreatic islets isolated from two independent groups of saline-treated NOD, NOD.*IL4*^{null}, and NOD.*IL10*^{null} female control mice (Fig. 3).

As previously reported by others (35, 39), pancreatic islets from standard NOD mice rendered IDDM resistant by CFA treatment were characterized by higher IL-4 mRNA levels than saline-treated controls (Fig. 3). CFA-treated NOD.*IL10*^{null} mice were also characterized by slightly higher intraislet IL-4 mRNA levels than observed in controls. This latter result is unlikely to be of pathogenic significance, because the level of IL-4 mRNA detected in islets of IDDM-susceptible saline-treated NOD mice was much higher than that observed in CFA-protected NOD.*IL10*^{null} mice. In contrast, intraislet IL-4 mRNA levels were not increased in either standard or IL-10-deficient NOD mice protected from IDDM by BCG treatment. Intraislet levels of IL-10 mRNA were actually reduced in both standard and IL-4-deficient NOD mice protected from IDDM by either CFA or BCG treatment. Thus, the inhibition of IDDM by CFA or BCG treatment is not associated with reciprocal compensatory intraislet increases in IL-4 or IL-10 in NOD mice genetically deficient in either of these Th2 cytokines.

CFA- and BCG-induced IDDM resistance in NOD, NOD.*IL4*^{null}, and NOD.*IL10*^{null} mice was associated with decreased intraislet levels of IFN-γ mRNA (Fig. 3). This may result from the fact that mRNA levels for the cytokine TGF-β, which can suppress IFN-γ production (40) were increased in NOD, NOD.*IL4*^{null}, and NOD.*IL10*^{null} mice protected from IDDM by either CFA or BCG treatment. However, arguing against a TGF-β-mediated suppression of IFN-γ production as being the mechanism by which CFA or BCG treatment inhibits IDDM development is the observation that NOD mice genetically deficient in IFN-γ remain disease susceptible (see Fig. 1).

*Insulitis is significantly decreased in CFA protected NOD, NOD.*IL4^{null}*, and NOD.*IL10^{null} *mice*

The data described above indicate that while intraislet Th1 to Th2 cytokine shifts are associated with CFA- and BCG-mediated IDDM protection in NOD mice, such deviations are unlikely to represent an obligatory mechanism by which these agents inhibit disease. Hence, we also examined whether CFA or BCG treatment might actually inhibit IDDM development by inducing the deletion, perhaps through AICD, of a significant fraction of β-cell autoreactive T cells either before or after they have entered the pancreatic islets. If quantitative decreases in numbers of β-cell autoreactive T cells accounted for the induction of IDDM resistance by CFA or BCG treatment, then protected mice should be characterized by lower insulitis levels than saline-treated controls. Hence, insulitis levels were compared among all CFA-, BCG-, and saline-treated mice depicted in Fig. 2 that remained free of overt

IDDM at 20 wk of age. Only one saline-treated standard NOD female remained free of overt IDDM at 20 wk of age. Thus, we also examined insulitis levels in two other 20-wk-old NOD females from our research colony that had not yet developed overt IDDM. As shown in Table I, the mean insulitis score in these three NOD control females (3.56 ± 0.36) was significantly higher than that observed in the CFA-treated group (1.83 ± 0.62). Similarly, significantly lower levels of insulitis were observed in NOD.*IL4*^{null} and NOD. *IL10*^{null} mice protected from IDDM by CFA treatment than in saline-treated controls. Hence, these evaluations of insulitis development indicate that CFA treatment most likely inhibits IDDM development in standard and Th2 cytokine-deficient NOD mice by inducing quantitative decreases in numbers of pathogenic T cells. In contrast, there were no significant differences in mean insulitis scores between saline- and BCG-treated NOD, NOD.*IL4*^{null}, and NOD.*IL10*^{null} mice remaining free of overt IDDM at 20 wk of age (data not shown). These collective data indicate that the mechanisms by which the nonspecific immunostimulatory agents CFA and BCG inhibit IDDM development in NOD mice are not completely identical. However, neither of the mechanisms involved is absolutely dependent upon induction of IL-4 or IL-10, or inhibition of IFN-γ.

