Functional studies of candidate genes contributing to Type 1 Diabetes in the NOD mouse

Marie Lundholm
Type 1 Diabetes (T1D) is an autoimmune disorder caused by both genetic and environmental factors. The non-obese diabetic (NOD) mouse is one of the best and most commonly studied animal models for T1D. This mouse strain spontaneously develops diabetes through a process that closely resembles the human pathogenesis. More than 20 insulin dependent susceptibility (Idd) loci have been identified in the NOD mouse, contributing to disease susceptibility; however the contribution of each of the various factors to disease pathogenesis is largely unknown.

The aim of this thesis was to identify and functionally characterize candidate genes mediating susceptibility to murine T1D.

Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) is a negative regulator of T-cell activation and has been shown to be associated with autoimmune diseases. Genetic analyses of the NOD mouse have identified the Ctx region as a major candidate for the Idd5.1 diabetes susceptibility locus and NOD mice have been found to display an impaired expression of CTLA-4 upon anti-CD3 stimulation in vitro. In Paper I, we showed that a novel locus (Ctex) in the distal part of the chromosome 1 together with the Idd3 (Il-2) locus on chromosome 3, constitute the major factors conferring the observed difference in CTLA-4 expression levels. Moreover, we also demonstrated that the defective expression of CTLA-4 in NOD T-cells can in part be overcome by the addition of exogenous interleukin-2 (IL-2). In Paper II, using congenic mice, we confirmed that the Ctex locus contributes to decreased expression of CTLA-4 observed in NOD mice and restricted the region of interest to a 28.8 Mb region containing the Cd3ζ gene. We also demonstrated a phenotypic correlation between strains carrying the NOD versus C57BL/6 alleles of Cd3ζ, respectively and showed that expression of CD3ζ is impaired in activated NOD CD4⁺ T cells. The NOD allele of the Cd3ζ region was found to confer impaired T cell activation and the defective CD3 signalling could be surpassed by PMA plus ionomycin stimulation, supporting the notion of CD3ζ as a prime candidate gene for Ctex.

NOD lymphocytes display relative resistance to various apoptosis-inducing signals, which have been proposed to contribute to the pathogenesis of diabetes. Resistance to dexamethasone-induced apoptosis in NOD immature thymocytes has been mapped to the Idd6 locus. In Paper III we restricted the Idd6 locus to an 8 cM region on the telomeric end of chromosome 6 using a set of congenic mice. In addition, we could confirm that the Idd6 region controls apoptosis resistance in immature thymocytes and restricted the control of apoptosis resistance to a 3 cM region within the Idd6 locus. In Paper IV, we further restricted the Idd6 locus to a 3 Mb region and excluded the region controlling resistance to apoptosis as directly mediating susceptibility to diabetes. We also showed that defective expression of the Lrmp/Jaw1 gene, encoding an endoplasmic reticulum resident protein, is controlled by the Idd6 locus making it the prime candidate for Idd6.

Together, these results contribute to the identification and functional characterization of candidate genes that may confer susceptibility to T1D in the NOD mouse. These results offer important insights into the pathophysiological processes underlying this disease.
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<td>Autoimmune disease</td>
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<tr>
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<td>Antigen presenting cell</td>
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<td>AICD</td>
<td>Activation induced cell death</td>
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<td>Autoimmune lympho-proliferative syndrome (human)</td>
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<td>B cell receptor</td>
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<td>C57BL/6 mouse strain</td>
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<tr>
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<td>C57BL/10 mouse strain</td>
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<tr>
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<td>Centimorgan</td>
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<td>Cytotoxic T lymphocyte antigen-4</td>
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<td>CY</td>
<td>Cyclophosphamide</td>
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<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>DN</td>
<td>Double negative (CD4⁻CD8⁻ thymocytes)</td>
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<tr>
<td>DP</td>
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<tr>
<td>Dxm</td>
<td>Dexamethasone</td>
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<tr>
<td>EAM</td>
<td>Experimentally induced autoimmune myocarditis</td>
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<td>ER</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>fCTLA-4</td>
<td>Full length form of CTLA-4</td>
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<tr>
<td>FLIP</td>
<td>Flice inhibitory protein</td>
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<td>FTOC</td>
<td>Fetal thymic organ culture</td>
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<td>GAD-65</td>
<td>Glutamatic acid decarboxylase-65</td>
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<td>Insulinoma-associated antigen-2</td>
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<td>ICOS</td>
<td>Inducible T cell co-stimulator</td>
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<td>ldd</td>
<td>Insulin dependent diabetes</td>
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<td>IFN-γ</td>
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<td>Interleukin</td>
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<td>IPEX</td>
<td>Immune dysregulation polyendocrinopathy enteropathy-X linked syndrome</td>
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<td>LADA</td>
<td>Latent autoimmune diabetes in adults</td>
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<tr>
<td>liCTLA-4</td>
<td>Ligand-independent form of CTLA-4</td>
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<tr>
<td>LOD</td>
<td>Logarithm of the odds</td>
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<td>Lrmp</td>
<td>Lymphoid restricted membrane protein</td>
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<td>LYP</td>
<td>Lymphoid tyrosine phosphatase</td>
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<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
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<tr>
<td>Mb</td>
<td>Megabase pairs</td>
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<td>MHC</td>
<td>Major histocompatibility complex</td>
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<td>MIDD</td>
<td>Maternally inherited diabetes and deafness</td>
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<td>MODY</td>
<td>Maturity onset diabetes of the young</td>
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<td>Abbreviation</td>
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<tr>
<td>MZ</td>
<td>Monozygotic twins</td>
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<td>NIDDM</td>
<td>Non-insulin-dependent diabetes mellitus (T2D)</td>
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<td>NK</td>
<td>Natural killer</td>
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<td>NKT</td>
<td>Natural killer T cell</td>
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<td>NOD</td>
<td>Non-obese diabetic (mouse strain)</td>
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<td>NRAMP</td>
<td>Natural resistance associated macrophage protein</td>
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<td>Programmed death-1</td>
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<td>QTL</td>
<td>Quantitative trait locus</td>
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<td>RAG</td>
<td>Recombinase activating gene</td>
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<td>Ribonucleic acid</td>
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<td>Soluble form of CTLA-4</td>
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<td>SLE</td>
<td>Systemic lupus erythrematosos</td>
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<td>SMLR</td>
<td>Syngenic mixed lymphocyte reaction</td>
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<td>SNP</td>
<td>Single nucleotide polymorphism</td>
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<td>SP</td>
<td>Single positive (CD4⁺ or CD8⁺ thymocytes)</td>
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<td>Type 1 Diabetes</td>
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<td>T2D</td>
<td>Type 2 Diabetes</td>
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<td>Transforming growth factor β</td>
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<td>T-helper type 1</td>
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<td>T-helper type 2</td>
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<td>TNF</td>
<td>Tumor necrosis factor</td>
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<td>T_reg</td>
<td>Regulatory T cell</td>
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<td>VNTR</td>
<td>Variable number of tandem repeats</td>
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LIST OF PAPERS

This thesis is based on the following papers, which will be referred to in the text by their roman numerals:


II Lundholm M, Mayans S, Motta V, Lofgren-Burstrom A, Holmberg D: The non-obese diabetic (NOD) mouse allele of Cd3ζ (Cd247) is associated with impaired TCR/CD3 mediated activation of T cells. Manuscript


* These authors contributed equally to this work.

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INTRODUCTION

1. The immune system
The immune system is the body’s defense against infectious microorganisms and other harmful agents. Thus, the word immunity means the state of protection from infectious disease. The immune system is amazingly effective, ensuring we rarely get ill even though we encounter potentially pathogenic microorganisms on a daily basis. Most pathogenic microorganisms are detected and destroyed within minutes or hours by the innate immune system. The key to innate immunity is the immediate availability of cells that can protect us against a wide range of pathogens. If the innate immune system fails to clear the pathogen, the adaptive immune system is activated. The adaptive immune response generates effector cells specific for a particular microorganism and produces memory cells which can prevent re-infection with the same microorganism.

1.2 The innate immune system
The innate immune system provides the first line of defense against invading microorganisms and helps prevent establishment of infections. The innate immune system is non-specific, has germline-encoded receptors and reacts similar to a variety of pathogens. The first and most important barriers against microorganisms are the epithelial surfaces. The skin, the mucous membranes and the ciliary respiratory lining act as a physical barrier between the internal milieu and the pathogen containing external environment. The surface epithelia also form a chemical barrier against infection by producing substances that are microbicidal or inhibit microbial growth. The most significant antibacterial substance is the enzyme lysozyme, which is secreted in tears and saliva. Other examples of barriers are the acidic pH of the stomach and body temperature that kills and/or inhibits growth of some pathogens. Together, the physical and chemical barriers form early effective defense mechanisms against infectious disease. If, however, these barriers are crossed, macrophages can immediately
recognize, ingest and kill the pathogens. Activated macrophages also secrete cytokines that induce an inflammatory response and subsequently attract neutrophiles to the site of infection.

1.3 The adaptive immune system
The adaptive immune system is activated when an innate immune response fails to eliminate a new infection. The adaptive immune response to a pathogenic microorganism takes some time to develop, hence the term “adaptive” immunity. Adaptive, or specific, immunity can recognize and selectively eliminate a particular pathogen; it can distinguish between two protein molecules that differ in only a single amino acid. The adaptive immune system has enormous diversity and is capable of recognizing billions of uniquely different structures on foreign antigen. Moreover, the adaptive immune system can store an immunological memory. Thus, upon a secondary encounter with the same antigen, the immune system can “remember” and react more rapidly and more efficiently. The adaptive immune system is also capable of self/non-self recognition, meaning that it normally only responds to foreign antigens. The main types of cells involved in the adaptive immune response are the lymphocytes. There are two major populations of lymphocytes, B and T lymphocytes that express unique antigen-binding receptors on their cell surfaces. The immunoglobulin in its membrane-bound form is referred to as the B-cell antigen receptor (BCR) while as a secreted protein it is called antibody. B lymphocytes develop and mature in the bone marrow, while activation and accumulation occurs in the secondary lymphoid organs; the lymph nodes and the spleen. Interaction of a B lymphocyte with antigen triggers its activation and differentiation into memory B cells and plasma cells that secrete antibodies. Secreted antibodies bind pathogens or their toxic products and constitute the main effector function of B cells in adaptive immunity. The antigen receptor expressed on T cells is known as the T-cell receptor (TCR) which consists of a heterodimer of either αβ or γδ-chains.
1.4 T cell development

T cell progenitors are generated in the bone marrow, just like B cells, but migrate to the thymus where they differentiate into mature T cells. During thymic development T cells pass through distinct developmental stages that can be defined by expression of certain cell surface molecules\(^1\). Thymocytes can be divided into four major subsets characterized by the expression of CD4 and CD8 co-receptor molecules. The most immature thymocytes CD4^-CD8^- (double negative, DN) express neither CD4 nor CD8, the more mature CD4^+CD8^- (double positive, DP) express both CD4 and CD8 and the most mature CD4^+ and CD8^+ (single positive, SP) thymocytes express either CD4 or CD8 on their surface. The surface expression of CD25 (IL-2 receptor \(\alpha\)-chain) and CD44 further divides the DN stage into four sequential stages, DN1 to DN4\(^2,3\). A schematic overview of T cell development is presented in Figure 1.

**Figure 1.** The different stages of T cell development in the thymus (adapted from Sebzada et al.\(^3\)).
Differentiation from the CD4−CD8− DN to the CD4+CD8+ DP stage is dependent on the expression of the pre-TCR which constitute an important checkpoint called β-selection\textsuperscript{4,5}. β-selection involves TCR-β gene rearrangement and association to a pre-Tα chain, forming the pre-TCR complex. Expression and signaling through this pre-TCR complex is necessary for survival and developmental progression from the DN to the more mature DP stage\textsuperscript{6}. In the DP stage TCR-α gene rearrangement takes place and the TCR-αβ complex is formed. TCR-αβ recognition of self-major histocompatibility (MHC) molecules is another checkpoint called positive selection\textsuperscript{7}. Thymocytes unable to bind to the MHC undergo apoptosis (death by neglect) and only those with low or moderate affinity/avidity interactions between the TCR and the self peptide/MHC complex will mature further\textsuperscript{8,9}. On the other hand, thymocytes recognizing the self peptide/MHC complex with high affinity/avidity are deleted through negative selection\textsuperscript{8,9}. Cells surviving selection at the DP stage finally differentiate to become SP CD4\textsuperscript{+} or CD8\textsuperscript{+} and enter the periphery as mature naïve T cells.

