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Commonality in the genetic control of type 1 diabetes in humans and NOD mice: Variants of genes in the IL-2 pathway are associated with autoimmune diabetes in both species

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The MHC region remains the major genetic determinant causing T1D in humans and NOD mice [1, 2] and the identification of non-MHC genes contributing T1D susceptibility has made significant progress. Of particular note, in 2007 it was demonstrated unequivocally that genome-wide association studies in humans could be used to discover novel genes causing autoimmune disease [3, 4], including T1D [5]. A conservative estimate of the number of genes influencing T1D susceptibility is at least 50 in both humans and NOD mice, with most regions having plausible candidate genes that have the potential to alter immune function. Importantly, overlap with T2D causal regions has not been observed [6] whereas overlapping associations are present with autoimmune diseases such as rheumatoid arthritis, Celiac disease, Graves' disease and systemic lupus erythematosus [5, 7, 8]. Humans and NOD mice have variations in some of the same T1D genes or gene pathways and there are data in both species that an early autoimmune response to insulin and other beta cell proteins that is caused by a partial failure in central and peripheral tolerance mechanisms precedes beta cell destruction [9, 10]. In addition to sharing structural aspects of the MHC class II molecules that confer T1D susceptibility or resistance [11], variation of the CTLA-4 gene, which negatively regulate T cells is also associated with T1D in both species. Gene variants encoding the interacting, ligand-receptor molecules IL-2 [12] and CD25 [13], which are essential for peripheral tolerance, are present in mice and humans, respectively. Supporting the hypothesis that gene variants altering T1D susceptibility can influence immune tolerance more generally, the success of a tolerance induction protocol for islet transplantation is altered by T1D alleles at IL-2, even when tested in the context of the non-autoimmune B6 genome [14].

Signalling through the IL-2 receptor is critical for the function of the FOXP3⁺CD25⁺CD4⁺ regulatory T cells that dampen immune responses [15] and it appears that their function is reduced in T1D patients and NOD mice [16–18]. In analysing the immune responses of IL-2 congenic mice, we observed a 2-fold increase in IL-2 production with the presence of a T1D

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resistance allele at *Ii2* [12]. Augmented FOXP3⁺CD25⁺CD4⁺ regulatory T cell function and decreased islet-specific effector CD8⁺ T cell proliferation in the pancreatic lymph nodes with subsequent migration to the islets also positively correlated with T1D resistance alleles. Our findings likely explain the observations that the IL-2 gene region also controls susceptibility to thymectomy-induced ovarian dysgenesis [19], a disease that is initiated by the reduction of FOXP3⁺CD25⁺CD4⁺ regulatory T cells caused by neonatal thymectomy [20]. These data are also consistent with the observation that neutralizing anti-IL-2 antibodies delivered *in vivo* can induce autoantibodies as well as cellular infiltrates in a variety of tissues [21]. We hypothesize that there has been selective pressure to maintain optimal levels of IL-2 production since excessive IL-2 could reduce immune responsiveness via the induction of overly potent regulatory T cells, while too little IL-2 increases the likelihood of autoimmune responses to reproductive or other organs because effector mechanisms are not held in check by regulatory T cells. Only small differences in IL-2 production are needed for significant biological effects and we showed haploinsufficiency at the IL-2 gene increased T1D susceptibility significantly [12].

The single nucleotide polymorphism (SNP), or SNPs, in the IL-2 gene that cause different levels of IL-2 production in mice having a susceptibility or resistance allele have not been defined [12]. However, from a sequence comparison of different T1D resistant and susceptible IL-2 haplotypes, the causative variant does not alter the coding region and is not within the well-defined promoter region. Disease-associated candidate SNPs are within introns and the extended 5' and 3' regions of the IL-2 gene and levels of IL-2 pre-mRNA correlate with genotype. Together, these data support the hypothesis that T1D-associated variants affect promoter accessibility and therefore transcription by altering the efficiency of chromatin remodelling in response to differentiation [12].

It remains a technical challenge to measure genotype-determined differences in protein or mRNA expression that are less than two-fold. Biological variation that occurs in inbred mouse strains, assay variation, and confounding influences in outbred populations from other variant loci that affect the pathway under examination can all blur a genotype phenotype relationship. One strategy to compare the effects of variant alleles on the expression of disease-associated genes is to study them in a heterozygous individual or animal using allele-specific assays. Such assays utilize properties conferred either by the known causative SNP or most associated SNP (or a SNP in linkage disequilibrium with this SNP) to discriminate which allele is producing the RNA product being measured. This experimental design ensures that the allelic products have arisen from cells subjected to identical environmental and activation conditions.

In studies of human CTLA-4 isoforms, we made use of an allele-specific assay known as hot-stop PCR [22] to assess the relative amounts of the full-length (4 exons) and the delta exon 3 isoforms present in individuals heterozygous for a susceptibility and resistant CTLA-4 allele [23]. This experimental approach confirmed results from a QPCR analysis demonstrating a less than 2-fold difference in the expression of the delta exon 3 isoforms levels in the two groups of homozygous donors. The hot-stop analysis also demonstrated that no difference was observed in the allele-specific expression of the full-length isoform.

