A SNP in the immunoregulatory molecule CTLA-4 controls mRNA splicing in vivo but does not alter diabetes susceptibility in the NOD mouse

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Abstract

CTLA-4 is a critical ‘checkpoint’ regulator in autoimmunity. Variation in CTLA-4 isoform expression has been linked to type 1 diabetes development in human and NOD mouse studies. In the NOD mouse, a causative link between increased expression of the minor isoform, ligand-independent CTLA-4, and a reduction in diabetes has become widely accepted. Altered splicing of CTLA-4 has been attributed to a SNP in Ctla4 exon2 (e2_77A/G). To investigate this link, we have employed NOD embryonic stem (ES) cells to generate a novel NOD transgenic line with the 77A/G single nucleotide polymorphism (SNP). This strain phenocopies the increase in splicing towards the liCTLA4 isoform seen in B10 Idd5.1 mice. Crucially, the SNP does not alter the spontaneous incidence of diabetes, the incidence of cyclophosphamide-induced diabetes or the activation of diabetogenic TCR transgenic CD4+ T cells after adoptive transfer. Our results show that one or more of the many other linked genetic variants between the B10 and NOD genome are required for the diabetes protection conferred by Idd5.1. With the NOD mouse model closely mimicking the human disease, our data demonstrate that knock-in transgenic mice on the NOD background can test causative mutations relevant in human diabetes.
The development of most autoimmune diseases, including type 1 diabetes, is influenced by both genetic and environmental factors. Such genetic susceptibility is complex. In addition to polymorphisms in MHC genes, there are many genetic variants which contribute to diabetes susceptibility, each of which contributes a relatively small additional risk. In man, genome wide association studies have identified more than 40 genetic traits which affect type 1 diabetes susceptibility; a similar number of genetic intervals have been identified in the NOD mouse model (1). Variation which alters the mRNA splicing of CTLA-4 has been implicated in diabetes susceptibility in both man and the NOD mouse model (2). CTLA-4 polymorphisms are also associated with type 1 diabetes susceptibility in dogs (3).

CTLA-4 is an important immunoregulatory molecule with an essential role in the function of regulatory T cells (4; 5) and a less well-defined role in effector T cells (6; 7). In humans two protein isoforms have been described, the canonical transmembrane homodimer (flCTLA4) and a soluble monomeric form (sCTLA4); both forms result from alternative mRNA splicing of the nascent CTLA-4 transcript and both bind to the Immunoglobulin superfamily ligands CD80 and CD86. In addition to these two forms, a third protein isoform, liCTLA-4 has been described in mice. This isoform results from alternative mRNA splicing in which exon 2 (encoding the ligand binding Ig domain) is spliced out to produce a predicted 72aa mature transmembrane protein capable of homo- or hetero-dimerization with li/flCTLA4 (8). Ueda et al. (2) found an association between lower expression levels of the soluble isoform of CTLA4 and susceptibility to autoimmune diseases, including type 1 diabetes, in humans and also showed that NOD mice but not NOD.B10Sn-Idd5 mice had lower levels of mRNA for the liCTLA4 isoform. Subsequent work by Wicker and colleagues has confirmed the close correlation between genetic variation in Cila4, variation in liCTLA4 mRNA expression and diabetes susceptibility in a number of NOD congenic mouse strains (9; 10). Indeed, the
protection from diabetes observed in NOD.B10Sn-Idd5.1 mice is now widely attributed to \( C\text{tla4} \), in particular to the A/G SNP at position 77 in \( C\text{tla4} \) exon 2 (11-13).