1.5 Tolerance

One of the most challenging tasks of the immune system is to recognize, respond to and eliminate foreign (non-self) antigens while not reacting harmfully to its own (self) antigens. This state of immunologic unresponsiveness is called tolerance. Self-tolerance is achieved through several immunological mechanisms which prevent potentially auto-reactive lymphocytes. Tolerance mechanisms can be divided into two main categories, central and peripheral tolerance.

Central tolerance

The negative selection of auto-reactive cells in the thymus constitutes the primary mechanism of generating self-tolerance\textsuperscript{10}. More than 95% of thymocytes are eliminated during thymic development to ensure central tolerance. Negative selection is dependent on expression of molecules that
affect the affinity/avidity between the TCR and the self peptide/MHC complex. Such molecules are co-stimulatory molecules, like CD80 (B7-1) and CD86 (B7-2), and thymic expression of peripheral self-antigens\textsuperscript{11, 12}. The crucial role for co-stimulatory molecules in tolerization of T cells in the thymus is evident from studies of mice where the B7 molecules are missing or blocked by antibodies\textsuperscript{13, 14}. T cells from such mice cause fatal multi-organ inflammation. The importance of intra-thymic expression of peripheral auto-antigens (promiscuous gene expression) is demonstrated in autoimmune regulator (AIRE) deficient mice\textsuperscript{15, 16}. \textit{Aire} knockout mice have strongly decreased expression of self antigens in the thymus, with consequent development of tissue-specific auto-antibodies and lymphoid infiltration of several peripheral organs\textsuperscript{15}. Furthermore, mutations in the human \textit{AIRE} gene cause a multi-organ autoimmune syndrome known as polyendocrinopathy candidiasis ectodermal dystrophy\textsuperscript{17}.

Another key function of the thymus in maintaining immunologic self-tolerance is the production of the natural regulatory T (T\textsubscript{reg}) cells\textsuperscript{10, 18}. T\textsubscript{reg} cells are a functionally mature T-cell subpopulation characterized by the expression of CD4 and CD25 (IL-2R\textalpha) and their development and function depends on the transcription factor Foxp3\textsuperscript{18-22}. Depletion of T\textsubscript{reg} cells from normal animals causes spontaneous development of various autoimmune diseases, such as autoimmune gastritis, thyroiditis and Type 1 Diabetes (T1D) and reconstitution of T\textsubscript{reg} cells prevents these disorders\textsuperscript{23, 24}. How T\textsubscript{reg} cells exert their regulatory function is not clear, but cell-to-cell contact mechanisms involving cytotoxic T lymphocyte antigen 4 (CTLA-4) and transforming growth factor beta (TGF-\textbeta) interactions have been proposed\textsuperscript{10, 23, 25}. 

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Peripheral tolerance

Even though central tolerance mechanisms are very efficient, a number of self-reactive cells overcome the thymus selection barrier. Indeed, auto-reactive T cells in the periphery are present in healthy individuals, suggesting that mechanisms of peripheral tolerance must be controlling these cells. Peripheral tolerance mechanisms include those that regulate the responding state of T cells intrinsically, such as ignorance, anergy and apoptosis and those that provide extrinsic controls like dendritic cells (DCs) and regulatory T cells (Figure 2).

**Figure 2.** Pathways to tolerance. Hematopoietic precursors migrate to the thymus where they undergo positive and negative selection. Most of the self-reactive T cells are deleted in the thymus, however the ones that progress to the periphery are controlled by peripheral tolerance mechanisms (adapted from Li and Boussiotis).
T-cell ignorance of self-antigens occurs if the self-antigens are not easily accessible or if the amount of self-antigen is too low to reach the threshold required to trigger a T-cell response. Anergy, a state of unresponsiveness to antigen, is a result of TCR ligation in the absence of co-stimulation or by signaling through alternative receptors such as CTLA-4 or programmed cell death 1 (PD-1). CTLA-4 is a well-characterized negative regulator of T-cell activation and direct evidence of its critical regulatory role comes from CTLA-4 deficient mice, which die around 3-4 weeks of age from multiorgan lymphocyte infiltration.

Activation induced cell death (AICD) is the most effective way for removal of auto-reactive T cells and termination of the immune responses to prevent lymphoid hyperproliferation. Repetitive TCR stimulation might trigger AICD and this process is mainly mediated through activation of the Fas/CD95 pathway and related death receptor pathways. The importance of Fas signaling in AICD has been established by the observation of lymphoproliferative disease and systemic autoimmunity in mice lacking Fas (lpr) or FasL (gld) function. Furthermore, defects in the human Fas pathway are associated with the autoimmune lymphoproliferative syndrome (ALPS).

Dendritic cells (DCs) are the key antigen presenting cells (APCs) involved in both the initiation of immune responses and the induction of T-cell tolerance. How DCs decide between immunity and tolerance and which mechanisms are involved in those processes are not completely understood. It has been suggested that production of cytokines such as IL-10 and TGF-β by DCs and crosstalk between Tregs and DCs are important mechanisms of tolerance induction.

Substantial evidence that Tregs are a central mechanism of peripheral tolerance has emerged during the last few years. However, much remains to be discovered for understanding their development, phenotype, peripheral
maintenance and function. Current views hold that Tregs actively suppress the activation and expansion of auto-reactive T cells, thereby preventing autoimmune disease\textsuperscript{23,40,41}. The IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome in humans and the X-linked recessive inflammatory disease in scurfy mutant mice are clear examples that deficiencies in natural Tregs, due to mutations in FOXP3, causes autoimmune disease\textsuperscript{42-44}. How the Tregs are maintained within the peripheral T cell pool is not clear, but cytokines like IL-2 and co-stimulatory molecules such as CD28 and B7 are important in the homeostasis of Treg cells\textsuperscript{40,45}. Natural Tregs constitutively express CTLA-4\textsuperscript{14,46,47}, in contrast to naïve T cells that only express this molecule after T cell activation. Recently it has been reported that CTLA-4 is absolutely required for Treg-suppressive function\textsuperscript{48,49}. The CTLA-4-mediated mechanism of suppression is not entirely known but down-regulation of CD80 and CD86 on APCs is one possible mechanism\textsuperscript{48,49}. Still, Tregs almost certainly use a variety of suppressive mechanisms for the control of an immune response, and just how crucial the different mechanisms are might depend on the environment and the specific immune response\textsuperscript{50}.

Recently, several studies have raised the possibility that Treg cells with suppressive capabilities can be generated in the periphery. Naïve CD4$^+$ CD25$^-$ Foxp3$^-$ cells can be induced by numerous factors to become regulatory T cells, but the main factors involved appear to be low-dose antigen presentation and TGF-β\textsuperscript{51}. These “induced” or “adaptive” Tregs are not clearly defined in terms of phenotype or function, although their inhibitory activities are thought to be mediated via cytokine-dependent pathways, predominately by TGF-β and IL-10\textsuperscript{52}. This is in contrast to the natural thymic derived Tregs which require cell-to-cell contact in order to mediate suppression. The relative importance of “natural” versus “induced” Tregs is not known and still needs to be established. Bluestone and Abbas\textsuperscript{52} suggested that the natural T reg function is mainly to prevent auto-reactive T-cell responses, while the induced Treg function primarily to suppress pathological immune responses.
2. **Autoimmune disease - when tolerance breaks**

One of the main functions of the immune system is the capability to distinguish between foreign and self antigens expressed by the body and to respond only to foreign antigens. The failure of the immune system to maintain immunological self-tolerance results in autoimmune disease (AID). AID affects about 5-7% of the population in Western countries and is a growing health and economical problem. There are two main groups of AID, those that are organ specific, like T1D and Hashimoto’s thyroiditis, and those that are systemic such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). The development of an AID is caused by a combination of genetic and environmental factors together with defective immune regulation\(^{53}\) (Figure 3).

**Figure 3.** Autoimmune disease is caused by a combination of genetic predisposition, environmental factors and defects in immunoregulatory mechanisms (adapted from Ermann and Fathman\(^{53}\)).
3. Diabetes

Diabetes mellitus is a chronic metabolic disorder characterized by high blood glucose levels (hyperglycemia). The two most common forms of diabetes are Type 1 Diabetes (T1D), 10-15% of all cases, and Type 2 Diabetes (T2D), 85-90% of all cases. T1D, also called insulin dependent diabetes mellitus (IDDM), is an autoimmune disease where the insulin producing \( \beta \)-cells found in the pancreatic islets of Langerhans are destroyed, leading to total insulin deficiency. The onset is generally during childhood or puberty and exogenous administration of insulin is an absolute requirement throughout the lifetime of those afflicted with T1D. In T2D, also referred to as non-insulin dependent diabetes mellitus (NIDDM), the pancreatic \( \beta \)-cells fail to produce adequate insulin (insulin deficiency) or the body can’t respond to the insulin being produced (insulin resistance). T2D occurs mostly in individuals over 30 years of age and is usually associated with obesity and life-style. Diabetes also includes the less common metabolic disorders, latent autoimmune diabetes in adults (LADA), maturity onset diabetes of the young (MODY) and maternally inherited diabetes and deafness (MIDD).

3.1 Pathology of T1D

T1D is a multi-factorial autoimmune disorder caused by both genetic and environmental factors. The risk of developing T1D in first degree relatives of patients with T1D is approximately 6%, which is 15 times higher than the prevalence in the general population (0.4%)\(^55\). In genetically identical monozygotic (MZ) twins the concordance rate is 35-70%\(^55\). These observations show that both genetic and environmental factors contribute to disease susceptibility.

The autoimmune destruction of the pancreatic \( \beta \)-cells is mediated by infiltrating lymphocytes, mainly CD8\(^+\) T cells\(^56\). Several years before clinical diagnosis of T1D, serum auto-antibodies specific for \( \beta \) cell auto-antigens such as insulin, glutamatic acid decarboxylase 65 (GAD65) and insulinoma-associated antigen-
2 (IA-2) can be detected. The presence of such auto-antibodies can predict future development of disease. When approximately 80-90% of the β-cells are destroyed clinical diabetes occurs\textsuperscript{57}. Although insulin is an efficient therapy for T1D, it is not a cure and many patients suffer from complications later in life, such as kidney failure and blindness.

### 3.2 Genetics of T1D

In humans, the genetic components conferring susceptibility to T1D are mainly determined by the human leukocytes antigen (HLA) locus. The HLA locus has been estimated to explain up to 40-50% of the familial clustering of T1D\textsuperscript{58}. The HLA region is a cluster of genes located within the major histocompatibility complex (MHC) on chromosome 6p21. This cluster is divided into HLA class I (A, B and C) and HLA class II (DP, DQ and DR) and the proteins encoded by these genes are extremely polymorphic and essential in self versus non-self immune recognition\textsuperscript{59}. Genes encoding the HLA class II, HLA-DR and -DQ molecules, show the strongest association to T1D\textsuperscript{60, 61}.

The HLA is necessary but not sufficient for the development of diabetes and explains less than 50% of the inherited disease risk, emphasizing a role for non-HLA genes in conferring susceptibility to T1D. Genome-wide linkage analysis and association studies have revealed 21 non-HLA loci that are associated with T1D\textsuperscript{62-65} but the identity of all the non-HLA genes are still not known. The insulin gene (\textit{INS}) on 11p15, \textit{PTPN22} on 1p13, \textit{CTLA-4} on 2q31, \textit{IL2RA} (CD25) on 10p15 and \textit{IFIH1} on 2q24 are some of the loci that provide convincing evidence for association with T1D\textsuperscript{65}. The strongest association with the insulin gene region is to a unique mini-satellite (VNTR) consisting of 14-15 tandem repeat sequences. Susceptibility to diabetes has been associated with short repeats (26 to 63 repeats), while long repeats (140 to 210 repeats) are dominantly protective\textsuperscript{59, 60}. It has been shown that this VNTR affects the transcription level of insulin in both the pancreas and thymus. The long repeats are associated with low \textit{INS} mRNA in the pancreas, but high transcription in
the thymus. This suggests that high levels of insulin expression in the thymus can contribute to central tolerance induction.

The \textit{PTPN22} gene encodes the lymphoid tyrosine phosphatase protein (LYP), which inhibits TCR signaling. An arginine to tryptophan substitution at position 620 of the Lyp protein (R620W) is associated with T1D. This substitution results in reduced T-cell activation which could lead to decreased deletion of auto-reactive T cells in the thymus and/or less effective T\textsubscript{reg} function in the periphery and thereby increased autoimmunity.

CTLA-4 is a negative co-stimulatory molecule that plays an important role in the down regulation of T-cell activation. CTLA-4 polymorphisms have been associated with several autoimmune diseases and numerous mechanisms have been suggested for CTLA-4s involvement in the autoimmune process. T1D susceptibility has been associated with a 3’non-coding region of \textit{CTLA-4}, with the CT60 polymorphism showing the strongest association. This CT60 polymorphism was correlated with lower levels of a splice variant encoding a soluble form of CTLA-4 (sCTLA-4). The reduced level of sCTLA-4 might increase T-cell activation and/or reduce T\textsubscript{reg} activity, increasing the risk for autoimmunity.

