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Pancreatic IL-4 Expression Results in Islet-Reactive Th2 Cells That Inhibit Diabetogenic Lymphocytes in the Nonobese Diabetic Mouse[1]

W. Scott Gallichan, Balaji Balasa, Joanna D. Davies, and Nora Sarvetnick[2]

When immunological tolerance breaks down, autoimmune destruction of insulin-producing β cells in the pancreas can cause insulin-dependent diabetes mellitus. We previously showed that transgenic nonobese diabetic (NOD) mice expressing IL-4 in the pancreas (NOD-IL-4 mice) were protected from insulitis and diabetes. Here we have characterized the avoidance of pathological autoimmunity in these mice. The absence of disease did not result from a lack of T cell priming, because T cells responding to dominant islet Ags were present. These islet Ag-specific T cells displayed a Th2 phenotype, indicating that Th2 responses could account for the observed tolerance. Interestingly, islet Ag-specific Th1 T cells were present and found to be functional, because neutralization of the Th2 effector cytokines IL-4 and IL-10 resulted in diabetes. Histological examination revealed that NOD-IL-4 splenocytes inhibited diabetogenic T cells in cotransfer experiments by limiting insulitis and delaying diabetes. Neutralization of IL-4 in this system abrogated the ability of NOD-IL-4 splenocytes to delay the onset of diabetes. These results indicate that IL-4 expressed in the islets does not prevent the generation of pathogenic islet responses but induces islet Ag-specific Th2 T cells that block the action of diabetogenic T cells in the pancreas. ***The Journal of Immunology*****, 1999, 163: 1696–1703.**

Insulin-dependent diabetes mellitus (IDDM)[3] is an autoimmune disease mediated by autoreactive T cells that infiltrate and destroy insulin-producing β cells in the islets of Langerhans. Nonobese diabetic (NOD) mice have been established as a murine model of autoimmune diabetes similar to that of human IDDM (1). In these mice, inflammatory cells usually infiltrate the pancreatic islets within the first few weeks of life, eventually leading to islet destruction and overt diabetes. The cellular infiltrate consists of CD4 and CD8 T cells, macrophages, and B cells. Adoptive transfer studies have demonstrated that both CD4 and CD8 T cells are required for clinical disease.

Differentiated CD4 T cells are divisible into two functional subsets according to the cytokines they secrete in response to antigenic stimulation (2): Th1 cells produce IFN-γ, and Th2 cells secrete IL-4 (among others), and these cytokines can counterregulate development of the opposing cellular subset. The manifestation of this process in NOD mice is that a period of local cytokine imbalance (3, 4) appears to cause the polarization and emergence of either a Th1 or a Th2 response (5–10). Polarization to a Th1 response induces rapid progression to IDDM (11), and the destructive insulitis of diabetes-prone NOD mice is associated with a relatively higher frequency of IFN-γ-producing cells and lower frequency of IL-4-producing cells than are found in mice protected from this disease (12). Whereas Th1 responses seem to produce disease, Th2-like responses have been associated with protection (7, 13–15), although homogeneous Th2 populations are unable to mediate protection from diabetogenic lymphocytes (11) and in fact can cause disease under certain conditions (16).

The prospect of counterregulating pathological autoimmune Th1 cells in diabetes by promoting a protective (Th2) phenotype has generated considerable interest (14, 17–21). Systemic administration of IL-4 to young diabetes-prone NOD mice reduced the incidence of diabetes (22–24), protection that was attributed to a reversal in CD4 T cell hyporesponsiveness and the capacity to produce IL-4 (22, 25). We have examined counterregulation originating from the target tissue by expressing IL-4 within the islets of NOD mice (NOD-IL-4 mice). Mice were free from insulitis and diabetes (18) despite the presence of autoreactivity (19); however, protection was abrogated when TCR diversity was restricted. Still unknown are whether protection from diabetes following IL-4 immunotherapy is due to the presence of islet Ag-specific Th2 cells and how the development of a Th2 phenotype regulates disease pathogenesis.

Therefore, we sought to characterize the islet Ag-specific T lymphocytes as well as their secreted factors in the lack of diabetogenicity in NOD-IL-4 mice. The islet cell autoantigens GAD65 and HSP65 were used to incite T cell responses (26, 27). Our results showed that NOD-IL-4 autoimmune T cells responded to the same determinants recognized in NOD mice. There was also a similar TCR Vβ 8.1 and/or 8.2 TCR usage by GAD65-specific T cells from NOD and NOD-IL-4 mice. Additionally, the cryptic existence of autoimmunity in NOD-IL-4 mice was made apparent by the presence of GAD65-specific Th2 cytokine-producing T cells that were capable of inhibiting diabetogenic cells. Neutralization of Th2 effector cytokines in adoptive transfers blocked this protection, eliciting disease and revealing the regulatory capacity of the T cell-derived IL-4 and IL-10.

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[3] Abbreviations used in this paper: IDDM, insulin-dependent diabetes mellitus; NOD, nonobese diabetic; tg, transgenic; SI, stimulation index; SFC, spot-forming cells; ELISPOT, enzyme-linked immunospot.

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Materials and Methods

Mice

Female NOD/*Shi* and NOD-IL-4 transgenic (tg) mice were raised and maintained in our animal housing facility. NOD.scid mice were kept in specific pathogen-free quarters according to our institutional guidelines.

Proliferative responses

Splenocytes from 5–8-wk-old NOD and NOD-IL-4 mice were prepared as suspensions in HL-1 serum-free medium (Hycor, Irvine, CA). For assay, the cells were placed in 96-well plates with GAD65 peptides (7 μM), GAD65, or HSP65 used as Ags at a 20 μg/ml final concentration and cultured for 5 days at 37°C (28). For proliferative responsiveness, splenocytes, thymocytes, and lymph node cells (5×10^5/well) were stimulated nonspecifically with anti-CD3 Ab (145-2C11 at 5 μg/ml; PharMingen, San Diego, CA). [^{3}H]Thymidine was added during the last 18 h of culture. The stimulation index (SI) was determined by dividing total counts from wells with Ag by background counts from wells lacking Ag (29).

