Genetic dissection of lymphopenia from autoimmunity by introgression of mutated *Ian5* gene onto the F344 rat

Daniel H. Moralejo[a]*, Hyunhee A. Park[a], Sara J. Speros[a], Armand J. MacMurray[a], Anne E. Kwitek[b], Howard J. Jacob[b], Eric S. Lander[c], Åke Lernmark[a]

[a] *Robert H. Williams Laboratory, Department of Medicine, University of Washington, Seattle, WA 98195, USA*
[b] *Human and Molecular Genetics Center, Medical College of Wisconsin, Milwaukee, WI 53226, USA*
[c] *Whitehead/MIT Center for Genome Research, Cambridge, MA 02139, USA*

Abstract

Peripheral T cell lymphopenia (*lyp*) in the BioBreeding (BB) rat is linked to a frameshift mutation in *Ian5*, a member of the Immune Associated Nucleotide (Ian) gene family on rat chromosome 4. This lymphopenia leads to type 1 (insulin-dependent) diabetes mellitus (T1DM) at rates up to 100% when combined with the BB rat MHC RT1 *u/u* genotype. In order, to better study the lymphopenia phenotype without possible confounding effects of diabetes or other autoimmune disease, we generated congenic F344.*lyp* rats by introgression of *lyp* on diabetes-resistant MHC RT1 *lv1/lv1* F344 rats. Analysis of thymic CD4 and CD8 T lymphocytes revealed no difference in the percentage of $CD4^-CD8^+$ and $CD4^+CD8^-$ subsets in *lyp/lyp* compared to +/+ F344 rats. The same subsets was however dramatically reduced in blood ($P=0.005$), spleen ($P=0.019$) and mesenteric lymph nodes (MLN) ($P<0.0001$). Compared to F344 +/+ rats double positive $CD4^+CD8^+$ T cells were increased only in *lyp/lyp* spleen ($P=0.034$) while double negative $CD4^-CD8^-$ were increased in thymus ($P=0.033$), spleen ($P=0.012$), MLN ($P<0.0001$), and peripheral blood ($P<0.0001$). There were no signs of inflammatory lesions in organs and tissues in F344.*lyp/lyp* rats examined at 120 days of age or older. We thus conclude that the lymphopenia phenotype was reconstituted by introgression of *lyp* on to F344 rats without subsequent development of organ-specific autoimmunity. The congenic F344.*lyp* rat should prove useful to dissect the mechanisms by which the *Ian5* frameshift mutation affects T cell selection, differentiation and maturation without organ-specific autoimmunity.
© 2003 Elsevier Ltd. All rights reserved.

Keywords: Lymphocytopenia; Insulitis; Autoimmunity; T-cell development; BB rat; F344 rat

1. Introduction

Diabetes in the BB rat is associated with autoimmunity not only to the pancreatic islet cells but also to other endocrine organ [1]; BB rats were found positive for autoantibodies to gastric parietal cells, thyroid colloid antigens, smooth muscle cells and thymocytes [2,3]. The genetic susceptibility to autoimmune diseases is notoriously complex; however, the BB rat has proven to be a very useful model to dissect the genetics of organ specific autoimmunity [4–7]. First, lymphopenia (*lyp*) on chromosome 4 is absolutely required for diabetes along with MHC RT1 *u/u*, on chromosome 20 [4,8,9]. To study the *Iddm1/lyp* locus in the absence of the other *Iddm* loci, we generated a congenic strain (DR.*lyp*), where lymphopenia (*lyp*) and *Iddm1* from a line of inbred diabetic prone BB rats (BBDP) was introgressed on to the genome of inbred diabetes-resistant BB rats (BBDR) by marker assisted selection [10]. This congenic rat strain has confirmed that a single locus is responsible for both T cell lymphopenia and spontaneous autoimmune diabetes. In the 6 years since we completed the congenic DR.*lyp* line, and in all recombinant animals developed from this strain, we have never had an animal develop diabetes without lymphopenia (*lyp*). The lymphopenia gene (*lyp*) has a recessive mode of inheritance and in specific pathogen free (SPF) condition type 1 diabetes occur in 100% of homozygous *lyp/lyp* BB rats but not in

* Corresponding author. Tel.: +1-206-543-0756; fax: +1-206-543-3169

E-mail address: moralejo@u.washington.edu (D.H. Moralejo).

lyp/+ or +/+ congenic DR.*lyp* animals [5,10]. In our previous cross–intercross analysis we showed that the MHC RT1 *lv1*/*lv1* or *u*/*lv1* was sufficient to protect lymphopenic *lyp*/*lyp* rats from developing diabetes [11], also demonstrating that RT1 *u*/*u* was a more important risk factor for thyroiditis than for lymphopenia [12]. The lymphopenia in diabetes prone BB rats [13,14] is due to a mutation in a novel (*Ian*) related gene, *Ian5* [5,6]. The frameshift mutation in *Ian5* is linked to a marked reduction in both CD4^{+} and CD8^{+} T cell subsets in the peripheral blood and secondary lymphoid organs. Along with this, the RT6.1 differentiation marker (also known as Art2a) is markedly reduced and at the same time there is an increase in Thy.1 positive T cells [15]. While the specific mechanism of lymphopenia is still unclear, in order to more fully examine the consequences of the *Ian5* mutation in different genetic background, we generated a congenic line F344.*lyp* rats in which the critical *lyp* region has been introgressed on to the diabetes-resistant F344 rat. To rapidly generate the F344.*lyp* rats (within 30 months breeding), we used marker-assisted selection (MAS). The aim of the present study was to test the hypothesis that introgression of the lympho-penia gene on to F344 rats would induce lymphopenia and tissue inflammation. That would depend on the F344 rat MHC RT1 *lv1*/*lv1*, and to produce a rat line in which the function of the defective rat *Ian5* gene could be studied without the possible confounding factor of background T1DM.

2. Materials and methods

2.1. Generation of F344.lyp/lyp rats

Since the expression of Type 1 diabetes in the BB rats has been shown to be altered by infection, all animals used for this study were kept under SPF conditions with standard light–dark cycles. The rats were fed with standard diet (LabDiet, PMI Nutrition International, LLC, Brentwood, MO, USA). Sentinel animals were negative for the following viral antibodies and parasites (Sendai virus, pneumonia virus of mice, sialodacryo-adenitis virus, rat corona virus, Kilham's rat virus, rat parvovirus type 1, *Mycoplasma pulmonis*, pinworms, and fur mites) during the period of the experiments. F344 rats were purchased from Charles River (Wilmington, MA, USA). The parental DR.*lyp*/*lyp* rats were selected from our 10th cycle of (BB) cross-intercross between DP and DR rats [10]. These rats have the *lyp* gene introgressed on to the inbred DR rats and are referred to as DR.*lyp* rats. The congenic line was generated by MAS in five cycles of cross–intercross breeding with whole genome scanning for F344 homo-zygosity at non-*Lyp* loci. At 25–30 days of age the rats were weaned and ear marked, a tail biopsy was taken to extract DNA for genotyping and at the same time peripheral blood was collected for T cell phenotyping.

