Interleukin-4 Deficiency Does Not Exacerbate Disease in NOD Mice

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To investigate the role of interleukin (IL)-4 in the regulation of autoimmune diabetes, we crossed the IL-4 knock-out mutation onto the NOD genetic background. This experiment was accelerated by typing for microsatellites linked to known diabetes susceptibility *(Idd)* loci, and included a control backcross of the wild-type 129/SvJ-derived IL-4 gene, the original target locus. We also crossed the mutation into the BDC2.5 transgenic line, a diabetes model that carries the rearranged T-cell receptor genes from a diabetogenic T-cell clone. The IL-4–null mutation did not accelerate or intensify insulitis in regular NOD mice or in the BDC2.5 transgenic model; it also had no effect on the timing or frequency of the transition to overt diabetes. These data indicate that IL-4 plays no required role in controlling the aggressiveness of murine diabetes. *Diabetes* 47:1207–1211, 1998

Immunoregulation, or its perturbation, is central to the pathogenesis of autoimmune diabetes. First, the malfunctioning of regulatory systems allows T-cells reactive to pancreatic islet β-cells to be generated in the thymus, and to persist and eventually expand in the periphery. Second, regulatory influences keep this potentially autoaggressive repertoire in check for some time, but they eventually break down and lose control; β-cell–reactive T-cells exist early on in prediabetic patients and NOD mice, and even insulitis is tolerated for a prolonged period, implying that the self-reactive T-cells are somehow prevented from immediately destroying their targets, even though they are in immediate contact with them, but that they eventually become destructive, after which diabetes ensues.

The profile of cytokines produced locally in the insulitic lesion is probably key to the unfolding of pathogenesis (1–3). Cytokines can have both regulatory and directly toxic influences; more generally, they reflect the balance of T-cell subsets present in the lesion. The relative contribution of CD4+ cells of the T-helper (Th)1 and Th2 phenotypes is thought to be of prime relevance, with the former being the more destructive and the latter often being invoked in a protective context.

Interleukin (IL)-4 is the prototypical Th2 cytokine. It favors humoral responses, particularly of the IgG1 and IgE isotypes,

by promoting the generation of Th2 cells and inhibiting differentiation along the Th1 pathway (4). It also has been implicated in the control of autoimmune diabetes (5–7); treatment of NOD mice with recombinant IL-4 prevented the evolution of insulitis to diabetes (5), and local expression of IL-4 in β-cells even blocked insulitis (8). Lymphocytes from NOD mice, in particular from females, have been reported to produce lower levels of IL-4 than those from some other inbred strains (5,9,10), perhaps because of a reduced population of a particular T-cell subset expressing natural-killer cell markers (9,11). It has been proposed that this deficiency is also key to diabetes development in humans (12).

Thus it seemed of interest to evaluate the effect of completely eliminating IL-4 on the disease pathogenesis in NOD mice by introducing the IL-4–null mutation onto the NOD genetic background. Would the loss of this regulatory cytokine, or the allied perturbation in Th1/Th2 balance, compromise immunoregulation and promote a raging diabetes?

RESEARCH DESIGN AND METHODS

Mice. The NOD/lt line used as a genetic background was originally obtained from Dr. E. Leiter (The Jackson Laboratory, Bar Harbor, ME) and was maintained in our facility by continued intercross. The BDC2.5 T-cell receptor (TCR) transgenic line has been previously described (13); in the crosses described here, transgenic animals used as breeders were at the 17th backcross to NOD. Mice carrying the IL-4–null mutation (14) were provided by Drs. R. Kuhn, W. Müller, and K. Rajewsky (Köln, Germany). In the Strasbourg facility, they were crossed with NOD mice for up to nine generations. At the third generation, to improve the efficiency of the backcross, we selected breeder animals that were homozygous for the NOD-derived alleles at the H-2 complex (by flow cytometry on blood lymphocytes, staining for H-2^b) and at *Idd3* and *Idd10* (according to simple sequence length polymorphism [SSLP], with primers for *D3Mit11* and *D3Nds11*, respectively) (15,16). Intercrosses to produce experimental animals were set up at the fourth and eighth generations (see figure legends). The IL-4–null mutation was followed in heterozygotes by Southern blots (*Eco*RI digests of genomic DNA, probed with a 0.9 kb *BamHI/KpnI* fragment from plasmid pT2-IL-4, revealing a wild-type band at 10.4 kb and a mutant band at 6.7 kb) (14). The intercrosses generated mice of three phenotypes: homozygous-mutant –/– as well as –/+ and +/+ controls.

