Molecular and cellular mechanisms contributing to the pathogenesis of autoimmune diabetes

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To my family…
“Nada do que foi será de novo
Do jeito que já foi um dia
Tudo passa, tudo sempre passará
A vida vem em ondas como mar...”

“Nothing of what once was will be again
As it was one day
It all moves on, everything will always move on
Live comes in waves like sea...”

“Como uma onda”
Caetano Veloso - “Noites do Norte”
Lulu Santos/Nelson Motta
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ABBREVIATIONS

ACAID  Anterior Chamber-Associated Immune Deviation model
AICD  Activation Induced Cell Death
ALPS  human Autoimmune Lympho-Proliferative Syndrome
α-GalCer  α-GalacosylCeramide
APC  Antigen Presenting Cell
APECED  Autoimmune Poly-Endocrinopathy-Candidiasis-Ectodermal Dystrophy
β2m  β2 microglobulin
B6  C57BL/6 mouse strain
B10  C57BL/10 mouse strain
cM  Centimorgans
CMJ  Cortico-Medullary Junction
CNS  Central Nervous System
CTLA-4  Cytotoxic T-Lymphocyte-associated Antigen 4
flCTLA-4  full length form of CTLA-4
liCTLA-4  ligand independent form of CTLA-4
sCTLA-4  soluble form of CTLA-4
DC  Dendritic Cell
DETCs  Dendritic Epidermal T Cells
DN  Double Negative CD4⁻CD8⁻ T cells
DP  Double Positive CD4⁺CD8⁺ T cells
EA chimeras  Embryo Aggregation chimeras
EAE  Experimental Autoimmune Encephalomyelitis
FTOC  Fetal Thymic Organ Culture
GEF  Guanine nucleotide Exchange Factor
GITR  Glucocorticoid-Inducible Tumor necrosis factor Receptor
Gly  Glycine
GR  Glucocorticoid Receptor
HLA  Human Leukocyte Antigen
ICOS  Inducible T-cell Co-Stimulatory molecule
Idd locus  Insulin-dependent diabetes susceptibility locus in mice
IDDM locus  Insulin-Dependent Diabetes Mellitus susceptibility locus in humans
IEL  Intestinal intra-Epithelial Lymphocytes
Ig  Immunoglobulin
iGb3  Isoglobotrihexosylceramide 3IL- Interleukin
INF-γ  Interferon γ
IPEX  Immune dysregulation Polyendocrinopathy Enteropathy-X linked syndrome
IRAG  Inositol 1,4,5-triphosphate Receptor Associated with cGMP kinase substract
Kb  kilobase pairs
LFA1  Lymphocyte Function associated Antigen 1
LOD  Logarithm of the Odds
LPS  Lipopolyssacharide
Lrmp  Lymphoid restricted membrane protein
Mbp  Megabase pairs
MEC  Medullary thymic Epithelial Cells
MHC  Major Histocompatibility Complex
MS  Multiple Sclerosis
NK  Natural Killer
NKT  Natural Killer-like T cells
NOD  Non-Obese Diabetic mouse strain
NOR  Non-Obese diabetes Resistant mouse strain
NRAMP  Natural Resistance Associated Macrophage Protein
OVA  Ovalbumin
PD-1  Programmed cell Death 1 molecule
QTL  Quantitative Trait Locus
Rag  Recombinase activating gene
RT-PCR  Real-Time reverse transcriptase Polymerase Chain Reaction
RW  Ragweed
SCID  Severe Combined Immuno-Deficiency
SLE  Systemic Lupus Erythrematosos
SNP  Single Nucleotide Polymorphism
SP  Single Positive CD4⁺ or CD8⁻ T cells
TCR  T-Cell Receptor
TEC  Cortical Thymic Epithelial cells
TGF-β  Transforming Growth Factor β
Th1  T-helper type 1
Th2  T-helper type 2
TNF  Tumor Necrosis Factor
Treg  Regulatory T cell
VNTR  Variable Number of Tandem Repeats
Type 1 diabetes is an autoimmune disorder determined both by genetic and environmental factors. The Non-obese diabetic (NOD) mouse is one of the best animal models of this disease. It spontaneously develops diabetes through a process resembling the human pathogenesis. The strong association of NOD Type 1 diabetes to the MHC region and the existence of other diabetes susceptibility loci are also in parallel with the human disease. The identity of the genetic factors and biological function mediated by these loci remain, however, largely unknown. Like in other autoimmune diseases, defects in tolerance mechanisms are thought to be at the origin of type 1 diabetes. Accordingly, defects in both central and peripheral tolerance mechanisms have been reported in the NOD mouse model.

Using a subphenotype approach that aimed to dissect the disease into more simple phenotypes, we have addressed this issue. In paper I, we analyzed resistance to dexamethasone-induced apoptosis in NOD immature thymocytes previously mapped to the Idd6 locus. Using a set of congenic mice carrying B6-derived Idd6 regions on a NOD background and vice-versa we could restrict the Idd6 locus to an 8cM region on the telomeric end of chromosome 6 and the control of apoptosis resistance to a 3cM region within this area. In paper II, further analysis of diabetes incidence in these congenic mice separated the genes controlling these two traits, excluding the region controlling the resistance to apoptosis as directly mediating susceptibility to diabetes. These results also allowed us to further restrict the Idd6 locus to a 3Mb region. Expression analysis of genes in this chromosomal region highlighted the Lrmp/Jaw1 gene as a prime candidate for Idd6. Lrmp encodes an endoplasmatic reticulum resident protein.

Papers III and IV relate to peripheral tolerance mechanisms. Several T cell populations with regulatory functions have been implicated in type 1 diabetes. In paper III, we analyzed NOD transgenic mice carrying a diverse CD1d-restricted TCR (Vα3.2β9), named 24αβNOD mice. The number of nonclassical NKT cells was found to be increased in these mice and almost complete protection from diabetes was observed. These results indicate a role for nonclassical NKT cells in the regulation of autoimmune diabetes. In paper IV, we studied the effects of introducing the diverse CD1d-restricted TCR (Vα3.2β9) in immunodeficient NOD Rag-/- mice (24αβNODRag-/- mice). This resulted in a surprising phenotype with inflammation of the ears and augmented presence of mast cells as well as spleenomegaly and hepatomegaly associated with extended fibrosis and increased numbers of mast cells and eosinophils in the tissues. These observations supported the notion that NKT cells constitute an “intermediary” cell type, not only able to elicit the innate immune system to mount an inflammatory response, but also able to interact with the adaptive immune system affecting the action of effector T cells in an autoimmune situation. In this context the 24αβNODRag-/- mice provide an appropriate animal model for studying the interaction of NKT cells with both innate and adaptive components of the immune system.
PUBLICATIONS


**Paper II:** The *Idd6* susceptibility locus controls defective expression of the *Lrmp* gene in non-obese diabetic (NOD) mice.
Nádia Duarte, Marie Lundholm and Dan Holmberg. Manuscript 2005

**Paper III:** Prevention of diabetes in non-obese diabetic mice mediated by CD1d-restricted nonclassical NKT cells.
Nádia Duarte* and Martin Stenström*, Susana Campino, Marie-Louise Bergman, Marie Lundholm, Dan Holmberg and Susanna L. Cardell. The Journal of Immunology, 2004, 173:3112-3118

**Paper IV:** CD1d-restricted nonclassical NKT cells provoke inflammation with mast cell recruitment in a NOD Rag-/- immunodeficient mouse model.
Nádia Duarte, Göran Roos and Dan Holmberg. Manuscript 2005

*These authors contributed equally to the work
INTRODUCTION

I-Mechanisms of tolerance

It is widely accepted that disruptions in immunological tolerance are at the origin of autoimmune diseases such as Type 1 diabetes. Indeed, it is logical that a common “side-effect” of a highly plastic adaptive immune system, with the ability to recognize virtually any foreign protein, would be the potential to respond to self-proteins. Mechanisms must exist in a healthy individual that ensure tolerance to self and prevent autoimmune tissue damage. Classically, tolerance mechanisms have been divided into two main categories: central tolerance mechanisms, which refer to the deletion of auto-reactive T cell clones as they develop in the thymus and peripheral tolerance mechanisms, which deal with auto-reactive T cells that escape thymic negative selection. In type 1 diabetes both defects in central tolerance (Kishimoto and Sprent, 2001; Lesage et al., 2002; Zucchelli et al., 2005) and in peripheral tolerance (Cameron et al., 1997; Colucci et al., 1997; Pop et al., 2005; Serreze and Leiter, 1988) have been reported.

Central tolerance

During thymic development, T cells are subjected to different selection events (Fig.1). First, immature pre-T cells must undergo β-selection, which involves the rearrangement of the β-chain of their T cell receptor (TCR) and association to a pre T-α chain. Successful assembly and cell surface expression of this pre-TCR complex is necessary for survival signals to be transmitted and for maturation to proceed (Fehling et al., 1995; Groettrup and von Boehmer, 1993; Mombaerts et al., 1992). A second checkpoint involves TCRαβ recognition of major histocompatibility molecules (MHC) presenting self antigens. Without such recognition T cells die by neglect (Janeway and Bottomly, 1994; Kisielow and von Boehmer, 1995; Surh and Sprent, 1994). Low to moderate affinity/avidity interaction between the TCRαβ and the self-peptide-MHC molecule complex presented by thymic cortical epithelial cells leads to positive selection of the T cell and to further maturation (Ashton-Rickardt and Tonegawa, 1994; Kisielow and Miazek, 1995; Surh and Sprent, 1994). On the other hand, if the self-peptide-MHC molecule complex is recognized with high affinity/avidity, the self-reactive T-cell is negatively selected and apoptosis is induced (Ashton-Rickardt and Tonegawa, 1994).
Figure 1: Schematic picture of T-cell development in the thymus. Thymic lymphoid precursors enter the thymus through large vessels at the cortico-medullary junction (CMJ) and undergo a program of proliferation, lineage commitment and MHC-restricted selection of T-cell receptor (TCR) $\alpha\beta$ (positive selection) under the influence of cortical thymic epithelial cells. Although negative selection caused by unsuccessful MHC-TCR interactions occurs in the cortex, the medulla is the main site for deletion of auto-reactive thymocytes. The different stages of development are distinguished mainly by the expression of CD4 and CD8 co-receptors. DN indicates double negative cells for CD4 and CD8 molecules, DP represents double positive cells for expression of both CD4 and CD8 co-receptors, and SP indicates single-positive cells for expression of CD4 or CD8 molecules. DN thymocytes go through different developmental stages that can be distinguished by expression of CD44 and CD25 molecules. The selected CD4 or CD8 T cells undergo final maturation in the medulla and are then exported to the peripheral T-cell pool as fully functional T cells. Adapted from (Crivellato et al., 2004).
The process of negative selection is an important mechanism of tolerance induction. Indeed, a variety of approaches have estimated that one-half to two-thirds of thymocytes which are positively selected undergo negative selection (Ignatowicz et al., 1996; Tourne et al., 1997; van Meerwijk et al., 1997). Reinforcing this notion, other studies have shown that tissue-restricted self antigens promiscuously expressed in the thymus are able to confer T-cell central tolerance (Derbinski et al., 2001; Klein et al., 2000; Kyewski et al., 2002; Sospedra et al., 1998). Accordingly, the AIRE gene (aire in mice) encodes a transcription factor mainly expressed in the medullary epithelial cells (MEC) of the thymic stroma and was shown to control promiscuous thymic transcription of peripheral tissue-specific antigens (Anderson et al., 2002; Nagamine et al., 1997). This gene is mutated in the autoimmune Polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) in humans (Nagamine et al., 1997). Expression of many tissue-specific genes in MECs was highly reduced in aire knock-out mice, with consequent development of tissue-specific auto-antibodies and lymphoid infiltration of several peripheral organs (Anderson et al., 2002). Furthermore, deletion of CD4+ lymphocytes specific for a neo-self-antigen under the control of the rat insulin promoter is abrogated in aire-deficient mice, implicating aire in negative selection events (Gotter and Kyewski, 2004; Liston et al., 2003). However, the restrained autoimmune disease observed in aire-deficient mice, and in AIRE-defective patients, points out the existence of operating peripheral tolerance mechanisms (Anderson et al., 2002; Ramsey et al., 2002). Moreover, auto-reactive T cells are normally found in a healthy individual, suggesting that central tolerance is the first but not the only barrier against auto-reactivity (Lohmann et al., 1996; Semana et al., 1999).

**Peripheral tolerance**

Peripheral tolerance mechanisms include different T cell-intrinsic mechanisms like ignorance, anergy, phenotypic skewing and apoptosis, as well as T-cell extrinsic mechanisms such as tolerogenic dendritic cells and regulatory T cells (Walker and Abbas, 2002).

**T cell-intrinsic tolerance mechanisms**

Ignorance concerns T-cells not responding to self-antigens either because these are not easily accessed or because the amount of antigen is not sufficient to reach the necessary threshold for T cell activation (Alferink et al., 1998; Kurts et al., 1998; Zinkernagel, 1996). Absence of co-stimulation has also been proposed to turn encounters with self-antigen into functional inactivation of T cells, a process usually designated as anergy. Currently, anergy is viewed as a result of
signaling through alternative receptors rather than simply due to the lack of co-stimulation. Several candidate molecules such as the cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed cell death 1 (PD-1) molecule have been implicated in this process (Lechner et al., 2001b; Perez et al., 1997; Walker and Abbas, 2002; Walunas and Bluestone, 1998). In addition, encounters with self-antigens might result in an inappropriate immune response that avoids pathogenic effects. An example of this so called phenotype skewing is the phenomenon of cytokine deviation. While T-helper type 1 (Th1) cytokines have been associated with aggressive autoimmune attack, T-helper type 2 (Th2) cytokines have been linked to downregulation of autoimmunity in experimental autoimmune encephalomyelitis (EAE) and type 1 diabetes (Bradley et al., 1999; Young et al., 2000). Phenotype skewing might also occur as result of altered lymphocyte trafficking which could determine which accessory molecules the T cell will meet and what type of response will be initiated (Kearney et al., 1994; Walker and Abbas, 2002). Activation induced cell death (AICD) of autoreactive T cell clones is another mechanism that prevents destruction of body tissues. A trigger for this process could be repetitive encounters with self-proteins and a key factor involved seems to be the ligation of Fas death receptor by its ligand (Watanabe-Fukunaga et al., 1992). Association of Fas and Fas ligand defects with lymphoproliferative lupus-like syndrome in MRL/lpr and gld mice and the findings of deficiencies in the Fas pathway in the human autoimmune lymphoproliferative syndrome (ALPS) have highlighted the importance of Fas signaling in AICD (Sobel et al., 1993; Suda et al., 1993). Additionally, CTLA-4, which is a negative regulator of T-cell activation, has been suggested to be involved in the regulation of apoptosis induction (Bergman et al., 2001; Colucci et al., 1997). Supporting this notion, Cttla4-deficient mice were observed to develop a lethal lymphoproliferative syndrome with multiorgan inflammation resulting in death at 4 weeks of age (Bergman et al., 2001; Tivol et al., 1995).

T cell- extrinsic tolerance mechanisms
Dendritic cells (DC) have also been implicated in the induction of T cell tolerance. They have been shown to be a very heterogeneous group capable of triggering potent immunogenic responses but also inducing peripheral tolerance to self with deletion of autoreactive clones or stimulation of T cells with regulatory capacities (Bonifaz et al., 2002; Hawiger et al., 2001; Kurts et al., 1997; Martin et al., 2003; Morgan et al., 1999). How dendritic cells decide whether to initiate an immune response or induce tolerance and what types of cells and molecules are involved in these processes is currently subject of intensive research. It has been suggested that different subsets of DCs are
responsible for the immunostimulatory and tolerizing activities. CD8+DCs appear to be particular efficient at cross-presentation of tissue-specific self antigens, although, solid experimental data for a specialized tolerizing peripheral DC subset is still lacking (Belz et al., 2002; den Haan et al., 2000; Jung, 2004). Others have proposed that immature DC cross-presenting tissue-specific antigens and expressing low levels of co-stimulatory molecules are involved in T cell deletion and tolerance induction, while mature DCs would have an enhancing immunogenic function (Bonifaz et al., 2002; Hawiger et al., 2001; Steinman and Nussenzweig, 2002). Support for this hypothesis came from experiments where delivery of antigen to DCs via the C-type lectin DEC 205 resulted in T cell tolerization but, when combined with a maturation signal, such as CD40 stimulation, resulted in T cell activation (Bonifaz et al., 2002; Hawiger et al., 2001). Furthermore, immature CD40 deficient DCs were able to trigger expansion of IL-10 producing antigen specific T cells with regulatory capacities (Martin et al., 2003). The production of tolerogenic cytokines such as IL-10 and TGFβ by DC in mucosal surfaces have also been suggested to be a mechanism of tolerance induction (Weiner, 2001). Interestingly, however, myeloid mucosal regulatory DCs have a mature phenotype (Akbari et al., 2002; Bell et al., 2001; Goddard et al., 2004; Kobayashi et al., 2004). In addition, epithelium-derived DCs with a mature phenotype continuously migrate into lymphoid tissues (Wilson et al., 2003). Taken together, this suggests that mature DCs originating from a tolerogenic environment or DCs that have developed in response to particular tissue factors may also play a role in tolerance induction.

