Brief Genetics Report

Further Mapping of the Idd5.1 Locus for Autoimmune Diabetes in NOD Mice

Salah-Eddine Lamhamedi-Cherradi, Olivier Boulard, Cristina Gonzalez, Najiby Kassis, Diane Damotte, Laure Eloy, Guy Fluteau, Matthieu Lévi-Strauss, and Henri-Jean Garchon

The *Idd5* locus for autoimmune diabetes in nonobese diabetic (NOD) mice has been mapped to the proximal half of chromosome 1 and appears to include two loci, *Idd5.1* and *Idd5.2*, *Idd5.1* being a candidate homolog of the human *IDDM12* locus. Using new recombinant congenic lines, we have reduced the *Idd5.1* interval to 5 cM at most, between *D1Mit279* and *D1Mit19* (not included). This interval now excludes the *Casp8* and *Cflar* (*Flip*) candidate genes. It still retains *Cd28* and *Ctla4* and also includes *Icos* (inducible costimulator). The previously reported differential expression of *Ctla4*, which is induced at a lower level in NOD than in B6-activated T-cells, was found independent of *Idd5.1* itself because *Ctla4* expression was induced at a low level in T-cells from *Idd5.1*-congenic mice. The *Idd5.1* locus protected against both spontaneous and cyclophosphamide-induced diabetes, but it did not prevent inflammatory infiltration of the islets of Langerhans. Furthermore, diabetogenic precursor spleen cells from prediabetic NOD and *Idd5.1*-congenic mice were equally capable of transferring diabetes to immunodeficient NOD.*scid/scid* recipient mice. The *Idd5.1* locus might affect a late event of disease development, subsequent to the onset of insulitis and possibly taking place in the islets of Langerhans. *Diabetes* 50:2874–2878, 2001

The *Idd5* locus was initially mapped to the proximal half of murine chromosome 1 (1,2). It was associated with susceptibility to both spontaneous and cyclophosphamide-induced diabetes (1) and also with inflammatory infiltration of the islets of Langerhans, including periinsulitis and insulitis (2,3). To further study the *Idd5* locus, we generated NOD mice congenic for a 30-cM interval of C57BL/6 (B6) origin extending from *D1Mit478* to *D1Mit26* (NOD-B6.Bcl2) (see Table 1). An intercross between heterozygous mice was performed at the 7th generation of backcrossing to confirm the existence of *Idd5* and to initiate its fine mapping. Progeny mice were genotyped, and development of spontaneous diabetes in female mice was monitored for 9 months. Incidence of diabetes was markedly influenced by genotypes at the *D1Mit18* (Fig. 1*A*) and *D1Mit5* (not shown) markers and at the proximal end of the selected region, but not at *D1Mit44* and the more distal loci (not shown). The protective effect of the B6 haplotype was partially dominant ($P = 0.008$). Similar results were obtained when diabetes was induced by injection of cyclophosphamide in progeny mice from heterozygous parents at the 11th generation of backcross. Genotypes at *D1Mit18* (Fig. 1*B*) and *D1Mit5* but not at more distal loci (not shown) significantly affected susceptibility to this form of diabetes, which is otherwise specifically induced in NOD mice (4). Interestingly, the protective effect of the B6 haplotype was dominant ($P = 0.008$).

The *Idd5* interval was recently circumscribed by the use of congenic and recombinant congenic mice (5). Evidence was also obtained for two loci, *Idd5.1* and *Idd5.2*, that were closely linked and both predisposing to autoimmune diabetes. Importantly, the human synteny equivalent to *Idd5.1* is on chromosome 2q33 and overlaps the IDDM12 locus for human type 1 diabetes (5).