By QPCR we observed that the B6 IL-2 allele makes approximately two fold more IL-2 pre-mRNA and mRNA than the NOD allele [12]. To confirm this difference, allele-specific pyrosequencing [24] was performed using cDNA derived from RNA isolated from activated (NOD × NOD.B6 *Idd3*)F1 T cells, which are heterozygous for the IL-2 gene [12]. We observed that the B6 allele was transcriptionally more active than the NOD allele with approximately 60-70% of the IL-2 mRNA derived from the B6 allele. When extrapolated to the homozygous genotypes, this equates to the approximately two-fold difference observed

by QPCR. However, the reliability of the pyrosequencing technique was dependent on having samples with a relatively high level of mRNA for the gene being measured. We therefore sought additional methods for analysing allele-specific expression differences in heterozygous samples.

We developed an allele-specific expression assay capable of analysing even rare pre-mRNAs and mRNA+pre-mRNAs based on the technique of Frommer *et al.* [25] to calculate the percentage methylation at CpG sites in genomic DNA by cloning, sequencing and counting the number of clones that had either a C or G at the analysed site. The steps of this assay are depicted in Figure 1. The most critical aspect of this approach is the necessity that the causal variant, or a variant associated with the causal variant, is located within the transcribed region of the gene of interest. Since variants causing expression differences can occur outside of the pre-mRNA (i.e. those associated with certain categories of epigenetic changes), additional sequencing may be needed to identify 'read-out' SNPs. Once the target sequence is selected, PCR primers flanking the SNP are used to amplify cDNA and genomic DNA (as a control for equivalent expression). The products are cloned into a vector and transformed cells are analysed by colony PCR. The variant present in each PCR product is determined by sequencing or conventional genotyping assays.

In figure 2, the cloning-based allele-specific expression assay was examined for its ability to produce results similar to those obtained with QPCR and allele-specific pyrosequencing in the case of IL-2 mRNA expression from T1D susceptible and resistant alleles. Counting the number of clones that contained either the NOD or B6 allele revealed that the B6 allele was more transcriptionally active, with on average 70% of the IL2 transcripts derived from the B6 allele. Since these results confirmed those obtained for IL-2 gene expression when assessing allele-specific expression with the pyrosequencing-based technique [12], this adaptation of the Frommer *et al.* [25] technique represents another method that can be used to assess relatively small expression differences caused by disease-associated variants. An additional advantage of this adapted protocol is that the statistical power to detect small expression differences can be increased by genotyping larger numbers of clones obtained from each heterozygous sample examined.

It is likely that many of the gene variants that contribute to the susceptibility of common diseases will only modestly alter gene function or expression. Based on the fact that small differences in the level of IL-2 cause large changes in T1D frequency in NOD mice, we expect that the CD25 gene variants associated with human T1D [13] will also produce relatively subtle expression changes. As is the case for mouse IL-2, T1D-associated SNPs within CD25 are outside of the coding regions of the gene and therefore likely to control the levels of mRNA expression. We are currently using allele-specific approaches to study the expression of the human gene encoding CD25 as well as other genes associated with T1D in humans and in NOD mice.

The importance of variation in the IL-2 pathway for human autoimmune disease is reinforced by the reports that Celiac disease is associated with IL-2 SNPs [8] and T1D has strong evidence for an IL-2 gene association, however this latter finding requires replication [3, 5]. While the results from the NOD mouse [12] would predict that the human susceptibility allele at *IL2* produces less IL-2, the pleiotropic aspects of the IL-2 pathway could still yield surprises when the alleles are studied in detail. Although studies of the functional consequences of common alleles associated with autoimmune diseases are in their infancy, they will surely increase our understanding of disease pathogenesis and thereby provide information necessary for the development of disease biomarkers as well as therapeutic and preventative approaches.

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Abbreviations used in this paper

Idd	insulin dependent diabetes
T1D	type 1 diabetes
SNP	single nucleotide polymorphism
QPCR	quantitative PCR of cDNA obtained from the reverse transcription of cellular RNA.

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Figure 1.
Workflow of a cloned-based allele-specific expression assay.

Figure 2.

Allele specific expression analysis of the IL-2 gene. Three (NOD × NOD.B6 *Idd3*)F1 mice were stimulated *in vivo* with 5 µg anti-CD3 (clone 2C11) for one hour, and cDNA was made from DNase treated total RNA extracted from whole spleen. The PCR product generated using primers flanking a NOD/B6 SNP in intron 2 of IL-2 (forward primer: AAAGAATGGCCCAACTTTCA, reverse primer: TTTCATTGGGACAAATAGATTTTACA) was cloned into a topo vector (Invitrogen), and colony PCR performed on the transformed cells. Restriction digestion with *RsaI* (New England Biolabs), which cuts the cloned PCR product of the NOD allele but not that of the B6, was used to score each clone.