Control animals carrying the 129 alleles of the wild-type IL-4 locus were generated by similar backcrosses, starting from 129/SvJ × C57Bl/6 (B6) mice and backcrossing repeatedly to NOD/lt, following the segregation of the normal loci by microsatellite SSLP analysis for the IL-4 locus, with primers for *D11Mit208* (Research Genetics, Huntsville, AL) at 33.0 cM on chromosome 11, linked to the IL-4 locus at 28.0 cM (Mouse Genome Database, release 3.2, www.informatics. jax.org/mgd). After several backcrosses (see figure legends), these control mice were intercrossed to generate animals carrying NOD/NOD, NOD/129, or 129/129 genotypes at the IL-4 locus.

Insulitis and diabetes. Insulitis was evaluated on hematoxylin/eosin-stained paraffin sections of pancreas, taken at several levels throughout the organ, as previously described (17). Fifty independent islets were scored for each mouse. For insulitis on the straight NOD background, pancreases were dissected at age 12 weeks; for insulitis in BDC2.5 TCR transgenic mice, they were dissected at age 5 weeks. Diabetes was monitored by the presence of glucose in urine (Uristix; Bayer Diagnostics, Mulgrave, Australia), with confirmation by blood glucose measurements (Glucofilm strips read in a Glucometer 3; Bayer Diagnostics). Animals were considered diabetic when they were positive by urine and with two consecutive blood glucose measurements >250 mg/dl.

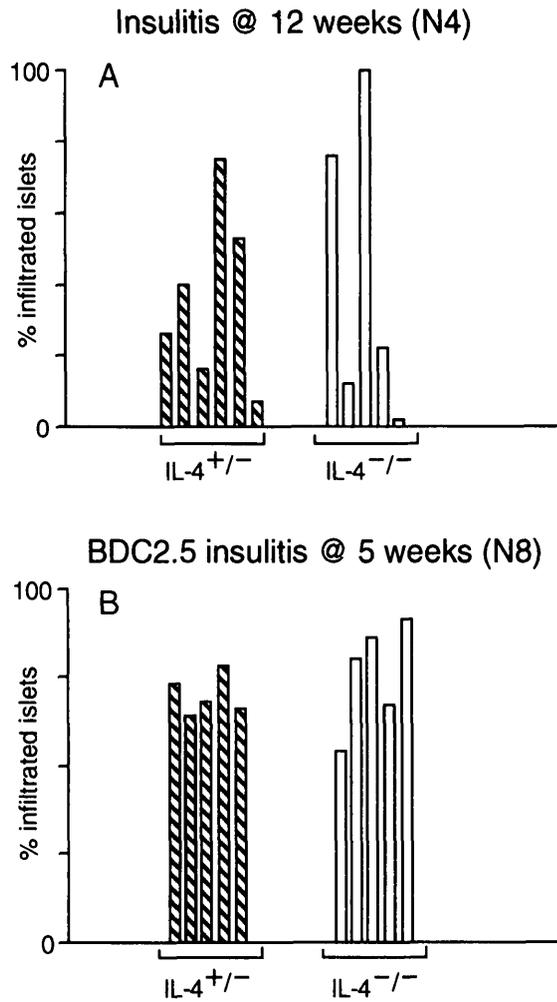## Insulitis @ 12 weeks (N4)

BDC2.5 insulitis @ 5 weeks (N8)

FIG. 1. Insulitis in IL-4–null and control animals. A: Insulitis in NOD mice at the fourth generation of backcross, either homozygous IL-4–null mutants or heterozygous controls. Pancreases were examined histologically at age 12 weeks, and insulitis was scored on several sections for each mouse. Each bar represents an individual mouse and shows the percentage of islets displaying infiltration. B: Mice carrying the BDC2.5 TCR transgene and heterozygous or homozygous for the IL-4–null mutation. Insulitis was scored as in A but at age 5 weeks because insulitis progresses faster in transgenic mice.

Circulating immunoglobulins. Levels of serum immunoglobulins, in the absence of immunization, were determined by a sandwich enzyme-linked immunosorbent assay (ELISA), as previously described (18), with polyclonal isotype-specific reagents (Southern Biotechnology, Birmingham, AL). These reagents efficiently detect the NOD alleles of IgG1 and IgG2a, as the levels measured were comparable with those of BALB/c serum.