While the correlation between diabetes protection and increased levels of liCTLA is suggestive, it does not demonstrate a causal link. Furthermore, there has been no direct test of the hypothesis that the exon 2 SNP is responsible for the alteration in mRNA splicing. We recently developed germline competent ES cells from the NOD strain (14). We therefore decided to directly test whether this SNP is responsible for altering splicing efficiency and diabetes susceptibility. We have derived a novel ‘knock-in’ transgenic strain on the NOD background which contains the candidate causative B10 SNP (\( C\text{tla4 e2_77A} \)). Analysis of this mouse strain demonstrates that the SNP is fully responsible for the change in alternative mRNA splicing and that the increased expression of liCTLA4 does not alter the susceptibility to diabetes.
Research Design and Methods

Mice
NOD/MrkTacDil, NOD.Cg-Tg(TcraBDC2.5,TcrbBDC2.5)1Doi/DoiJ, NOD.Cg-N(Foxp3-I-EGFP) and NOD 77A (official name NOD-Ctla4^{tm1(77A)Holm}) mice as well as their crosses were bred under specific pathogen free conditions at the Biological Services Unit of the Department of Pathology, Cambridge, UK. Mice had unrestricted access to standard laboratory food and water and were housed in open or air filtered cages. All animal work was carried out in accordance with UK Home Office project and personal licences and was approved by the Ethical Review Committee of the University of Cambridge.

ES cell culture and blastocyst injection.

Clone 16 NOD ES cells and their derivatives were maintained in N2B27 medium supplemented with 1 μM of the MAPK inhibitor PD0325901 and 3 μM of the glycogen synthase kinase-3 inhibitor CHIR99021 (2i; both synthesized in the Division of Signal Transduction Therapy, University of Dundee, Dundee, UK) plus LIF (25 ng/mL, produced in house). We injected 10–15 trypsinized NOD ES cells into each C57BL/6 blastocyst and then transferred the blastocysts to the uterus of a pseudopregnant F1 female mouse at 2.5 days post coitum. We assessed the resulting chimeras for germline transmission by mating them with NOD mice.
Gene targeting

The NOD BAC CH29-120A16 was obtained from the Children’s Hospital Research Center, Oakland, Oakland CA. A 10,047bp SpeI-SacI fragment which contained CTLA-4 was cut from the BAC sequence and inserted into a SacI-XbaI cut pNEB193 vector. This 10,047bp fragment was in turn cut with SacI-KpnI and KpnI-PacI, producing two fragments which contained the 5' and 3' arms of the CTLA-4 gene respectively, which were subcloned into pNEB193. The 3' arm was modified by insertion of a selection cassette into the NsiI site in intron 2. The selection cassette consisted of a loxP flanked Neomycin resistance gene and a Herpes Simplex Virus Thymidine Kinase gene, both driven by an enhancerless thymidine kinase promoter from plasmid, pNeoTKloxP (Peter McKinnon, St. Jude Children's Research Hospital, Memphis). The 5' ARM was modified by substitution of a 236bp DraIII-KpnI fragment containing a single G→A point mutation at position 77 of exon 2. The two arms were rejoined at the KpnI site the resulting plasmid linearised with NotI and 10^7 clone 16 male NOD ES cells (14) cells transfected by electroporation. Transfected cells were subjected to G418 selection in 2i media and any surviving cells were cultured as individual clones in 96 well plates.

Homologous recombinants were identified by PCR screening with a 3 primer assay which amplified distinct products from targeted and endogenous loci (primers P1+P3+P4; see Table 1). Recombinants were confirmed by Southern blotting with both a 5' flanking and internal probe and the co-integration of the targeted 77A mutation confirmed by sequencing. The loxP flanked cassette was removed by transient transfection with a CAGGS-Cre-Ires-Blasticidin plasmid (Sandra Gomez-Lopez, Cambridge Stem Cell Institute). 24 hours after transfection cells were exposed to Blastocidin for 24hrs and candidate clones were picked from surviving cells. The structure of the recombined locus was confirmed by sequencing.
Monitoring of glucose levels

Urine glucose levels were measured using indicator strips and readings of >28mM glucose in the urine were considered diabetic (rated according to the colour-glucose-scale provided by the manufacturer). Glucose readings were taken between 15:00 and 18:00 hours in the afternoon, except where stated otherwise. Only female mice were used for spontaneous diabetes incidence studies.

