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Moralejo, Daniel H.; Fuller, Jessica; Rutledge, Elizabeth A.; Van Yserloo, Brian; Ettinger, Ruth A.; Jensen, Richard; Osborne, William; Kwitek, Anne; Lernmark, Åke

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BB rat *Gimap* Gene Expression in Sorted Lymphoid T and B Cells

Daniel H. Moralejo[1], Jessica M. Fuller[2,3], Elizabeth A. Rutledge[4], Brian Van Yserloo[3], Ruth A. Ettinger[3], Richard Jensen[3], William Osborne[5], Anne Kwitek[6] and Åke Lernmark[2,3]

[1]Dept. of Comparative Medicine, University of Washington 1959 N.E. Pacific St., Box 357710, Seattle, Washington, 98195, USA

[2]Dept. of Clinical Sciences, Lund University, Clinical Research Center, Entrance 72, Building 91:10, Malmö, 20502, Sweden

[3]Dept. of Medicine, University of Washington, 1959 N.E. Pacific St., Box 357710, Seattle, Washington, 98195, USA

[4]Salish Kootenai College, Department of Life Sciences, 58138 Hwy 93, Pablo, MT 59855, USA

[5]Dept. of Pediatrics, University of Washington, 815 Mercer St., Box 358055, Seattle, 98109, USA

[6]Dept. of Internal Medicine, University of Iowa, 375 Newton Rd, 3111B MERF, Iowa City, IA, 52242, USA

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Corresponding author:

Daniel H. Moralejo
University of Washington
Department of Comparative Medicine
1959 NE Pacific St.
Box 357710
Seattle, WA 98195
Phone: 206-221-4587
Fax: (206) 543-3169
Email: moralejo@u.washington.edu

ABSTRACT

The *Gimap* gene family has been shown to be integral to T cell survival and development. A frameshift mutation in *Gimap5,* one of seven members of the *Gimap* family, results in lymphopenia and is a prerequisite for spontaneous type 1 diabetes (T1D) in the BioBreeding (BB) rat. While not contributing to lymphopenia, the *Gimap* family members proximal to *Gimap5,* encompassed within the *Iddm39* quantitative trait locus (QTL), have been implicated in T1D. We hypothesized that expression of the *Gimap* family members within the *Iddm39* QTL, during thymocyte development as well as in peripheral T and B cell subsets, may contribute to T1D. Quantitative real time (qRT) PCR analysis of cell sorted subpopulations showed that expression of *Gimap4* was reduced in DR.$^{lyp/lyp}$ rat double negative, double positive and CD8 single positive (SP) thymocytes while expression of *Gimap8, Gimap6,* and *Gimap7* was reduced only in CD8 SP thymocytes. Interestingly, expression of the entire *Gimap* gene family was reduced in DR.$^{lyp/lyp}$ rat peripheral T cells compared to non-lymphopenic, non-diabetic DR.$^{+/+}$ rats. With the exception of *Gimap6*, the *Gimap* family genes were not expressed in B cells from spleen and mesenteric lymph node (MLN). Expression of *Gimap9* was only detected in hematopoietic cells of *non* B cell lineage such as macrophage, dendritic or NK cells. These results suggest that the lack of the Gimap5 protein in the DR.$^{lyp/lyp}$ congenic rat was associated with impaired expression of the entire family of *Gimap* genes and may regulate T cell homeostasis in the peripheral lymphoid organs.

INTRODUCTION

The GTPase immunity-associated protein (*Gimap*) gene family in the rat is comprised of eight genes located within 150 Kb on rat chromosome (RNO) 4 [1, 2]. The seven functional *Gimap* genes in rat; *Gimap8, Gimap9, Gimap4, Gimap6, Gimap7, Gimap1* and *Gimap5* are conserved across multiple vertebrates including human and mouse as well as in *Arabidopsis thaliana* [2-4]. These small GTPases regulate pro- and anti- apoptotic T cell pathways [5-12] as well as thymocyte maturation and differentiation [7, 13] and are characterized by novel guanosine diphosphate/guanosine triphosphate (AIG1) and coiled-coil domains, common binding motifs and variable C termini [2, 4].