Here, we have further narrowed down the *Idd5.1* interval using new recombinant congenic lines. The R1 line harbored a segment of B6 origin (Table 1) between *D1Mit478* and *D1Mit178* (both markers included) that overlaps with that defined by the *Idd5*R467 recombinant congenic strain (5). As shown on Fig. 2*A*, R1 mice had a significantly reduced incidence of spontaneous diabetes ($P = 1 \times 10^{-5}$) that was approximately half of that of their NOD littermates. These mice were also protected from diabetes induced by cyclophosphamide ($P = 9 \times 10^{-5}$) (Fig. 3*A*). Additional recombinant congenic strains were selected (Table 1). The R6 and R16.14 subcongenics retained various segments of B6 origin in the proximal part of the R1 segment. These mice had an incidence of diabetes similar to that of NOD mice (Fig. 2*B*). Conversely, the R67 and R39 recombinants, which retained a B6 chromosomal segment in the distal part of the R1 interval, were protected from diabetes ($P < 1 \times 10^{-5}$ for both R67 and R39) (Fig. 2*A*).

Altogether, we now map the *Idd5.1* locus to the interval defined by the R39 recombinant congenic strain. The

From the Institut National de Santé et de Recherche Médicale (INSERM) U25, Hôpital Necker-Enfants malades, Paris, France; and the Service d'Anatomie Pathologique, Hôpital Européen Georges Pompidou, Paris, France.

S.-E.L.-C. is currently affiliated with the Department of Molecular and Cellular Engineering, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania.

Address correspondence and reprint requests to Henri-Jean Garchon, INSERM U25, Hôpital Necker, 161 rue de Sèvres, 75743 Paris Cedex 15, France. E-mail: garchon@necker.fr.

S.-E.L.-C and O.B. contributed equally to this work.

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mAb, monoclonal antibody; PCR, polymerase chain reaction; RFLP, restriction fragment–length polymorphism.

TABLE 1
Mapping the *idd5.1* interval with congenic and recombinant congenic lines

Marker	Gene	Genetic distance (cM)*	MGD distance†	NOD.B6-Bcl2	R1	R6	R16.14	R67	R39
D1Mit213		—	25.7	NOD	NOD	NOD	NOD	NOD	NOD
D1Mit478		0.18 (4/2274)	30.5	**B6**	**B6**	**B6**	NOD	NOD	NOD
D1Mit18		2.2 (51/2274)	29.7	**B6**	**B6**	**B6**	NOD	NOD	NOD
D1Mit530		0.31 (7/2274)	32.1	**B6**	**B6**	**B6**	**B6**	NOD	NOD
D1Mit74		0.35 (8/2274)	31.3	**B6**	**B6**	**B6**	**B6**	NOD	NOD
D1Mit414		0.13 (3/2274)	32.1	**B6**	**B6**	**B6**	**B6**	**B6**	NOD
	Cflar‡	—	30.1	—§	**—**	—	**—**	**—**	—
D1Nds27	*Casp8*	—	30.1	**B6**	**B6**	—	**B6**	**B6**	NOD
D1Mit161		0.09 (2/2274)	27.0	**B6**	**B6**	NOD	**B6**	**B6**	NOD
D1Mit279		0.22 (5/2274)	33.3	**B6**	**B6**	NOD	**B6**	**B6**	NOD
D1Mit302		0 (0/2274)	32.8	**B6**	**B6**	NOD	NOD	**B6**	**B6**
D1Mit22		0.09 (2/2274)	32.8	**B6**	**B6**	NOD	NOD	**B6**	**B6**
D1Mit479		0.11 (2/1811)	32.8	**B6**	**B6**	NOD	NOD	**B6**	**B6**
	Cd28	—	30.1	**B6**	**B6**	NOD	NOD	**B6**	**B6**
D1Nds25		—	—	**B6**	**B6**	NOD	NOD	**B6**	**B6**
	Ctla4	—	30.1	**B6**	**B6**	NOD	NOD	**B6**	**B6**
	Icos	—	32.0	**B6**	**B6**	NOD	NOD	**B6**	**B6**
D1Mit249		0 (0/1811)	32.8	**B6**	**B6**	NOD	NOD	**B6**	**B6**
D1Mit301		0.55 (10/1811)	32.8	**B6**	**B6**	NOD	NOD	**B6**	**B6**
D1Mit303		0.66 (12/1811)	34.8	**B6**	**B6**	NOD	NOD	**B6**	**B6**
D1Mit300		0 (0/1811)	32.8	**B6**	**B6**	NOD	NOD	**B6**	**B6**
D1Mit480		0.11 (2/1811)	32.8	**B6**	**B6**	NOD	NOD	**B6**	**B6**
D1Mit5		0.50 (9/1811)	32.8	**B6**	**B6**	NOD	NOD	**B6**	**B6**
D1Mit156		0 (0/1811)	32.8	**B6**	**B6**	NOD	NOD	**B6**	**B6**
D1Mit21		1.0 (8/784)	32.8	**B6**	**B6**	NOD	NOD	**B6**	**B6**
D1Mit178		1.8 (14/784)	34.8	**B6**	**B6**	NOD	NOD	**B6**	**B6**
D1Mit19		5.4 (18/334)	36.9	**B6**	NOD	NOD	NOD	NOD	NOD
	Nramp‡	—	39.2	**—**	—	—	—	—	—
D1Mit44		2.4 (8/334)	50.3	**B6**	NOD	NOD	NOD	NOD	NOD
D1Mit48		3.3 (11/334)	54.0	**B6**	NOD	NOD	NOD	NOD	NOD
D1Mit11		1.5 (5/334)	58.7	**B6**	NOD	NOD	NOD	NOD	NOD
	Bcl2	0.9 (3/334)	59.8	**B6**	NOD	NOD	NOD	NOD	NOD
D1Mit26		5.7 (19/334)	62.1	**B6**	NOD	NOD	NOD	NOD	NOD
D1Mit30			70.0	NOD	NOD	NOD	NOD	NOD	NOD