The notion of suppressor T cells, able to induce tolerance upon other T cells in a dominant way, has been around for more than 30 years. Yet, their existence has only recently gained substantial evidence with the recognition and characterization of the CD4+ T cell subset expressing high amounts of IL-2Rα (CD25) and the transcription factor FoxP3 of the forkhead-winged-helix family. These CD4+CD25+ cells are referred to as “natural” regulatory T cells (Tregs) and constitute a unique lineage of T cells (Gershon et al., 1972; Hori et al., 2003; Sakaguchi and Sakaguchi, 1988; Takahashi et al., 1998). First described as CD4+CD45RBlowCD25+, this T cell subset was shown to dominantly suppress activation and proliferation of effector T cells with prevention of autoimmune manifestations (Sakaguchi, 2005; Sakaguchi and Sakaguchi, 1988; Shevach, 2000; Takahashi et al., 1998; Takahashi et al., 2000). The identification of mutations in the transcription factor Foxp3 as being the causal factor of an X-linked recessive inflammatory disease in scurfy mutant mice and subsequently of the human X-linked immunodeficient syndrome IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) has been a
major contribution to the characterization of regulatory T cells (Bennett et al., 2001; Brunkow et al., 2001; Chatila et al., 2000; Wildin et al., 2001). Foxp3 was found to be crucial for the development and function of natural CD25+CD4+ regulatory T cells and still remains as the marker that best defines this population (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003). Recently, it has been demonstrated that Foxp3 expression by developing thymocytes acts as a lineage specification factor for this regulatory population (Fontenot et al., 2005; Fontenot and Rudensky, 2005). Induction of Foxp3 expression in peripheral non-regulatory T cells in both humans and mice under standard physiological conditions has been suggested to be a relatively rare process (Fontenot and Rudensky, 2005). Nevertheless, in a transgenic mouse model on a Rag-/- background without pre-existing CD4+CD25+Foxp3+ regulatory T cells, and where all the T cells showed specificity for the male transplantation antigen (DBy), tolerance to male skin graft was associated with transforming growth factor β (TGF-β) dependent induction of Foxp3 expression upon antigen stimulation in the presence of saturating non-depleting CD4 antibodies (Cobbold et al., 2004). In addition, oral exposure to antigen has been reported to induce TGF-β dependent development of CD4+CD25+Foxp3+ T cells and confer tolerance to Th2 allergic responses (Mucida et al., 2005). Peripheral generation of regulatory T cells has been therefore suggested to occur as consequence of sustained suboptimal antigenic stimulation, with TGF-β contributing by increasing the threshold for T cell activation (Graca et al., 2005; Waldmann et al., 2005).

This natural regulatory T cell subset is able to suppress other T cell populations, mainly by direct cell contact with effector cells but also by secreting suppressive cytokines such as TGF-β or IL-10 to interfere with T cell activation (von Boehmer, 2005). IL-2 is thought to be required for in vitro and in vivo activation of regulatory T cells and for sustaining CD25 expression. Both the CTLA-4 receptor and glucocorticoid-inducible tumor necrosis factor receptor (GITR) are constitutively expressed in the Treg population with contentious implications for its maintenance and function (Sakaguchi, 2005). Besides being involved in autoimmunity, CD4+CD25+Foxp3+ regulatory T cells have been implicated in suppression of tumor immunity and induction of tolerance in organ transplantation studies (Sakaguchi, 2005).

The discovery of CD4+CD25+ regulatory T cells has also promoted investigations of immune-regulation mediated by other T cell populations in different situations. It has been observed that chronic stimulation in vivo or culture under certain conditions could result in the generation of CD4+ T cells anergic in terms of proliferation in vitro and secreting high
levels of IL-10 (Buer et al., 1998). This cell group was also observed to have suppressor activities on other lymphocytes (Barrat et al., 2002; Groux et al., 1997; O'Garra et al., 2004). Other cell populations implicated in immuno-regulation can be included in the “unconventional” T cell group. These T cells present unusual oligoclonal TCRs, have a tissue-specific distribution, are apparently autoreactive and are able to either promote inflammation or immunoregulation depending on the conditions they are presented with. Within this group we can include the group of γδT cells localized to the epidermis called dendritic epidermal T cells (DETCs), CD8αα+ αβ intestinal intraepithelial lymphocytes (IELs) T cells and the CD1d-restricted Natural Killer-like (NKT) cells (Bendelac et al., 1995; Hayday and Tigelaar, 2003; Lehuen et al., 1998; Leishman et al., 2002; Poussier et al., 2002). It was proposed that these particular T cell subsets may act as local gate-keepers (Hayday and Tigelaar, 2003).

A particular characteristic of both the “natural” CD4+CD25+ regulatory T cells and “unconventional” T cells like NKT is the high specificity for self-antigens. Indeed it has been suggested that both T cell group are positively selected during thymic development based on the intermediate reactivity to self-antigens (Bendelac et al., 1995; Fontenot and Rudensky, 2005; Hayday, 2000; Lin et al., 1999; Rocha et al., 1992). In this scenario, central tolerance is not only important for deletion of potential self-reactive T cell clones but also crucial for selection of regulatory T cells with vital roles in tolerance induction in the periphery (Fig.2).
Figure 2 - Link between central and peripheral tolerance by thymus production of cells with regulatory functions. Thymocytes with a high avidity self-reactive TCR are negatively selected and undergo apoptosis. Some self-reactive thymocytes with an intermediate avidity for self-peptide ligands upregulate FoxP3 in response to increase signal strength or duration of a TCR signal in combination with an unknown signal. Upon FoxP3 induction, thymocytes commit to the natural regulatory T cells lineage. In a parallel situation, TCR recognition of self-glycopeptides presented by the CD1d molecule on CD4^+CD8^+ (DP) thymocytes are necessary for Natural killer-like (NKT) cells to undergo further maturation. Selected regulatory T cells and NKT cells will then migrate to the periphery where they will regulate either directly or indirectly a great number of immune responses.
II- Autoimmune diabetes: when tolerance fails

The Greek origin of the word “diabetes” is siphon or pipe-like while the Latin word for honey/sweet is “mellitus”. In reality, one of the first methods for diagnosing diabetes mellitus was by tasting the urine of diabetic patients for sweetness. Such urine is excreted in large volumes and is accompanied by unquenchable thirst in advance stages of disease, leading a seventeenth century English surgeon to call diabetes “the pissing evil” (Bliss, 1982). Nevertheless, as early as 600 years before Christ, two Indian physicians, Chakrata and Susruta were able to differentiate between two forms of the disease (LeRoith et al., 2000). Nowadays, diabetes is considered as a syndrome and comprises a heterogeneous collection of disorders with different types and etiologies in spite of the similar pathogenic effects after onset. Even so, conventionally, two major types of diabetes are considered, type 1 and type 2 diabetes. Briefly, type 2 diabetes is caused by a combination of environmental and genetic factors, which leads to insulin resistance and insulin deficiency. It comprises about 90 to 95% of all the diabetes cases and it is usually associated with obesity and onset later in life. Type 1 diabetes mellitus on the other hand, is an autoimmune disease and includes 5 to 10% of the total diabetes cases. Its onset usually occurs during puberty or adolescence with outburst of severe symptoms like hyperglycemia as a consequence of absolute insulin deficiency due to immune mediated destruction of the insulin producing β-cells on the pancreatic islets of Langerhans. Administration of exogenous insulin is required throughout life and there is proneness to ketosis even in the basal state (LeRoith et al., 2000). Type 1 diabetes incidence is increasing among western societies and although insulin is an efficient therapy for the disease, late complications like kidney failure and blindness are difficult to avoid.

Akin to all autoimmune diseases, a primary cause of Type 1 diabetes pathogenesis is the disruption of tolerance. Genetic predisposition and environmental factors are believed to affect the immune system resulting in impairment of tolerance (Anderson and Bluestone, 2005). Unquestionably, the Non-obese diabetic (NOD) mouse constitutes an invaluable tool for the study of pathogenic mechanisms leading to the disruption of tolerance and, consequently, type 1 diabetes.
The NOD mouse model

The NOD mouse strain was described as a model of autoimmune diabetes in Japan in the late 70s and remains to date the only mouse model to develop spontaneous type 1 diabetes (Makino et al., 1980). The disease in this mouse strain progresses in a process very similar to the human diabetes, starting with infiltration of perivascular and peri-islet regions (peri-insulitis) of the pancreatic islet of Langerhans in early stages, followed by selective T-cell mediated destruction of insulin producing β-cells. In the NOD mice the first infiltrating cells appear around 3-4 weeks of age and consist mainly of antigen presenting cells (APC), such as dendritic cells and macrophages, followed by T cells, B cells and NK cells (Delovitch and Singh, 1997; Kanazawa et al., 1984). Final destruction of β-cells occurs around 4-6 months of age and it is thought to constitute a second stage of the disease. Indeed, infiltration of pancreatic islets (insulitis) can occur without progression to overt diabetes. For example, insulitis occurs in all NOD mice but diabetes develops mostly in females with an incidence of 80-90%, compared to the 40-60% of diabetes scores in male colonies. In other murine models insulitis is observed but it does not develop into overt diabetes (Poirot et al., 2004; Robles et al., 2003). In addition, the presence of anti-β cell autoantibodies does not always predict clinical hyperglycemia in humans (Tisch and McDevitt, 1996). The switch from this stage of “respectful” insulitis to diabetes appears to be a very tightly regulated process and both environmental and genetic factors have been suggested to be involved (Gonzalez et al., 1997; Robles et al., 2003). The difference between sexes observed in NOD has been related to the influence of sex hormones, with studies indicating that the incidence of diabetes decreases with androgen treatment of females and increases by castration of males (Fitzpatrick et al., 1991; Fox, 1992; Makino et al., 1981). Still, exactly how sex hormones could influence the outburst of overt diabetes is unclear. Interestingly, however, many other human autoimmune diseases such as reumatoid arthritis, systemic lupus erythrematosos and multiple sclerosis occur at substantially higher frequencies in females than in males (Grossman et al., 1991).

T cells play a major role in autoimmune diabetes. Transfer experiments of different cell populations have shown that T cells are necessary and sufficient for the induction of diabetes in healthy mice (Bendelac et al., 1987; Miller et al., 1988; Wicker et al., 1988). Furthermore, diabetes does not occur in immuno-deficient mice, such as NOD.Scid-/- and NOD.Rag-/- mice which lack T and B lymphocytes, neither in MHC class I and class II deficient NOD mice lacking CD8+ and CD4+ T cells respectively (Katz et al., 1993; Prochazka et al., 1992; Serreze et al., 1994a; Soderstrom et al., 1996; Wicker et al., 1994a). CD4+T cells have been proved essential both at early and later stages of disease development. Thus, these cells were able to transfer the disease
into healthy mice and treatment with anti-CD4 antibody prevented onset of diabetes in NOD mice (Christianson et al., 1993; Shizuru et al., 1988). Moreover, CD4+T cells can also directly mediate destruction of the β-cells themselves (Anderson and Bluestone, 2005). In turn, CD8+T cells were implicated early in the disease development, perhaps by causing sufficient islet destruction to prime a more robust CD4+T cell response or by playing an effector function themselves (Serreze et al., 1997; Wang et al., 1996; Wicker et al., 1994a; Wong et al., 1996). B lymphocytes primarily acting as antigen presenting cells rather than autoantibody producers are known to contribute to pathogenesis (Serreze et al., 1998b; Wong et al., 2004). Nonetheless, repetitive administration of anti-mouse IgM was shown to suppress B cell development and result in reduction of diabetes incidence in the NOD mouse (Andersson et al., 1991; Forsgren et al., 1991a). One recent study has suggested that the passive transfer of auto-antibodies from mother to offspring may influence diabetes development (Greeley et al., 2002). Defects in dendritic cell maturation and macrophage function have also been reported and claimed to play a role in the disease (Maree et al., 2005; Peng et al., 2003; Piganelli et al., 1998; Serreze et al., 1993). Furthermore, NOD mice have been shown to have low levels of NK cell activity (Kataoka et al., 1983; Ogasawara et al., 2003).

Differences exist between type 1 diabetes progression in humans and in the NOD mouse strain. For example, the precipitating causes of diabetes in humans may differ from the NOD mice and there is no sex-related difference in incidence of human diabetes which may be due to the fact that onset of the disease occurs, in general, much earlier in humans than in mice. In addition only a few diabetic patients exhibit all the clinical features of NOD mice, including progressive hearing loss, haemoletic anaemia widespread deficiencies in innate immunity and a polyglandular spectrum of autoimmune syndromes affecting thyroid and salivary glands (Leiter and von Herrath, 2004). However, striking similarities subsist, justifying the wide use of NOD mouse strains as a model for autoimmune diabetes, particularly when a genetic approach is in use.

**Genetics of type 1 diabetes**

Type 1 diabetes is a complex genetic disease, resulting from a multifaceted interaction between genetic factors and environmental factors. The genetic components conferring susceptibility to the disease are numerous and most of them only contribute slightly to the disease risk. These genetic factors may differ considerably between individuals. There is significant familial clustering of type 1 diabetes, with an average prevalence risk in siblings of 6% compared to 0.4% in general Caucasian population. The main genetic component identified is the susceptibility locus *IDDM1*,


situated within the MHC gene region or human leukocyte antigen (HLA) as it is called in humans, located on chromosome 6p21. The HLA gene cluster has been estimated to explain up to 40-50% of the familial clustering of type 1 diabetes. The genes in the HLA region can be divided into four families, classes I, II, III, IV. The strongest genetic association to type 1 diabetes occurs with class II gene alleles and there is good evidence of involvement of particular alleles in the DQA1, DQB1 and DRB1 loci with type 1 diabetes. However, the strong linkage disequilibrium between the loci makes the study of the individual contribution of each locus very difficult. Clearly, some combinations of HLA-DQ and DR alleles are associated with susceptibility and some with protection from the disease (Table 1) (Pociot and McDermott, 2002; Pugliese et al., 1995; Thomson, 1988; Thorsby, 1997; Todd and Wicker, 2001; Undlien et al., 2001). An important factor determining the risk of the HLA alleles seems to be the particular amino-acids that determine the structure and action of certain peptide-binding pockets in the HLA molecule. In particular, it was observed that aspartic acid at residue 57 on the pocket 9 of the HLA-DQB1 molecule is encoded by DQB alleles protecting from diabetes (for example DQ6 molecule) while alanine, valine or serine aminoacids at the same position characterize susceptibility alleles (for example DQ8 and DQ2 molecules) (Chao et al., 1999; Cucca et al., 2001; Latek et al., 2000; Lee et al., 2001; McDevitt, 2001; Todd and Wicker, 2001).

Table 1 - Type 1 diabetes-associated HLA class II alleles and haplotypes. Relative risk is given for the combined DQ-DR haplotype and refers to high-risk populations. Adapted from (Pociot and McDermott, 2002)

<table>
<thead>
<tr>
<th>HLA-DQ alleles</th>
<th>HLA-DR alleles</th>
<th>Relative risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susceptible haplotypes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1<em>0301-B1</em>0302</td>
<td>DRB1*04</td>
<td>2.5-9.5</td>
</tr>
<tr>
<td>A1<em>0501-B1</em>0201</td>
<td>DRB1*0301</td>
<td>2.5-9.5</td>
</tr>
<tr>
<td>A1<em>0501-B1</em>0302</td>
<td>DRB1<em>301/DRB1</em>04</td>
<td>12.0-32.0</td>
</tr>
<tr>
<td>A1<em>0301-B1</em>0201</td>
<td>DRB1<em>301/DRB1</em>04</td>
<td>12.0-32.0</td>
</tr>
<tr>
<td>A1<em>0301-B1</em>0402</td>
<td>DRB1<em>04/DRB1</em>801</td>
<td>4.0-15.0</td>
</tr>
<tr>
<td>A1<em>0301-B1</em>0201</td>
<td>DRB1*701</td>
<td>8.0-13.0</td>
</tr>
<tr>
<td>A1<em>0301-B1</em>0201</td>
<td>DRB1*901</td>
<td>5.5</td>
</tr>
<tr>
<td>A1<em>0301-B1</em>0401</td>
<td>DRB1*04</td>
<td>3.5-4.5</td>
</tr>
<tr>
<td>A1<em>0301-B1</em>0303</td>
<td>DRB1*901</td>
<td>2.0-4.5</td>
</tr>
<tr>
<td>Protective haplotypes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1<em>0102-B1</em>0602</td>
<td>DRB1*1501</td>
<td>0.03-0.2</td>
</tr>
<tr>
<td>A1<em>0103-B1</em>0603</td>
<td>DRB1*1301</td>
<td>0.05-0.25</td>
</tr>
<tr>
<td>A1<em>0301-B1</em>0301</td>
<td>DRB1*04</td>
<td>0.2-0.5</td>
</tr>
<tr>
<td>A1<em>0501-B1</em>0301</td>
<td>DRB1*1101</td>
<td>0.05-0.5</td>
</tr>
</tbody>
</table>
Several studies have also implicated other HLA class II alleles as well as class I and class IV genes in predisposition to type 1 diabetes (Pociot and McDermott, 2002). The current view for the region is consistent with a model in which multiple loci, including but not limited to those within the HLA class II region, contribute susceptibility to type 1 diabetes (Onengut-Gumuscu and Concannon, 2002).