Peripheral T cell lymphopenia in the BioBreeding (BB) diabetes prone (DP) rat is linked to a single nucleotide deletion in *Gimap5* (*Iddm2; lyp*) resulting in a premature stop codon and truncation of the full-length protein [4, 14]. This truncation results in loss of *Gimap5* protein expression [16] and is a prerequisite for onset of spontaneous type 1 diabetes (T1D) in the DP rat [4, 15]. Normal *Gimap5* transcript and protein levels were restored in the F344.$^{lyp/lyp}$ rat containing a 150-kb P1 artificial chromosome (PAC) transgene harboring the wild-type *Gimap5* allele [16]. While the *Gimap5* mutation was not associated with T1D in humans [17], the uncommon SNP in the polyadenylation signal of human *GIMAP5* was found to be associated with risk for high IA-2 autoantibody levels in T1D patients [18] implicating a possible role in islet autoimmunity. The common SNP at this location was associated with systemic lupus erythematosus underscoring the possible contribution of *GIMAP5* to autoimmunity [19].

Positional cloning of *Gimap5* was in part due to generation of the DR.*lyp* congenic rat line with 2 Mb of DP DNA, encompassing *Gimap5,* introgressed onto the BB diabetes resistant (DR) genetic background [4, 15, 20]. DR.$^{lyp/lyp}$ rats, homozygous for the DP DNA introgression, are 100% lymphopenic and 100% develop diabetes by 84 days of age [15]. In contrast, 34 Mb of F344 DNA, introgressed proximal to *Gimap5* in the DR.$^{lyp/lyp}$ rat, generates rats that are 100% lymphopenic but protected from T1D development (DRF.$^{f/f}$) [15, 21]. Development of DRF.$^{f/f}$ congenic sublines revealed two quantitative trait loci (QTL) encompassed within the 34 Mb F344 DNA interval on RNO4, each of which confer partial protection from spontaneous T1D in the DR.$^{lyp/lyp}$ rat [21]. The first, *Iddm38* (previously referred to as Region 1), is a 670 Kb interval encompassing the *TCR Vβ* and *Trypsin* gene sequences. The second, *Iddm39* (previously referred to as Region 2) maps to within the 340 Kb proximal to *Gimap1* and *Gimap5*. Thus, it cannot be excluded that the *Gimap* genes proximal to *Gimap1; Gimap8, Gimap9, Gimap4, Gimap6* and *Gimap7* play a role in onset of T1D in the DR.$^{lyp/lyp}$ rat.

Gimap8, Gimap9, Gimap4, and *Gimap7* are transcribed in the same direction while *Gimap6* runs opposite to the rest of the family. Each gene is separated by approximately 12 Kb of non-coding sequence. *Gimap8,* unique with three AIG1 binding domains [1, 2, 7, 22], localizes to the endoplasmic reticulum, Golgi and mitochondria and may play an anti-apoptotic role in immune cells [8]. *Gimap6* and *Gimap7* are transmembrane proteins containing weak endoplasmic reticulum (ER) localization signals [2]. *Gimap4,* a cytosolic lymphoid specific signaling molecule, is associated with *β*-selection during maturation of CD4-CD8- double negative (DN) thymocytes into CD4+CD8+ double

positive (DP) thymocytes [5, 23], positive selection of T cells and accelerated T cell apoptosis [5, 7, 24]. In activated CD4+ cord blood cells, expression of *Gimap4* is down-regulated in response to IL-4, a Th2 promoting cytokine, and up-regulated by IL-12, a stimulator of IFN-γ production and promoter of Th1 differentiation [25].

Our previous study of the *Gimap* family showed that all seven members are differentially expressed in DR.$^{+/+}$ and DR.$^{lyp/lyp}$ spleen, thymus and mesenteric lymph node (MLN) [1]. However, as whole organs, not pure populations of cells were studied, it remains 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. To investigate these *Gimap* gene expression variations in sorted subpopulations of cells, we performed quantitative real time (qRT) PCR analysis on purified T and B cells from DR.$^{+/+}$ and DR.$^{lyp/lyp}$ rat thymus, spleen and MLN. We chose to study lymphocytes from MLN in the BB rats because their defective intestinal permeability may permit passage of intestinal environmental antigens initiating an autoimmune response, triggering diabetes and changes in expression of Gimap genes.

1. Graham, S., Courtois, P., Malaisse, W.J., Rozing, J., Scott, F.W., Mowat, A.M.I. 2004. Enteropathy precedes type 1 diabetes in the BB rat. *Gut, 53*: 1437-1444.
2. Malaisse, W.J., Courtois, P., Scott, F.W. 2004. Insulin-dependent Diabetes and Gut Dysfunction: The BB Rat Model. *Horm Metab Res, 36*: 585-594.
3. Vaarala, O. 2008. Leaking gut in type 1 diabetes. *Current Opinion in Gastroenterology, 24*: 701-706.