2.2. Detection of diabetes

Starting from 50 days of age, DR.*lyp* and their progeny being the F344 *lyp*/*lyp*, *lyp*/+ or +/+ were weighed daily on a Sartorius Electronic scale (Model BL 1500, Sartorius GmbH, Göttingen, Germany). Since the growth rate differs between male and female rats, they were weighed and comparisons were stratified by sex. The onset of type 1 diabetes in DR.*lyp* rats is preceded by 1–3 days of decreased weight gain [16] and by blood glucose levels equal to or exceeding 13 mmol/l (Exac-Tech Glucose Meter, Medisense Inc. Bedford, MA, USA). Blood glucose levels were checked in rats that did not gain weight. An animal was considered diabetic if the morning blood glucose levels equaled or exceeded 13 mmol/l and the rats remained hyper-glycemic the morning of the following day.

2.3. Genetic typing by radioactive PCR

SSLP markers were used to define the *lyp* region flanked by R236 and Npy [10] by PCR performed with radioactively labeled primers and products visualized on acrylamide gels as detailed elsewhere [4]. One primer was end labeled with ^{32}P-ATP using T4 kinase according to standard protocols. Twenty-five nanograms of genomic DNA were denatured at 95 °C for 4 min and then snap cooled on ice for 2 min. The denatured samples were amplified in a 10 µl PCR reaction using Ampli Taq DNA polymerase. The primer concentrations were 100 nM of each of the two unlabeled primers and 20 nM of one end-labeled primer. PCR products were mixed with loading buffer consisting of xylene cyanol and bromophenol blue dyes in 100% formamide, denatured for 5 min on a 95 °C heating block, and electrophoresed on 7% polyacrylamide gels for 3 h at 120 W. Gels were wrapped in Saran Wrap and exposed directly to film for 4–16 h at −80 °C. Autoradiographs were independently scored by two people, who were unaware of the rat's phenotypes.

2.4. Genotyping by Li-Cor

Genomic DNA was amplified in a 5 µl PCR reaction using Taq polymerase (Sigma, St Louis, MO, USA). The PCR reactions are as follows: 25 ng/µl genomic DNA, 100 nM forward and reverse primers, the forward primer was coupled to fluorescence labeled dATP (IRD700-dATP) (LI-COR, Lincoln, NE, USA). Conditions for PCR cycles are 1 cycle at 94 °C for 2 min, 35 cycles at 94 °C for 30 s, 55 °C for 35 s and 72 °C for 30 s and 1 cycle at 72 °C for 3 min. Annealing temperature was defined according to the melting tem-perature of the primers and ranges between 52 °C and

60 °C. A single dye automated DNA sequencer from (LI-COR, Lincoln, NE, USA) driven by pentium computer was used to detect the PCR products. Reactions were analyzed on a 6% acrylamide gel on a LI-COR 4000L sequencer (LI-COR, Lincoln, NE, USA), using 18-cm long gel apparatus, 0.25-mm thickness. Electrophoresis parameters were set at 950 V, 36.4 mA and 35 W. The gel was pre-run until electrical parameters remained constant. The fluorescence was excited at 685 nm. PCR products were mixed with 2 µl of loading dye (95% formamide, 0.01% bromophenol blue, and 0.01% xylene cyanol) and then denatured at 94 °C for 2 min before loading. At the end of the run the gel image is saved in LI-COR 16-bit format, together with an associate gel file where the molecular weight standards used were identified with their position on the gel. The gel image and information file were transferred automatically by an FTP procedure in an incoming repertory of SUN Unix computer to be processed by SAGA which is an automated genotyping software. Scoring was done independently by two people, who were unaware of the rat's phenotypes.

2.5. Genome wide scanning, sequence and restriction enzyme analysis of the F344 rat Immune associated nucleotide (Ian5)

In order to confirm introgression of the *lyp* region on to the F344 rat, total genome scans were performed. The MAS strategy was used, and for each backcross generation the genome scanning was performed with 150 simple sequence repeat (SSR) genetic markers. Primer pairs were designated for amplification of the rIan5-coding exons 2 and 3 [5]. The exon 3 carrying the single nucleotide deletion [5], and the single nucleotide polymorphism (SNP) by restriction enzyme assay were used as a marker for the introgressed fragment from chromosome 4. Primers 5′-GGGAAGGAGCTTCCTAGTG G-3′ (nt 219–238) and 5′-GCTGCTCCTCCCCAGA AG-3′ (nt 586–569) were used for PCR fragment amplification. The PCR products, with the exception of the control, were digested with BsrI, analyzed by electrophoresis in a 4.0% agarose gel (SeaKem LE agarose, BioWhittaker Molecular Applications, Rockland, ME, USA), and stained with ethidium bromide.

2.6. Detection of lymphopenia

One hundred microliters of blood was diluted 1:1 in 0.1 Mol/l EDTA in PBS. The erythrocytes were lysed at room temperature for 6 min in 0.16 M NH_4Cl and washed by centrifugation at $400 \times g$ for 5 min in PBS supplemented with 4% BSA (pH 7.4). The cells were incubated for 30 min at room temperature in the dark with fluorescein isothiocyanate (FITC)-conjugated mouse anti-rat TCR-specific (R73) reagent

(Pharmingen, San Diego, CA, USA) 1:200 in PBS with 4% bovine serum albumin (BSA). After two washes with 200 µl of 4% BSA in PBS, the cells were fixed for 20 min at room temperature in 200 µl of 0.5% paraformaldehyde (PFA) in the dark and centrifuged at $400 \times g$ for 10 min and the supernatant discarded. The pellets were resuspended in 300 µl of 0.1% propidium iodide solution and store at 4 °C. Fraction of TCR+ cells among 10,000 leukocytes was determined on a FACScan (Coulter EPICS ELITE, FL, USA). An animal was considered lymphopenic if <25% of the leukocytes were TCR+.

2.7. Differential counts

At about 180 days of age, an equal number of rats of each genotype and gender were euthanized and 5–10 ml of blood was obtained by heart puncture. The blood was diluted 1:1 in PBS containing 0.1 Mol/l EDTA and 1% (w/v) BSA (pH 7.4) and analyzed by automated differential counting (Cell Dyn Model # 3500, Abbott, Santa Clara, CA, USA) of white blood cells (WBC).