HLA however, accounts for less than 50% of the inherited disease risk which indicates a substantial role for non-HLA genes in conferring susceptibility to autoimmune diabetes. Indeed other susceptibility regions have been reported in several association and linkage studies and about 18 Insulin-dependent diabetes mellitus (IDDM) susceptibility loci have now been reported (Table 2).

Apart from the HLA region, only the IDDM2 and IDDM12 loci have been unequivocally linked or associated with diabetes. IDDM2 is localized in the insulin-gene region on chromosome 11p15. Association has been found between diabetes and a unique minisatellite (VNTR) which arises from tandem repetition of a 14-15 base pair (bp) oligonucleotide sequence located in the 5’ regulatory region of the insulin-gene. The repeats vary from about 26 to over 200bp. Susceptibility to diabetes has been associated with short repeats (26-63 bp), whereas longer repeats (141-209 bp) seem to confer dominant protection (Pociot and McDermott, 2002). Recent data suggests that the VNTR may modulate the transcription level of insulin in the pancreas and thymus. Longer repeats have been associated with low transcription levels in pancreas, but high transcription in thymus, when compared to smaller repeats (Pugliese and Miceli, 2002). The third known locus, IDDM12 is localized on chromosome 2q33 in the T-cell costimulatory receptors CD28, CTLA-4 and inducible T-cell co-stimulator (ICOS) region. The prime candidate gene for this locus is CTLA-4, a known negative regulator of T cell activation, with polymorphisms particularly in the exon 1 (49 G>A) SNP found to be associated to the disease (Larsen et al., 1999; Marron et al., 1997; Nistico et al., 1996). This polymorphism in CTLA-4 has also been found to be associated with Grave’s disease and other immune-mediated diseases (Ahmed et al., 2001; Yanagawa et al., 1995). Also, one hundred single nucleotide polymorphisms have been identified in this region in a mutation screening. Fine mapping studies based on this data provided evidence for allelic variation in the non-coding region, at 6.1 kilobase pairs (Kb) 3’ of the gene influencing mRNA expression and alternative splicing of the human CTLA-4 gene (Ueda et al., 2003). Moreover, the CTLA-4 gene is known to give rise to a soluble form identified in humans, which has been suggested to contribute to autoimmune pathogenesis (Magistrelli et al., 1999; Oaks and Hallett, 2000).
## Table 2. Susceptibility loci to human type 1 diabetes*. Adapted from (Pociot and McDermott, 2002)

<table>
<thead>
<tr>
<th>Locus</th>
<th>Chromosome</th>
<th>Markers</th>
<th>Candidate genes proposed</th>
<th>references</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDDM1</td>
<td>6p21.3</td>
<td>HLA genes</td>
<td>HLA locus</td>
<td>1,2,3,4,5,6</td>
</tr>
<tr>
<td>IDDM2</td>
<td>11p15.5</td>
<td>VNTR in 5' of insulin gene</td>
<td>VNTR in insulin gene</td>
<td>5,7,8,9,10,11,12,13,14</td>
</tr>
<tr>
<td>IDDM3</td>
<td>15q26</td>
<td>D15S107</td>
<td>No candidate genes analyzed</td>
<td>15,16,17</td>
</tr>
<tr>
<td>IDDM4</td>
<td>11q13</td>
<td>D11S1296-FGF3 (fibroblast growth factor 3)</td>
<td>Genes encoding Zinc finger protein 162 (ZFM1), Fas-associated death domain protein (FADD) and Low-density lipoprotein receptor related protein (LRP5)</td>
<td>18,19,20,21,22,23,24</td>
</tr>
<tr>
<td>IDDM5</td>
<td>6q25</td>
<td>D6S476-D6S473</td>
<td>Genes encoding Manganese superoxide dismutase (SOD2) and small ubiquitin-like modifier 4 protein (SUMO4)</td>
<td>18,20,25,26,27,28,29</td>
</tr>
<tr>
<td>IDDM6</td>
<td>18q12-q21</td>
<td>129, II-1043 and 56-D18S487</td>
<td>Candidate gene deleted in colorectal carcinoma (DCC) ZNF236 gene encoding a kruppel-like zinc-finger protein Gene encoding Anti-apoptotic molecule-bcl-2</td>
<td>30,31,32,33,34,35</td>
</tr>
<tr>
<td>IDDM7</td>
<td>2q31</td>
<td>D2S326, D2S152, D2S1391</td>
<td>Interleukine-1 gene cluster Genes encoding Homeo box D8 (HOXD8) Glutamic acid decarboxylase 1 (GAD1) UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3 (GALNT3) and Neurogenic differentiation protein (NEUROD)</td>
<td>5,18,26,36,37,38,39,40,41,42,43,44,45</td>
</tr>
<tr>
<td>IDDM8</td>
<td>6q27</td>
<td>D6S446-D6S281</td>
<td>Genes encoding insulin-like growth factor-II receptor (IGF2R) and TATA box-binding protein</td>
<td>5,16,18,20,28,46,47,48,49</td>
</tr>
<tr>
<td>IDDM9</td>
<td>3q21-q25</td>
<td>D3S1303, D3S1279</td>
<td>No candidate genes analyzed</td>
<td>18,35,46</td>
</tr>
<tr>
<td>IDDM10</td>
<td>10p11-q11</td>
<td>D10S191-D10S220</td>
<td>GAD2 gene encoding Glutamic acid decarboxylase 65</td>
<td>5,18,46,50,51,52</td>
</tr>
<tr>
<td>IDDM11</td>
<td>14q23.3-q31</td>
<td>D14S67</td>
<td>Gene encoding α-endosulfine (ENSA) SFL1L gene-a negative regulator of Notch signaling pathway</td>
<td>53,54,55,56</td>
</tr>
<tr>
<td>IDDM12</td>
<td>2q33</td>
<td>D2S72-CTLA4-D2S116</td>
<td>Genes encoding T-cell costimulatory receptor CD28, Cytotoxic T-lymphotye-associated protein 4 (CTLA-4) and Inducible T-cell co-stimulator (ICOS)</td>
<td>5,35,42,57,58,59,60,61</td>
</tr>
<tr>
<td>IDDM13</td>
<td>2q34</td>
<td>D2S137-D2S164</td>
<td>Gene encoding insulinoma associated protein (IA-2) Insulin-like growth factor binding protein 2 (IGFBP2), Insulin-like growth factor binding protein (IGFBP5), and Natural resistance associated macrophage protein (NRAMP)</td>
<td>5,42,62,63,64,65</td>
</tr>
<tr>
<td>IDDM15</td>
<td>6q21</td>
<td>D6S283-D6S158</td>
<td>No candidate genes analyzed</td>
<td>5,28,66,67</td>
</tr>
<tr>
<td>IDDM16</td>
<td>14q32.3</td>
<td>D14S292-D14S293</td>
<td>Immunoglobulin heavy chain (IGH) region</td>
<td>68</td>
</tr>
<tr>
<td>IDDM17</td>
<td>10q25</td>
<td>D10S1750-D10S1773</td>
<td>Gene encoding theTNF receptor superfamily, member 6 (FAS) AMACO gene encoding A-domain containing protein similar to matrilin and collagen</td>
<td>5,69,70,71</td>
</tr>
<tr>
<td>IDDM18</td>
<td>5q31.1-q33.1</td>
<td>IL12B</td>
<td>Gene encoding Interleukin IL-12B</td>
<td>72,73,74,75,76</td>
</tr>
</tbody>
</table>
Table 2 references


IDDM2 - 5 (Cox et al., 2001), 7 (Bell et al., 1984), 8 (Julier et al., 1991), 9 (Lucassen et al., 1993), 10 (Owerbach and Gabbay, 1993), 11 (Julier et al., 1994), 12 (Bennett et al., 1995), 13 (Bennett et al., 1996), 14 (Hanahan, 1998)

IDDM3 - 15 (Field et al., 1994), 16 (Luo et al., 1995), 17 (Zamani et al., 1996)

IDDM4 - 18 (Davies et al., 1994), 19 (Hashimoto et al., 1994), 20 (Luo et al., 1996), 21 (Lucassen et al., 1993), 22 (Owerbach and Gabbay, 1993), 23 (Julier et al., 1994), 24 (Bennett et al., 1995), 25 (Bennett et al., 1996), 26 (Hanahan, 1998)

IDDM5 - 15 (Field et al., 1994), 16 (Luo et al., 1995), 18 (Davies et al., 1994), 26 (Pociot et al., 1994), 27 (Davies et al., 1996), 28 (Delepine et al., 1997), 29 (Guo et al., 2004)

IDDM6 - 30 (Merriman et al., 1997), 31 (Merriman et al., 1998), 32 (Merriman et al., 2001), 33 (Komaki et al., 1998), 34 (Holmes et al., 1999), 35 (Laine et al., 2004)

IDDM7 - 5 (Cox et al., 2001), 18 (Davies et al., 1994), 26 (Pociot et al., 1994), 36 (Pociot et al., 1992), 37 (Mandrup-Poulsen et al., 1994), 38 (Copeman et al., 1995), 39 (Owerbach and Gabbay, 1995), 40 (Bergholdt et al., 1995), 41 (Metcalfe et al., 1996), 42 (Esposito et al., 1998), 43 (Kristiansen et al., 2000a), 44 (Bergholdt et al., 2000), 45 (Kristiansen et al., 2000b)

IDDM8 - 5 (Cox et al., 2001), 16 (Luo et al., 1995), 18 (Davies et al., 1994), 20 (Luo et al., 1996), 28 (Delepine et al., 1997), 46 (Mein et al., 1998), 47 (Owerbach, 2000), 48 (McCann et al., 2004), 49 (Owerbach et al., 2004)

IDDM9 - 18 (Davies et al., 1994), 35 (Laine et al., 2004), 46 (Mein et al., 1998)

IDDM10 - 5 (Cox et al., 2001), 18 (Davies et al., 1994), 46 (Mein et al., 1998), 50 (Rambrand et al., 1997), 51 (Reed et al., 1997), 52 (Chistiakov et al., 2004)

IDDM11 - 53 (Field et al., 1996), 54 (Heron et al., 1999), 55 (Harada et al., 1999), 56 (Saltini et al., 2004)

IDDM12 - 5 (Cox et al., 2001), 35 (Laine et al., 2004), 42 (Esposito et al., 1998), 57 (Nisticò et al., 1996), 58 (Noble et al., 1996), 59 (Johnson et al., 2001), 60 (Ihara et al., 2001), 61 (Ueda et al., 2003)

IDDM13 - 5 (Cox et al., 2001), 42 (Esposito et al., 1998), 62 (Morahan et al., 1996), 63 (Larsen et al., 1999), 64 (Owerbach et al., 1997), 65 (Slager et al., 2002)

IDDM15 - 5 (Cox et al., 2001), 28 (Delepine et al., 1997), 66 (Concannon et al., 1998), 67 (Nerup and Pociot, 2001)

IDDM16 - 68 (Field et al., 2002)

IDDM17 - 5 (Cox et al., 2001), 69 (Verge et al., 1998), 70 (Nolsoe et al., 2000), 71 (Eller et al., 2004)

IDDM18 - 72 (Huang et al., 2000), 73 (Hall et al., 2000), 74 (Morahan et al., 2001), 75 (Adorini, 2001), 76 (Bergholdt et al., 2004)

*IDDM14 was not accepted as being associated to type 1 diabetes*
Understanding the genetic factors that underlie complex diseases such as type 1 diabetes could provide the possibility of early prediction and allow for therapeutic intervention before onset of overt disease. However, there are different factors complicating genetic studies in humans such as heterogeneity in the population, difficulties in distinguishing environmental contributions from genetic factors, problems in recruitment of individuals for studies and problems establishing consistent diagnosis with proper medical registers. One strategy to overcome these obstacles has been through the use of animal models like inbred mouse strains, where the environmental factors can be controlled. Moreover, controlled crosses between susceptible and resistant inbred strains are also achievable, allowing the study of particular susceptibility loci and determination of inheritance modes. In addition, since only two alleles will segregate at each locus, the problem of genetic allelic heterogeneity is overcome. The use of several different strains also permits the identification of additional susceptibility loci (Wakeland et al., 1997; Wakeland et al., 1999). Moreover, functional studies can be performed in animal models and the contribution of a particular genetic factor to diabetes can be verified in vivo.

**NOD mouse genetics**

Adding to the clinical characteristics described above, the genetic component of disease observed in the NOD mouse parallels in complexity to the human disease, with several susceptibility loci being identified through outcrosses with different inbred mouse strains. Genetic mapping and linkage analysis have revealed the presence of more than 20 susceptibility loci to diabetes (termed insulin dependent diabetes-\(Idd\)) in the NOD mouse strain. Further analysis of each susceptibility locus through construction of congenic NOD mice have lead to sub-division of many \(idds\), as it became clear that susceptibility to diabetes resulted from contributions and interactions of several genetic factors, even within small regions of association (Table 3). Some of the susceptibility regions identified overlap with susceptibility loci to other autoimmune diseases, indicating that they could represent general factors in autoimmunity (Johansson et al., 2003).

As in humans, the main genetic contribution to diabetes in the NOD mouse strain is conferred by class II MHC genes located on chromosome 17 within the \(idd1\) locus. The NOD mouse is homozygous for a unique H-2 haplotype (H-2\(^{g7}\)) that contains a non-productive I-E\(_\alpha\) gene and encodes an I-\(\alpha^d/I-\beta^{g7}\) heterodimer in which the histamine and aspartic acid found at positions 56 and 57 in most I-\(\beta\) chains are replaced by proline and serine, respectively (Acha-Orbea and McDevitt, 1987; Hattori et al., 1986; Todd et al., 1987). The role of this MHC-haplotype in susceptibility to diabetes has been demonstrated in transgenic and congenic NOD mice where
presence of a non-NOD H-2 haplotype lead to decrease in diabetes incidence (Tisch and McDevitt, 1996; Yamamura et al., 1992). The similarities in the binding pockets of the NOD MHC class II I-\(\text{A}\)\(\beta\)\(^{g7}\) molecule and human HLA-DQ8 and -DQ2 class II molecules advocate that similar autoantigen presentation events may underlie diabetes in mice and humans (Lee et al., 2001). Studies with the 4.1-TCR-transgenic NOD mice that contain a specific CD4\(^{+}\)T cell clone expressing a highly diabetogenic TCR have provided evidence for a relationship between central tolerance and MHC class II haplotypes. Thymocytes expressing the 4.1-TCR were found to undergo central deletion in H-2\(^{g7/b}\), H-2\(^{g7/k}\), H-2\(^{g7/q}\) and H-2\(^{g7/nb1}\) NOD mice resistant to type 1 diabetes (Schmidt et al., 1997). It has been suggested that protective MHC class II alleles in NOD mice afford resistance to type 1 diabetes by tolerizing a group of highly pathogenic MHC promiscuous, 4.1-like CD4\(^{+}\)T cells, which play a critical role in diabetes (Yang and Santamaria, 2003). A possible role for protective MHC class II molecules in the selection of regulatory T cells is yet to be determined.