While not contributing to lymphopenia, we tested the hypothesis that a *Gimap* family member, other than *Gimap5 and Gimap1*, is associated with T1D to explain the iddm39 QTL. Clarifying *Gimap* gene expression in specific tissue-cells populations will aid in our understanding of the role of the *Gimap* family not only in thymocyte development, T cell survival in the periphery, or both, but also in T1D.

MATERIALS AND METHODS

DR.*lyp* Rats.

The DR.*lyp* (BBDR.BBDP-(*D4Rhw17- SS99306861*)(*D4Rhw11-D4Rhw10*)/*Rhw*) congenic rat line was derived from animals with two independent recombination events developed from our previously described introgression of the lymphopenia locus by cyclic cross-intercross breeding of BBDP with BBDR rats [26]. The first recombination event was flanked by simple sequence length polymorphism (SSLP) marker D4Rhw11 (75.81 Mb) and the second flanked by SSLP marker D4Rhw10 (77.81 Mb) [15]. Thus, the DP DNA in the DR.*lyp* rat line encompasses the *lyp* critical interval from D4Rhw6 (76.83 Mb) to IIsnp3 (77.16 Mb) [4]. In addition, the DR.*lyp* congenic rat line used in the present study also contains BBDP DNA at D4Rat102 (66.22 Mb) and D4Rat26 (69.18 Mb). The DR.*lyp* congenic rat line is kept in heterozygous sister-brother breeding and produces Mendelian proportions of the DR.$^{lyp/lyp}$ (25%), DR.$^{lyp/+}$ (50%) and DR.$^{+/+}$ (25%) genotypes. DR.$^{lyp/lyp}$ rats are 100% lymphopenic and 100% diabetic.

Housing.

Rats were housed in a specific pathogen–free facility at the University of Washington, Seattle, Washington, on a 12-h light/dark cycle with 24-h access to food (Harlan Teklad, Madison, WI) and water. The University of Washington Rodent Health Monitoring Program was used to track infectious agents via a quarterly sentinel monitoring system. The University of Washington institutional animal use and care committee approved all of the protocols used in the present study.

Genotyping Chromosome 4.

Between 25 and 30 days of age, 2 mm tail snips were obtained and DNA isolated as previously described [21]. The isolated DNA samples were diluted to 25 ng/μL in ddH_2O and 2μL of this genomic DNA used in the following 10 μL reaction: 2 μL 5X reaction buffer (Promega, Madison, WI), 0.2 μL 10 mmol/L dNTP (Promega), 1.0 μL 1 μmol/L IRDye 700 labeled primer (LiCor Biosciences, Lincoln, NE), 0.5 μL 20 μmol/L unlabeled reverse primer (Invitrogen, Carlsbad, CA), 0.20 μL GoTaq DNA Polymerase (Promega) and 4.1 μL ddH_2O. The PCR protocol was 95 °C for 5 min, 95 °C for 20 sec, 62 °C for 20 sec, 72 °C for 30 sec, steps 2–4 repeated 30 times, then 72 °C for 3 min. PCR products were diluted to 25% with STOP solution (LiCor Biosciences) and analyzed using a NEN Global IR2 DNA Analyzer System (Model 4200S-2) with a 6.5% gel matrix (LiCor Biosciences).

Cell Preparation and Purification.

Rat thymocytes, spleen, and MLN were minced in PBS containing 10% FCS. The single cell suspensions from spleen were washed in lysing buffer (BD Pharm Lyse™) (BD

Biosciences, San Diego, CA) to eliminate red blood cells. B cell suspension were purified by positive selection of $CD45^+$ and $CD45RA^+$ cells with the respective biotinylated monoclonal antibody (mAb) from BD Bioscience Pharmingen (San Diego, CA) using streptavidin-conjugated MACS microbeads according to the manufacturer's instructions Miltenyi MicroBead (Invitrogen, Carlsbad, CA).

FACS Sorting Of Lymphocyte Subsets.

