Genetic Loci Contributing to Spontaneous Autoimmune Diabetes

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GENETIC LOCI CONTRIBUTING TO SPONTANEOUS AUTOIMMUNE DIABETES

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Abstract

Background and Aims: Spontaneous type 1 diabetes (T1D) in the BioBreeding (BB) rat mimics human T1D as the rats experience weight loss, polydipsia, polyuria, ketoacidosis, onset during puberty and insulin-dependency within a day after diagnosis. Because the DP rat develops T1D spontaneously, it is a prime laboratory animal for dissecting the genetics of T1D susceptibility without the need for external manipulation. The BB rat is comprised of two separate substrains; the diabetes prone (BBDP) and the diabetes resistant (BBDR). Failure to express the *Gimap5* protein is associated with lymphopenia (*lyp*) and linked to T1D in the BBDP rat. In an intercross between F1(BBDP x F344) rats we identified a rat with a recombination event on rat chromosome (RNO) 4, allowing us to fix 34Mb of F344 between D4Rat253 and D4Rhw6 in the congenic DR.*lyp* rat line with two Mb of BBDP DNA, encompassing the *Gimap5* mutation, introgressed on the DR genetic background. The aim of this thesis was to characterize the F344 DNA introgression, test the hypothesis that the introgression would result in 1) no effect on T1D development or 2) protection from T1D, generate congenic sublines and positionally clone and characterize the resulting candidate genes on rat RNO4.

Material and Methods: The F344 fragment in the DRF.$^{f/f}$ rat line was fixed onto the DR.*lyp* background in a total of nine backcross and seven intercross matings. To generate DRF.$^{f/f}$ congenic sublines, DRF.$^{f/f}$ rats were crossed to inbred BBDR or DR.$^{lyp/lyp}$ rats and the offspring genotyped, phenotyped for lymphopenia and monitored for T1D. Positional candidate genes were then subjected to coding sequence analysis, cDNA sequencing and/or quantitative real-time (qRT) PCR expression analysis.

Results: DRF.$^{f/f}$ rats, homozygous for the F344 allele, were lymphopenic but did not develop T1D while all (100%) DR.$^{lyp/lyp}$ rats developed T1D by 83 days of age. Generation of congenic sublines revealed that reduction of the DRF.$^{f/f}$ F344 DNA fragment by 26 Mb (42.52 Mb-68.51 Mb) retained complete T1D protection. Further dissection revealed that a 2 Mb interval of F344 DNA (67.41-70.17 Mb) (*Iddm38*) resulted in 47% protection while retaining <1 Mb of F344 DNA at the distal end (*Iddm39*) resulted in 28% protection, both of which significantly delayed onset. Comparative analysis of T1D frequency in the DRF.$^{f/f}$ congenic

sublines refined the *Iddm38* and *Iddm39* intervals to approximately 670 Kb between SNP SS105325016 and D4Rat26 and 340 Kb proximal to *Gimap5,* respectively. Coding sequence analysis revealed *TCR Vβ 8E, 12* and *13* as candidate genes in *Iddm38* and *Znf467* and *Atp6v0e2* in *Iddm39*. Quantitative RT-PCR analysis of whole organ as well as in FACS sorted thymocytes and peripheral T-cells stained with CD4 and CD8 monoclonal antibodies showed a reduction in expression of four out of five *Gimap* genes located within the *Iddm39* interval, in addition to *Gimap5,* in DR.$^{lyp/lyp}$ spleen and mesenteric lymph nodes (MLN) when compared to DR.$^{+/+}$.

Conclusions:

Our data demonstrates that introgression of a 34 Mb region of the F344 genome, proximal to the mutated *Gimap5* gene, renders the congenic DR.$^{lyp/lyp}$ rat T1D resistant despite being lymphopenic. Generation of congenic sublines revealed that spontaneous T1D in the BB rat is controlled, in part, by at least *two* genetic loci, *Iddm38* and *Iddm39*, in addition to the *Gimap5* mutation on RNO4. Coding sequence analysis revealed *TCR Vβ 8E, 12 and 13* as candidate genes in *Iddm38* and *Znf467* and *Atp6v0e2* as candidate genes in *Iddm39*. Quantitative RT-PCR expression analysis suggests that the lack of the Gimap5 protein in the DR.$^{lyp/lyp}$ congenic rat impairs expression of the entire *Gimap* gene family and regulates T-cell homeostasis in the peripheral lymphoid organs. The molecular identification and characterization of the genetic factors protecting from T1D in the DRF.$^{f/f}$ congenic rat line should prove critical to disclose the mechanisms by which T1D develops in the BB rat.

List of Publications Included in this Thesis

This thesis is based on the following four original papers, referred to in the text by their Roman numerals.

I. **J.M. Fuller**, A.E. Kwitek, T.J. Hawkins, D.H. Moralejo, W. Lu, T.D. Tupling, A.J. MacMurray, G. Borchardt, M. Hasinoff, Å. Lernmark**.** *Introgression of F344 Rat Genomic DNA on BB Rat Chromosome 4 Generates Diabetes-Resistant Lymphopenic BB Rats.* Copyright 2006 American Diabetes Association. From Diabetes®, Vol. 55, 2006; 3351-3357. Erratum in: Diabetes 56:549, 2007. Reprinted with permission from The American Diabetes Association.

II. **J.M. Fuller**, M. Bogdani, T.D. Tupling, R.A. Jensen, R. Pefley, S. Manavi, L. Cort, E.P. Blankenhorn, J.P. Mordes, Å. Lernmark, A.E. Kwitek. *Genetic Dissection Reveals Diabetes Loci Proximal to the Gimap5 Lymphopenia Gene.* Physiological Genomics 10:89-97, 2009. Used with permission.

III. E.A. Rutledge, **J.M. Fuller**, B. Van Yserloo, D.H. Moralejo, R.A. Ettinger, P. Gaur, J.L. Hoehna, M.R. Peterson, R. Jensen, A.E. Kwitek, Å. Lernmark. *Sequence Variation and Expression of the Gimap Gene Family in the BB rat.* Experimental Diabetes Research 2009:835650. Used with permission.

IV. D.H. Moralejo, **J.M. Fuller**, E.A. Rutledge, B. Van Yserloo, R.A. Ettinger, R.A. Jensen, A. Kwitek and Å. Lernmark. *BB rat Gimap Gene Expression in Sorted Lymphoid T and B Cells.* (Submitted manuscript)

Note: *During the period in which this thesis was developed I contributed to a number of other publications related to the subject or methods used in this investigation. The references to these works are provided in the appendix.*

List of Abbreviations

BB	BioBreeding rat
BBDP	Diabetes prone BioBreeding rat
BBDR	Diabetes resistant BioBreeding rat
DN	Double negative
DP	Double positive
F344	Fischer rat
Gimap	GTPase immune associated protein
GTP	Guanosine tri-phosphate
IL	Interleukin
ILR	Interleukin receptor
INF	Interferon
IRF	interferon regulatory factor
KRV	Kilham rat virus
lyp	lymphopenia
MHC	Major histocompatibility complex
MLN	Mesenteric lymph node
PAC	P1-derived artificial chromosome
PCR	Polymerase chain reaction
QTL	Quantitative trait loci
RNO	Rat chromosome
SNP	Single nucleotide polymorphism
SP	Single positive
SSLP	Simple sequence length polymorphism
STAT	Signal transducer and activator of transcription
STZ	Streptozotocin
T1D	Type 1 diabetes
TCR Vβ	T-cell receptor variable beta
TLR	Toll-like receptor
T_{reg}	Regulatory T-cell
WF	Wistar Furth

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1 Introduction

Spontaneous autoimmune type 1 diabetes mellitus (T1D) in the BioBreeding (BB) diabetes prone (DP) rat mimics human T1D as the rats experience weight loss, polydipsia, polyuria, ketoacidosis and onset during puberty. As in human T1D, islets are infiltrated by mononuclear cells at the time of clinical onset and insulitis is associated with a rapid onset of hyperglycemia due to a complete loss of islet β-cells (for a review see [1]) and the development of complete insulin-dependency within a few days after diagnosis [2]. In human T1D, a number of critical steps in the disease process need to be clarified. For example, the genetic etiology is not fully understood. While HLA is the major susceptibility factor, other genetic factors or loci have been identified through linkage (sib-pair) [3] or association (case-control) [4, 5] approaches. In addition, whether environmental etiological factors, such as viruses, are able to trigger the islet autoimmunity that precedes clinical diagnosis is still a matter of debate [6, 7].

Because the BBDP rat develops T1D spontaneously, it is a prime laboratory animal for dissection of the genetic physiology of T1D susceptibility and pathology as well as to study physiological responses and phenotypes without the need for external manipulation. The mechanisms by which the BB rat develops T1D are not fully understood. Autoimmune phenomena associated with the rapid onset of hyperglycemia include the appearance of insulitis, transient development of islet autoantibodies and the ability to prevent or induce T1D in the BBDP rat as well as related diabetes resistant (BBDR) rats has been well documented during the past 25 years. Prior to clinical onset, there are not only immune but also fundamental metabolic abnormalities, including altered carbohydrate and lipid metabolism that may contribute to β-cell death and induction of autoimmune β-cell destruction [8, 9]. Identifying the genetic factors associated with disease development in the BB rat will aid in the identification of signaling pathways of importance to the etiology and pathogenesis of T1D in humans.

1.1 Insulitis

As in human T1D, BBDP rat islets are infiltrated by mononuclear cells (insulitis) leading to a rapid and destructive loss of islet β-cells. Insulitis, accompanied by T-cell infiltration and the presence of eosinophils [10-12] as well as other immune cells, precedes clinical onset. In early studies of BBDP rats, insulitis could be detected as early as four weeks of age [13]. As is the case in humans, there is little or no peri-insulitis prior to the clinical onset [14]. Our breeding program to yield the present DR.$^{lyp/lyp}$ congenic line of BB rats [15], with 2 Mb of BBDP DNA introgressed onto the BBDR genetic background, has resulted in an animal which develops insulitis the day of onset or just before and no longer develops islet autoantibodies [16], as has been described in outbred, heterogeneous colonies of BB rats [17]. In this rapid onset form of T1D, there appears to be little time for antibodies to be formed or they appear only around the time of diagnosis [18].

Four stages of insulitis have been defined. The first shows infiltration of macrophages to the pancreas which can present as low-grade vasculitis, ductilitis or both. Stage two reveals the first appearance of lymphocytic infiltration surrounding the islets, comprised mostly of major histocompatibility complex (MHC) class II expressing macrophages [19]. Stage two is accompanied an islet upregulation of MHC class I expression. Stage three shows further macrophage infiltration with T-cell and NK-cell recruitment into the islets without change in islet morphology. Islets also begin to show an upregulation of MHC class II antigens. The fourth, or end-stage, reveals B-cell recruitment accompanied by distorted β-cell morphology or islets devoid of β-cells [19, 20]. At clinical onset, the majority of β-cells will present at stages three and four with invading lymphocytes largely expressing MHC class II antigens and interleukin receptor 2 (ILR2) [21].

1.2 Clinical Onset

As in human T1D, onset of T1D in the BBDP rat is accompanied by polydipsia, polyuria, glycosuria, hyperglycemia, ketoacidosis and onset during puberty [13]. DR.$^{lyp/lyp}$ rats gain body weight similar to DR.$^{+/+}$ littermates from <40 days of age up until the 1-2 days preceding the day of T1D onset. Two days preceding clinical onset, DR.$^{lyp/lyp}$ rats stop gaining weight followed by a return to normal weight gain the day proceeding the day of onset (Fig.1A). On

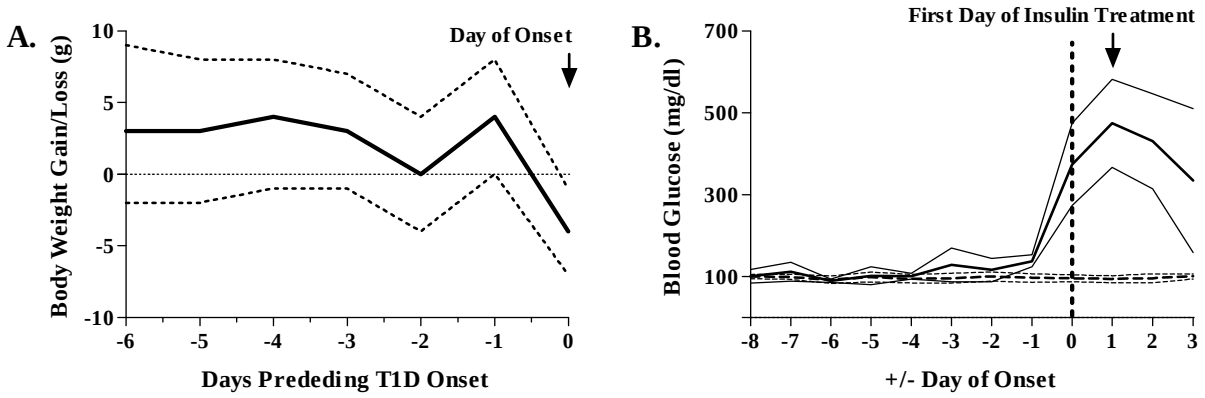

Figure 1. Panel A. *Growth Preceding T1D Onset.* DR.$^{lyp/lyp}$ rat (n=34) mean body weight gain or loss (g) ± SD in the six days leading up to onset of T1D. Males and Females were combined. **Panel B.** *Blood Glucose as a Diagnostic Measurement of T1D Onset.* Mean blood glucose readings ± SD leading up to and following the day of T1D onset are shown. Dashed lines indicate DR.$^{+/+}$ (n=8) rats and solid lines indicate DR.$^{lyp/lyp}$ (n=34). The day of onset (day 0) is indicated with a vertical dashed line. Males and females were combined.

the day of onset, both male and female DR.$^{lyp/lyp}$ rats lose between one and eleven grams of body weight accompanied by an overnight progression from normoglycemia to hyperglycemia (Fig.1B). DR.$^{lyp/lyp}$ rats will lose >20% of their body weight within 3-11 days of onset if not treated with insulin [2].

1.3 Diabetes Complications

As in humans, BBDP rats are prone to physiological complications associated with long term T1D and insulin dependence. BBDP rats develop diabetic polyneuropathy with impaired nerve fiber regeneration and central pontine myelinolysis, nephropathy combined with hepatic fatty changes, encephalopathy with hypoglycemic brain damage and vascular disease [22, 23]. In the exocrine pancreas, diabetic BBDP rats develop pancreatitis, eosinophilic infiltrates, granulomatous lesions and interstitial inflammation [24]. In addition, BBDP rats may be prone to stomach erosions, cataracts and testicular atrophy [23].

1.4 Diabetes Induction

1.4.1 Poly I:C and ART2.1 Monoclonal Antibodies

Administration of Poly I:C alone [25] or in conjunction with the T_{reg} depleting cytotoxic DS4.23 ART2.1 (formerly known as RT6) monoclonal antibody [26] induces T1D in BBDR rats as well as RT1.B/$D^{u/u}$ related rats [27-29]. Poly I:C, an activator of innate immunity, is a synthetic double stranded RNA that induces β-cell apoptosis through activation of the Toll-Like Receptor (TLR)-3 and Interferon Regulatory Factor (IRF)-3 signaling pathways [30]. Depleting ART2.1, a regulatory surface protein found on T-cells [31], essentially renders the BBDR rat lymphopenic like its related BBDP counterpart that is prone to spontaneous T1D development. Such treatment of RT1.B/$D^{u/u}$ related WF rats fails to induce disease [32] and points towards the existence of a genetic predisposition in the BB rat that can be phenotypically manifested upon immune perturbation. In crosses between WF and either BBDP or BBDR rats, a quantitative trait locus (QTL) important for induced T1D (*Iddm14*-previously designated *Iddm4*) was mapped to rat chromosome (RNO)-4 [27, 33].

1.4.2 Kilham Rat Virus

Kilham Rat Virus (KRV) is a small single stranded DNA parvovirus reported to induce T1D in BBDR rats treated with KRV alone as well as RT1.B/$D^{u/u}$ related LEW1.WR1 rats and PVG.RT1 rats in combination with Poly I:C [34-36]. As WF rats are resistant to induction of T1D by KRV, the pathogenesis is not RT1.B/$D^{u/u}$ dependant [35]. KRV induces T1D through activation of an innate immune response against β-cells. While the mechanism still needs to be elucidated, KRV has been shown to upregulate both the TLR9 and signal transducer and activator of transcription (STAT)-1 signaling pathways along with macrophage derived cytokine production leading to a proinflammatory immune response [37-39].