Cytokine production. Cytokine production by lymph node cells from mutant mice and control littermates was assessed essentially according to Kopf et al. (19). Briefly, 10^6 lymph node CD4$^+$ T-cells (95% pure after magnetic depletion of B- and CD8$^+$ T-cells) were stimulated with plate-bound anti-$\alpha\beta$ TCR (coated with purified monoclonal antibody H57–597 at 10 µg/ml) in 1 ml of Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 50 µmol/l 2-mercaptoethanol, and IL-2 (100 U/ml). In some wells, IL-4 (40 U/ml) was added in addition to IL-2. After this initial culture, the cells were washed and restimulated in vitro by plate-bound anti-$\alpha\beta$ TCR for 24 h in the presence of IL-2 (100 U/ml). Supernatants were collected and cytokine levels determined by ELISA, as described (20).

RESULTS

The IL-4 knock-out mutation replaced the first two exons of the IL-4 gene with a neomycin-resistance expression cassette, resulting in a total inability to produce IL-4 in homozygous-mutant animals (14). It was originally generated in
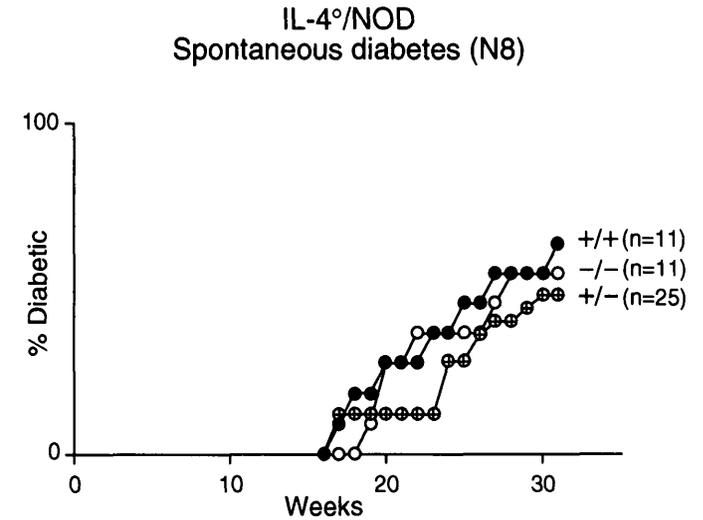
IL-4°/NOD
Spontaneous diabetes (N8)

FIG. 2. Diabetes in IL-4–null and control animals. After eight generations of backcrossing the IL-4–null mutation onto the NOD background, a large cohort of littermates was produced by intercrossing; mice of the three resulting genotypes were tested for diabetes.

embryonic stem cells derived from the 129/SvJ mouse strain, and was then crossed onto a mixed 129 × B6 F2 genetic background (14). We repetitively crossed the IL-4-null mutation onto the NOD/lt background, selecting for the mutant allele by Southern blotting at each generation. We also enhanced the efficiency of the backcross by typing third-generation mice by flow cytometry and SSLP, and selecting those that carried only NOD alleles at the major histocompatibility complex (MHC) and the Idd3 and Idd10 loci, the latter two being the non-MHC loci known to have the most profound influence on diabetes development (15,16). Heterozygous-mutant mice were intercrossed at different stages of the backcross and evaluated for various disease parameters. We first examined the incidence of insulitis in 12-week-old homozygous mutants and controls in an intercross at four generations of NOD backcross. As illustrated in Fig. 1A, insulitis was similar in the two types of animals, with no evident increase in severity in the homozygous mutants. In addition, the histological appearance of the islet infiltrates was comparable in the two cases (not shown).

We complemented our assessment of the mutation's effect on insulitis in straight NOD mice by examining BDC2.5 TCR transgenic animals (13) carrying the IL-4-null mutation. The transgenic line has a predominance of T-cells expressing a β-cell-specific diabetogenic TCR (21), promoting early insulitis but delayed diabetes when added onto the NOD genetic background (22). Equally strong insulitis was detected at an early age in the mutant and control BDC2.5 transgenics (Fig. 1B). Thus in both contexts in which we introduced the mutation, insulitis was present and was not notably enhanced.

