Immunogenetics of Type 1 Diabetes

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1. Introduction

Type 1 diabetes (T1D), also known as Insulin dependent diabetes mellitus (IDDM) is an incurable multi-factorial autoimmune disorder. The disease is characterized by the loss of insulin producing beta cells of the pancreas resulting in abnormal metabolism of glucose which may lead to ketoacidosis and several other complications like retinopathy, nephropathy and even cardio-vascular diseases and pre-mature deaths (Pociot & Mcdermott, 2002). World-wide disease affects 1 in 300-400 children (Todd, 1995). Population based data from South India shows the incidence of T1D for four year period to be 10.5/100,000/ year (Ramachandran et al., 1996). Similar prevalence of type 1 diabetes has been observed in North India. A study from district of Karnal in North India reported the prevalence to be 10.20/100,000 population, with a higher prevalence in urban (26.6/100,000) as compared to rural areas (4.27/100,000) (Kalra et al. 2010). T1D develops as a result of complex interaction of many genetic and environmental factors leading to autoimmune destruction of the insulin producing pancreatic beta cells. While 20 genomic intervals have been implicated for the manifestation of the disease (Pociot & Mcdermott, 2002), role of an intricate network of the products of these genes cannot be ruled out. However, unravelling different factors involved and how they interact in integrated networks is like solving a jigsaw puzzle which is the aim of our studies. Basic problem with T1D patients is that by the time they first report to the physician, most of their pancreatic beta cells are already destroyed which leaves the clinicain with no option but to give daily insulin injections. So, there is a need to identify the prediabetics before the onset of the disease and device ways to inhibit autoimmunity in them. Following sections will show the work done in our laboratory to understand the intricate networks in which the genes involved in immune responses interact and their implications.

2. Role of Major Histocompatibility Complex (IDDM1)

The Major Histocompatibility Complex (MHC) region on chromosome 6p21.31 has been shown to have major role in predisposition to get type 1 diabetes. It is also called IDDM1.

2.1 Genes and proteins of the Major Histocompatibility Complex (MHC)

The human MHC, Human Leukocyte Antigen (HLA) system is the most polymorphic system of the human genome with more than 5000 alleles. The alleles of HLA loci are co-dominant i.e. both the alleles at a particular locus are equally expressed. The genes of HLA code for
glycoprotein molecules which are expressed on nucleated cells and are responsible for the recognition of non-self from self. The function of MHC molecules is to present exogenous and endogenous antigens in the form of peptides to the T cells for subsequent immune response to take place. The gene map of the MHC region of man on chromosome 6p21.3 shows that it spans about 4 megabases (3,838,986 bp to be precise). It is the most gene-dense region of the human genome with 224 genes of which 128 are known to be expressed. 40% of the expressed genes in this region have immune related functions (Horton et al., 2004).

There are two types of MHC molecules: MHC Class-I and Class-II which differ from each other in their constituents as well as their functions.

### 2.1.1 MHC class-I genes and proteins

MHC class-I genes are expressed on all nucleated cells in the form of cell surface glycoproteins. Function of MHC class-I molecules is to present antigenic peptides to CD8+ cytotoxic T cells (CTLs). The classical class-I genes in humans are HLA-A, HLA-B and HLA-C. All these genes are very polymorphic with 1519 alleles for HLA-A locus, 2069 alleles for B-locus and 1016 alleles for C-locus and these numbers are increasing with the discovery of new alleles everyday.

The MHC class-I molecule is a hetero-dimer of a heavy alpha chain (about 40-45 KDa) and the light chain, beta 2 microglobulin ($\beta_2$m) of 12 KDa (Bjorkman et al., 1987). While the genes for the heavy chains i.e. the alpha chains are encoded on chromosome 6, the gene for $\beta_2$m is encoded on human chromosome 15. The alpha chain of the MHC class-I molecule has three domains alpha 1 ($\alpha_1$), alpha 2 ($\alpha_2$) and alpha 3 ($\alpha_3$). Alpha 1 ($\alpha_1$) and alpha 2 ($\alpha_2$) domains are the most polymorphic domains since they constitute the peptide binding groove of the MHC molecule. The genes encoding MHC class-I alpha chain have 8 exons with second and the third exons of the alpha chain gene being most polymorphic since they code for the $\alpha_1$ and $\alpha_2$ domains. The peptides that are presented by the MHC molecules have allele specific motifs, which means that certain peptides can be presented by certain MHC molecules. The affinity of the peptide to bind to the peptide binding groove is determined by the anchors present on the peptide binding groove where the peptides go and bind through hydrogen bonds. Specific motifs on the peptides determine which peptides would bind to which MHC molecule (Falk et al., 1991, Garrett et al., 1989).

### 2.1.2 MHC class-II genes and proteins

MHC class-II glycoproteins in humans are HLA-DR, -DP and -DQ. The MHC class-II molecule is a heterodimer of two polypeptide chains: an alpha (25-33 KDa) and a beta chain (24-29KDa) (Brown et al., 1993, De Vries & Van Rood, 1985). Unlike MHC class-I, both alpha and beta chains of the class-II molecule are encoded on chromosome 6. DRB1 gene encodes DR beta chain while DRA1 encodes DR alpha chain with 966 DRB1 alleles and 3 DRA1 alleles. Similarly DQB1 and DPB1 encode beta chains of DQ and DP molecules with 144 and 145 alleles respectively and DQA1 and DPA1 encode the alpha chains of DQ and DP molecules with 35 and 28 alleles respectively (Robinson et al., 2009).

While HLA class-I molecules are expressed on all nucleated cells, HLA class-II molecules are expressed on antigen presenting cells like macrophages, dendritic cells, B cells, thymic epithelium and activated T cells (Holling et al., 2004). The function of MHC class-II molecules is to present antigenic peptides to the CD4+ T helper cells (Th cells) which in turn initiate a cascade of immunological events resulting in activation of CD8+ cytotoxic T cells.
When a non-self antigen is presented to CD4+ T helper cells, they get activated and secrete certain cytokines like Interferon gamma and TNF-alpha in case of Th1 cells and IL-4, IL-5 and/or IL-6 in case of Th2 cells. While the cytokines secreted by Th1 cells activate the cytotoxic T cells which have already seen the antigen in the context of HLA class-I, Th2 cytokines activate the B cells to become plasma cells which make the antibodies against antigen they have seen. Thus an immune response takes place which varies in strength depending on the host factors and the peptides being presented.