*Intermarker distance determined from the ratio of recombinants to meiosis in our progeny mice; †distance from the centromere (in cM) according to the Mouse Genome Database; ‡location according to ref. 5; §not genotyped.

centromeric boundary of this interval lies between the *D1Mit279* and *D1Mit302/D1Mit22* markers. This location therefore rules out the segment comprising the region between *D1Mit478* and *D1Mit302/D1Mit22* that was previously part of the *Idd5.1* interval (5). The distal boundary of R39 is more loosely limited and maps between the *D1Mit178* and *D1Mit19* markers, and thus it cannot be distinguished with currently available markers from the distal boundary defined by the *Idd5*R467 line (5). As a result, the size of the *Idd5.1* interval ranges from 3.0 to 5.0 cM.

The newly restricted *Idd5.1* locus excludes *Cflar* and *Casp8*, two important candidate genes (5). It still retains the two costimulatory receptors *Cd28* and *Ctla4*. No coding polymorphism between NOD and B6 was identified in these two genes (6; S.-E.L.-C., O.B., unpublished observations). By endonuclease restriction of both genomic DNA (Southern blot analysis) and polymerase chain reaction (PCR)-amplified intronic sequences, restriction fragment–length polymorphism (RFLP) between the two strains were characterized (see RESEARCH DESIGN AND METHODS). Typing of recombinant haplotypes for these RFLPs allowed for the placement of *Ctla4* telomeric to *Cd28*.

Such noncoding polymorphisms could have a regulatory function that results in differential expression of *Ctla4* or *Cd28*. Indeed, *Ctla4* is induced in activated T-cells at a lower level in NOD than in B6 mice (6) (Fig. 4). This is consistent with a contribution of the *Ctla4* gene to the autoimmune predisposition of NOD mice, because *Ctla4* is viewed as a negative regulator of T-cell activation (7), and *Ctla4*-null mice develop early lethal pleiotropic dysimmune manifestations (8). However, activated T-cells from R67 congenic mice expressed *Ctla4* at a low level, like their NOD parent (Fig. 4). The decrease of inducible *Ctla4* expression in NOD T-cells is therefore unlikely to be determined at the *Ctla4* locus itself or at a closely linked gene included in the *Idd5.1* interval.

Inducible costimulator (*Icos*), which facilitates T-cell activation, is another important regulatory molecule homologous to *Ctla4* and *Cd28* (9). The *Icos* gene was located in the vicinity of *Cd28* and *Ctla4* (10). A polymorphic dinucleotide repeat was identified in the second intron of the *Icos* sequence. Using this marker, *Icos* was located in the R39 interval, telomeric to *Cd28* and tightly linked to *Ctla4*, *D1Nds25*, and *D1Mit249*. Because the expression and nucleotide sequence of *Icos* have not yet been characterized in NOD mice, it is an important candidate that warrants additional investigation.