1.4.3 Streptozotocin

Multiple low doses of the naturally occurring antimicrobial agent streptozotocin (STZ), a glucose analog with a D-glucopyranose and methylnitrosourea group, induces T1D in multiple rat strains, including the BB rat [2, 40]. Strain dependant susceptibility to STZ

induced T1D appears to correlate with innate interleukin (IL)-2 and interferon (IFN)-γ production and is enhanced by depletion of ART2.1+ T-cells [40]. Streptozotocin is predominantly cytotoxic to pancreatic β-cells and its effect is elicited through transport into the β-cell by GLUT2 where it fragments DNA through generation of superoxide or hydroxyl radicals resulting in DNA degradation [41]. GLUT2 is a passive transmembrane glucose carrier protein located on β-cells that is essential for glucose uptake and glucose stimulated insulin secretion. In addition to DNA degradation, STZ also appears to preferentially decrease GLUT2 mRNA expression [42]. Interestingly, GLUT2 autoantibodies were reported in newly diagnosed T1D patients suggesting reduction or loss of glucose transport may participate in onset of disease [43].

1.5 Peripheral T-Cell Lymphopenia

Thymocyte maturation in the thymus and migration of mature T-cells to the periphery is accompanied by a series of changes in membrane marker expression. Briefly, CD4+CD8+ double positive (DP) thymocytes undergo positive and negative selection in the thymus to establish T-cells that will recognize harmful antigens without causing self-reactivity and autoimmunity. Once thymocytes leave the thymus they will undergo further phenotypic changes. Expression of Thy-1 will decrease whereas expression of ART2.1 and CD45 will increase. Early studies showed that both non-diabetic and acutely diabetic BBDP rats were immunodeficient in that the number of peripheral splenic and lymph node W3/13+ cells (T-lymphocytes), W3/25+ cells (helper CD4+ T-cells) and OX8+ cells (cytotoxic CD8+ T-cells) [44, 45]. Since then, virtually all strains of BBDP rats have been shown to be severely T-cell lymphopenic (the total T-cell count reduced by more than 85%), where the reduction of CD8+ T-cells is more profound than CD4+ T-cells [46, 47].

Studies suggested that lymphopenia in the BBDP rat was either caused by intrathymic T-cell apoptosis [48], impaired post-thymic development and /or a reduced lifespan of peripheral T-cells [49-51] While the lifespan of peripheral BBDP rat T-cells is decreased, increased mitotic activity in activated T-cells appears to compensate for decreased viability as the number of peripheral T-cells is not decreased even after thymic involution [50, 51]. It is now known that lymphopenia is caused by a frameshift mutation in the *Gimap5* (*Ian4, Ian4l1, Ian5*) gene [52, 53]. *Gimap5* belongs a family of GTPase Immune Associated protein (*Gimap*)

encoding genes predominately expressed in organs associated with immune tolerance such as thymus, spleen and lymph nodes [52, 54, 55]. The primary *Gimap5* defect is detected in the total number of peripheral CD4+ and CD8+ T-cells with the overall expression of *Gimap5* in DR.$^{lyp/lyp}$ rat thymocytes reduced when compared to DR.$^{+/+}$ with a normal copy of the gene [54]. *Gimap5* deficient T-cells exhibit an increased cell size and lower activation threshold via the NF-κb and MEK dependant pathways [56]. In addition, *Gimap5* deficient T-cells exhibit a "promiscuous" and/or "partial "activation before proceeding to apoptosis in the periphery [57, 58].

1.6 Metabolism

As T1D is a metabolic disorder, caused by dysregulation of the immune system, we investigated tissue temperatures of pre-diabetic DR.$^{lyp/lyp}$ rats longitudinally. The study revealed that though brain, brown adipose tissue and lower back temperatures were unchanged throughout the test period, DR.$^{lyp/lyp}$ rat core body temperature was decreased as early as nine days prior to T1D onset. When administered a β3-adrenergic agonist, DR.$^{lyp/lyp}$ rats showed an exaggerated response, suggesting that the rats are hypersensitive in response to adrenergic stimuli [8]. The reasons for this could be multiple; first, adrenergic input in the pre-diabetic DR.$^{lyp/lyp}$ rat could be insufficient to maintain the body temperature. Second, stress and inflammation have been reported to decrease sensitivity of β-adrenergic receptors [59, 60].

Calorimetric analyses of the DR.$^{lyp/lyp}$ rats further aides in understanding metabolic events preceding onset of T1D. Decreasing respiratory quotient suggests that lipid oxidation is favored over carbohydrate metabolism as the animal progresses towards hyperglycemia [61]. The DR.$^{lyp/lyp}$ rat pre-diabetic metabolic state has not been extensively studied but there are reports in mice and humans that indicate a role for liver metabolism in the disease progress. For instance, *Gimap5* knockout mice have been generated, showing a severe liver phenotype [62] and in humans, phoshatidylcoline metabolism is implicated as patients progress to overt T1D [63]. These findings provide a link between the autoimmune process and altered metabolism prior to hyperglycemia.

1.7 Mapping Disease Loci

As with most common human diseases, T1D in the BB rat is multifactorial, i.e. caused by a complex interaction between genetic factors and environmental stimuli. Inbred strains of multifactorial disease are generally developed by selective breeding, to enrich for the genes predisposing to disease. There are multiple methods of genetic mapping using animal crosses. The most common is the F1 backcross or the F2 intercross. By this method, one crosses one inbred strain with another to generate a first filial or F1 generation of offspring. All offspring from this cross are heterozygous at all places in the genome, having 50% of their genetic material from the maternal inbred strain and 50% from the paternal inbred strain. The F1 offspring are either backcrossed to the 'disease' strain or crossed with other F1 siblings. The offspring of the former cross would be either homozygous for the disease allele or heterozygous at any locus in the genome. The offspring of the F2 intercross could have three possible genotypes at any given locus, following Mendel's Laws of inheritance: 25% homozygous for the 'disease' strain, 50% heterozygous and 25% homozygous for the 'control' strain. These crosses have led to the identification of nearly 40 genomic loci in the BB rat that harbor disease susceptibility genes. Although several of these are overlapping and may actually encompass the same gene, it is also possible that multiple susceptibility genes are located within a single QTL.

Once a disease locus is identified, positional cloning efforts ensue to identify a disease susceptibility gene. Because complex disease is caused by combinations of multiple genes, the first approach often taken to go from locus to gene is to isolate a single locus in an alternate genomic background, or to generate consomic or congenic strains [64, 65]. A consomic strain is one where an entire chromosome containing a QTL is substituted from the susceptible strain to the resistant strain, or reciprocally, from the resistant strain onto the susceptible strain. A congenic strain involves the substitution of just the genomic interval encompassing the QTL. To generate either of these strains, one performs successive backcrosses combined with marker assisted breeding to ensure the donor allele is present for the entire chromosome or QTL interval in each backcross while the background genome is fixed for the recipient's genome. The donor fragment is then fixed by intercrossing to generate a new strain that differs from the parental strain by only a relatively small interval of the genome encompassing the disease locus of interest. Therefore, any phenotypic difference must be due to genetic

differences in the introgressed fragment. This provides excellent genetic control strains for physiological studies and provides the resource for gene identification by positional cloning.

Many crosses have been performed using the BB rat, as a disease strain, crossed to many different control or non-diabetic strains, leading to the identification of many mapped loci contributing to T1D susceptibility and time to onset of disease. Confirming previous reports, and similar to human and mouse, the MHC region (*Iddm1*) was among the first loci identified in linkage studies [66]. The MHC RT1.B/D$^{u/u}$ T1D susceptibility haplotype was identified in crosses of BB rats with both F344 and LEW rats. In addition to the RT1.B/D$^{u/u}$ haplotype, genome wide linkage analysis identified multiple QTL following crosses of inbred BB rats to WF rats (*Iddm11-14, Iddm20* and *Iddm24*) [29, 33, 67, 68], DA rats (*Iddm16*) [69] and SHR rats (*Iddm8-10*, *Iddm15* and *Iddm18*) [69-71]. These genetic factors therefore seem to be dependent on the rat strain used in the cross, i.e. influenced by a differing genomic background. To date, several of these loci have been captured in congenic strains and successful gene identification is forthcoming.

1.8 Genetic Predisposition

1.8.1 MHC RT1B.D$^{u/u}$ (*Iddm1)*

The foremost predictor of T1D predisposition in the BB rat as well as humans is the class II MHC haplotype RT1.B/D$^{u/u}$, an ortholog to human HLA-DQ/DR [72], located on RNO20. RT1.B/D$^{u/u}$ is also linked to development of thyroiditis [73-76] as well as spontaneous T1D development in the KDP and LEW.1.AR1/Ztm-*Iddm* rat strains. The MHC gene region itself is highly conserved between species [72, 77]. Class II MHC proteins, expressed on the surface of antigen presenting cells such as macrophages, dendritic cells and B-cells, mediate self and non-self discrimination by presenting short polypeptides to CD4+ T-cells (T helper). The RT1.B/D$^{u/u}$ haplotype is not unique to the BB rat but shared by other rat strains including WF, LOU, AO and WAG [72] and is thus necessary but not sufficient for T1D development.

1.8.2 *Gimap5* (*Iddm2*).

Peripheral T-cell lymphopenia (<15% normal T-cell count, with low representation of CD5+, CD4+, CD8+, and ART2.1+ subsets) in the BBDP rat is linked to a single nucleotide deletion in the *Gimap5* gene that results in a premature stop codon and truncation of the full-length protein [52, 53]. Analysis of protein expression suggests that the *Gimap5* mutation is a null allele since there is no evidence that even a truncated protein is made [52, 78]. *Gimap5* belongs to a family of at least seven *Gimap* encoding genes, characterized by GTP binding motif (AIG1) domains [55], all of which are differentially expressed in DR.$^{+/+}$ and DR.$^{lyp/lyp}$ T and B lymphocytes, as well as macrophages and dendritic cells [47, 53, 79, 80]. *Gimap5* contains a predicted coiled-coil and transmembrane domains and has been shown to localize to the endoplasmic reticulum, Golgi and mitochondrial membrane [55, 81-85]. The truncation results in loss of both the AIG1 and putative transmembrane domains. Seven *Gimap5* mRNA's have been characterized encoding two distinct proteins with up to eleven potential transcription start sites and putative YY1, Sp1 and MED-1 (TATA-less) promoters [86]. Interestingly, two of the seven mRNA's link *Gimap5* and the first two 3' untranslated exons of *Gimap1* into one full length mRNA.

The positional cloning and subsequent identification of the *Gimap5* gene on RNO4 was in part established through generation of the DR.*lyp* congenic rat line along with recombination events following our method of marker assisted breeding of BBDP with F344 rats. [52, 87]. Analysis of the *lyp* phenotype in a F344 DNA recombinant rat made it possible to define the *lyp* critical interval as a region of approximately 33 Kb between D4Rhw6 (76.83 Mb) and IIsnp3 (77.16 Mb) containing *Gimap1*, *Gimap5*, and *Gimap3* (formerly known as *Ian2*, *Ian5*, and *Ian4,* respectively) [52, 88]. Normal *Gimap5* transcript and protein levels can be rescued in a P1-derived artificial chromosome (PAC) transgenic rat [78]. However, potential contributions to lymphopenia and/or T1D from the other *Gimap* genes are still unknown. Similarly, how the mechanisms by which reduced *Gimap5* transcript levels and the absence of the Gimap5 protein [52, 78, 82] contribute to lymphopenia and T1D are still being elucidated [56, 81, 89-91].

The predicted structures of the Gimap proteins show common sequences and motifs, such as GTP-binding domains in the N-terminal half, but with differing C-terminal ends [52, 55]. Some C-terminal regions are consistent with transmembrane domains as in the case of

Gimap1 and Gimap5, while others, as in Gimap9 and Gimap4, predict coiled coil domains [55, 92]. Both GIMAP4 and GIMAP7 in human Jurkat cells [55] localize to the endoplasmic reticulum and Golgi apparatus while mouse Gimap3 from murine IL-3 dependent 32D myeloid precursor cells was expressed at the outer mitochondrial membrane [84]. Conflicting reports show that GIMAP5, from human primary T-cells [81] and from GIMAP5 transfected 293T cells [85] localizes to the centrosome, Golgi apparatus or endoplasmic reticulum (ER), whereas Gimap5, cloned from Rat2 fibroblasts, localizes to a distinct subcellular fraction that is neither mitochondrial nor ER [90]. Gimap proteins may therefore have similar function, but at different subcellular locations. At this time, there is a paucity of information as to the expression of the *Gimap* genes in specific cell types.

1.9 Other Autoimmune Abnormalities

1.9.1 Thyroiditis

Both thyroiditis and enteropathy is over represented in individuals with T1D and this is also true for the BB rat [93-95]. BB rat thyroiditis presents in both BBDP and BBDR strains, although at a lower frequency in BBDR rats, and is associated with the RT1.B/D$^{u/u}$ haplotype [76]. An early sign of emerging thyroiditis in the BB rat is an increased number of dendritic cells in the thyroid well before autoantibodies to thyroglobulin are detected [96]. BB rat thyroiditis is also associated with CD4+ and CD8+ T lymphocytic infiltration of the tissue [95, 97] and with upregulated levels of IL-10, IL-2, IL-12p40 and IFN-γ in the thyroid [98, 99]. Hypothyroidism is inducible in the BB rat through excess intake of iodine as well as administration of IL-1β or ART2.1 monoclonal antibodies in combination with poly I:C [100-102]. Administration of thyroglobulin has proven efficient to induce tolerance in humans, but neither administration of L-thyroxine or thyroglobulin has any effect on lymphocytic infiltration of the BB rat thyroid [103, 104]. However, neonatal treatment with iodine or with T3 in early adolescence seemingly reduces prevalence of autoimmune thyroiditis [105].

1.9.2 Gut Inflammation

A growing body of evidence suggests that intestinal microflora play a role in the pathogenesis of several diseases, among which human and BBDP rat T1D is mentioned. Increased numbers of lymphocytes in the intestinal epithelium and dysregulation of disaccharidase processes and peroxidase activity preceding T1D are characteristics of BB rat enteropathy however the mechanisms by which enteropathy develops are not clear. Suggested mechanisms involve increased intestinal permeability (leaky gut) that allows harmful agents to enter the body and induce autoreactivity. Alternately, decreased intestinal Glucagon-Like Peptide (GLP)-1 has also been discussed as a defect that could potentially influence insulin secretion (reviewed in [106]). Conflicting reports suggest treatment with antibiotics decreases incidence of T1D in BBDP rats; leading to the conclusion that gut flora is indeed a part of T1D pathogenesis [107]. However, this is a paradox in that introduction of lactobacilli has been suggested to prevent autoimmune disease in both humans and rodents (reviewed in [108-111].

1.10 Type 1 Diabetes Prevention

1.10.1 Bone Marrow Transplant

Wildtype RT1.B/D$^{u/u}$ compatible bone marrow rescues both lymphopenia and T1D in the BBDP rat [112-115]. T-cells arise from bone marrow derived hematopoietic stem cells and, via the lymphatic system, migrate to the thymus to differentiate into mature CD4+ and CD8+ single positive T-cells. The null mutation in the *Gimap5* gene leads to premature apoptosis of CD4+ and CD8+ T-cells upon leaving the thymus and is the primary cause of lymphopenia in the BBDP rat [52, 53]. Early bone marrow transplantation studies using genetically heterogeneous colonies of BB rats showed reconstitution of BBDP rats with WF or BBDR bone marrow resulted in only partial protection from T1D [112]. Transplantation studies in DR.$^{lyp/lyp}$ and DR.$^{+/+}$ rats show DR.$^{+/+}$ bone marrow completely rescues lethally irradiated DR.$^{lyp/lyp}$ rats from lymphopenia, insulitis and T1D [113]. The inverse (DR.$^{lyp/lyp}$ bone marrow into DR.$^{+/+}$ recipients) results in an intermediate lymphopenia phenotype (41.4 ± 2.1% and 54.8 ± 2.5% R73 positive T-cells in two separate experiments) [113], similar to previous reports [112]. In addition, DR.$^{lyp/lyp}$ bone marrow transplanted into DR.$^{+/+}$ rats does not result

in development of T1D suggesting that either the DR.$^{+/+}$ rat has irradiation resistant cells that participate in the pathogenesis of T1D or that the DR.$^{lyp/lyp}$ rat has an underlying propensity for development of T1D independent of the *Gimap5* mutation.

1.10.2 Thymectomy

While ineffective in 60 day old rats [116], thymectomy in young (20-30 day old) BBDP rats protects from T1D [116, 117] supporting the role of an immune system defect in the pathogenesis of T1D. Complete thymectomy shows no adverse side effects aside from initial discomfort at the surgery site. As in protection from T1D by splenocyte transfer, thymectomy allows for breeding of BBDP females without the complications associated with diabetic pregnancy and/or poor neonate nurturing and does not affect incidence or time to onset of T1D in offspring [117].

1.10.3 Adoptive T-cell Transfer

Adoptive transfer of CD4+CD25+ T regulatory (T_{reg}) cells to lymphopenic BBDP or leukodepleted BBDR rats can rescue the animal from T1D [118-121]. It has been shown that loss of *Gimap5* does not impact the numbers of functional T_{reg} thymocytes, however, once leaving the thymus, these cells fail to thrive, rendering BBDP animals deficient in this essential T-cell subpopulation [119]. In addition, whole organ splenocyte cells isolated from RT1.B/D$^{u/u}$ compatible WF or BBDR rat injected intraperitoneally into BBDP rats at 25-30 days of age protects from T1D allowing breeding of BBDP females without complications associated with diabetic pregnancy and/or poor neonate nurturing [114, 117, 122]. Protection from T1D by splenocyte transfer does not affect incidence or time to onset of T1D in offspring [117].