Single cell suspensions of rat thymocytes, spleen and MLN cells were washed three times in PBS containing 10% FCS and then incubated for 30 min at 4°C with primary mAbs in the dark. Cells were washed twice and after the final wash filtered through a 40-µm cell-strainer immediately before cell sorting. The following mouse anti rat mAbs obtained from BD Bioscience Pharmingen, were used R-phycoerythrin (R-PE)-labeled anti-CD3 (G4.18), Cy-chrome labeled anti-CD4 (OX35), FITC labeled anti-CD8 (OX8), and FITC labeled anti-alpha-beta T cell receptor (R73). Cell populations were sorted on a FACSAria cell sorter (BD Biosciences, Mountain View, CA). Purities were checked by flow cytometry to be between 90 and 99.9%.

RNA Isolation.

FACS sorted thymus, spleen and MLN cells from 45-50 day old rats were homogenized in RNA lysis solution (Stratagene, La Jolla, CA or Qiagen, Valencia, CA) using a Kinematica Polytron PT 10/35 (Brinkmann, Westbury, NY) then aliquoted to minimize degradation from freezing and thawing. Total RNA was isolated using either RNeasy (Qiagen) or Absolutely RNA Miniprep Kit (Stratagene) followed by treatment with

DNase according to the manufacturer's instructions.

Quantitative RT-PCR.

Quantitative RT-PCR was performed on an Mx4000® Multiplex QPCR System (Stratagene) with samples run in duplicate using approximately 10 ng of total RNA. Twenty-five μL reactions were run using a Brilliant® Single-Step qRT-PCR Kit (2.5 μL 10x core RT-PCR buffer, 5.0 mmol/L $MgCl_2$, 400 nmol/L each primer, 200 nmol/L each probe, 0.8 mmol/L dNTP mix, 75 nmol/L passive reference dye, 2.0 units Stratascript RT, 1.25 units SureStart *Taq* DNA-polymerase). The PCR cycling conditions were as follows; 45°C for 30 min, 95°C for 10 min, and 40 cycles of 95°C for 30 sec, 60°C for 1 min. Resulting Ct values were converted to copies, normalized to GAPDH and expressed as the mean ± standard error of the mean (SEM). Probes were positioned in the 3' regions of the transcripts where there is more variation between the different *Gimap* genes and subjected to BLAST alignment to ensure specificity. The primers and probes used for each gene are detailed elsewhere [1]. Primers were obtained from Integrated DNA Technologies (Coralville, IA) or Qiagen Operon (Valencia, CA). Fluorescently labeled probes were obtained from Integrated DNA Technologies. Representative qRT-PCR products for each gene were run on an agarose gel to check for primer pair binding specificity. These PCR amplicons (used to convert to copy number) were quantitated in triplicate wells with the PicoGreen® RNA Quantitation Kit (Molecular Probes, Eugene, OR) using standards supplied by the manufacturer. Results from each assay were validated and normalized against GAPDH. The standard curves showed the following reaction efficiencies; *Gimap8*: 96% ± 3 SD, *Gimap9*: 94% ± 1 SD, *Gimap4*: 92% ± 2

SD, *Gimap6*: 90% ± 3 SD, *Gimap7*: 91% ± 4 SD, *Gimap1*: 94% ± 2 SD, *Gimap5*: 92% ± 2 SD, *Lr8*: 94% ± 3 SD, and GAPDH: 92% ± 1 SD.

Statistics.

Analyses of *Gimap* gene expression were carried out using linear mixed effect models [27] with SPLUS (Insightful Corp., Seattle, WA). Comparisons of expression between cell types consisted of twelve rats with two repeated measures for each rat. Comparison between DR.$^{lyp/lyp}$ and DR.$^{+/+}$ genotypes consisted of six rats from three litters consisting of one DR.$^{lyp/lyp}$ and one DR.$^{+/+}$ rat in each litter and two repeated measures for each rat. All of these analyses contained random effects for each of the three litters and two measures for each rat nested within those litters. Likelihood ratio tests were used to test the need for random intercepts. The significance for the fixed effects were assessed by *F* test for the coefficient or the group of coefficients [28] A Bonferroni correction was applied to *p*-values to adjust for multiple comparisons and adjusted values were considered significant at $p < 0.05$.

RESULTS

Thymic T cells.

