UNIVERSITY OF PÉCS

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Regulation of B cell development

PhD thesis

JUDIT KNISZ

Thesis supervisors:

Dr. Paul B. Rothman
M.D.
Dr. John D. Colgan
Ph.D.
Dr. Wilhelm Márta
Ph.D.

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INTRODUCTION

Differentiation of cells from multipotent and pluripotent progenitors is essential for the development of multicellular organisms. In turn, there is great therapeutical potential in manipulation and control of such differentiation processes for cancer. The hematopoietic system is one of the best-studied systems of the differentiation process and has established that all cell types within the mammalian lymphoid system originate from a common progenitor found in the bone marrow. Understanding the genetic and molecular mechanisms that control the development of lymphocytes from this progenitor is important for therapeutics of cancers originating in lymphocytes such as B and T cell lymphomas. A detailed understanding of lymphoid cell development would further our understanding of the molecular basis for these pathologic conditions. Lymphopoiesis is a well-understood differentiation process mainly because there are well defined existing methods for in vitro differentiation in culture. Lymphopoiesis studies have not only led to a deeper understanding of lymphoid cell development, but have also provided key insight into malignant processes in which genes responsible for the differentiation of lymphocytes were targets in cellular transformation. As the sole source of immunoglobulin, B lymphocytes (or B cells) are an essential component of the adaptive immune system.

B cell development

B cells, like all hematopoietic cells, are produced from self-renewing hematopoietic stem cell within the fetal liver and postnatal bone marrow. Throughout life, the self renewing, pluripotent hematopoietic stem cells (HSCs) regenerates all blood cell types in a stepwise process by differentiating into a progenitor cell with a gradual restricted developmental potential ().

An early step of hematopiesis in bone marrow is the generation of multipotent progenitors (MPPs) through HSCs. MPPs give rise to either a lymphoid-primed multipotent progenitors (LMPPs) or common myleoid progenitors (CMPs), which in turn can differentiate into an erythroid and a myeloid cell. LMPPs seed the thymus and the bone marrow where they differentiate into lymphocytes. The differentiation process transits through the common lymphoid progenitor (CLP) stage. CLP is currently viewed as a bone marrow intermediate, primarily progenitor of B lymphocytes and NK cells but can give rise to dendritic cells (DC) and has residual T lineage potential, but lacks myeloid potential. Cells than proceed through early B cell development that includes the

pre-pro-B cell, pro-B cell, pre-B cell, immature B cell, mature B cell, and plasma cell stages. These stages are defined by progression of immunoglobulin gene DNA recombination, expression of cell surface markers, and expression of transcriptional regulators.

Production and Immunological Characterization of Justy Mice

Our laboratory has been characterizing a novel mutant mouse strain called *Justy*, which was created via random, genome-wide mutagenesis at Ingenium Pharmaceuticals. Male C3HeB/FeJ (C3H) mice were injected with ENU and mated with wild-type C3H females. An F1 male, excluded from carrying a potentially dominant mutation, was mated with wild-type C3H females producing G2 offsprings. G2 females were crossed with the F1 male to produce G3 offspring. Flow cytometric analysis of peripheral blood from F3 progeny identified mice lacking cells expressing CD45R/B220 (B220), a marker found almost exclusively on B cells [19]. G3 founder was crossed to wild-type C3H mice and the progeny used for brother-sister matings to recover mice that were homozygous for the causal mutation [65]. The mutant strain was named *Justy* (just T cells). Serum immunoglobulin was not detected in these mice and injection of LPS or Ficoll failed to induce IgM synthesis as observed in control mice. Spleens from *Justy* mice are abnormally small in size and weight; histologically, this correlates with hypoplasia and reduced cell numbers in white pulp regions. Total B cells in *Justy* splenocytes are decreased ~500-fold relative to wild type. B cells are undetectable in *Justy* lymph node cells and the frequency of B1 and B2 cells in *Justy* peritoneal exudate cells is greatly reduced.

Reciprocal bone marrow transplantation demonstrated that *Justy* bone marrow stroma can support B cell development from wild-type progenitors and the developmental defect caused by the *Justy* mutation is intrinsic to B cell progenitors.

The mutation is associated with the Gon4l gene

Simple sequence length polymorphisms (SSLPs) analysis showed a complete association between the developmental block and a region on mouse chromosome 3 (chr 3) between SSLPs D3Mit49 and D3Mit175. Sequence analysis of the candidate region revealed a T to A transversion at nucleotide position 88703382 in chr 3 within intron 24 of the mouse gene Gon4l (gon4-like), Mutagenesis of *C. elegans* identified the prototypical *Gon4l* ortholog, named *gon-4*. Characterization of four mutant alleles, each probably being null, demonstrated that gon-4 is required solely for cell divisions that generate somatic gonadal organs. Each of two independent mutagenesis screens of zebrafish identified a recessive loss-of-function allele for the *Gon4l* ortholog *Udu*.

Homozygosity for either allele results in abnormal gastrulation, cell death in the central nervous system and disruption of early erythropoiesis. Although *Gon4l* orthologs have been identified in vertebrate genomes as well, we know very little about its function.