The \textit{IL2RA} gene encodes the \(\alpha\)-chain of the IL-2 receptor (CD25) and the expression of IL-2RA on T\textsubscript{regs} is critical for their function. The mechanisms by which \textit{IL2RA} allelic variants are involved in T1D susceptibility is not known, but effects on IL-2RA expression could affect the suppressive role of T\textsubscript{reg}.

The interferon-induced helicase (\textit{IFIH1}) encodes an RNA helicase that is involved in the innate antiviral response. The association between viral infection and T1D susceptibility and the widespread expression of the IFIH1
transcript in lymphoid tissues suggests a functional role of the *IFIH1* gene in the autoimmune process of T1D\textsuperscript{59}.

Recent reports from genome-wide association (GWA) studies and analyses have confirmed several of the susceptibility genes previously identified in linkage and association studies as well as provided evidence for several novel susceptibility genes and regions\textsuperscript{62-65, 77}. However, these novel regions require further investigation to determine and confirm plausible candidate genes. With the continuation of GWA studies and analyses on larger materials using denser genotyping arrays it is likely that most of the genetic basis for T1D will be revealed in the near future. This further underscores the importance on focusing on studies aimed at understanding the contribution of these genetic factors to the pathogenesis of disease. For these types of studies the use of animal models is an invaluable tool. Identifying the molecular mechanisms underlying T1D could reveal potential therapeutic targets.
4. The NOD mouse

The non-obese diabetic (NOD) mouse spontaneously develops autoimmune diabetes and is one of the best and most widely studied animal models for T1D. Since its establishment by Makino et al.\textsuperscript{78} in the late 1970s the NOD mouse has provided essential information regarding the pathogenesis of autoimmune diabetes. The disease progress in this mouse strain shares many similarities to human T1D, which highlights the NOD mouse as an excellent model of autoimmune diabetes.

4.1 Pathology of T1D in the NOD mouse

As in humans, T1D in the NOD mouse is a multi-factorial autoimmune disease for which susceptibility is determined by both environmental and genetic factors. Both in humans and in NOD mice the autoimmune process starts with mononuclear infiltration of perivascular and peri-islet regions of the pancreatic islets of Langerhans (peri-insulitis). In the NOD mouse this begins at around 3-4 weeks of age. These infiltrates then progress and invade the islets (insulitis) and around 10 weeks of age most of the mice display severe insulitis. The infiltrating cells are mainly CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells, but DCs, macrophages, NK cells and B cells are also present in the islets\textsuperscript{79-81}. At around 4 to 6 month of age, when more than 90% of the insulin producing β cells are destroyed, overt diabetes with symptoms like hyperglycemia and glycosuria develops\textsuperscript{82} (Figure 4). Almost all NOD mice develop insulitis, but a number of mice never progress to overt diabetes. In a typical high-incidence NOD colony, 80-90% of females and 40-50% of males become diabetic between 3 and 7 months of age. Still, the incidence varies a lot between different NOD colonies. In a worldwide survey, diabetes ranged from 20% to 100% in females and 1% to 65% in males at 30 weeks of age\textsuperscript{83}. This huge difference in diabetes incidence between colonies is thought to be dependent on environmental factors, like diet, viral infection and temperature, and to some extent genetic drift\textsuperscript{84-86}. The difference in diabetes incidence between NOD males and females has been suggested to be influenced by sex hormones since castration of males increases the diabetes
incidence and androgen treatment of females prevents diabetes\textsuperscript{87,88}. This female gender bias is not observed in human T1D. However, in other human autoimmune diseases like SLE and RA more women are affected than men\textsuperscript{89}.

\textbf{Figure 4.} Pancreatic islets from NOD mice, stained for insulin (red) and the T cell marker anti-CD3 (green). A: Non-infiltrated islet. B: Infiltration of lymphocytes surrounding the islet (peri-insulitis). C: Lymphocyte infiltration (insulitis) resulting in β-cell destruction. D: Heavily infiltrated islet. Overt diabetes occurs when more than 90\% of the insulin producing β cells is destroyed.

T cells are essential for the development of diabetes in the NOD mouse demonstrated by the absence of disease in athymic nude NOD-\textit{nu/nu} mice\textsuperscript{90} and in MHC class I and class II deficient NOD mice lacking CD8\textsuperscript{+} and CD4\textsuperscript{+} T cells, respectively\textsuperscript{91-93}. Additional proof of a T cell requirement in the disease process is shown in transfer experiments where T cells are necessary and sufficient for the development of diabetes\textsuperscript{94, 95}. CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells are important in the early stages of disease development but also play a crucial role as final effectors in the destruction of pancreatic β cells\textsuperscript{94, 96-99}. The pathogenic CD4\textsuperscript{+} T cells normally display a type 1 phenotype (CD4\textsuperscript{+} Th1) marked by the production of interleukin 2 (IL-2), interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α) and tumor necrosis factor beta (TNF-β). These type-1 cytokines in turn activate CD8\textsuperscript{+} T cells that interact specifically with β cells and destroy them via a CD95/CD95L- and/or perforin-dependent mechanism\textsuperscript{100-102}. In addition, it has been shown that the individual cytokines
TNF-α, TNF-β and IFN-γ inhibit insulin synthesis and secretion as well as mediate islet β cell destruction when added in combination\textsuperscript{100, 103}.

B cells are not required at the effector stage of NOD T1D, as T cells from diabetic NOD mice transfer diabetes to B-cell depleted NOD mice\textsuperscript{104}. However, converging data suggests that B cells play an important role as APCs for the initiation of T cell mediated autoimmune diabetes in the NOD mouse\textsuperscript{105-107}.

Macrophages and DCs are the two major subsets infiltrating the islets during the early stage of insulitis. Exactly how macrophages and DCs are involved in the development of diabetes is not fully understood, but it has been shown that they have an essential role as APCs in the initial step of the autoimmune process\textsuperscript{108-110}.

Several β cell autoantigens have been identified in the NOD mouse and the two most studied, as in human T1D, are insulin and GAD65\textsuperscript{111-115}. The presentation of β cell antigens is complex since pancreatic β cells do not express MHC class II or co-stimulatory molecules. Data suggests that autoantigens are presented by APCs, most likely DCs, to naïve T cells in the draining pancreatic lymph nodes (PLNs)\textsuperscript{116, 117}. Moreover, removal of the PLNs in young NOD mice protects against diabetes demonstrating the crucial role of PLNs in selection and activation of auto-reactive T cells\textsuperscript{118}.

In addition to diabetes, NOD mice have other autoimmune traits such as sialitis, myocarditis, prostatitis and late in life autoimmune hemolytic anemia. NOD mice are also susceptible to the experimental induction of a variety of autoimmune diseases, including experimental autoimmune thyroiditis, colitis-like wasting disease, encephalomyelitis and manifestations of SLE. These data suggest that the NOD mouse has a general defect in tolerance to self-proteins leading to autoimmune responses\textsuperscript{114, 119}.
4.2 Genetics of T1D in the NOD mouse

An ultimate goal when studying genetics of a disease is to find and understand how each gene variant alters disease pathogenesis and thereby create the possibility of early prediction and therapeutic intervention before onset of overt disease. The NOD mouse constitutes an invaluable tool for understanding the contribution of genetic factors to T1D pathogenesis. One of the advantages of using animal models is that the environmental influence can be controlled such that the disease phenotype can be attributed to mainly genetic factors. Moreover, the effects of individual susceptible and protective alleles can be evaluated in experimental genetic studies. In addition, by studying crosses between inbred strains, the problem of genetic allelic heterogeneity is efficiently circumvented, since only two alleles segregate at each locus. The use of several different strains also increases the chance of identifying additional susceptibility or protective loci\(^{120,121}\).

More than 20 chromosomal regions referred to as insulin dependent diabetes (\textit{Idd}) loci contribute to T1D development in the NOD mouse (Table 1)\(^{122}\). However, most of the loci lack identification of a disease associated gene. As in humans, the MHC class II molecule is the major genetic risk factor for susceptibility to T1D in the NOD mouse\(^{123,124}\). NOD mice have a unique MHC haplotype (\textit{K}^d, \textit{I}-\textit{A}^g7, \textit{I}-\textit{E}^\text{null}, \textit{D}^b), termed H-2\(^g7\), which maps to the \textit{Iddl} susceptibility locus on chromosome 17. This unusual MHC haplotype does not express an I-E molecule because of a defective E\(\alpha\) locus\(^{125}\). Moreover, the I-\textit{A}^g7 molecule has proline and serine at positions 56 and 57, instead of the histidine and aspartic acid found in most murine I-\textit{A}\(\beta\) chains\(^{126}\). This unique structure significantly alters the repertoire of MHC binding peptides and is thought to contribute to the diversity and large number of auto-reactive T cells observed in the NOD mouse\(^{127-129}\). Interestingly, a non-aspartic acid at position 57 of the human HLA-DQ\(\beta\) chain is also associated with diabetes susceptibility, suggesting that similar autoantigen presentation events may underlie diabetes in both mice and humans\(^{130,131}\).
In NOD mice the incidence of diabetes and insulitis can be prevented by the presence of a non-NOD MHC haplotype, demonstrating the central role of this H-2\textsuperscript{g7} haplotype in the development of T1D\textsuperscript{132, 133}. In addition, the contribution of MHC susceptibility alleles is dose-dependent since NOD mice heterozygous for the MHC locus develop diabetes with a lower penetrance\textsuperscript{122, 134, 135}.

When the MHC of the NOD mouse is expressed in the non-autoimmune prone mouse strains C57BL/10 (B10) and C57BL/6 (B6), no insulitis or diabetes is observed\textsuperscript{136-138}. This demonstrates that disease associated MHC genes are not sufficient for diabetes development and emphasizes the involvement of non-MHC genes in diabetes pathogenesis. Identification of non-MHC genes and the biological functions they mediate has proven difficult. Only a few Idd genes, in more than 20 Idd loci identified, have been defined and suggested that genetic variants are involved in diabetes susceptibility. Analysis of congenic NOD mice has also revealed that regions once thought to have a single Idd gene actually have several closely linked T1D genes, leading to sub-division of many Idds (Table 1)\textsuperscript{122}.
Table 1. The *Idd* regions.