Next, diabetes incidence was compared in mutant and control NOD animals intercrossed after several more generations of backcross (Fig. 2). The curves of diabetes incidence were superimposable for mice of all three genotypes. Neither the timing nor the penetrance of diabetes was affected by the IL-4-null mutation. Similarly, introduction of the mutation into the BDC2.5 TCR transgenic line failed to induce particularly early or frequent diabetes (data not shown). This latter finding should be viewed in the context of previous
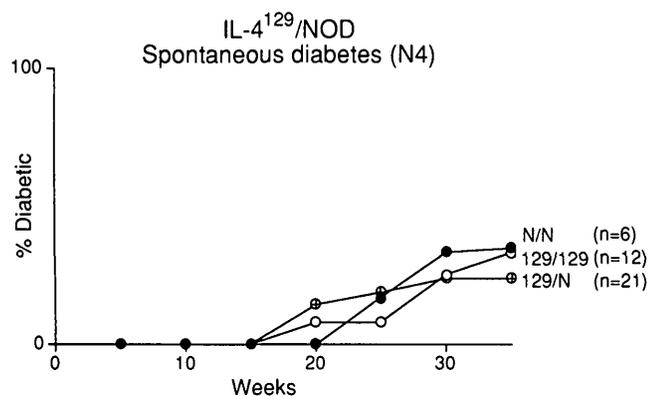
IL-4^{129}/NOD Spontaneous diabetes (N4)

N/N (n=6)
129/129 (n=12)
129/N (n=21)

FIG. 3. Diabetes in an IL-4^{129} control line. After four generations of backcrossing the IL-4 locus from the 129 strain onto the NOD background, a large cohort of littermates was produced by intercrossing; mice of the three resulting genotypes were tested for diabetes.

results establishing that it is possible to exacerbate disease dramatically in BDC2.5 animals by either genetic manipulation or drug treatment. (22–24).

Despite the mutation's apparent lack of effect, we felt it important to compare disease parameters in a control line constructed by backcrossing the wild-type IL-4 locus from the 129 strain onto the NOD background. This was to avoid the pitfall seen with many knock-out backcross experiments—the attribution of a certain effect to an engineered mutation when, in fact, it is due to a linked gene carried along during the backcross (see 25,26). It was conceivable that the IL-4 deficiency actually had an accelerating effect on diabetes, but that this influence was masked in our experiments by an accompanying resistance locus from the 129 genome cosegregating with the mutation. An artifact due to a linked gene is particularly plausible in the case of the IL-4 gene because it is close to the *Idd4* locus on murine chromosome 11 (27,28). Therefore, the wild-type 129 allele at the IL-4 locus was carried through several generations of backcross, typing each generation by SSLP with the closely linked *D11Mit208* microsatellite marker. As demonstrated in Fig. 3, we observed no effect of linked 129 loci after this backcross. (Note that these control animals were less backcrossed than the IL-4–null mutants used for assaying diabetes to ensure that they would contain at least as many linked genetic elements from the 129 genome.)

Given the lack of effect of the IL-4 mutation on disease in both diabetes models, we felt it important to verify that the mutation had the same effects on immunological parameters when carried on the NOD genetic background as has been documented on the original 129 × B6 background—in particular, the depression of functions normally attributed to Th2 cells. We compared the capability of CD4$^+$ T-cells from IL-4–deficient mice and control littermates to produce cytokines after anti-TCR stimulation in vitro (Table 1). There was, of course, no IL-4 produced by the cultures from homozygous mice; these also showed a strongly reduced ability to produce IL-5. The decrease in IL-5 was largely reversed when IL-4 was added in the first phase of the cultures, indicating that T-cells from the mutant mice were still able to differentiate to Th2-like cells when appropriately stimulated. The impact of the IL-4 deficiency was also apparent in the levels of circulating immunoglobulin isotypes in the absence of deliberate immunization (Fig. 4). There was a substantial reduction of circulating IgG1 in the mutants ($P < 0.0001$), consistent with the documented role of IL-4 in promoting switching to IgG1 (4).

DISCUSSION

Even though somewhat unexpected, the results were quite clear: the complete absence of IL-4 did not influence the severity of disease in conventional NOD mice nor in a TCR transgenic model of diabetes. Insulitis appeared unaffected, and diabetes was neither accelerated nor more prevalent. These findings match recent observations on experimental autoimmune encephalomyelitis, another autoimmune disease commonly attributed to Th1 cells and one that was also not affected by the IL-4–null mutation (29).