1.10.4 Intrathymic Islet Transplantation

Islets from RT1.B/D$^{u/u}$ compatible WF rats, implanted intrathymically into neonatal, spontaneously T1D prone BBDP rats (but not ART2.1 depleted BBDR rats), prevent insulitis, native recipient β-cell autoimmune destruction and T1D [123, 124]. In addition, islets implanted intrathymically from both RT1.B/D$^{u/u}$ compatible WF rats or incompatible LEW rats rescues serum glucose levels in acutely diabetic BBDP rats [125]. As mature T-cells

rarely recirculate through the thymus, it is thought to be immunologically privileged and islets survive without the need for immunosuppression. While the islets remain protected from the immune system, intrathymic islet transplantation does not protect from development of thyroiditis. Prevention of T1D in the BBDP rat may be due to deletion or functional inactivation of autoreactive T-cells in the thymus and suppression of the autoimmune response [123, 125, 126].

1.10.5 Insulin Treatment

Administration of hypoglycemic doses of insulin to pre-diabetic BBDP rats prevents or delays development of T1D, insulitis and impaired glucose tolerance but not autoimmune thyroiditis [127-129]. One mechanism by which insulin therapy may protect from T1D development in the BBDP rat is that the exogenous insulin itself acts as an antigen, essentially inducing immune tolerance. While this may be the case, studies have shown that activated splenic T-cells from insulin protected, ART2.1+ depleted BBDR rats retain the ability to transfer T1D suggesting that autoreactive T-cells are still present in insulin protected rats [130]. Another more likely mechanism is that the hypoglycemic state induced by high dose insulin administration suppresses endogenous insulin secretion, lowers β-cell metabolic activity and induces β-cell rest with less macrophage recruitment, expression of β-cell specific autoantigens and lowered cytokine expression. By either mechanism, protection appears to be specific to the insulin B chain versus the insulin A chain [131]. While prophylactic insulin therapy trials in high-risk relatives of T1D patients showed no effect on T1D development [132], the insulin doses were low and may not have been sufficient to protect from disease development.

1.10.6 Diet

BBDP rats fed a hydrolyzed casein-based diet from weaning are partially protected from development of insulitis and T1D [133-135]. BBDP rats are prone to increased gut permeability and, as in human TID and celiac disease, a propensity towards IFN-γ related gut inflammation [133, 134, 136-138]. IFN-γ is a proinflammatory cytokine that drives MHC class II expression, a sign of immune activation, and production of Th1, cells associated with the development of insulitis in both the BBDP rat and humans [97, 139-141]. Prior to any indication of insulitis, the mesenteric lymph nodes (MLN) of BBDP rats show a lack of Th2

cells and a predisposition towards increased Th1 cell proliferation. BBDP rats fed a hydrolyzed casein-based diet show decreased IFN-γ production in MLN along with decreased IFN-γ and increased Th2 and Th3 cytokine production in the few monocytes found infiltrating the pancreas [134, 142]. Interestingly, neonates fed T1D promoting diets such as wheat or cereal based diets, along with dams milk, show either a delay in onset or complete protection from T1D suggesting a role of the gut in the early development of immunity to dietary antigens [135].

2 Aims

The overall aim of the present thesis was to dissect the genetics of spontaneous autoimmune type 1 diabetes in the BB rat.

Study I

In our approach to dissect the genetics of spontaneous T1D in the BB rat [143-145], we relied heavily on generating F2 (BBDP X F344) rats to maximize recombination events within the genome. One rat (see Table 1 in [79] for details), showed a recombination event within the *lyp* critical interval that left *Gimap5* and *Gimap1* as BBDP and the remaining *Gimap* genes, contained within a 34 Mb DNA fragment, as F344. The aim of **Paper I** was to secure introgression of the 34 Mb F344 locus onto the congenic DR.*lyp* rat line through a series of marker assisted crosses, intercrosses and backcrosses and to test whether introgression of the F344 genome proximal to *Gimap5* resulted in 1) no effect on T1D development or 2) protection from T1D. The first outcome would underscore lymphopenia and the *Gimap5* mutation as a diabetogenic factor. The alternative outcome would identify a diabetogenic factor independent of lymphopenia.

Study II

Generation of the DRF.$^{f/f}$ congenic rat line in **Paper I** with 34 Mb of F344 DNA introgressed between D4Rat253 and D4Rhw6 into the congenic DR.$^{lyp/lyp}$ genetic background resulted in a lymphopenic but non-diabetic rat. Protection from T1D in the DRF.$^{f/f}$ congenic rat line led us to conclude that spontaneous T1D in the BB rat is controlled, in part, by a diabetogenic factor(s) independent of the *Gimap5* mutation (76.84 Mb) on RNO4. The aim of **Paper II** was to cross the DRF.$^{f/f}$ rat to BBDR and DR.$^{lyp/lyp}$ produce recombinant sublines that could be assessed for both the lymphopenia and T1D phenotypes as well as perform candidate gene coding sequence analysis to identify causal gene sequence variants related to the T1D phenotype.

Study III

While excluded from participating in development of peripheral T-cell lymphopenia, the *Gimap* family members located within the *Iddm39* QTL may play a role in development of T1D in the DR.$^{lyp/lyp}$ rat. Candidate coding sequence analysis in **Paper II** did not detect any causal gene sequence variants within the *Iddm39 Gimap* genes however it is possible that there are additional genetic factors controlling gene expression (transcription) that may be important for development of disease. The aim of **Paper III** was to perform *Gimap* family cDNA sequencing in DR.$^{+/+}$ and DR.$^{lyp/lyp}$ rats, examine *Gimap* gene expression across multiple tissues using quantitative real time (qRT) PCR and quantify mRNA expression of all annotated and putative *Gimap* genes in DR.$^{+/+}$ and DR.$^{lyp/lyp}$ rat thymus, spleen and MLN.

Study IV

Expression analysis of the *Gimap* family in **Paper III** showed that all seven members are differentially expressed in DR.$^{+/+}$ and DR.$^{lyp/lyp}$ spleen, thymus and MLN [54]. However, as whole organs, not pure populations of cells were studied, it remained uncertain to what extent the observed differences were due to the cellular composition of these organs which are markedly affected by the reduction of T-cells and a concordant increase in B-cells and monocytes. The aim of **Paper IV** was to investigate *Gimap* gene expression variations using qRT-PCR analysis on sorted subpopulations of purified T- and B-cells from DR.$^{+/+}$ and DR.$^{lyp/lyp}$ rat thymus, spleen and MLN.

3 Methods

3.1 DR.$^{lyp/lyp}$ Rats

The parental DR.*lyp* rat line used to generate the DRF.$^{f/f}$ congenic and congenic sublines (**Papers I & II**) and for analysis of *Gimap* gene family expression (**Papers III & IV**) was derived from two independent recombination events identified following introgression of the BBDP *Gimap5* lymphopenia (*lyp*) gene interval onto the BBDR genetic background [143]. The first recombination event was flanked by simple sequence length polymorphism (SSLP) marker D4Rhw10 (77.81 Mb) and the second flanked by D4Rhw11 (75.81 Mb). The parental line was analyzed after continuous backcrosses to BBDR rats and further fine-mapping revealed additional BBDP DNA carried between markers *D4Rhw17* (61.77 Mb) and *SS99306861* (70.17 Mb). In **Paper II**, care was taken to remove this BBDP interval through eight marker-assisted backcross generations with inbred BBDR/Rhw rats. The resulting congenic subline, defined as BBDR.BBDP-(*D4Rhw11-D4Rhw10*)/Rhw, is thereafter referred to as DR.$^{lyp/lyp}$. The remainder of the genome represents BBDR as verified by genome wide scanning (data not shown). The DR.*lyp* congenic rat lines are kept in heterozygous sister-brother breeding and produce Mendelian proportions of DR.$^{lyp/lyp}$ (25%), DR.$^{lyp/+}$ (50%) and DR.$^{+/+}$ (25%).

3.2 BBDR Rats

BBDR/Rhw rats (non-lymphopenic and T1D resistant) used to generate the DRF.$^{f/f}$ congenic and congenic sublines (**Papers I & II**) had been sister/brother mated for 59 generations when used in these studies.

3.3 F344 Rats

In **Paper I**, the single female F344 rat was obtained from Charles River Laboratories, Wilmington, MA.

3.4 Breeding

In **Paper I**, the one male rat containing the recombination event within the *lyp* critical interval that left *Gimap1* and *Gimap5* as BBDP and the remaining *Gimap* genes as F344, was crossed to a female BBDR. The recombination was introgressed onto the congenic DR.*lyp* rat line through backcrosses to DR.$^{lyp/lyp}$ rats and intercrosses within the line itself to check the lymphopenia and T1D phenotypes (**Paper I**, Fig.2). The 34 Mb F344 genomic DNA fragment was fixed onto the DR.*lyp* background in a total of nine backcross and seven intercross matings.

To generate additional congenic sublines to narrow the 34 Mb F344 DNA interval in the DRF.$^{f/f}$ rat in **Paper II**, we established F2(DRF.$^{f/f}$ x BBDR) intercrosses and typed 13 genetic markers on RNO4 to identify recombinant offspring. Two separate recombination events reduced the proximal end of the F344 DNA fragment in the congenic DRF.$^{f/f}$ rat line and generated the DRF.A (Flanked by BBDR DNA at D4Rat253 and D4Rhw8) and DRF.B (D4Got33-D4Rhw8) congenic sublines (Fig.2). Additional intercrosses within the DRF.A and DRF.B sublines generated the DRF.C (D4Rat102-D4Rhw8) and DRF.D (D4Rat102-D4Rhw8) sublines respectively (Fig.2).

To generate recombination events in that would reduce the proximal end of the F344 DNA fragment while retaining the *Gimap5* mutation, we established F2(DRF.$^{f/f}$ x DR.$^{lyp/lyp}$) intercrosses. A recombination event indentified at D4Rat27 in a male rat generated the DRF.E (D4Rat153-D4Rat27) congenic subline (Fig.2). Following crosses of the DRF.E male progenitor to a female BBDR, a recombination event was identified that generated the DRF.G (D4Arb11-D4Rat27) subline. Two separate recombination events identified in additional crosses of the F2(DRF.E x BBDR) to DR.$^{lyp/lyp}$ generated the DRF.F (D4Rat253-D4Rat27) and DRF.H (D4Rat102-D4Rat27) sublines (Fig.2).

Three separate recombination events identified in F2(DRF.$^{f/f}$ x BBDR) offspring again reduced the distal end of the F344 DNA fragment and generated the DRF.I (D4Rat102-D4Rhw8), DRF.K (D4Rat27-D4Rhw8) and DRF.L (D4Got59-D4Rhw8) congenic sublines. Following crosses of the DRF.$^{f/f}$ to the BBDR, a fourth recombination was identified that became the progenitor of the DRF.J (D4Rat26-D4Rhw8) subline (Fig.2).

In all cases (DRF.A-L), the recombinant rat was crossed to a BBDR rat to produce additional animals and the offspring intercrossed to generate lymphopenic rats homozygous for the F344 DNA recombination that could be followed for the lymphopenia and T1D phenotypes. Once established, the congenic sublines were subjected to high resolution sequencing analysis to characterize breakpoints between genetic markers. The DR.$^{lyp/lyp}$, DRF.$^{f/f}$ and DRF.D congenic lines and subline are currently being held in heterozygous sister/brother breeding and are available upon request.

3.5 Housing

For **Papers I-IV**, rats were housed in a specific pathogen–free facility at the University of Washington, Seattle, WA on a 12-h light/dark cycle with 24-h access to food (Harlan Teklad, Madison, WI) and water. The institutional animal use and care committee approved all protocols and the University of Washington Rodent Health Monitoring Program used to track infectious agents.

3.6 Diabetes Diagnosis

To monitor T1D development in **Papers I & II**, lymphopenic rats were weighed daily (Sartorious, Edgewood, NY) starting at 40 days of age and blood glucose concentration measured (Ascensia Contour; Bayer, Leverkusen, Germany) if the rat did not gain weight compared with the previous day. T1D was diagnosed as a glucose concentration >200 mg/dl for two consecutive days, after which insulin therapy was initiated.

3.7 Antibody Phenotyping

To analyze lymphopenia in **Papers I & II**, two drops of tail vein blood were obtained between 25 and 30 days of age for peripheral blood T-cell phenotyping of R73, CD4 and CD8 antibody positive cells. The samples were diluted in 10 ml Gey's solution, centrifuged for 10 min at 500 x g after 20 min on ice, and washed again by centrifugation in 2 mL 4% (w/v) BSA in PBS (BSA-PBS). Cells were then re-suspended in 300 µl BSA-PBS, 100 µl aliquots centrifuged for 5 min at 500 x g and the supernatant removed. The aliquots were re-suspended in 100 µl PE-labeled R73, FITC-labeled CD8 and CY5-labeled CD4, diluted 1:200 (R73, CD8) or 1:150 (CD4) in 4% BSA-PBS. The cells were incubated in the dark for 10 min and then washed by centrifugation in 100 µl BSA-PBS. The supernatant was removed and the cells re-suspended in 200 µl PBS and FACS analyzed the same day. The fraction of fluorescent R73 positive T-cells among mononuclear cells was determined on a BD Facscan (Beckton Dickenson, San Jose, CA). The instrument was compensated with BBDR quality control blood samples and the proportion of R73+ cells was determined from light scatter-gated mononuclear cells. Among the R73+ cells in **Paper II**, we determined the proportion of CD4+ and CD8+ cells. Results are expressed as median percent and interquartile range.

3.8 Genotyping Chromosome 4

To perform the single sequence length polymphorphism (SSLP) genotyping in **Papers I-IV**, 5 mm tail snips were obtained between 25-30 days of age and digested overnight with 12.5 µL Proteinase K (Promega, Corp., Madison, WI) in 500 µl SET (1% SDS in 150 mmol/L NaCl, 5 mmol/L EDTA and 50 mmol/L Tris, pH 8.0) at 55 ^{o}C. The digested samples were centrifuged at 20,800 x g for 15 min, the supernatant transferred to 500 µL cold isopropanol and the DNA pellet collected by centrifugation at 20,800 x g for 5 min. The isopropanol was discarded and the DNA pellet washed by centrifugation in cold 70 % EtOH twice. The pellet was air dried for 15 min and resuspended in TE (10 mmol/L Tris, 1 mmol/L EDTA, pH 7.4) at 55 ^{o}C overnight. The samples were diluted to 25 ng/µL in ddH_2O and 2 µL of this genomic DNA used per one of the following 10 µL reactions. *Lyp region primers* : 1 µL 10X reaction buffer (Promega), 0.8 µL MgCl (Promega), 0.2 µL 10 mmol/L dNTP's (New England BioLabs, Beverly, MA), 0.5 µL of 1 µmol/L IRDye 700 labeled primer (LiCor Biosciences, Lincoln, NE), 0.5 µL of 20 µmol/L unlabeled reverse primer (Qiagen, Valencia, CA), 0.1 µL Taq DNA

Polymerase (Promega), 0.04 µL 10mg/ml BSA (New England BioLabs) and 4.4 µL ddH_2O. *SSLP primers outside of the lyp region:* 1µL 10X reaction buffer (Promega), 0.8 µL MgCl (Promega), 0.2 µL 10mmol/L dNTP's (New England BioLabs), 0.5 µL 1µmol/L M13 labeled forward primer (Qiagen), 0.5 µL of 20 µmol/L unlabeled reverse primer (Qiagen), 0.1 µL Taq DNA Polymerase (Promega), 1 µL M13-700 (LiCor Biosciences) and 3.9 µL ddH_2O.

All samples were then amplified using the following standard PCR protocol: 95 ^oC 5 min, 95 ^oC 20 sec, 60 ^oC 20 sec, 72 ^oC 30 sec, steps 2-4 repeated 30 times, 72 ^oC 3 min. Samples were kept at 4^oC until use. PCR products were diluted to 25% with STOP solution (LiCor Biosciences) and analyzed using a NEN Global IR^2 DNA Analyzer System (Model 4200S-2) with a 6.5% gel matrix (LiCor Biosciences).