MHC however, is not sufficient for disease development, which demonstrates the polygenic nature of type 1 diabetes (Rose and Mackay, 1998). The genetic factor(s) in each Idd locus and the biological functions they mediate remain largely unknown. Some candidate genes have been proposed. Like in human diabetes the Ctla-4 gene has been implicated in susceptibility to autoimmunity in the NOD mouse and proposed to constitute the genetic factor in the Idd5.1 locus (Colucci et al., 1997; Hill et al., 2000; Ueda et al., 2003; Wicker et al., 2004). The expression of CD28 and CTLA-4 was observed to be lower in NOD mice when compared to B6 mice after T-cell activation with anti-CD3 antibody (Colucci et al., 1997). Recently, full-length forms of CTLA-4 (flCTLA-4) as well as two other forms, ligand-independent (liCTLA-4) and soluble CTLA-4 (sCTLA-4) were characterized (Wicker et al., 2004). A polymorphism in exon 2 of Ctla-4 observed between NOD and B6 mouse strains was suggested to affect expression of liCTLA-4 and possibly constitute the genetic basis of Idd5.1 (Wicker et al., 2004). The Icos gene, which independently or together with the ctla-4 gene may lead to the effect of the Idd5.1 locus, has been also considered a candidate for this locus (Wicker et al., 2004). Interestingly, contrary to flCTLA-4, the expression of ICOS was higher on activated NOD cells compared to B6 and B10 T cells (Greve et al., 2004). Also, congenic NOD mice for the Idd5.1 region were more susceptible to myelin oligodendrocyte glycoprotein 35-55-induced murine experimental autoimmune encephalomyelitis (EAE) (Greve et al., 2004). Lower expression of ICOS and reduced production of IL-10 was observed in T cells from NOD.Idd5.1 congenic mice compared to NOD T cells, and
this was suggested to be responsible for the susceptibility to EAE observed in the congenic animals (Greve et al., 2004). IL-10 has been demonstrated to have an inhibitory role in EAE progression (Bettelli et al., 1998). The influence of IL-10 on type 1 diabetes on the other hand seems to depend on both the location and time point where it is released during the disease (Lee et al., 1996; Moritani et al., 1994; Pennline et al., 1994; Zheng et al., 1997). The opposite effects of alleles in the Idd5.1 locus in the two autoimmune diseases may reflect different roles for costimulatory pathways in inducing autoimmune responses depending upon the origin of the target antigen. As for the Idd5.2 locus, a functional nonsynonymous polymorphism (glycine$^{169}$>aspartic acid$^{169}$) is known to distinguish the NOD and B10 Nramp1 alleles, which made this gene, coding for natural resistance associated macrophage protein (NRAMP), the most appealing candidate for the locus (Hill et al., 2000; Vidal et al., 1995; Wicker et al., 2004). In addition, the $\beta_2$-microglobulin ($\beta2m$) gene located on the Idd13 chromosomal region has been linked to diabetes risk and genes encoding the IL-2 and IL-21 cytokines were suggested to be good candidates for the Idd3 locus (Denny et al., 1997; Hamilton-Williams et al., 2001; King et al., 2004; Marron et al., 2002; Serreze et al., 1998a). Recently 3 polymorphisms were identified in the Vav3 gene which may account for Idd18 (Anderson and Bluestone, 2005; Maier et al., 2005). This gene belongs to the Vav family of Rho-guanine nucleotide exchange factors (GEFs), which are thought to be involved in the control of a diverse array of signaling pathways emanating from antigen receptors in lymphocyte (Swat and Fujikawa, 2005). Furthermore, the EWI-101 (Cd101) gene, encoding an egg-white implanted (EWI) immunoglobulin subfamily member was proposed as a candidate gene for the Idd10 locus (Penha-Goncalves et al., 2003; Yamaji et al., 2005). This CD101 molecule is expressed at the surface of several immune cells and it is involved in T cell proliferation through its effect on interleukin-2 (IL-2) production (Bagot et al., 1997; Rivas et al., 1995; Soares et al., 1997; Soares et al., 1998). In a recent study, the immune signaling molecule 4-1BB was reported to be encoded by the Idd9.3 susceptibility locus and have a primary function in the etiology of autoimmune diabetes (Cannons et al., 2005). T cells from NOD mice showed decreased IL-2 secretion as well as decreased proliferation in response to costimulation with 4-1BB in comparison to T cells from B10 mice (Cannons et al., 2005).
<table>
<thead>
<tr>
<th>Locus</th>
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<th>NOD allele confers</th>
<th>Diabetes -resistant strain used for mapping</th>
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<td>B10</td>
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<td>NON.H2g7</td>
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<tr>
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<td>B6</td>
<td>43</td>
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<td>C3H, PWK</td>
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<td>C3H</td>
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<td>17</td>
<td>susceptibility</td>
<td>B6</td>
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Table 3 references

*Bid1* - 1 (Hattori et al., 1986), 2 (Prochazka et al., 1987), 3 (Wicker et al., 1987), 4 (Prochazka et al., 1989), 5 (Ikegami et al., 1990), 6 (Todd et al., 1991), 7(Ikegami et al., 1995), 8 (Yui et al., 1996), 9 (Ikegami et al., 2003),
*Bid2* - 2 (Prochazka et al., 1987), 4 (Prochazka et al., 1989), 10 (Ghosh et al., 1993), 11 (McAleer et al., 1995)
*Bid3* - 6 (Todd et al., 1991), 8 (Yui et al., 1996), 9 (Ikegami et al., 2003), 10 (Ghosh et al., 1993), 11 (McAleer et al., 1995), 12 (Wicker et al., 1994b), 13 (Wicker et al., 1995), 14 (Lord et al., 1995), 15 (Denny et al., 1997), 16 (McDuffie, 1998), 17 (Lyons et al., 2000)
*Bid4* - 6 (Todd et al., 1991), 8 (Yui et al., 1996), 10 (Ghosh et al., 1993), 18 (Gill et al., 1995), 19 (Grattan et al., 2002)
*Bid4.1* - 19 (Grattan et al., 2002)
*Bid4.2* - 19 (Grattan et al., 2002)
*Bid5* - 6 (Todd et al., 1991), 8 (Yui et al., 1996), 10 (Ghosh et al., 1993), 20 (Cornall et al., 1991), 21 (Hill et al., 2000), 22 (Eaves et al., 2002)
*Bid5.1* - 21 (Hill et al., 2000), 23 (Lamhamedi-Cherradi et al., 2001)
*Bid5.2* - 21 (Hill et al., 2000)
*Bid5.3* - 24 (Fox et al., 2000), 25 (Ivakine et al., 2005)
*Bid6* - 8 (Yui et al., 1996), 10 (Ghosh et al., 1993), 11 (McAleer et al., 1995), 26 (de Gouyon et al., 1993), 27 (Rogner et al., 2001), 28 (Carnaud et al., 2001), 29 (Bergman et al., 2003)
*Bid7* - 10 (Ghosh et al., 1993), 11 (McAleer et al., 1995), 16 (McDuffie, 1998)
*Bid8* - 10 (Ghosh et al., 1993), 30 (Liston et al., 2004)
*Bid9* - 10 (Ghosh et al., 1993), 11 (McAleer et al., 1995), 13 (Wicker et al., 1995), 22 (Eaves et al., 2002), 31 (Rodrigues et al., 1994)
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*Bid11* - 35 (Morahan et al., 1994), 36 (Brodnicki et al., 2000)
*Bid12* - 35 (Morahan et al., 1994)
*Bid13* - 16 (McDuffie, 1998), 37 (Serrezee et al., 1994b), 38 (Serrezee et al., 1998a)
*Bid13.1* - 38 (Serrezee et al., 1998a)
*Bid13.2* - 38 (Serrezee et al., 1998a)
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*Bid16.1* - 43 (Pomerleau et al., 2005)
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*Bid17* - 9 (Ikegami et al., 2003), 16 (McDuffie, 1998), 44 (Podolin et al., 1997), 45 (Podolin et al., 1998)
*Bid18* - 9 (Ikegami et al., 2003), 16 (McDuffie, 1998), 34 (Lyons et al., 2001), 45 (Podolin et al., 1998)
*Bid19* - 27 (Rogner et al., 2001), 46 (Melanitou et al., 1998)
*Bid20* - 27 (Rogner et al., 2001)
*Bid21* - 47 (Hall et al., 2003)
*Bid22* - 41 (Mathews et al., 2003)
*Bid23* - 42 (Deruytter et al., 2004)
*Bid24* - 42 (Deruytter et al., 2004)

*B6 stands for C57BL/6, B10 represents C57BL/10, NON stands for non-obese non diabetic and NOR means non-obese resistant mice
One way to address the problem of how a particular locus may contribute to disease is to employ a sub-phenotype approach. This approach consists of dissecting the disease into simpler phenotypes related to potentially pathogenic impairments in immune function that if relevant to the disease would be controlled by one or a few susceptibility loci. Since type 1 diabetes is an autoimmune disease, immunological features reflecting dysfunctions in the mechanisms of tolerance induction have been studied using this approach. Thus, it was observed that the idd5 locus may be controlling resistance of NOD peripheral lymphocytes to cyclophosphamide-induced apoptosis as well as NOD thymocyte resistance to γ-irradiation-induced apoptosis (Bergman et al., 2001; Colucci et al., 1997). In addition, Idd5.3 was identified as a locus that in conjunction with Idd13 is able to control islet invasiveness (Fox et al., 2000; Ivakine et al., 2005). In a recent study using both genotyping mapping and congenic NOD and Non-obese diabetes resistant (NOR) mice, Idd4, Idd5 and Idd9 loci were found to control susceptibility to cyclophosphamide-induced type 1 diabetes (Ivakine et al., 2005). In that study Idd4 and Idd9 were shown to be interacting and to play a role in controlling insulitis development. While Idd5 and Idd9 were shown to prevent insulitis progression, Idd4 seemed to protect invasive insulitis from converting to diabetes. Interestingly, the Idd4 locus clearly showed a sex-specific effect in controlling cyclophosphamide-induced type 1 diabetes (Ivakine et al., 2005). Other functions for the Idd4 locus have been proposed. It was reported that a sub-locus of Idd4, the Idd4.2, was responsible for the T-cell hypore sponsiveness observed in NOD mice upon cross-linking of the T-cell receptor/CD3 complex (Grattan et al., 2002). Furthermore, another group has suggested that other subintervals within the Idd4 locus are controlling the defect in IL-12p40 expression observed in NOD macrophages (Simpson et al., 2003). Our recent observations that the enlargement in the marginal zone B cell population observed in the NOD mouse is controlled by a quantitative trait locus located in the Idd11 gene region, is another example of successfully applying the subphenotype approach (Rolf et al., 2005).
Central tolerance defects associated with type 1 diabetes

Defects in selection events during T cell development in the thymus have been suggested to contribute to the autoimmune set-up in type 1 diabetes. Diabetic patients have been reported to have reduced expression of insulin in the thymus, which could lead to defects in thymic selection of reactive clones (Pugliese and Miceli, 2002). Moreover, recent studies in mice have shown that the development of insulin autoantibodies, insulitis and diabetes is dependent on native insulin gene sequences, indicating that insulin can be a primary target of autoimmunity (Nakayama et al., 2005). In mouse chimeras between NOD and B6, presence of insulitis was found to correlate with MHC chimerism in lymphoid cells and thymus cortical regions, implicating thymic selection events in the development of diabetes (Forsgren et al., 1991b). In that study, all individuals displaying NOD or NOD/B6 thymic cortical regions developed insulitis, while no insulitis was observed in chimeras with only B6 thymic cortical regions. Furthermore, analysis of crystal structure of the NOD I-A^β^ molecule indicates that, on one hand, it may be intrinsically unstable, which results in poor peptide binding, possibly leading to poor negative selection of autoreactive clones or poor positive selection of regulatory T cells in response to self-antigens in the thymus (Kanagawa et al., 1998; Ridgway et al., 1999). On the other hand, the ability of NOD H-2^β^ haplotype to provide susceptibility to type 1 diabetes, while introgression of the H-2^β^ MHC onto the NOD backgrounds substantially exacerbates thyroiditis, suggests that specific peptide presentation might explain the role of MHC class II molecules (Wicker, 1997). Conversely, abnormal negative selection has been observed in NOD mice, with reports of the semi-mature heat stable antigen high single positive (HSA^+^ SP) T cells located in the thymic medulla being resistant to TCR/CD28-mediated apoptosis induced by anti-CD3 or superantigen treatment in vitro and in vivo (Kishimoto and Sprent, 2001). This effect was independent of NOD MHC class II molecules. However these results could not be replicated by others, possibly due to different methods used for thymocyte purification (Villunger et al., 2003). In another set up using a transgenic mouse model, the fate of high avidity CD4^+^T cells recognizing a pancreatic β-cell self antigen (HEL) was followed in autoimmune susceptible and autoimmune resistance backgrounds with the same H-2 haplotype. Non-MHC genes were observed to be responsible for the resistance to clonal deletion of the autoreactive T cells in the thymus of NOD mice (Lesage et al., 2002). The impact of NOD background genes on negative selection has also been explored in a recent study using fetal thymic organ cultures (FTOCs) derived from NOD and C57BL/6.H2^β^ mice to compare the maturation of the β-cell self antigen specific BDC2.5 transgenic CD4^+^ T cell. Resistance to clonal deletion in differentiating thymocytes expressing the BDC2.5 specificity as well as a reduced ability to divert
autoreactive thymocytes into an alternative pathway of differentiation that results in T cells of the CD8αα phenotype was observed in the NOD background (Zucchelli et al., 2005). In the same study, genetic mapping attributed these differences to regions located on Ch1 and Ch3, while microarray analysis pointed to combined effects of genes involved in regulating apoptosis and cell survival. Other studies comparing NOD and B6 mice have found no evidence for defects in negative selection of DP thymocytes in the thymic cortex of NOD and have attributed the development of autoreactive diabetogenic T cells mainly to an inordinate positive selection due to the low activation threshold observed for the NOD DP thymocytes (Kwon et al., 2005).
III-Regulatory T cells in type 1 diabetes

Loss of immunoregulation is at the root of autoimmune diabetes. It has been at least 15 years since T cells with suppressor functions were suggested to be involved in the onset of type 1 diabetes (Boitard et al., 1989; Hutchings and Cooke, 1990). With the recent validation of suppressor T cells, now rehabilitated as regulatory T cells, studies of different T cell population involved in regulation of diabetes have had a revival. Three T cell subsets have been described in NOD mice, the CD4⁺CD62L⁺ T cells, the CD4⁺CD25⁺ T cells and the Natural Killer-like (NK) T cells.

The CD4⁺CD62L⁺ and CD4⁺CD25⁺ T cell populations
A subset of CD4⁺ T cells, expressing the L-selectin CD62L usually present in naïve lymphocytes has been described as being able to protect from diabetes when co-transferred with diabetogenic T cells into an immunodeficient NOD.Scid mouse. In this model, protection from diabetes was attributed to modification of the homing ability of diabetogenic T cells to pancreatic islets, with observations of lower numbers of effector T cells in the infiltrates and attenuation of the infiltration pattern (Lepault and Gagnerault, 2000). These CD4⁺CD62L⁺ T cells did not produce any IL-4 or IL-10, showing that protection from diabetes was independent of these cytokines (Lepault and Gagnerault, 2000). However, the CD4⁺CD62L⁺ subset is rather large and 50% of the CD45RBlowCD4⁺CD25⁺ regulatory T cells express the CD62L marker. It is therefore possible that the protective effects observed with this cell population are a reflection of the presence of CD4⁺CD25⁺ regulatory cells within the subset. Accordingly, other studies have demonstrated that CD4⁺CD25⁺ can regulate autoimmune diabetes and have implicated the CD4⁺CD25⁺CD62L⁺ T cells as being particularly effective in in vivo experiments due to high expression of the chemokine CCR7, which in combination with CD62L makes is essential for T cell entry into peripheral lymph nodes (Salomon et al., 2000; Szanya et al., 2002; Wu et al., 2002). Indeed, the current notion is that when CD4⁺CD25⁺ regulatory T cells are transferred into the NOD mouse, they are capable of preventing or suppressing diabetes only if they are able to home to and proliferate in pancreatic lymph nodes through recognition of agonistic TCR ligands (Jaeckel et al., 2005; Tang et al., 2004; Tarbell et al., 2004). In addition, analysis of CD28- or B7-deficient mice have shown that costimulation through the CD28 receptor and B7-1, B7-2 ligands is essential for generation and maintenance of regulatory T cells in both the thymus and the periphery (Sakaguchi, 2005). NOD mice with CD28 or B7 deficiency or a B7 blockade consistently manifested accelerated development of type 1 diabetes, which substantiates the effect of regulatory T cell deficiency in the onset of the disease (Salomon et al., 2000). Also, in a DP-BB rat model of
accelerated diabetes where the disease is induced by transfer of purified T cells from diabetic DR-BB rats, co-transfer with CD4⁺CD25⁺ T cells prevented diabetes (Lundsgaard et al., 2005). Nevertheless, defects in the number and function of this regulatory population in unmanipulated NOD mice have been difficult to assess. A numerical defect in the number of CD4⁺CD25⁺ regulatory T cells in NOD mice compared to other mouse strains has been suggested (Wu et al., 2002). In contrast to previous reports of lower numbers of regulatory T cells in diabetic patients, a recent study in humans has failed to detect any difference in frequency of CD4⁺CD25⁺ T cells between aged matched healthy control subjects and patients (Brusko et al., 2005; Kukreja et al., 2002). Still, CD4⁺CD25⁺ regulatory T cells from diabetic patients were observed to be functionally deficient in their ability to suppress proliferation of effector T cells and in the production of a number of cytokines (Brusko et al., 2005). The difficulty in distinguishing the regulatory compartment from generally activated T cells that expresses the CD25 receptor at the surface has hampered these studies. To circumvent this problem, authors have determined the transcription levels of Foxp3, an unquestionable marker for this subset of regulatory T cells. Recent observations point to an age-dependent decline of T cells expressing Foxp3 and TGFβ1 from the CD4⁺CD25⁺ pool in pancreatic lymph nodes of NOD female mice, while in NOD males the levels of these cells remained constant (Pop et al., 2005). In that study the authors proposed that the progressive decline in the percentage of FoxP3- and TGFβ1-coexpressing CD4⁺CD25⁺ regulatory T cells contributes to the development of aggressive insulitis in female NOD mice. An association study in a Japanese population revealed a weak association of a polymorphism in the promoter/enhancer region of the Foxp3 gene with type 1 diabetes (Bassuny et al., 2003). However, a similar study with the Sardinian population using a larger sample set failed to find association between variations in the Foxp3 gene and type 1 diabetes (Zavattari et al., 2004). While CD4⁺CD25⁺ T cells are unquestionably able to suppress diabetes in several disease models, it remains to be determined whether a defect in this particular population underlies pathogenesis in un-manipulated mice or in diabetic patients.