Cytokine, Ab, and spot-forming cell (SFC) analysis

For cytokine analysis, splenocytes were cultured in 24-well plates for 5 days with Ag. Expanded splenocytes were then harvested, purified over Ficoll gradients, and restimulated nonspecifically on plates coated with anti-CD3 Ab. Supernatants from these cultures were analyzed for IL-2, IL-4, IL-10, and IFN-γ. IFN-γ levels were determined using capture mAb R46A2 and biotinylated detection Ab XMG1.2 (PharMingen). IL-10 was quantitated using capture mAb and biotinylated detection Ab (PharMingen). IL-4 and IL-2 levels were measured in a bioassay using NK cells and the anti-IL-2 or anti-IL-4 Abs, respectively (28). The limit of detection in this system was between 2 and 5 pg/ml. Levels of IFN-γ in the pancreas were determined by ELISA analysis of the supernatants from frozen and homogenized pancreata from five NOD and NOD-IL-4 mice.

The relative frequency of Ag-specific T cells secreting IFN-γ or IL-4 was determined by using the enzyme-linked immunospot (ELISPOT) assay and then identifying SFCs. In brief, Ag-specific splenocytes expanded in bulk culture were added to a multiscreen-HA (MAHA S45; Millipore, Bedford, MA) plate that had been coated with cytokine capture Abs as above. Con A was added at 5 μg/ml, and plates were incubated for 24 h to quantify IFN-γ or 40 h for IL-4. After washing, biotinylated detection Abs were added, and the plates were incubated at 4°C overnight. Bound secondary Abs were visualized using HRPO-streptavidin (Dako, Carpinteria, CA) and 3-amino-9-ethylcarbazole. Abs R46A2/XMG1.2-biotin and 11B11/24G2-biotin (PharMingen) were used for capture and detection of IFN-γ and IL-4, respectively. Relative frequencies of cells producing IFN-γ and IL-4 were determined by counting SFCs for individual mice and expressed as SFC/10^6 GAD65-expanded lymphocytes. Results represent individual mice.

For detection of GAD65-specific Abs, 96-well plates were coated with GAD65 protein at 5 μg/ml in PBS. Wells were blocked with 1% BSA in PBS, and diluted serum from individual 8–10-wk-old mice was applied. Bound Abs were detected with appropriate HRP-conjugated goat anti-mouse Ig (PharMingen).

Adoptive transfers

Splenocytes from female NOD-IL-4 mice or their nondiabetic female NOD littermates were used directly or after GAD65 stimulation as donor cells for adoptive transfers. For the individual transfers, 15×10^6 cells from NOD or NOD-IL-4 mice were i.v. injected into 8- to 12- wk-old NOD.scid females. In cotransfer experiments, NOD.scid mice received $3–5 \times 10^6$ splenocytes from newly diagnosed diabetic female NOD mice plus splenocytes from female NOD-IL-4 mice or their nondiabetic female NOD littermates. Groups were either untreated or treated with 1 mg of anti-IL-4 or both anti-IL-4 (11B11; American Type Culture Collection (ATCC), Manassas, VA) and IL-10 (JES2A5; ATCC) mAb every other day for the 2 wk after transfer. Control groups received rat IgG (YCATE 55 (30)) only. The development of diabetes was monitored weekly by measuring blood glucose values.

Generation and characterization of GAD65-specific hybridomas

GAD65-specific T cell hybridomas were created by fusing spleen cells from NOD or NOD-IL-4 mice with those from the BW5147α^-/β^- cell line by following the protocol described earlier (31). Splenocytes were first stimulated in vitro in the presence of GAD65 and then expanded with rIL-2 (20 U/ml). GAD65-responsive hybridoma cells were identified by IL-2 production using the NK bioassay. Briefly, hybridoma cells were plated at 5×10^4 cells/well, along with 8×10^5 irradiated (1500 rad) NOD splenocytes, in the absence or presence of 20 μg/ml GAD65. After a 24-h incubation, culture supernatants were harvested and tested for IL-2. Hybridomas specific for GAD65 were analyzed by flow cytometry for TCR Vβ expression using a panel of labeled mAb specific for TCR Vβ and CD4 (PharMingen).

Histologic analysis

Lymphocytic infiltration of the islets was evaluated on hematoxylin and eosin-stained paraffin sections of the pancreas taken at several sites throughout the organ. Sections were scored for the presence of insulitis as follows: 0, absence of inflammation; 1, presence of peri-insulitis; 2, presence of insulitis; or 3, presence of severe insulitis that has destroyed islets (28). An insulitis score representing the overall severity of inflammation was derived by taking an average of the grade of insulitis.

Statistical analysis

Statistical analysis was determined by the χ^2, log-rank, or Student's *t* test, using Statview software (Abacus Concepts, Berkeley, CA) where appropriate.

Results

T lymphocytes from NOD-IL-4 mice are not tolerant to islet Ags

Although NOD-IL-4 mice are protected from the spontaneous insulitis and diabetes of their non-tg NOD counterparts, this protection was partially reversed by injecting diabetes-inducing cyclophosphamide (19). This finding suggests that islet Ag-specific T cells were present in vivo. Therefore, we tested NOD-IL-4 mice for the presence of autoreactive T cells in lymphocyte proliferation assays. As shown (Fig. 1), splenocytes from NOD and NOD-IL-4 mice 8 wk of age or older proliferated in response to GAD65 and HSP65, and responses were generally higher in NOD-IL-4 mice (Fig. 1*A*).