3.9 Whole Genome Scan

To ensure the genomic background of the DRF.$^{f/f}$ congenic rat line in **Paper I** was fixed for BBDR, we genotyped 144 SSLPs spanning the entire rat genome at the N6-N8 generations with an average genome coverage of 10 cM. At the N11 generation, the number of SSLPs was reduced to 46 and only covered those chromosomes not fully fixed for BB (at a 10 cM resolution). To confirm that the genomic background of the new DR.$^{lyp/lyp}$ strain was fixed for BBDR **in Paper II**, we genotyped 95 SSLPs at the N4 and N6 generations, including D4Rat26 and D4Rat102, at an average coverage of 20 cM. All SSLPs were amplified and genotypes determined using fluorescent genotyping on an ABI 377, as outlined in detail elsewhere [146]. Briefly, each forward primer for the SSLP is synthesized with a 5' tail containing the universal M13 primer sequence (5' TGTAAAACGACGGCCAGT-SSLP-f 3'). The PCR reaction is a 2-step reaction containing the primers specific for the SSLP and an additional fluorophore-labeled M13 primer. The initial amplification steps incorporate and amplify the SSLP specific primers, thus incorporating the M13 tail. The latter rounds incorporate the labeled M13 dye-conjugate primer, allowing for detection on the ABI 377. Multiple fluorophores are used, in combination with different PCR product sizes, to allow multiplexing of 6 SSLPs at the gel electrophoresis level.

3.10 Histology

For analysis of insulitis and thyroiditis (**Papers I and II**), pancreas and thyroid sections (5 μm) were cut and stained with hematoxylin and eosin. Scoring of pancreatic inflammation, insulitis and thyroiditis was carried out on coded sections by two independent investigators as follows (duplicate for each animal); **Paper I** (pancreas and thymus): +0 no infiltration, +1 infiltration with mononuclear cells around blood vessels or ducts, +2 occasional mononuclear cells infiltration, +3 distinct mononuclear cell infiltration, +4 mononuclear cell infiltration with little recognizable normal tissue [147]. **Paper II** (pancreas only): +0 no inflammatory cells, +1 inflammatory cells around ducts and vessels only, +2 inflammatory cells around the islets, +3 inflammatory cells inside the islets without change in islet morphology, +4 inflammatory cells inside the islets with distorted islet morphology. Grades 2-4 were always accompanied by inflammatory cells around ducts and vessels.

3.11 Bioinformatics

In **Papers I & II**, candidate coding sequences were identified from a) known and reference gene sequences, b) comparative analysis of rat, mouse and human mRNA, rat spliced EST and gene prediction algorithms found at the University of California Santa Cruz Rat Genome Browser (UCSC) (http://genome.ucsc.edu/index.html, Assembly November 2004) or c) previous studies [148]. In all cases it was required that the candidate coding sequence have rat, mouse or human mRNA or EST evidence along with identifiable AG/GT exon boundaries. SSLP markers for genotyping critical intervals were found at the Rat Genome Database (http://rgd.mcw.edu/). All other primers were designed using Primer3 (Massachusetts Institute of Technology, http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

In **Papers III & IV**, predicted protein sequences were aligned using T-coffee (http://www.ch.embnet.org/software/TCoffee.html). Structure and topology of proteins was defined using HMMTOP (http://www.ensim.hu./hmmtop/index.html) or Protein Predict (http://cubic.bioc.columbia.edu/pp/). Subcellular locations were predicted using PSORT (http://psort.nibb.ac.jp/form2.html).

The nomenclature of the *Gimap* gene family in **Papers I-IV** follows the official names determined by the rat nomenclature committee (Lois J. Maltais, Mouse Genome Database (MGD), Mouse Genome Informatics Web Site, The Jackson Laboratory, Bar Harbor, Maine, http://www.informatics.jax.org) and is different from previous publications [52, 55, 149, 150].

3.12 Sequencing

To identify the single nucleotide polymorphisms in candidate coding sequences (cSNPs) or those that would be useful to characterize breakpoints (SNPs) in the DRF.$^{f/f}$ congenic sublines in **Papers I & II**, we generated 900-1500bp PCR products of both the forward and reverse strand genomic sequence from BBDR/Rhw (DR), BBDP/Rhw (DP) and F344/Rhw (F344) rat genomic DNA. Primer pairs spanned the full length of each individual exon including the flanking intronic sequences.

In **Paper III**, thymus cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's specifications. Amplified PCR products were cloned into pCRII with the TOPO-TA cloning kit (Invitrogen) and 5' and 3' RACE (Rapid Amplification of cDNA Ends) carried out with a Marathon cDNA Amplification kit (K1802-1, Clontech, Palo Alto, CA). These plasmids were transformed into XL1Blue (Stratagene, La Jolla, CA) by electroporation of Top10 cells (Invitrogen) and purified using a GenElute Plasmid Maxiprep Kit (Sigma, St. Louis, MO) or Plasmid Maxi Kit (Qiagen).

In all cases**,** samples were sequenced using ABI BigDye Terminator v3.1 Cycle Sequencing Mix (Applied Biosystems, Foster City, CA) and analyzed on an ABI 3730XL sequencer (Applied Biosystems) at the University of Washington Biochemistry Sequencing Core in Seattle, WA. The resulting genome survey sequences (GSSs) generated for **Papers I & II** and mRNA sequences generated for **Paper III** were submitted for official naming to Genbank at The National Center for Biotechnology Information (NCBI). DR.$^{+/+}$ and DR.$^{lyp/lyp}$ *Gimap* mRNA Genbank accession numbers are DQ125335 – DQ125353. All SNPs were submitted to the Database of Single Nucleotide Polymorphisms (dbSNP) at NCBI and assigned an individual NCBI assay ID (SS) identifier (**Paper II**, Supplementary Tables A & B).

3.13 Quantitative RT-PCR

In **Papers III & IV**, *Gimap* gene expression was analyzed using qRT-PCR performed on an Mx4000® Multiplex QPCR System (Stratagene). Total RNA or Poly A+ RNA from thymus, spleen and MLN was isolated from whole organ (**Paper III)** or from FACS Aria (BD Biosciences, Mountain View, CA) sorted cell populations stained with R-phycoerythrin (R-PE)-labeled anti-CD3 (G4.18), Cy-chrome labeled anti-CD4 (OX35) FITC labeled anti-CD8 (OX8), FITC labeled anti alpha-beta T-cell receptor (R73) or $CD45^+$ and $CD45RA^+$ biotinylated monoclonal antibody stained cells separated using streptavidin-conjugated MACS microbeads (Invitrogen) (**Paper IV**).

Each twenty-five µl qRT-PCR reaction was run in triplicate using a Brilliant® Single-Step qRT-PCR Kit (Stratagene). *Gimap* gene probes were positioned in the 3' regions of the transcripts where there is more variation, subjected to BLAST alignment to ensure specificity and duplexed with rat cyclophilin (NM_017101) as an internal control. Representative qRT-PCR products for each gene, from each tissue, were run on an agarose gel to check for primer pair binding specificity. Results from each assay were validated and normalized against cyclophilin (**Paper III**) or GAPDH (**Paper IV**).

3.14 Statistical Analysis

Univariate analyses of lymphocyte phenotyping are shown as median percent (interquartile range) of R73 antibody positive cells. In each new DRF subline group in **Paper II** (A-D, E-H and I-L) the nonparametric K-sample test on the equality of medians was used to test the null hypothesis that the K samples were drawn from populations with the same median percent positive cells. When comparing medians between groups ($DR.^{lyp/lyp}$, $DRF.^{f/f}$, A-D, E-H and I-L), *p*-values are reported with a Bonferroni correction for multiple comparisons. In the case of two samples, the chi-squared test statistic was calculated with a continuity correction. Survival time in days (median (interquartile range)) is presented for each genetic line along with the percentage of rats that become diabetic. Survival time to onset of T1D was calculated using the log-rank test. Statistical analyses were performed using Stata 8 (StataCorp. 2003. Stata Statistical Software: Release 8, StataCorp LP, College Station, TX)

and Graphpad Prism 5 (La Jolla, CA) used to generate the graphs. All two-tail statistical tests were judged with statistical significant at $p<0.05$.

To compare *Gimap* and *Lr8* gene expression across multiple tissues in **Paper III**, data was first normalized to cyclophilin then scaled and expressed as a percentage of DR.+/+ *Gimap5* MLN, the highest expressing gene overall. For analysis of *Gimap* expression in DR.$^{lyp/lyp}$, DR.$^{lyp/+}$ and DR.$^{+/+}$ rat thymus, spleen and MLN, 15 rats (5 rats per genotype) were used from 5 litters consisting of 1 rat genotype from each litter. MLN data for 1 litter was missing leaving 12 rats from 4 litters for analysis. Comparisons from bone marrow and kidney are not shown due to very low expression and high error in these tissues. Comparisons of expression between cell types in **Paper IV** consisted of twelve rats with two repeated measures for each rat. Comparisons between DR.$^{lyp/lyp}$ and DR.$^{+/+}$ rats consisted of six rats from three litters with one DR.$^{lyp/lyp}$ and one DR.$^{+/+}$ rat in each litter and two repeated measures for each rat. In both **Papers III & IV**, pair-wise comparisons of the individual ratios were carried out using linear mixed effects models in S-PLUS (Insightful Corp., Seattle, WA) with a random intercept for each litter. A conditional *F*-test was implemented to test the significance of terms in the fixed effects models. A Bonferroni correction was applied to *p*-values to adjust for multiple comparisons and adjusted values were considered significant at $p<0.05$.

4 Results

4.1 Papers I and II.

4.1.1 BBDR and DR.$^{lyp/lyp}$ Rats

The congenic DR.$^{lyp/lyp}$ (BBDR.BBDP-(*D4Rhw11-D4Rhw10*)/Rhw) rats developed in **Paper II** with 2 Mb of BBDP DNA introgressed between D4Rhw11 (75.81 Mb) and D4Rhw10 (77.81 Mb) on the BBDR genetic background (Fig.2) were lymphopenic with 13% (11-14) (median percent (interquartile range)) R73+ cells (n=80) (Fig.3). In contrast to DR.$^{lyp/lyp}$ rats, BBDR rats (not shown) had 60% (57-69) R73+ cells (p<0.001) (Fig.3). Among the R73+ cells, 75% (73-76) were CD4+ and 22% (21-24) were CD8+; the CD4/CD8 ratio being 3.4 (3.1-3.6) (n=75) (p=0.99, p<0.001 and p<0.001 respectively compared to DR.$^{lyp/lyp}$ rats) (Percent CD4+ and CD8+ cells and the CD4/CD8 ratio for DR.$^{lyp/lyp}$ rats are shown in **Paper II**, Fig.2B-D).

Of the 39 DR.$^{lyp/lyp}$ rats followed to T1D onset in **Papers II**, all (100%) developed T1D by 83 days with a median of 56 (53-62) (median (interquartile range)) days (Fig.4). The appearance of lymphopenia and T1D in DR.$^{lyp/lyp}$ rats was associated with the 2 Mb of BBDP DNA, encompassing the *Gimap5* mutation, introgressed on RNO4 (Fig.2).

4.1.2 DRF.$^{f/f}$ Rats

In an intercross of F1(BBDP x F344) offspring, we discovered a recombination event proximal to *Gimap1* that left *Gimap1* and *Gimap5* as BBDP and the remaining *Gimap* family members as F344. The strategy to fix the F344 recombinant fragment was to combine complete genotyping of the *lyp* critical interval with phenotyping for lymphopenia and T1D. Whole-genome scan and SSLP analyses showed that F344 DNA had been introgressed between D4Rat153 (42.53 Mb) and D4Rhw6 (76.83 Mb) on RNO4 (Fig.2). Sequencing

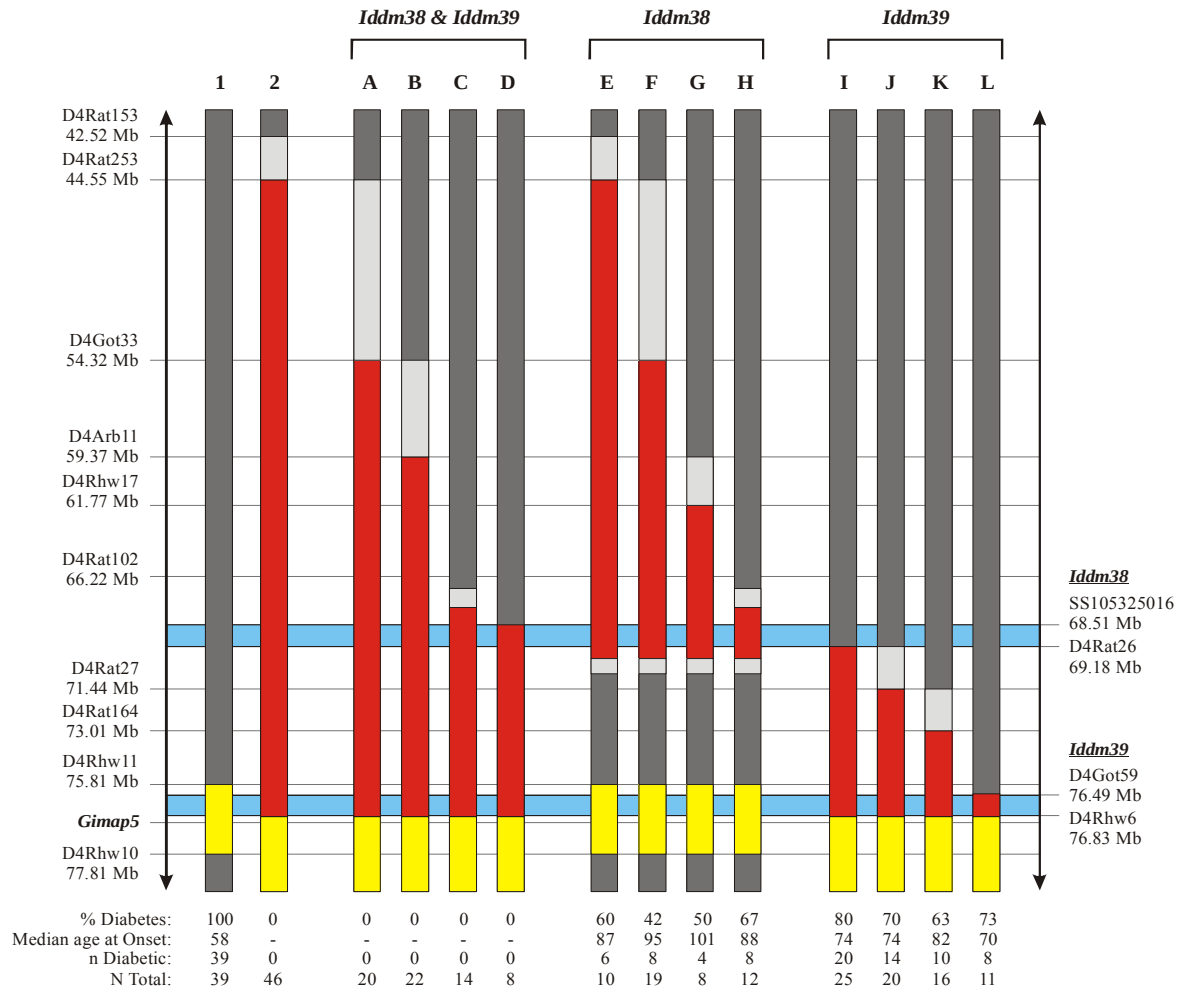

Figure 2. *Chromosome 4 Map.* The color code is as follows; red represents F344/F344, dark grey DR/DR, yellow DP/DP and light grey is unknown. The light blue areas represent the two critical intervals; *Iddm38* from SNP SS105325016 to SSLP marker D4Rat26 containing the *TCR Vβ* and *trypsin* genes and *Iddm39* from D4Got59 to D4Rhw6. All non-diabetic recombinant interval bearing rats (DRF.A-L) were tracked to between 145-312 days of age. See Fig.6 for fine mapping of the DRF.H F344 DNA interval.

across the right-most breakpoint revealed the recombination event to be within D4Rhw6, just proximal to *Gimap1*. The congenic line, referred to as DRF.$^{f/f}$, have retained BBDP DNA from D4Rhw8 (76.84 Mb) to the end of RNO4 (data not shown). During rescue of the F344 DNA fragment in the DRF.$^{f/f}$ line, rats heterozygous for the F344 fragment were generated (DRF.$^{b/f}$) as well as rats that did not contain the recombination fragment (DRF.$^{b/b}$). On RNO4, DRF.$^{b/b}$ rats genetically mimic the DR.$^{lyp/lyp}$ (Fig.2) and thus provided an internal control for comparison of the lymphopenia and T1D phenotypes between the two lines.