Cyclophosphamide (CTX) injections

All mice were weighed and tested for hyperglycaemia prior to injections. Only healthy male mice were injected intraperitoneally with 20 mg/ml CTX solution at a dose of 250mg/kg. Mice were held in a supine position and injections were made in the lower left quadrant of the abdomen. Following injection, mice were tested for hyperglycaemia every 24 hours for 4 weeks.

Quantitative PCR

For template generation, cells were lysed using the QIAshredder kit and RNA was extracted using the Rneasy kit DNA contaminants were removed using the TURBO DNA free kit and the mRNA was reverse transcribed using the SuperScript III First Strand Synthesis System. RNA sequence bias was avoided by using Oligo (dT)20 primers for cDNA synthesis. RNA content in all reactions was optimised to maximise cDNA concentration.

Quantitative PCR assays were performed in triplicate or quadruplicate with water controls included on every plate and housekeeping gene controls included for every sample. Reactions
contained 10μl of the relevant primer mix (2x), 10μl of GoTaq qPCR Master Mix and approximately 1ng of cDNA template. Primer sequences are shown in Table 2.

Samples were analysed using the Applied Biosystems 7500 Fast Real-Time PCR System calibrated for SYBR green detection. All plates were run using the same amplification program: 95°C for 15min, (94°C for 15s, 60°C for 30s, 72°C for 30s) × 40 cycles followed by a dissociation stage. The threshold for detection was set within the linear phase of the logarithmic amplification plot.

**CFSE labelling**

For CFSE labelling, CD4+ splenocytes were incubated with 1μM CFSE in PBS at a concentration of 10^7 cells/ml. Cells were incubated for 15 minutes at 37°C and washed twice with cold IMDM. Labelled cell pellets were re-suspended in IMDM at a concentration of 5x10^6 cells/ml and injected into the tail veins of recipient mice (10^6 cells per mouse).

**Flow cytometry and cell sorting**

Cells were labelled with anti-CD4 (RM4-5) supplemented with 2.4G2 anti-FcγRII/III (produced in house). Labelled cells were analysed using the Cytek DxP8 flow cytometer.

Cells for use in qPCR were isolated using the MoFlo cell sorter and cells for CFSE labelling were isolated using magnetic-activated cell sorting of anti-CD4(L3T4) conjugated magnetic beads.

**Cell culture**
For T cell activation, culture dishes were coated with 10μg/ml anti-CD3 antibody (145-2C11 produced in house) overnight. Purified T cells were cultured in IMDM medium supplemented with 10% FCS, 100 U/ml Penicillin, 100 μg/ml Streptomycin, 2mM glutamine and 5μg/ml anti-CD28 antibody (37.51 also produced in house).

Results

Generation of a NOD transgenic bearing the \textit{Ctla4} exon 2 SNP 77A

NOD strain ES cells were maintained in ground state by culture in 2i medium (14) and transfected with a \textit{Ctla4} targeting construct. The construct was prepared using the NOD bacterial arteficial chromosome (BAC) CH29-120A16 as an isogenic DNA source and contains a single G→A point mutation at position 77 in exon 2 (see Research Design and Methods). The final genomic structure (R2, Fig. 1A) retains a single loxP site within intron 2. 23 male chimeras were produced by injecting clone 3E1/11 ES cells into C57BL/6 blastocysts. 11 chimeras, chosen for strong NOD coat color contribution, were backcrossed to our NOD colony; 54% of offspring were ES cell derived and 31.25 % of these carried the targeted \textit{Ctla4}^{77A} allele (difference in allelic transmission not significant). The breeding scheme for producing incidence cohorts for analysis is shown in Fig.1B. Heterozygous 77A/G NOD offspring were intercrossed to produce both 77A/A and 77G/G homozygotes; from here on referred to as NOD 77A and NOD 77G. These first generation homozygotes were again intercrossed to produce sufficient female offspring for study (Incidence cohort I). In this way, the experimental and control cohorts shared all ES derived genetic material except \textit{Ctla4} and its linked genomic segment. A second cohort set was produced after a further backcross to our NOD colony, with the gender of the NOD parent reversed (Fig. 1B).
First generation 77A homozygotes were also crossed to our NOD.Cg-BDC2.5 and NOD.Cg-
FoxP3\textsuperscript{EGFP} colonies. Sib-mating of the offspring produced 77A homozygotes carrying the
relevant transgene. FoxP3\textsuperscript{EGFP} was also made homozygous whereas the BDC2.5 TCR transgenes were maintained by screening.