2.8. T cell purification

Rats under anesthesia were bled and thymus, spleen, and mesenteric lymph nodes removed into sterile RPMI-1640 medium with 10% (v/v) FCS, made into single cell suspensions between two frosted glass slides and filtered over a cell 100 µm strainer nylon mesh. T cells were purified using nylon wool fiber columns (Polysciences, Inc., Warrington, PA, USA) followed by incubation at 37 °C for 1 h. The flow through which contains non-adherent cells was collected. Peripheral blood lymphocytes were purified from a total of 5–10 ml blood obtained by heart puncture and diluted in an equal volume of PBS supplemented with 0.1 Mol/l EDTA. The fresh anti-coagulated blood was transferred to a 50 ml conical centrifuge tube containing an equal volume of PBS with 1% (w/v) BSA. Five ml of both Histopaque-1077 and Histopaque-1119, respectively (Sigma, St Louis, MO, USA) per 10 ml of blood diluted in PBS was slowly layered from underneath the blood. Multiple gradients were centrifuged at 18–20 °C for 30 min at $900 \times g$ with no brake. The cells at the upper opaque layer interface were transferred to another pre-wetted 50 ml centrifuge tube and washed at 4 °C by centrifugation at $400 \times g$ for 10 min. The isolated cells were collected in 96-well round bottom polystrene plates (Costar, Corning, NY, USA), rinsed and resuspended to a final concentration of 5×10^6 cell/ml in PBS containing 1% BSA and 0.02% sodium azide.

2.9. Flow cytometry

Approximately 1×10^6 cells were stained with fluorescent-labeled antibodies in 100 µl staining buffer

(PBS containing 4% BSA) for 30 min on ice in 96-well round bottom plates. Cells were washed twice in staining buffer before fixing for 20 min on ice in PBS containing 1% PFA. The cells were resuspended in 300 μl PBS and analyzed by flow cytometry. Two and three-color flow cytometric analysis was performed on a FACS-440 (Becton Dickinson Immunocytometry Systems, Sunnyvale, CA, USA) equipped with a helium–neon and argon laser. Acquisition was performed using the Consort 30 program, and analysis was done with Lysis 1(Becton Dickinson). The flow cytometer was calibrated with QC3 (FITC/PE/Cy-Chrome) beads. Forward and side light-scatter were used for identification of lymphoid cells and exclusion of contaminating dead cells. The following panel of monoclonal antibodies were purchased from (Pharmingen, San Diego, CA, USA) to be used for analysis of T cell subsets: R-phycoerythrin (R-PE)-labeled anti-CD3 (G4.18), Cy-chrome labeled anti-CD4 (OX35), FITC labeled anti-CD8 (OX8), Peridinin chlorophyll-a protein (PerCP) labeled anti-CD90 (Thy-1), and FITC labeled anti alpha-beta T cell receptor (R73).

2.10. Histology

Multiple organs and tissues were removed and fixed in 4% (w/v) PFA in PBS. The specimens were washed three times in 50% ethanol for 1 h each and preserved in 70% ethanol before embedding in paraffin and 5 μm sections cut and stained with hematoxylin and eosin. Coded sections were evaluated under light microscope for infiltration of mononuclear cells. The degree of infiltration was scored on coded slides (duplicate for each animal) by two independent investigators according to the following scale: 0, no infiltration; +1, infiltration with mononuclear cells around blood vessels or ducts; +2, occasional mononuclear cells infiltrating the parenchyma; +3, distinct mononuclear cell infiltration; +4, mononuclear cell infiltration, only with little recognizable normal tissue.

2.11. Statistics

Mean and standard error of the mean (SEM) was calculated for the number of rat indicated. Student's *t*-test or one way ANOVA test for paired comparisons were used and differences were considered significant at $P < 0.05$.

3. Results

3.1. Generation of F344.lyp rats by speed congenic breeding

In order to study the effect of the *Ian5* (*lyp*) mutation on a F344 genetic background, we first established a line

of F344.*lyp* congenic rats using MAS at each backcross. Initially, DR.*lyp/lyp* rats were mated with F344 rats to generate an intercross. A male rat from this intercross was then backcrossed to a female F344 rat, generating N2 rats. In the N2 generation a total genome scan was performed and one male N2 *lyp/+* rat with the largest number of F344 alleles (that is, with the genome closest to our desired goal) was selected and crossed with a female F344 rat. Continuing with the same strategy, we produced an N5 generation and again used a total genome scan to select those animals to be used for inbreeding by sister–brother intercross. As described below, those animals carried only the F344 alleles of markers outside the *lyp* region, and so the N5 *lyp/+* rats were intercrossed to generate F1 rats, the founders of our line of F344.*lyp* congenic rats. As expected, these offspring carried the *lyp/lyp*, *lyp/+* and *+/+* genotype in a 1:2:1 ratio.

3.2. Genome scanning

In the progeny of F344.*lyp* rats of the 5th cycle of introgression (N5) all tested loci were present as F344 alleles except for those markers immediately flanking the *lyp* gene, which has been selected to maintain the *lyp* haplotype D4Mit5 (R236), D4Mgh24, D4Mit6 and D4Mit24 (Npy). The distance between the flanking markers of *lyp* (D4Mit5 and D4Mit24) is estimated to be about 1 cm. The critical *lyp* interval in F344.*lyp* rat harbors four *Ian* family members (Fig. 1), but the rat *Ian3* gene was excluded as a candidate gene for *lyp* [5]. The sequence of the introgressed *lyp* fragment confirmed the presence of a single-nucleotide frameshift deletion in the *Ian5* gene (Fig. 2 a), with one SNP downstream of the deletion (Fig. 2 b). Digestion of PCR products from F344.*+/+* animals produced three fragments (168 bp, 136 bp, and 64 bp), and PCR products from F344.*lyp/lyp* produced two fragments (232 bp and 136 bp) (Fig. 2b). The second BsrI recognition site is not present in F344.*lyp/lyp* rats due to a silent SNP (ACTGG–ATTGG) within the IAN-5 gene.

3.3. Growth of F344.lyp rats

A total of 10 rats of each *lyp* genotype of F344.*lyp* rats were weighed daily until 170–180 days of age (data not shown). There was no statistically significant difference in body weight gain between *lyp/lyp, lyp/+* and *+/+* F344 rats. All rats were normoglycemic and did not develop any sign of diabetes. These results indicate that the *lyp* allele of *Ian5* neither affect growth nor induce diabetes in the F344.*lyp* rats.

