

# Congenetic mapping and functional analysis of a second component of the MHC-linked diabetogenic gene (*Idd16*)

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## Abstract

By using a congenic non-obese diabetic (NOD) mouse strain that possesses a recombinant major histocompatibility complex (MHC) from a diabetes-resistant sister strain, the CTS mouse, we have previously mapped a second component of the MHC linked susceptibility gene (*Idd16*) to the <11.8-centiMorgan (cM) segment of chromosome 17 adjacent to, but distinct from class II A and E genes (*Idd1*). To further localise and characterise *Idd16*, CTS-derived non-diabetogenic genetic interval, identified in our previous study, was transferred onto the NOD background, and a new recombinant with recombination breakpoint at 1.3-cM proximal to the previous congenic chromosome was obtained among 49 intercrosses of heterozygous mice. The frequency of type 1 diabetes in mice possessing the new recombinant chromosome was lower than that in the parental NOD strain, as in the case of the previous congenic strain. The tumor necrosis factor  $\alpha$  gene (*Tnf*), a strong candidate gene for type 1 diabetes, is located within the newly localized *Idd16* interval. Although our previous study indicated that the sequences of exons and exon-intron junctions of *Tnf* in the NOD mouse were identical to those in the control mouse, defective expression of TNF $\alpha$  may well contribute to *Idd16* effect. We therefore determined plasma TNF $\alpha$  levels in NOD and CTS mice. TNF $\alpha$  levels under basal conditions and after stimulation with interferon gamma (IFN-gamma) and lipopolysaccharide (LPS), however, were comparable among NOD, CTS and control mice. These data suggest that *Idd16* is located in the <10.5-cM segment of chromosome 17 adjacent to *Idd1*, and that defective expression of TNF $\alpha$  may not be responsible for *Idd16* effect, at least under the condition used in the present study.

**Key Words:** Type 1 diabetes, NOD mouse, Congenic mouse, Tumor necrosis factor  $\alpha$ , MHC, *Idd16*.

## Introduction

Type 1 diabetes is caused by the auto-immune destruction of pancreatic beta cells in genetically susceptible individuals. Both in humans and animal models, inheritance of type 1 diabetes is

polygenic,<sup>1,2</sup> and major genetic susceptibility has been mapped to MHC.<sup>3</sup> In the non-obese diabetic (NOD) mouse, an animal model of type 1 diabetes,<sup>4</sup> the development of type 1 diabetes is strongly linked to the MHC region on chromosome 17.<sup>1,3</sup> More specifically, a unique class II MHC, a rare I-A<sup>g7</sup> molecule and defective expression of I-E molecule in the NOD mouse, appear to be responsible for MHC-linked susceptibility to the disease (*Idd1*).<sup>3,5-7</sup> The existence of additional susceptibility gene(s) linked to MHC has recently been

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indicated by analysis of NOD mice congenic for MHC with the same class II MHC as the NOD mouse, but different alleles outside the class II MHC. This gene, *Idd16*, is responsible for up to 90% variation in the incidence of type 1 diabetes in the NOD mouse.<sup>1</sup> By mapping the boundaries of the congenic segments, *Idd16* has been localised to the <11.8-centiMorgan (cM) region on chromosome 17 adjacent to, but distinct from class II A and E genes.<sup>1</sup> To further localise *Idd16*, we identified a new recombinant chromosome in the *Idd16* interval and established a new congenic strain with this chromosome. Preliminary data on the incidence of type 1 diabetes in this strain suggested the presence of *Idd16* in the newly determined *Idd16* interval. This interval contains a strong candidate gene, tumour necrosis factor  $\alpha$  gene (*Tnf*), which encodes TNF  $\alpha$ . Although our previous study indicated that the sequences of exons and exon-intron junctions of *Tnf* in the NOD mouse were identical to those in the control mouse,<sup>1</sup> defective expression of TNF $\alpha$  may well contribute to *Idd16* effect. In fact, endogenous TNF $\alpha$  level was reported to be much lower in the NOD mouse than in other mouse strains,<sup>8</sup> and the administration of TNF $\alpha$  prevented insulinitis and diabetes in the NOD mouse.<sup>9,10</sup> We therefore determined plasma TNF $\alpha$  levels in NOD and CTS mice in comparison with control strains.