There are about 50,000-100,000 MHC molecules on each cell. Most MHC molecules are occupied by self peptides and the T cells are tolerized against them during thymic education so that auto-immune responses do not take place, however, sometimes something goes awry and there is a break in the tolerance resulting in recognition of self as non-self by the immune system resulting in an auto-immune response. This could be due to low expression of some antigens in the thymus which may result in self-reactive T cells to reach the peripheral circulation. Or it could be due to escape of self-reactive T cells from clonal deletion during T cell development.

2.2 MHC and Type 1 diabetes
Despite so much polymorphism, significant increase of one or more alleles of HLA in a disease population as compared to healthy controls, suggests functional implications due to their role in antigen presentation. We observed a significant increase of DRB1*03:01 (p<10⁻⁶, Odds Ratio (OR)=11.0), DRB1*04:01 (p<0.01, OR=6.4) and DRB1*04:05 (p<0.03, OR=5) in the patients (Figure 1a) using high resolution typing method of polymerase chain reaction followed by hybridization with sequence specific oligonucleotide probes (PCR-SSOP) (Rani et al., 2004, Rani et al., 1999).

**a** shows the homozygosity and heterozygosity of predisposing and protective alleles significantly increased or reduced in the T1D patients as compared to controls. Homozygous DRB1*03:01/03:01, heterozygous DRB1*03:01/04:05, DRB1*03:01/04:01 and DRB1*03:01/X were significantly increased and DRB1*04:03/X and DRB1*07:01/X were significantly reduced in the T1D patients as compared to controls.
Our results were in concordance with earlier studies in North Indians (Gupta et al., 1991, Kanga et al., 2004, Mehra et al., 2002, Sanjeevi et al., 1999, Witt et al., 2002). However, we also observed DRB1*07:01 (p<7x10^{-6}, OR=0.16), DRB1*04:03 (p<0.02, OR=0.25) and DRB1*04:04 (p<0.05, OR=0.2) to be significantly decreased in the patients as compared to controls. We did not find any significant reduction of HLA-DR2 haplotype DRB1*15:01-DQB1*06:02 which has been shown to confer strong protection from T1D in most ethnic groups (Baisch et al., 1990, Pugliese et al., 1995), probably because this haplotype has been found with a low frequency of only 1.06% in North Indians (Rani et al., 1998). On the other hand, we observed a marginally reduced frequency of DRB1*15:06 in patients as compared to controls, which did not remain significant when p was corrected for the number of alleles tested for DRB1 locus (Rani et al., 2004).

Figure 1b shows the homozygosity and heterozygosity of DRB1*03:01 and DRB1*04 alleles significantly increased in T1D. Homozygous DRB1*03:01 (p<10^{-7}, OR=14.54), heterozygous DRB1*03:01/*04:05 (p<0.03, OR=10.9) and DRB1*03:01/*04:01 (p<0.01, OR=13) were significantly increased in the patients as compared to controls who lacked this heterozygous combination. Heterozygous 03:01/X (i.e. any other allele) (p<0.04, OR=1.89) was also significantly increased in the patients as compared to controls. Heterozygous DRB1*04:03/X (p<0.04, OR=0.22) and DRB1*07:01/X (p<10^{-7}, OR=0.066) were significantly reduced in the T1D patients as compared to controls suggesting their protective role. Significant protection has been shown to be associated with DRB1*04:03 allele in a Belgian study of diabetes (Van Der Auwera et al., 1995). DRB1*03:01, DRB1*04:01 and DRB1*04:05 have also been shown to be associated with T1D patients in Sardinians, black population from Zimbabwe, Lithuanians, Czecks, Lebanese, Brazilians and African Americans (Alves et al., 2009, Cucca et al., 1995, Ei Wafai et al., 1993, Garcia-Pacheco et al., 1992, Skrodeniene et al., Tait et al., 1995, Weber et al.). Cucca et al suggested that amino acid position β74 and β86 in DR beta chain are the key residues in the P4 and P1 pockets of the peptide binding groove of HLA-DR molecules (Cucca et al., 2001). A combined presence of Asp, Glu and Val in positions β57 (P9), β74 (P4) and β86 (P1) in protective DRB1*04:03 has been shown to be different from high risk DRB1*04:05 which has Ser, Ala and Gly at these positions. However, in the North Indians we observed DRB1*03:01 to be at highest risk and this allele has Asp, Arg, and Val in the three positions (Figure 2). A less predisposing allele in North Indians, DRB1*04:01 has Asp, Ala, Gly and the protective DRB1*04:04 and DRB1*07:01 have Asp, Ala, Val and Val, Gln and Gly in the three positions respectively. Thus, Asp, Arg and Val in DRB1*03:01 is entirely different from Val, Gln, and Gly in DRB1*07:01 which seems to be important in our study since all the four DR4 alleles are present in less than 10% of the patients or control samples. In essence, these data suggest that it is probably not β74 and β86 alone, rather an integration of all the pockets of the peptide binding groove that determines which peptide of an auto-antigen would bind to the MHC molecule and result in auto-aggression based on the thymic education.

We also studied the alleles of DQB1 locus. DQB1*02:01 which is linked to DRB1*03:01 was significantly increased (p<1x10^{-8}, OR=5.08) in patients (Figure 3a). However DQB1*03:02 and DQB1*03:07, alleles linked with DRB1*04:01, DRB1*04:03, DRB1*04:04 and DRB1*04:05 were not significantly increased in the patients because two of these alleles DRB1*04:01 and DRB1*04:05 were increased in the patients and the other two DR4 alleles DRB1*04:03 and DRB1*04:04 were significantly reduced in the patients. DQB1*03:01 (p<6x10^{-4}, OR=0.27) and
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DRB1*0301 DRB1*0701
DRB1*0401
DRB1*0405
DRB1*0404
DRB1*0403
P9-β57D
P9-β57D P9-β57D
P9-β57D P9-β57D
P9-β57D
P4-β74R
P4-β74A
P4-β74A
P4-β74A
P4-β74Q
P4-β74E
P1-β86V
P1-β86V
P1-β86V
P1-β86G
P1-β86G
P1-β86G

Fig. 2. Peptide binding groove of the predisposing and protective HLA-DRB1 alleles showing positions β57 (P9), β74 (P4) and β86 (P1) for predisposing DRB1*03:01, DRB1*04:01 and DRB1*04:05 and protective DRB1*07:01, DRB1*04:04 and DRB1*04:03 alleles.