The other T cell population implicated in regulation of type 1 diabetes is known as natural killer-like (NKT) cells due to co-expression of cell surface receptors characteristic of natural killer cells with an αβT cell receptor. This cell population is known to be numerically and functionally deficient in NOD mice and several lines of evidence point to it as exerting an important role in the regulation of type 1 diabetes.
NKT cells

What are they?

NKT cells were first identified in mice as a distinct subset of T cells that expressed the $\alpha\beta$-T-cell receptor at intermediate levels and lacked expression of the CD4 and CD8 co-receptors. A high frequency of V$\beta$8 usage was observed among these cells (Budd et al., 1987; Ceredig et al., 1987; Fowlkes et al., 1987). Later it was discovered that this cell population was a potent source of immunoregulatory cytokines such as IL-4 and INF-$\gamma$ (Zlotnik et al., 1992). In parallel, others have identified an $\alpha\beta$-T cell population also expressing intermediated levels of TCR and biased towards V$\beta$8 usages, that co-expressed a natural killer cell marker, the C-type lectine NK1.1, and NKT became the nomenclature for this cell population. This NKT population was shown to comprise a CD4$^+$ T cell compartment besides the CD4$^-$CD8$^-$ (DN) subset both producing high levels of cytokines (Arase et al., 1993; Osman et al., 2000; Yoshimoto and Paul, 1994). Additional studies lead to the recognition that NKT cells are restricted to the MHC class-I-like molecule CD1d, that presents conserved endogenous and exogenous glycolipids (Fischer et al., 2004; Kinjo et al., 2005; Mattner et al., 2005; Zhou et al., 2004). Furthermore, these cells showed preferential use of an invariant TCR$\alpha$ chain consisting of V$\alpha$14-J$\alpha$281 (also known as J$\alpha$18) rearrangements, a bias toward V$\beta$8 usage and co-expressed other NK cell receptors such as members of the NKrpl and Ly49 family of inhibitors. In addition, NKT cells presented an activated phenotype with high expression of CD69 and CD44 receptors and low expression of the CD62L molecule (Godfrey et al., 2004). Moreover, NKT cells were found to be positively selected in the thymus by CD1d-expressing bone-marrow-derived cells, possibly CD4$^+$CD8$^+$ (DP) thymocytes, which supported the notion that they constitute an independent T cell lineage (Bendelac et al., 1995; Coles and Raulet, 2000). The definition of NKT cells became thorny due to lack of NK1.1 expression in several mouse strains and the unavoidable use of a blend of surrogate markers to identify them. Furthemore, it was observed that several T cells with NKT cell characteristics failed to express the NK1.1 marker (Godfrey et al., 2004). Indeed, the expression of NK1.1 was observed to depend on maturity, activation state and tissue location, as well as on the genetic background (Benlagha et al., 2002; Chen and Paul, 1998; Eberl et al., 1999; Gapin et al., 2001; Hammond et al., 2001; Lantz et al., 1997; Matsuda et al., 2000; Pellicci et al., 2002). The identification of a CD1d-reactive glycolipid antigen, the marine sponge derived agent (2S, 3S, 4R)-1-O-(\alpha-D-galactopyranosyl)-N-hexacosanoyl-2-amino-1,3,4-octadecanetriol), usually referred to as \(\alpha\)-galacosylceramide (\(\alpha\)-galCer), with the ability to potently activate the V$\alpha$14-J$\alpha$18 NKT cells allowed for a more precise identification and characterization of
these cells (Kawano et al., 1997). Nowadays fluorochrome-conjugated, tetrameric complexes of CD1d loaded with α-GalCer are widely used to identify NKT cells. A similar cell population has been identified in other species including rats, primates and humans (Dellabona et al., 1994; Kashiwase et al., 2003; Matsuura et al., 2000; Motsinger et al., 2003; Porcelli et al., 1993). Part of NKT cells in humans express TCR V regions orthologous to mouse Vα14 and Vβ8, including an invariant Vα24/Jα18 rearrangement on the TCRα chain co-expressed with the Vβ11 TCRβ-chain (Dellabona et al., 1994; Porcelli et al., 1993). However, this population is less frequent in humans than the Vα14-Jα18 NKT cells in mice and can be present among CD8+ T cells (Ishihara et al., 1999; Metelitsa et al., 2001).

Besides the Vα14-Jα18 NKT-cell population that makes up the major and most well defined subset, several studies have recognized greater heterogeneity within this cell compartment. A group of CD1d-restricted non-Vα14-Jα18 NKT cells that use a somewhat diverse αβ-TCR repertoire have been identified in mice and seem to be a considerable fraction of CD4+ T cells in MHC class II deficient mice (Behar et al., 1999; Cardell et al., 1995; Chiu et al., 1999; Park et al., 2001). These cells are not strictly polyclonal since they include expanded populations that express either Vα3.2-Jα9/Vβ8 or Vα8/Vβ8 TCRs (Park et al., 2001). Nevertheless this subset also includes DN cells, and contains both NK1.1+ and NK1.1- T cells. In addition, these lymphocytes express several NK markers such as Ly49 family members, have an activated phenotype, produce large amounts of cytokines upon stimulation and have been suggested to preferentially express the α2 integrin CD49b over the CD69 molecule (Behar and Cardell, 2000; Stenstrom et al., 2004). On the other hand, while Vα14-Jα18 NKT cells require endosomal targeting of CD1d molecules, the non-Vα14-Jα18 NKT cells most likely recognize CD1d molecule presenting ligands loaded in the secretory pathway and cannot be identified through the use of α-GalCer-CD1d tetramers (Chiu et al., 1999). Due in part to difficulties with its definition, this cell population is far less well characterized and it is not yet clear to what extent it overlaps in phenotype and function with the Vα14-Jα18 NKT population. It has been proposed to name this cell population nonclassical NKT cells in contrast to the classical Vα14-Jα18 NKT cell population (Table 4). In humans, CD1d-dependent T cells with diverse TCR and reactive to α-GalCer have also been identified. These cells were non Vα24-Jα18 CD8α+CD8β+ NKT cells and showed CD8 dependent cytotoxicity in vitro (Gadola et al., 2002). Moreover, the majority of CD1d-reactive T cells isolated from human BM or hepatitis C virus-infected livers had a diverse TCR (Baron et al., 2002; Exley et al., 2001).
NKT cells distribute preferentially through the liver, spleen, thymus and bone marrow with different subsets having predominance in each of these organs (Hammond et al., 1999; Stenstrom et al., 2004). Tissue-specific microenvironments have been suggested to modulate phenotype and function of NKT cells (Yang and Santamaria, 2003). Little is known about the factors that direct NKT cells into their specific locations. The lymphocyte function associated antigen 1 (LFA1) has been reported to be involved in the transport of Vα14-Jα18 NKT cells into the liver (Emoto et al., 1999; Ohteki et al., 1999). Migration of NKT cells to sites of inflammation and infection was also described after injection of glycolipids (Mempel et al., 2002). Moreover, migration to the spleen in response to macrophage inflammatory protein 2 was observed during induction of tolerance after injection of antigen into the interior chamber of the eye (Faunce et al., 2001; Kronenberg and Gapin, 2002). Similarly, accumulation of NKT cells in the lungs after cryptococcal infection was observed to be dependent on monocyte chemotactic protein 1 (Kawakami et al., 2001).

**Table 4** - Classification of NKT cells. Adapted from (Godfrey et al., 2004)

<table>
<thead>
<tr>
<th></th>
<th>Classical NKT cells</th>
<th>Non-classical NKT cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CD1d dependent</strong></td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td><strong>α-GalCer reactive</strong></td>
<td>yes</td>
<td>no*</td>
</tr>
<tr>
<td><strong>TCR α-chain</strong></td>
<td>Vα14-Vα18 (mice)</td>
<td>Diverse, but some Vα3.2-Jα9, Vα8 (mice)</td>
</tr>
<tr>
<td></td>
<td>Vα24-Vα18 (humans)</td>
<td></td>
</tr>
<tr>
<td><strong>TCR β-chain</strong></td>
<td>Vβ8.2, Vβ7 and Vβ2 (mice)</td>
<td>Diverse, but some Vβ8.2 (mice)</td>
</tr>
<tr>
<td></td>
<td>Vβ11 (humans)</td>
<td></td>
</tr>
<tr>
<td><strong>NK1.1 (CD161)</strong></td>
<td>+ (resting mature)</td>
<td>+/-</td>
</tr>
<tr>
<td></td>
<td>-/low (immature or post-activation)</td>
<td></td>
</tr>
<tr>
<td><strong>Other surface markers</strong></td>
<td>CD69+ CD49b-low</td>
<td>CD69+ CD49b&lt;sup&gt;high&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Subsets</strong></td>
<td>CD4&lt;sup&gt;+&lt;/sup&gt; and DN (mice)</td>
<td>CD4&lt;sup&gt;+&lt;/sup&gt; and DN (mice)</td>
</tr>
<tr>
<td></td>
<td>CD4&lt;sup&gt;+&lt;/sup&gt;, CD8&lt;sup&gt;+&lt;/sup&gt; and DN (humans)</td>
<td></td>
</tr>
<tr>
<td><strong>IL-4 production</strong></td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>INF-γ</strong></td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

* In humans some CD1d restricted non-Vα24/Vβ11 T cells reactive to α-GalCer have been identified
Where do they come from?

The origin of NKT cells has been the subject of much controversy (Kronenberg and Gapin, 2002). Nevertheless, through the use of CD1d tetramers loaded with α-GalCer it was clearly demonstrated that the thymus is both necessary and sufficient for their development. Vα14-Jα18 NKT can develop in vitro from FTOCs and are absent from the spleen and the liver of nude mice that cannot differentiate a functional thymus (Chun et al., 2003; Pellicci et al., 2002; Pellicci et al., 2003). Furthermore, injection of immature thymocytes into the thymus, but not through an intravenous route, could populate the liver and spleen of recipient mice with classical NKT cells (Gapin et al., 2001). It has also been observed that DP thymocytes can differentiate into classical NKT cells only in the presence of CD1d (Matsuda and Gapin, 2005). It has been suggested that thymic NKT cells undergo 3 stages of development, going from CD44lowNK1.1- (stage 1) to CD44highNK1.1- (stage 2) and CD44highNK1.1+ (stage 3) with up regulation of other NK lineage receptors (such as Ly49G2, Ly49C, Ly49I, CD94, NKG2D, Ly6C and 2B4) and the IL2/IL15Rβ chain (CD122) in this later phase as well (Benlagha et al., 2002; Gadue and Stein, 2002; Gapin et al., 2001; Pellicci et al., 2002). IL-15 seems to be required for classical NKT cell proliferation and maintenance (Matsuda et al., 2002; Ranson et al., 2003). Recently, using CD1d tetramer enrichment, HSAhigh cells representing the earliest stages in NKT cell development were characterized (Benlagha et al., 2005). These cells were observed at very low frequencies, showed Vβ8 bias, appeared to go through a non-dividing DPlow and CD4+ stage, and expressed a CD44lowNK1.1- phenotype (Benlagha et al., 2005). Most of Vα14-Jα18 NKT cells are exported before the expression of the NK markers, which indicates that full maturation can occur in the periphery (Benlagha et al., 2002; Pellicci et al., 2002). In support of its status as a specific T cell lineage, it was shown that NKT cells are positively selected by CD1d expressing DP thymocytes rather than by the cortical epithelial cells usually required for selection of conventional T cells (Bendelac et al., 1995; Coles and Raulet, 2000; Wei et al., 2005). Recently it was demonstrated that a glycoprophospholipid structurally similar to α-GalCer, the isoglobotrihexosylceramide 3 (iGb3, an endogenous ligand generated in lysosomal compartments) seems to be required for positive selection of Vα14-Jα18 NKT cells. Lack of NKT-cell precursors was observed in the thymus of β-hexosaminidase-b-deficient mice (Hexb-/-), which is the enzyme required for the generation of iGb3. The iGb3 molecule is also capable of stimulating mature NKT cells in the periphery, reflecting selection for self-reactivity (Zhou et al., 2004). Speculations have arisen regarding whether this could represent a stress signal important for the immune system to recognize and simultaneously contribute to host defense by providing specificity for the recognition of unusual glycoprophospholipids present in certain types of bacteria (Kronenberg and Rudensky,
Microbial α-glycuronosylceramides found in the cell wall of Gram^- LPS^- bacteria and mycobacterial phosphatidylinosiolmannosides were recently shown to be presented by CD1d molecules to NKT cells (Fischer et al., 2004; Kinjo et al., 2005; Mattner et al., 2005). Negative selection upon encounter with high avidity antigen or abundant self-antigen has also been suggested to occur, but at an earlier stage of development and within a precise time-frame. A balance between activating and inhibiting NK receptors seems to be important for NKT cell development (Matsuda and Gapin, 2005). Other specific requirements for NKT cell development appear to involve transcription factors such as NF-κB family members and T-box expressed in T cells (T-bet), as well as signaling molecules such as the Src family kinase Fyn and the adaptor signaling lymphocyte activation molecule-associated protein (SAP) that interacts with Fyn (Chung et al., 2005; Kronenberg and Rudensky, 2005; Matsuda and Gapin, 2005; Nichols et al., 2005; Pasquier et al., 2005).

**What do they do?**

An immunoregulatory role for NKT cells was early on suggested due to the observation that these cells could rapidly produce large amounts of both Th1 and Th2 cytokines like INF-γ, IL-2 and IL-4, IL-10, IL-13 and TGF-β upon TCR stimulation (Arase et al., 1993; Yoshimoto and Paul, 1994; Zlotnik et al., 1992). Also, this ability to become activated fast renders them good candidates to be involved in the first line of defense against infections, besides modulating the downstream development of acquired immune responses mediated by T and B cells. Indeed, they can promptly stimulate activation of dendritic cells, NK cells and other cell types which is reminiscent of an innate immune response.

**NKT cells in suppression of immune responses**

The ability of NKT cells to suppress cell-mediated immune responses has been demonstrated in several different models. In the anterior chamber-associated immune deviation model (ACAID) in mice, introduction of an antigen in the anterior chamber of the eye, an immune privileged site, results in local and systemic inhibition of antigen-specific inflammatory T cell responses. Studies in CD1d knock out mice that lack NKT cells have shown that these cells are critical for ACAID. IL-10 secretion by NKT cells and induction of CD8^+ T cells with regulatory capacities, possibly as consequence of exposure to IL-10 are mediating tolerance in this model (Nakamura et al., 2003; Sonoda et al., 1999; Sonoda et al., 2001). In addition, in a mouse corneal allograft model NKT cells were required for tolerance induction also by recruitment of downstream allospecific
regulatory T cells (Sonoda et al., 2002). This suggests possible interactions between different regulatory populations. In support of this, suppression of NKT cell activity by CD4^+CD25^+ T cells has been reported in mice and humans (Azuma et al., 2003; Nishikawa et al., 2003). A role for NKT cells in allograft tolerance induction has also been proposed in two models of cardiac allografts following tolerogenic immunotherapy. In both models, suppression by these cells was only found when associated with immunological conditioning regimes (Higuchi et al., 2002; Seino et al., 2001). A similar role for NKT cells was observed in a model of xenograft tolerance (Ikehara et al., 2000). Bone-marrow derived NKT cells were also observed to inhibit graft versus-host disease which was critically dependent on IL-4 production (Zeng et al., 1999). In all of these models no exogenous stimulation was provided. It is possible that weak stimulation by endogenous glycolipids stimulates a tolerogenic response, while stronger stimulation by exogenous ligands may lead to a different response (Godfrey and Kronenberg, 2004).