<table>
<thead>
<tr>
<th><em>Idd</em> Region</th>
<th>Non-NOD strain</th>
<th>R or S</th>
<th>Chr</th>
<th>Region Mb</th>
<th>Interval Size Mb</th>
<th>Reference</th>
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| *Idd1*       | B10            | R      | 17  | 34.133 - 35.405 | 1.27            | Reviewed in Wicker et al., 1995 
|              |                |        |     |           |                  | 124       |
| *Idd2*       | B10            | R      | 9   | 32.308 - 98.698 | 66.39           | Pearce, 1998 |
| *Idd3*       | B6             | R      | 3   | 36.627 - 37.277 | 0.65            | Yamanouchi et al., 2007 |
| *Idd4.1*     | NOR            | R      | 11  | 69.76 - 71.152  | 1.39            | Ivakine et al., 2006 |
| *Idd4.3*     | C57L           | R      | 11  | 44.553 - 55.855 | 11.30           | Litherland et al., 2005 |
| *Idd5.1*     | B10            | R      | 1   | 60.833 - 62.840 | 1.96            | Wicker et al., 2004 |
| *Idd5.2*     | B10            | R      | 1   | 73.984 - 75.465 | 1.48            | Wicker et al., 2004 |
| *Idd5.3*     | B10            | R      | 1   | 66.530 - 70.084 | 3.55            | Hunter et al., 2003 |
| *Idd5.4a*    | B10            | S      | 1   | 77.143 - 147.307| 70.16           | Hunter et al., 2007 |
| *Idd5.4b*    | B10            | S      | 1   | 152.632 - 157.938| 5.31            | Hunter et al., 2007 |
| *Idd6.1*     | C3H            | R      | 6   | 146.378 - 149.517| 3.14            | Hung et al., 2006 |
| *Idd6.2*     | C3H            | R      | 6   | 143.560 - 146.378| 2.82            | Hung et al., 2006 |
| *Idd6.6*     | B6             | R      | 6   | 137.404 - 146.386| 8.99            | Bergman et al., 2003 |
| *Idd6.3*     | C3H            | R      | 6   | 146.262 - 147.388| 1.13            | Hung et al., 2006 |
| *Idd7*       | B6             | S      | 7   | 21.0 - 43.0     | 22.0            | Serreze et al., 2008 |
| *Idd7*       | B10            | S      | 7   | Peak at 19.997  |                 | Ghosh et al., 1993 |
| *Idd7*       | NON            | S      | 7   | Peak at 19.997  |                 | McAlee et al., 1995 |
| *Idd8*       | B10            | S      | 14  | Peak at 21.66   |                 | Ghosh et al., 1993; Liston et al., 2004 |
| *Idd9.2*     | B10            | R      | 4   | 144.968 - 149.098| 4.13            | Stegmann et al., 2000 Unpublished data from Wicker et al |
| *Idd9.3*     | B10            | R      | 4   | 149.300 - 150.522| 1.22            | Cannons et al., 2005 |
| *Idd10*      | B6             | R      | 3   | 99.699 - 100.577| 0.88            | Penha-Goncalves et al., 2003; Unpublished data from Wicker et al |
| *Idd11*      | B6             | R      | 4   | 125.017 - 132.983| 7.97            | Brodnicki et al., 2006 |
| *Idd12*      | B6             | R      | 14  | Peak at 35.170  |                 | Liston et al., 2004; Morahan et al., 1994 |
| *Idd13*      | NOR            | R      | 2   | 114.118 - 158.330| 44.21           | Chen et al., 2007; Serreze et al., 1998 |
| *Idd13 - B2m*| NOR            | R      | 2   | 114.118 - 130.275| 16.16           | Chen et al., 2007 |
| *Idd13*      | NOR            | R      | 2   | 121.973 - 134.812| 12.84           | Chen et al., 2007 |
| *Idd14*      | B6             | S      | 13  | 25.424 - 120.284| 94.86           | Brodnicki et al., 2003 |
| *Idd15*      | NON            | R      | 5   | Peak at 8.798   |                 | McAlee et al., 1995 |
| *Idd16*      | B6             | R      | 17  | 26.318-29.405   | 3.09            | Deruytter et al., 2004 |
| *Idd17*      | B6             | R      | 3   | 79.484 - 87.106 | 7.62            | Podolin et al., 1997 |
| *Idd18.1*    | B6             | R      | 3   | 108.993 - 109.597| 0.60            | Lyons et al., 2001; Unpublished data from Wicker et al |
| *Idd18.2*    | B6             | S      | 3   | 100.95 - 108.222| 7.27            | Lyons et al., 2001; Unpublished data from Wicker et al |
| *Idd19*      | C3H            | S      | 6   | 117.439 - 128.468| 11.03           | Morin et al., 2006; Rogier et al., 2001 |
| *Idd20*      | C3H            | R      | 6   | 83.595 - 91.990 | 8.39            | Morin et al., 2006 |
| *Idd21.2*    | ABH            | S      | 18  | 64.618 - 74.588 | 9.97            | Hollis-Moffatt et al., 2005 |
| *Idd21.3*    | ABH            | R      | 18  | 0 - 21.671     | 21.67           | Hollis-Moffatt et al., 2005 |
| *Idd22*      | ALR            | R      | 8   | Peak at 90.626  |                 | Mathews et al., 2003 |
| *Idd23*      | B6             | R      | 17  | 3.924 - 26.318  | 22.39           | Deruytter et al., 2004 |
| *Idd24*      | B6             | R      | 17  | 35.340 - 44.938 | 6.10            | Deruytter et al., 2004 |
| *Idd25*      | NOR            | R      | 4   | Peak at 133.341 |                 | Reifsnyder et al., 2005 |
| *Idd26*      | NOR            | R      | 1   | 19.802 - 40.319| 20.52           | Reifsnyder et al., 2005 |
| *Idd27*      | CBA            | R      | 7   | 86.521 - 127.029| 40.51           | Chen et al., 2005 |
| *Not assigned* | C57L          | R      | 7   | 117.936 - 152.524| 34.59           | Chen et al., 2005 |
Table 1 describes the location and size of the Idd regions affecting the frequency of diabetes in NOD mice. Resistant (R) and Susceptible (S) refer to the diabetes phenotype associated with the Idd allele present in the non-NOD strain (adapted from Ridgway et al.\textsuperscript{122}).

The two most extensively studied Idds are Idd3 and Idd5. NOD.B6.Idd3 congenic mice, with a protective Idd3 allele from B6 mice, show a diabetes incidence of 25\% as compared to 80\% in the parental NOD strain\textsuperscript{167,168}. Fine-mapping and positional cloning studies have reduced the Idd3 locus to a 650 kb interval in the proximal region of mouse chromosome 3, a region which contains Il2, Il21 and several other genes\textsuperscript{73,168,169}. The main candidate gene for the Idd3 locus is Il2, which is an important cytokine for development and function of T\textsubscript{reg}\textsuperscript{170-173}. Yamanouchi et al.\textsuperscript{73} demonstrated, by analyzing IL-2 congenic mice, that the B6 allele of Il2 produces approximately 2-fold more IL-2 mRNA than the NOD allele. Sequencing and haplotype analysis revealed several candidate single nucleotide polymorphisms (SNPs) within the 5\` region of the NOD Il2 promoter that collectively could influence the reduced levels of IL-2 production observed in the NOD mouse\textsuperscript{73}. In addition, this IL-2 difference between NOD and B6 was confirmed in a study showing that the B6 allele is transcriptionally more active than the NOD allele\textsuperscript{174}. Furthermore, a recent report suggests that the increased production of IL-2 by CD4\textsuperscript{+} effector T cells from NOD.B6 Idd3 congenic mice might protect against T1D by promoting the expansion and function of T\textsubscript{reg} locally in the pancreas and simultaneously inhibiting T\textsubscript{H}17 cells\textsuperscript{175}. The newly identified T\textsubscript{H}17 cells produce IL-17 and seem to play a central role in mediating autoimmunity and inducing tissue inflammation\textsuperscript{176-178}. Together, these findings strongly support Il2 as the Idd3 gene. Nevertheless, the expression of IL-21, a cytokine produced by activated T cells, has been reported to be higher in NOD mice compared with non-autoimmune strains. This increased IL-21 expression is correlated with lymphopenia and compensatory homeostatic expansion, which may drive autoimmunity in the NOD mouse\textsuperscript{179}. Moreover, it has been demonstrated that
IL-21 contributes to the generation of pathogenic T\textsubscript{H}17 cells\textsuperscript{180} and inhibits T\textsubscript{reg} suppressor function\textsuperscript{181}. Thus, \textit{Il21} is also a likely candidate gene for the \textit{Idd3} locus.

The \textit{Idd5} region on mouse chromosome 1 has been sub-divided into four loci, \textit{Idd5.1}, \textit{Idd5.2}, \textit{Idd5.3} and \textit{Idd5.4}\textsuperscript{142,143,167,182}. T1D resistant alleles at \textit{Idd5} result in 40\% diabetes compared with control NOD mice that have a diabetes frequency of 80 \%\textsuperscript{167}. Congenic strain analysis has reduced the \textit{Idd5.1} locus to a 2.1 Mb region containing four genes including the candidate genes \textit{Ctla-4} and \textit{Icos}\textsuperscript{142}. The \textit{Ctla-4} gene is particularly interesting since it has been implicated in T1D susceptibility in humans\textsuperscript{71,183}. A SNP in mouse exon 2 of \textit{Ctla-4} affects the expression of the ligand-independent CTLA-4 (liCTLA-4) and the NOD allele mediates significantly lower levels of liCTLA-4 compared to the protective B10 allele\textsuperscript{71,142}. The liCTLA-4 isoform mediates negative signaling in T cells via binding and dephosphorylation of the CD3ζ chain. This suggests that higher expression of liCTLA-4, as observed in mice with the diabetes-resistant allele of \textit{Ctla-4}, could inhibit T cell activation and/or expansion and thereby protect against diabetes\textsuperscript{184}. ICOS (Inducible T cell co-stimulator), the other main candidate gene for the \textit{Idd5.1} locus, is a costimulatory molecule that is up-regulated on T cells upon activation and provides a positive signal for T cell activation\textsuperscript{185}. Activated T cells of NOD origin have been reported to express higher levels of ICOS compared to B6 and B10 T cells. This ICOS expression difference between NOD and diabetes resistant strains is controlled by the \textit{Idd5.1} region. However, it is not known if this is directly controlled by polymorphisms in the \textit{Icos} gene or by other molecules genetically regulated by the \textit{Idd5.1} locus, questions which deserve further investigation\textsuperscript{142,186}. In addition, recently Hawiger \textit{et al.}\textsuperscript{187} showed that NOD ICOS knockout mice were completely protected from TID, suggesting that ICOS is essential for the induction of the autoimmune process that leads to diabetes.
The **Idd5.2** locus is localized to a 1.52 Mb interval of chromosome 1 containing around 45 genes. *Slc11a1 (Nramp1)* is the prime candidate gene in this region because there is a known functional missense polymorphism (glycine$^{169}$ > aspartic acid$^{169}$) distinguishing the NOD and B10 *Slc11a1* alleles$^{142,167}$. The NRAMP1 protein, a divalent cation transporter in phagosomes, helps the killing of certain intracellular pathogens. NOD mice express a functional NRAMP1 protein which mediates resistance to infections by intracellular pathogens such as *Salmonella*, while B10 mice have a non-functional NRAMP1 protein$^{188,189}$. In NOD mice when *Slc11a1* is silenced by RNA interference, a reduced frequency of T1D was observed and susceptibility to *Salmonella* infection increased$^{190}$. This strongly supports *Slc11a1* as the causative gene in Idd5.2. Moreover, it has been demonstrated recently that NRAMP1 is expressed on DCs$^{191}$ and that the NOD-derived functional protein enhanced the antigen processing and presentation which may activate auto-reactive T cells leading to autoimmune diabetes$^{192}$.

The **Idd5.3** locus, which is located between **Idd5.1** and **Idd5.2**, is a 3.55 Mb region containing 11 genes$^{143}$. Using genome wide microarray expression analysis, *Acadl* was highlighted as the major candidate gene in the **Idd5.3** locus since it was the only gene in this interval that was highly differentially expressed between NOD and NOD.B10 **Idd5/3** congeneric mice. *Acadl* encodes an enzyme, acyl-coenzyme A dehydrogenase which is involved in the fatty acid β-oxidation. The NOD allele of *Acadl* has lower mRNA expression compared with the B10 allele, however, what implications this might have on the diabetes pathogenesis remains to be determined$^{193}$.

Analysis of congeneric mice revealed a fourth locus on chromosome 1, the **Idd5.4** locus. The **Idd5.4** region is distal of **Idd5.2**, comprises about 78 Mb and intriguingly it is the B10 allele that confers susceptibility to T1D$^{143}$. *Daf1* (*Cd55*) is a promising candidate gene in the **Idd5.4** region since NOD CD4$^+$ T cells express more DAF on their cell surface compared with NOD.B10 **Idd5/3**
T cells, however, additional congenic strains will be required to reduce the region and confirm candidate genes.

Interestingly, remarkably strong and complex interactions between the four Idd.5 loci have been shown. For example, susceptibility alleles at Idd5.4 (B10 alleles) could completely mask the effect of protective alleles at Idd5.3 and Idd5.2 and protective alleles at Idd5.1 could fully mask susceptibility alleles at Idd5.4. This illustrates not only the complexity of gene-gene interactions but also the advantage of using congenic mice to discover gene interactions and masking effects that are probably hidden in conventional association studies in humans.

MHC class II molecules, CTLA-4 and the IL-2/CD25 pathway have all been implicated as major contributors to T1D susceptibility in both humans and the NOD mouse. This indicates that similar immune pathways are involved in disease pathogenesis in humans and NOD mice and underscores the value of studying the NOD mouse for identifying therapeutic targets for prevention of T1D.

4.3 Immunological defects in the NOD mouse

Immunoregulation is a dynamic process and several immunological mechanisms contribute to the establishment of immune homeostasis/tolerance. NOD mice have been shown to display a number of immunological defects that may contribute to the development of autoimmunity.

4.3.1 Thymic selection defects

Impaired thymic selection in NOD mice has been suggested to contribute to the development of auto-reactive NOD T cells. As discussed above, the unique NOD I-A\(^{\text{g7}}\) structure has been shown to be intrinsically unstable resulting in poor self-peptide binding which may lead to reduced efficiency of negative selection of potentially auto-reactive T cells. The insulin B chain segment
9-23 is one example of a weak MHC binding peptide in NOD mice that can trigger autoreactivity\textsuperscript{129}. In addition, it has been demonstrated that thymic insulin expression in mice plays an important role in deleting insulin-specific T cells\textsuperscript{195}.