Expression of the five *Gimap* genes within the *Iddm39* QTL; *Gimap8, Gimap9, Gimap4, Gimap6* and *Gimap7*, was compared to *Gimap1*, *Gimap5* and *Lr8,* a gene unassociated with the *Gimap* family but located within the 33 Kb lymphopenia critical interval (Figure I). The number of thymocytes, sorted based on double staining with CD4 (T helper) and CD8 (T cytotoxic) monoclonal antibodies did not differ between DR.$^{+/+}$ and DR.$^{lyp/lyp}$ rats

(Figure II). The sorted cells showed a high degree (>90%) of purity (Figure II, Panels A-D). Expression of *Gimap4* in CD4-CD8- 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 (Figure III). It should be noted that all statistical tests have been 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.

Spleen and Mesenteric Lymph Node Peripheral T cells.

Peripheral T cells from spleen and MLN were triple stained with CD3 (T cell receptor), CD4 and CD8 monoclonal antibodies. 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 (Figure II, panel C and D). Correcting for litter and multiple testing, we found that the expression of all *Gimap* genes in CD4+ splenic T cells did not differ between DR.$^{+/+}$ and DR.$^{lyp/lyp}$ rats (Figure IV). In contrast, the expression of all of the *Gimap* genes within the *Iddm39* QTL was reduced in DR.$^{lyp/lyp}$ CD8+ splenic T cells.

The expression of *Gimap4* and *Gimap9* genes in the MLN CD4+ T cells showed no difference between DR.$^{lyp/lyp}$ and DR.$^{+/+}$ rats, however, there was a reduction in *Gimap8*, *Gimap6* and *Gimap7*. The MLN CD8+ T cells showed a reduction in expression of

Gimap8, *Gimap4* and *Gimap7* while there was no difference in *Gimap9* and *Gimap6* (Figure IV). As in the thymus, *Gimap1* and *Lr8* in the lyp critical interval did not express appreciably in either DR.$^{+/+}$ or DR.$^{lyp/lyp}$ rat peripheral T cells (Figure IV).

Spleen and MLN derived B cells.

Hematopoietic B and *non* B cells (including T cells, macrophages, dendritic or NK cells) from spleen and MLN were double stained with CD45 (hematopoietic) and CD45RA (B cell) monoclonal antibodies and sorted based on staining with CD45RA (+/-). 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 (Figure 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. The comparisons which remained statistically significant after correction for litter and multiple testing are indicated (Figure V). 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 (Figure I), *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 (Figure V).

DISCUSSION

The *Gimap5* mutation within the *lyp* critical interval has been documented to result in peripheral T cell lymphopenia in the DR.$^{lyp/lyp}$ rat [4, 14, 22]. In our previous qRT-PCR analysis we showed reduced expression of all seven *Gimap* genes; *Gimap8, Gimap9, Gimap4, Gimap6, Gimap7, Gimap1* and *Gimap5* in DR.$^{lyp/lyp}$ rat spleen and MLN when compared to DR.$^{+/+}$ while only four; *Gimap9*, *Gimap4, Gimap1*, and *Gimap5* were reduced in thymus [1]. The present study resolves these previously observed expression differences to specific cell types both during thymocyte development and to mature T cells in the periphery. As lymphopenia impacts the cellular composition of the spleen and MLN, but not necessarily the thymus, the present qRT-PCR analysis of sorted cells further substantiates the *Gimap5* frameshift mutation as the primary defect in the periphery.

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 a significant difference in 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 the possible role of the *Gimap* genes in T cells as well as their possible role in the pathogenesis of T1D.

The possible role of the *Gimap* genes in either protection from T cell apoptosis, such as *Gimap5* [6, 7, 12] and *Gimap8* [8], or to induce apoptosis such as *Gimap4* [7] 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 [7]. 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.

The major finding from the analysis of CD3+ spleen and MLN T cells sorted for either CD4 or CD8 positivity was that all the *Gimap* genes, except *Gimap9* and *Gimap1,* whether in the *Iddm39* QTL or the *lyp* critical interval showed reduced expression in the cells from the DR.$^{lyp/lyp}$ rats. 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 than in CD4 or CD8 SP thymocytes. The study of lymphocytes from MLN is justified because it is well known that the defective intestinal permeability associated with BB rats may permit

passage of environmental antigens initiating an autoimmune response and triggering T1D (1. Graham, S., Courtois, P., Malaisse, W.J., Rozing, J., Scott, F.W., Mowat, A.M.I. 2004. Enteropathy precedes type 1 diabetes in the BB rat. *Gut, 53*: 1437-1444.