DQB1*03:02 which was not significantly increased in the patients, showed a significant increase in heterozygous combination with DQB1*02:01 (p<2x10^{-5}, OR=34.16). In fact none of the controls had DQB1*02:01/*03:02 heterozygous combination. In a Swedish study, DQA1*0301/DQB1*0302 and heterozygous combinations of DQA1*0301/DQB1*0302 and DQA1*0201/DQB1*0501 have been shown to confer the highest susceptibility (Sanjeevi et al., 1995).

Some critical residues within the peptide binding sites of HLA-DQ beta chain have been proposed to play a crucial role in conferring predisposition to and protection from the diseases (Nepom & Kwok, 1998, Sheehy, 1992, Todd et al., 1987). Several studies have suggested that aspartic acid at DQβ residue 57 confers protection while DQB1 alleles with alanine at that position (DQB1*02:01 and DQB1*03:02) and DQA1 with arginine at position 52 (R52) confer susceptibility (Badenhoop et al., 1995, Chauffert et al., 1995, Todd et al., 1987). However, an individual can be either homozygous or heterozygous for alleles carrying
Fig. 3. Distribution of HLA-DQB1 alleles in T1D patients and controls. a shows DQB1*02:01 was significantly increased and DQB1*03:01 and DQB1*05:03 were significantly reduced in T1D patients as compared to controls. b. Homozygous and heterozygous DQB1 alleles in T1D. Homozygous DQB1*02:01/*02:01 and heterozygous DQB1*02:01/*03:02 were significantly increased and DQB1*03:02/X were significantly reduced in T1D patients as compared to controls (Rani et al., 2004).

Asp^{57} in DQB1 or Arg^{52} in DQA1. Our in-depth investigation revealed that when DR3 homozygosity was considered along with codon 57 of DQB1 and codon 52 of DQA1, the only combination that was significantly increased in the patients group as compared to the controls was DRB1*03:01,03:01-DQB1*XX-DQA1*RR, suggesting that DRB1*03:01 association is primary since the DQB1 and DQA1 alleles which are in linkage disequilibrium with DRB1*03:01 have non-Asp57 (DQB1*X) and Arg52 (DQA1*R), respectively (Rani et al., 1999).

3. Insulin linked polymorphic region in T1D (IDDM2)

Insulin linked polymorphic region (IDDM2) consists of a highly polymorphic stretch of 14-15 base pair repeats of DNA lying 365 bp upstream of the initiation of transcription of the insulin (INS) gene. IDDM2 has been shown to have a role in transcription of insulin in thymus. Several forms of IDDM2 have been reported based on the number of repeats (Bell et al., 1981, Kennedy et al., 1995). These INS-variable number of tandem repeats (VNTR) are divided into three different classes based on their sizes: class-I (26-63 repeats), Class II (about 85 repeats) and class III (141-209 repeats) (Bell et al., 1982, Bennett et al., 1995, Rotwein et al., 1986). T1D is associated with class I homozygosity (Bell et al., 1981, Bennett et al., 1995, Kennedy et al., 1995, Lucassen et al., 1993). We studied INS-VNTR Class-I and Class-III alleles based on typing for Insulin gene 1127 Pst I site (3’end) by PCR-RFLP as described by Pugliese et al (Pugliese et al., 1997).

Table 1 shows the frequencies of Insulin VNTR in T1D patients and healthy controls. While the frequency of class-I VNTR was increased significantly in the patients, class-III VNTR was decreased in them as compared to the controls. However, when the genotypes were studied, class I homozygosity was considerably increased in the patients as compared to controls,
giving an Odds ratio of 7.8. Class I, III heterozygosity was significantly reduced in the patients (Rani et al., 2004).

<table>
<thead>
<tr>
<th>INS-VNTR</th>
<th>DIABETES</th>
<th>CONTROLS</th>
<th>p value</th>
<th>OR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>Class I</td>
<td>108</td>
<td>98.2</td>
<td>85</td>
<td>89.47</td>
</tr>
<tr>
<td>Class III</td>
<td>64</td>
<td>58.2</td>
<td>87</td>
<td>91.57</td>
</tr>
<tr>
<td>Genotypes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Class I, I</td>
<td>46</td>
<td>41.8</td>
<td>8</td>
<td>8.42</td>
</tr>
<tr>
<td>Class I, III</td>
<td>62</td>
<td>56.4</td>
<td>77</td>
<td>81.05</td>
</tr>
<tr>
<td>Class III, III</td>
<td>2</td>
<td>1.8</td>
<td>10</td>
<td>10.52</td>
</tr>
</tbody>
</table>

Table 1. INS-VNTR allele frequencies and Genotype frequencies in T1D patients and controls.

### 3.1 Simultaneous presence of predisposing HLA-DRB1 and INS-VNTR alleles

MHC and VNTR are encoded on two different chromosomes. However, they may have integrated roles in manifestation of T1D due to the functional implications of these genes. So, we studied if simultaneous presence of the predisposing alleles of the two genes had any role to play in manifestation of T1D.

Our investigation revealed that homozygous Class-I INS-VNTR along with homozygous or heterozygous DRB1*03:01 were significantly increased in the T1D patients (p<1x10^-8) with a Relative Risk of 70.81 (Rani et al., 2004). In fact, none of the controls had homozygous Class-I INS-VNTR along with DRB1*03:01 in homozygous or heterozygous state. This combination gives a positive predictive value (PPV) of 100% with a specificity of 100% and sensitivity of 32.63% since only 32.63% of the patients showed this combination. Since DRB1*03:01 homozygosity is significantly increased in the patients, homozygous DRB1*03:01 and heterozygous DRB1*03:01 only with DRB1*04:01 and DRB1*04:05 along with heterozygous I, III-INS-VNTR may also be considered as predisposing since it gives a relative risk of 10.55 (Rani et al., 2004).