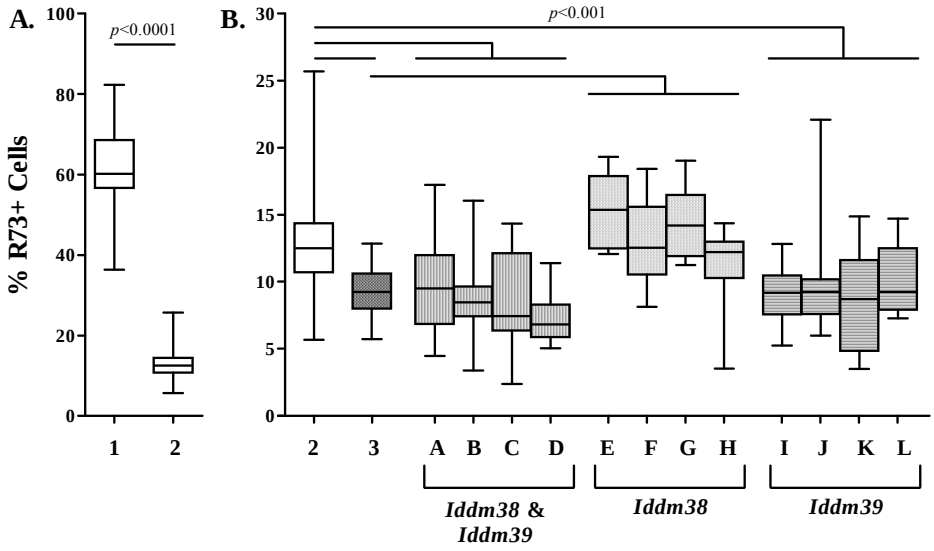

Figure 3. *Percent R73+ TCR Phenotyping*. Column designations are as follows; **Panel A.** 1) BBDR n=75 2) DR.$^{lyp/lyp}$ n=80. **Panel B.** 2) DR.$^{lyp/lyp}$ n=80, 3) DRF.$^{f/f}$ n=20, A) DRF.A n=23, B) DRF.B n=33, C) DRF.C n=24, D) DRF.D n=9, E) DRF.E n=12, F) DRF.F n=19, G) DRF.G n=9, H) DRF.H n=15, I) DRF.I n=31, J) DRF.J n=20, K) DRF.K n=22 and L) DRF.L n=11. Box plots indicate median and interquartile range with error bars indicating total range.

DRF.$^{f/f}$ rats had less R73+ cells (9% (8-11)) compared to DR.$^{lyp/lyp}$ rats (p<0.001; Fig.3). The median percent CD4+ and CD8+ cells and the CD4/CD8 ratio did not differ between DR.$^{lyp/lyp}$ and DRF.$^{f/f}$ rats (**Paper II**, Fig.2B-D).

All (88/88) DRF.$^{f/f}$ rats generated in **Papers I & II** that were monitored to a median age of 162 (152-174) days showed *complete* protection from T1D (Fig.4). The age at onset of T1D in DRF.$^{b/b}$ rats significantly differed from DR.$^{lyp/lyp}$ rats in that the median age at onset was 66 (60-72) days (χ^2_1=24, p<0.0001) (n=93) (Fig.4). The frequency of T1D in DRF.$^{b/b}$ rats was also 100%. In contrast, T1D frequency in DRF.$^{b/f}$ rats was 63% (102/163) with a median age at onset of 82 (74-97) days (χ^2_1=215, p<0.0001 compared to DR.$^{lyp/lyp}$ rats) when followed to a median age of 180 (151-190) days.

Evaluation of pancreatic mononuclear cell infiltration in DRF.$^{f/f}$ rats followed to >150 days of age showed that 92% (59/64) remained free of mononuclear cell inflammation in and around the islets while in 8% (5/64), insulitis was observed (**Paper I**, Table 2). In contrast, while the thyroid had a normal appearance (grade +0) in young DRF.$^{f/f}$ rats (Fig.5), 75% (39/52) of

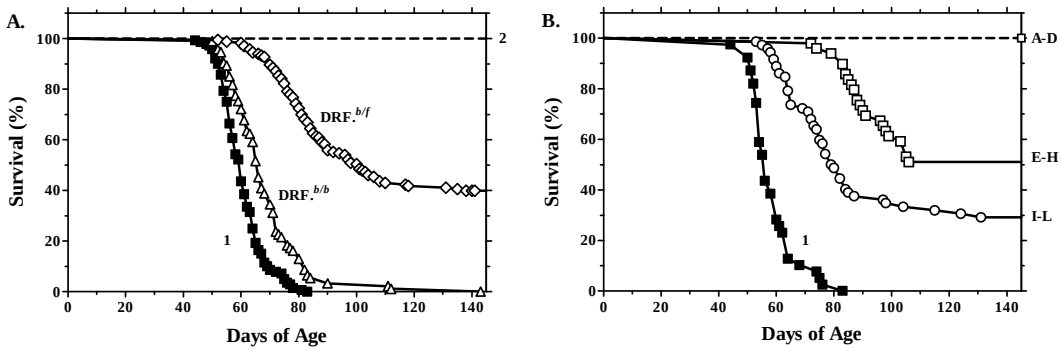

Figure 4. *Diabetes Incidence as Percent Survival*. Line designations are as follows; **Panel A.** Solid squares represent DR.$^{lyp/lyp}$ rats (1) (n=39), dashed line represents DRF.$^{f/f}$ (2) (n=73), open triangles are DRF.$^{b/b}$ (n=93) and open diamonds are DRF.$^{b/f}$ (n=163). **Panel B.** The solid squares again represents DR.$^{lyp/lyp}$ rats (1) (n=39), dashed line represents DRF.A-D (n=63), open circles represent the DRF.E-H (n=55) and open squares represent DRF.I-L (n=72). All non-diabetic recombinant interval bearing rats were tracked to between 145-312 days of age.

DRF.$^{f/f}$ followed to >150 days of age showed thyroiditis, the score varying from +1 to +4 (**Paper I**, Table 2). A 150 day-old DRF.$^{f/f}$ rat having grade +4 thyroiditis is shown (Fig.5).

4.1.3 DRF.$^{f/f}$ Congenic Sublines

Proximal reduction of the 34 Mb F344 DNA fragment by 10-26 Mb (DRF.A-D).

Recombination events generated in F2(DRF.$^{f/f}$ x BBDR) offspring reduced the proximal end of the 34 Mb F344 DNA fragment in the DRF.$^{f/f}$ line by up to 26 Mb and generated four congenic sublines, DRF.A, B, C and D (Fig.2). Further sequencing analysis revealed the DRF.D congenic subline was flanked by BBDR DNA at SS105325016 (Fig.2, Fig.6).

While the median percent R73+ and CD4+ cells did not differ between the DRF.A, B, C and D lines, a difference was observed in CD8+ cells and the CD4/CD8 ratio (p=0.02 for both). DRF.A-D rats were all lymphopenic with 8% (7-11) R73+ cells (n=89) when grouped (Fig.3). The percentage of R73+ cells did not differ from DRF.$^{f/f}$ rats but was significantly lower than in the DR.$^{lyp/lyp}$ (p<0.001). DRF.A-D rats also differed from DR.$^{lyp/lyp}$ and DRF.$^{f/f}$ rats in CD4+ and CD8+ cells (p<0.001 for both) (**Paper II**, Fig.2B-D).

All (64/64) DRF.A-D rats monitored to a median age of 196 (167-199) days showed *complete* protection from T1D (Fig.4). The DRF.D subline retains the shortest F344 recombinant fragment conferring complete protection from T1D (encompassing both *Iddm38* and *Iddm39*)

and defines the proximal border of *Iddm39* (Fig.2, Fig.6). Evaluation of pancreatic mononuclear cell infiltration showed 97% of 375 islets inspected in 5 non-diabetic rats from DRF.B exhibited inflammatory cells only around ducts and vessels (grade +1) (**Paper II**, Table 1). The data in these rats suggest that *complete* T1D protection is provided within the 8 Mb of F344 DNA conserved between SS105325016 (68.51 Mb) and D4Rhw6 (76.83 Mb) (Fig.2).

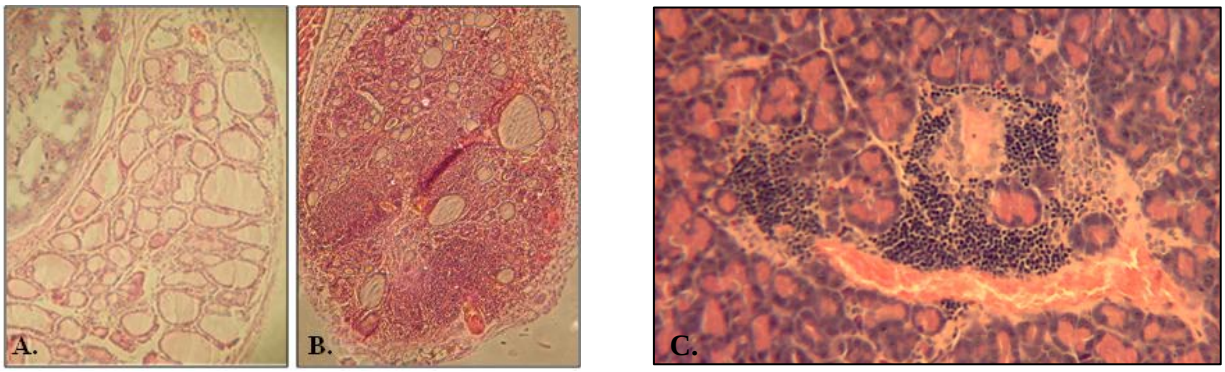

Figure 5. *Thyroid and Pancreas Histology.* **Panel A.** Thyroid histology was normal (grade +0) in the 56 day old DRF.$^{f/f}$ rat shown in panel A. **Panel B.** Severe thyroiditis (grade +4) was observed in the 163 day old DRF.$^{f/f}$ rat shown in panel B. **Panel C.** Pancreatic mononuclear cell infiltration around ducts and vessels (grade +1) is shown in one DRF.E recombinant subline rat followed to >200 days without developing T1D. Magnification is x100.

Retaining 3 Mb F344 DNA at D4Rat26 (DRF.E-H).

Recombination events generated in F2(DRF.$^{f/f}$ x DF.$^{lyp/lyp}$) offspring reduced the distal end of the 34 Mb F344 DNA fragment in the DRF.$^{f/f}$ line to 28 Mb, 26 Mb, 11 Mb and 3 Mb (Fig.2). The DRF.H subline is flanked by BBDR DNA at rs8168904 (67.41 Mb) and SS99306861 (70.17 Mb) and retains the shortest F344 recombinant fragment encompassing only *Iddm38* (Fig.2, Fig.6).

The median percent R73+, CD4+ and CD8+ cells, the CD4/CD8 ratio and the survival time to the onset of T1D did not differ between the DRF.E, F, G and H lines. DRF.E-H rats were all lymphopenic with 13% (11-16) R73+ cells (n=55) when grouped (Fig.3). The percentage of R73+ cells did not differ from DR.$^{lyp/lyp}$ rats but was significantly higher than in DRF.$^{f/f}$ rats (p<0. 001) (Fig.3).

When taken together, T1D frequency in DRF.E-H rats was 53% (26/49) with a median age of onset of 90 (84-104) days (χ^2_1=120, p<0.001 compared to DR.$^{lyp/lyp}$ rats) (Fig.4). Evaluation of pancreatic mononuclear cell infiltration showed 82% of 891 islets inspected in 21 non-diabetic DRF.E-H rats were grade +1, 11% grade +2 and 7% grade +4 (**Paper II**, Table 1). One DRF.E recombinant interval bearing rat with inflammatory cells only around ducts and vessels (grade +1) is shown in Fig.5. The results from these rats suggest that *partial* T1D protection is located within the 3 Mb of F344 DNA encompassed by DRF.H (Fig.2, Fig.6).

Retaining 340 Kb F344 DNA between D4Rhw6 and D4Rhw2 (DRF.I-L).

Recombination events generated in F2(DRF.$^{f/f}$ x BBDR) offspring reduced the proximal end of the 34 Mb F344 DNA fragment in the DRF.$^{f/f}$ line to 9 Mb, 8 Mb, 5 Mb and 340 Kb (*Iddm39*) (Fig.2). The DRF.L subline defines the *Iddm39* interval (Fig.2, Fig.6).

The median R73+, CD4+ and CD8+ cells, the CD4/CD8 ratio and the survival time to the onset of T1D did not differ between the DRF.I, J, K and L lines. DRF.I-L rats were all lymphopenic with 9% (7-11) R73+ cells (n=84) when grouped (Fig.3). The percentage of R73+ cells did not differ from DRF.$^{f/f}$ rats but was significantly lower than in DR.$^{lyp/lyp}$ rats (p<0.001) (Fig.3). DRF.I-L rats also differed from DR.$^{lyp/lyp}$ rats in CD8+ cells and the CD4/CD8 ratio (p<0.001 and p=0.008 respectively) (**Paper II**, Fig.2B-D).

When taken together, T1D frequency in DRF.I-L rats was 72% (52/72) with a median age of onset of 75 (64-82) days (χ^2_1=91.7, p<0.001 compared to DR.$^{lyp/lyp}$ rats) (Fig.4). Evaluation of pancreatic mononuclear cell infiltration showed 77% of 594 islets inspected in 15 non-diabetic DRF.I-L rats were grade +1, 12% grade +2 and 11% grade +4 (**Paper II**, Table 1). The results from these rats suggest that *partial* T1D protection can be explained by retention of 340 Kb F344 DNA at the distal end of the 34 Mb F344 DNA fragment between D4Got59 (76.49 Mb) and D4Rhw6 (Fig.2, Fig.6).

4.1.4 *Iddm38*

The DRF.D congenic subline with *complete* protection from T1D, flanked by BBDR DNA at SS105325016 (68.51 Mb), defined the proximal border of *Iddm38* (Fig.6). Further comparisons of the non-diabetic DRF.D rat line with the DRF.I congenic subline (with 80%

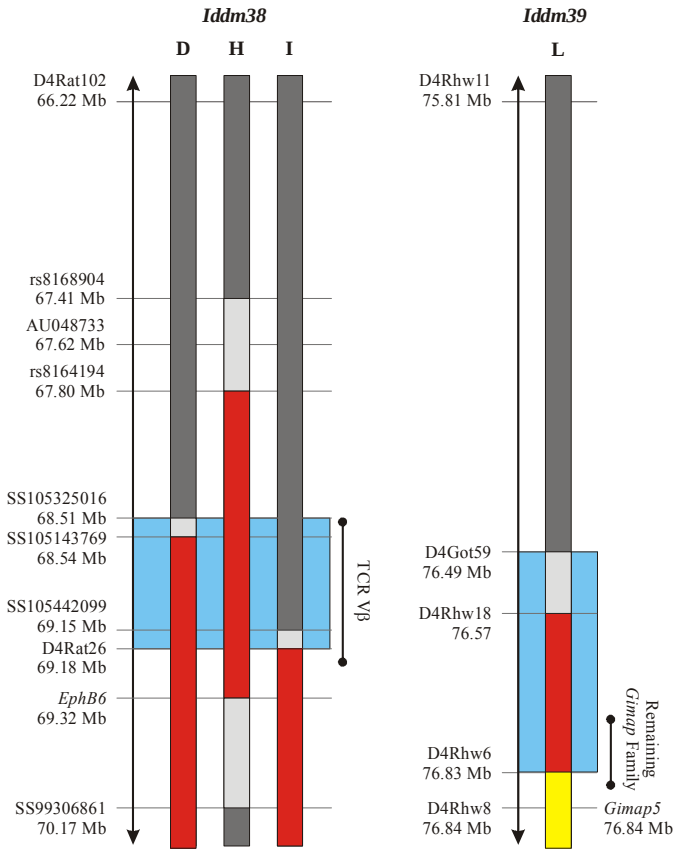

Figure 6. *Fine Mapping of Critical Intervals.* The color code is identical to Fig.2; red represents F344/F344, dark grey DR/DR, yellow DP/DP and light grey is unknown. *Iddm38*, illustrated in light blue, is defined between the proximal flanking DR SNP SS105325016 in the DRF.D congenic subline and the proximal flanking F344 SSLP marker D4Rat26 in the DRF.I congenic subline. AU048733 is the proximal *Iddm14* marker. *Iddm39*, also illustrated in light blue, is defined by the interval between markers D4Got59 and D4Rhw6. *Iddm38* and *Iddm39* are scaled within themselves but not to each other.

T1D) showed that the leftmost F344 SSLP marker D4Rat26 (69.18 Mb), in the DRF.I rat line, defined the distal border of the *Iddm38* interval (Fig.6).

Of the thirty-five potential candidate coding sequences identified in *Iddm38*, thirty-one were sequenced in BBDP, BBDR and F344 (**Paper II**, Supplementary Table B). Eight of these genes; *TryX3, Prss2,* and *TCR Vβ's 6, 8.3, 8E, 12, 13* and *20* had non-synonymous cSNPs between BBDP, BBDR and F344. *TCR Vβ6, 8.3, 8E, 12, 13* and *20* are six of twenty-four T-cell receptor variable beta genes located within the 670 Kb *Iddm38* interval on RNO4. *TryX3* and *Prss2* are part of a family of eleven trypsin genes also located within the *Iddm38* interval. Of the eight genes, only *Vβ8E, 12,* and *13* had cSNPs that were unique to both BBDP and BBDR (BB) when compared to F344 and the BN genome sequence (BN/Hsdmcwi) (UCSC) (Table 1).