The responses were measured to previously described immunodominant determinants of GAD65 (32). The peptides GAD202–221 (p14) and GAD217–236 (p15) consistently stimulated T cells from both groups of mice (Fig. 1*A*). However, proliferative responses to GAD78–97 (p6) (not shown) or to the originally reported immunodominant GAD65 epitopes GAD247–263 (p17) and GAD509–526 (p34) (26) did not reach significant levels (i.e., <2 SI) (Fig. 1). When lymphocytes from pancreatic lymph nodes that drain the pancreas were also examined for their reactivity to islet Ags, responses resembled those of the splenocytes (not shown). Additionally, IL-2 was produced in the proliferation assays in response to HSP65 and GAD65 (Fig. 1*B*).

Predominant TCR Vβ 8.1 and/or 8.2 usage among GAD65-specific T cells in NOD and NOD-IL-4 mice

To determine whether protection from diabetes correlated with changes in the peripheral T cell repertoire, we compared TCR Vβ profiles and usage in protected NOD-IL-4 mice and non-tg NOD mice. Fig. 2 shows that both groups of mice had similar profiles of TCR Vβ used by CD4 T cells in the spleen, pancreatic lymph nodes, or mesenteric lymph nodes. The same was true for CD8 T cells (data not shown). The results are representative of 3 separate experiments. Next, to examine TCR Vβ usage by islet-Ag-specific T cells, T cell hybridomas specific for GAD65 were generated. Analysis of these hybridomas by flow cytometry revealed that Vβ 8.1 and/or 8.2 predominated in TCRs recognizing GAD65 of both NOD-IL-4 (five of seven hybridomas) and non-tg mice (four of eight hybridomas) (Table I). These results may reflect the preferential Vβ usage of T cells from NOD-IL-4 and NOD mice (Fig. 2) and/or the expansion of Vβ 8.1 and/or 8.2 GAD65-specific T cells during the generation of the hybridomas. Some of the GAD65-specific hybridomas produced IL-2 in the absence of GAD65, suggesting an inherent level of autoreactivity.

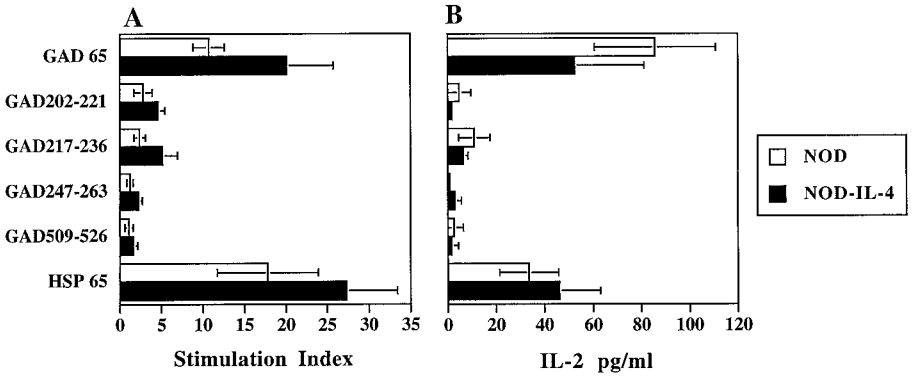

FIGURE 1. Islet Ag-responsive T cells exist in NOD-IL-4. Splenocytes from 8-wk-old female NOD or NOD-IL-4 mice were cultured for 5 days in the presence of GAD65, HSP65, or GAD65 peptides. Proliferation was determined by measuring the amount of [^{3}H]thymidine incorporated (*A*) over the last 18 h of culture and is expressed as the SI. Culture supernatants were sampled 48 h after Ag administration, and IL-2 levels (*B*) were measured in a NK-1 bioassay. Each group contained three mice stimulated individually with Ag. Error bars represent the SD. The background levels of lymphocyte proliferation for NOD-IL-4 and NOD mice were 3,470 and 10,327, respectively.

Pancreatic IL-4 expression restores IL-4-producing capacity in NOD T cells

An important similarity between human and murine IDDM is a thymic and peripheral T cell hyporesponsiveness following TCR stimulation, a factor contributing to the development of disease (25). This defect, coincident with the onset of insulitis, is mediated by reduced IL-2 and IL-4 production (22). Systemic treatment of NOD mice with IL-4 reverses this defect, resulting in a decreased incidence of diabetes (22, 25). To test whether pancreatic IL-4 also reversed this defect, thymic and peripheral lymphocytes from NOD and NOD-IL-4 female mice were stimulated with Con A or anti-CD3. Neither thymic nor splenic T cell proliferative responses nor IL-2 or IL-4 production profiles were altered by pancreatic expression of IL-4 in either group (not shown). We then examined responses of T lymphocytes in the draining lymph nodes of the pancreas for effects of pancreatic IL-4. Anti-CD3 stimulation resulted in elevated IL-2 and IL-4 responses by T cells from NOD-IL-4 mice compared with NOD mice (Fig. 3, *B* and *D*); however, proliferative and IFN-γ responses were unchanged (Fig. 3, *A* and *C*). Thus, pancreatic IL-4 seems to maintain the IL-4-producing capacity of T cells within the draining lymph nodes, cells that may be recent immigrants from the pancreas.