## Materials and methods

### *Mice*

NOD/Shi, CTS/Shi and NOD.CTS-H-2 congenic mice were maintained at Shionogi Aburahi Laboratories. All mice were housed under sterile, specific, pathogen-free conditions. Mice were monitored for the development of diabetes by testing for urinary glucose with Tes-Tape (Eli Lilly Co.,

Indianapolis, IN) and were classified as diabetic after producing consistent Tes-Tape values of  $\geq 3+$ . The cumulative incidence of type 1 diabetes in female NOD mice was 91% at 30 weeks of age.

### *Congenic strain mapping for further localisation of Idd16*

To obtain a new recombinant within the *Idd16* interval,<sup>1</sup> MHC heterozygotes of NOD.CTS-H-2 mice were intercrossed at N24 generation and resulting N24F1 mice were screened for new recombinants. These F1 mice were genotyped with markers in the *Idd16* region. The following Map Pairs<sup>TM</sup> (Research Genetics, Huntsville, USA) were used: *D17Mit144*, *D17Mit30*, *D17Mit61*, *D17Mit81*, *D17Mit134*, *D17Mit147*, *D17Mit192*, *D17Mit13*, *D17Nds3*, *D17Mit104*, *D17Mit105*, *D17Mit176*. A new recombinant obtained was backcrossed to NOD to obtain multiple heterozygous mice of both sexes, and these were then intercrossed to produce N25F1 mice, and the incidence of type 1 diabetes was monitored.

### *Quantitative determination of serum TNF $\alpha$*

The basal serum level of TNF $\alpha$  was measured in NOD, CTS and other control laboratory strains at 9 weeks of age, by ELISA for mouse TNF $\alpha$  (Genzyme, Cambridge, USA). Serum level of TNF $\alpha$  after stimulation was measured in the same strains primed with i.v. injection of 10,000 units murine interferon gamma (IFN-gamma) followed after 3 hours by i.v. injection of 5  $\mu$ g lipopolysaccharide (LPS). Two hours later, serum was collected and TNF $\alpha$  was measured by ELISA.

## Results

MHC heterozygotes in the previously reported congenic mouse, NOD.CTS-H-2 (R1), at N16 generation were repeatedly backcrossed to NOD mice and the progeny were genotyped with markers in the *Idd16* region, to detect a new recombinant in the *Idd16* interval. In order to increase the possibility of obtaining new recombinants within the interval, MHC heterozygotes were intercrossed at N24 generation and 49 F1

mice were produced. Genotyping of the markers in *Idd16* interval revealed that one mouse was a new recombinant (R2) with recombination breakpoint at 1.3-cM proximal to that in R1 (Fig. 1). The mouse was backcrossed to NOD, and the incidence of type 1 diabetes was compared between mice with and without CTS-derived congenic interval. The frequency of type 1 diabetes was lower (50% vs. 83% at 30 weeks of age) and age at onset was older (177 vs. 127 days) in mice with CTS-derived congenic

**Fig. 1** CTS-derived non-diabetogenic genetic interval identified in our previous study<sup>1</sup> was transferred onto the NOD background by eight successive backcrosses, and MHC heterozygotes were intercrossed at N24 generation. A new recombinant (R2) was found in one out of 49 mice at N24F1 generation. R2 had a recombination breakpoint at 1.3-cM proximal to that in R1. The frequency of diabetes at 30 weeks in female mice was 83% (mean age at onset 130 days) in NOD, 0% in CTS, 44% (173 days) in R1 and 33% (170 days) in R2. Numbers in parentheses are the location based on the consensus map (cM).