Inhibition of IDDM by BCG or CFA treatment is IFN-γ dependent

For the reasons described above, we considered it unlikely that CFA or BCG treatment inhibits IDDM development in standard NOD mice as well those genetically deficient in IL-4 or IL-10 by diminishing production of the Th1 cytokine IFN-γ in pancreatic islets. However, to more rigorously test this question, we determined whether CFA or BCG treatment inhibited IDDM development in NOD.*IFN-γ*^{null} mice. It was reasoned that if either treatment inhibits IDDM in standard or Th2 cytokine-deficient NOD mice by reducing intraislet levels of IFN-γ, then these protocols should also exert highly protective effects in the NOD.*IFN-γ*^{null} stock. However, BCG treatment failed to inhibit IDDM development in NOD.*IFN-γ*^{null} mice (Fig. 4). Thus, the mechanism by which BCG treatment inhibits IDDM development in NOD mice actually requires the presence of IFN-γ, which heretofore was thought to contribute exclusively to pathogenic processes. Interestingly, CFA treatment also inhibited IDDM development much less effectively in NOD.*IFN-γ*^{null} females (Fig. 4) than in standard NOD mice or those genetically deficient in IL-4 or IL-10 (see Fig. 2). This indicated that the mechanism by which CFA treatment blocks IDDM in NOD mice also operates most efficiently under conditions where IFN-γ can be produced. Hence, while there are some differences, there is also overlap in the mechanisms by which CFA and BCG treatment inhibit IDDM development in NOD mice.

Table I. *Insulitis is significantly decreased in CFA protected NOD, NOD.*IL4^{null}*, and NOD.*IL10^{null} *mice*[a]

Strain	MIS ± SEM in Saline-Treated Controls	MIS ± SEM in CFA-Treated Group
NOD	3.56 ± 0.36 ($n = 3$)[b]	1.83 ± 0.62[c] ($n = 8$)
NOD.*IL4*^{null}	3.65 ± 0.36 ($n = 2$)	1.40 ± 0.34[c] ($n = 10$)
NOD.*IL10*^{null}	3.67 ± 0.47 ($n = 2$)	1.59 ± 0.68[c] ($n = 7$)

[a] Females from the indicated strains were treated with saline or CFA at 4 wk of age. Mean insulitis scores (MIS) in mice remaining free of overt IDDM at 20 wk of age were determined as described in *Materials and Methods.*

[b] Value based on one saline-treated control remaining free of IDDM through 20 wk of age plus two additional age-matched IDDM-free NOD females identified in the research colony.

[c] Significantly less ($p < 0.05$, Student's t test) than controls.

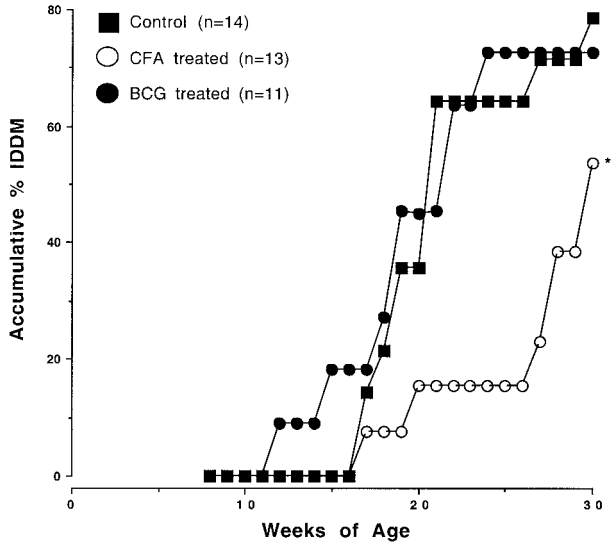

FIGURE 4. The mechanism by which BCG and, to a lesser extent, CFA treatment inhibits IDDM development in NOD mice requires the presence of IFN-γ. Female NOD.*IFN-γ*^{null} mice were injected in a rear foot pad at 4 wk of age with CFA, BCG, or saline and subsequently monitored for IDDM development. ∗, Marginal statistical difference ($p = 0.06$, by Kaplan Meier life table analysis) from saline-treated controls.