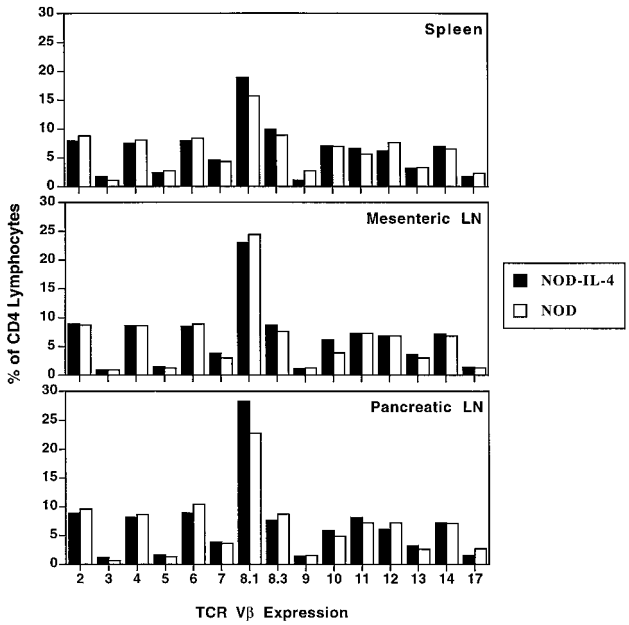

FIGURE 2. TCR Vβ T cell repertoire in NOD-IL-4 mice is similar to that in NOD mice. Lymphocytes from the spleen and mesenteric or pancreatic lymph nodes of 7-wk-old female mice were assessed by FACS for CD4 and TCR Vβ expression. Individual mice were evaluated, and results shown are representative of three separate experiments. Vβ 5 represents 5.1 and/or 5.2, and Vβ 8.1 represents 8.1 and/or 8.2.

Islet Ag-specific lymphocytes in NOD-IL-4 mice exhibit a Th2 phenotype

To test islet Ag-specific T cells from NOD-IL-4 mice for the acquisition of a Th2 phenotype, cytokine profiles of GAD65-specific T cells were analyzed. GAD65 is a major IDDM autoantigen, and modulation of responses to this Ag can dictate disease outcome (14, 21). First, supernatants of splenocytes cultured with GAD65 for 48 h were assessed for levels of IL-2 produced. NOD-IL-4 mice produced significantly lower amounts of IL-2 per stimulation unit than their non-tg littermates (3.4 $\pm$ 1.9 vs 12.7 $\pm$ 6.8, respectively; $p \leq 0.005$) (Fig. 4, *A* and *C*). Second, amounts of the Th1

Table I. *Predominant usage of Vβ 8.1/8.2 TCR by GAD65-specific T cells*[a]

T Cell Hybridoma	TCR Vβ Expression	Thymidine Uptake	
		No Ag	GAD65
NOD-IL-4			
1F12	8.1, 8.2	3,768	20,092
1B5	8.1, 8.2	32,089	62,198
3E5	8.1, 8.2	20,822	72,054
1D6	8.1, 8.2	2,624	45,908
3B4	8.1, 8.2	3,728	107,055
3D11	4	22,447	56,550
2B4	5.1, 5.2	11,339	60,023
NOD			
6G2	8.1, 8.2	931	107,261
7F7	8.1, 8.2	5,313	32,816
7C4	8.1, 8.2	3,754	25,545
8B9	8.1, 8.2	1,638	36,834
7D1	8.3	10,537	56,361
6G5	8.3	1,110	87,419
6H7	10b	2,865	33,742
8B8	4	7,920	117,082

[a] IL-2 secretion by T cell hybridomas upon 24-h incubation with GAD65. The TCR Vβ expression by the respective hybridoma was determined by FACS.

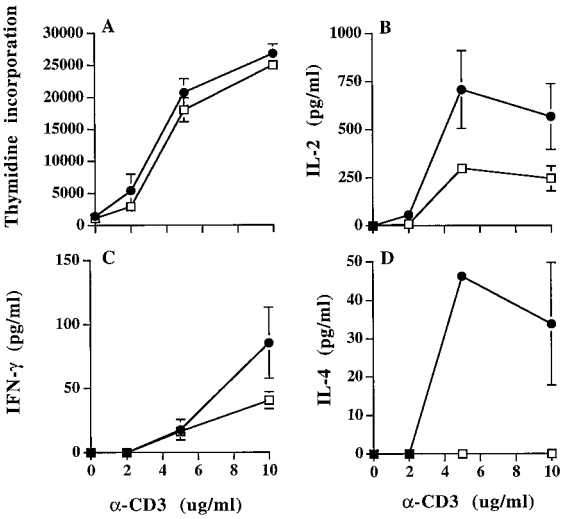

FIGURE 3. Pancreatic IL-4 restores cytokine production to lymphocytes draining the pancreas. Lymphocytes from the pancreatic lymph nodes of three NOD (□) or NOD-IL-4 (●) mice were pooled and stimulated in triplicate with anti-CD3. Culture supernatants were analyzed for proliferation (*A*), IL-2 (*B*), IFN-γ (*C*), and IL-4 (*D*) after 48 h of stimulation.

(IFN-γ) and Th2 (IL-4) cytokines were measured after splenocytes were stimulated with GAD65 for 5 days and then restimulated nonspecifically with anti-CD3 mAb for 48 h. With this protocol, splenocytes from NOD-IL-4 mice produced significantly higher levels of IL-4 than the non-tg group (6780 ± 3100 pg/ml vs 1921 ± 1116 pg/ml, respectively; $p \leq 0.002$) (Fig. 4*B*), but the amounts of IFN-γ were similar in both groups (4716 ± 2021 pg/ml vs 5703 ± 3409 pg/ml). Thus, the ratios of IL-4 to IFN-γ were significantly higher in NOD-IL-4 mice (1.6 ± 0.9 vs 0.45 ± 0.3 for non-tg mice; $p \leq 0.02$), indicating a preferential islet-specific Th2 response. More importantly, this preference increased by the time mice reached 8 wk of age (Fig. 4*D*). The responses to HSP65 were similar (not shown). The levels of IL-10 produced from splenocytes that were stimulated with GAD65 and subsequently with anti-CD3 were also significantly higher in NOD-IL-4 mice (299 ± 25 pg/ml vs 102 ± 26 pg/ml for non-tg littermates; $p \leq 0.001$), also suggesting the maintenance of a Th2 phenotype.