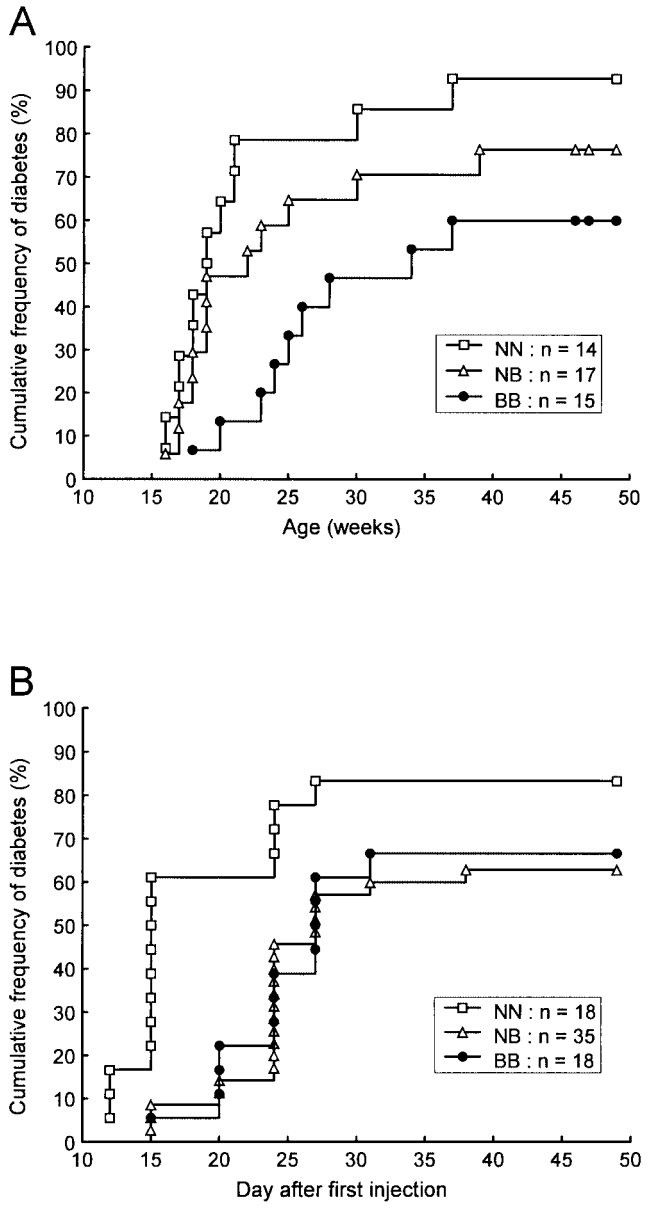

FIG. 1. Influence of the *D1Mit18* marker on the cumulative frequency of spontaneous (*A*) and cyclophosphamide-induced (*B*) diabetes in intercrossed congenic mice. Heterozygous mice at the 7th (*A*) or 11th (*B*) generation of backcrossing for the [*D1Mit18-D1Mit26*] interval were intercrossed. Progeny mice were genotyped for markers spanning the interval and were monitored for development of spontaneous (*A*) or cyclophosphamide-induced (*B*) diabetes.