If we add all these predisposing combinations i.e. simultaneous presence of homozygous or heterozygous HLA-DRB1*03:01 along with homozygous (Class-I, I) or heterozygous (I, III) VNTR class-I and III, 50.53% of the patients as compared to only 1.4% of the controls had these combinations giving a relative risk of 48.67. This combination gives a PPV of 97.96% with a specificity of 98.6% and sensitivity of 50.5% since only 50.5% of the patients showed this combination. Thus, our results showed that: (1) homozygous or heterozygous DRB1*03:01 along with homozygous Class-I INS-VNTR and (2) homozygous DRB*03:01 and heterozygous DRB1*03:01 only with DRB1*04:01 or DRB1*04:05 with heterozygous Class-I/III INS-VNTR may be used to predict a pre-diabetic before the onset of the disease in North Indian high risk group (Rani et al., 2004). However, typing a larger cohort may be required to confirm such a major increase in risk.

Pathogenesis of T1D is extremely complex. Significant association with HLA-DRB1*03:01 and INS-VNTR Class-I may have functional implications. Increase in frequency of particular MHC allele suggests that these molecules may be preferentially presenting certain autopeptides to the T cells resulting in subsequent autoimmune responses. Studies on INS-VNTR, however, have shown that class-III alleles are associated with 2 to 3 fold higher
mRNA levels of insulin than Class-I in thymii of fetuses, suggesting poor expression of thymic INS expression resulting in poor thymic education for insulin in people with homozygous Class-I,II and class-I,III VNTRs resulting in break of tolerance in predisposed individuals. However, higher expression of insulin in the thymii of individuals with homozygous class-III,III may be able to facilitate immune tolerance induction, as a mechanism for dominant protective effect of Class-III alleles (Pugliese et al., 1997, Vafiadis et al., 1997).

Our results are contrary to that of Veijola et al. (Veijola et al., 1995) on Finnish children who showed that both 5' and 3' INS loci showed an association with T1D in non-DR3/non-DR4 patients (Veijola et al., 1995). However, in our studies only 9.47% of the non-DR3/DR4 patients were homozygous for Class-I INS-VNTR as compared to 4.2% controls and this difference was not significant statistically. Julier et al. (Julier et al., 1991), on the other hand, had reported that the risk contributed by the INS region was increased in DR4-positive patients. Again, in our study only 6.32% of the patients as compared to 1.39% of controls had INS-VNTR class-I homozygosity with DRB1*04:01 and DRB1*04:05 alleles and this difference was not significant statistically.

4. Cytokine genes

Cytokines are the coordinators of the immune system that interact in integrated networks and functions of one cytokine may be modulated or substituted by another (Bidwell et al., 1999). A cascade of cytokines are involved in pro-inflammatory auto-immune responses in T1D. Single nucleotide polymorphisms (SNPs) in different pro-inflammatory and anti-inflammatory cytokine genes at certain defined regions have been shown to be associated with differential amount of their production (Asderakis et al., 2001, Awad et al., 1998, Bittar et al., 2006, Burzotta et al., 2001, Fishman et al., 1998, Louis et al., 1998, Pociot et al., 1993). Pro-inflammatory cytokines and their integrated influences are known to regulate complex immune responses during autoimmune destruction of tissues (Rabinovitch, 1994). Hence it is necessary that they are studied and analysed in context of each other and not in isolation from each other. We had reported for the first time the integration and interaction of TNF-α gene with other cytokine genes and HLA-DRB1 and B loci alleles (Kumar et al., 2007).

We studied the cytokine gene polymorphism using XIIIth International Histocompatibility Workshop’s (IHWC, Heidelberg kit) and One lambda’s cytokine typing kits (Canoga Park, CA, USA) based on Polymerase Chain reaction (PCR) with sequence specific primers (PCR-SSP). PCR-SSPs were done for IFN-γ(A874T) (14), TNF-α (G308A) (15), IL-6 (G174C) (9), IL-10 (A1082G, T819C, C592A) (16), and TGFβ1 (Tcdn10C, Gcdn25C) (11). T→C substitution in nucleotide 29, codon 10 of the first exon of TGFβ1, changes the amino acid Leu → Pro. Similarly G→C substitution in nucleotide 74, codon 25 of first exon of TGFβ1, changes the amino acid Arg → Pro. However, since we are studying the SNPs in the two codons, we will refer to the SNPs in codons 10 and 25 hereafter. and not the resultant amino acids to avoid any confusion and to maintain consistency with the other SNPs.

Our results showed that the high producing genotype of TNF-α-308GA and AA were significantly increased and low producing genotype GG was significantly reduced in TID patients as compared to controls (p < 7 x10^-6). None of the other cytokine genes showed any significant difference between the patients and controls.
4.1 Simultaneous presence of TNF-α genotypes with IFN-γ, IL-6, IL-10 and TGF-β1 genotypes and haplotypes

TNF-α, IFN-γ, IL-10, IL-6 and TGF-β1 genes are localized on different chromosomes. TNF-α is encoded on chromosome 6p21.3, IFN-γ is encoded on 12q14, IL-10 is encoded on 1q31-q32, IL-6 is encoded on 7p21 and TGF-β1 is encoded on 19q13.2. However, the products of these genes interact in integrated networks. Since only TNF-α showed a significant association with T1D, we studied whether simultaneous presence of TNF-α genotypes with different genotypes of the other cytokines in an individual could suggest an interaction between these cytokine genes.