To examine the phenotypes of individual islet-specific lymphocytes, cytokines produced by GAD65-expanded splenocytes were examined by ELISPOT. The consequent islet Ag-specific responses in NOD-IL-4 mice were dominated by IL-4-producing lymphocytes (Fig. 4*E*). NOD-IL-4 mice produced fewer GAD65-specific IFN-γ-producing lymphocytes but not to a significant extent. Overall, the relative frequency of IL-4- to IFN-γ-producing lymphocytes was 25 times higher in NOD-IL-4 mice, denoting a Th2-dominated autoimmune repertoire characterized by decreased IL-2 expression and increased numbers of IL-4- and IL-10-producing islet-Ag-specific T cells. In line with this, the levels of IFN-γ in the pancreas were similar in NOD-IL-4 mice and their non-tg littermates (Fig. 5).

Because Th1 and Th2 cells differentially influence the development of IgG Ab subclasses, subclass analysis of GAD65-specific Abs was determined. Even though quantities of total Ig specific for GAD65 were comparable in NOD-IL-4 mice and their non-tg littermates (not shown), measurement of serum IgG1, IgG2a, IgG2b, and IgG3 Abs specific for GAD65 determined that amounts of all IgG subclass anti-GAD65 Abs, except IgG2a, were higher in NOD-IL-4 mice than in non-tg littermates (Fig. 6), correlating with the Th2-dominated autoimmune response of the former to islet Ags.

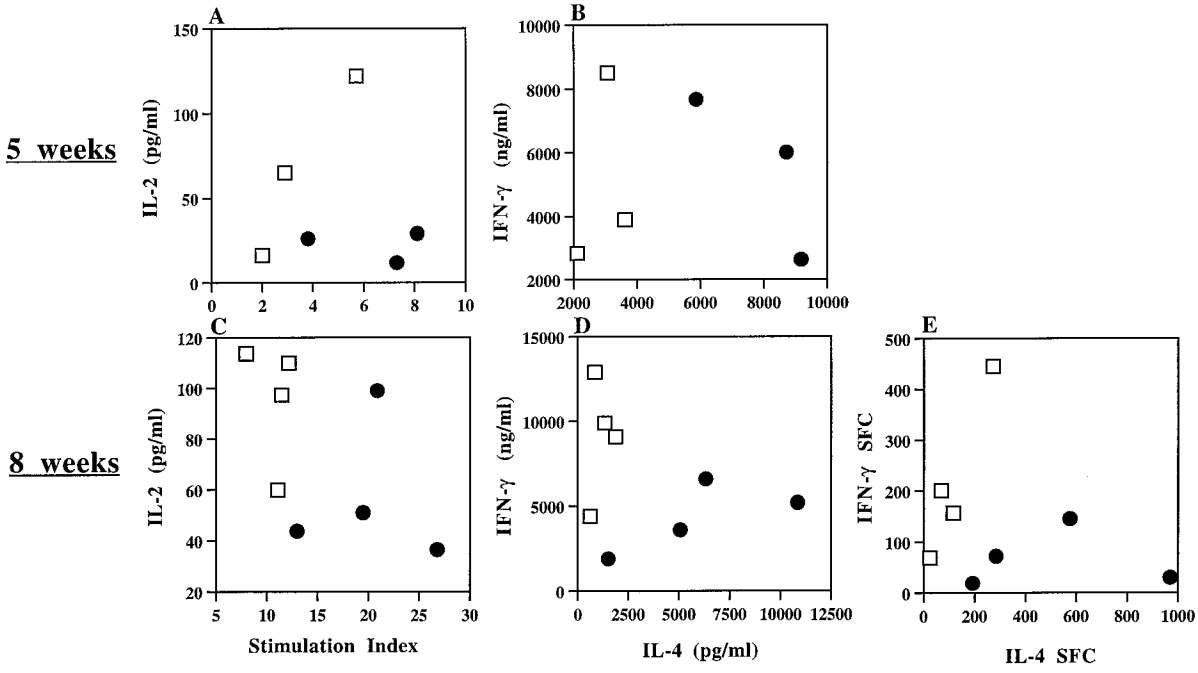

FIGURE 4. GAD65-specific T cell responses from NOD-IL-4 mice display a Th2 phenotype. Splenocytes from individual female NOD (□) and NOD-IL-4 (●) mice at 5 (*A* and *B*) and 8 (*C* and *D*) wk of age were cultured with GAD65. Proliferation (*A*) and IL-2 (*C*) production were determined as described in Fig. 1. To assess the cytokines produced from GAD65-specific T cells, GAD65 expanded splenocytes were stimulated nonspecifically through the TCR complex using anti-CD3 mAb. Culture supernatants were evaluated for IFN-γ by ELISA (*B*) and IL-4 (*D*) in a bioassay at 48 and 72 h, respectively. The number of individual GAD65 expanded T cells producing IL-4 or IFN-γ was determined by ELISPOT (*E*) and expressed as SFC.

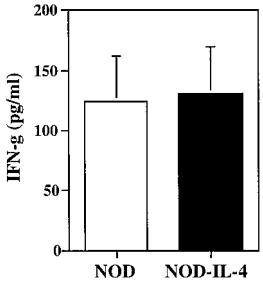

FIGURE 5. Intrapancreatic levels of IFN-γ are similar in NOD and NOD-IL-4 mice. The pancreata from five NOD or NOD-IL-4 mice were freshly frozen, homogenized, and assessed for IFN-γ levels by ELISA. Error bars represent the SD.