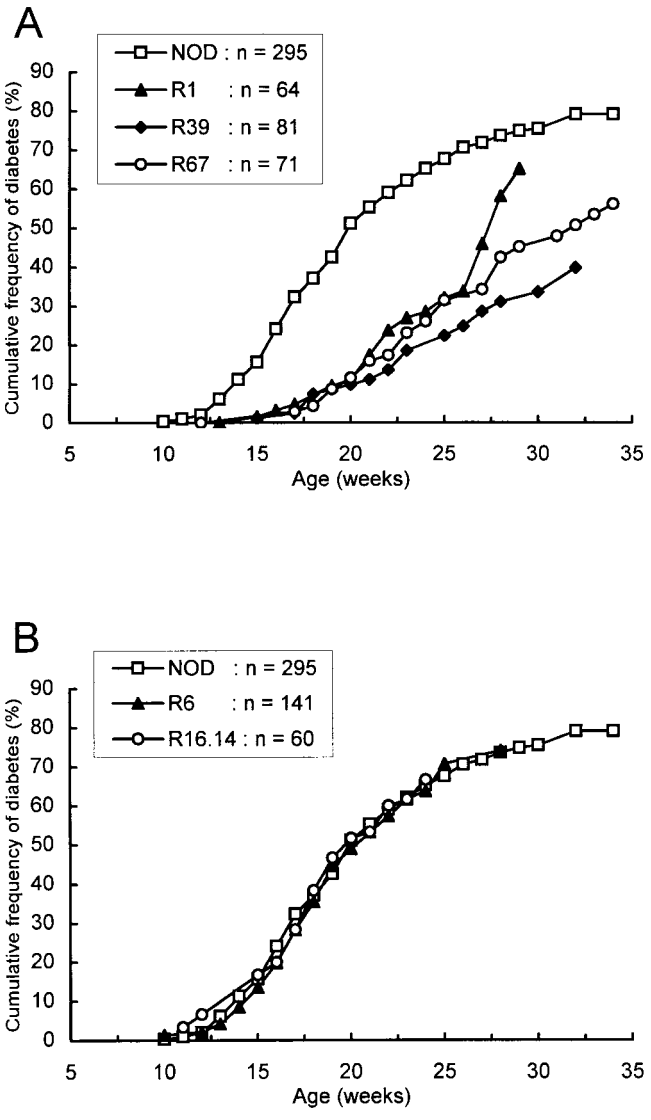

FIG. 2. Cumulative incidence of spontaneous diabetes in resistant (*A*) and sensitive (*B*) recombinant congenic mice and in NOD control mice.

The *Idd5* region defined by the large *Idd5*R8 segment did not affect inflammatory infiltration of the islets of Langerhans, as determined from tissue sections stained with hematoxylin and eosin (5). This was also the case for the smaller *Idd5.1* interval harbored by the R67 recombinant congenic line (data not shown). Transfer experiments were performed to determine whether diabetes protection occurred before or after the entry of these inflammatory cells into the islets of Langerhans. Prediabetic spleen cells from NOD or R67 donor mice were infused into immunodeficient NOD.*scid/scid* recipient mice. As shown in Fig. 3*B*, spleen cells from both donors were equally capable of transferring diabetes. This finding is consistent with an association of *Idd5.1* with resistance to induction of diabetes by cyclophosphamide, because this drug is thought to require the presence of insulitis to be effective. The *Idd5.1* gene might therefore act at a late stage of disease development, subsequent to the onset of insulitis and possibly taking place in the islets of Langerhans. For example, it might affect the late differentiation of inflammatory cells into cytotoxic cells or modulate β-cell sensitivity to autoimmune aggression.

With the rapid progress of the Mouse Genome Sequencing program, the current size of the *Idd5.1* interval makes the identification of the *Idd5.1* gene a feasible task. Construction and sequencing of a contig covering the region of interest should be carried out concurrently with the search for DNA polymorphisms. Knowing the site of expression of *Idd5.1* should be helpful in testing correlations between these polymorphisms and quantitative variation of candidate gene expression. Such quantitative variation could be the basis of *Idd5.1* if the pathogenic DNA alteration maps outside the coding region.