Another reported defect of NOD thymocytes is the altered sensitivity to undergo negative selection. Kishimoto and Sprent\textsuperscript{196} showed that negative selection mediated by anti-CD3 or superantigen in both \textit{in vitro} and \textit{in vivo} assays was defective in NOD thymocytes. This defect in negative selection was independent of I-A\textsuperscript{g7} expression and affected both the Fas-dependent and Fas-independent apoptosis pathways.

Non-MHC genes from the NOD mouse have also been shown to be responsible for resistance to clonal deletion of high affinity auto-reactive T cells in the thymus\textsuperscript{197}. This insensitivity of NOD thymocytes to undergo clonal deletion resulted in increased auto-reactive T cells in the periphery with subsequent breakdown of T cell tolerance to the islet antigens.

Support for NOD thymocytes being less sensitive to clonal deletion also comes from a study where fetal thymic organ cultures (FTOCs) derived from NOD and B6.H\textsuperscript{2g7} mice expressing the BDC2.5 clonotype were analyzed. In that study, Zuccelli \textit{et al.}\textsuperscript{198} showed that the NOD genetic background conferred resistance to clonal deletion in differentiating thymocytes expressing the BDC2.5 specificity as well as a reduced ability to divert auto-reactive thymocytes into an alternative pathway of differentiation.

In another study, resistance to thymic deletion in NOD was reported to be a consequence from aberrant up-regulation of the pro-apoptotic protein Bim during \textit{in vivo} encounter with high-avidity autoantigen\textsuperscript{148}.

Indications that defects in positive selection of NOD thymocytes also play a critical role for the development of autoimmunity are observed in NOD↔B6 allophenic mouse chimeras. In these chimeric mice, insulitis was found to correlate with the proportion of NOD cells in the thymic cortex region where positive selection takes place\textsuperscript{199}.
4.3.2 Apoptosis resistance

Apoptosis is an important physiological process of cell death that contributes to the immunological homeostasis and elimination of undesirable cells. This process is a tightly regulated event that ends with the generation of apoptotic bodies that are efficiently phagocytosed without initiating an inflammatory reaction\textsuperscript{200}. Apoptosis plays a key role at several stages of central tolerance, peripheral regulation and termination of immune responses and a large number of molecules are involved in controlling these apoptosis pathways\textsuperscript{34}. Evidence suggests that defective regulation of apoptosis could contribute to the pathogenic mechanisms of autoimmune diseases\textsuperscript{36,201}. In NOD mice the development of TID may be influenced by defects in mechanisms that control both thymocyte, as mentioned above, and peripheral T cell apoptosis.

It has been shown that activated NOD T cells are resistant to apoptosis after IL-2 deprivation when compared to other non-autoimmune strains\textsuperscript{202}. This increased resistance to apoptosis was observed early in life and suggested to be dependent on the anti-apoptotic Bcl-x protein that was increased in activated T cells of NOD mice\textsuperscript{202-204}. Immature CD4\textsuperscript{+}CD8\textsuperscript{+} DP NOD thymocytes are reported to have an increased resistance to dexamethasone (Dxm)-induced apoptosis \textit{in vivo}\textsuperscript{205}. Through F2 intercross studies the resistance to Dxm-induced apoptosis of DP NOD thymocytes was mapped to a telomeric region on chromosome 6, overlapping with the previously known \textit{Idd6} locus\textsuperscript{206}. In addition, NOD mice showed an impaired proliferation of immature thymocytes after Dxm treatment compared with B6 mice. This aberrant proliferation was most evident in the CD4\textsuperscript{+}CD8\textsuperscript{lo} CD8\textsuperscript{+} cells differentiating from the DN to the DP stage and appears to be controlled by the \textit{Idd6} locus as well\textsuperscript{207}. Furthermore, NOD lymphocytes were shown to be relatively resistant to cyclophosphamide (CY)- and \textgamma-irradiation-induced apoptosis\textsuperscript{208,209}. These apoptosis resistant traits are controlled by the \textit{Idd5} region on chromosome 1, suggesting involvement in diabetes pathogenesis\textsuperscript{208,210}. Moreover, T cells from
Ctla-4−/− deficient mice displayed a similar resistance to γ-irradiation-induced apoptosis as observed in the NOD mice, supporting the notion of CTLA-4 as a major key player in the pathogenesis of autoimmune diabetes208.

Some studies have also suggested that resistance to TCR-induced activation-induced cell death (AICD) exists in NOD mice. This phenotype was characterized by persistent levels of the anti-apoptotic protein c-FLIP and reduced expression of pro-apoptotic molecules like caspase-8 and Fas/FasL211,212.

4.3.3 Impaired T cell activation

A range of defects in NOD T cell activation have been described. One example is the defective syngenic mixed lymphocyte reaction (SMLR) response that occurs at the time of insulitis, around 4-6 weeks of age, in NOD mice213. This age-related defect is observed in the CD4+ T cell population and characterized by low IL-2 production214. Another T cell activation defect observed in NOD mice is the T cell proliferative hyporesponsiveness upon TCR stimulation215. This proliferative unresponsiveness in vitro after activation through the TCR is linked to ldd4 on mouse chromosome 11 and mediated by low levels of IL-2 and IL-4216-218. In addition, it was shown that the NOD T cell hyporesponsiveness was associated with defective TCR-mediated activation along the PKC/Ras/MAPK pathway219.

4.3.4 Th1/Th2 imbalance

CD4+ T helper cells can be divided into distinct subsets on the basis of the cytokines they produce. Th1 T cells produce proinflammatory cytokines such as IL-2, IFN-γ and TNF-α and primarily support cell-mediated immune responses. In contrast, immunoregulatory Th2 T cells secrete cytokines like IL-4, IL-5 and IL-10 and help promote selected humoral responses. Many studies have suggested that a functional imbalance between the Th1 and Th2 subsets plays a role in diabetes pathogenesis123. A simplified generalized view is that Th1 effector response is associated with disease progression in NOD mice and
Th2 cells have a suppressive role in the development of diabetes\textsuperscript{220}. For example, administration of the cytokine IL-12\textsuperscript{221}, an inducer of Th1 responses, has been shown to accelerate disease in NOD mice, while injection of IL-4\textsuperscript{218} or IL-10\textsuperscript{222}, which promote Th2 development and function, protects against diabetes. However, the IL-12\textsuperscript{223} and IL-4\textsuperscript{224} knockout NOD mice have no change in diabetes incidence, emphasizing the oversimplification of the Th1/Th2 imbalance view. Nevertheless, NOD mice have an impaired Th1/Th2 balance that occurs around 4-6 weeks of age and is mirrored by a high IFN-\(\gamma\)/IL-4 expression ratio in the islet-infiltrating T cells\textsuperscript{225}. This early cytokine bias is predictive of the onset of destructive insulitis\textsuperscript{226} and may also explain why some T1D treatments are most effective when administrated to NOD mice before 3 weeks of age\textsuperscript{227}. There are probably many events that contribute to this imbalance between the Th1 and Th2 subsets in the NOD mouse and they are currently under extensive investigation\textsuperscript{123}.

### 4.3.5 Defective regulatory T cells?

As mentioned earlier, the CD4\textsuperscript{+} CD25\textsuperscript{+} Foxp3\textsuperscript{+} regulatory T cells play an indispensable role in controlling immune and autoimmune processes. During recent years there has been an explosion of reports proposing a role for T\(_{\text{regs}}\) in the diabetes pathogenesis of NOD mice\textsuperscript{119}. Nonetheless, whether or not NOD mice have a deficiency in T\(_{\text{regs}}\) is still controversial. Some studies have demonstrated altered numbers and/or function of T\(_{\text{regs}}\) in NOD mice\textsuperscript{14, 228-230}, at the same time as a number of other groups found no significant changes within the T\(_{\text{reg}}\) pool\textsuperscript{231-234}. The majority of reports, however, have shown an age-related decline in the suppressor function of T\(_{\text{regs}}\) associated with the progression of disease\textsuperscript{230, 233-238}. Interestingly, a recent report by Diane Mathis and colleagues\textsuperscript{239} demonstrated that the deficient T-cell regulation observed in NOD is not a result of impaired T\(_{\text{regs}}\) but rather due to an over-reactivity of T effector cells, which turns them relatively insensitive to suppression.
4.3.6 Impaired co-stimulation

Complete T cell activation requires not only binding of the TCR to the MHC-peptide complex but also additional signaling through co-stimulatory molecules. **CD28**, a protein expressed by naïve and activated T cells, is one of the best characterized co-stimulatory receptors. CD28 binds to CD80 (B7-1) and CD86 (B7-2) on APCs and mediates a positive signal for inducing and maintaining a T cell response\textsuperscript{240,241}. NOD B7-1/2 and CD28 knockout mice have a more rapid onset and severity of diabetes, implicating a role for the CD28/B7 pathway in the autoimmune process. Disruption of the CD28/B7 pathway is thought to result in a Th1 immune response and a defect in the number and function of CD4\textsuperscript{+}CD25\textsuperscript{+} regulatory T cells\textsuperscript{14,242}.

**CTLA-4** is another co-stimulatory molecule that binds to the same ligands as CD28, although with much higher affinity\textsuperscript{243}. CTLA-4 is expressed in T cells upon activation but, in contrast to CD28 inhibits T cell activation\textsuperscript{30,244}. As mentioned above, direct evidence for CTLA-4 as an important negative regulator of T cells was demonstrated in CTLA-4 deficient mice which die at 3-4 weeks of age from massive lymphocytic infiltration and tissue destruction in critical organs\textsuperscript{31,32}. Exactly how CTLA-4 inhibits T cell responses is not clear, but potential mechanisms include ligand competition with CD28 and recruitment of serine/threonine and tyrosine phosphatases to inhibit TCRζ chain phosphorylation, and immune synapse formation\textsuperscript{245}. There is plenty of evidence suggesting that CTLA-4 mediates regulation intrinsically on CTLA-4 expressing cells however, T cell non-autonomous effects have also been proposed. Bachmann \textit{et al.} showed that bone marrow chimeras generated from CTLA-4\textsuperscript{-/-} and wild-type CTLA-4\textsuperscript{+/+} donors are protected from lymphoproliferation, demonstrating that CTLA-4 deficient cells can be controlled by other cells expressing CTLA-4\textsuperscript{246}. Recently, it was demonstrated that T\textsubscript{regs} are critically dependent on CTLA-4 for \textit{in vivo} regulatory function and that the suppressive function might be mediated through CD80 and CD86 down regulation on APCs\textsuperscript{48,49}.
Several studies have supported a direct role for CTLA-4 in NOD disease progression. CTLA-4Ig (a soluble CD28 antagonist) treatment of NOD mice at the onset of insulitis inhibited diabetes development, presumable by blocking co-stimulatory molecules on APCs. In the BDC2.5 TCR transgenic NOD mouse model, blocking CTLA-4 with monoclonal antibodies before the development of insulitis resulted in a clear acceleration of diabetes. These data suggest a role of CTLA-4 signaling at very early stages of disease initiation.

As discussed above, Ctl-a-4 is the main candidate gene in the Idd5.1 region and data suggests that it is the ligand-independent isoform that is responsible for the Idd5.1 disease susceptibility. The full-length CTLA-4 isoform expression is not controlled by the Idd5.1 region. The role of the full-length CTLA-4 isoform in NOD pathogenesis is not known, although it seems to be of importance since blocking the full-length protein increases the diabetes incidence. Furthermore, anti-CD3 activated T cells from NOD mice have been shown to have a reduced full-length CTLA-4 expression compared to other mouse strains. In addition, impaired CTLA-4 expression in NOD mice could be associated with low levels of CD86 on NOD T cells and APCs.

ICOS is another co-stimulatory molecule in the CD28 family that appears to be dysregulated in NOD mice. ICOS is expressed on activated T cells, bind its ligand ICOSL and mediates a positive signal to T cells promoting IL-10 production and regulating the Th2 pathway. NOD activated T cells have a higher expression of ICOS on their cell surface compared with T cells from diabetes-resistant B6 and B10 mice. This increased ICOS expression appears to be correlated with increased production of IL-10 by NOD T cells. Treatment with anti-ICOS in the BDC 2.5 TCR transgenic mouse resulted in a decrease in T_{regs} and acceleration of diabetes. On the other hand, deletion of ICOS in NOD mice led to complete protection from diabetes and an unchanged number of T_{regs}. 

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**PD-1** (program death-1) is a co-stimulatory molecule expressed on activated T cells that negatively regulates T cell activation. This PD-1 inhibition of T cell responses is mediated by an immunoreceptor tyrosine-based motif located in the cytoplasmic tail of PD-1 that blocks downstream phosphorylation events\(^\text{253}\). Treatment of NOD mice at different ages with either anti- PD-1 or anti-PD-L1 (PD-1 ligand) resulted in an accelerated development of diabetes\(^\text{254}\). Moreover, expression of PD-L1 by pancreatic β-cells protects against diabetes\(^\text{254, 255}\).