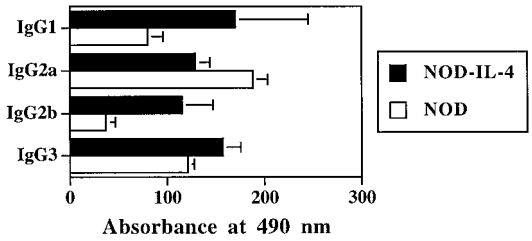

FIGURE 6. Anti-GAD65 Ab responses in NOD-IL-4 mice display a Th2 profile. Sera from five NOD and NOD-IL-4 mice (8–10 wk old) were analyzed individually by ELISA for IgG subclasses specific for GAD65. Error bars represent the SD between individual mice.

NOD-IL-4 T cells inhibit the development of insulitis

NOD-IL-4 mice do not develop insulitis or diabetes, despite their islet Ag-specific T cells. Additionally, despite a strong Th2 response, these mice do have potentially diabetogenic Th1 cells, and cyclophosphamide treatment may enhance their activity (19). Therefore, we asked whether the absence of disease derived from the ability of splenocytes from NOD-IL-4 to inhibit diabetogenic T cells. To address this question, infiltration and destruction of islets were documented after adoptive transfer of T cells from recently diabetic mice into NOD and NOD-IL-4 recipients. By 7 days posttransfer, diabetic splenocytes had infiltrated and initiated islet destruction in NOD mice, whereas NOD-IL-4 recipients had primarily peri-insulitis and overall a significantly reduced level of insulitis ($p \leq 0.0001$) (Table II). Because T cell responses to GAD65 are believed to directly contribute to diabetes (26), GAD65-stimulated splenocytes from NOD mice were similarly transferred into NOD and NOD-IL-4 mice. Again, NOD mice developed severe insulitis and islet destruction, but NOD-IL-4 mice remained significantly ($p \leq 0.0001$) resistant at day 7 (Table II).

Next, we tested whether the inability of diabetogenic T cells to initiate insulitis in NOD-IL-4 mice was related to a regulatory effect of systemic NOD-IL-4 splenocytes. Splenocytes from recently diabetic mice were cotransferred with GAD65-stimulated or unstimulated NOD or NOD-IL-4 splenocytes into NOD.scid mice at a ratio of 1 to 2, respectively. By 7 days posttransfer, recipients of diabetic and NOD-IL-4 splenocytes showed significantly ($p \leq 0.01$) reduced levels of insulitis compared with recipients of diabetic and NOD splenocytes (Table II). Similarly, when diabetic splenocytes were cotransferred with GAD65-stimulated splenocytes from NOD-IL-4 mice, insulitis was significantly ($p \leq 0.0001$) reduced compared with recipients of GAD65-stimulated NOD splenocytes. These observations suggest that T cells in NOD-IL-4 mice regulate diabetes by impeding the accumulation of diabetogenic T cells in the islet.

The Th2 phenotype is responsible for regulating diabetogenic T cells

Because lymphocytes in NOD-IL-4 mice displayed an islet Ag-specific Th2 phenotype, we reasoned that Th2 cytokines might regulate potentially diabetogenic T cells. When we adoptively transferred NOD-IL-4 splenocytes into NOD.scid mice coincident with neutralization of Th2 (IL-4 and IL-10) cytokines and monitored the animals weekly, none of the anti-IL-4-treated mice developed diabetes over a 15-wk period. In contrast, mice receiving a combination of anti-IL-4 and anti-IL-10 mAb developed diabetes by 10 wk of age (Table III). These results confirm that T cells with diabetogenic potential are present in NOD-IL-4 mice. Moreover, control of these T cells in NOD-IL-4 mice is dependent on the Th2 cytokines IL-4 and IL-10.

Finally, to learn whether Th2 cytokines were also capable of regulating splenocytes from recently diabetic NOD mice (see Table II), splenocytes from that source were cotransferred with NOD or NOD-IL-4 splenocytes at 1:5 ratios into NOD.scid recipients. The results in Table III show that splenocytes from NOD-IL-4 mice significantly ($p \leq 0.05$) delayed the onset of IDDM induced by diabetogenic splenocytes. NOD.scid mice receiving the cotransfers were then treated either with anti-IL-4 alone or with a combination of anti-IL-4 and anti-IL-10 mAbs. Both treatments

Table II. *NOD-IL-4 splenocytes inhibit the development of insulitis*[a]

Group	Donor Splenocytes From	Recipients	Total No. of Islets/ Group	Grade of Insulitis (%)				Insulitis Score
				0	1	2	3	
A	Diabetic	NOD	77	12	39	26	23	1.6
	Diabetic	NOD-IL-4	48	65	29	6	0	0.41*
B	GAD65-stimulated	NOD	75	15	12	32	41	2.0
	GAD65-stimulated	NOD-IL-4	89	69	19	11	1	0.45*
C	Diabetic + NOD	NOD.scid	48	15	29	45	11	1.5
	Diabetic + NOD-IL-4	NOD-scid	88	48	33	19	0	0.71**
D	GAD65-stimulated-NOD + diabetic	NOD.scid	41	18	24	35	23	1.6
	GAD65-stimulated-NOD-IL-4 + diabetic	NOD.scid	47	69	19	13	0	0.45**

[a] Pancreatic islets were scored for insulitis by hematoxylin and eosin staining 7 days following adoptive transfer of 5×10^6 recently diabetic NOD splenocytes (group A); 1.5×10^7 GAD65 stimulated NOD splenocytes (group B); 3×10^6 diabetic plus 1.5×10^7 NOD or NOD-IL-4 (group C); or 1×10^7 GAD65 stimulated plus 5×10^6 recently diabetic NOD splenocytes (group D).

* $p < 0.0001$; ** $p < 0.01$ (χ^2 test).