TCR stimulation induces lower levels of AICD among NOD CD4^{+} *T cells producing Th2 than Th1 cytokines*

While unlikely to represent the mechanism of protection, the question remained as to why Th1 to Th2 cytokine shifts are observed

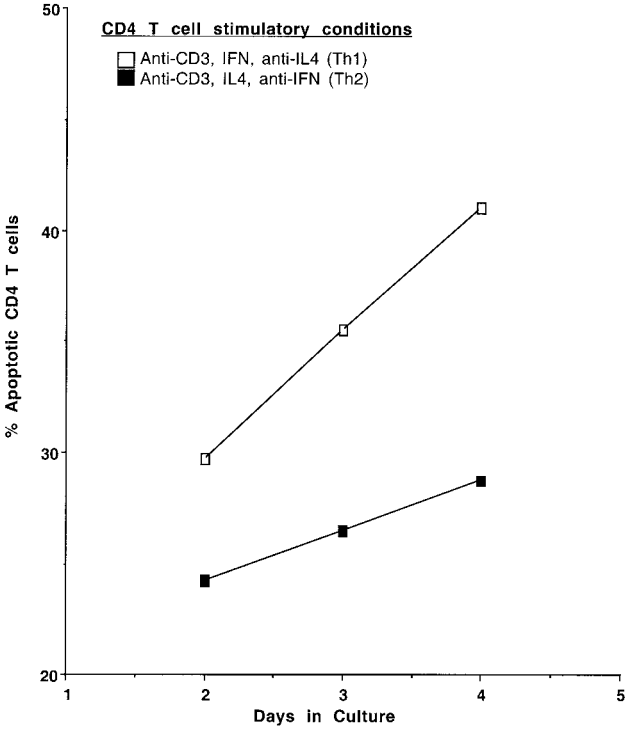

FIGURE 5. TCR stimulation induces lower levels of AICD among NOD CD4^{+} T cells producing Th2 than Th1 cytokines. Purified NOD CD4^{+} T cells were stimulated through the TCR with anti-CD3 in the presence of the indicated cytokine/Ab combinations designed to induce a Th1 or a Th2 response. At the indicated time points, flow cytometric techniques were used to quantify by TUNEL staining the proportion of NOD CD4^{+} T cells that underwent apoptotically mediated AICD under each culture condition. Similar results were obtained in two other experiments.

in islets of standard NOD mice that have been made IDDM resistant by CFA treatment. In nonautoimmune strains it has been reported that Th2 are less prone than Th1 cells to AICD (6, 7). This suggested the possibility that $CD4^+$ T cells producing Th1 cytokines may indeed be important contributors to autoimmune IDDM in NOD mice, but they are more easily deleted by AICD pathways triggered by CFA treatment than those producing Th2 cytokines. Thus, we determined whether AICD rates varied in NOD $CD4^+$ T cells that underwent TCR-mediated stimulation under conditions designed to promote either a Th1 or a Th2 response. It should be noted that we used anti-IL-4 in conjunction with IFN-γ, rather than IL-12, to elicit Th1 responses, because this combination would result in the maximal cosuppression of Th2 activity. At all time points, lower levels of apoptosis were observed among NOD $CD4^+$ T cells that had undergone anti-CD3 stimulation under Th2 vs Th1 cytokine conditions (Fig. 5).

Surviving $CD4^+$ T cells underwent secondary anti-CD3 stimulation to determine their cytokine production profiles. As shown in Fig. 6*A*, these analyses demonstrated that production levels of the Th1 cytokine IFN-γ did not correlate with the ability of NOD $CD4^+$ T cells to undergo AICD. Interestingly, higher levels of IFN-γ were produced by NOD $CD4^+$ T cells initially activated under Th2 rather than Th1 conditions. This might be explained by the fact that NOD $CD4^+$ T cells are predisposed to Th1 responses (reviewed in Refs. 3 and 4) and a report that ongoing Th1 responses can actually be enhanced by IL-4 (41). However, resistance to AICD did correlate with NOD $CD4^+$ T cells acquiring an enhanced ability to produce the Th2 cytokines IL-4 (Fig. 6*B*) and/or IL-10 (Fig. 6*C*). If there are similar disparities in apoptotic sensitivity in vivo, then islet-infiltrating $CD4^+$ T cells that are producing Th2 cytokines might be preferentially protected from the deletional events that inhibit IDDM development in CFA-treated NOD mice. Such a difference in T cell death rates could result in an unmasking process that has a secondary appearance of being a Th1 to Th2 cytokine shift.