2. Malaisse, W.J., Courtois, P., Scott, F.W. 2004. Insulin-dependent Diabetes and Gut Dysfunction: The BB Rat Model. *Horm Metab Res, 36*: 585-594.

3. Vaarala, O. 2008. Leaking gut in type 1 diabetes. *Current Opinion in Gastroenterology, 24*: 701-706.) We considered the DR.$^{lyp/lyp}$ rat gut dysfunction may impact expression of Gimap genes in MLN. Our data showed that in MLN T cells all the *Gimap* genes, except *Gimap9* and *Gimap1,* showed reduced expression in the cells from DR.$^{lyp/lyp}$ rats.

Ake will change/expand these comments!!!!!!!!!!!!!!!

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.

Our observation in sorted peripheral CD4+ and CD8+T cells was that the loss of the Gimap5 protein is associated with a reduction in expression of all the *Gimap* family members (except *Gimap1* and *Gimap9*) 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. It is tempting to speculate that the coordinate expression of *Gimap8, Gimap4, Gimap6,*

Gimap7 and *Gimap5* 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. 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 nature of the *Gimap5* frame shift mutation leading to the absence of the Gimap5 protein [4, 16] suggests that the loss of this anti-apoptotic protein contributes to lymphopenia. The importance of the *Gimap5* mutation for the 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. As the *Gimap5* mutation is comparable to a null mutation, i.e. no Gimap5 protein is made [4, 16] it is of interest to note that the *Gimap5* knock out mouse that we recently reported [29] 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 as demonstrated in the present study. As many of these proteins have been implicated in

cell survival [7, 12, 30], 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 [4, 14, 22].

In conclusion, a frameshift mutation in *Gimap5* results in lymphopenia and precludes onset of spontaneous T1D in the diabetes prone BB rat. Reduced expression of all seven *Gimap* genes in peripheral lymphoid organs suggests that the lack of Gimap5 protein in the DR.$^{lyp/lyp}$ congenic rat line impairs expression of the entire *Gimap* gene family and regulates T cell homeostasis in the peripheral lymphoid organs. The major findings in the present study both confirm our initial observations as well as resolve *Gimap* gene expression in specific thymic lineages as well as peripheral T cell and B cell subsets**.** Further **e**lucidating the role of the *Gimap* family, specifically *Gimap5*, in T cell survival, development and/or activation will aid in our understanding of the pathways in onset of spontaneous T1D in the BB rat and ultimately the pathways leading to development of human disease.

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REFERENCES

1. Rutledge, E.A., Fuller, J.M., Van Yserloo, B., Moralejo, D.H., Ettinger, R.A., Gaur, P., Hoehna, J.L., Peterson, M.R., Jensen, R., Kwitek, A.E. *et al.* 2009. Sequence variation and expression of the Gimap gene family in the BB rat. *Exp Diabetes Res, 2009***:** 835650.
2. Krucken, J., Schroetel, R.M., Muller, I.U., Saidani, N., Marinovski, P., Benten, W.P., Stamm, O., Wunderlich, F. 2004. Comparative analysis of the human gimap gene cluster encoding a novel GTPase family. *Gene, 341***:** 291-304.
3. Reuber, T.L., Ausubel, F.M. 1996. Isolation of Arabidopsis genes that differentiate between resistance responses mediated by the RPS2 and RPM1 disease resistance genes. *Plant Cell, 8***:** 241-9.
4. MacMurray, A.J., Moralejo, D.H., Kwitek, A.E., Rutledge, E.A., Van Yserloo, B., Gohlke, P., Speros, S.J., Snyder, B., Schaefer, J., Bieg, S. *et al.* 2002. 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, 12***:** 1029-39.
5. Schnell, S., Demolliere, C., van den Berk, P., Jacobs, H. 2006. Gimap4 accelerates T-cell death. *Blood, 108***:** 591-9.
6. Nitta, T., Takahama, Y. 2007. The lymphocyte guard-IANs: regulation of lymphocyte survival by IAN/GIMAP family proteins. *Trends Immunol, 28***:** 58-65.
7. Nitta, T., Nasreen, M., Seike, T., Goji, A., Ohigashi, I., Miyazaki, T., Ohta, T., Kanno, M., Takahama, Y. 2006. IAN family critically regulates survival and development of T lymphocytes. *PLoS Biol, 4***:** e103.
8. Krucken, J., Epe, M., Benten, W.P., Falkenroth, N., Wunderlich, F. 2005. Malaria-suppressible expression of the anti-apoptotic triple GTPase mGIMAP8. *J Cell Biochem, 96***:** 339-48.
9. Dalberg, U., Markholst, H., Hornum, L. 2007. Both Gimap5 and the diabetogenic BBDP allele of Gimap5 induce apoptosis in T cells. *Int Immunol, 19***:** 447-53.
10. Carter, C., Dion, C., Schnell, S., Coadwell, W.J., Graham, M., Hepburn, L., Morgan, G., Hutchings, A., Pascall, J.C., Jacobs, H. *et al.* 2007. A natural hypomorphic variant of the apoptosis regulator Gimap4/IAN1. *J Immunol, 179***:** 1784-95.
11. Hernandez-Hoyos, G., Joseph, S., Miller, N.G., Butcher, G.W. 1999. The lymphopenia mutation of the BB rat causes inappropriate apoptosis of mature thymocytes. *Eur J Immunol, 29***:** 1832-41.
12. Pandarpurkar, M., Wilson-Fritch, L., Corvera, S., Markholst, H., Hornum, L., Greiner, D.L., Mordes, J.P., Rossini, A.A., Bortell, R. 2003. Ian4 is required for mitochondrial integrity and T cell survival. *Proc Natl Acad Sci U S A, 100***:** 10382-7.
13. Filen, J.J., Filen, S., Moulder, R., Tuomela, S., Ahlfors, H., West, A., Kouvonen, P., Kantola, S., Bjorkman, M., Katajamaa, M. *et al.* 2008. Quantitative proteomics