**Fig. 2** Life table of NOD.CTS-H-2 female mice with CTS allele (heterozygous or homozygous for the CTS-derived congenic interval) or without CTS allele (NOD homozygous) in *Idd16* region. It shows a significant difference between the survival curve of mice with CTS-derived congenic interval and that of mice without ( $p < 0.05$ ). Mice were monitored for the development of diabetes by testing for urinary glucose with Tes-Tape (Eli Lilly Co., Indianapolis, IN) and were classified as diabetic after producing consistent Tes-Tape values of  $\geq 3+$ . Statistical analysis was performed using Wilcoxon rank sum test.

interval than in those without. Preliminary data in mice homozygous for CTS in the interval also suggested lower frequency (33%) and later onset (170 days) of the disease. Life table analysis demonstrated a significant difference between the survival curve of mice with CTS-derived congenic interval and that of mice without ( $p < 0.05$ , Fig. 2). These data suggest that CTS-derived congenic interval in the new congenic line (R2)

contained a resistance allele at the *Idd16* locus.

Basal  $\text{TNF}\alpha$  level was below the detection limit in all strains used. After stimulation, serum  $\text{TNF}\alpha$  level was  $25.1 \pm 8.8$  ng/ml in NOD mice,  $37.5 \pm 8.9$  ng/ml in CTS mice,  $23.2 \pm 6.6$  ng/ml in BALB/c mice and  $37.0 \pm 10.4$  ng/ml in C57BL/6 mice.

## Discussion

The NOD mouse spontaneously develops autoimmune type 1 diabetes with striking similarity to human type 1 diabetes.<sup>4</sup> Identification of susceptibility genes in the NOD mouse will clarify the molecular mechanisms leading to the destruction of the pancreatic beta cells and will increase our understanding of the genetics and pathogenesis of this disease, in order to develop effective methods of prediction and prevention.

Type 1 diabetes in the NOD mouse, however, is inherited as a complex polygenic trait, making identification of susceptibility genes difficult. Construction of congenic mouse strains and sequencing of candidate genes within congenic intervals is one of the best ways of fine mapping and identification of the responsible genes for these loci.<sup>1,11-16</sup> In this study, the congenic strategy was used to fine map *Idd16*. A new recombinant (R2), which had a recombination breakpoint at 1.3-cM proximal to that in the previous congenic line (R1), was obtained in intercrosses of mice heterozygous for R1. Despite the replacement of a 1.3-cM interval with NOD chromosome, the incidence of type 1 diabetes was still low as compared with the NOD parental strain, suggesting that *Idd16* is located in the <10.5-cM segment of chromosome 17 adjacent to *Idd1*.

Cytokines are peptide molecules synthesized and secreted by activated lymphocytes, macrophages/monocytes, and cells outside the immune system. Recently, several reports have been published on the relationship between cytokines and type 1 diabetes.<sup>9-10,17-19</sup> TNF $\alpha$  is a lymphokine that plays an important role in the regulation of the immune response as part of the cytokine network, and its gene lies within the *Idd16* interval. Administration of TNF $\alpha$  is reported to prevent insulinitis and

diabetes in the NOD mouse.<sup>9,10,20</sup> *In vitro*, co-existence of TNF $\alpha$  was reported to increase the destructive action of IL-1 on rat islet beta cells.<sup>21</sup> More recently, an age-dependent difference in the effect of TNF $\alpha$  on the development of type 1 diabetes has been reported,<sup>22</sup> suggesting distinct effects on the diabetogenic process depending upon the developmental stage of the target tissue or cell. These reports suggest that the impaired regulation of TNF $\alpha$  plays an important role in the development of type 1 diabetes.

Although our previous study indicated that the sequences of exons and exon-intron junctions of *Tnf* in the NOD mouse were identical to those in the control mouse,<sup>1</sup> defective expression of TNF $\alpha$  may well contribute to *Idd16* effect. To examine this possibility, we measured serum TNF $\alpha$  level using an ELISA for mouse TNF $\alpha$ . TNF $\alpha$  levels under basal conditions and after stimulation with IFN-gamma and LPS were comparable among NOD, CTS and control mice, suggesting that defective expression of TNF $\alpha$  may not be responsible for *Idd16* effect. Further studies in different conditions, however, are necessary to clarify the relationship between susceptibility to type 1 diabetes and defective expression of TNF $\alpha$ .

In summary, congenic mapping and functional analysis of TNF $\alpha$  suggested that *Idd16* is located in the <10.5-cM segments of chromosome 17 adjacent to *Idd1*, and that defective expression of TNF $\alpha$  may not be responsible for *Idd16* effect, at least under the condition used in the present study.

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