Table III. *Th2 cytokines are responsible for regulating diabetogenic T cells in NOD-IL-4 mice*[a]

Donor Splenocytes			Incidence of Diabetes (at wks)				
Nondiabetic	Diabetic	Ab Treatment	6	7	8	9	10
NOD-IL-4	—	Control	0/3	0/3	0/3	0/3	0/3
NOD-IL-4	—	α-IL-4	0/3	0/3	0/3	0/3	0/3
NOD-IL-4	—	α-IL-4/α-IL-10	0/3	1/3	1/3	3/3	—
NOD	NOD	Control	2/7	5/7	7/7	—	—
NOD-IL-4	NOD	Control	0/4	0/4	3/4	3/4	4/4*
NOD-IL-4	NOD	α-IL-4	0/5	3/5	5/5	—	—
NOD-IL-4	NOD	α-IL-4/α-IL-10	1/4	2/4	4/4	—	—

[a] Splenocytes (15×10^6) from 8 to 10-wk-old nondiabetic NOD-IL-4 or NOD mice were adoptively transferred into NOD.scid mice, with or without splenocytes (3×10^6) from recently diabetic NOD mice. Groups of mice were treated every other day for 2 wk following transfer with α-IL-4 (11B11) and/or α-IL-10 (JES2A5) or control IgG Ab. Mice were scored as diabetic when blood glucose values reached greater than 300 mg/dL. The anti-IL-4 mAb (11B11) were found effective in transplantation model (30) and anti-IL-10mAb (JES2A5) was found effective in autoimmune diabetes (50).

* $p < 0.05$ (log-rank test).

abolished the delay in diabetes observed when NOD-IL-4 splenocytes were cotransferred along with diabetogenic splenocytes (Table III). Thus, cells in NOD-IL-4 mice inhibited the pathogenicity of diabetogenic T cells, and the Th2 cytokine IL-4 was essential for this process.

Discussion

As demonstrated here, pancreatic expression of IL-4 promotes the establishment of immunoregulatory islet Ag-specific Th2 cells. Recognition of an individual islet Ag (GAD65) by T cells from tg mice of which the pancreata make IL-4 showed mostly TCR Vβ 8.1 and/or 8.2 usage but was no different from that observed in non-tg NOD mice. These T lymphocytes in NOD-IL-4 mice forestalled diabetes and inhibited insulitis, and the Th2 cytokines IL-4 and IL-10 were critical for regulating and maintaining this nonpathologic state.

Previously, we showed that IL-4 expression in pancreatic β cells completely prevented diabetes in NOD mice despite the presence of autoreactivity (18, 19). Here we found further that IL-4 expression in β cells induced functionally active islet Ag-specific Th2 cells. Moreover, as in NOD mice, the T cells in NOD-IL-4 mice recognized the islet Ags GAD65 and HSP65. Responses to the previously described T cell determinants of GAD65 (32), i.e., GAD202–221 and GAD217–236, were also similar to those seen in NOD mice, showing that the failure to recognize at least these dominant epitopes was not responsible for immune protection in this situation. Thus, although it appears that dominant determinant recognition was unchanged in our NOD-IL-4 mice, only extensive epitope specificities of T cells will determine whether IL-4 induces T cell responses to additional epitopes.

According to more than one report, systemic administration of IL-4 limits insulitis and IDDM by reversing CD4 T cell hyporesponsiveness and potentiating Th2 cell function (17, 22, 33). When we re-examined this issue in NOD-IL-4 mice, no reversal in hyporesponsiveness of thymic or splenic T cells was found. This may not be unexpected, considering the pancreatic source of IL-4. However, lymphocytes in the draining pancreatic lymph nodes of NOD-IL-4 mice did produce more IL-4 and IL-2 after TCR stimulation than those of their non-tg littermates. This conclusion supports the contention that pancreatic IL-4 also corrects an inherent defect in NOD mice by promoting the generation of Th2 responses (17).

Next, we analyzed cytokine expression to establish whether islet Ag-specific Th2 cell responses were maintained in NOD-IL-4 mice. IL-2 levels were found to be lower, and IL-4 and IL-10 cytokines dominated the lymphocyte responses to GAD65 in NOD-IL-4 mice. There was also a significant increase in the numbers of GAD65-specific IL-4-producing T cells. However, IFN-γ responses and pancreatic IFN-γ levels were unchanged from those of their non-tg counterparts, suggesting that the protection from diabetes in NOD-IL-4 mice did not simply result from decreased numbers of effector T cells. The maintenance of a Th2 response to islet Ag is consistent with findings by Cameron et al. (17), which showed IL-4 production from islet-infiltrating cells of IL-4-treated mice, as well as our recent observations showing Th2-biased HSP65 and GAD65 responses in diabetes-resistant recipients of HIV-IL-4-transduced islet grafts (28). Additionally, GAD65-specific Ab isotype profiles in NOD-IL-4 mice supported the association of protection with a Th2-biased response, as previously reported after islet Ag (GAD65)-induced tolerance (14, 34–36). Subsistence of autoimmune Th2 T cells in these mice is likely due to tg IL-4 imparting an increased capacity of T cells to produce IL-4 (17), supporting Th2 T cell development during Ag priming in the draining lymph nodes (37). However, the fact that IFN-γ responses were undiminished indicates the development of a diverse autoimmune T cell repertoire in NOD-IL-4 mice. Regardless, these results indicate that the Th1 phenotype that is causative of disease in humans and NOD mice (38) can be counterregulated by the local expression of IL-4, leading to the maintenance of a balanced Th1/Th2 repertoire that regulates disease.