**NKT cells in autoimmunity**

The role of NKT cells in autoimmunity was first suggested by the observation that this cell population was numerically and functionally deficient in the thymus, spleen, bone marrow and liver of the NOD mouse model of type 1 diabetes (Baxter et al., 1997; Godfrey et al., 1997; Gombert et al., 1996). Moreover, increasing the number of NKT cells by adoptive transfer or via the introduction of Vα14-Jα281 transgene reduced diabetes progression (Baxter et al., 1997; Hammond et al., 1998; Lehuen et al., 1998). Conversely, NKT cell deficiency in CD1d^-/- NOD mice exacerbates diabetes development (Wang et al., 2001). NKT cells from NOD mice have also been reported to secrete less IL-4 cytokine after stimulation, but nevertheless stimulation by repetitive administration of α-GalCer do protect from diabetes (Gombert et al., 1996; Hammond et al., 2001; Hammond et al., 1998; Hong et al., 2001; Naumov et al., 2001; Poulton et al., 2001; Sharif et al., 2001; Wang et al., 2001).

The NKT cell deficiency in the NOD mouse was shown to be controlled by multiple loci (Esteban et al., 2003; Jordan et al., 2004; Matsuki et al., 2003). Two of the most important loci controlling the number of these cells are Nkt1 located on the tip of chromosome 1, overlapping with a region postulated to confer lupus susceptibility (Containing the Sl21, Nba2 and Lbw7 loci) and Nkt2 on chromosome 2 that overlaps with the diabetes susceptibility locus Idd13 (Esteban et al., 2003; Jordan et al., 2004). Several candidate genes contributing to this deficiency have been analyzed, but besides β^2m that was proposed as a candidate gene for the Idd13 locus, no other gene localizes to regions identified through congenic or linkage analyses (Hamilton-Williams et al., 2001; Jordan et al., 2004).
The β2m molecule was shown to associate with CD1d in the endoplasmic reticulum forming a complex that travels to the cell surface where the cytoplasmic tail of CD1d binds to an adaptor protein resulting in the targeting of the complex to the late endosomes. Therefore β2m is necessary for proper antigen presentation to classical NKT cells (Ohteki and MacDonald, 1994). The absence of NKT in β2m knock out mice strengthens the evidence for positive selection by CD1d molecules (Ohteki and MacDonald, 1994).

Using different approaches, other studies have demonstrated that the defect in NKT cells is intrinsic to the T-cell lineage. Reconstitution of T-cell compartment of immunodeficient NOD.Scid or Balb/c.Scid mouse strains with T-cell-committed thymic precursor cells from NOD or NOR mice, which shares about 88% of NOD genome including the MCH locus and the NKT cell deficiency, resulted in a deficient NKT cell population. In contrast, normal NKT cells developed when NOD.Scid or Balb/c.Scid mice were reconstituted with AKR mice-derived thymic precursors (Yang et al., 2001). A recent study has reported that NKT cell deficiency in both embryonic and adult NOD mice appears after positive selection by DP thymocytes and is visible from embryonic day 18 to day 1 after birth, suggesting a defect in selection of this regulatory cell subset (Wagner et al., 2005).

Protection from diabetes has been associated with IL-4 secretion and skewing towards a Th2 type of immune response against islet autoantigens. Protection from diabetes via an IL-4 and/or IL-10 mechanism and a Th2 polarized response has been observed in the Vα14-Jα18 NKT transgenic NOD mice and in Vα14-Jα18 NKT cell transfers into immunodeficient mice as well as in mice treated with α-GalCer (Hammond et al., 1998; Hong et al., 2001; Laloux et al., 2001; Sharif et al., 2001). However, the abundance of NKT cells in pancreatic lymph nodes compared with other peripheral lymph nodes and their presence in islets of Langerhans suggests that these cells might interfere with the autoimmune response locally (Laloux et al., 2002). Indeed, it was recently shown that NKT cells can inhibit T cell differentiation into effectors cells in a model where naïve diabetogenic T cells from BDC2.5 transgenic Ca-deficient mice (Ca deficiency leads to absence of endogenous T cells) were transferred into NOD recipients containing or not (Vα14Ca-/- or Ca-/mice, respectively) NKT cells. It was observed that expansion and full differentiation of the anti-β T cells into Th1 effector lymphocytes was inhibited by NKT cells, thus, preventing islet destruction (Beaudoin et al., 2002). NKT cells however, could not prevent diabetes when autoagressive BDC2.5 T lymphocytes are fully differentiated prior to transfer. Further studies where separation between BDC2.5 T cells and NKT cells was performed in transwell cell cultures revealed that inhibition of the diabetogenic T cells required cell-to-cell contact (Novak et al., 2005). Cytokine blockage by
antibodies as well as knockout mice had virtually no effect showing that cytokines, even IL-4, in this system play little role in diabetes protection (Novak et al., 2005). NKT cells were also reported to recruit tolerogenic dendritic cells into pancreatic lymph nodes, and to locally control the frequency and function of the dendritic cell subsets (Chen et al., 2005; Naumov et al., 2001). α-GalCer stimulation was observed to lead to an increase in the numbers of tolerogenic myeloid DC in pancreatic lymph nodes and transfer of DC from pancreatic lymph nodes protected recipient mice from type 1 diabetes (Naumov et al., 2001). Recent studies have suggested that accumulation and maturation of dendritic cells in pancreatic lymph nodes leads to recruitment and tolerance of effector T cells with a consequent decrease in diabetes development (Chen et al., 2005). In humans, deficiencies in NKT cell numbers have been reported to occur in diabetic patients in some studies but these results are somewhat controversial (Kukreja et al., 2002; Lee et al., 2002; Oikawa et al., 2002; Wilson et al., 1998). Discrepancies between studies can be attributed to differences in control and patient populations, different methods of detection of NKT cells, and problems related to comparing analyses of peripheral blood of humans to analysis of lymphoid tissues in mice. In fact, even though NKT cell numbers are deficient in several organs, their numbers are normal or even increased in the blood of NOD mice compared to other mouse strains (Berzins et al., 2004).

NKT cells have been observed to mediate protection in other autoimmune diseases, namely in experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS) caused by immunizing susceptible rodents with myelin-derived antigens (Furlan et al., 2003; Jahng et al., 2001; Singh et al., 2001). Results were nevertheless controversial with α-GalCer stimulation being reported to have different consequences in EAE development, raging from protection to acceleration of the disease (Jahng et al., 2001; Miyamoto et al., 2001; Pal et al., 2001; Singh et al., 2001). Discrepancies are probably due to use of different models, timing of experiments, or dose/route of α-GalCer administration (Furlan et al., 2003; Jahng et al., 2001). Protection however has been associated with a shift towards the Th2 type of response to central nervous system (CNS) antigens, while exacerbation of the disease has been related to an opposing effect (Jahng et al., 2001; Pal et al., 2001). The role of IL-4 and INF-γ in mediating protection or contributing to disease is somewhat obscure (Godfrey and Kronenberg, 2004). In Vα14-Jα18 transgenic mice, enhanced numbers of NKT cells lead to an impaired EAE induction without exogenous NKT cell stimulation and protection was associated with a reduction of INF-γ production by autoreactive effector T cells (Mars et al., 2002). In MS patients, decrease in Vα24-Jα18 mRNA in peripheral blood was reported
and NKT cell lines from patients in remission showed a Th2 cytokine bias compared to similar cell lines from patients in relapses, suggesting a regulatory function for NKT cells (Araki et al., 2003).

NKT cells also seem to play a role in regulating Systemic lupus erythematosos (SLE), though in some experimental models they have been shown to exacerbate disease. CD1d deficiency was associated with exacerbated SLE in a hydrocarbon oil-induced model and more skin disease in the lupus-prone MRL lpr/lpr mice (Yang et al., 2004; Yang et al., 2003b). An earlier study failed to concur with such observations, probably due to genetic contamination during backcrossing (Chan et al., 2001). Repeated α-GalCer stimulation was observed to ameliorate dermatitis in MRL lpr/lpr mice, but no effect on lupus nephritis was observed (Yang et al., 2003a). However, NKT cells were shown to promote autoimmunity in the SLE mouse model (NZBxNZW)F1 mice (Zeng et al., 2000; Zeng et al., 2003). Increases in NKT cell numbers were observed after disease onset and in vivo treatment with anti-CD1d antibody had beneficial effects in this model (Godfrey and Kronenberg, 2004; Zeng et al., 2000; Zeng et al., 2003).

Adding to the dual role of NKT cells in autoimmunity, they were shown to be partially protective in a model of colitis induced by dextran sodium sulfate, but in a hapten oxazalone induced colitis model where disease occurs due to Th2 type of responses, NKT cells and particularly IL-13 secretion were required for disease development (Heller et al., 2002; Saubermann et al., 2000).

**NKT cells in anti-tumor immune responses**

CD1d-restricted NKT cells are also involved in antitumor immune responses (Swann et al., 2004). Evidence exists pointing to a function of NKT cells in recruiting and promoting a response by downstream effectors in an INF-γ dependent manner, rather than being involved in direct killing of tumor cells. α-GalCer may enhance both natural killer (NK) and cytotoxic T lymphocyte (CTL) activity and both effector cells have been implied in α-GalCer-induced tumor immunity. Also, NKT cells may boost tumor immunity by promoting antigen presenting cell (APC) activation and IL-12 production. In humans, deficiencies in NKT cell number or function have been reported in some types of cancer. α-GalCer stimulation can kill or inhibit growth of tumor cells in vitro and activate NK cells through an IL-12 dependent mechanism (Godfrey and Kronenberg, 2004).
NKT cells in immunity against infectious diseases

NKT cells also contribute to immunity against infections caused by bacteria, viruses and protozoan parasites using different mechanisms including bystander activation of other lineages such as NK cells, the regulation of Th1/Th2 subsets, direct cytotoxic activity, and help for antibody formation against lapidated antigens. Recruitment to inflammation sites is likely to be driven by cytokines such as tumor necrosis factor (TNF), while activation probably occurs through TCR recognition of CD1d-presented autologous glycolipids. In some cases, CD1d-restricted NKT cells can have detrimental effects and contribute to pathogenesis. A role for background genes have been suggested in modulating such responses (Godfrey and Kronenberg, 2004; Hansen and Schofield, 2004).

NKT cells in allergic responses

Interestingly, recent studies have implicated NKT cells as playing an effector role in airway hypersensitivity. In mice, this type of response can be induced by sensitization with antigen followed by intranasal antigen challenge. Ovalbulmin (OVA)-immunized B6 Jα18/-/- mice that lacked the classical NKT cells exhibited impaired airway hypersensitivity and airway eosinophilia, decreased IL-4 and IL-5 production in bronchoalveolar lavage fluid and reduced OVA specific IgE compared with wild type mice littermates. Adoptive transfer of Vα14-Jα18 NKT cells fully reconstituted allergic asthma in these mice, and anti-CD1d antibody treatment blocked NKT amplification of eosinophil recruitment to airways as well as Th2 cytokine and IgE production after OVA challenge (Lisbonne et al., 2003). In another report, airway hypersensitivity did not develop in either Balb/c CD1d/-/- and Balb/c Jα18/-/- mice that lack NKT cells. Moreover, when Vα14-Jα18 NKT cells from wild-type mice but not from IL-4 or IL-13 knockout mice were adoptively transferred into Jα18/-/- mice, the response was restored (Akbari et al., 2003). Another study using a common environmental allergen, ragweed (RW), revealed that CD1d/-/- mice have significantly lower pulmonary eosinophilia and bronchial mucus production following RW sensitization and local challenge when compared to wild-type Balb/c mice. In the CD1d/-/- mice, the reduction of allergic reaction was associated with an impaired allergen-driven IL-4 production by spleen and draining lymph node cells and reduced eotaxin production in the lung. In addition, stimulation with α-GalCer enhanced airway eosinophilia, IL-4 secretion and eotaxin production in wild-type but not in CD1d/-/- mice (Bilenki et al., 2004). The role of Th2 cytokines in atopic diseases was highlighted in a recent study where overproduction of Th2 cytokines by transgenic introgression of a 120kb region on mouse chromosome 11 where several cytokine genes are clustered, lead to spontaneous atopic dermatitis and airway inflammation reminiscent of an asthmatic response against environmental allergens (Lee
and Flavell, 2004). In a physiological response to an airway allergen, activation of NKT cells and consequent secretion of IL-4 and IL-13 cytokines appeared to be necessary for asthma induction by promoting and enhancing the action of Th2-biased T cells in the lungs. In humans however, the role of NKT cells in allergy deserves further investigation. One study failed to observe differences in NKT cell numbers between atopic and non-topic individuals but then again, assessment of cell numbers was performed in peripheral blood that might not exactly reflect the situation in other tissues (Prell et al., 2003).

In contact hypersensitivity, NKT cells through IL-4 secretion were shown to lead to a cascade that involves activation of the B-1 type of B cells and mast cells which resulted in vascular permeability and recruitment of effector T cells to the site in the skin where the antigen was located (Campos et al., 2003). NKT cells were also observed to exacerbate arteriosclerosis though the exact mechanism of action is not well defined (Tupin et al., 2004).

Both Regulatory FOXP3 expressing CD4⁺CD25⁺ T cells and NKT cells constitute unique lineages of T cells that are positively selected for self-reactivity in the thymus and play immunoregulatory roles in several situations. However, while the CD4⁺CD25⁺ regulatory T cells generally have suppressive actions with down-regulation of immune responses, NKT cells were observed to promote an immune response in several situations that could be either beneficial or detrimental. NKT cells thus seem to be key players in different set ups of immune responses, being able to interact with a wide range of cells, including other regulatory T cells, from both innate and adaptive immune systems either directly or through cytokine production.
AIMS OF THIS STUDY

The overall aim of this thesis was to try to understand the mechanisms leading to the break of
tolerance underlying type 1 diabetes in the NOD mouse model. More particularly, we have
addressed two main questions:

1- What is the genetic factor in the Idd6 susceptibility locus that is contributing to
type 1 diabetes?
2- What is the role of non-classical NKT cells in autoimmune diabetes development?
METHODOLOGY

Genetic mapping and quantitative trait loci (QTL) analysis

Analysis of a complex trait involves choosing phenotypically distinct parental strains. Genetic mapping studies can be performed in second-generation cohorts such as F2 and backcross, where the phenotype will reflect the genetic reassortment of the two parental strains. Genetic markers for the mouse genome that differ between the two strains can then be used to genotype the F2 or backcross generation and be analyzed for co-segregation with the phenotype.

Some phenotypes have a quantitative rather than qualitative distribution, meaning that they cannot be sorted into distinct categories, but vary more or less continuously. While type 1 diabetes can be studied based on the presence or the absence of the disease, most immunological traits that have been analyzed in the NOD mouse present a quantitative distribution. Such traits can be studied under the assumption that the phenotype in a given individual is caused by the quantitative contribution of several loci, called quantitative trait loci (QTL). To identify associations between the genetic markers and the quantitative phenotype, the MAPMAKER/QTL software (Lander et al., 1987) was used. The identification of QTLs is based on methods of linear regression of the phenotypic values on the genotype, which allows inferring the variance explained by a given QTL (Paterson et al., 1988). The chromosomal location of the QTL can be estimated using the interval-mapping method, that calculates association of the trait to loci located between genetic markers. The maximum likelihood odds (LOD-score analysis) for a QTL at each point can then be estimated and plotted against a framework linkage map. This enables the generation of a probability curve along the chromosome, representing the statistical significance of the genetic association at each point (Lander and Botstein, 1989). QTL mapping requires a progeny in which the alleles controlling the trait segregate and the trait values display a normal distribution. Genetic analysis in F2 progenies permits detection of alleles that control the trait in recessive, dominant and additive fashions. Backcross generations however, only allow for detection of dominant alleles.
**Congenic mouse strains**

Congenic mouse strains are very useful to confirm and to narrow down the region of linkage obtained in genetic mapping experiments. Also, the contribution of the individual locus to the disease phenotype can be evaluated. To develop congenic mouse strains, the donor strain carrying the detected region contributing to protection from disease will be continuously backcrossed to the disease susceptible recipient strain. In this way the congenic mice will differ from the susceptible parental strain only in the particular region that is derived from the donor strain. Therefore the contribution of that region to disease protection can be assessed. The congenic region can be further narrowed down by backcrossing the congenic strain to the recipient strain, and subsequently screening for recombination within the inserted region.