The 77A mutation in C\textit{tla-4} exon 2 controls mRNA splicing and increases liCTLA-4 expression

We examined the effect of the 77A mutation on CTLA-4 mRNA splicing by measuring the
mRNA levels of each CTLA-4 isoform in the T cells of NOD 77A Foxp3\textsuperscript{EGFP} and NOD 77G
Foxp3\textsuperscript{EGFP} mice. Three different T cell populations, conventional T cells (CD4\textsuperscript{+}Foxp3\textsuperscript{−}),
regulatory T cells (CD4\textsuperscript{+}Foxp3\textsuperscript{+}) and \textit{in vitro} activated T cells (CD4\textsuperscript{+}FoxP3\textsuperscript{−} stimulated with
anti-CD3 + anti-CD28) were investigated. CTLA-4 mRNA was most abundant in regulatory
T cells which in comparison to \textit{in vitro} activated T cells and conventional T cells contained
six fold and sixty seven fold more CTLA-4 mRNA, respectively (Fig. 2A). Analysis of the
different CTLA-4 isoforms revealed that the ligand independent and soluble isoforms are rare
compared to the full length isoform which represents between 86 and 99% of the total CTLA-
4 mRNA (Fig. 2 B,C,D).

In all analysed T cell populations, the introduction of the 77A mutation resulted in a
significant increase of liCTLA-4 mRNA. A two-fold increase in liCTLA-4 was observed in
conventional and regulatory T cells and a fourfold increase was observed in \textit{in vitro} activated
cells (Fig. 2C). Considering that all isoforms are spliced from the same pre-mRNA, any
increases in exon 2 splicing were expected to decrease the abundance of full length and soluble isoforms (Fig. 2 B,D). These results directly demonstrate, for the first time, that the 77A mutation in CTLA-4 exon 2 enhances the effectiveness of the exonic splicing silencer motif. Given the importance of CTLA-4 in immune regulation, the immunological relevance of this change in mRNA expression was explored.

The 77A mutation and associated increases in liCTLA-4 expression do not affect spontaneous or induced diabetes development in the NOD mouse

Spontaneous diabetes incidence in female mice represents the most powerful immunological readout of the NOD model and we therefore investigated spontaneous incidence in our gene targeted NOD colonies. The first cohort study was conducted on animals which contained equal amounts of NOD ES cell derived and ‘colony NOD’ derived genetic material (Fig. 1B). The main onset of diabetes in this cohort occurred around 15 weeks of age and the total incidence levelled off between 60 and 70%. The 77A mutation had no significant effect on spontaneous diabetes development (Fig. 3A). Mice backcrossed a second time, now containing only 25% ES cell derived genetic material, were investigated in a second incidence study (Fig. 3B). With diabetes onset at 12 weeks of age and a total incidence above 80% the backcrossed NOD 77A and 77G mice mirrored the diabetes incidence of the originator colony. Despite the increased cohort size, no difference between the NOD 77A and NOD 77G cohorts could be distinguished (Fig. 3B). The 77A mutation and altered levels of liCTLA-4 do not therefore affect spontaneous diabetes development. The difference in incidence between the initial and backcrossed cohorts may be due to variation in the environment as all mice were moved from open cages into individually ventilated cages 20 weeks into the first study. Also, the possibility that both initial cohorts were affected by
genetic or epigenetic changes occurring during ES cell culture, and lost after the second backcross, cannot be ruled out.