3.4. Differential counts of WBC

None of the heterozygous parents used in the intercross was lymphopenic, while 25% (F344.*lyp/lyp*) of
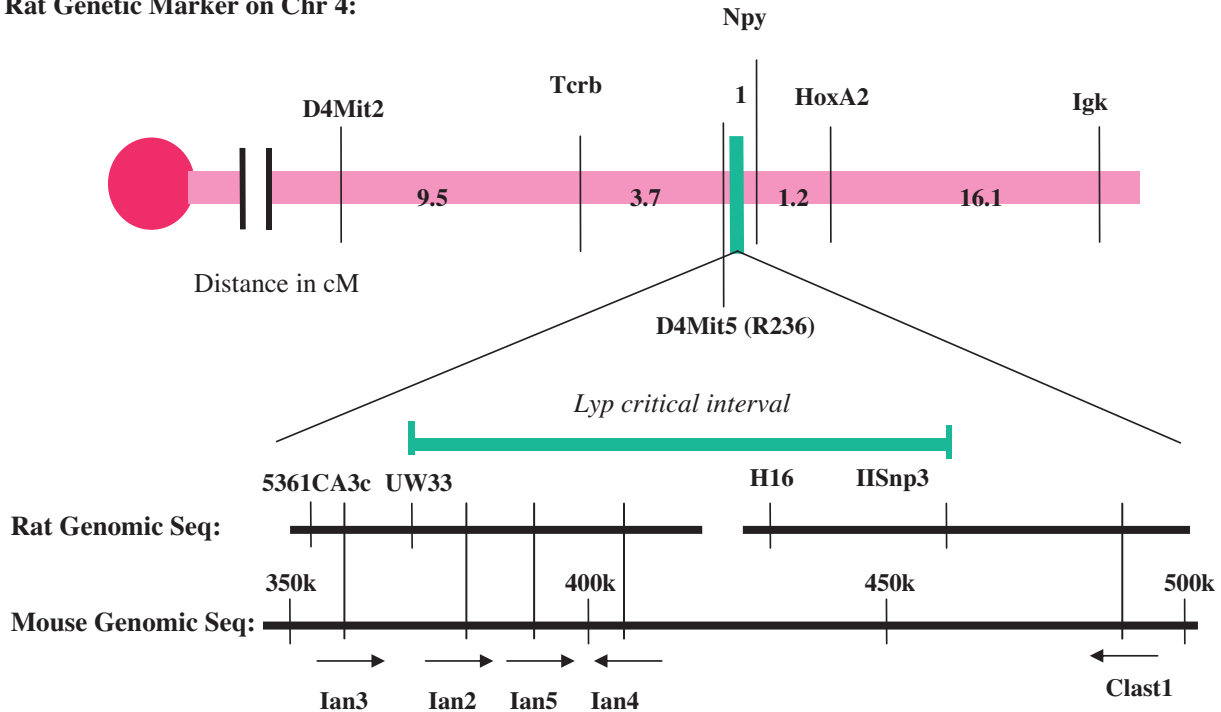
Rat Genetic Marker on Chr 4:

Fig. 1. Genetic localization of the lymphopenia region. Physical map of the rat *lyp* gene region on chromosome 4 with genetic markers integrated. Distance between markers may not be strictly to scale. The bottom part shows an expanded view of the *lyp* critical interval, showing the location of known genes in the rat based on assembled sequence contigs of rat genomic DNA. A 13-kb long rat genomic sequence contig include the rat *Ian5* gene. Position coordinates shown are those from mouse sequence supercontig Mm6_WIFeb01_100.

the F2 generation rats developed lymphopenia. We observed a significant reduction in the total number of WBC for *lyp/lyp* compared to the wild type +/+ F344 rats ($P=0.002$) and the lymphocyte count was markedly reduced as well (Table 1). Interestingly, there was also a significant increase in the total number of neutrophils and monocytes in *lyp/lyp* compared to +/+ F344 rats ($P=0.006$ and $P=0.01$), respectively (Table 1).

3.5. Flow cytometry of thymus cells

The analysis of thymocytes revealed no significant difference in the percentages of CD4$^+$CD8$^-$ subsets between *lyp/lyp*, *lyp/+* and +/+ F344 rats. CD4$^-$CD8$^+$ T cells were increased (ANOVA=0.044) in the *lyp/lyp* compared to the +/+ F344 rats (Table 2). However there was a reduction of CD4$^+$CD8$^+$ ($P=0.02$) and an increase of CD4$^-$CD8$^-$ ($P=0.033$) T cells in the *lyp/lyp* compared to that of +/+ F344 rats. The alpha-beta TCR subset was reduced in *lyp/lyp* compared to the +/+ F344 rats ($P=0.016$) but no difference was found for CD90$^+$ and CD3$^+$ T cells (Table 2 and Fig. 3).

3.6. Flow cytometry of spleen cells

Analyses of the spleen T cells showed a marked difference in the percentage of R73$^+$, CD90$^+$ and CD3$^+$ T cells between *lyp/lyp*, *lyp/+* and +/+ F344 rats

(ANOVA<0.0001) (Fig. 3). The percentage of CD4$^+$CD8$^-$ T cells was markedly reduced in the *lyp/lyp* rats ($P<0.0001$) (Table 2) while the percentage of double negative CD4$^-$CD8$^-$ was elevated in the *lyp/lyp* compared to the +/+ F344 rats ($P=0.0116$) (Table 2).

3.7. Flow cytometry of mesenteric lymph node cells

The percentages of CD4$^+$CD8$^-$, CD4$^-$CD8$^+$ T cells were dramatically decreased while CD4$^-$CD8$^-$ T cells were increased by a factor of two in the MLN of *lyp/lyp* compared to *lyp/+* and +/+ F344 rats. These three subsets showed the same degree of significance (ANOVA $P<0.0001$) (Table 2). CD3$^+$, CD90$^+$, and R73$^+$ T cells showed a dramatic difference in the percentage between *lyp/lyp*, *lyp/+*, and +/+ F344 rats (ANOVA <0.0001) (Fig. 3).