Discussion

Many Ag-specific and nonspecific immunostimulatory protocols that inhibit IDDM development in NOD mice have been reported to do so by eliciting a Th1 to Th2 cytokine shift among β-cell autoreactive T cells (reviewed in Refs. 2–4). However, most of these studies have documented associative rather than causative

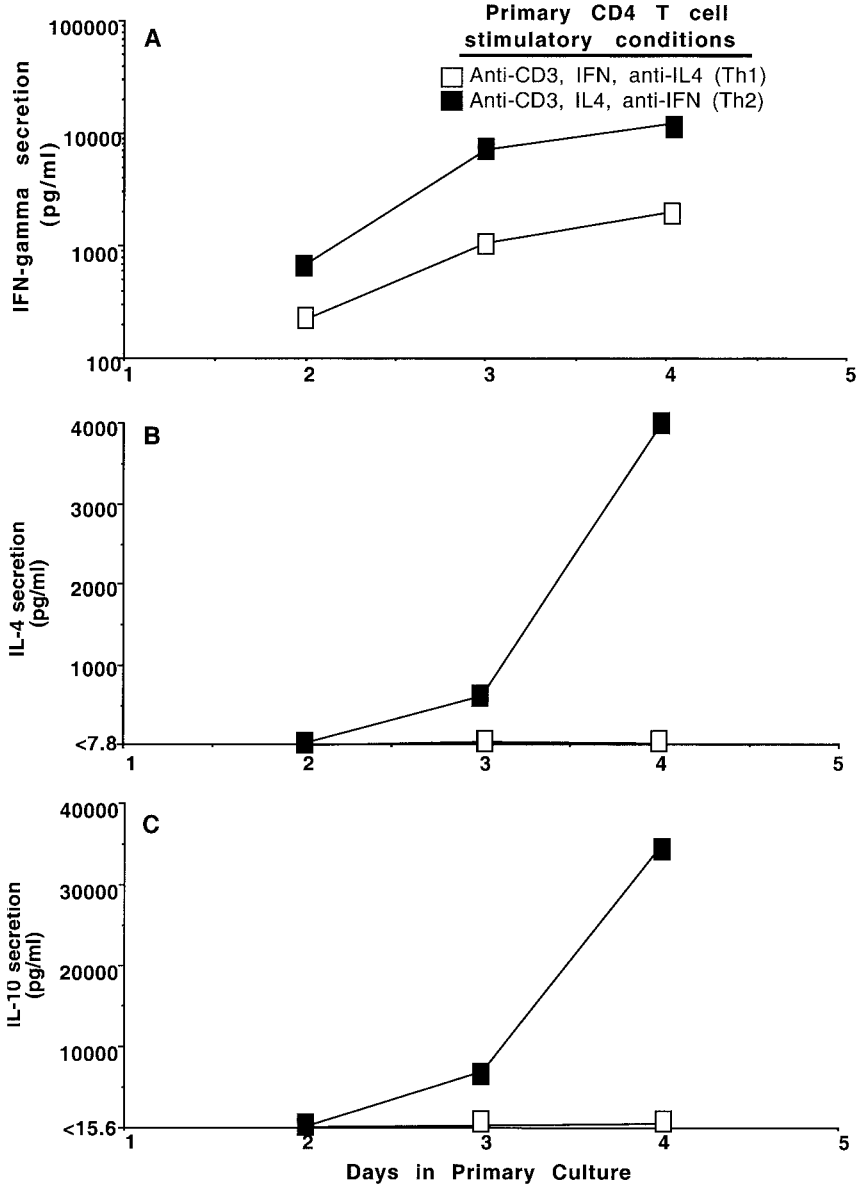

FIGURE 6. Resistance of NOD $CD4^+$ T cells to AICD correlates with an enhanced ability to produce Th2 cytokines. Purified NOD $CD4^+$ T cells underwent primary anti-CD3-mediated stimulation under the indicated conditions designed to promote a Th1 or a Th2 response. An aliquot of cells was then assessed for AICD rates as shown in Fig. 6. The remaining $CD4^+$ T cells were restimulated for 24 h with anti-CD3 alone, and culture supernatants were analyzed by ELISA for IFN-γ (*A*), IL-4 (*B*), and IL-10 (*C*) concentrations. Similar results were obtained in two other experiments.