To explore the possibility of only certain functions of the immune system being linked to the 77A mutation, we compared the cyclophosphamide (CTX) induced diabetes incidence in male NOD 77A and NOD 77G mice. CTX is thought to induce diabetes by depleting mature lymphocytes in the periphery which leads to the preferential repopulation of lymphoid niches by diabetogenic effector T cells and hence the preclusion of regulatory T cells (15; 16). 12 week old mice received a single intraperitoneal injection of 250 mg/kg CTX and urinary glucose was monitored for 3 weeks. In both strains the fastest rate of diabetes development was reached on day 10 post injection and a total of 70-80% of mice developed diabetes by day 21 (Fig. 3C). No effect of the 77A mutation was observed, suggesting that the capacity of diabetogenic T cells to repopulate lymphoid niches was not affected by the 77A mutation or physiological changes in liCTLA-4 (Fig. 3C). These data appear at odds with the published result of Stumpf et al. (17) who did see a reduction in CTX-induced diabetes in NOD background mice with similarly increased liCTLA4. There are a number of experimental differences however. One of these was that younger mice were given two injections of 200 mg/kg CTX seven days apart (17). We therefore used this protocol to determine whether the 77A mutation conferred any protection from diabetes onset. We could find no evidence for this (data not shown); indeed the majority of mice given one injection of 200mg/kg CTX at 8-10 weeks developed diabetes by 7 days later (see Discussion for further consideration of these differences).

**Physiological increases in liCTLA-4 mRNA have no measurable effect on T cell activation, proliferation or regulatory function**
To directly assess the effect of liCTLA-4 mRNA variation on immune function, we used BDC2.5 transgenic NOD 77A and NOD 77G control mice. T cells in these mice express the T cell receptor (TCR) from BDC2.5, a diabetogenic T cell clone (18). When transferred to adult NOD mice, BDC2.5 transgenic T cells undergo antigen-driven proliferation in the pancreatic lymph node (19). CD4⁺ T cells were isolated from BDC2.5 transgenic NOD 77A and NOD 77G mice, in vitro labelled with the proliferation dye CFSE and adoptively transferred into wild type NOD recipients. After 72 hours cells were recovered from the pancreatic lymph nodes of recipient mice and analysed by flow cytometry (Fig. 4). In all recipient mice, a proportion of transferred T cells were dividing, visualised by the typical pattern of division peaks (Fig. 4B,C,D,E). To directly compare the proliferative properties of 77A and 77G derived BDC2.5 T cells, proliferation profiles were averaged and overlaid (Fig. 4F). No significant effect of increased liCTLA-4 levels on T cell proliferation could be observed. Alternative analysis of this data, comparing the number of cells that remained undivided also showed no significant impact of liCTLA-4 mRNA (data not shown). These data strongly suggest that the TCR activation threshold which represents the cell’s immunological state, as well as the dynamics of cell proliferation, is not affected by the 77A induced changes to liCTLA-4 mRNA.

We also assessed the function of regulatory T cells using an in vivo adoptive transfer model. T cells from BDC2.5 mice induce rapid diabetes when transferred to NOD-SCID recipients; the co-transfer of CD25hi Tregs can prevent or delay diabetes onset in the NOD-SCID mice (20). BDC2.5 transgenic NOD 77A mice were crossed with the NOD.Cg-FoxP3EGFP strain. This enabled the preparation of Treg-depleted effector populations (CD4⁺GFP⁻) and Tregs (CD4⁺CD25hiGFP+) from the same donors. Co-transfer of Tregs with effector T cells in a ratio of 1:3 delayed but did not prevent diabetes onset (Fig. 5). However, there was no
discernible difference between the effectiveness of the Tregs from 77A and 77G genotypes (Fig. 5). This experimental model reinforces the conclusion that there is no difference in the ability of 77A effectors to cause diabetes and shows that effectors from 77A and 77G genotype NOD mice are equally responsive to regulation by cognate Tregs of the same CTLA4 genotype (Fig. 5).