3.8. Flow cytometry of peripheral blood

We also observed reduced percentages of CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ T cells in the *lyp/lyp* rats compared to that of *lyp/+* ($P<0.0001$ and $P=0.005$), and +/+ ($P<0.0001$ and $P=0.004$) F344 rats (Table 2). The percentage of CD4$^-$CD8$^-$ T cells was strikingly increased in the *lyp/lyp* rats compared to that of *lyp/+* ($P<0.0001$) and +/+ ($P<0.0001$) F344 rats (Table 2). CD3$^+$ and R73$^+$ T cells subsets was reduced, and CD90$^+$
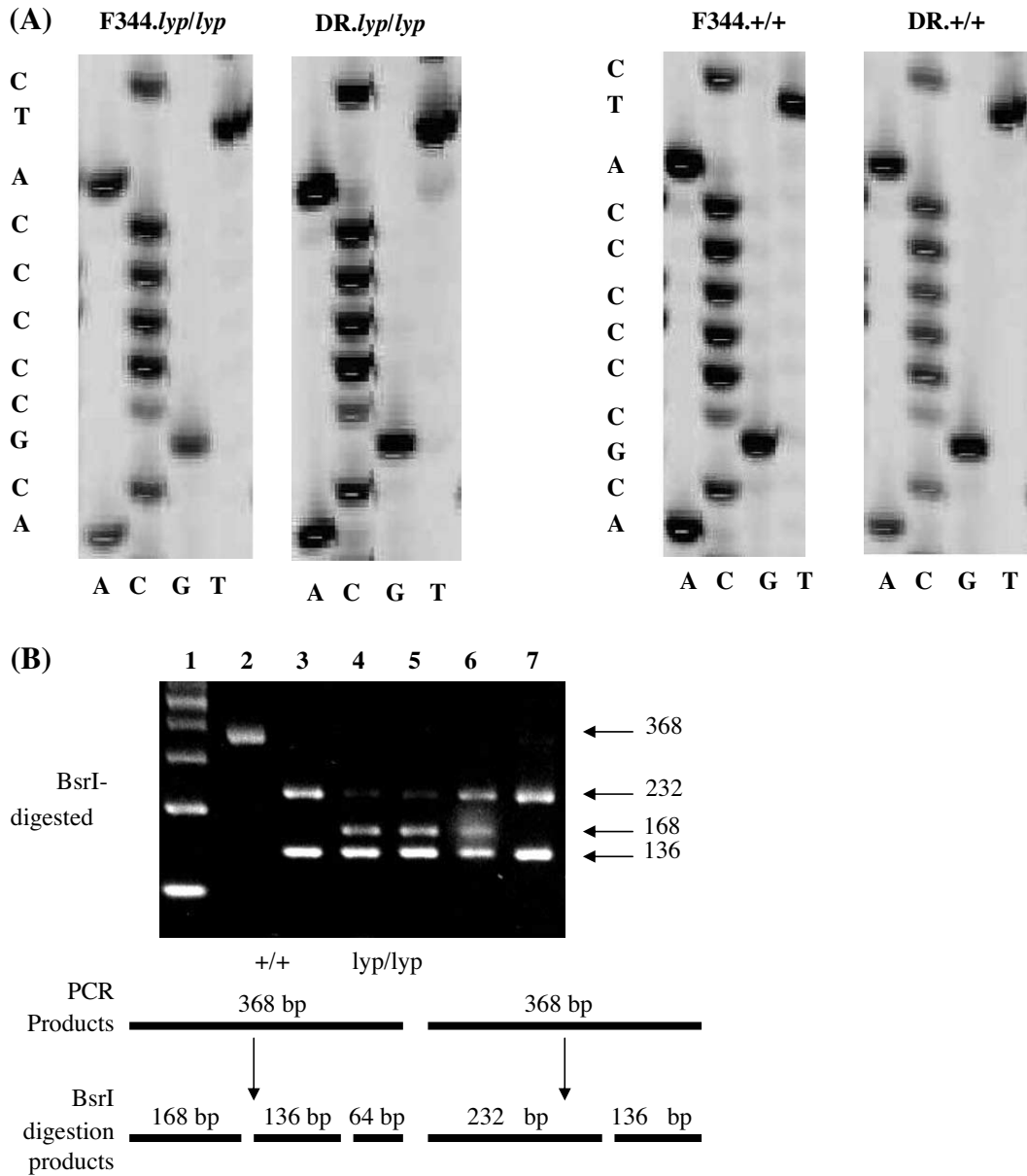
Fig. 2. (a) Sequence of the F344.*lyp/lyp* rat Immune associated nucleotide (*Ian5*). A representative sequencing trace of DNA from F344.*lyp/lyp* compared with DR.*lyp/lyp*, F344.+/+, and DR.+/+ rats. (b) Genotyping of r*Ian5* by PCR-RFLP analyses. Lane 1, 100 bp marker; uncut PCR product in lane 2; digestion of PCR products from BBDR.*lyp/lyp* and BBDR.+/+ rats in lane 3 and 4; and F344.+/+, F344.*lyp*/+, and F344.*lyp/lyp* rats in lane 5, 6, and 7, respectively. The 64-bp fragment is not represented in the figure.

T cell population increased in *lyp/lyp* compared to that of *lyp*/+ and +/+ F344 rats (Fig. 3).

3.9. Histology

Histology of various organs did not reveal any evidence of infiltration by mononuclear cells in any of the organs and tissues examined. The thymus of the lymphopenic rats showed largely normal appearance. The spleen of *lyp/lyp* F344 rats showed a diminution of white pulp.

4. Discussion

The lymphopenic BB rat develops striking abnormalities in T cells associated not only with diabetes, but also with a more generalized loss of tolerance [17]. In this work we have shown that lymphopenia was effectively

Table 1

Differential counts (cells/µl) of white blood cells in the congenic F344.*lyp* rats

| | F344 rat genotypes | | | P value | | | |
	+/+ (a)	*lyp*/+(b)	*lyp*/*lyp*(c)	a vs. b	b vs. c	a vs. c	One-way ANOVA
WBC	2864 ± 204 (11)	3135 ± 247 (11)	1786 ± 232 (11)	n.s.	0.0007	0.0023	0.0005
Neutrophils	543 ± 41 (11)	630 ± 60 (11)	868 ± 99 (11)	n.s.	n.s.	0.0064	0.0083
Lymphocytes	2153 ± 201 (11)	2311 ± 187 (11)	383 ± 32 (11)	n.s.	<0.0001	<0.0001	<0.0001
Monocytes	38 ± 12 (11)	55 ± 12 (11)	78 ± 7 (11)	n.s.	n.s.	0.0096	0.0402
Eosinophils	41 ± 5 (11)	43 ± 8 (11)	54 ± 6 (11)	n.s.	n.s.	n.s.	n.s.
Basophils	35 ± 6 (11)	41 ± 5 (11)	40 ± 7 (11)	n.s	n.s.	n.s.	n.s.
Platelets	571 ± 27 (11)	523 ± 20 (11)	585 ± 9 (11)	n.s.	0.0092	n.s.	n.s.

Mean values ± SEM for the number of rats indicated. The results are shown as 10^{-3} cells/µl for platelets. P value <0.05 is considered significant.