links between such Th1 to Th2 cytokine shifts and the induction of IDDM resistance. In this study, which used two newly developed stocks of NOD mice that are genetically deficient in IL-4 or IL-10, we found that while the induction of IDDM resistance by CFA and BCG treatment is associated with intraislet Th1 to Th2 cytokine shifts, this is a secondary outcome rather than the cause of disease resistance. Hence, while Th1 and Th2 cytokine shifts may be observed among β-cell autoreactive T cells of NOD mice protected from overt IDDM by various immunomodulatory therapies, it cannot automatically be assumed that this is the cause of disease resistance.

Rather than resulting from a Th1 to Th2 cytokine shift, our data indicate that the induction of IDDM resistance by CFA treatment most likely results from quantitative decreases in levels of islet-infiltrating T cells. Interestingly, while containing fewer T cells, islets from CFA-protected NOD and NOD.*IL10*^{null} mice were characterized by higher IL-4 mRNA levels than those from saline-treated controls. This suggested that the process that underlies the deletion of β-cell autoreactive T cells in CFA-treated NOD mice might preferentially spare those producing Th2 cytokines. Indeed, as previously observed in nonautoimmune prone strains (6, 7), we found that NOD CD4$^+$ T cells producing Th2 cytokines are preferentially protected from apoptotically mediated AICD. As a result, the enhanced Th2 cytokine levels observed in pancreatic islets of NOD mice rendered IDDM resistant by CFA treatment most likely represent a secondary unmasking event that is not the cause of disease protection.

While also not dependent upon Th2 cytokine induction, BCG treatment appears to block IDDM development in NOD mice through a mechanism that only partially overlaps that elicited by CFA treatment. This conclusion is partially based on the finding that unlike CFA, the inhibition of IDDM development in BCG-treated NOD, NOD.*IL4*^{null}, and NOD.*IL10*^{null} mice was not associated with reductions in islet-infiltrating T cell levels. Our data suggested that one mechanism by which BCG treatment might inhibit IDDM development in NOD, NOD.*IL4*^{null}, and NOD.*IL10*^{null} mice is by reducing mRNA transcripts for the Th1 cytokine IFN-γ. We reasoned that if this was correct, BCG treatment would effectively block IDDM development in IFN-γ-deficient NOD mice. Thus, we were surprised by the fact that BCG treatment had absolutely no IDDM-protective effect in NOD.*IFN-γ*^{null} mice. Furthermore, the IDDM-protective effects of CFA treatment were also greatly attenuated in NOD.*IFN-γ*^{null} mice. These results indicate that the inductive phase of the mechanism by which BCG and, to a lesser extent, CFA treatment, inhibits IDDM development in standard NOD mice is actually dependent upon the presence of IFN-γ, which was previously only thought to exert pathogenic effects. In contrast, the fact that IDDM spontaneously develops at a high rate in NOD.*IFN-γ*^{null} mice indicates that once generated, the pathogenic effector functions of β-cell autoreactive T cells are not dependent upon the presence of IFN-γ.

Our data suggest that the reduced intraislet levels of IFN-γ observed in CFA and BCG protected NOD, NOD.*IL4*^{null}, and NOD.*IL10*^{null} mice may be a secondary consequence of TGF-β induction. However, it is possible that the increased intraislet level of TGF-β directly contributes to IDDM resistance in BCG-treated NOD mice. In CFA-treated NOD mice, an elevation of intraislet TGF-β may confer an additional level of IDDM protection beyond that afforded by a reduction in β-cell autoreactive T cell levels. These possibilities are supported by a previous report that an NOD-derived CD4$^+$ T cell clone capable of inhibiting IDDM development did so through the release of TGF-β (18). Similarly, both a transgenic and a gene therapy system that increase pancreatic TGF-β levels have been shown to inhibit IDDM development in NOD mice (42, 43). One way that TGF-β has been proposed to inhibit IDDM development in NOD mice is by inducing a change in the type of APC presenting β-cell Ags to autoreactive CD4$^+$ T cells from B lymphocytes to a myeloid population (44).