Discussion

A number of previous studies have provided evidence for the hypothesis that increased levels of mRNA splicing to the liCTLA4 isoform have functional consequences in T cells and, in congenic NOD strains, reduce the incidence of spontaneous and/or induced diabetes (8-10; 17). We believe that our data (Fig. 3) clearly contradicts the conclusion that a modest (2-4-fold) increase in liCTLA4 levels produced by the G→A single nucleotide substitution at exon 2 position 77 alters the spontaneous or induced diabetes susceptibility in NOD mice. For the most part, significant experimental differences easily account for the differing conclusions of our study with those previously published. In the work of Wicker and her colleagues, NOD congenic strains were painstakingly derived with introgressed genomic DNA from the Idd5.1 region from the CAST and SWR strains in addition to the original B10Sn-Idd5.1 strain (10). NOD.CAST-Idd5.1 had a greater increase in liCTLA4 mRNA than the B10 congenic while NOD.SWR-Idd5.1 mirrored the NOD level; the presence of a single copy of CAST-Idd5.1 was sufficient to reduce the spontaneous incidence of diabetes by nearly half but two copies of SWR-Idd5.1 made no difference to diabetes susceptibility. However, in all the NOD congenics which shared the Cta4 77A variant, it was accompanied by a very large number of other linked genetic variants differing from the NOD genome, so that the correlation was
suggestive but by no means conclusive. In our mice, the only known genetic differences between our two cohorts are the 77A variant and the presence of a loxP site in Ctla4 intron 2.

Araki et al. (10) also found that NOD mice transgenic for a CD2-driven liCTLA4 cDNA developed diabetes at a reduced frequency. Here, the link of function to expression of liCTLA4 mRNA and protein is direct and the mice are 100% NOD genetically except for the transgene. However, the transgenic construct produced an approximate 2000-fold increase in the liCTLA4 mRNA level and it is plausible that such an overexpression might have adventitious consequences, for example by sequestering miRNAs. A more physiological increase in the level of liCTLA4 mRNA was produced by transgenesis with a C57BL/6 BAC clone encoding Ctla4. The BAC was modified so that exon 2 was flanked by loxP sites. Intercrossing with a Vav1-Cre deleter strain should produce mice whose hemopoetic cells contain a Cre-recombined transgene, capable of only producing liCTLA4 (17). These mice, termed NOD-Tg-Cre, were shown to have a 2-fold increase in liCTLA4 mRNA in CD4+CD25hiCD62L− cells. Surprisingly, Stumpf and colleagues (17) do not describe the effects of the transgene on the spontaneous incidence of diabetes. The most directly comparable experiments reported are reduced incidence of pancreatic infiltration (scored by histology) in 20 week-old pre-diabetic mice and a reduced incidence of diabetes in 8 week-old mice treated with a 2 dose CTX regime. Neither experiment reports the gender of the mice used so we cannot be sure we are making correct comparisons, however we saw no significant difference in immune cell infiltration in 12 week-old females (data not shown) and no difference in diabetes incidence in CTX-treated males (Fig. 3C). The BAC transgenic was produced by microinjection into NOD embryos, however the Vav1-Cre transgene was made in (CBA/Ca×C57F7BL/10)F1 mice (21). Some non-NOD genomic DNA is present in the NOD-Tg-Cre mice even though the Vav1-Cre strain had been backcrossed to NOD. It also
seems likely that the USCF colony has a lower incidence of diabetes; their NOD mice took 16 days to develop diabetes after 2 injections of CTX whereas we find diabetes starts at about 1 week after a single injection when given at 8-10 weeks of age. In addition, we have to conduct our insulitis screen at 12 weeks since 40-50% of our colony is diabetic by 20 weeks of age (Fig. 3B). These probable differences in diabetes incidence could be the result of genetic or environmental factors or a combination of both.