Hence IL-4 does not appear to have a unique role in controlling the aggressiveness of the autoimmune process. As in other cases of negative results on knock-out mice, it is not possible to distinguish whether IL-4 actually has no role under the usual disease conditions, or whether it has some role, but other cytokines are capable of compensating for its absence. In either case, these data do not support the notion that reduced production of IL-4 by NOD mice is a major determinant of disease (5,9,11). One might argue, of course, that there could be a threshold of IL-4 production below which NOD mice already fall and under which a further decrease would be inconsequential. We think such a view rather unlikely as *1*) the disease in NOD mice is clearly slow and con-

TABLE 1
Cytokine production by CD4$^+$ cells after TCR stimulation in vitro (secondary cultures)

IL-4 added in first culture	IL-4		IL-5		γ-Interferon	
	−	+	−	+	−	+
IL-4$^{+/+}$						
Experiment 1	150	440	266	720	2010	653
Experiment 2	190	476	436	1060	1050	20
IL-4$^{-/-}$						
Experiment 1	<5	<5	15	448	710	434
Experiment 2	<5	<5	9	492	800	10

CD4$^+$ lymph node T-cells were purified and stimulated with plastic-bound anti-TCR in the presence or absence of exogenous IL-4. After 5 days, the cells were restimulated in vitro, and released cytokines were measured by ELISA. Results of two independent experiments are shown; all values are in arbitrary units, relative to the standard curve.
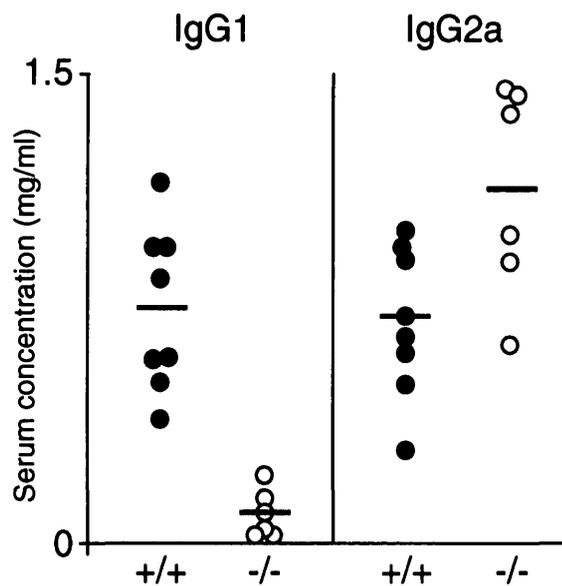
IgG1   IgG2a

FIG. 4. Natural immunoglobulin isotypes in IL-4 deficient and control animals. Mice at the eighth generation of backcross to NOD were intercrossed, and serum immunoglobulins of the IgG1 and IgG2a isotypes were measured in matched littermates by ELISA.

trolled—a control that can be lifted by manipulations such as cyclophosphamide induction—and there is room for NOD disease to worsen; and *2*) elimination of IL-4 has clear effects on different immunological parameters on the NOD background, just as it did in other backgrounds.

Our data also have a bearing on the respective role of Th1 and Th2 lymphocytes in the diabetes process. There is a general consensus that Th1 cells are the major disease effectors, but whether Th2 cells have a protective effect or not is still debated (1,2,30). For example, cotransfer of polarized Th1 and Th2 cells did not inhibit the ability of the Th1 population to provoke diabetes (20,31). Our observations would tend to support this conclusion: the reduction of Th2 cells, which is a by-product of the IL-4-null mutation, did not lift protection and enhance terminal β-cell destruction. This interpretation must be tempered, though, by the fact that the IL-4 knock-out mice do not show a complete absence of Th2-like cells. Described effects of Th2 cells directly linked to IL-4 production, such as IgG1 and IgE levels or mucosal responses, are severely impaired in the mutants (14,19,32), but the production of other Th2-secreted cytokines (e.g., IL-5, IL-10), though reduced, remains detectable (19,33,34). Prototypic Th2-linked phenomena—such as the susceptibility to *Leishmania major* infection—are not completely abrogated (33,35). With this caveat, however, our data do not support the concept that Th2 cells provide dominant protection against β-cell destruction in the insulitic lesion.

These data will also need to be taken into account in evaluating the proposed role of CD1-restricted NK T-cells in the control of diabetes (9,11,12), a role that has been envisioned to involve IL-4.

In conclusion, we were unable to detect a required role for IL-4 in two murine models of diabetes. However, the animals described here should prove useful in identifying regulatory factors that in lieu of or in conjunction with IL-4 habitually control the progression of diabetes in the NOD model.

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