### 4.3.7 Defects in multiple subsets

Even though T1D is a T cell mediated disease and a lot of effort has been made, including this thesis, to understand the role of T cells in the development of disease, NOD mice have defects in numerous other subsets that may contribute to diabetes susceptibility\(^\text{104}\). Some examples are, defective macrophage maturation and function\(^\text{256}\), impaired function and number of NKT cells\(^\text{257-259}\), activation defects of NK cells\(^\text{260-262}\), DC “hyperinflammatory” phenotype\(^\text{263-265}\) and B cell hyperresponsiveness\(^\text{266}\).
AIMS OF THIS THESIS

The general aim of this thesis was to identify and functionally characterize candidate genes potentially mediating susceptibility to T1D in the NOD mouse.

The strategy has been to identify abnormalities in the immune system of NOD mice that could contribute to the loss of tolerance and progression to autoimmunity. These NOD specific phenotypes were genetically mapped and candidate genes were subsequently identified and characterized.

**Paper I**
The aim was to determine the genetic factor(s) contributing to the defective CTLA-4 expression in NOD mice.

**Paper II**
The aim was to test the hypothesis that the NOD alleles of the CD3ζ mediate impaired CTLA-4 expression resulting from defective T cell activation.

**Paper III**
The aim was to examine the genetic control of resistance to dexamethazone (Dxm) induced apoptosis in NOD thymocytes.

**Paper IV**
The aim was to identify the genetic factor(s) in the *Idd6* susceptibility locus contributing to T1D in the NOD mouse.
RESULTS AND DISCUSSION

5. Research strategy
T1D is a complex multi-factorial disease and despite the identity of multiple genetic susceptibility loci, the biological functions mediated by individual *Idd* loci are largely unknown. In our studies the strategy has been to identify specific immunological traits in the NOD mouse that are likely to contribute to pathogenesis and therefore be considered as subphenotypes of diabetes. Such NOD-defined traits were then genetically analyzed by combining genome-linkage analysis with congenic mapping. Phenotypes that map to an already defined *Idd* region would represent possible contributors to the disease process and novel regions might constitute new *Idd* loci. The biological functions controlled by these loci may provide relevant information for a rational search of candidate genes. Functional characterization of candidate genes could identify “diabetes genes” and result in important insights into the pathophysiological processes underlying the disease.

6. CTLA-4 expression in NOD mice (paper I)
Cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4), also known as CD152, is one of the most well characterized negative co-stimulatory molecules. The importance of CTLA-4 as a negative regulator of immune response is demonstrated in CTLA-4 deficient mice which develop a rapidly lethal lymphoproliferative disorder with multi-organ lymphocyte infiltration at a young age. The *Ctla-4* gene is located on mouse chromosome 1 and on human chromosome 2 and has been implicated in susceptibility to T1D in both humans and NOD mice. There are four splice variants for the *Ctla-4* gene: full-length (flCTLA-4), soluble (sCTLA-4), ligand-independent (liCTLA-4) and a transcript coding only for exons 1 and 4 (Figure 5).
Figure 5. The various splice forms of the Cita-4 gene. The Cita-4 gene consists of four exons that can give rise to four different splice variants. Humans express the liCITA-4, exon 1-4 CITA-4 and sCITA-4 isoforms, while mice also express the liCITA-4 (adapted from Teft et al.267).

In humans, higher levels of the sCITA-4 mRNA in resting CD4^+ T cells have been shown to correlate with protection from both T1D and autoimmune thyroid disease^{71}. The reduced level of sCITA-4 mRNA produced in the CT60 disease susceptible genotype has been suggested to enhance T-cell activation and/or reduce T_{reg} activity, thereby increasing the risk of autoimmunity^{69,70}.

In the NOD mouse, CTLA-4 constitutes a major candidate for the Idd5.1 diabetes susceptibility locus^{142}. Ueda et al.^{71} have reported that a SNP in exon 2 of Cita-4 affects the level of the alternatively spliced liCITA-4. This liCITA-4 lacks the B7.1/B7.2 ligand-binding domain (Figure 5) and inhibits T-cell proliferation and cytokine production by dephosphorylating the CD3ζ^{184}. Higher expression levels of liCITA-4 mRNA are associated with protection from T1D in diabetes-resistant NOD.Idd5 congeneric mice^{71,142}. The liCITA-4
has been shown to be highly expressed in resting T cells and is rapidly downregulated upon T cell activation and therefore this isoform is speculated to control survival and/or homeostasis of naïve T cells\textsuperscript{184,245}.

\textit{\textit{fl}CTLA-4} is upregulated in T cells following activation and we have previously shown that activated NOD T cells have an impaired expression of \textit{fl}CTLA-4 compared to non-autoimmune strains\textsuperscript{210}. The importance of \textit{fl}CTLA-4 expression levels in diabetes pathogenesis is demonstrated by the accelerated disease progression in NOD mice treated with anti-CTLA-4 antibodies recognizing \textit{fl}CTLA-4\textsuperscript{248}. Moreover, \textit{fl}CTLA-4 is constitutively expressed on CD4\textsuperscript{+} CD25\textsuperscript{+} regulatory T cells\textsuperscript{14,46,47} and is essential for their suppressive function\textsuperscript{48,49}. In \textit{Paper I} we confirmed that \textit{fl}CTLA-4 has reduced expression in both activated CD4\textsuperscript{+} and CD8\textsuperscript{+} NOD T cells. Moreover, as previously reported\textsuperscript{182}, using NOD.Idd5 congenic mice we showed that it was not the \textit{Cita-4} gene itself or gene(s) located in the \textit{Idd5} region that controlled the aberrant \textit{fl}CTLA-4 expression observed in the NOD mouse. Because \textit{fl}CTLA-4 expression could play a role in disease development of the NOD mouse we genetically mapped the control of \textit{fl}CTLA-4 expression. Using a F2(NODxB6) cohort we identified a novel region in the distal part of chromosome 1, called \textit{Ctex}, and a region on chromosome 3 overlapping with the \textit{Idd3/Ii-2} locus controlling the \textit{fl}CTLA-4 expression. The \textit{Ctex} locus was not found to map to any known \textit{Idd}, however it overlapped the QTLs for NKT-cell numbers\textsuperscript{268} and the region that controls increased IgG serum levels of NOD mice compared with B6 mice\textsuperscript{269}. The \textit{Ctex} region also overlapped the NOD mouse lupus susceptibility gene Babs2/Bana3 controlling production of antinuclear autoantibodies (ANA)\textsuperscript{270}. Moreover, Zuchelli \textit{et al.}\textsuperscript{198} reported that genes on chromosome 1 and chromosome 3, overlapping with the control of \textit{fl}CTLA-4, influenced resistance of NOD thymocytes to negative selection. Together, these findings emphasize the \textit{Ctex} locus as an important region, potentially affecting diabetes susceptibility in the NOD mouse.
The suggestive linkage of control of flCTLA-4 expression to the Idd3/Ii2 region was confirmed in NOD.Idd3 congenic mice displaying an intermediate flCTLA-4 expression compared to B6 and NOD mice (Paper I). This is in agreement with the fact that IL-2 has been shown to play an important role in the expression of flCTLA-4 upon T-cell activation. Thus, IL-2 deficient mice are unable to induce any detectable surface flCTLA-4 following anti-CD3 activation. IL-2 is also essential for development and function of Treg. It has been shown that the T1D resistant B6 allele of Ii2 produces approximately 2-fold more IL-2 mRNA than the NOD allele. In addition, hemizygous IL-2 KO NOD mice, which have only one functional Ii2 allele, have a 50% decrease in IL-2 production and an increased T1D frequency. The protective effect of increased IL-2 levels was correlated with improved Treg function and inhibition of Th17 cells. Moreover, administration of low-dose IL-2 promoted Treg survival and protected NOD mice from developing diabetes.

Interestingly, we observed that addition of exogenous IL-2 could in part overcome the difference in CTLA-4 expression between B6 and NOD after in vitro anti-CD3 activation of spleen cells. On the other hand, co-stimulation with anti-CD28 could not upregulate CTLA-4 expression in NOD mice to B6 levels (Paper I). One can speculate, in view of the recent reports demonstrating the critical role of CTLA-4 for Treg function that IL-2 may influence CTLA-4 expression on Tregs thereby affecting their suppressive function.

ICOS is a positive regulator of T-cell activation, belonging to the same B7/CD28 family as CTLA-4. In parallel with lower levels of flCTLA-4 expression, activated NOD T cells have been reported to express higher levels of ICOS compared with B6 and B10 T cells. This increased expression of ICOS by activated NOD T cells is controlled by the Idd5.1 locus which also confers lower liCTLA-4 expression in NOD mice. It has been suggested that high ICOS levels could be a consequence of low expression levels of the
liCTLA-4 in NOD T cells\textsuperscript{142, 186}. In concordance with this, Riley et al.\textsuperscript{272} showed that CTLA-4 engagement reduced ICOS cell surface expression.

In Paper I and Paper II we confirmed that ICOS levels are controlled by the \textit{Idd.5} locus and showed that the \textit{Ctex} and \textit{Idd3} regions do not influence ICOS expression levels.

7. \textit{Cd3ζ} as a candidate gene (Paper II)

The \textit{Ctex} region identified in Paper I is large and contains around 200 genes. Nevertheless, \textit{Cd3ζ} (\textit{Cd247}) was a promising candidate gene as it is involved in TCR mediated activation of T cells and located close to the highest linkage peak in the mapping study. CD3ζ is one of the five invariant polypeptide chains of the TCR/CD3 complex and functions as an amplifier of the TCR signaling cascade\textsuperscript{273}. The TCR/CD3 complex consists of two CD3ζ polypeptide chains forming a homodimer and each ζ-chain has three intracytoplasmic immunoreceptor tyrosine-based motifs (ITAMs) (Figure 6). CD3ζ is one of the first and most heavily tyrosine-phosphorylated proteins following TCR engagement\textsuperscript{274, 275}. CD3ζ is also essential for efficient surface expression of the TCR/CD3 complex, as demonstrated in T cells from CD3ζ deficient mice which expressed little or no surface CD3 and TCR\textsuperscript{276}.

In Paper II, the aim was to test the hypothesis that the NOD allele of CD3ζ mediates impaired CTLA-4 expression upon T-cell activation. Since CD3ζ is important for T-cell signaling we analyzed CTLA-4 expression in activated sorted CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells. We showed that the impaired CTLA-4 expression following anti-CD3 activation is due to an intrinsic defect of NOD T cells, emphasizing CD3ζ as a candidate gene.
Figure 6. Overview of signal transduction during T-cell activation. Upon TCR and CD28 co-receptor engagement, the first step in the signaling cascade is activation of LCK and FYN which phosphorylate the ITAMs of CD3ζ- and CD3ε-chains. Signaling results in the activation of various signal-transduction pathways which leads to the formation of several products. NF-κB, NFAT and activated MAPK are three key molecules that have important influences on transcription. Complete T-cell activation is indicated by activation of the transcription factors NF-κB and NFAT, transcription of cytokine genes, secretion of cytokines, proliferation of T cells and recruitment of various cells of the adaptive and innate immune system. Following T-cell activation, several inhibitory mechanisms operate (shown in red), including CTLA-4 (adapted from Michal Baniyash).
Next we analyzed the CD3ζ protein expression after anti-CD3 activation and showed that CD4+ T cells from B6 mice expressed more CD3ζ compared with CD4+ T cells from NOD mice.

As recently demonstrated, CTLA-4 plays a key role in Treg suppressive function and we therefore analyzed the expression of CTLA-4 in this subset after anti-CD3 activation. In Paper II we showed that activated NOD CD4+FoxP3+ T cells displayed a lower expression of CTLA-4 compared with Tregs from B6 mice. This altered expression of CTLA-4 in Tregs from NOD mice may influence their inhibitory function. Nonetheless, several studies have reported that the NOD strain might not have inborn Treg defects but rather a decline in the suppressive function of Tregs with the progression of disease. In addition, a recent report showed that the impaired T-cell regulation in NOD was a result of the inability of effector T cells to be regulated and not due to defective Tregs. This Treg/Effector imbalance appeared to be intrinsic to the NOD genome, did not vary with age and was not a consequence of the autoimmune process. Interestingly, Cuda et al. demonstrated that the murine lupus susceptibility locus Sle1a, which overlaps with the Ctex region, plays a major role in T-cell tolerance through Treg regulation via multiple mechanisms. Our data showed that the impaired upregulation of CTLA-4 following activation was evident in both regulatory and effector CD4+ T cells which could potentially affect both the NOD Treg suppressive function and the reported overreactivity of NOD effector cells.