The data reported here are the most direct (and simple) test of the functional effects of the modest increase in liCTLA4 mRNA produced by the exon 2 77A SNP and we contend that the incidence data are conclusive in showing that this mRNA splicing change does not alter diabetes susceptibility. The clear implication is that one or more of the >700 other genetic differences between the NOD and C57BL10/Sn-Idd5.1 strains (9), possibly affecting other genes e.g. Icos, are required for the change in diabetes incidence previously reported (9; 10). We do not exclude that the exon 2 77A SNP may be necessary for the diabetes phenotype but neither it nor the splicing change are sufficient. This study demonstrates that NOD ES cells can provide a probative test of candidate mutations in diabetes susceptibility. Further studies using NOD ES cells carrying multiple or single genetic differences could resolve the ambiguities which surround causative mutations in genetic susceptibility to diabetes and provide models for probing mechanisms of protection, in many cases directly relevant to human disease (22).

Author contributions: F.J. and N.H. designed and performed experiments, analysed data and wrote the manuscript. K.J. and W.M. performed experiments and reviewed/edited the manuscript. J.N and A.C contributed to experimental design and interpretation and reviewed/edited the manuscript.
Acknowledgements

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Table 1: List of genomic screening primers.

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Table 2: List of qPCR primers.

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Figure Legends

Figure 1.

**Generation of the NOD-Ctla4<sup>tm1(77A)Holm</sup> strain and pedigree.**
A. The targeting construct and recombination strategy in NOD ES cells. R1 shows the structure of the *Ctla4* locus after the first recombination and R2 after Cre deletion. The actual sequence difference from NOD germline is shown in red below.  
B. The pedigree of NOD 77A and NOD 77G control strains beginning with NOD ES cell.C57BL/6 chimeras; numbers of animals at each step are shown, where a subset were used for breeding, the number bred is in brackets.

Figure 2.

**The 77A mutation increases liCTLA-4 isoform expression in T cells.**
CD4<sup>+</sup>Foxp3<sup>-</sup> and CD4<sup>+</sup>Foxp3<sup>+</sup> splenocytes were isolated from 7-9 wk old female NOD 77A Foxp3<sup>EGFP</sup> (triangles, n=10) and NOD 77G Foxp3<sup>EGFP</sup> (circles, n=13) mice. For *in vitro* activation, CD4<sup>+</sup>Foxp3<sup>-</sup> cells were cultured with anti-CD3/anti-CD28 for 48 hours. qPCR analysis reveals total CTLA-4 expression (A) as well as the contribution flCTLA-4 (B), liCTLA-4 (C) sCTLA-4 (D) to the total CTLA-4 mRNA pool in both mouse strains. Statistical analysis was performed using the non-parametric Mann-Whitney test. Median values are indicated as black bars. The data are pooled from 3 independent experiments

Figure 3.

**Spontaneous and induced diabetes incidence is not affected by increased expression of liCTLA-4.** Spontaneous diabetes incidence in female mice of the initial cohort (A) and the backcrossed cohort (B) was determined through weekly urine glucose measurements. No significant difference in spontaneous incidence was found in either cohort (Log-rank, A: p=0.4697, B: p=0.329).  
C. In 12 wk old male mice, diabetes was induced by a single injection of 250mg/kg CTX. Urine glucose measurements were taken daily for three weeks. No significant difference in induced incidence was found (Log-rank, p=0.8646). In all incidence studies mice with two consecutive readings of >28mM glucose in the urine were considered diabetic. Numbers of animals are shown in figures. A and B show both replicates, data in C are pooled from 2 experiments.
Figure 4.