<table>
<thead>
<tr>
<th>Other cytokines Genotype / haplotype</th>
<th>TNF-α GA/AA</th>
<th></th>
<th>TNF-α GG</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TID No. (%)</td>
<td>Controls No. (%)</td>
<td>p (OR 95% CI)</td>
<td>TID No. (%)</td>
</tr>
<tr>
<td>IFN-γ Int +874</td>
<td>N=235 N=128</td>
<td></td>
<td>N=235 N=128</td>
<td></td>
</tr>
<tr>
<td>AA (L)</td>
<td>41 (17.4)</td>
<td>9 (7.0)</td>
<td>0.003*</td>
<td>2.79 (1.25-6.42)</td>
</tr>
<tr>
<td>TA+TT (H)</td>
<td>56 (23.8)</td>
<td>15 (11.07)</td>
<td>0.003*</td>
<td>2.39 (1.24-4.66)</td>
</tr>
<tr>
<td>IL-6 -174</td>
<td>N=235 N=127$</td>
<td></td>
<td>N=235 N=127$</td>
<td></td>
</tr>
<tr>
<td>CC (L)</td>
<td>11 (4.7)</td>
<td>1 (0.78)</td>
<td>0.03*</td>
<td>4.3 (1.21-14.56)</td>
</tr>
<tr>
<td>GG+GC (H)</td>
<td>86 (36.6)</td>
<td>23 (18.1)</td>
<td>0.0001*</td>
<td>2.61 (1.5-4.56)</td>
</tr>
<tr>
<td>IL-10 Haplotypes*</td>
<td>N=235 N=128</td>
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<td>N=235 N=128</td>
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<tr>
<td>Low secretor</td>
<td>45 (19.2)</td>
<td>16 (12.7)</td>
<td>0.068</td>
<td>1.65 (0.86-3.22)</td>
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<tr>
<td>High Secretor</td>
<td>52 (22.1)</td>
<td>8 (6.25)</td>
<td>0.0001*</td>
<td>4.26 (1.9-10.1)</td>
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<td>TGF-β1 Haplotypes*</td>
<td>N=235 N=128</td>
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<td>N=235 N=128</td>
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<tr>
<td>Low secretor</td>
<td>8 (3.4)</td>
<td>1 (0.8)</td>
<td>0.11</td>
<td>3.17 (0.87-12.11)</td>
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<td>High Secretor</td>
<td>89 (37.8)</td>
<td>23 (18.0)</td>
<td>0.00004*</td>
<td>2.8 (1.6-4.86)</td>
</tr>
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</table>

N=Total number of samples studied. $Number of control samples studied for IL-6 were 127, one sample could not be typed due to PCR failure. TNF-α GA/AA have been combined as high secretor genotypes.

# Corrected p value (pc) not significant, @ Corrected p value (pc) significant,

*IL-10 : haplotype combinations -1082/-819/-590 : GCC,GCC; GCC,ACC; GCC,ATA= high secretors; ACC,ACC; ACC,ATA = Low secretors.

TGF-β1 haplotype combinations Cdn10/Cdn25 : TG,TG; TG,CG; TG,CC; CG,CG =High secretors, CG,CC; CC,CC = Low secretors.

Table 2. Simultaneous presence of TNF-α genotypes with IFN-γ, IL-6, IL-10 and TGF-β1 genotypes and haplotypes (Kumar et al., 2007).
Table 2 shows the simultaneous presence of high and low secreting genotypes of TNF-α, along with IFN-γ, IL-6, IL-10 and TGF-β1 genotypes and haplotypes. When IFN-γ was studied by itself, it did not show any significant difference between patients and controls. However, when studied in the context of TNF-α -308G/A, both low and high secretor genotypes, IFN-γ (+874AA and TA+TT respectively) along with high secretor genotypes of TNF-α -308 GA+AA were significantly increased in patients as compared to controls, suggesting its effect is rather neutral. However, low producer genotypes of TNF-α -308GG along with low producer genotype of IFN-γ +874 AA seems to be protective. Interestingly, 66.7% of the patients who had low producer genotype of TNF-α -308GG had high producer genotype of IFN-γ +874 TA+TT. Hence, in the absence of high secretor genotype of TNF-α, IFN-γ may have a role in autoimmune destruction of pancreatic beta cells. IFN-γ acts singularly as well as synergistically with other inflammatory stimuli to induce NO production which can be cytotoxic and thus has been implicated in pathogenesis of certain autoimmune and inflammatory diseases (Mccartney-Francis et al., 1993).

Similarly, promoter SNPs of IL-6 -174G/C did not show any significant difference between patients and controls, but when studied in the context of TNF-α -308G/A, high producer genotypes, IL-6 -174 GG+GC (Fishman et al., 1998) were increased in patients with TNF-α -308 GA+AA. Kristiansen and Mandrup-Poulsen (Kristiansen & Mandrup-Poulsen, 2005) have shown that IL-6 promotes islet inflammation but is unable to promote β-cell destruction for which other pro-inflammatory cytokines are needed. The other pro-inflammatory cytokine playing a role in destruction β-cells in the present scenario could be

<table>
<thead>
<tr>
<th>HLA-B-DRB1-haplotypes</th>
<th>Patients N=210</th>
<th>Controls N=91</th>
<th>p</th>
<th>OR (95% C.I.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B<em>8-DRB1</em>03</td>
<td>69</td>
<td>3</td>
<td>0.003</td>
<td>3.55</td>
</tr>
<tr>
<td>B<em>8-Non DRB1</em>03</td>
<td>2</td>
<td>1</td>
<td></td>
<td>0.639</td>
</tr>
<tr>
<td>B<em>50 – DRB1</em>03</td>
<td>41</td>
<td>3</td>
<td>0.003</td>
<td>7.11</td>
</tr>
<tr>
<td>B<em>50 – Non-DRB1</em>03</td>
<td>6</td>
<td>4</td>
<td></td>
<td>0.355</td>
</tr>
<tr>
<td>B<em>58 – DRB1</em>03</td>
<td>36</td>
<td>5</td>
<td>0.003</td>
<td>2.83</td>
</tr>
<tr>
<td>B<em>58 – Non DRB1</em>03</td>
<td>3</td>
<td>8</td>
<td></td>
<td>0.165</td>
</tr>
<tr>
<td>NonB8/B50/58-DRB1*03</td>
<td>35</td>
<td>6</td>
<td></td>
<td>2.83</td>
</tr>
<tr>
<td>NonB8/B50/B58/non DRB1*03</td>
<td>38</td>
<td>59</td>
<td>0.003</td>
<td>18.1</td>
</tr>
</tbody>
</table>

* No. of patients /Controls with the haplotypes shown in the first column.