To verify that the defective upregulation of CTLA-4 is controlled by the NOD allele of the Ctex/Cd3ζ locus we constructed congenic mice over the Ctex region. Analysis of these mice confirmed that the Ctex/Cd3ζ locus contributes to the low expression of CTLA-4 observed in NOD mice.

Since impairment in CD3ζ would most likely affect CD3-mediated signaling we analyzed the level of proliferation and production of the cytokines IL-2,
IFN-γ and IL-4 upon anti-CD3 stimulation. Using NOD.C1R11 congenic mice, having the B6 allele of Ctx/Cd3ζ on the NOD genetic background, we showed that the NOD allele of the Ctx/Cd3ζ region conferred impaired cytokine secretion and proliferation. The phorbol ester PMA plus calcium ionophore ionomycin trigger stimulation of T cells by bypassing cross linking of the TCR/CD3 complex directly activating protein kinase C-θ (PKC-θ) and raising the calcium levels, respectively\(^\text{279}\) (Figure 6). Using PMA plus ionomycin activation we demonstrated that both the defective cytokine and CTLA-4 expression could be restored which sustains our hypothesis. Interestingly, reports have showed that reduced CD3ζ expression mediates T-cell dysfunction, involving hypoproliferation and reduced cytokine expression\(^\text{280-282}\). Together, these data supports Cd3ζ as the prime candidate gene for the Ctx locus.

It has previously been reported that NOD T cells display a proliferative hyporesponsiveness upon TCR activation, which is mediated by reduced IL-2 and IL-4 production\(^\text{215, 218, 219, 283}\). This hypoproliferation of NOD T cells is associated with a block in Ras activation and defective signaling along the PKC/Ras/MAPK pathway\(^\text{219}\). Several sequential changes in TCR-proximal signaling events, including differential activation of the Fyn-TCRζ-Cbl pathway may explain this block in Ras activation\(^\text{284}\). The genetic control of this T-cell hyporesponsive trait was mapped to gene(s) in the Idd4.2 region on chromosome 11\(^\text{216, 217}\). Analyses of Idd4 congenic mice showed that a B6 allele at this locus could partially restore the T-cell proliferation response\(^\text{217}\). This is compatible with our results demonstrating that the B6 allele of the Ctx/Cd3ζ region could also only partially restore the B6 cytokine and proliferation pattern. Moreover, the less pronounced effects on cytokines and proliferation compared with CTLA-4 expression in the NOD.C1R11 mice may result from different downstream signaling of CD3ζ and interaction with other genetic factors including the Idd4.2. The overlapping phenotypes controlled by the Ctx/Cd3ζ and Idd4.2 regions is not a surprise since T-cell signaling is a
complex process and NOD T-cell hyporesponsiveness is most probably a polygenic trait manifested by pleiotropic, epistatic, and/or penetrance effects\textsuperscript{217}.

The murine \textit{Cd3ζ} gene is composed of 9 exons and spans approximately 79 kb (www.informatics.jax.org). The first intron $> 66$ kb has SNPs that differ between the NOD and B6 mouse strains. The mouse strain DBA/2 has the NOD allele whereas C3H/HeJ and Balb/c mice have the B6 allele of \textit{Cd3ζ}. In \textbf{Paper II}, we showed that the NOD/DBA allele of CD3ζ mediates defective CTLA-4 expression upon anti-CD3 activation \textit{in vitro} compared to the B6/C3H/Balb allele. This suggests that polymorphisms in the Ctx/Cd3ζ region confer altered CTLA-4 expression following TCR activation independent of genetic background and also independent of an ongoing autoimmune process.

Analysis of the diabetes incidence in NOD.\textit{C1R11} congeneric mice is ongoing, for evaluation of the direct impact of the Ctx/Cd3ζ locus on diabetes development. However, activated NOD.\textit{C1R11} spleen cells showed highly increased IL-4 production, even more so than the B6 mouse strain, which could potentially affect the pathogenic profile of islet-infiltrating cells\textsuperscript{149}. It has been shown that administration of rIL-4 \textit{in vivo} protects NOD mice from insulitis and diabetes by correcting the Th1 bias present in the NOD mouse\textsuperscript{218,285}. Moreover, \textit{in situ} expression of IL-4 within the pancreatic β cells induced a local immunosuppressive effect preventing the mice from developing insulitis and diabetes\textsuperscript{286}. Interestingly, decreased IL-4 production in activated T cells from patients with new onset IDDM has been reported\textsuperscript{287}. In view of this, high IL-4 levels in NOD.\textit{C1R11} mice might have protective effects on the development of diabetes.

CD3ζ has previously been implicated in autoimmunity. Studies have demonstrated altered expression and function of CD3ζ in patients with both SLE\textsuperscript{281, 288-290} and RA\textsuperscript{280, 291}. Several factors and mechanisms may contribute to the reduction of CD3ζ expression in patients with such pathologies and further
studies are required to understand these mechanisms (reviewed in 277, 292). A recent report suggested that reduced CD3ζ expression caused by genetic variations in the CD3ζ gene can have functional consequences manifested by autoimmunity293. These authors showed that defective CD3ζ expression was associated with polymorphisms in the CD3ζ 3′-untranslated region (UTR) in SLE patients and healthy controls. Additionally, performing a family-based study they demonstrated that the haplotype carrying the low-expression variants predisposes to SLE293.

Further insights into how impairment of CD3ζ might contribute to autoimmunity stems from the analysis of CD3ζ knockout (ZKO) mice. These ZKO mice276, 294-296 have greatly reduced surface TCR expression and impaired T-cell development. Thus, it has been shown that low TCR signaling caused by the CD3ζ deficiency resulted in aberrant T-cell selection, including positive selection of autoreactive T cells296. Moreover, T cells from ZKO mice have very low cell surface expression of CTLA-4/CD28 and abnormal regulation of cytokine production297. Interestingly, complete CD3ζ deficiency in humans showed similar defects in T-cell development and function as those observed in ZKO mice. The lack of a CD3ζ protein due to a mutation in the CD3ζ gene caused severe combined immunodeficiency (SCID) by preventing normal TCR assembly and surface expression298.
8. Dexamethazone-induced apoptosis and the Idd6 susceptibility locus (Paper III)

Apoptosis play an essential role during both thymocyte development and T-cell mediated immune responses. NOD mice display resistance to several in vivo and in vitro treatments known to induce programmed cell death in T cells and this defective apoptosis induction may contribute to the development of autoimmunity in this mouse strain. In line with this, Leijon et al. demonstrated that immature DP NOD thymocytes showed enhanced resistant to in vivo glucocorticoid (dexamethazone)-induced apoptosis. Glucocorticoids (GCs) are a class of steroid hormones which regulate a variety of important biological functions, including the ability to induce T lymphocyte apoptosis (Figure 7).

![Diagram showing glucocorticoid regulation](image)

**Figure 7.** Immunoregulation by glucocorticoids (GCs). GCs enter the cells and bind to the intracellular GC receptor (GR) after which the GC-GR complex translocates to the nucleus for positive and negative gene regulation. Thymocytes and T cells can respond to different levels of GC stimulation by either undergoing apoptosis or growth stimulation (adapted from Jondal et al.).
The adrenal gland is the primary site for GC synthesis but there is also strong evidence for the production of endogenous GCs in the thymus, most likely by thymic stromal cells\textsuperscript{300-303}. Independent signaling through the glucocorticoid receptor (GR) or through the TCR has been reported to induce apoptosis, while simultaneous stimulation through both receptors may result in reduced cell death\textsuperscript{304}. It has also been demonstrated that inhibition of thymic GC biosynthesis causes an increase in thymocyte apoptosis, suggesting that GR signaling can alter thymocyte selection\textsuperscript{305}. In support of this, King et al.\textsuperscript{304} showed that the GR antisense transgenic mice with reduced GR expression had lower thymic cellularity and were more resistant to GC-induced thymocyte apoptosis, implying that physiological levels of GR signaling are important for thymocyte survival in association with positive selection. Based on these findings it can be suggested that the weak signaling through the GC pathway observed in NOD mice by Leijon et al. and in \textbf{Paper III}, indirectly affects TCR-based selection. Nonetheless, the issue of thymus-derived GCs and the involvement of these hormones in thymocyte selection remain controversial. Okret and colleagues\textsuperscript{306} showed that transgenic mice with reduced GR expression had increased thymocytes instead of diminished thymocytes numbers as reported by King et al.\textsuperscript{304}. Moreover, thymocytes derived from GR knockout mice showed no significant differences in cell number or thymic selection, thus thymocyte development appeared to be normal in the absence of GR signaling\textsuperscript{307, 308}. The GR knockout thymocytes, on the other hand, were completely resistant to GC-induced apoptosis. Like GR knockout mice, it has been demonstrated that T cell specific GR knockout mice are resistant to GC-induced apoptosis while having normal thymocyte numbers and distribution\textsuperscript{309, 310}. The contradictory results using genetically modified forms of GR in different mouse models could be due to the fact that GCs can mediate both positive and negative effects in the thymus depending on, for instance, the local GC concentration, the responsiveness in the thymocyte population and the age of the mice\textsuperscript{300}. In addition, GCs can affect signaling transduction pathways...
independent of the GR by interfering with numerous cellular processes such as second-messenger cascades, cellular Ca$^{2+}$ levels and phosphorylation events$^{311}$.

Using quantitative trait loci mapping of a F2 cohort between NOD and B6, Penha-Goncalves et al.$^{206}$ demonstrated that the Idd6 susceptibility locus controls resistance in NOD DP thymocytes to dexamethazone (Dxm)-induced apoptosis. In Paper III, we have refined the Dxm resistant phenotype by performing a time-course experiment measuring the Dxm-induced apoptosis in both NOD and B6 mice. 12 hours after Dxm treatment we observed a peak in thymocyte apoptosis which also coincided with the maximum difference between the two strains. Genetic mapping confirmed the previously reported$^{206}$ association between the Dxm resistant phenotype and the Idd6 locus. The strongest linkage, with a maximum LOD score value of 6.5, was obtained for the distal markers D6Mit14 and D6mit15 on chromosome 6.

To confirm and further define the genetic mapping results, as well as to evaluate the contribution of the Idd6 locus to TID development, we constructed three mouse strains congenic over the Idd6 region. The N.B-Idd6 and N.B-Idd6-15 congenic strains contained either a 9 or a 3 cM, respectively, B6-derived region on the NOD genetic background and the reverse B.N-Idd6 congenic strain carried a 9 cM NOD-derived region on the B6 genetic background (Figure 8 and Paper III). Analysis of diabetes development in the N.B-Idd6 congenic strain demonstrated that the most distal 9 cM region on chromosome 6 protects from diabetes and thus includes the Idd6 locus. This region overlaps with the previously mapped Idd6 region$^{136}$.
**Figure 8.** *Idd6* congenic strains. The B.N-*Idd6* and the N.B-*Idd6* strains contain a 9 cM NOD-derived (white bars) or B6-derived (black bars), respectively, chromosomal segment telomeric to the D6Mit291 marker on chromosome 6. The N.B-*Idd6*-15 strain contains a 3 cM B6-derived chromosomal region telomeric to the D6Mit200 marker. NOD.C3H *Idd6* VIII strain constructed by Rogner et al.\(^{162}\) contains a 5 cM C3H/HeJ-derived (striped bar) chromosomal region telomeric to the D6Mit113 marker. Genetic distances were retrieved from the mouse genome informatics database (MGI).

Furthermore, analysis of Dxm-induced apoptosis in the N.B-*Idd6*-15 congenic strain showed that the 3 cM region telomeric to marker D6Mit200 was sufficient to restore the B6 parental phenotype. The reverse congenic B.N-*Idd6* containing a 9 cM NOD-derived region displayed an apoptosis resistant phenotype similar to the NOD mouse. These results restricted the control of apoptosis resistance to a 3 cM interval on distal chromosome 6 (Figure 8) and indicated that allelic variation in this region confers altered apoptosis induction upon Dxm treatment, independent of genetic background and also independent of an ongoing autoimmune process.
9. The *Lrmp* gene and the *Idd6* susceptibility locus (Paper IV)

To further refine the *Idd6* locus defined in Paper III, we measured the diabetes incidence in the N.B-*Idd6*-15 congenic strain containing a 3 cM B6-derived region distal on chromosome 6. This congenic mouse line showed a similar incidence of diabetes compared to littermate controls (Paper IV), excluding the 3 cM chromosomal segment at the tip of chromosome 6 from directly contributing to diabetes. This data restricted the diabetes susceptibility region to an 8 cM interval spanning markers D6Mit291 and D6Mit15.