Vβ8E had two coding sequence base pair deletions; one at position 260 of the mRNA, relative to the ATG start site, in BBDR and one at position 273 in BBDP, both of which

Table 1. Sequencing and Detection of *TCR Vβ* Coding SNP's in *Iddm38*

Gene Name:	***Start:***	***mRNA Position:***	***BBDP:***	***BBDR:***	***F344:***	***BN:***	***A.A Change:***
TCR Vβ8E	68.81 Mb	240	G	T	G	G	Syn
		260	C	-	C	C	SVYFCASS… 87-94 LCGSTQQ*
		273	-	T	T	T	CASS… 91-94 WPA*
TCR Vβ13	68.83 Mb	15	T	T	C	T	Syn
		21	C	C	T	T	Syn
		42	C	C	T	T	Syn
		116	***T***	***T***	C	C	S 39 F
		122	***C***	***C***	G	G	W 41 S
		145	***C***	***C***	G	G	E 49 Q
		150	***C***	***C***	A	A	R 50 S
		176	***G***	***G***	C	C	A 59 G
		211	***A***	***A***	G	G	E 71 K
		256	-	-	***T***	-	AVRT…. 86-89 CCED*
		394	C	C	T	T	Syn
		409	C	C	T	T	Syn
TCR Vβ12	68.83 Mb	206	***G***	***G***	T	T	L 69 R
		460-463	-	***AAAG***	-	-	154*
		477	-	***A***	-	-	159*
		509	-	***A***	***A***	-	NTVL... 168-188 KYSI...

* = STOP, Syn = Synonymous amino acid change

independently resulted in a premature stop codon in the hypothetical protein at amino acid position 94 (Table 1). *Vβ13* had a unique BB coding sequence haplotype across six cSNPs from mRNA position 116 to 211, all of which produced amino acid substitutions when compared to F344 and the BN genome sequence (UCSC). Analysis of *Vβ13* database mRNA sequences showed this BB haplotype to be shared by LEW (AF213508), BUF (AF213507) and AS (AF213506). In addition, a unique T base pair insertion at position 256 in F344 rats resulted in a premature stop codon. In *Vβ12*, one out of the four cSNPs resulted in a leucine (L) to arginine (R) substitution at amino acid position 69 in BB (DP and DR) (Table 1). Further sequencing analysis revealed that this amino acid substitution was not unique to BB but in fact shared by BDIX, BUF, LEW, and SHR (data not shown).

Table 2. Sequencing and Detection of Coding SNP's in *Iddm39*

Gene Name:	***Start:***	***mRNA Position:***	***BBDP:***	***BBDR:***	***F344:***	***BN:***	***A.A Change:***
Zfp467†	76.52 Mb	1024	***T***	C	***T***	C	P 342 S
		1726	***A***	T	T	T	S 576 T
Atp6v0e2	76.62 Mb	190	***C***	A	A	A	S 64 R

† = missing first 70bp of exon 4

4.1.5 *Iddm39*

Of the thirteen potential candidate coding sequences identified in *Iddm39*, twelve were sequenced in BBDP, BBDR and F344 (**Paper II**, Supplementary Table C). Two of these genes, *Zfp467* and *Atp6v0e2,* had non-synonymous cSNPs between BBDP, BBDR and F344 (Table 2). *Zfp467,* one of three zinc finger proteins located within the *Iddm39* interval, had two amino acid substitutions in the hypothetical protein sequence one of which was unique to BBDP*;* a serine (S) to threonine (T) at position 1726 relative to the ATP start site. *Atp6v0e2,* an H+ ATP vascular proton pump, had one amino acid substitution unique to BBDP; a serine to arginine (R) at position 190.

4.2 Papers III & IV

4.2.1 *Gimap* Family cDNA Sequencing

Thymus cDNA sequencing of the *Gimap* genes located within the *Iddm39* QTL (Fig.7) in **Paper III** showed three cSNPs in *Gimap4* at positions 216, 510 and 618, relative to the ATG start site, and two nucleotides deleted at position 922-923 in DR.$^{lyp/lyp}$ rats as compared to DR.$^{+/+}$ rats (Table 3). The first three cSNPs resulted in synonymous amino acid changes in the hypothetical protein sequence, while the deletion resulted in a frameshift mutation in the last three predicted amino acid residues and eliminated the normal stop codon at position 311. 3' RACE from DR.$^{lyp/lyp}$ thymus cDNA showed that the reading frame continued for another 21 amino acids before generating a new stop codon (**Paper III**, Table **2**). This same frameshift mutation was also identified in F344/Rhw (non-lymphopenic) (Table 3). One cSNP was identified in *Gimap7* at position 603, relative to the ATG start site, between DR.$^{+/+}$ and

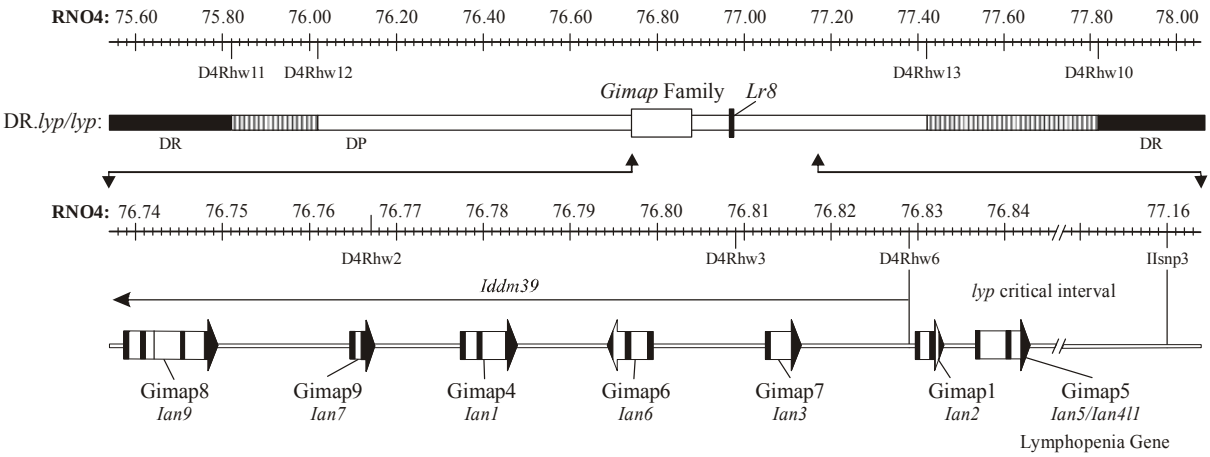

Figure 7. *The Gimap Gene Family on RNO4.* An expanded map of the 2 Mb of DP DNA in the congenic DR.*lyp* rat line is shown along with the *Gimap* family chromosomal locations on RNO4. The 33 Kb lymphopenia critical interval is indicated between the SSLP markers D4Rhw6 and IIsnp3. Arrows indicate the orientation of the full length mRNA with exons shown in black and introns in white. *Ian* aliases are in parentheses underneath the corresponding *Gimap* name.

DR.$^{lyp/lyp}$ that resulted in a synonymous amino acid change in the hypothetical protein sequence (Table 3). No cSNPs were found in *Gimap8*, *Gimap9,* and *Gimap6*.

Thymus cDNA sequencing analysis of *Gimap1* and *Gimap3,* located inside the *lyp* critical interval (Fig.7), showed a non-synonymous cSNP between DR.$^{lyp/lyp}$ and DR.$^{+/+}$ rats in *Gimap1* (Table 3). The cSNP produced a methionine (M) to threonine (T) substitution at amino acid 251, located near the C-terminus and outside the predicted GTP binding domains. Sorting intolerant from tolerant (SIFT) analysis (http://blocks.fhcrc.org/sift/SIFT.html) predicted the T substitution to be tolerated at this position and was not predicted to affect protein function. Attempts were made to amplify *Gimap3* from DR. $^{+/+}$ and DR.$^{lyp/lyp}$ rat thymus cDNA, however, no specific PCR products were obtained. Genomic sequencing of the putative ortholog of mouse *Gimap3* from base pair positions 76,846,091to 76,852,162 on RNO4 (the orthologous DNA interval to mouse *Gimap3*) in DR. $^{+/+}$ and DR.$^{lyp/lyp}$ rats revealed repetitive single or dinucleotide repeats throughout the region that likely resulted in early termination of the sequencing reactions. As such, no specific PCR products could be generated. Comparative analysis of the Brown Norway (BN/Hsdmcwi) (UCSC), with the mouse *Gimap3* database sequence (UCSC) failed to establish an open reading frame. The multiple repetitive elements added additional difficulty in locating potential exons or transcripts. No rat EST evidence could be found in the region orthologous to mouse *Gimap3* and in human, *GIMAP3* is annotated as a pseudogene. Lastly, no evidence of a *Gimap3*

transcript was found in northern blots of DR.$^{+/+}$ and DR.$^{lyp/lyp}$ or from qRT-PCR of DR.$^{+/+}$ rat thymus or spleen (data not shown). Therefore, *Gimap3* is likely a pseudogene in rat.

Table 3. *Gimap* Family Thymus cDNA Sequencing

Gene Name:	***Start (Mb):***	***mRNA Position:***	***DR.$^{+/+}$***	***DR.$^{lyp/lyp}$***	***F344:*** †	***A.A Change:***
Gimap8	76.74	-96	C	T		5' UTR
		-11	T	C		5' UTR
Gimap9	76.77	928	C	T		3' UTR
D4Rhw2	**76.77**					
Gimap4	76.78	216	A	G	A	Syn
		510	G	A	G	Syn
		618	G	A	G	Syn
						YLN*
		922-923	TA	--	--	308
						LELI….
Gimap6	76.79	No SNPs				
Gimap7	76.81	603	G	A		Syn
D4Rhw6	**76.82**					
Gimap1	76.83	752	T	C		M 251 T
Gimap5	76.84	252	C	--		IFES….
						85
						SSSQ….
		523	C	T		L 175 -
IIsnp3	**77.16**					

* = STOP, † = From genomic sequencing, Syn = Synonymous amino acid change

4.2.2 Predicted Protein Alignment

Alignment of the Gimap family predicted protein sequences in the DR.$^{+/+}$ rat in **Paper III** showed predicted GTP binding domains and conserved box characteristics for all of the Gimap proteins with the most divergent regions located near the C-terminal ends (**Paper III**, Fig.2). Gimap1 and Gimap5 were predicted to contain transmembrane domains while Gimap4 and Gimap9 were predicted to contain coiled coil domains. Gimap8, Gimap7, and Gimap6 were predicted to have neither transmembrane nor coiled coil domains. Gimap8 was larger than the other Gimap proteins, containing 688 amino acids and three repeated GTP binding domains (**Paper III**, Fig.2).

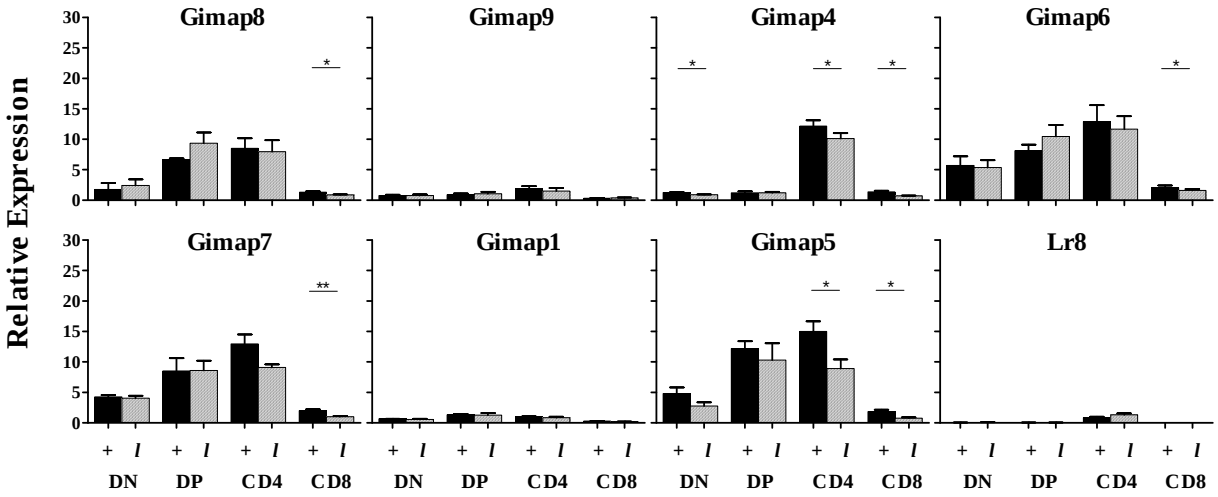

Figure 8. *Gimap Gene Expression in DR.$^{+/+}$ and DR.$^{lyp/lyp}$ Sorted Thymic Cells.* The mean ± standard error of the mean (SEM) is shown for *Gimap* family expression in CD4 and CD8 FACS sorted thymocytes following normalization to GAPDH. All statistical tests have been corrected for both litter and multiple comparisons. DN is double negative (CD4-CD8-) and DP is double positive (CD4+CD8+). Black columns represent DR.$^{+/+}$ rats (+, n=3) and hatched columns represent DR.$^{lyp/lyp}$ (*l*, n=3). Significant differences are * for $p<0.05$ and ** for $p<0.001$. Genes appear in the order at which they appear on RNO4.

4.2.3 Expression Across Multiple Tissues

In the DR.$^{+/+}$ rat (**Paper III**, Fig.3), all of the *Gimap* genes showed a higher level of expression in MLN, thymus and spleen as compared to bone marrow and kidney ($p<0.0001$). In the MLN, *Gimap4*, *Gimap5* and *Gimap8* expressed significantly higher than *Gimap9* ($p<0.0001$) while in kidney, *Gimap4* and *Gimap8* expressed significantly higher than *Gimap1* and *Gimap9* ($p<0.001$) (**Paper III**, Fig.3). Overall, *Lr8*, a gene unassociated with the *Gimap* family but also within the 2 Mb of BBDP DNA in the congenic DR.$^{lyp/lyp}$ rat line, expressed predominantly in the spleen, an expression pattern unique relative to the *Gimap* family.

4.2.4 Expression in Thymus

In **Papers III and IV**, expression analysis of the five *Gimap* genes within the *Iddm39* QTL; *Gimap8, Gimap9, Gimap4, Gimap6* and *Gimap7*, was compared to *Gimap1*, *Gimap5* and *Lr8*, located within the *lyp* critical interval (Fig.7). Quantitative RT-PCR expression analysis of the thymus in **Paper III** showed *Gimap4*, *Gimap9*, *Gimap1* and *Gimap5* were significantly underexpressed in DR.$^{lyp/lyp}$ rat as compared to DR.$^{+/+}$ (**Paper III**, Fig.4). In contrast, *Gimap7* expression in thymus was higher in DR.$^{lyp/lyp}$ as compared to DR.$^{+/+}$ while *Gimap8*, *Gimap6*

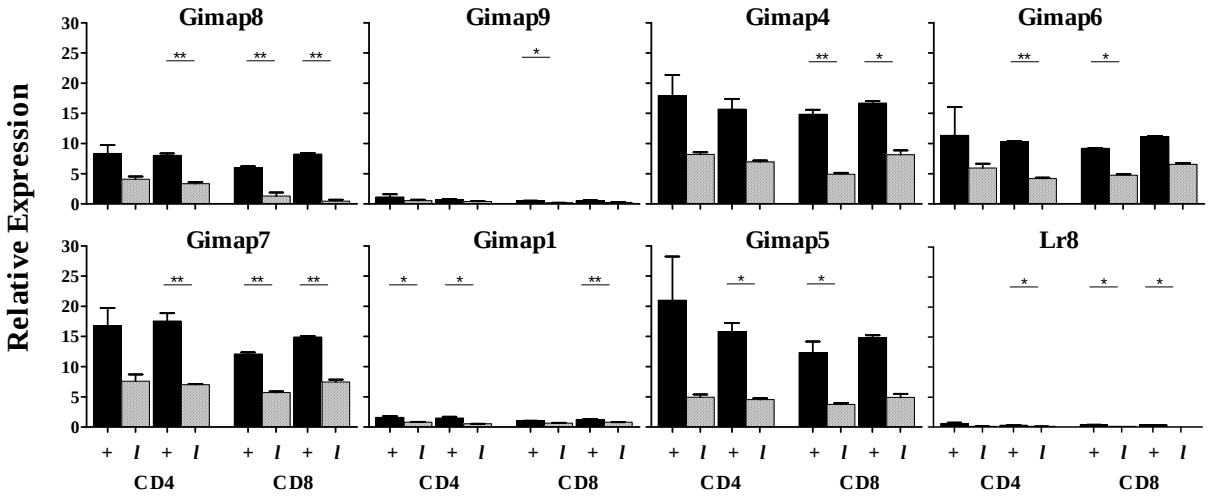

Figure 9. *Gimap Gene Expression in T cells from Spleen and MLN.* The mean ± standard error of the mean (SEM) is shown for *Gimap* family expression in CD4+ and CD8+ FACS sorted T cells (CD3+) from spleen (Spl) and MLN following normalization to GAPDH. All statistical tests have been corrected for both litter and multiple comparisons. Black columns represent DR.$^{+/+}$ rats (+, n=3) and hatched columns represent DR.$^{lyp/lyp}$ (*l*, n=3). Significant differences are * for $p<0.05$ and ** for $p<0.001$. Genes appear in the order at which they appear on RNO4.

and *Lr8* showed no differential expression. We observed the same expression pattern whether the data were normalized to cyclophilin or to total RNA (data not shown). Data from bone marrow and kidney is not shown due to the very low expression in these tissues relative to cyclophilin.