**Transgenic animals**

Using transgenic techniques, the contribution of a particular genetic sequence implicated in disease can be evaluated within the specific genomic background of the animal. The generation of transgenic animals by standard methods can be briefly described in the following steps: Double-stranded DNA components of the transgene are combined enzymatically to yield a transgene expression cassette; the transgene cassettes are inserted into plasmid vectors and cloned; these transgene-bearing plasmids are transfected into cultured eukaryotic cells to evaluate expression of the transgene; plasmid-free transgene fragments are introduced directly into embryonic pronuclei by microinjection and manipulated embryos are placed in the reproductive tract of a pseudopregnant recipient mouse. Finally, the genomic DNA of live-born pups is analyzed for the presence of the transgenic DNA sequence.

**Real-Time PCR**

Real time reverse-transcriptase polymerase chain reaction (RT-PCR) is a technique widely used to quantify differences in messenger RNA (mRNA) expression. Compared to other methods that detect the final amplified product at the end-point, real-time PCR allows the reproducible determination of the initial amount of template (Freeman et al., 1999). In addition it requires small amounts of mRNA and can discriminate closely related mRNAs. The real-time PCR system is based on the detection and quantification of a fluorescent reporter (Livak et al., 1995). The signal increases in direct proportion to the amount of PCR product in a reaction. By recording the amount of fluorescence emitted in each cycle, it is possible to monitor the PCR reaction during exponential phase, where the first significant increase in the amount of PCR
product correlates to the initial amount of target template. The higher the starting copy number of nucleid acid target, the sooner a significant increase in fluorescence is observed. There are several fluorescence-monitoring systems for DNA-amplification. In this thesis TaqMan hydrolysis probes were used. This type of probes are oligonucleotides longer than the primers, containing a fluorescent dye usually on the 5’ base and a quenching dye typically on the 3’ base. While the probe is intact the quencher will absorb all the fluorescence emitted by the dye. TaqMan probes are designed to anneal to an internal region of a PCR product, in this case cDNA obtained by reverse-transcriptase-PCR. When the polymerase replicates the template on which the probe is bound, its 5’ exonuclease activity cleaves the probe which ends the activity of the quencher (Holland et al., 1991). The reporter dye will then start to emit fluorescence which increases in each cycle proportionally to the rate of probe cleavage. The assay uses universal thermal cycling parameters and PCR reaction conditions.

**Flow cytometry and cell sorting**

A powerful tool for defining and enumerating lymphocytes is the flow cytometer (FACS, fluorescent-activated cell sorter) which detects and counts individual cells passing in a stream through a laser beam. Individual cells within a mixed population are first tagged with specific monoclonal antibodies labeled with fluorescent dyes. Then the mixture of labeled cells is forced in a saline solution through a nozzle, creating a fine stream of liquid containing cells spaced singly at intervals. As each cell passes through a laser beam it scatters the laser light, and the dye molecules will be excited and will emit fluorescence. Photomultiplier tubes detect the scattering of light, which gives information on the size and granularity of the cell, and emissions from the different fluorescent dyes which gives information on the expression of specific proteins by each cell.

A flow cytometer can be equipped to sort the identified cells. In this cell sorter, the signals passed back to the computer are used to generate an electric charge, which is passed from the nozzle through the liquid stream at the precise time that the stream breaks into droplets, each containing a single cell. Droplets containing a charge can then be deflected from the main stream of droplets as they pass between plates of opposite charge, so that positively charged droplets are attracted to a negative charged plate, and vice-versa. In this way, specific subpopulations of cells, distinguished by the binding of the labeled antibody, can be purified from a mixed population of cells.
DISCUSSION

I-The genetic factor in the *Idd6* susceptibility locus (Papers I and II)

**Brief review on the subphenotype approach**

The identification of the biological functions underlying the diabetogenic effects of individual *Idd* loci has proven to be a challenging task. In our studies we have been following a subphenotype strategy to address the issue. The first step in this approach consists of identifying particular traits in the NOD mouse which could be hypothesized to relate to pathogenesis, and be considered as subphenotypes of diabetes. Next we analyze whether the genetic control of each subphenotype is correlated with the genetic control of type 1 diabetes. If a particular susceptibility locus correlates with the subphenotype, we hypothesize that the biological function mediated by the genetic factor in such loci might be linked to the trait under study.

One interesting immunological trait we have observed was the extended survival of NOD lymphocytes *in vitro*, when embryonic aggregation (EA) chimeras between NOD and C57BL/6 mice were constructed (Leijon et al., 1994). Subsequent experiments showed that immature DP NOD thymocytes were partially resistant to apoptosis induced by dexamethazone (a synthetic glucocorticoid) treatment (Leijon et al., 1994). Considering that DP cells are subject to selection events crucial for induction of central tolerance and that a function for glucocorticoids in T-cell development has been suggested (King et al., 1995; Sacedon et al., 2000), we reasoned that the phenotype could be contributing to pathogenesis by affecting thymic selection. A recent study using a conditional rat glucocorticoid receptor (GR) transgene specifically expressed in mouse T cells, showed that in adrenalectomize (to exclude systemic glucocorticoids) transgenic mice there is a dramatic increase in thymocyte death by apoptosis (Pazirandeh et al., 2005). The increased death was observed both *in vivo* and *in vitro* and could be reversed by the GR antagonist RU486. These results provided evidence for the presence of thymic-produced glucocorticoids, which were able to trigger thymocyte apoptosis in such system. Several other papers have indicated the presence or production of endogenous glucocorticoids in the thymus, most likely by thymic stromal cells (Jenkinson et al., 1999; Lechner et al., 2001a; Lechner et al., 2000; Pazirandeh et al., 1999; Vacchio and Ashwell, 1997; Vacchio et al., 1998; Vacchio et al., 1994). Independent signaling through the TCR or through the glucocorticoid receptor has been reported to induce apoptosis, while stimulation through both receptors may result in reduced cell death (King et al., 1995). These studies suggest that GR signaling can antagonize TCR signaling thereby inhibiting TCR-dependent thymocyte...
deletion. In support, GR antisense transgenic mice with reduced GR expression had reduced thymocyte numbers, suggesting that physiological levels of GR signaling are important for thymocyte survival in association with positive selection (King et al., 1995). According to this model, we could argue that the deficient NOD response to signaling through the glucocorticoid pathway should indirectly affect TCR-based selection. The role of glucocorticoids during thymic development is nevertheless a controversial subject. In another model, glucocorticoids have been suggested to mediate thymocyte apoptosis induced by T-cell activation and blockade of glucocorticoid signaling was observed to enhance survival (Brewer et al., 2002; Brewer et al., 2003). In contrast, other groups using GR knockout mice have failed to observe evidence for a role of glucocorticoids during thymocyte development and it was reported that T-cell development proceeds normally in the absence of GR signaling (Purton et al., 2000; Purton et al., 2002; Reichardt et al., 1998). In stress response, the increase in glucocorticoid levels clearly results in thymocyte apoptosis (Sapolsky et al., 2000). It is possible that under physiological conditions, glucocorticoids play a more subtle but nonetheless important role. Recently, glucocorticoids were shown to strongly activate the IL7-R-α-chain on T cells and endogenous glucocorticoids may play a regulatory role in this activation (Franchimont et al., 2002).

Genetic mapping provided a tool to test the relevance of the apoptosis resistance trait for type 1 diabetes. NOD and C57BL/6 (B6) mice were crossed and 110 animals from the resulting F2 generation were analyzed for resistance to dexamethazone induced apoptosis. The animals were then genotyped for DNA markers linked to previously identified Idd loci. Association was found between resistance to glucocorticoid induced apoptosis and the Idd6 susceptibility locus which validated our interest in studying this trait (Penha-Goncalves et al., 1995).

In paper I we have refined the resistance to apoptosis phenotype by following the kinetics of dexamethazone induced apoptosis in B6 and NOD mice. We observed a peak in thymocyte apoptosis at 12 h after treatment, which coincided with the biggest difference in apoptotic cell levels between NOD and the B6 mouse strain (Paper I, fig 1). Genetic mapping once again confirmed association between this phenotype and a genetic factor located within the Idd6 region. The strongest linkage with a maximum LOD score of 6.5 was obtained for markers D6Mit14 and D6Mit15 located on the distal part of chromosome 6 (Paper I, fig 2). This region quantitatively controlled 34% of the variance of the apoptosis resistance trait in the F2 progeny (53% was attributed to genetic factors), and the NOD allele controlled the trait in a dominant fashion.
Analysis of *Idd6* congenic mouse strains

In order to evaluate the contribution of the *Idd6* locus to diabetes and to confirm our mapping results we have developed three congenic mouse strains (Paper I, Table 1). First we have constructed a NOD.B6(A)-*Idd6* (N.B-*Idd6*) congenic mouse strain containing a 8cM B6-derived chromosomal segment telomeric to marker D6Mit291. A sub-congenic mouse strain, the NOD.B6(B)-*Idd6* (N.B-*Idd6*-15) was further developed carrying a 3cM B6-derived region telomeric to the marker D6Mit200. We have also constructed a congenic mouse strain on the B6 background, the B6.NOD-*Idd6* (B6.N-*Idd6*) congenic mouse strain containing an 8cM NOD-derived region telomeric to marker D6Mit291.

Analysis of diabetes development in the N.B-*Idd6* congenic mouse strain revealed a 22% reduction in diabetes frequency in females and a 50% reduction of the disease in males (Paper I, fig 3). Therefore we have localized a diabetes susceptibility locus within an 8cM region on chromosome 6, which includes the previously mapped *Idd6* locus (Ghosh et al., 1993).

In addition, analysis of the N.B-*Idd6*-15 congenic mouse strain demonstrated that the 3cM B6-derived region telomeric to marker D6Mit200 was sufficient to restore the apoptosis phenotype of NOD thymocytes. Conversely, the 8cM NOD-derived region carried by the B6.N-*Idd6* conferred a NOD-like phenotype to mice that otherwise have a B6 genetic background (Paper I, fig 4). Indeed, the apoptosis levels after dexamethazone treatment in N.B-*Idd6*-15 congenic mice were similar to the high levels observed in B6 mice, while the B6.N-*Idd6* mice showed a resistance to the apoptosis phenotype resembling what was detected in NOD. These results restricted the control of apoptosis resistance to a 3cM interval on distal chromosome 6 and indicated that this region could control the apoptosis phenotype independently of the genetic background.

In order to further refine the *Idd6* locus we have analyzed diabetes development in the N.B-*Idd6*-15 congenic mice. We have observed that the B6-derived 3cM region at the distal part of chromosome 6 is not sufficient to confer protection from diabetes (Paper II, fig 1). Similar to the wild-type littermate controls, 90% of N.B-*Idd6*-15 female and 44% of male mice developed diabetes before 45 weeks of age. This data excluded the 3cM chromosomal segment at the distal part of chromosome 6 from containing a direct contributor to type 1 diabetes. Consequently, the phenotype of resistance to apoptosis induction mapped to this region is not directly contributing susceptibility to diabetes. A limitation of the phenotype approach is the possibility that, as we observe herein, the gene(s) controlling the trait is closely linked to the susceptibility locus but does not constitute the etiological factor itself. Nevertheless, this analysis allowed for the restriction of the *Idd6* locus to the 5cM
region between markers D6Mit291 and D6Mit200. The possibility that a gene(s) located in the 3cM distal portion of chromosome 6 and controlling apoptosis induction might indirectly contribute to type 1 diabetes cannot be formally rejected. Although this most distal part of chromosome 6 so far has not been identified as an Idd by itself, interactions with a genetic factor(s) in the Idd6 locus may still be necessary for the protection from diabetes observed in N.B-Idd6 mice. Analysis of diabetes progression in a subcongenic mouse strain containing a 5cM congenic region we defined as containing the Idd6 locus and excluding the telomeric 3cM segment could provide the answer. In the literature several examples of subdivision and interaction between Idd loci have been described. The Idd1 locus has been divided into two loci, Iddl and Idd16 which in turn was observed to be constituted by at least two etiological factors (Babaya et al., 2002; Pomerleau et al., 2005). Moreover, two susceptibility loci were identified within each of the Idd4 and Idd13 regions (Grattan et al., 2002; Serreze et al., 1998a). In addition, the Idd5 as well as the Idd9 were demonstrated to contain 3 individual Idd loci (Fox et al., 2000; Hill et al., 2000; Lyons et al., 2000). Idd10 was also shown to contain two additional locus named Idd17 and Idd18 (Podolin et al., 1998; Podolin et al., 1997). Furthermore it is becoming evident that genes with similar function tend to group together in the genome. The Idd5 locus is a clear example, the Ctla4, Cd28 and Icos genes implied in regulation of T cell activation, map together in the 1.5cM region defined as the Idd5.1 locus (Wicker et al., 2004).

Several other Idd6 congenic strains have been described and it was reported that the Idd6 region from both B6 and C3H/Hej mouse strains partially protected from type 1 diabetes (Carnaud et al., 2001; Rogner et al., 2001). Interestingly, Rogner et al. 2001, by analyzing a set of Idd6 congenic strains between NOD and C3H/Hej reported that distal region of chromosome 6 defined by markers D6Mit113 and D6Mit304 mediates the protective effect of the Idd6 locus. Extrapolating from this data in combination with our results, we can hypothesize that the Idd6 locus is restricted to the 2cM region telomeric to the D6Mit113 and centromeric to the D6Mit200 marker. In the physical map as retrieved from the Celera database, this region corresponds to approximately 3Mbp (Paper II, table 1). Analysis of candidate genes in this region became a feasible task.
Candidate gene analysis

In the 3Mbp region corresponding to the Idd6 locus, 17 annotated genes were identified using the Celera database (Paper II, table 2). We decided to analyze the thymic expression of these genes in NOD and B6 mice using real-time PCR. From this experiment one gene, the Lrmp/Jaw1 gene encoding a lymphoid restricted membrane protein (Behrens et al., 1994; Snyder et al., 1997) showed significantly decreased expression in NOD compared to B6 mouse strains (Paper II, fig 2). Analysis of other mouse strains, namely C3H/Hej and Balb/c demonstrated that the reduced expression of this gene is specific to NOD mice (Paper II, fig 3). Moreover, when expression was analyzed in the Idd6 congenic mice, Lrmp mRNA levels were shown to be controlled by the Idd6 region itself (Paper II, fig 4). Indeed, the expression of Lrmp in N.B-Idd6 congenic mice was significantly higher than what was observed in NOD mice, while in the N.B-Idd6-15 mouse strain that excludes the Idd6 locus, levels of lrmp mRNA were similar to NOD. Conversely, reverse B6 congenic mice carrying a NOD-derived Idd6 region, displayed expression levels similar to NOD mice, indicating that the lower expression of Lrmp is not a result of the autoimmune inflammatory process of the NOD mouse strain. In addition, analysis of sorted thymic populations indicates that Lrmp expression is higher in the most immature DN and DP thymocyte populations (Paper II, fig 5). These results support the notion that lower Lrmp expression could be a susceptibility factor for type 1 diabetes.

Another gene, the 4930469P12Rik (P12Rik) gene, encoding a growth hormone-inducible soluble protein provisionally denominated as Ghiso (Manenti et al., 2004), showed a tendency for higher expression in B6 mice when compared to NOD (Paper II, fig 2). However, difference was not significant due to the high variation in gene expression observed within the B6 strain and suggests the influence of environmental factors. Furthermore, while the C3H/Hej mouse strain revealed higher expression of the gene, the Balb/c mouse strain had similar P12Rik mRNA expression levels to those of NOD (Paper II, fig 3). Although this gene cannot be excluded, we consider the Lrmp gene a more plausible candidate for the Idd6 locus.
The *Lrmp* gene and the *Idd6* locus

The *Idd6* locus overlaps with the pulmonary adenoma susceptibility locus 1 (*Pas1*) identified in crosses between A/J and B6 mouse strains (Manenti et al., 2004; Wang et al., 2003). Polymorphisms in the *Lrmp* gene were shown to co-segregate with mouse lung tumour susceptibility (Wang et al., 2003). Moreover, susceptible alleles in the *Pas1* locus and in particular a proline to leucine non-conservative variation in the *Lrmp* gene, were linked with acute inflammatory response (AIR) (Maria et al., 2003). A recent study has revealed specific NOD mutations in the *Lrmp* in addition to the polymorphisms identified in *Pas1* susceptibility mouse strains such as A/J and Balb/c (Grimm et al., 2003). Since we have observed that Balb/c shows higher expression levels of *Lrmp* in comparison to NOD, particular NOD mutations might be influencing gene expression in our study.

The *Lrmp* gene encodes a type II integral membrane protein resident in the endoplasmic reticulum and its coiled-coil domain is particularly conserved between mice and humans (Behrens et al., 1994). Expression of *Lrmp* has been observed in organs of the immune system and higher levels were detected in thymocytes as well as in pre-T and pre-B cell lines (Behrens et al., 1994). In agreement with our results, *Lrmp* gene expression seems to be down-regulated during differentiation of T and B cells, suggesting a possible role for the gene during T-cell development (Behrens et al., 1994).