**Antigen specific T cell activation and proliferation are not affected by increased expression of liCTLA-4.** A. CD4+ splenocytes were isolated from 8-9 wk old male 77A (triangles, n=14) or 77G (circles, n=13) control BDC2.5 NOD mice; the data are pooled from 3 experiments. Sorted cells of either genotype were CFSE labelled and injected i.v. into 6-8 week old male wt NOD mice. 72 hours post transfer, cells were recovered from the pancreatic LNs of recipient mice. Recovered cells were stained with anti-CD4-APC and analysed. Panels B and C show results from an example mouse. B. Cells are gated for FSC/SSC. C. CD4+CFSE+ cells gated from panel B were analysed for CFSE intensity. D. E. Individual division profiles for each mouse are summarised for NOD 77A T cells (D) and NOD 77G T cells (E). The dividing cells within each peak are displayed relative to the total number of dividing cells. F. Average division profiles of NOD 77A (closed triangles, solid line) and NOD 77G (open circles, dotted line) transfers closely correlate (Pearson r=0.988, p=0.0016).

Figure 5

**Increased expression of liCTLA4 does not impair regulatory T cell activity in an adoptive diabetes transfer model.** T cells were isolated from 12 week old female 77A or 77G Foxp3

EGFP

-BDC2.5 NOD mice. 2x10^5 CD4+GFP- Teffector cells were injected (i.v.) alone (open symbols, dotted lines: 77A genotype= squares, 77G=circles) or together with 0.7x10^5 CD4+CD25

hi

GFP+ Treg cells from the same mice (closed symbols, solid lines: 77A genotype= squares, 77G=circles) into 8 week old NOD SCID mice. Following the adoptive transfer, blood glucose levels were tested daily between 11:00 and 13:00 hours for 2 weeks. Mice with two consecutive readings of >13.9mM glucose in the blood were considered diabetic. Tregs from both 77A and 77G genotypes delayed the onset of diabetes significantly (TeffA vs Treg+effA; p=0.0005. TeffG vs Treg+effG; p=0.0024 ; Log-Rank test) but there was no significant difference between the potency of the effectors or Tregs from the different CTLA4 genotypes.
Figure One

A

Exon 1  Exon 2 LoxP Neo HSVtk LoxP Exon 3  Exon 4

pTarg

Ctla4

R1

R2

TAT TCA CCA TCA CAC

B

Chimera: 3E1/11 → B6

NOD 77A/G  NOD 77G/G  B6xNOD F1

Incidence cohort II  Incidence control cohort II

Incidence cohort I  Incidence control cohort I
Figure Two

A. Total CTLA-4 as a fraction of b2m ex vivo CD4+Foxp3- CD4+Foxp3+ in vitro

B. liCTLA-4 as percentage of total CTLA-4 mRNA ex vivo CD4+Foxp3- CD4+Foxp3+ in vitro

C. flCTLA-4 as percentage of total CTLA-4 mRNA ex vivo CD4+Foxp3- CD4+Foxp3+ in vitro

D. solCTLA-4 as percentage of total CTLA-4 mRNA ex vivo CD4+Foxp3- CD4+Foxp3+ in vitro

Graphs show statistical significance:
- p=0.049
- p=0.052
- p=0.015
- p=0.0012
- p=0.0002
- p=0.0002
- p=0.0015
- p=0.007

Different symbols represent 77A and 77G genotypes.
Figure Three

A

Log-rank p = 0.4697

B

Log-rank: p = 0.329

C

Log-rank: p = 0.852
Figure Four

A. CD4+ cells → Flow analysis → CFSE label → PanLN → 72 hours

B. Flow cytometry analysis

C. Graph showing Counts vs. CFSE

D. Graph showing Percentage of all dividing cells vs. Division peak

E. Graph showing Percentage of all dividing cells vs. Division peak for multiple samples

F. Graph showing Pearson correlation: r=0.988 (p=0.0016)