While not representing the mechanism of action elicited by treatment with the nonspecific immunostimulatory agents CFA and BCG, other protocols that inhibit IDDM development in NOD mice may well do so by eliciting Th1 to Th2 cytokine shifts within β-cell autoreactive T cell populations. For example, one therapy entailing immunization with peptides derived from the candidate β-cell autoantigen glutamic acid decarboxylase inhibits the development of IDDM in standard NOD mice, but not in the same NOD.*IL4*^{null} stock used in the current study (32). Collectively, these past studies coupled with our current findings indicate that while some immunomodulatory protocols do indeed block IDDM development in NOD mice through an enhancement of Th2 cytokine production by β-cell autoreactive T cells, in some cases such a cytokine shift is a consequence rather than the cause of protection. Hence, great care should be taken in interpreting the pathological significance of any Th1 to Th2 cytokine shifts that may occur among islet-infiltrating T cells of NOD mice protected from the development of overt IDDM by various immunomodulatory protocols. Furthermore, the alternative interpretation that Th1 to Th2 cytokine shifts among β-cell autoreactive T cells of NOD mice made IDDM resistant by immunostimulation can be an outcome rather than the cause of protection may also be applicable to other autoimmune diseases.

Acknowledgments

We thank Holly Savage for excellent animal care assistance, and Darryl Glubrecht for help with the RT-PCR assays.

References

1. Serreze, D. V. 1998. The identity and ontogenic origins of autoreactive T lymphocytes in NOD mice. In *NOD Mice and Related Strains: Research Applications in Diabetes, AIDS, Cancer, and Other Diseases.* E. H. Leiter and M. A. Atkinson, eds. Landes Bioscience, Austin, p. 71.
2. Rabinovitch, A. 1994. Immunoregulatory and cytokine imbalances in the pathogenesis of IDDM: therapeutic intervention by immunostimulation? *Diabetes 43:613.*
3. Tisch, R., and H. McDevitt. 1996. Insulin-dependent diabetes mellitus. *Cell 85: 291.*
4. Delovitch, T. L., and B. Singh. 1997. The nonobese diabetic mouse as a model of autoimmune diabetes: immune dysregulation gets the NOD. *Immunity 7:291.*
5. Hosken, N. A., K. Shibuya, A. W. Heath, K. M. Murphy, and A. O'Garra. 1995. The effect of antigen dose on CD4$^+$ T helper cell phenotype development in a T cell receptor-$\alpha\beta$-transgenic model. *J. Exp. Med. 182:1579.*
6. Varadhachary, A. S., S. N. Perdow, C. Hu, M. Ramanarayanan, and P. Salgami. 1997. Differential ability of T cell subsets to undergo activation-induced cell death. *Proc. Natl. Acad. Sci. USA 94:5778.*
7. Zhang, X., T. Brunner, L. Carter, R. W. Dutton, P. Rogers, L. Bradley, T. Sato, J. C. Reed, D. Green, and S. L. Swain. 1997. Unequal death in T helper (Th)1 and Th2 effectors: Th1, but not Th2, effectors undergo rapid Fas/FasL-mediated apoptosis. *J. Exp. Med. 185:1837.*
8. Serreze, D. V., and E. H. Leiter. 1991. Development of diabetogenic T cells from NOD/Lt marrow is blocked when an allo-H-2 haplotype is expressed on cells of hematopoietic origin, but not on thymic epithelium. *J. Immunol. 147:1222.*
9. Serreze, D. V., J. W. Gaedeke, and E. H. Leiter. 1993. Hematopoietic stem cell defects underlying abnormal macrophage development and maturation in NOD/Lt mice: defective regulation of cytokine receptors and protein kinase C. *Proc. Natl. Acad. Sci. USA 90:9625.*
10. Carrasco-Marin, E., J. Shimizu, O. Kanagawa, and E. R. Unanue. 1996. The class II MHC class I-A^{g7} molecules from non-obese diabetic mice are poor peptide binders. *J. Immunol. 156:450.*
11. Ridgway, W. M., M. Fasso, A. Lanctot, C. Garvey, and C. G. Fathman. 1996. Breaking self-tolerance in nonobese diabetic mice. *J. Exp. Med. 183:1657.*
12. Piganelli, J. D., T. Martin, and K. Haskins. 1998. Splenic macrophages from the NOD mouse are defective in ability to present antigen. *Diabetes 47:1212.*
13. Milich, D. R., J. E. Jones, A. McLachlan, R. Houghton, G. B. Thornton, and J. L. Hughes. 1989. Distinction between antigenicity and tolerogenicity among HBcAg T cell determinants: influence of peptide-MHC interaction. *J. Immunol. 143:3148.*
14. Ucker, D. S., J. Meyers, and P. S. Obermiller. 1992. Activation-driven T cell death. II. Quantitative differences alone distinguish stimuli triggering nontransformed T cell proliferation or death. *J. Immunol. 149:1583.*