Homology has been detected between *Lrmp* and two other genes, the *Irag* gene found in bovine smooth muscle and the mouse *Mrvil* gene. These genes have been implicated in modulation of intracellular calcium levels. The *Irag* gene encodes an inositol 1,4,5-triphosphate (IP3) receptor associated with cGMP kinase substrate, its also located in the endoplasmic reticulum and has a structure similar to *Lrmp* (Schlossmann et al., 2000). Phosphorilation of the IRAG protein by a cGMP dependent protein kinase Ib (cGKIb) leads to a decrease in the elevation of intracellular calcium levels which had been stimulated by IP3 (Ammendola et al., 2001; Schlossmann et al., 2000). The *Mrvil* gene is thought to represent the murine version of *Irag* (Shaughnessy et al., 1999). It is possible that *Lrmp* might have a similar function in modulating calcium levels. The endoplasmic reticulum is known to store and release intracellular calcium ions (Berridge, 1993; Clapham, 1995). Calcium (Ca$^{2+}$) is a very important intracellular cell messenger and intracellular waves of calcium have been implicated in modulation of activation and proliferation of T cells (Lewis, 2001). In addition, Ca$^{2+}$ has been implicated in apoptosis, and the anti-apoptotic Bcl-2
molecule was shown to be able to modify the Ca2+ handling properties of the endoplasmic reticulum (Berridge et al., 1998; He et al., 1997).

Recently, it was shown that the thymus is essential for the observed diabetes protection in the NOD.C3H 6.VIII congenic mouse, which contains a 5cM-C3H derived chromosomal segment at the telomeric end of chromosome 6 (Rogner et al., 2001). In addition, transfer experiments into immunodeficient NOD.Scid mice revealed that CD4+CD25+ regulatory T cells from NOD.C3H 6.VIII congenic mice were more efficient in preventing transfer of disease by diabetogenic spleenocytes than control NOD mice (Rogner et al. personal communication). Whether the effect in modulation of regulatory T cell activity is controlled by genes in the 3Mb region we have defined as corresponding to the Idd6 locus is yet unknown.

In conclusion, we have restricted the Idd6 locus to a 3Mbp pair region located between the markers D6Mit113 and D6Mit200 on chromosome 6, and suggested that the low expression of the Lrmp gene controlled by the Idd6 region could be contributing susceptibility to type 1 diabetes. It would be interesting to see whether the difference in expression we observed at mRNA level is also detected at the protein level. A more detailed analysis of polymorphisms in the Lrmp gene between NOD and other mouse strains could be helpful in determining mutations with a possible functional consequence. The direct contribution of the Lrmp gene to diabetes could only be accessed by construction of a knock-in NOD animal, where expression of Lrmp would be restored and its impact on diabetes progression analyzed.
II-The role of non-classical NKT cells (Papers III and IV)

Analysis of non-classical NKT cells in the context of autoimmune diabetes

Little is known about the role of non-classical NKT cells. Cardell’s group have established a TCR transgenic B6 mouse expressing TCR genes derived from a CD1d auto-reactive T cell hybridoma with the diverse-type TCR (Skold et al., 2000). This VIII24 hybridoma resulted from a CD4⁺ population in MHC class II-deficient mice using Vα3.2 and Vβ9 TCR rearrangements and reactive to endogenous CD1d on splenocytes and different CD1d-transfected cell lines (Cardell et al., 1995). The expression of the transgene in B6 mice directed the development of NKT cells that were mainly DN, CD44high, CD62low, CD122⁺ and NK1.1⁺ (Skold et al., 2000). A comparison of this transgenic animal expressing a diverse CD1d-restricted TCR with transgenic mice expressing the invariant Vα14-Jα18 rearrangements, revealed that non-classical NKT cells contrary to the invariant NKT cells expressed low CD69 levels but high CD49b and were low producers of IL-4 (Stenstrom et al., 2004). Most of the IL-4 secreting cells were found in the thymus, while CD49b expressing cells which secreted low levels of IL-4, higher levels of INF-γ and expressed a diverse TCR were most frequent in the spleen. In other studies, CD49b⁺CD4⁺ T or CD49b⁻NK1.1⁺ T cell populations were implied in the prevention of autoimmune diseases (Fritz and Zhao, 2001; Gonzalez et al., 2001). It is possible that these T cell populations include non-classical NKT cells that are contributing to immunoregulation, similar to what has been described for the invariant Vα14-Jα281 NKT cells.

To address the issue of whether the two different subsets of NKT cells have identical immunoregulatory roles we introduced the Vα3.2 and Vβ9 transgenic TCR into the NOD mouse and analyzed its impact on type 1 diabetes progression (Paper III). First we verified that the expression of the transgenic TCR lead to an increased presence of NKT cells. As is shown in figure 1 from paper III, transgenic cells were present mainly in the DN and also in the CD4⁺ T cell compartment in both the thymus and the spleen of the 24αβNOD transgenic mice. Furthermore, these cells had a surface phenotype of non-classical NKT cells, as can be seen by the expression of CD44, CD122, Ly49G2 and CD49b and low expression of the CD69 activation marker (Paper III, figure 2). These transgenic non-classical NKT cells where most abundant in the spleen and liver and interestingly they represented more than 20% of the T cells present in the pancreatic lymph nodes (Paper III, figure 3). The cytokine secretion analysis showed a low IL-4 and high INF-γ production profile, both in the thymus and in the spleen (Paper III, figure 4). Strikingly, the 24αβNOD transgenic animals
were almost completely protected from diabetes (Paper III, figure 5). Since only low secretion of IL-4 was detected, the protection from diabetes is not likely to be mediated through production of this cytokine and consequent shifting to a Th2 type of immune response. Recently, it was suggested that transgenic NOD Vα14-Jα281 NKT cells were able to prevent diabetes by direct cell contact with diabetogenic naïve T cells at the pancreatic lymph nodes, inhibiting their functional maturation into effector cells (Beaudoin et al., 2002; Novak et al., 2005). Since the 24αβNOD transgenic mice non-classical NKT cells were significantly represented in pancreatic lymph nodes, we can speculate that a similar mode of action can lead to protection from diabetes in this model. In addition, NOD NKT cells were shown to be defective in TCR-mediated and IL-12-induced activation and IFN-γ response, suggesting that IFN-γ secretion may be contributing to protection from diabetes (Falcone et al., 1999).

Analysis of insulitis in the 24αβNOD transgenic animals demonstrated the presence of autoaggressive cells in the pancreatic islets of Langerhans though the lymphocyte infiltration was overall less severe in comparison to what was observed in NOD mice (Paper III, figure 6). This indicates that the protective effect of these non-classical cells may be mediated at later stages in the autoimmune process. Additionally, we observed that transgenic non-classical NKT cells were able to inhibit the development of diabetes induced in NOD. Scid animals by transfer of diabetogenic spleen cells from a diabetic NOD mouse (Paper III, figure 7).

We demonstrated that non-classical NKT cells like the classical Vα14-Jα281 NKT can modulate autoimmunity. It remains unanswered whether the CD1d-restricted NKT cell subsets are equally efficient and whether they use the same mode of action to mediate this regulatory effect.
Non-classical NKT cells as effector cells mediating inflammation in an immunodeficient mouse model

The introduction of the Vα3.2 and Vβ9 transgenic TCR into a NOD.Rag-/- immunodeficient background, where mature T and B cells other than the transgenic NKT cells are absent, resulted in a peculiar phenotype characterized by an increase in size of the spleen and liver and signs of dermatitis in the ears (Paper IV, figure 1). NKT cells in these animals were present mainly in the DN compartment in both the thymus and the spleen (Paper IV, figure 2). In addition, expression of CD49b, Ly49G2, CD122 and CD25 seemed to be increased in T cells of the 24αβNODRag-/- mice while CD69 was barely detected (Paper IV, figure 3). However, when cytokine production was measured, increased secretion of both INF-γ and IL-4 was observed (Paper IV, figure 4). Interestingly a different cytokine secretion profile was detected in the 24αβNODRag-/- animals, where less cytokine seemed to be expressed per T cell. Moreover, both cytokines were produced by a non-T cell population, which indicated activation of the innate immune system. Histological analysis of the abnormal organs revealed extensive fibrosis in the spleen which correlated with increased numbers of mast cells concentrated in the fibrotic tissue (Paper IV, figure 5).

Mast cells have been demonstrated to stimulate fibrosis through secretion of fibrogenic cytokines as well as by proteases which are chemotatic for fibroblasts and able to stimulate matrix metalloproteins (Cairns and Walls, 1997; Gruber, 2003; Johnson et al., 1998; Li and Baek, 2002; Lindstedt et al., 2001). Moreover, mast cells also produce a large range of cytokines including IL-4 (Mekori, 2004). It is possible that NKT cells, directly or through cytokine production, stimulate secretion of IL-4 by mast cells. Consistent with stimulation of an innate response, increased presence of granulocytes and particularly eosinophils, was also detected (Paper IV, figure 5). Interestingly, extended extra-medullary hematopoiesis was observed, both in the NODRag-/- control animals and 24αβNODRag-/-Tg mice.

In the liver, the organ where non-classical NKT cells are most abundant, increased size correlated with leukocyte infiltration around the hepatic central veins where increased fibrosis and mast cell concentration could also be observed (Paper IV, figure 6). In the leukocyte infiltrates, large numbers of granulocytes and again eosinophils were observed. Non-classical NKT cells were shown to be mediating liver injury in response to transgenic expression of partial copies of the hepatitis B virus genome in the liver (Baron et al., 2002). These NKT cells were not reactive to α-GalCer tetramers, produced both IL-4 and INF-γ and did not express the Vα14 TCR rearrangement. Furthermore, non-
classical NKT cells were observed to be present in the liver in significant numbers in wild type mice (Baron et al., 2002). In a model consistent with the autoreactivity observed for these cells, it was suggested that expression of the hepatitis B virus could lead to enhanced presentation of endogenous glycolipids by CD1d, which might result in activation of NKT cells (Baron et al., 2002; Bendelac et al., 1997).

The presence of the transgenic NKT cells on the NOD immunodeficient background seemed to stimulate the innate immune system and induce an inflammatory process with some similarities to a Th2 type of response. Histological analysis of the ears of these mice also demonstrated concentrations of leukocytes in the dermis and an enlarged presence of mast cells (Paper IV, figure 7). Cutaneous inflammation associated with mast cell recruitment has been observed to be induced by an unidentified population of αβ TCR cells and regulated by TCRγδ dendritic epidermal cells (Girardi et al., 2002). This inflammatory reaction was observed in TCRγδ KO mice on the NOD background but not on the B6 background (Girardi et al., 2002). We can therefore speculate that NKT cells might act as effector T cells, mediating skin inflammation. In this context, NKT cells in our Rag-/- transgenic mouse model might localize to the ears, where due to the absence of regulatory γδT cells they induce dermatitis. This cutaneous dermatitis could occur as result of the deficient function of NKT cells on the NOD background, which would be prone to induce an inflammatory response. In support, introduction of a Vα14-Vβ8.2 TCR transgene into B6.Rag-/- animals lead to an increase in classical NKT cells, without any report of other abnormalities (Kawano et al., 1997; Taniguchi et al., 1996). It remains to be addressed whether the phenotype we observed in the 24αβNOD.Rag-/- transgenic mouse is due to this particular transgenic NKT population. Introgression of the transgenic non-classical NKT cells in an immunodeficient B6.Rag-/- animal would provide answers to these questions.

Furthermore, NKT cells have been implicated as effector cells in different murine models of induced airway hyperreactivity (Akbari et al., 2003; Bilenki et al., 2004; Lisbonne et al., 2003). This effect was associated with the secretion of both IL-4 and IL-13. In addition, NOD mice in comparison to allergy prone Balb/c mice developed a stronger Th2-mediated pulmonary inflammatory response, with eosinophil recruitment, after allergen sensitization (Araujo et al., 2004). This inflammatory reaction was ameliorated in CD1d -/- NOD mice (Araujo et al., 2004).
Interestingly, an immunodeficient syndrome in humans, named the Omenn syndrome has been describe to relate to mutations in both Rag-1 and Rag-2 genes which retain some recombination activity (Omenn, 1965; Villa et al., 1998). In patients suffering from Omenn syndrome the presence of activated oligoclonal T cells using a highly restricted TCR repertoire and presenting a skewed Th2 phenotype leads to generalized, exudative erythrodermia, lymphoadenopathy, hepato and splenomegaly as well as hypeeosinophilia and elevated serum IgE (Santagata et al., 2000). The similarities between the human syndrome and the phenotype we observed in our 24αβNOD.Rag-/- mice substantiate our interest in understanding the mechanisms leading to pathogenesis in this model.

NKT cells have been demonstrated to interact with several other cells of the immune system, including dendritic cells and other regulatory T cell populations (Azuma et al., 2003; Chen et al., 2005; Naumov et al., 2001; Nishikawa et al., 2003; Santagata et al., 2000). The 24αβNOD.Rag-/- mouse model supports the notion that interactions with other T cell populations might be required to modulate the function of NKT cells. Recent studies have revealed that in a model of type 1 diabetes induced by transfer of hemagglutinin (HA)-specific CD8 T cells into recipient control or Vα14 transgenic mice expressing HA under the control of the rat insulin promoter (Ins-HA), elevated frequencies of the classical NKT cells lead to exacerbation of disease (Griseri et al., 2005). Priming of NKT cells in this case, occurred in the popliteal lymph nodes in the presence of their specific antigen and CFA. The prodiabetogenic effects of NKT cells in this model were associated with increased Th1 response to self-antigens resulting in activation, proliferation and differentiation of the CD8 transgenic T cells into cytotoxic cells. The ambivalent role of NKT cells in type 1 diabetes as well as in other autoimmune situations indicates that these cells might interact in singular ways with distinct lymphocyte subsets according to the immunological context of the response. Indeed, different cell types express particular CD1d isoforms and may present distinct glycolipids to different CD1d-reactive T cells, which may have dissimilar roles according to their anatomical location (Behar et al., 1999; Durante-Mangoni et al., 2004; Exley et al., 2000; Porcelli and Modlin, 1999).

These observations strengthen the concept that NKT cells are not regulatory T cells per se, but constitute an “intermediary” cell type that is able to elicit the innate immune system to mount an inflammatory immune response but also interact with the adaptive immune system both.
modulating the action of effector T cells in an autoimmune context and, possibly, being controlled back by encounters with other lymphocytes. Non-classical NKT cells might have a similar role to that of classical NKT cells, being possibly distinct in tissue distribution and range of glycolipids recognized.
CONCLUDING REMARKS

The contribution of the non-MHC loci to type 1 diabetes susceptibility remains largely elusive. A promising strategy to address this issue is through the combination of immunological phenotype analysis with candidate genes analysis in the Idd regions. Construction of congenic mice constitutes a powerful tool to define the locus and assess its impact on disease progression.

In this thesis we have restricted the Idd6 to a 3Mb region downstream of the D6Mit113 and upstream of the D6Mit200 marker. From analysis of candidate genes in this region we propose that low expression of the lymphoid restricted membrane protein gene (Lrmp) observed in NOD mice is controlled by the Idd6 locus and constitutes a susceptibility factor for type 1 diabetes. We suggest that the Lrmp gene might play a role during T cell development, perhaps by modulating intracellular calcium signaling. We have also shown that resistance to dexamethasone-induced apoptosis of NOD thymocytes is controlled by a gene(s) localized in a 3cM region down of the Idd6 locus. Though the phenotype is not directly influencing diabetes, an indirect contribution is possible through interactions with a gene(s) in the Idd6 region.

Different T cell populations have been implicated in the regulation of type 1 diabetes. NKT cells, which are numerically and functionally deficient in NOD mice have also been shown to be able to restore protection from disease. The CD1d-restricted NKT cell population, however, constitutes a heterogeneous population. The role of non-classical NKT cells expressing a diverse type of TCR is to date largely unknown.

In this thesis we have shown that non-classical CD1d-restricted NKT cells can mediate protection from type 1 diabetes through intervention in the later stages of disease. We have also observed that when introduced in an immunodeficient NOD.Rag-/- mouse model, these cells promoted spontaneous inflammation with eosinophil and mast cell recruitment and extensive fibrosis in the spleen and liver, as well as dermatitis in the ears. These observations indicate that non-classical NKT cells are not only able to modulate the action of effector T cells in an autoimmune situation, but are also able to stimulate the innate immune system to mount an inflammatory immune response. The pathogenesis of this inflammatory response suggests that interactions with other lymphocytes are necessary to modulate the activity of NKT cells. The 24αβNODRag-/- mice provide a good animal model for the study of interactions of NKT cells with both innate and adaptive components of the immune system.
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Fim/The end
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