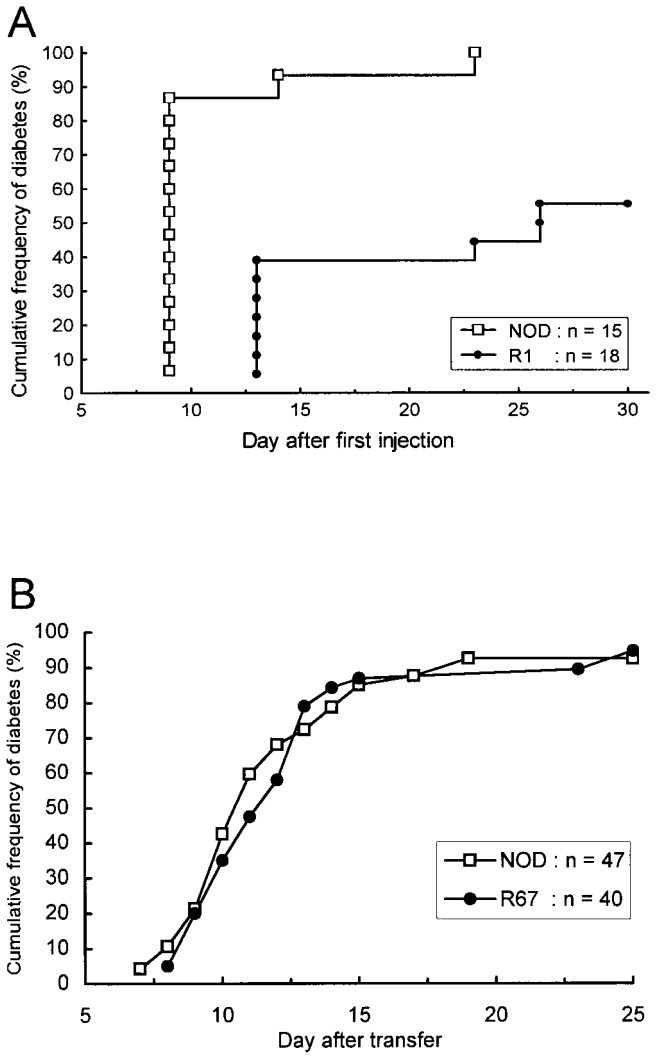

FIG. 3. *A*: Resistance of *Idd5.1* (R1) congenic mice (●) to cyclophosphamide-induced diabetes compared with NOD controls (□). *B*: Transfer of diabetes into immunodeficient NOD.*scid/scid* mice by diabetogenic precursor spleen cells from NOD (□) and R67 congenic mice (●).

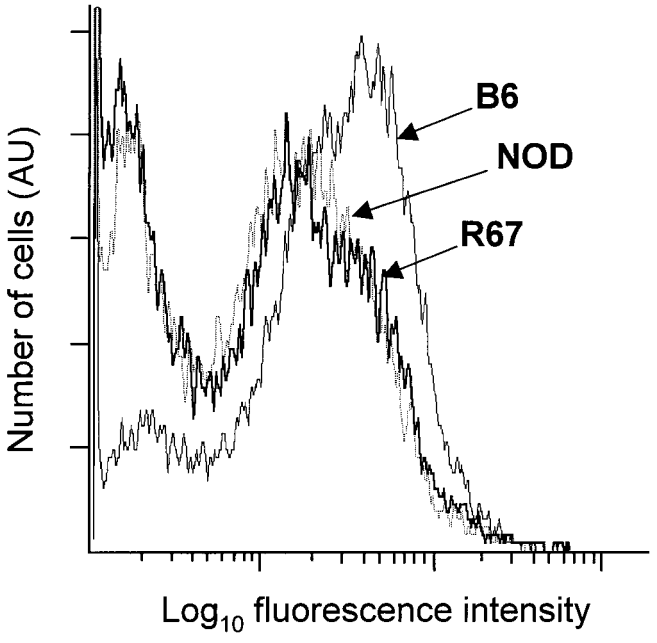

FIG. 4. Ctla4 expression in activated T cells from NOD (dotted line), B6 (thin line) and R67 (thick line) mice. Spleen cells were stimulated for 2 days by ligation of CD3. Cells were then harvested, permeabilized, and stained with a phycoerythrin-tagged anti-Ctla4 mAb and analyzed by fluorocytometry. These profiles are representative of three NOD mice (mean ± SD, 60.0 ± 1.3% of Ctla4-positive cells), three B6 (80.5 ± 1.2) and four R67 (55.1 ± 1.7). The differences between B6 and NOD or B6 and R67 were significant (*P* value of Student's *t* test 4×10^{-5} and 4×10^{-6}, respectively). Two additional experiments yielded similar results.