Table 2

T cell subsets in thymus, spleen, mesenteric lymph nodes and peripheral blood in *lyp*/*lyp*, *lyp*/+ and +/+ F344 rats

| | F344 rat genotypes | | | P value | | | |
	+/+ (a)	*lyp*/+ (b)	*lyp*/*lyp* (c)	a vs. b	b vs. c	a vs. c	One way ANOVA
Thymus							
CD3	44 ± 13 (9)	65 ± 9 (13)	48 ± 8 (25)	n.s	n.s	n.s	n.s
$CD4^+CD8^+$	78 ± 3 (9)	59 ± 4 (13)	66 ± 3 (25)	0.002	n.s	0.0196	0.007
$CD4^-CD8^+$	7 ± 1.8 (9)	25 ± 6 (13)	16 ± 3 (25)	0.0362	n.s	n.s	0.0445
$CD4^+CD8^-$	7 ± 1.9 (9)	6 ± 1.5 (13)	7 ± 1.0 (25)	n.s	n.s	n.s	n.s
$CD4^-CD8^-$	8 ± 1.5 (9)	14 ± 1 (13)	12 ± 0.8 (25)	0.0124	n.s	0.0329	0.0186
Spleen							
CD3	46 ± 4 (9)	55 ± 7.1 (10)	13 ± 1.4 (11)	n.s	<0.0001	<0.0001	<0.0001
$CD4^+CD8^+$	19 ± 5 (9)	22 ± 4 (10)	37 ± 6 (11)	n.s	n.s	0.0338	0.0414
$CD4^-CD8^+$	22 ± 4 (9)	18 ± 4 (10)	11 ± 1 (11)	n.s	n.s	0.0186	n.s
$CD4^+CD8^-$	46 ± 4.1 (9)	45 ± 3 (10)	17 ± 2 (11)	n.s	<0.0001	<0.0001	<0.0001
$CD4^-CD8^-$	17 ± 1 (9)	16 ± 2 (10)	31 ± 4 (11)	n.s	0.0058	0.0116	0.0018
Mesenteric Lymph nodes							
CD3	65 ± 4 (9)	59 ± 5 (9)	18 ± 3 (20)	n.s	<0.0001	<0.0001	<0.0001
$CD4^+CD8^+$	3 ± 1.2 (9)	4 ± 1.2 (9)	1 ± 0.5 (20)	n.s	0.0391	n.s	n.s
$CD4^-CD8^+$	24 ± 2.1 (9)	21 ± 1.4 (9)	2 ± 0.2 (20)	n.s	<0.0001	<0.0001	<0.0001
$CD4^+CD8^-$	22 ± 6.1 (9)	37 ± 1.6 (9)	11 ± 2 (20)	0.0332	<0.0001	0.0368	<0.0001
$CD4^-CD8^-$	44 ± 7 (9)	39 ± 3 (9)	86 ± 2 (20)	n.s	<0.0001	<0.0001	<0.0001
Blood							
CD3	60 ± 7 (10)	63 ± 6 (11)	8 ± 1.6 (8)	n.s	<0.0001	<0.0001	<0.0001
$CD4^+CD8^+$	8 ± 3.0 (10)	9 ± 1.9 (11)	4 ± 1.9 (8)	n.s	n.s	n.s	n.s
$CD4^-CD8^+$	15 ± 2 (10)	20 ± 3 (11)	6 ± 1.5 (8)	n.s	0.0053	0.0048	0.0063
$CD4^+CD8^-$	49 ± 6 (10)	50 ± 5 (11)	12 ± 2 (8)	n.s	<0.0001	<0.0001	<0.0001
$CD4^-CD8^-$	30 ± 5 (10)	26 ± 8 (11)	83 ± 4 (8)	n.s	<0.0001	<0.0001	<0.0001

The results are shown as percentage of cell subsets. Mean values ± SEM for the number of rats indicated. P value <0.05 is considered significant.

established in the F344.*lyp*/*lyp* congenic rats by introgression of the mutated form of the novel rat *Ian5* gene [5]. Lymphopenia occurred in 100% of the F344 rats homozygous for *lyp*/*lyp* but not in *lyp*/+ or +/+ congenic F344.*lyp* animals. As expected, the lymphopenia phenotype in the new congenic line was reconstituted without a subsequent development of diabetes.

Since the age of onset of Type 1 diabetes in DP BB rats ranges from 60–90 days [11] and thyroiditis from 60–170 days of age [12] we examined the animals for diabetes from 60 days of age, and thyroiditis or any other autoimmune disease until 180 days of age. The rats were healthy and did not develop any signs of diabetes, thyroiditis, tissue infiltration, inflammation or any other autoimmune disease. Our results therefore support the notion that *lyp* alone does not cause diabetes or thyroiditis which are both dependent on the *u*/*u* (or similar) genotype [12]. More importantly, there was no
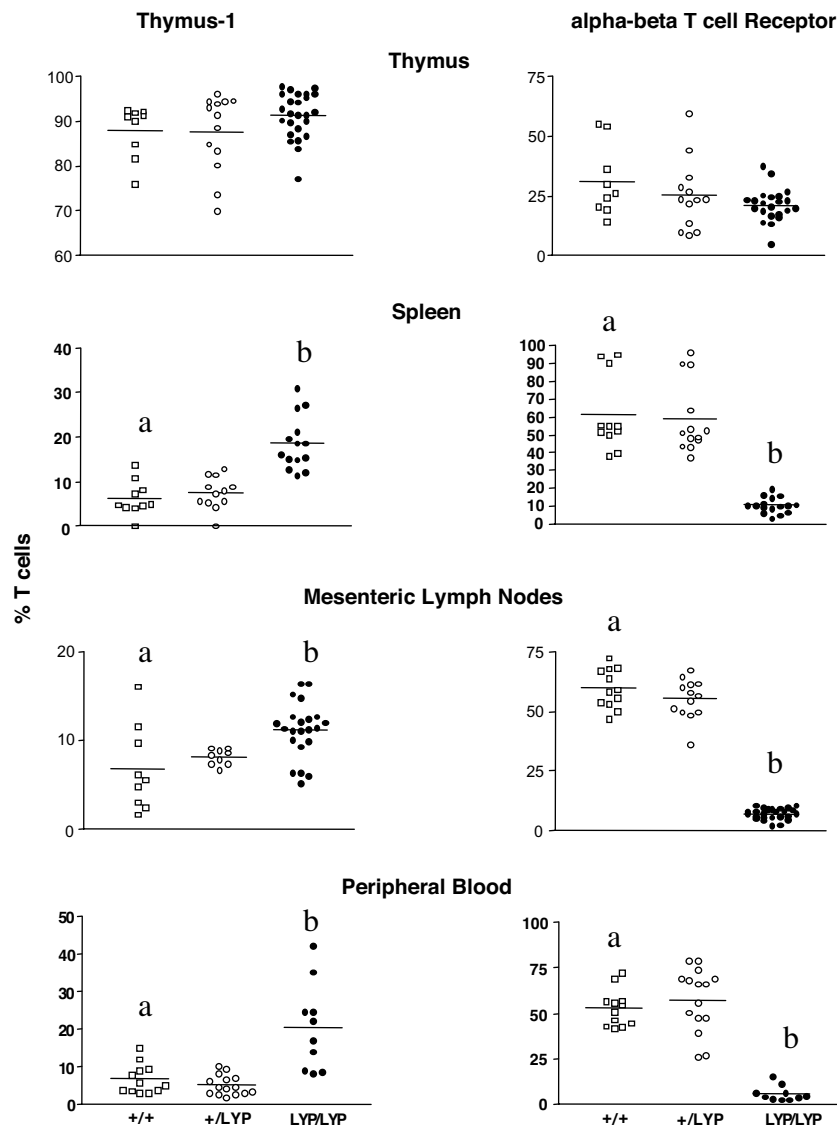
Fig. 3. Scatterplots of alpha-beta T cell receptor and Thy-1 in thymus, spleen, MLN, and peripheral blood of (9–12) F344.+/+, (13–15) F344.*lyp*/+, and (10–25) F344.*lyp*/*lyp* rats. Values are expressed as percentages of positive cells are shown. The scale levels differ between assay to illustrate their ranges in the phenotyped rats. *P* values are for spleen, MLN, and peripheral blood for Thymus-1 (CD90) and (R73) between *lyp*/*lyp* (b) and +/+ (a) F344 rats (<0.0001, <0.0001, =0.0032, <0.0001, <0.0001, <0.0001) respectively.