Table 3. Comparison of HLA-B-DRB1 haplotypes showing significant association, between patients and controls (Kumar et al., 2007)
TNF-α in patients with GA and AA genotypes and IFN-γ in patients with TNF-α GG genotype. High producer genotype IL-6 -174 GG+GC along with low producer genotype of TNF-α -308GG seems to be protective. Different haplotypes of IL-10 based on SNPs in the promoter region have been shown to be associated with quantity of IL-10 production in-vitro (Asderakis et al., 2001, Stanilova et al., 2006). The frequency of low producer haplotype of IL-10 ATA (haplotype with positions -1082/-819/-590) has been shown to be increased in the adult onset patients in Japan, with no significant differences between T1D patients and controls (Ide et al., 2002). Reynier et al (Reynier et al., 2006) did not see any significant association of IL-10-1082G/A with T1D in French population. However, they did observe a significant association of IL-10 -1082 polymorphism to be associated with GAD and IA-2 antibody at clinical onset. In our study also, we did not observe any significant difference between T1D patients and controls when IL-10 was studied by itself. However, simultaneous presence of high producer genotypes of TNF-α -308 GA+AA and high producer haplotypes of IL-10 in the patients may have a role in recruitment of islet specific CD8+ T cells and thus may have a role in insulitis through ICAM-1 dependent pathway. In non-obese diabetic (NOD) mice (animal model for human Type 1 diabetes) pancreatic IL-10 has been shown to hyper-induce ICAM-1 expression on vascular endothelium. However, in the absence of ICAM-1, insulitis and diabetes could be prevented, thus providing evidence that IL-10 is sufficient to drive pathogenic autoimmune responses and accelerated diabetes via an ICAM-1 dependent pathway (Balasa et al., 2000). Presence of IL-10 during early stages of IDDM has also been shown to favor the generation of effector CD8+ T cells leading to accelerated diabetes in NOD mice (Balasa et al., 2000). Treatment of young mice with anti-TNF-α and anti-IL-10 mAb has also been shown to prevent diabetes and insulitis (Lee et al., 1996, Yang et al., 1994).

Similarly, when TGF-β1 was studied by itself, no significant difference was observed between patients and controls. A significant increase of high producer haplotypes of TGF-β1 with TNF-α -308 GA+AA and a significant decrease of high producer haplotypes of TGF-β1 with TNF-α -308GG in T1D patients as compared to controls was observed. These results show that different cytokines work in concert with each other and may alter or modulate their functions depending on the milieu. TGF-β1 has been shown to be an extremely potent chemotactic factor in-vitro which influences monocyte recruitment and accumulation via increased expression of α and β integrins (Wahl et al., 1993). It has been shown to rapidly and transiently up-regulate α-4 integrin dependent adhesion of leukocyte cell lines and peripheral blood lymphocytes (Bartolome et al., 2003). α-4 integrin, in turn, has been shown to play a prominent role in the spontaneous development of insulitis and diabetes in NOD mice (Yang et al., 1997). Increased levels of TGF-β1 have been associated with destruction of pancreatic beta cells and pathogenesis of diabetic complications (Korpinen et al., 2001). Hence, in the presence of high secretors of TNF-α, the high secretor genotypes of TGF-β1 may have a role both in destruction of pancreatic beta cells as well as in migration of CD4+ and CD8+ T cells into the pancreas (Insulitis) through α-4 integrin, which act against pancreatic beta cells along with Nitric oxide mediated cytotoxicity. Under these circumstances, TGF-β1 may not be able to arrest the pro-inflammatory functions of TNF-α and IFN-γ.

So, our data provides circumstantial evidence justifying the presence of high secretor genotypes of TNF-α -308GA and AA along with high secretor genotypes of IL-6, IL-10 and TGF-β1 and provide an immunogenetic basis for the autoimmune responses in T1D. The data suggest that the beta cell destruction in T1D may be mediated by both CD4+ T helper
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cells and CD8+ cytotoxic T cells recruited through Integrins and ICAM-1 dependent pathways in the pancreas for which cytokine genes seem to play a pivotal role.

4.2 TNF-α and HLA genes

TNF-α gene is very closely linked to the MHC. Deng et al (Deng et al., 1996) observed that the TNF-α associations in Caucasians and Chinese of Taiwan, may be due to its being in linkage disequilibrium with DR3-DQB1*0201 haplotype. We too had observed DRB1*03:01, DRB1*04:01 and DRB1*04:05 to be associated with T1D in North Indians (Rani et al., 2004). Hence, we wanted to study if the TNF-α association was independent of these alleles or due to linkage disequilibrium (LD) between TNF-α -308A and the predisposing DRB1 alleles. Interestingly, the LD analysis showed that both TNF-α -308A as well as TNF-α -308G alleles are in LD with DRB1*03:01, the most predisposing HLA-DRB1 allele, suggesting that the effect of TNF-α -308A is not because of its being in LD with HLA-DRB1*03:01, the predisposing MHC allele.

Since TNF-α locus is very closely linked to HLA-B locus, we also studied the alleles of B-locus for a possibility of TNF-α -308A allele being in LD with one of the B-locus alleles. Surprisingly we observed LD between TNF-α -308G with B*08 and TNF-α -308A allele with HLA B*50:01 and B*58:01. All the three B-locus alleles B*08:01, B*50:01 and B*58:01 were in linkage disequilibrium with DRB1*03:01 (Table 3). Because of HLA-B*08 being in LD with TNF-α -308G, B*08:01-DRB1*03:01 haplotype was also in LD with TNF-α -308G (Table 4).

<table>
<thead>
<tr>
<th>HLA-B-TNF-α-DRB1- haplotypes</th>
<th>Number of haplotypes observed 2N=418</th>
<th>Haplotype frequencies</th>
<th>Dallele#</th>
</tr>
</thead>
<tbody>
<tr>
<td>B<em>8- TNF-α -308A-DRB1</em>03</td>
<td>19</td>
<td>0.045</td>
<td>-0.013</td>
</tr>
<tr>
<td>B<em>8- TNF-α -308G-DRB1</em>03</td>
<td>75</td>
<td>0.179</td>
<td>0.015</td>
</tr>
<tr>
<td>B<em>50- TNF-α -308A-DRB1</em>03</td>
<td>42</td>
<td>0.1</td>
<td>0.0329</td>
</tr>
<tr>
<td>B<em>50- TNF-α -308G-DRB1</em>03</td>
<td>32</td>
<td>0.076</td>
<td>-0.0023</td>
</tr>
<tr>
<td>B<em>58- TNF-α -308A-DRB1</em>03</td>
<td>33</td>
<td>0.079</td>
<td>0.0141</td>
</tr>
<tr>
<td>B<em>58- TNF-α -308G-DRB1</em>03</td>
<td>28</td>
<td>0.067</td>
<td>-0.0032</td>
</tr>
</tbody>
</table>

Table 4. Linkage disequilibrium analysis of HLA-B- TNF-α -DRB1 haplotypes prevalent in T1D patients from North India (Kumar et al., 2007).