In **Paper IV**, thymocytes were sorted based on double staining with CD4 and CD8 monoclonal antibodies. The number of cells sorted did not differ between DR.$^{+/+}$ and DR.$^{lyp/lyp}$ rats and showed a high degree of purity (>90%) (**Paper IV**, Fig.2). Expression of *Gimap4* in CD4-CD8- double negative (DN), CD4 single positive (SP), and CD8 SP thymocytes differed between DR.$^{+/+}$ and DR.$^{lyp/lyp}$ rats ($p<0.05$ for all) while expression of *Gimap8* ($p<0.05$), *Gimap6* ($p<0.05$) and *Gimap7*($p<0.001$) only differed in CD8 SP cells (Fig.8). It should be noted that all statistical tests were corrected for both litter and multiple comparisons. Within the *lyp* critical interval, *Gimap5* expression was reduced in DR.$^{lyp/lyp}$ rat CD4 and CD8 SP thymocytes when compared to DR.$^{+/+}$ ($p<0.05$ for both). *Gimap9, Gimap1* and *Lr8* were not appreciably expressed at any stage of T-cell development in the thymus (Fig.8).

4.2.5 Expression in Spleen and MLN

Quantitative RT-PCR expression analysis of all of the *Gimap* genes in **Paper III** showed reduced expression of all of the *Gimap* genes in DR.$^{lyp/lyp}$ rat spleen and MLN as compared to DR.$^{+/+}$ (**Paper III**, Fig.4). In peripheral T-cells from spleen and MLN, triple stained with CD3 (T-cell receptor), CD4 and CD8 monoclonal antibodies in **Paper IV**, the number of sorted cells gated for CD3 and then sorted based on staining with CD4 and CD8 did not differ between DR.$^{+/+}$ and DR.$^{lyp/lyp}$ rats and showed a high degree of purity (**Paper IV**, Fig.2). Correcting for litter and multiple testing, we found that the expression of all *Gimap* genes (except *Gimap1*) in CD4+ splenic T-cells did not differ between DR.$^{+/+}$ and DR.$^{lyp/lyp}$ rats (Fig.9). In contrast, the expression of all of the *Gimap* genes (except *Gimap1*) within the *Iddm39* QTL was reduced in DR.$^{lyp/lyp}$ CD8+ splenic T-cells. In sorted MLN CD4+ T-cells, *Gimap9* and *Gimap4* showed no differential expression between DR.$^{lyp/lyp}$ and DR.$^{+/+}$ rats however, there was an apparent reduction in expression of *Gimap8*, *Gimap6* and *Gimap7* (Fig.9). MLN CD8+ T-cells showed a reduction in expression of *Gimap8*, *Gimap4* and *Gimap7* with no difference in expression of *Gimap9* and *Gimap6* (Fig.9). As in the thymus, *Gimap1* and *Lr8* within the *lyp* critical interval did not express appreciably in either DR.$^{+/+}$ or DR.$^{lyp/lyp}$ rat peripheral T-cells (Fig.9).

To determine *Gimap* gene expression in peripheral B-cells in **Paper IV**, hematopoietic B and *non* B-cells (including T-cells, macrophages, dendritic or NK- cells) from spleen and MLN were sorted based on double stained with CD45 (hematopoietic) and CD45RA (B-cell) monoclonal antibodies. With the exception of *Gimap6*, the *Gimap* family in did not express appreciably in DR.$^{+/+}$ or DR.$^{lyp/lyp}$ rat B-cells (CD45+CD45RA+) from spleen or MLN (**Paper IV**, Fig.V.). Expression of the *Gimap* genes within the *Iddm39* QTL; *Gimap8, Gimap9, Gimap4, Gimap6* and *Gimap7* was reduced in *non* B-cells (CD45+CD45RA-) from DR.$^{lyp/lyp}$ rat spleen and MLN. Interestingly, *Gimap9* was expressed in both DR.$^{+/+}$ and DR.$^{lyp/lyp}$ rat *non* B-cells suggesting that *Gimap9* is expressed in hematopoietic cells of *non* T- or B-cell lineage such as NK, macrophage or dendritic cells. Within the *lyp* critical interval (Fig.7), *Gimap5* showed a similar pattern with little or no expression in B-cells and a reduced expression in DR.$^{lyp/lyp}$ rat *non* B-cells (p<0.05). As in the thymus, spleen and MLN, *Gimap1* did not express appreciably in either DR.$^{+/+}$ or DR.$^{lyp/lyp}$ rat B or *non* B-cells (**Paper IV**, Fig.V.).

5 Discussion

In **Paper I** we made the unexpected observation that 34 Mb of F344 DNA introgressed proximal to the *Gimap5* mutation on the DR.$^{lyp/lyp}$ congenic background retained lymphopenia but fully protected the rats from spontaneous T1D. The study presented in **Paper I** is the first to establish that peripheral T-cell lymphopenia, due to a null mutation in the *Gimap5* gene, can be disconnected from the T1D phenotype in the BB rat. Protection from T1D in the DRF.$^{f/f}$ congenic rat line led us to conclude that spontaneous T1D in the BB rat is controlled, in part, by a diabetogenic factor(s) independent of the *Gimap5* mutation on RNO4. The major finding in **Paper II** was that the 34 Mb F344 DNA fragment harbors at least *two* genetic loci affecting spontaneous T1D in the BB rat. The two intervals; *Iddm38* and *Iddm39*, were refined to 670 Kb located between SS105325016 and D4Rat26 and a 340 Kb interval located between D4Got59 and D4Rhw6, proximal to *Gimap5,* respectively. Retaining F344 DNA at *Iddm38* or *Iddm39* results in partial T1D protection (47% and 28%, respectively) and delays time to onset while retaining F344 DNA at both *Iddm38* and *Iddm39* results in *complete* T1D protection. This suggests the interval is a compound QTL, although it remains to be determined if combining just these two regions recovers complete protection.

The fact that only two genes in the *Gimap* family, *Gimap1* and *Gimap5,* remain BBDP downstream of D4Rhw6 further underscores the finding that the cause of lymphopenia is due to the frameshift mutation in the *Gimap5* gene [53, 79]. The consequence of this mutation is not only a marked reduction in *Gimap5* transcripts [47, 53, 86] but more importantly in an apparent complete obliteration of *Gimap5* protein expression [78]. The absence of a normal gene product affirms the notion that the *Gimap5* mutation effectively is a null allele and represents a spontaneous knock-out of the *Gimap5* protein. Our genomic and cDNA sequencing of *Gimap1* in **Papers II & III** showed a coding variation between DR.$^{+/+}$ and DR.$^{lyp/lyp}$ however there is no indication that this gene contributes to the lymphopenia phenotype. It is therefore of interest that the 34 Mb of F344 genomic DNA that we introgressed onto the DR.*lyp* is, if anything, more lymphopenic than the parental DR.$^{lyp/lyp}$ rat. The congenic DRF.$^{f/f}$ rat is completely resistant to T1D indicating that T1D and lymphopenia are genetically distinct in the DR.$^{lyp/lyp}$.

Using our approach of marker assisted breeding [52, 66, 87] the DR.$^{lyp/lyp}$, DRF.$^{f/f}$ and DRF.A-L congenic sublines generated for **Papers I & II** retained the *Gimap5* mutation and, as expected, were lymphopenic. Thus, it was of interest to observe that the severity of lymphopenia differed among groups. Comparative analysis of R73+ cells in DRF.A-L congenic subline rats with the parental DRF.$^{f/f}$, the F344.$^{lyp/lyp}$ [87] and the WF.$^{lyp/lyp}$ (Fuller et al., *unpublished observations*) congenic rat lines suggests that retention of BBDP DNA downstream of D4Rhw13 (77.41 Mb) results in appearance of the severe lymphopenia phenotype. In addition, re-appearance of partial T1D penetrance in the DRF.I-L congenic sublines with significantly lower percent R73+ cells compared to DR.$^{lyp/lyp}$ rats suggests that severity of lymphopenia does not correlate with protection from T1D in the DRF.$^{f/f}$ congenic rat line.

While the mechanisms by which the *Gimap5* null allele results in lymphopenia remains to be determined, the identification of the *Gimap* family of proteins and the potential to generate specific antibody reagents [78] should make it feasible to better dissect the series of events that preclude survival of T-cells past the thymus. It cannot be excluded that cellular events preceding the onset of T1D may compromise the function of T-cells that survive the *Gimap5* null allele [151]. If that is the case however, the process would have to be specific for T1D since all of the DRF.$^{f/f}$ rats that we followed until 150 days in **Paper I** developed thyroiditis while insulitis was conspicuously absent. Two recent studies have shown that adoptive transfer of DR.$^{+/+}$ T_{reg} cells to DR.$^{lyp/lyp}$ rats protect the recipients from T1D [118, 119]. It is possible that such cells regulating diabetogenesis may be affected by the *Gimap5* null allele more than other thymic T-cells [119]. The congenic DRF.$^{f/f}$ rats should be particularly instrumental in this regard since these animals are severely lymphopenic but do not develop T1D, therefore proving useful in studies of these and other T-cell subsets in the absence of signals or events eventually leading to T1D.

Interestingly, the absence of T1D in the DRF.$^{f/f}$ and DRF.A-L congenic sublines rats in **Papers I & II** did not correlate with normal pancreas histology. While only 1% of the islets in 145 day or older non-diabetic DRF congenic subline rats had grade +4 inflammation, almost 20% of more than 1900 islets inspected had islets were scored at grade +1 suggesting that the F344 DNA introgressions fail to protect the pancreas from low-grade mononuclear cell infiltration around ducts and vessels. As such, the genetic factors of F344 origin in *Iddm38*

and *Iddm39* appear important for preventing β-cell death but not the underlying autoimmunity.

Although fewer studies have been published on the lymphocytic thyroiditis in the BB rat, compared to insulitis and T1D, several important observations have been made. First, spontaneous lymphocytic thyroiditis varies between different BBDP sub lines [152]. Second, IL1-β treatment induced thyroiditis in both BBDP and BBDR rats [153]. Based on crosses between BBDR, BBDP, Lewis and F344 rats, we showed that two susceptibility factors for T1D--the *Gimap5* null allele and the MHC--also appeared to be risk factors for thyroiditis [154]. While the *Gimap5* null allele was absolutely required for T1D, it only conferred risk for thyroiditis. Also, in contrast to T1D, RT1.B/D$^{u/u}$ conferred dominant susceptibility to thyroiditis [154, 155]. The DRF.$^{f/f}$ congenic rats developed in **Paper I** support this notion since they developed thyroiditis in the presence of lymphopenia but in the absence of T1D. These data suggest that it should be possible in the future to map both MHC and non-MHC genetic factors in the DRF.$^{f/f}$ rats that are important to the spontaneous development of thyroiditis and underscores the importance of the BB rat in dissecting the genetics of spontaneous autoimmune T1D .

Although the BBDR rat has a number of genetic factors that make it prone to develop T1D, there is no spontaneous disease; rather, the rats must be induced to be lymphopenic in order to develop T1D. Our DR.*lyp* congenic has the *lyp* allele from the BBDP strain - a single gene defect resulting in spontaneous lymphopenia - on the non-lymphopenic BBDR genomic background. Therefore, congenic DR.$^{lyp/lyp}$ have the same T1D susceptibility factors found in the BBDR, with spontaneous lymphopenia due to the genetic defect in *Gimap5*. These observations have lead to the notion that, while spontaneous T1D is controlled by the *Gimap5* null allele, the BBDR rat has all genetic factors necessary for T1D development; it simply requires immunological perturbants to develop disease [27]. In contrast, our DR.$^{lyp/lyp}$ rats are 100% lymphopenic and all develop T1D between 46 and 81 days of age in our current generations. Hence, all the genetic factors necessary for spontaneous T1D development are present in our congenic DR.*lyp* rat line.

The *Iddm38* QTL is encompassed within *Iddm14*, a locus associated with induction of T1D in the BBDR rat by administration of poly I:C and cytotoxic DS4.23 anti-ART2.1 (formerly RT6) monoclonal antibodies [27] and it is attractive to consider that *Iddm14* could itself

account for the regulation of spontaneous T1D. The approach to map *Iddm14* differs from our approach to dissect the spontaneous onset type of T1D but the present study may be of help to identify hypothetical genetic factors in *iddm14* independent of modifying genetic factors. Indeed, after inducing T1D with the KRV, a parvovirus cytopathic to T-cells but not β-cells, it was reported that not only did T1D segregate with *Iddm14* but also with a locus on RNO17 (*Iddm20*) [33]. Indeed, mapping of the *Iddm38* interval (68.51-69.18 Mb) to within the same 2.5 Mb location as *Iddm14* [156] may narrow the *Iddm14* QTL to the 670 Kb between SS105325016 and D4Rat26. Recent analysis of *Iddm14* have suggested *TCR Vβ4* [156, 157], *Vβ1* and *Vβ13* [157] as candidate genes. The *TCR Vβ* gene sequences encode the variable segment of the extracellular β-polypeptide chain that, combined with the α-polypeptide chain, forms the T-cell receptor antigen-binding site crucial for T-cell signaling.

Although sequence differences were found between BB and WF in these recent studies, we did not see them in our analyses of cSNPs in *Vβ1* or *Vβ4*. However, we did detect non-synonymous cSNPs where BBDP and BBDR were the same allele, but different from F344 and the BN database sequence (UCSC) in *Vβ8E*, *Vβ12* and *Vβ13*. The *Vβ12* polymorphism was not unique to the BB rat. During maturation of T-cells from DN to DP, a functional *TCRβ* chain is rearranged which covalently binds to a pre-TCR (essentially a non-rearranged *TCRα* chain). T-cells arranged in this manner are allowed to proliferate and mature in a process known as *β*-selection [158]. *TCR Vβ8E* (as BBDP or BBDR) and *Vβ13* (as F344) contain SNPs that generate truncations of the full-length *TCRβ* proteins and thus, theoretically, would not progress past early *β*-selection in the thymus and not be represented in the periphery. The *Vβ13* gene would be expressed in BBDR and BBDP rats as well as in WF and BN rats [157] but not in F344 because of a truncation mutation at amino acid 89. While the amino acid sequence of BBDR would be identical to that of BBDP, these two rats differ from WF and BN with a protective *Vβ13* haplotype [157]. Furthermore, *Vβ13* alleles have been shown to alter CD4/CD8 ratios on T-cells that bear one or the other allele of *Vβ13* [159]. It is therefore tempting to speculate that *Vβ13* expression on T-cells, be it CD4 or CD8, may be important to T-cell mediated β-cell death in both spontaneous and induced T1D in rats. It would be of interest to examine T1D phenotypes in DRF $^{f/f}$ rats in response to poly I:C and cytotoxic DS4.23 anti-ART2.1 monoclonal antibodies [27] or viral infection to resolve the significance of TCR *Vβ13* in both spontaneous and induced T1D. The amino acid substitutions in *Tryx3* and *Prss2* are also of potential interest as trypsin genes have been associated with exocrine cell dysfunction and pancreatitis [160].

Iddm39 also harbors several potential candidate genes. Zinc finger proteins have been associated with the regulation of immune response pathways [161] including the TLR pathways, negative regulation of macrophage activation [162] and in transient neonatal diabetes [163]. *Zfp467* itself has been characterized as a nuclear export protein and possible transcription regulator [164]. While the function of the replication initiator, *Repin1*, another zinc finger protein located within the *Iddm39* QTL, remains unknown, variations in a 3' untranslated triplet repeat have been associated with increased VLDL cholesterol, serum insulin and severity of metabolic syndrome [165]. Sequencing of the *Repin1* triplet T repeat showed BBDP with 27 repeats differing from both BBDR and F344 with 21 repeats (data not shown). As such, it cannot be excluded that *Repin1* may contribute to spontaneous BB rat T1D.

While we can exclude the involvement of the members of the *Gimap* family remaining within the *Iddm39* QTL (*Gimap8, Gimap9, Gimap4, Gimap6* and *Gimap7*) in the development of lymphopenia, we cannot exclude that they may play a role in the development of T1D. Coding sequence analysis of these family members in **Papers II & III** revealed that only *Gimap4* had genetic differences, specifically a two base pair deletion, that would result in a non-synonymous amino acid change between the non-diabetic DR.$^{+/+}$ and the T1D susceptible DR.$^{lyp/lyp}$. Although the effect of this variation is unknown, we did discover this same deletion in the non-lymphopenic, T1D resistant F344 rat. F344 DNA introgressed through this interval in the DRF.$^{f/f}$ rat protects from onset of T1D suggesting that the deletion mutation in *Gimap4* is not deleterious. In addition, the predicted protein sequences of both human (AK001972) and mouse (NP_778155.2) Gimap4 show that the 23 C-terminal amino acids are similar to those of DR.$^{lyp/lyp}$ (data not shown). It is therefore unlikely that the *Gimap4* two base pair deletion mutation in the DR.$^{lyp/lyp}$ rat is functionally relevant to development of T1D or lymphopenia, rather it is likely an additional natural isoform [166].