sign of any other autoimmune disease, which suggests that the *lv1*/*lv1* MHC genotype in F344 rats is resistant to spontaneous organ-specific autoimmunity. Since both the sexes of the *lyp*/*lyp* rats gained weight normally as compared to *lyp*/+ and +/+ F344 rats we also conclude that *lyp* does not have any effect on body weight.

As expected, we noted a profound reduction in the number of peripheral T lymphocytes in the F344.*lyp*/*lyp* rats, which indicates that the lymphopenia phenotype was successfully reconstituted by introgression of the critical *lyp* gene region on to F344 rats. The single nucleotide mutation in the *Ian5* gene [5] that causes a truncated protein suggests that the expression of the *Ian5* gene should affect the T cell subsets in our F344 congenic-line.

We observed a significant reduction in the total number of peripheral leukocytes. This can be explained by the fact that 70% of the leukocytes are lymphocytes and any significant reduction in the lymphocyte count would decrease the total number of leukocytes. Therefore from our data we conclude that earlier studies on diabetic BB rats showing a large increase in eosinophils, along with smaller increases in neutrophils, monocytes and lymphocytes at the time of onset of overt diabetes is associated with a significant leukocytosis [18], and due to the lymphopenia phenotype in the F344.*lyp*.

The *Lyp* mutation dramatically reduces the life span not only of the peripheral T cells, but also of the CD4 and CD8 single positive thymocytes by inducing or failing to prevent spontaneous premature apoptosis [19].

The differences in percentages of CD4$^+$CD8$^+$ and CD4$^-$CD8$^-$ subsets between *lyp/lyp* and +/+ F344 rats indicate an abnormal differentiation of T cells in the F344.*lyp/lyp* rat thymus (Table 2). These findings were not consistent with earlier studies showing that the frequency of CD4$^+$CD8$^+$ and CD4$^-$CD8$^-$ subsets was not different between BBDP and BBDR [20], and BBDP/Ed and congenic lymphopenic PVG-*lyp/lyp* (RT1u) and (RT1c) rat thymocytes [19]. The increase in the percentage of CD4$^-$CD8$^+$ T cells in the thymus of F344.*lyp* rat is however not consistent with earlier studies showing a reduction in thymic CD8 single positive T cells. It should be noted that the extent of reduction in CD8 thymocyte numbers caused by the *lyp* defect might be influenced by other genes in the BB rat [19]. Differences were reported in the RT6$^+$, Thy-1$^+$, and CD25$^+$ T cell subsets in the congenic line F344.*lyp*RT1^u [21]. Our congenic line carrying the RT1.*lv1/lv1* showed an increase in the percentage of Thy-1$^+$ in spleen, MLN, and peripheral blood. We speculated that this difference is caused solely by the lymphopenia gene. Since in our study we used congenic lymphopenic rats, which have only the short *lyp* region from the BB rat, and there is no possibility of interference of other genes from the BB rat we conclude that the *lyp* mutation does cause an increase in the percentages of double negative CD4$^-$CD8$^-$ and a reduction of double positive CD4$^+$CD8$^+$ T cells in the thymus.

The observed reduction of splenic and MLN CD4$^+$CD8$^-$ T cell subset and increase in immature CD4$^-$CD8$^-$ T cells in the *lyp/lyp* compared to +/+ F344 rats support previous reports of severe T cell lymphopenia in spleen and lymph nodes of BBDP rats [20]. The CD4/CD8 ratio in MLN was constitutively higher in *lyp/lyp* compared to +/+ F344 rats.

Previous studies showed that the number of CD8$^+$ T cells in the peripheral blood was more severely reduced than that of CD4$^+$ T cells [22,23]. Others have reported the near total absence of CD8$^+$ T cytotoxic/suppressor population in both diabetic and diabetes-prone BB/Wor rats [24]. Interestingly, our F344.*lyp/lyp* rats showed a decrease in the percentages of CD4$^+$, CD8$^+$ (ANOVA $P<0.0001$, $P=0.006$), and an increase of CD4$^-$CD8$^-$ (ANOVA $P<0.0001$) T cells in the peripheral blood of *lyp/lyp* compared to that of the *lyp/+* and +/+ F344 rats. The CD4$^-$CD8$^+$ T cells in the BB rats are considered to have autoreactive properties that are essential for the development of Type 1 diabetes [23]. In our study lymphopenic rats revealed low CD4$^+$ and CD8$^+$ T cells, and increased CD4$^-$CD8$^-$ T cells in the peripheral blood indicating that these T-lymphocyte abnormalities of congenic Fischer rats segregate with the lymphopenia gene and are therefore not related to diabetes risk. The Fischer rats are protected from developing diabetes by the presence of protective genes [25,26].

The histology of the thymus was normal in the F344 *lyp/lyp* rats compared to that of the controls. Previous studies comparing the thymus of BB rat with that of the WF rat revealed a normal BB rat thymus histology and normal percentages of T lymphocyte subsets [3,27]. Histology of the spleen revealed marked diminution of the white pulp supporting previous studies that the effect of *lyp* is more pronounced in the spleen, lymph nodes, and peripheral blood [24,28].