Other studies using congenic strains from crosses of NOD and C3H/HeJ mice have localized the *Idd6* locus to a 5 cM region at the tip of chromosome 6 (Figure 8). Moreover, Hung *et al.* recently subdivided the *Idd6* locus into three loci, *Idd6.1, Idd6.2*, and *Idd6.3*, using NOD.C3H congenic mice. The *Idd6.1* and *Idd6.3* regions identified by these authors correspond to the 3 cM region at the tip of chromosome 6 and the *Idd6.2* locus to a region from marker D6Mit113 at 144.4 Mb to the upstream of marker D6Mit15 at 147.3 Mb. As we could now exclude the telomeric 3 cM region from contributing directly to diabetes susceptibility in the NOD/B6 strain combination, we focused our analysis on an approximately 3 Mb region spanning the interval between the markers D6mit113 and D6Mit15 on chromosome 6 (Figure 8 and Paper IV). This region corresponds approximately to the *Idd6.2* region identified by Hung *et al.*

In the 3 Mb chromosomal region, twelve confirmed genes were analyzed using quantitative real-time PCR (Paper IV). From analysis of RNA expression of these 12 candidate genes only one gene, the lymphoid restricted membrane protein encoding gene (*Lrmp*) \(^{312, 313}\), was expressed at significantly different levels in thymocytes from NOD and B6 mice. NOD thymocytes showed significantly decreased expression of Lrmp compared to B6 thymocytes but also compared to C3H/Hej and BALB/c thymocytes, making it a plausible candidate gene for the *Idd6.2* locus. Furthermore, analysis of Lrmp expression
in thymocytes from *Idd6* congeneric mice demonstrated that control of transcript levels of this gene lies within the *Idd6.2* region. The expression of Lrmp in N.B-*Idd6* congeneric mice was significantly higher compared to the NOD control mice, while in the N.B-*Idd6*-15 congeneric mice, excluding the *Idd6.2* locus, Lrmp levels were similar to NOD. In addition, the reverse congeneric B.N-*Idd6* mouse strain, carrying the NOD allele at the *Idd6.2* locus, displayed expression levels similar to NOD. This indicated that the lower Lrmp expression observed in the NOD mouse was independent of genetic background and not a result of an ongoing autoimmune inflammatory process.

The gene 4930469P12Rik (*P12Rik*), also located in the *Idd6.2* region, which encodes a growth hormone-inducible soluble protein showed a huge variation in expression in thymocytes from B6 but not from NOD origin. However, the expression level did not significantly differ between the strains and the BALB/c mouse strain expressed similar *P12Rik* RNA levels as the NOD mouse (data not shown). Even though this gene cannot be formally excluded, the *Lrmp* is a more plausible candidate for the *Idd6.2* locus.

The *Lrmp/Jaw1* gene encodes a type II integral membrane protein localized to the endoplasmic reticulum (ER) of primarily lymphocytes. The protein contains a coiled-coil domain in the middle third that is highly conserved between mice and humans, implying that this region may be functionally important. Lrmp is highly expressed in thymocytes and pre-T and -B cell lines, but has reduced or no expression in mature T-cell lines and plasma B cells. This down regulation of T and B cells during differentiation suggests a role for Lrmp in lymphoid development. This is in agreement with our results showing that Lrmp expression is enhanced in immature thymic populations, hinting that the gene is involved in T-cell development (*Paper IV*). We also demonstrated that the Lrmp expression was higher in immature compared to mature B cells, although the strain specific differences were less pronounced.
Lrmp has been shown to have homology with two other genes, *Irag* and *Mrvi1*. The *Irag* gene encodes an inositol 1,4,5-triphosphate (InsP$_3$) receptor associated cGMP kinase substrate found in many bovine smooth muscle, such as the aorta, trachea and uterus. IRAG is also located in the ER and has a similar structure to Lrmp, including the central coiled-coil structure. IRAG has been proposed to have a role as a modulator of intracellular calcium levels. Thus, phosphorylation of IRAG by a cGMP dependent protein kinase I$\beta$ (cGKI$\beta$) inhibits InsP$_3$-induced intracellular calcium release. Calcium is an essential second messenger which controls a wide range of cellular processes, including T-cell proliferation and activation as well as apoptosis.

The *Mrvi1* gene encodes a protein that like Lrmp is a type II membrane protein localized in the ER. Mrvil most likely represents the murine-lymphoid form of IRAG. By analogy it is therefore possible that Lrmp might play a role in the control of intracellular calcium levels.

The *Idd6.2* locus overlaps with the susceptibility locus for pulmonary adenoma (*Pas1*) identified in crosses between lung tumor-resistant B6 mice and lung tumor-susceptible A/J mice. Candidate gene analysis of the *Pas1* locus showed polymorphisms in the *Lrmp* gene that resulted in alteration in the amino acid sequence between the B6 and A/J strains. These polymorphisms were demonstrated to cosegregate with mouse lung tumor susceptibility. Moreover, alleles in the *Pas1* locus are associated to acute inflammatory response (AIR). In the *Lrmp* gene, the proline to leucine substitution at codon 537 showed a highly significant linkage with the AIR phenotype. This proline allele for the *Lrmp* gene was also linked to the *Pas1* susceptibility phenotype.

NOD *Lrpm* revealed 13 nucleotide polymorphisms, of which seven predicted an alteration in the amino acid sequence in comparison to C3H/Hej and B6. Four of these polymorphisms were those observed in the *Pas1* susceptibility
mouse strains A/J and BALB/c\textsuperscript{319, 321}. In addition, the authors observed three other coding polymorphisms that were specific for NOD\textsuperscript{321}. Since we detected higher Lrmp expression in BALB/c mice compared to NOD mice, the NOD-specific mutations might be influencing gene expression in our study.

In **Paper III**, we demonstrated that the 3 cM region on the tip of chromosome 6 controls the resistance to Dxm-induced apoptosis in immature thymocytes observed in the NOD mouse. In **Paper IV**, the diabetes incidence of the N.B-\textit{Idd6}-15 excluded the apoptosis resistance phenotype from directly contributing to diabetes susceptibility. However, it does not exclude the possibility that gene(s) in the distal 3 cM region of chromosome 6 might indirectly, through interactions with other genes, contribute to T1D. Studies with congenic mice have demonstrated interaction between \textit{Idd} loci. The most striking example is the strong and complex interactions between the four \textit{Idd5} loci on chromosome 1\textsuperscript{143}. Moreover, in the NOD/C3H strain combination, Hung \textit{et al.}\textsuperscript{144} identified the \textit{Idd6.1} and \textit{Idd6.3} loci located in the distal region of chromosome 6. These susceptibility loci roughly correspond to the region controlling the Dxm-apoptosis resistance phenotype. In fact, whether the region we define in **Paper IV** containing the \textit{Idd6.2} locus can mediate protection against diabetes by itself in an NOD/B6 strain combination remains to be established.

Interestingly, the \textit{Idd6} locus overlaps with another autoimmune locus, the experimental autoimmune myocarditis locus \textit{Eam}\textsuperscript{322}. The A.SW mice, which are susceptible to experimentally induced autoimmune myocarditis (EAM), also demonstrated reduced sensitivity to Dxm-induced thymocyte apoptosis compared with the EAM resistant B10.S strain. In addition, these authors identified a locus on proximal chromosome 1, overlapping with the diabetes susceptibility locus \textit{Idd5}, which was likely to influence susceptibility to EAM. The \textit{Idd5} locus has been shown to control resistance of peripheral NOD lymphocytes to cyclophosphamide (CY)-induced apoptosis\textsuperscript{210}, and again like the NOD mice, A.SW lymphocytes showed diminished sensitivity to CY-
induced apoptosis\textsuperscript{322}. This suggests that polymorphisms at these loci influence apoptosis and might control susceptibility to various autoimmune diseases.

The \textit{Idd6} locus has previously been shown to control low rates of proliferation in immature NOD thymocytes\textsuperscript{207} and recently, it has been reported that the \textit{Idd6} NOD.C3H-congenic mouse strains displayed a down-regulation of the \textit{Toll-like receptor 1 (Tlr1)} gene and reduced expression of \textit{Tnf-\alpha} and \textit{IL-6}, both known to be controlled by TLR1\textsuperscript{323}. The down-regulation of \textit{Tlr1} was also shown to be associated with lower proliferation of stimulated CD4\textsuperscript{+} T cells\textsuperscript{323}.

Furthermore, it has recently been demonstrated that \textit{Idd6} alleles modulate the efficiency of CD4\textsuperscript{+} CD25\textsuperscript{+} T\textsubscript{regs}\textsuperscript{324}. In transfer experiments, NOD.C3H \textit{Idd6} CD4\textsuperscript{+} CD25\textsuperscript{+} T cells showed significantly higher suppressive activity than control NOD T\textsubscript{regs}. Moreover, local changes in the NOD.C3H \textit{Idd6} islet infiltrate suggested that T\textsubscript{regs} interact with other cell types either in the islet itself or the draining lymph nodes, modulating the aggressiveness of the autoimmune response to \textit{\textbeta} cells\textsuperscript{324}.

In summary, the T1D-associated \textit{Idd6} locus controls various cellular phenotypes and overlaps with other disease susceptibility loci implying that this region is a multi-gene locus with important immune functions. Definitive proof regarding the implications of the \textit{Lrmp} gene, or any other gene in this region, for diabetes development can only be assessed by functional testing, for example by construction of knock-in or knock-out NOD animals\textsuperscript{321}. 

\textsuperscript{58}
CONCLUDING REMARKS

The incidence of T1D, particularly in children aged 0-14, is increasing worldwide\textsuperscript{325, 326}. Sweden has the third highest incidence in the world, after Finland and Sardinia\textsuperscript{326}. With the accelerating discovery of diabetes susceptibility genes it is likely that most of the genetic basis for T1D will be revealed in the near future. However, little is known about the contribution of such genetic factors on the pathogenesis of disease. The NOD mouse, which spontaneously develops diabetes and is one of the most commonly used animal models for T1D research, constitutes an invaluable tool for studying association between genetic factors and pathogenic mechanisms contributing to the disease process. Identification of molecular and cellular mechanisms underlying T1D could enable discovery of better treatments or even prevention of the disease.

In this thesis we have used the strategy of characterizing and genetically mapping possible disease associated traits in the NOD mouse in combination with candidate gene analysis. The findings of this thesis can be summarized as follows:

We have demonstrated that a novel locus, \textit{Ctex}, in the distal part of chromosome 1, together with the \textit{Idd3/Il-2} locus on chromosome 3, control the defective expression of flCTLA-4 observed in activated NOD T cells. Moreover, we showed that exogenous IL-2 can partially restore the deficiency of NOD T cells to upregulate flCTLA \textit{in vitro}. Additionally, flCTLA-4 expression is not associated with ICOS expression in NOD mice. Instead, as previously shown\textsuperscript{186}, the \textit{Idd5.1} region controls ICOS expression levels. By the use of congenic mice we restricted the region controlling flCTLA-4 expression to a 28.8 Mb region containing the \textit{Cd3\zeta} candidate gene. We also demonstrated that impaired CTLA-4 expression is conferred by the NOD allele of \textit{Cd3\zeta} on various genetic backgrounds and showed that activated NOD CD4\textsuperscript{+} T cells had
defective CD3ζ expression. Furthermore, the NOD allele of the Ctex/Cd3ζ region was shown to confer defective T-cell activation which could be surpassed by PMA plus ionomycin stimulation. Based on these findings we suggest that the allelic variants of the NOD Cd3ζ gene might contribute to defective T-cell activation and potentially susceptibility for T1D in the NOD mouse.

The Idd6 region was restricted to an 8 cM interval spanning markers D6Mit291 and D6Mit15 on chromosome 6, by congenic mapping. Combining our findings with results from congenic strain analyses between NOD and C3H/HeJ mice, directed our further research to a 3 Mb region roughly corresponding to the previously defined Idd6.2 locus\textsuperscript{144}. From analyses of candidate genes in this region, the Lrmp gene encoding a lymphoid restricted membrane protein was shown to be expressed at lower levels in NOD compared to B6 mice. Moreover, we demonstrated that Lrmp is expressed at high levels in immature thymic populations and that the Lrmp levels are controlled by the Idd6.2 region. From these data we hypothesize that the Lrmp gene plays a role during T-cell development, possibly through modulating intracellular calcium levels, and constitutes a susceptibility factor for T1D. In addition we also showed that the 3 cM region on the tip of chromosome 6, roughly overlapping with the Idd6.1 and Idd6.3 loci, controls resistance to the Dxm-induced apoptosis in immature thymocytes observed in the NOD mouse.
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