The development of GAD65-specific Th2 cells in NOD-IL-4 and NOD mice was characterized by the high recovery of Vβ 8.1 and/or 8.2 TCR-bearing hybridomas. Analysis of more GAD65-specific T cell hybridomas from NOD-IL-4 splenocytes would substantiate this notion. This finding was not surprising, considering the fact that Vβ 8-bearing T cells constitute ~25% of the NOD T cell repertoire. Surprisingly, some but not all of these hybridomas displayed spontaneous autoreactivity as well as specificity for GAD65, which is suggestive of different lineages. This spontaneous autoreactivity may be related to the inherent property of islet Ag-specific NOD T cells that can respond to I-Ag7 class II MHC molecules devoid of peptide, as suggested earlier (39). Interestingly, we previously found that double-tg mice expressing the BDC2.5 Vβ4 TCR (recognizing an as-yet-unidentified islet Ag) and IL-4 rapidly developed diabetes (19). In addition, Th2 cells specific for islet Ag or myelin basic protein caused diabetes or experimental autoimmune encephalomyelitis in immunodeficient hosts via IL-10-dependent pathway (16, 40). Thus, the presence of restricted populations of Th2 cells can actually contribute to disease rather than prevent it, indicating that populations of lymphocytes with additional specificities are required for protection.

In examining the mechanism of regulation in NOD-IL-4 mice, the dominant protective effect of T cells was found to be abolished through neutralization of Th2 cytokines. Although no disease developed when NOD.scid recipients of NOD-IL-4 splenocytes were treated with anti-IL-4 alone, within 10 wk of combined anti-IL-10 and anti-IL-4 treatment, the mice developed diabetes. Thus, potentially diabetogenic (Th1) T cells in NOD-IL-4 mice do have the capacity to cause full-blown diabetes but are apparently regulated in vivo by cytokines produced from pancreatic IL-4-generated islet Ag-specific Th2 cells. To substantiate the regulatory ability of NOD-IL-4 splenocytes, we performed cotransfers with diabetogenic lymphocytes. Splenocytes from NOD-IL-4 mice were able to significantly delay the development of diabetes caused by diabetogenic lymphocytes following cotransfer into NOD.scid mice. This delay was due to the ability of NOD-IL-4 splenocytes to inhibit insulitis, an effect that was abrogated by treatment with anti-IL-4 Ab. Thus, IL-4 plays an essential role in inhibiting disease-causing lymphocytes, a finding consistent with that of Seddon and Mason (41), who have recently shown that IL-4 is required by regulatory cells to block disease in a CD8-independent model of autoimmune thyroiditis. The mode of action by which IL-4 controls diabetogenic T cells is unclear but may be due to TGF-β (24, 42), which is secreted by activated T cells in the presence of IL-4 (43). Although IL-4 appears sufficient to regulate diabetogenic lymphocytes, complete regulation of potentially diabetogenic Th1 T cells in NOD-IL-4 mice appears to also require IL-10. A reasonable explanation may be that the induction of diabetes by splenocytes from recently diabetic mice is largely mediated by activated CD4 T cells, whereas in NOD-IL-4 mice the participation of both CD8 and CD4 T cells may be required to reach a sufficient level of damage to initiate disease. In support is the fact that IL-10 therapy can block the development of diabetes in mice older than 5 wk (44). We suggest that IL-4 may be directly involved in inhibiting disease-causing lymphocytes, whereas IL-10 limits the activation of potentially diabetogenic CD8 T cells. Although future experiments would resolve this issue, it is important to note that neonatal expression of the IL-10 transgene in the islets of pancreas in NOD mice accelerated their diabetes via CD8 T cell-dependent pathway, circumventing the requirement for CD4 T cells and B cells (29). Therefore, depending on the timing, concentration, and site of IL-10 expression, this cytokine functions as an immunostimulatory or immunosuppressive molecule in autoimmune diabetes of NOD mice.

We have shown here that islet expression of IL-4 in NOD mice confers complete protection via Th2 immune deviation. However, disruption of the IL-4 gene in NOD mice did not accelerate diabetes (45). This apparent lack of effect of the IL-4 gene disruption on IDDM may reflect already low levels of endogenous IL-4 that are produced by lymphocytes in wild-type NOD mice. These basal levels may normally be insufficient to generate islet-specific Th2 cells that regulate IDDM in NOD mice, unless IL-4 secretion is induced following GAD65 treatment (21, 35, 36), tg expression of IL-4 in the islets (current study and Ref. 18), or i.p. injection of exogenous IL-4 (17). Similar to IL-4-deficient NOD mice, IL-10-deficient NOD mice also fail to develop accelerated diabetes. These mice succumb to cyclophosphamide-induced diabetes (B. Balasa and N. Sarvetnick, unpublished observations). Interestingly, when the IFN-γ gene was disrupted in NOD mice (IFN-γ-deficient NOD mice), the mice readily developed diabetes, albeit with a slower onset (46). This apparent lack of effect on NOD diabetes in mice deficient in IFN-γ (46) may be due to the fact that these mice are still capable of generating Th1 cells under the influence of IL-12. This proposed compensatory mechanism might substitute for the apparent absence of IFN-γ in IFN-γ-deleted NOD mice. The study of mice deficient for IFN-γ and IL-12 genes would resolve the role of Th1 subset on spontaneous diabetes of NOD mice.

The ability of IL-4 to allow the development of functional tolerance is reminiscent of recent studies demonstrating that the lack of disease in NOD mice after Ag-induced tolerance is associated with the development of a Th2 autoimmune environment (14, 21, 36, 47–49) and the capacity to regulate diabetogenic T cells (5, 35). The fact that potentially diabetogenic Th1 T cells were still present in NOD-IL-4 mice indicated that immune deviation to a Th2 phenotype was not solely responsible for the absence of disease and signaled that Th1 T cells were actively regulated in vivo. We found that the Th2 cytokines IL-4 and IL-10 were responsible for this regulation, with IL-4 being sufficient to block diabetogenic T cells in the presence of a diverse autoimmune response. Establishing that regulation of diabetogenic T cells is an inherent property of an IL-4-shaped Th2 autoimmune response has the potential for providing a route of immunotherapeutic intervention in the diabetic process.

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