In conclusion, our data demonstrate that introgression of *lyp* is an efficient approach to cause lymphopenia but proved insufficient to induce frank autoimmune disease development in the F344 rat. The F344.*lyp* rats therefore represent a unique line in which lymphopenia is expressed in the absence of diabetes or other apparent autoimmunity. The F344.*lyp/lyp* rats represent a perfect tool to study lymphopenia independent of autoimmune abnormalities and should be useful to determine whether the Ian5 gene is a key factor in T-cell development. If the mechanisms of *Ian5*-associated T cell development can be clarified in this rat, it should be possible to demonstrate whether (as we expect) similar mechanisms are applicable to human T-cell development. Finally the understanding of the genomic organization of the Ian gene family and the possible interaction between these genes during T cell differentiation may help to elucidate the possible role of T cells in human type 1 diabetes and other autoimmune disorders.

References

[1] Bieg S, Lernmark Å. Animal models for insulin-dependent diabetes mellitus. In: Volpé R, editor. Autoimmune endocrinopathies. Totowa (NJ): Humana Press; 1999;113–40.

[2] Elder M, Maclaren N, Riley W, Mcconnell T. Gastric parietal cell and other autoantibodies in the BB rat. Diabetes 1982;4:313–8.

[3] Maclaren NK, Elder ME, Robbins VW, Riley WJ. Autoimmune diatheses and T lymphocyte immunoincompetences in BB rats. Metabolism 1983;32:92–6.

[4] Jacob H, Pettersson A, Wilson D, Lernmark Å, Lander ES. Genetic dissection of autoimmune type 1 diabetes in the BB rat. Nat Genet 1992;2:56–60.

[5] Macmurray AJ, Moralejo DH, Kwitek AE, Rutledge EA, Van Yserloo B, Gohlke P et al. Lymphopenia in the BB rat model of Type 1 diabetes is due to a mutation in a novel Immune-associated nucleotide (Ian)-related gene. Genome Res 2002; 12:1029–39.

[6] Hornum L, Romer J, Markholst H. The diabetes-prone BB rat carries a frameshift mutation in Ian4, a positional candidate of Iddm1. Diabetes 2002;51:1972–9.

[7] Martin AM, Blankenhorn EP, Maxson MN, Zhao M, Leif J, Mordes JP et al. Non-major histocompatibility complex-linked diabetes susceptibility loci on chromosomes 4 and 13 in a back-cross of the DP-BB/Wor rat to the WF rat. Diabetes 1999; 48:50–8.

[8] Colle E, Guttmann RD, Seemayer T. Spontaneous diabetes mellitus syndrome in the rat. J Exp Med 1981;154:1237–42.

[9] Markholst H, Andreasen B, Eastman S, Lernmark Å. Diabetes segregates as a single locus in crosses between inbred BB rats prone or resistant to diabetes. J Exp Med 1991;174:297–300.

[10] Bieg S, Koike G, Jiang J, Klaff L, Pettersson A, Macmurray AJ et al. Genetic isolation of the Lyp region on chromosome 4 in the diabetes prone BioBreeding (BB) rat. Mamm Genome 1998; 9:324–6.

[11] Pettersson A, Jacob H, Lernmark Å. Lessons from the animal models: the BB rat. In: Palmer JP, editor. Diabetes prediction, prevention and genetic counseling in IDDM. New York: John Wiley & Sons Ltd; 1996.

[12] Pettersson A, Wilson D, Daniels T, Tobin S, Jacob JJ, Lander ES et al. Thyroiditis in the BB rat is associated with lymphopenia but occurs independently of diabetes. J Autoimmun 1995;8:493–505.

[13] Poussier P, Nokhooda AF, Sima AF, Marliss EB. Lymphopenia in the spontaneously diabetic "BB" Wistar rat: Passive transfer of insulitis in the presence of donor rat lymphopenia. Clin Invest Med 1981;4.

[14] Poussier P, Nakhooda AF, Falk JA, Lee C, Marliss EB. Lymphopenia and abnormal lymphocyte subsets in the "BB" rat: relationship to the diabetic syndrome. Endocrinology 1982; 110:1825–7.

[15] Thiele HG, Haag F. The RT6 system of the rat: developmental, molecular and functional aspects. Immunol Rev 2001;184:96–108.

[16] Markholst H, Eastman S, Wilson D, Fisher L, Lernmark Å. Decreased weight gain in BB rats before the clinical onset of insulin-dependent diabetes. Diabetes Res Clin Pract 1993;21:31–8.

[17] Ramanathan S, Poussier P. BB rat lyp mutation and Type 1 diabetes. Immunol Rev 2001;184:161–71.

[18] Eastman S, Markholst H, Wilson D, Lernmark Å. Leukocytosis at the onset of diabetes in crosses of inbred BB rats. Diabetes Res Clin Pract 1991;12:113–23.

[19] Hernandez-Hoyos G, Joseph S, Miller NG, Butcher GW. The lymphopenia mutation of the BB rat causes inappropriate apoptosis of mature thymocytes. Eur J Immunol 1999; 29:1832–41.

[20] Jung CG, Kamiyama T, Agui T. Elevated apoptosis of peripheral T lymphocytes in diabetic BB rats. Immunology 1999;98:590–4.

[21] Hornum L, Lundsgaard D, Markholst H. An F344 rat congenic for BB/DP rat-derived diabetes susceptibility loci Iddm1 and Iddm2. Mamm Genome 2001;12:867–8.

[22] Naji A, Silvers WK, Kimura H, Bellgrau D, Markmann JF, Barker CF. Analytical and functional studies on the T cells of untreated and immunologically tolerant diabetes-prone BB rats. J Immunol 1983;130:2168–72.

[23] Groen H, Klatter FA, Brons NH, Mesander G, Nieuwenhuis P, Kampinga J. Abnormal thymocyte subset distribution and differential reduction of CD4+ and CD8+ T cell subsets during peripheral maturation in diabetes-prone BioBreeding rats. J Immunol 1996;156:1269–75.

[24] Woda BA, Like AA, Padden C, Mcfadden ML. Deficiency of phenotypic cytotoxic-suppressor T lymphocytes in the BB/W rat. J Immunol 1986;136:856–9.

[25] Klaff LS, Koike G, Jiang J, Wang Y, Bieg S, Pettersson A et al. BB rat diabetes susceptibility and body weight regulation genes colocalize on chromosome 2. Mamm Genome 1999;10:883–7.

[26] Kwitek AE, Speros S, Tschannen M, Fuller J, MacMurray A, Majewski B et al. A diabetes resistance locus identified in a cross between the BB and F344 rat strains. FASEB J 2002;16:A864.

[27] Elder ME, Maclaren NK. Identification of profound peripheral T lymphocyte immunodeficiencies in the spontaneously diabetic BB rat. J Immunol 1983;130:1723–31.

[28] Hosszufalusi N, Chan E, Granger G, Charles MA. Quantitative analyses comparing all major spleen cell phenotypes in BB and normal rats: autoimmune imbalance and double negative T cells associated with resistant, prone and diabetic animals. J Autoimmun 1992;5:305–18.