15. Girao, C., Q. Hu, J. Sun, and P. G. Ashton-Rickardt. 1997. Limits to the differential avidity model of T cell selection in the thymus. *J. Immunol. 159:4205.*
16. Akhtar, I., J. P. Gold, L.-Y. Pan, J. L. M. Ferrara, X.-D. Yang, J. I. Kim, and K.-N. Tan. 1995. $CD4^+$ β islet cell-reactive T cell clones that suppress autoimmune diabetes in nonobese diabetic mice. *J. Exp. Med. 182:87.*
17. Pakala, S. V., M. O. Kurrer, and J. D. Katz. 1997. T helper 2 (Th2) T cells induce acute pancreatitis and diabetes in immune-compromised nonobese diabetic (NOD) mice. *J. Exp. Med. 186:299.*
18. Zekzer, D., F. S. Wong, L. Wen, M. Altieri, T. Gurlo, H. von Grafenstein, and R. S. Sherwin. 1997. Inhibition of diabetes by an insulin reactive CD4 T-cell clone in the nonobese diabetic mouse. *Diabetes 46:1124.*
19. Poulin, M., and K. Haskins. 2000. Induction of diabetes in nonobese diabetic mice by Th2 T cell clones from a TCR transgenic mouse. *J. Immunol. 164:3072.*
20. Hultgren, B., X. Huang, N. Dybdal, and T. A. Stewart. 1996. Genetic absence of γ-interferon delays but does not prevent diabetes in NOD mice. *Diabetes 45:812.*
21. Kanagawa, O., G. Xu, A. Tevaarwerk, and B. A. Vaupel. 2000. Protection of nonobese diabetic mice from diabetes by gene(s) closely linked to IFN-γ receptor loci. *J. Immunol. 164:3919.*
22. Serreze, D. V., C. M. Post, H. D. Chapman, E. J. Johnson, B. Lu, and P. B. Rothman. 2001. Interferon-γ receptor signaling is dispensable in the development of autoimmune type 1 diabetes in NOD mice. *Diabetes. In press.*
23. Rapaport, M. J., D. Zipris, A. H. Lazarus, A. Jaramillo, D. V. Serreze, E. H. Leiter, P. Cyopick, and T. L. Delovitch. 1993. Thymic T cell proliferative unresponsiveness in autoimmune NOD mice. II. IL-4 reverses NOD thymic T cell anergy and prevents the onset of diabetes. *J. Exp. Med. 178:87.*
24. Cameron, M. J., G. A. Arreaza, P. Zucker, S. W. Chensue, R. M. Strieter, S. Chakrabarti, and T. L. Delovitch. 1997. IL-4 prevents insulitis and insulin dependent diabetes mellitus in nonobese diabetic mice by potentiation of regulatory T helper-2 cell function. *J. Immunol. 159:4686.*
25. Pennline, K. J., E. Roque-Gaffney, and M. Monahan. 1994. Recombinant human IL-10 prevents the onset of diabetes in the nonobese diabetic mouse. *Clin. Immunol. Immunopathol. 71:169.*
26. Mueller, R., T. Krahl, and N. Sarvetnick. 1996. Pancreatic expression of interleukin-4 abrogates insulitis and autoimmune diabetes in nonobese diabetic (NOD) mice. *J. Exp. Med. 184:1093.*
27. Wogensen, L., M.-S. Lee, and N. Sarvetnick. 1994. Production of interleukin 10 by islet cells accelerates immune-mediated destruction of β cells in nonobese diabetic mice. *J. Exp. Med. 179:1379.*
28. Dalton, D. K., S. Pitts-Meek, S. Keshav, I. S. Figari, A. Bradley, and T. A. Stewart. 1993. Multiple defects of immune cell function in mice with disrupted interferon-γ genes. *Science 259:1739.*
29. Kuhn, R., K. Rajewsky, and W. Muller. 1991. Generation and analysis of interleukin-4 deficient mice. *Science 254:707.*