reveals GIMAP family proteins 1 and 4 to be differentially regulated during human T helper cell differentiation. *Mol Cell Proteomics*.
14. Hornum, L., Romer, J., Markholst, H. 2002. The diabetes-prone BB rat carries a frameshift mutation in Ian4, a positional candidate of Iddm1. *Diabetes, 51***:** 1972-9.
15. Fuller, J.M., Kwitek, A.E., Hawkins, T.J., Moralejo, D.H., Lu, W., Tupling, T.D., Macmurray, A.J., Borchardt, G., Hasinoff, M., Lernmark, A. 2006. Introgression of F344 rat genomic DNA on BB rat chromosome 4 generates diabetes-resistant lymphopenic BB rats. *Diabetes, 55***:** 3351-7.
16. Michalkiewicz, M., Michalkiewicz, T., Ettinger, R.A., Rutledge, E.A., Fuller, J.M., Moralejo, D.H., Van Yserloo, B., MacMurray, A.J., Kwitek, A.E., Jacob, H.J. *et al.* 2004. Transgenic rescue demonstrates involvement of the Ian5 gene in T cell development in the rat. *Physiol Genomics, 19***:** 228-32.
17. Payne, F., Smyth, D.J., Pask, R., Barratt, B.J., Cooper, J.D., Twells, R.C., Walker, N.M., Lam, A.C., Smink, L.J., Nutland, S. *et al.* 2004. Haplotype tag single nucleotide polymorphism analysis of the human orthologues of the rat type 1 diabetes genes Ian4 (Lyp/Iddm1) and Cblb. *Diabetes, 53***:** 505-9.
18. Shin, J.H., Janer, M., McNeney, B., Blay, S., Deutsch, K., Sanjeevi, C.B., Kockum, I., Lernmark, A., Graham, J., Arnqvist, H. *et al.* 2007. IA-2 autoantibodies in incident type I diabetes patients are associated with a polyadenylation signal polymorphism in GIMAP5. *Genes Immun, 8***:** 503-12.
19. Hellquist, A., Zucchelli, M., Kivinen, K., Saarialho-Kere, U., Koskenmies, S., Widen, E., Julkunen, H., Wong, A., Karjalainen-Lindsberg, M.L., Skoog, T. *et al.* 2007. The human GIMAP5 gene has a common polyadenylation polymorphism increasing risk to systemic lupus erythematosus. *J Med Genet, 44***:** 314-21.
20. Moralejo, D.H., Park, H.A., Speros, S.J., MacMurray, A.J., Kwitek, A.E., Jacob, H.J., Lander, E.S., Lernmark, A. 2003. Genetic dissection of lymphopenia from autoimmunity by introgression of mutated Ian5 gene onto the F344 rat. *J Autoimmun, 21***:** 315-24.
21. Fuller, J.M., Bogdani, M., Tupling, T.D., Jensen, R.A., Pefley, R., Manavi, S., Cort, L., Blankenhorn, E.P., Mordes, J.P., Lernmark, A. *et al.* 2009. Genetic Dissection Reveals Diabetes Loci Proximal to the Gimap5 Lymphopenia Gene. *Physiol Genomics*.
22. Dion, C., Carter, C., Hepburn, L., Coadwell, W.J., Morgan, G., Graham, M., Pugh, N., Anderson, G., Butcher, G.W., Miller, J.R. 2005. Expression of the Ian family of putative GTPases during T cell development and description of an Ian with three sets of GTP/GDP-binding motifs. *Int Immunol, 17***:** 1257-68.
23. Fehling, H.J., von Boehmer, H. 1997. Early alpha beta T cell development in the thymus of normal and genetically altered mice. *Curr Opin Immunol, 9***:** 263-75.
24. Poirier, G.M., Anderson, G., Huvar, A., Wagaman, P.C., Shuttleworth, J., Jenkinson, E., Jackson, M.R., Peterson, P.A., Erlander, M.G. 1999. Immune-associated nucleotide-1 (IAN-1) is a thymic selection marker and defines a novel gene family conserved in plants. *J Immunol, 163***:** 4960-9.
25. Filen, J.J., Filen, S., Moulder, R., Tuomela, S., Ahlfors, H., West, A., Kouvonen, P., Kantola, S., Bjorkman, M., Katajamaa, M. *et al.* 2009. Quantitative proteomics