However, B*50:01-DRB1*03:01 and B*58:01-DRB1*03:01 haplotypes were in LD with TNF-α -308A allele. We observed 48.8 % of the patients had B*08/non-B*08/non-B*50:01/non-B*58:01-TNF-α-308G-DRB1*03:01 haplotypes as compared to 34 % with B*50:01/ B*58:01-TNF-α -308A-DRB1*03:01 haplotypes. With this in-depth analysis, it becomes clear that the effect of TNF-α -308A allele is not because of its being in LD with DRB1*03:01, B*08:01, B*50:01 or B*58:01, but due its functional implications and its integrated effect with other cytokines. In conclusion, while the MHC may be involved in auto-antigen presentation, TNF-α and other cytokines play an integrated role in destruction of the pancreatic beta cells though enrichment and recruitment of autoantigen specific CD4+ and CD8+ T cells which have immunogenetic bases (Figure 6).

5. Vitamin D receptor

Vitamin D Receptor (VDR) is a ligand dependent transcription factor that belongs to the super family of the Nuclear Hormone Receptors (Evans, 1988). The ligand for VDR is
Vitamin D3 i.e., 1,25-(OH)₂D₃ which mediates its biological actions through VDR. When 1,25-(OH)₂D₃ binds to VDR, it induces conformational changes in VDR promoting its heterodimerization with Retinoid X Receptor (RXR), followed by translocation of this complex into the nucleus. The RXR-VDR heterodimer in turn binds to the vitamin D₃ responsive elements (VDRE) in promoter regions of vitamin D responsive genes (Boonstra et al., 2001). This results in the regulatory function of Vitamin D₃. In the absence of classical responsive elements, 1,25-(OH)₂D₃ may controls the expression of some genes like cytokine genes by targeting inducible transcription factors like NFAT in IL-2 in a sequence specific manner (Takeuchi et al., 1998). 1,25-(OH)₂D₃ has been shown to have an important immunomodulatory role since it represses expression of Th1 cytokines like IL-2 (Alroy et al., 1995, Bhalla et al., 1984), IFN-γ (Cippitelli & Santoni, 1998) and IL-12 (D’ambrosio et al., 1998) and up regulates the production of Th2 cytokines IL-4 and TGF-β1 (Cantorna et al., 1998). 1,25-(OH)₂D₃ has been shown to have an important immunomodulatory role since it represses expression of Th1 cytokines like IL-2 (Alroy et al., 1995, Bhalla et al., 1984), IFN-γ (Cippitelli & Santoni, 1998) and IL-12 (D’ambrosio et al., 1998) and up regulates the production of Th2 cytokines IL-4 and TGF-β1 (Cantorna et al., 1998). It has been shown to enhance the development of TH2 cells via a direct effect on naive CD4⁺ cells (Boonstra et al., 2001). Besides, 1,25-(OH)₂D₃ has also been shown to modulate the expression of HLA class-II alleles on monocytes and human bone cells (Rigby et al., 1990, Skjodt et al., 1990).

Studies have shown that administration of Vitamin D3 in NOD mice, before the onset of Insulitis, can effectively prevent the disease progression. However, when administered after the establishment of insulitis, vitamin D₃ was not as effective. Similarly, in humans too, vitamin D supplementation in early childhood has been shown to reduce the incidence of TID (Hypponen et al., 2001, Jones et al., 1998). Since 1,25-(OH)₂D₃ mediates its effect through VDR, we studied the VDR gene polymorphisms and their interaction with the most predisposing MHC alleles to investigate their role, if any, in the pathophysiology of TID.

The VDR SNPs studied include the T>C SNP in exon2 initiation codon detected with FokI restriction enzyme (Gross et al., 1996), the A>G SNP detected with BsmI (Morrison et al., 1992) and G>T SNP detected with Apal (Faraco et al., 1989) located in Intron 8, and a silent C>T SNP (Durrin et al., 1999) detected with TaqI, located in Exon 9. These SNPS were studied using PCR amplification and restriction digestion by the aforesaid enzymes as described earlier (Faraco et al., 1989, Hustmyer et al., 1993). We also studied the interaction between VDR alleles and predisposing HLA alleles using LD based statistics (Zhao et al., 2006) and subsequently sequenced the promoter region of the predisposing MHC allele to detect the VDRE sequence which has been shown to modulate the expression of the HLA alleles (Ramagopalan et al., 2009), suggesting the functional implications of the statistically significant interaction (Israni et al., 2009). We further provided documentary evidence that expression of HLA class-II molecules was being modulated by vitamin D₃.

### 5.1 VDR FokI, BsmI, Apal and TaqI genotypes and haplotypes in T1D patients

While there were no significant differences in the genotypes of Apal and TaqI in patients and controls. FokI ‘ff’ was significantly increased in the patient group as compared to controls and BsmI ‘bb’ was significantly decreased in the patient group. However, these differences did not remain significant after Boneferroni’s correction (Israni et al., 2009).

Haplotype analysis was carried out for the four restriction sites studied in the VDR gene in patients and controls using SHÉsis program (http://202.120.7.14/analysis/myAnalysis.php) (Shi & He, 2005). Additionally, Famhap (http://famhap.meb.uni-bonn.de ) was used to confirm the frequencies of the haplotypes. Haplotype FBAₜ and fBAT were significantly increased in T1D patients and fBAT was significantly reduced in them as compared to controls.
5.2 Gene to gene interaction of VDR haplotypes with predisposing HLA alleles

Simultaneous presence of different VDR haplotypes along with the predisposing HLA alleles was studied in patients. Interestingly, simultaneous presence of haplotypes FBAT and FbaT along with the predisposing DRB1 alleles was significantly increased while the same haplotypes were protective when associated with non-predisposing alleles of DRB1. Similar results were obtained with other haplotypes like FBAT, fBAT and fbaT in association with the predisposing HLA-DRB1 alleles (Israni et al., 2009).

To study the interaction between two unlinked loci i.e., VDR and the predisposing HLA-DRB1 alleles, we used LD based statistics as described by Zhao et al (Zhao et al., 2006). The analysis revealed that F and T alleles in the exons 2 and 9 for FokI and TaqI restriction sites respectively showed significant interactions with predisposing HLA-DRB1 allele DRB1*03:01 (Israni et al., 2009).