RESEARCH DESIGN AND METHODS

Mice and genetic analysis. NOD and C57BL6/J (B6) mice were bred in our animal facilities under specific pathogen-free conditions, in keeping with the European Union legislation on animal care. NOD mice congenic for the portion of chromosome 1 derived from the B6 strain and comprising the region between *D1Mit478* and *D1Mit26* (both markers included) were bred by iterative backcrossing with NOD parents and by genotypic selection of heterozygous mice at each generation. The R1 homozygous line was established by appropriate sister-brother matings at the 10th generation of backcrossing. Other recombinant congenic lines were derived from the R1 line after a large (1,490 progeny mice) backcross of R1 heterozygous mice with NOD parents. The R16.14 was a secondary recombinant derived from one of these primary recombinant congenic lines.

Diabetes assessment and transfer experiments. Development of spontaneous diabetes was followed weekly by testing urinary levels of glucose with glukotest (Roche). Mice were classified as diabetic after producing two consecutive tests of 3+ or higher. Diabetes was also induced in 8- to 10-week-old mice by two intraperitoneal injections of cyclophosphamide (200 mg/kg) on days 0 and 14. Diabetes onset was then monitored daily for 30 days. For transfer experiments, donor spleen cells were prepared from 10-week-old prediabetic NOD or R67 congenic mice. T-cells were enumerated with a fluorescein isothiocyanate–labeled anti-CD3 monoclonal antibody (mAb) (clone 145-2C11). An equivalent of 2×10^6 T-cells was injected intravenously into immunodeficient NOD.*scid/scid* recipient mice. Incidences of diabetes were compared with the log-rank test.

Polymorphisms and genetic map. Microsatellite markers that are polymorphic between NOD and B6 were drawn from the Massachusetts Institute of Technology database (11) (see website at www-genome.mit.wi.edu) and, for *D1Nds25* and *D1Nds27*, from the recent report by Hill et al. (5). Orders and distances between loci were those of the Mouse Genome Database (12) (see website at www.informatics.jax.org). Previously unordered markers in the *Idd5* interval were ordered by the typing of recombinant haplotypes and minimization of the number of recombination events.

RFLPs of *Ctla4* and *Cd28* genes between NOD and B6 strains were typed by Southern blot hybridization with cDNA probes, using standard protocols (13). The *Ctla4* probe was a partial cDNA probe (position 324–922, Genbank accession number X05719). An RFLP was identified with *Xba*I (14 kb in NOD, 8 kb in B6). The *Cd28* probe was also a partial cDNA probe (position 60–676, Genbank accession number M34563). An RFLP was identified with *Bgl*II (3.2 kb in NOD, 0.8 kb in B6).

PCR-RFLPs were also characterized in the *Ctla4* gene by amplification of genomic DNA using oligonucleotide primers (forward and reverse: position 324–343 and 922–901 of the cDNA, sequence CACAGAGAAGAATACAGTGG and GCTCTCTGTTCTGCTCCTTAGC, respectively) located in the second and third exons, respectively. The resulting PCR product (~2 kb) was cut with *Hin*fI and migrated on a 2% agarose gel. A polymorphic fragment between NOD (~240 nt) and B6 (~220 nt) was visualized by ethidium bromide staining.

A CA/GA complex repeat was identified in the second intron of the *Icos* gene by inspection of available sequence data (Genbank accession number AF327185). It was amplified with the following primers: forward ATCTCCAA GACTTCTCCCAC and reverse AATGAGCTGCTGTCAACTAC. Polymorphic PCR products were separated by agarose gel electrophoresis.

Typing of recombinant haplotypes for the above polymorphisms led to this most likely order:

cen-(*D1Mit302/D1Mit22*)-(*D1Mit479/Cd28*)-(*D1Nds25/Ctla4/Icos/D1Mit249*)-*D1Mit301*-tel.

T-cell activation and Ctla4 staining. Cell suspensions were prepared from spleens of 6-week-old mice and cultured at 2×10^6 cells/ml with soluble anti-CD3 mAb (clone 145-2C11) at 0.5 μg/ml. After 48 h, cells were harvested, fixed with 2.5% formaldehyde, permeabilized with 0.1% saponin, and stained with a phycoerythrin-conjugated anti-Ctla4 mAb (clone UC10-4F10; Pharmingen). Flow cytometry was performed on a FACScan (Becton Dickinson). At

least 30,000 events were acquired and analyzed with CellQuest software. Ctla4 expression in activated T-cells was detected by gating large blast cells using forward and side-scatter profiles.

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