reveals GIMAP family proteins 1 and 4 to be differentially regulated during human T helper cell differentiation. *Mol Cell Proteomics, 8***:** 32-44.
26. Bieg, S., Koike, G., Jiang, J., Klaff, L., Pettersson, A., MacMurray, A.J., Jacob, H.J., Lander, E.S., Lernmark, A. 1998. Genetic isolation of iddm 1 on chromosome 4 in the biobreeding (BB) rat. *Mamm Genome, 9***:** 324-6.
27. Pinheiro, J.C. 2000. Mixed-Effects Models in S and S-Plus. Springer-Verlag, New York.
28. Lehmann, E.L. 1986. Testing Statistical Hypotheses. . John Wiley and Sons, , New York.
29. Schulteis, R.D., Chu, H., Dai, X., Chen, Y., Edwards, B., Haribhai, D., Williams, C.B., Malarkannan, S., Hessner, M.J., Glisic-Milosavljevic, S. *et al.* 2008. Impaired survival of peripheral T cells, disrupted NK/NKT cell development, and liver failure in mice lacking Gimap5. *Blood, 112***:** 4905-14.
30. Sandal, T., Aumo, L., Hedin, L., Gjertsen, B.T., Doskeland, S.O. 2003. Irod/Ian5: an inhibitor of gamma-radiation- and okadaic acid-induced apoptosis. *Mol Biol Cell, 14***:** 3292-304.

Figure Legends

Figure I: ***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 *lyp* 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.

Figure II. ***T cell Profile and Purity.*** Scatter plots are shown from one DR.$^{+/+}$ and one DR.$^{lyp/lyp}$ rat FACS sorted T cells from thymus, spleen and MLN following staining with CD4 and CD8 monoclonal antibodies. Cells from spleen and MLN were first gated based on staining with a CD3 monoclonal antibody. The percent gated cells are shown within each quadrant. Panels A-D show the purity of sorted cells from one DR.$^{lyp/lyp}$ rat.

Figure III. ***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. 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.

Figure IV. ***Gimap Gene Expression in Peripheral T cells.*** The mean ± standard error of

the mean (SEM) is shown for *Gimap* family expression in CD4+ and CD8+ FACS sorted T cells from spleen (Spl) and MLN following normalization to GAPDH. Cells from spleen and MLN were first gated based on staining with a CD3 monoclonal antibody. 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.

Figure V. ***Gimap Gene Expression in Peripheral B cells.*** The mean ± standard error of the mean (SEM) is shown for *Gimap* family expression in FACS sorted hematopoietic B cells (CD45+CD45RA+) and *non* B cells (CD45+CD45RA-) from spleen and MLN after normalization to GAPDH. Black columns represent DR.+/+ rats (+, n=3) and hatched columns represent DR.$^{lyp/lyp}$ (*l*, n=3). Significant differences are * for $p<0.05$. Genes appear in the order at which they appear on RNO4.