The candidate gene coding sequence analysis in **Papers II** and *Gimap* gene cDNA analysis in **Paper III** made it possible for us to identify causal gene sequence variants that could then be analyzed *in vitro* to provide evidence for biological function(s) related to the T1D phenotypes associated with genetic factors in either *Iddm38, Iddm39*, or both. It is possible that there are additional genetic factors controlling gene expression (transcription) that may be important for development of spontaneous T1D in the BB rat. There are numerous examples of non-coding

mutations linked to levels of expression and/or disease mechanism. A single SNP located in a polyadenylation signal of human *GIMAP5* was associated with IA-2 autoantibodies in T1D patients [167] as well as risk for systemic lupus erythematosus [168] for example. In addition, the role of microRNA's cannot be excluded although it is of interest to note that no microRNA's could be identified in either *Iddm38* or *Iddm39* via the miRNA track (miRBase) found at UCSC.

Quantitative RT-PCR analysis of *Gimap* gene expression performed in **Paper III** revealed that all the *Gimap* genes were predominantly expressed in organs associated with immune tolerance; thymus, spleen and MLN, consistent with previous findings of a role of the *Gimap* gene family in lymphocyte development [169]. Interestingly, expression of all seven *Gimap* genes; *Gimap8, Gimap9, Gimap4, Gimap6, Gimap7, Gimap1* and *Gimap5* was reduced in DR.$^{lyp/lyp}$ rat spleen and MLN when compared to DR.$^{+/+}$ while only four; *Gimap9*, *Gimap4, Gimap1*, and *Gimap5* were reduced in thymus. Quantitative RT-PCR analysis of sorted thymocytes in **Paper IV** showed that three of the *Gimap* genes within the *Iddm39* QTL; *Gimap8, Gimap6* and *Gimap7* as well as the *Gimap5* gene itself were expressed in DN and DP thymocytes with no significant difference in expression between DR.$^{+/+}$ and DR.$^{lyp/lyp}$ rats. Interestingly, while these genes were turned on during maturation of DN to DP thymocytes, no impact of the *Gimap5* mutation on gene expression was observed, as was seen in the periphery.

Upon transition from DP to SP thymocytes, expression of four of the *Iddm39* genes; *Gimap8, Gimap4, Gimap6* and *Gimap7* as well as *Gimap5* increased in CD4 SP thymocytes and showed the highest overall expression as compared to the other three stages of thymocyte development however only *Gimap4* and *Gimap5* showed expression variation between DR.$^{+/+}$ and DR.$^{lyp/lyp}$ rats. These same five *Gimap* genes were poorly expressed in CD8 SP thymocytes and all showed significant differential expression between DR.$^{+/+}$ and DR.$^{lyp/lyp}$ rats. We believe this apparent reduction in expression of *Gimap8, Gimap4, Gimap6, Gimap7* and *Gimap5* in DR.$^{lyp/lyp}$ CD8+ cells before leaving the thymus is a significant observation that may aid in understanding possible role of the *Gimap* genes in T-cells as well as their possible role in the pathogenesis of T1D.

The role of the *Gimap* genes in either protection from T-cell apoptosis, such as *Gimap5* [82, 150, 170] and *Gimap8* [149], or to induce apoptosis such as *Gimap4* [150] would perhaps

explain the severe lack of CD8+ T-cells in peripheral blood and tissues. *Gimap4* and *Gimap5* interact with the Bcl-2 family of signal transduction molecules to regulate the mitochondrial mediated T-cell apoptosis pathway [150]. *Gimap5,* predominantly characterized as a membrane bound T-cell survival protein, co-precipitates with Bcl-2 and Bcl-xL, members of the Bcl-2 family known to promote T-cell survival [150]. *Gimap4* localizes to the cytoplasm and interacts with Bax, a member of the Bcl-2 family known to facilitate T-cell apoptosis [150]. This *Gimap4*-Bax interaction may initiate the mitochondrial mediated apoptosis pathway (reviewed in [170]). The lower observed expression of *Gimap4* in the DR.$^{lyp/lyp}$ CD4+ SP thymocytes would potentially explain that more CD4+ and less CD8+ T-cells are detected in peripheral blood and tissues. It is however not understood why CD8+ cells are expressing less of all the *Gimap* genes, whether from DR.$^{+/+}$ or DR.$^{lyp/lyp}$ rats, or how this low expression impacts the ability of CD8+ T-cells to survive in the periphery.

Our observation in whole organ as well as sorted peripheral CD4+ and CD8+ splenic and MLN T-cells in **Papers III & IV** was that the loss of the Gimap5 protein was associated with a reduction in expression of all the *Gimap* family members, whether in the *Iddm39* QTL or the *lyp* critical interval, suggesting that despite an increase in expression from the thymus to the periphery, CD8+ T-cells from the DR.$^{lyp/lyp}$ rat are prone to die. While the *Gimap* genes showed the highest expression levels overall in cells sorted from the spleen and MLN, the difference in *Gimap* gene expression between DR.$^{+/+}$ and DR.$^{lyp/lyp}$ appeared more pronounced then in CD4 or CD8 SP thymocytes. It should be noted that the same number of cells were sorted from both the spleen and the MLN whether the cells were from a DR.$^{+/+}$ (typical sorting time for CD8+ T-cells was 90 minutes) or DR.$^{lyp/lyp}$ (typical sorting time for CD8+ T-cells was 4 hours) rat. The higher level of expression of all the *Gimap* genes, except *Gimap9* and *Gimap1*, in peripheral compared to thymic CD8+ cells suggests that only the highest *Gimap* expressing cells survive the transition from the thymus to the periphery.

It is tempting to speculate that the coordinate expression of the entire *Gimap* family may be due to the absence of the Gimap5 protein. However, analyses of the Gimap5 amino acid sequence show no indication that this protein would represent a transcription factor. The alternative explanation would be that *Gimap8, Gimap4, Gimap6, Gimap7* and *Gimap5* share a common transcriptional element. Bioinformatic analyses of our own and available database sequences have failed to detect a common promoter region or other sequences that would indicate the presence of a common regulator. Another hypothesis for reduction in DR.$^{lyp/lyp}$ rat

Gimap gene transcripts is a difference in organ composition from those of DR.$^{+/+}$ rats. Reduced T-cells numbers in DR.$^{lyp/lyp}$ rats could lead to a difference in cellular composition, specifically a concordant increase in B-cells, and may explain the lower observed expression of all of the *Gimap* genes. The absence of *Gimap* gene expression in B-cells in **Paper IV** indicates that this hypothesis is not sufficient to explain the apparent reduction in expression of all of the *Gimap* genes in the periphery. Further studies are therefore required to uncover the mechanisms by which *Gimap8, Gimap4, Gimap6, Gimap7* and *Gimap5* are regulated in a coordinate fashion in CD4+ and CD8+ cells from DR.$^{lyp/lyp}$ rats.

The importance of the *Gimap5* mutation for DR.$^{lyp/lyp}$ rat lymphopenia was amply illustrated by the marked reduction in expression of the *Gimap5* in the *non* B-cells sorted from the spleen or the MLN. It is not clear why *Gimap5* transcript levels are reduced as the single cytosine residue deletion results in a frameshift mutation and a premature truncation in the protein. One hypothesis is that during protein synthesis, the incomplete (truncated) protein may destabilize the RNA/protein complexes and cause mRNA degradation [171, 172]. As the *Gimap5* mutation is comparable to a null mutation, i.e. no Gimap5 protein is made [52, 78] it is of interest to note that the *Gimap5* knock out mouse that we recently reported [62] resulted in impaired thymocyte maturation as well as survival of peripheral CD4 and CD8 positive T-cells. *Gimap5* deficiency also blocked natural killer and NKT-cell differentiation which could be restored on transfer of *Gimap5* deficient bone marrow into a wild-type environment. Although the phenotype of the *Gimap5* knockout mouse is comparable to that of the DR.$^{lyp/lyp}$ rat, it remains to be clarified if the other *Gimap* genes are affected in the knockout mouse as demonstrated in **Papers III & IV**. As many of these proteins have been implicated in cell survival [82, 85, 150], it cannot be excluded that the concomitant reduction in several of the *Gimap* genes outside the *lyp* critical interval may contribute to the severe lymphopenia observed in the periphery [47, 52, 53]. As the function of Gimap proteins remains rather poorly defined, it would be a useful addendum if in vitro knockdown experiments were designed to test the functional changes that might derive from reduced expression of *Gimap5*. These types of experiments could determine if the mutation is significant in functional terms or if the altered expression the most critical feature.

In conclusion, the present study of the BB rat has been used to dissect the genetics of spontaneous autoimmune T1D. Characterization of the lymphopenic DRF.$^{f/f}$ congenic rat line with *complete* protection from T1D in **Paper I** led us to conclude that spontaneous T1D in the

BB rat is controlled, in part, by a diabetogenic factor(s) independent of the *Gimap5* mutation on RNO4. Generation of recombinant sublines in **Paper II** revealed that the 34 Mb F344 fragment, introgressed onto the DR.$^{lyp/lyp}$ rat, harbors at least *two* genetic loci; *Iddm38* and *Iddm39,* each conferring partial protection from T1D. Coding sequence analysis within the two intervals showed *TCR Vβ 8E, 12 and 13* as candidate genes in *Iddm38* and *Znf467* and *Atp6v0e2* as candidate genes in *Iddm39*. Quantitative RT-PCR expression analysis of the *Gimap* family members remaining within the *Iddm39* interval in **Papers III & IV** suggest that the lack of the Gimap5 protein in the DR.$^{lyp/lyp}$ congenic rat impairs expression of the entire *Gimap* gene family and regulates T cell homeostasis in the peripheral lymphoid organs. Further molecular identification and characterization of the genetic factors protecting from T1D in the DRF.$^{f/f}$ congenic rat line should prove critical to disclose the mechanisms by which T1D develops in the BB rat.

6 Sammanfattning På Svenska

Bakgrund och målsättning: Spontan typ 1diabetes (T1D) i den BioBreeding (BB) råttan liknar human T1D eftersom BB råttan som bär risk för diabetes (DP råttan) visar viktminskning, polydipsi, polyuria, ketoacidos, diagnos under puberteten och insulin-beroende kort tid efter diagnos. Eftersom DP råttan utvecklar diabetes spontant representerar den ett viktigt försöksdjur för att dissekera T1D's genetik utan att djuren behöver behandlas för att utveckla diabetes. BB råttan representerar två huvudtyper, diabetes risk (DP) och diabetes resistent (DR). En oförmåga att uttrycka proteinet Gimap5 i DP råttan är associerat med lymfopeni (*lyp*) som i sin tur är genetiskt kopplad till T1D. I en korsning mellan F1(BBDP x F344) råttor identifierades en råtta med en överkorsning på kromosom 4, som gjorde det möjligt att fixera 34 Mb av F344 gener mellan markörerna D4Rat253 och D4Rhw6 i en kongen DR.*lyp* stam. Denna kongena DR stam innehåller bara 2 Mb DP DNA inklusive Gimpa5 gene mutationen all annan genetiskt material är BB DR. Målsättningen med denna avhandling var att karakterisera det F344 DNA som fixerats i DR BB råttan och testa hypotesen att F344 genetiska faktorer resulterar i 1) ingen effekt på diaebtesutveckling eller 2) en skyddande effekt på diabetesutveckling. Målsättningen var också att med hjälp av positionskloning karakterisera kandidatgener på råttans kromosom 4 som påverkar risken för T1D.

Material och metoder: F344 genomiskt DNA i den kongena DRF.$^{f/f}$ råttan fixerades i den kongena DR.*lyp* råttan i ett totalt antal av nio "backcross" och sju "intercross". Kongena DRF.$^{f/f}$ råttor korsades sedan med inavlade BBDR eller DR.$^{lyp/lyp}$ råttor. Avkomman genotypades för T1D risk gener och fenotypen bestämdes för lymfopeni och om råtttan utvecklade diabetes. Kandidat gener som identifierats p g a sitt läge i genomet sekvensbestämdes eller analyserades med cDNA sekvensering eller kvantitativ real-tids PCR.

Resultat: DRF.$^{f/f}$ rats, homozygota för F344 alleler, hade lymfopeni men utvecklade inte T1D. Alla (100%) DR.$^{lyp/lyp}$ rats utvecklade T1D innan 83 dagars ålder. Avel för att få fram kandidatgener som skulle kunna förklara varför F344 alleler skyddar mot T1D visade att en reduktion av F344 fragmentet med 26 Mb (42.52 Mb-68.51 Mb) bevarade den skyddande effekten. Ytterligare avel gjorde det möjligt att reducera F344 DNA fragmentet till 2 Mb

(67.41-70.17 Mb). Detta fragment (*iddm38*) visade sig innehålla genetiska faktorer som innehöll 47% skydd mot T1D. Ett mindre, mer distalt fragment (*iddm39*) resulterade in 28% skydd mot T1D. Både *iddm38* och *iddm39* gjorde att råttorna utvecklade T1D vid senare ålder. Jämförande analys av T1D frekvens i de olika kongena stammarna förfinade *iddm38* och *iddm39* till respektive c:a 670 Kb och 340 Kb proximalt om *Gimap5*. Analys av kodande sekvenser visade att *TCR Vβ 8E, 12 and 13* skulle kunna vara kandidatgener i *iddm38* och *Znf467* samt *Atp6v0e2* i *Iddm39*. Kvantitativ RT-PCR analys av hela organ såväl som FACS sorterade thymocyter eller perifera T celler som färgats med monoklonala antikroppar mot CD4 eller CD8 visade en reduktion i expression av samtliga fem *Gimap* gener i *iddm39* intervallet förutom den reduktion visades för *Gimap1* och *Gimap5.*

Konklusioner: Föreliggande resultat visar att introgression av 34 Mb F344 genom proximalt om den muterade *Gimap5* genen framkallar resistens mot T1D trots att den kongena DR.*lyp* råttan hade lymfopeni. Utveckling av ytterligare substammar visade att spontan diabetes i BB råttan kontrolleras delvis av två genetiska loci, *Iddm38* och *Iddm39*, bägge oberoende av *Gimap5* mutation på RNO4. Analys av kodande sekvenser visade att *TCR Vβ 8E, 12 and 13* är kandidatgener i *Iddm38* och *Znf467* samt *Atp6v0e2* i *Iddm39*. Dessa resultat antyder att frånvaron av Gimpa5 proteinet i DR.$^{lyp/lyp}$ kongena råttor nedsätter expressionen av hela familjen av *Gimap* gener och därigenom reglerar T cellers överlevnad i perifera lymfoida organ. Framtida identifiering och karakterising av de genetiska faktorer som skyddar BB råttan från T1D i den kongena DRF.$^{f/f}$ råttan kommer att göra det möjligt att förklara mekanismerna på vilket sätt T1D spontant utvecklas i BB råttan.

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9 Appendix

The following are publications related to the subject or methods used in my investigation in which I have collaborated during the development of this thesis

- J. Mordes, L. Cort, E. Norowski, J. Leif, **J.M. Fuller**, D. Greiner, Å. Lernmark, E. Blankenhorn. *Analysis of the Iddm14 Rat Diabetes Susceptibility Locus in Multiple Rat Strains: Identification of a Susceptibility Haplotype in the Tcrb-V Locus.* Mammalian Genome 2009 Mar;20(3):162-9. Epub 2009 Feb 10.

- J.E. Blevins, J. Overduin, **J.M. Fuller**, D.E. Cummings, K. Matsumoto, D.H. Moralejo. *Normal Feeding and Body Weight in Fischer 344 Rats Lacking the Cholecystokinin-1 Receptor Gene.* Brain Research 2009 Feb 19;1255:98-112. Epub 2008 Dec 16.

- L. Åkesson, R.W. Gelling, R. Jensen, K. Ogimoto, **J.M. Fuller**, R. Pefley, S. Manavi, Å. Lernmark, M.W. Schwartz. *Increased Lipid Oxidation Heralds Diabetes Onset in DR.lyp/lyp Rats.* Experimental and Clinical Endocrinology & Diabetes 2008 Aug;116(8):475-80. Epub 2008 May 9.

- I.R. Sweet, O. Yanay, L. Waldron, M. Gilbert, **J.M. Fuller**, T.D. Tupling, Å. Lernmark, W.R. Osborne. *Treatment of Diabetic Rats with Encapsulated Islets.* Journal of Cellular & Molecular Medicine 2008 Dec;12(6B):2644-50. Epub 2008 Mar 28.

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