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Fig. 4. HLA-DRB1*03:01 promoter sequence from 3 subjects suffering from T1D and 3 normal healthy individuals homozygous for DRB1*03:01. Important regulatory elements like S-box, X-box, Y-box, CCAAY-box, TATA-box and VDRE are highlighted. Star (*) in the last row shows homology and dots (.) shows nucleotide substitution in one or more samples at that particular site and dashes(-) represent gaps inserted to minimize the homology. (Israni et al., 2009).
5.3 Sequence analysis of HLA DRB1*0301 promoter region
Amongst the predisposing HLA-DRB1 alleles, majority of the patients (85.9%) had DRB1*03:01. Thus, we sought to look for the VDRE in the promoter region of the allele. The promoter regions of 3 T1D subjects and 3 healthy controls homozygous for HLA-DRB1*03:01 were amplified and sequenced to determine the VDRE variants in the North Indian population. Sequences were aligned using ClustalW2, and the presence of a VDRE was confirmed in-silico using JASPAR_CORE version 3.0 database using default conditions (Sandelin et al., 2004). Figure 4 shows the HLA-DRB1*03:01 promoter sequences showing the localization of vitamin D response element (VDRE) in the promoter region of HLA-DRB1*0301 from the 6 subjects.
Interestingly, the alignment showed exactly the same sequence of VDRE in the promoter region of HLA-DRB1*03:01 which has been shown to influence the expression of HLA DRB1*15:01 by Ramagopalan et al (Ramagopalan et al., 2009) suggesting the bases for interaction of VDR with HLA-DRB1*03:01

5.4 Altered expression of HLA-DRB1*03:01 by 1,25-(OH)₂D₃ (Calcitriol)

5.4.1 Flow cytometry
To study if vitamin D3 administartion would alter the expression of MHC class-II, we stimulated HLA-DRB1*03:01 homozygous B-lymphoblastoid cell (B-LCL) line VAVY (International Histocompatibility Workshop cell line Number IHW09023) with 100nM of calcitriol (Sigma) for 24 hours and stained with anti-HLA DR-PE antibody (BD Biosciences) and acquired on BD-LSR to study the expression of HLA-DR on stimulated and unstimulated B-LCL The data was analysed using WinMDI 2.9 software. The results showed significantly higher expression of HLA-DR in the B-LCL stimulated with calcitriol as compared to the unstimulated one (Figure 5A and B).

5.4.2 Real time PCR
We also studied the levels of transcripts for HLA-DRB1 in B-LCL VAVY after 24 hour stimulation with calcitriol and compared it to unstimulated B-LCL using real time PCR. The data shows 1.89 fold increase in the HLA-DRB1 transcripts from B-LCL stimulated with calcitriol as compared to the unstimulated one. These results were confirmed on peripheral blood mononuclear cells (PBMCs) derived from a normal healthy control homozygous for HLA-DRB1*03:01.
Our results showed enhanced expression of HLA-DR on the B-LCLs stimulated with calcitriol as compared to the unstimulated one confirming that indeed the interaction of VDR with HLA-DRB1*03:01 is occurring through the VDRE present in the promoter region of the gene. Based on the earlier studies and the present data one can speculate that in the absence of required amount of Vitamin D in early life in the predisposed individuals with HLA-DRB1*03:01, the expression of the allele may be impaired in the thymus (Ramagopalan et al., 2009) resulting in escape from thymic deletion of autoreactive T cells leading to T1D manifestations.

6. Conclusions
Our studies show that simultaneous presence of DRB1*03:01 along with homozygous INS-VNTR class-I was significantly increased ( p < 10⁻⁸) in T1D patients, giving a relative risk of 70.81 (Rani et al., 2004). INS-VNTR class-I has been shown to be associated with lower
expression of Insulin in thymii of fetuses as compared to Class-III alleles (Pugliese et al., 1997, Vafiadis et al., 1997) which may be responsible for poor thymic education for insulin resulting in autoimmunity against pancreatic beta cells. Our studies provide additional evidence based on the statistically significant interaction between the predisposing HLA allele and high producer alleles of VDR which may be detrimental for the manifestation of T1D in the absence of 1,25-(OH)₂D₃ in early childhood and/or in-utero and this interaction is mediated by VDRE present in the promoter region of DRB1*03:01 (Israni et al., 2009). With poor thymic education for insulin and HLA-DRB1*03:01 protein, environmental factors like viral infections, vitamin D deficiency and some milk proteins may be involved in initiation of the autoimmune responses against the pancreatic beta cells. While HLA class-II molecules may be involved in auto-antigen presentation to T helper cells, higher producing genotypes of pro-inflammatory cytokines like IFN-gamma and TNF-alpha may be involved in enhancing the cell mediated immune responses through proliferation of CD4⁺ and CD8⁺ T cells, while higher producing genotypes of IL-10 and TGF-beta may have a role in recruitment of these autoreactive T cells in the pancreas through ICAM-1 and Integerin dependent pathways. Final destruction of pancreatic beta cells may occur through CD4⁺ and CD8⁺ T cells and nitric oxide production since IFN-γ may act singularly as well as synergistically with other inflammatory stimuli to induce NO production which can be cytotoxic and thus may have a role in pathogenesis of T1D (Figure 6). Future studies should focus on developing approaches to inhibit autoimmunity before the onset of the disease.
Fig. 6. Conclusions of our studies. Predisposing genetic factors like MHC, INS-VNTR and VDR may be involved in poor thymic education for insulin and HLA-DRB1*03:01 protein resulting in recognition of self proteins as non-self by T cells. These genetic factors along with environmental factors like viral infections, vitamin D deficiency and some milk proteins may be involved in initiation of the autoimmune responses against the pancreatic beta cells. While HLA class-II molecules may be involved in auto-antigen presentation to T helper cells, higher producing genotypes of pro-inflammatory cytokines like IFN-γ and TNF-α may be involved in the cell mediated immune responses. Higher producing genotypes of IL-10 and TGF-beta, may have a role in recruitment of the autoreactive CD4+ and CD8+ T cells in the pancreas through ICAM-1 and Integerin dependent pathways respectively. Final destruction of pancreatic beta cells may occur through CD4+ and CD8+ T cells and nitric oxide production since IFN-γ may act singularly as well as synergistically with other inflammatory stimuli to induce NO production which can be cytotoxic and thus may have a role in pathogenesis of T1D.

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8. References