30. Kuhn, R., J. Lohler, D. Rennick, K. Rajewsky, and W. Muller. 1993. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell 75:263.*
31. Serreze, D. V., H. D. Chapman, D. S. Varnum, M. S. Hanson, P. C. Reifsnyder, S. D. Richard, S. A. Fleming, E. H. Leiter, and L. D. Shultz. 1996. B lymphocytes are essential for the initiation of T cell mediated autoimmune diabetes: analysis of a new "speed congenic" stock of NOD.*Ig*μ^{null} mice. *J. Exp. Med. 184:2049.*
32. Tisch, R., B. Wang, and D. V. Serreze. 1999. Induction of GAD65-specific Th2 cells and suppression of autoimmune diabetes at late stages of disease is epitope-dependent. *J. Immunol. 163:1178.*
33. Qin, H.-Y., M. W. J. Sadelain, C. Hitchon, J. Lauzon, and B. Singh. 1993. Complete Freund's adjuvant-induced T cells prevent the development and adoptive transfer of diabetes in nonobese diabetic mice. *J. Immunol. 150:2072.*
34. Gerling, I. C., D. V. Serreze, S. W. Christianson, and E. H. Leiter. 1992. Intrathymic islet cell transplantation reduces β-cell autoimmunity and prevents diabetes in NOD/Lt mice. *Diabetes 41:1672.*
35. Rabinovitch, A., W. L. Suarez-Pinzon, O. Sorensen, R. C. Bleackley, and R. F. Power. 1995. IFN-γ gene expression in pancreatic islet-infiltrating mononuclear cells correlates with autoimmune diabetes in nonobese diabetic mice. *J. Immunol.* 154:4874.
36. Serreze, D. V., and E. H. Leiter. 1988. Defective activation of T suppressor cell function in nonobese diabetic mice: potential relation to cytokine deficiencies. *J. Immunol. 140:3801.*
37. Christianson, S. W., L. D. Shultz, and E. H. Leiter. 1993. Adoptive transfer of diabetes into immunodeficient NOD-*scid/scid* mice: relative contributions of $CD4^+$ and $CD8^+$ T lymphocytes from diabetic versus prediabetic NOD.NON-*Thy1*^{a} donors. *Diabetes 42:44.*
38. Wang, B., A. Gonzalez, P. Hoglund, J. D. Katz, C. Benoist, and D. Mathis. 1998. Interleukin-4 deficiency does not exacerbate disease in NOD mice. *Diabetes 47:1207.*
39. Shehadeh, N. N., F. LaRosa, and K. J. Lafferty. 1993. Altered cytokine activity in adjuvant inhibition of autoimmune diabetes. *J. Autoimmun. 6:291.*
40. Letterio, J. J., and A. B. Roberts. 1998. Regulation of immune responses by TGF-β. *Annu. Rev. Immunol. 16:137.*
41. Oriss, T. B., S. A. McCarthy, M. A. K. Campana, and P. A. Morel. 1999. Evidence of positive cross-regulation on Th1 by Th2 and antigen presenting cells: effects on Th1 induced by IL-4 and IL-12. *J. Immunol. 162:1999.*
42. Moritani, M., K. Yoshimoto, S. F. Wong, C. Tanaka, T. Yamaoka, T. Sano, Y. Komagata, J.-I. Miyazaki, H. Kikutani, and M. Itakura. 1998. Abrogation of autoimmune diabetes in nonobese diabetic mice and protection against effector lymphocytes by transgenic paracrine TGF-β1. *J. Clin. Invest. 102:499.*
43. Piccirillo, C. A., Y. Chang, and G. J. Prud'homme. 1998. TGF-β1 somatic gene therapy prevents autoimmune disease in nonobese diabetic mice. *J. Immunol. 161:3950.*
44. King, C., J. Davies, R. Mueller, M.-S. Lee, T. Krahl, B. Yeung, E. O'Conner, and N. Sarvetnick. 1998. TGF-β1 alters APC preference, polarizing islet antigen responses toward a Th2 phenotype. *Immunity 8:601.*