AIMS OF THE STUDY

The main goals of the present study were:

1. To analyze the genetic locus associated with the observed phenotype.
2. To analyze *Gon4l* gene product at mRNA and protein level.
3. To identify target genes regulated by Gon4l.
4. Analysis of earlier precursors during B cell development.
5. To define the in vitro differentiation potential of bone marrow cells from *Justy* mice.
6. Analysis of the mouse *Gon4l paralog* gene.
7. To analyze the role of Socs7 in TSLP and IL-7 signaling

METHODS

Mice

Justy mice were generated at Ingenium (Germany) by introducing point mutations in the male C3HeB/FeJ (C3H) germline via injection N-ethyl-N-Nitrosourea. Mutations were screened by positional cloning. Socs7 KO mice were created as described before. For experiments, 129/Bl6 mixed KO mice as well as wild-type 129/Bl6 F2 hybrid mice were used.

Flow Cytometric Analysis and Cell Sorting

Single cell suspensions were prepared from spleen, thymus or bone marrow by removing red blood cells and debris using lympholyte or by lysing red blood cells with ACK buffer followed by passage through a 70μM cell strainer. Cells were resuspended in stain buffer (PBS containing 3% FBS) and incubated with anti-mouse CD16/32 (eBioscience) to prevent nonspecific antibody binding. Cells were incubated with fluorochrome- or biotin-conjugated antibodies for 30 min on ice and then washed 3 times with stain buffer. Optimal antibody concentrations for staining were determined by titration experiments. To label biotin-conjugated antibodies, cells were resuspended in stain buffer plus the appropriate concentration of streptavidin- or avidin-conjugated fluorophore, incubated for additional 30 min on ice and then washed 3 times with stain buffer. Flow cytometric analysis was performed using either an LSR II or a FACSDiVa (Becton Dickinson). Collected data was analyzed with FlowJo software (TreeStar). Cell yields for different fractions were calculated from post-sort analysis of collected data. Total events in the appropriate gate were divided by the total events in the forward

scatter versus side scatter gate; this percentage was then multiplied by total yield of splenocytes or bone marrow cells as determined by counting viable cells using a hemacytometer. Cell populations were sorted using a FACSDiVa. For isolation of pre-pro-B and pro-B cells from bone marrow, a negative selection step was performed prior to sorting. Cells were incubated with rat monoclonal antibodies (Ter-119, anti-CD5, anti-Ly6C and anti IgM), washed and then incubated with magnetic beads coated with sheep anti-rat antibodies (Dynal). Beads and unwanted cells were selected out and cells in the supernatant were recovered, stained with antibodies and then sorted. For sort-purification, antibodies specific for the following cell-surface markers were used: B220, CD43, CD11c, CD49b, CD19, HSA and BP-1. For sort-purification 8-12 week-old mice were used, 6-8 mice per sort.

RNA Purification

Mouse organs were quick frozen in liquid N_2 and homogenized in 1ml of TriZol (Invitrogen) per 50mg of tissue using a PowerGen Homogenizer (Fisher). Homogenetes were centrifuged for 10 min at 14000 rpm and supernatants were incubated for 5 min at room temperature (RT). Samples were shaken for 15 sec after the addition of 200µl of chloroform per 1ml of starting TriZol and incubated for 2-3 minutes at room temperature. After centrifugation for 20 min at 14000 rpm at 4^{o}C the aqueous phase was transferred to a new tube and 500µl of isopropanol was added per 1ml of starting TriZol. After incubation for 10 min at room temperature samples were centrifuged for 10 min at 10000rpm at 4^{o}C. Pellets were dried and resuspended in nuclease free water. Samples were treated with DnaseI (Roche) and cleaned up using RNeasy mini columns (Qiagen) following the manufacturer's instruction.

Sort purified cells were homogenized in 800µl of TriZol by vortexing. After, the same procedure was used as described above. Pellet was resuspended in 21.5 µl of nuclease free water and was treated with 1 µl of DNase I (Invitrogen) in the presence of 10µl of 10x DNase buffer for 15 min at room temperature. The reaction was stopped by adding 2.5µl of 25mM EDTA (pH 8.0). 25µl out of this mix was used for reverse transcription.

Primers and RT-PCR Analysis

Isolated RNA was reverse-transcribed using the SuperScript III First-strand cDNA Synthesis Kit (Invitrogen). Conventional PCR was performed using Taq enzyme (Qiagen) and BioRad MyCycler thermal cycler according to the manufacturers'

instructions. Quantitative real-time PCR was performed using POWER SYBR Green Master Mix and the ABI PRISM 7700 Detection system (Applied Biosystems) to obtain cycle threshold (Ct) values for target and internal reference cDNA levels. Ct values correspond to the PCR cycle at which fluorescent signals above baseline fluorescence are detected; baseline was determined using fluorescence readings collected during the first 12-15 cycles of PCR amplification. Target cDNA levels were normalized to an internal reference using the equation $2^{-[\Delta Ct]}$, where ΔCt is defined as $Ct_{target} - Ct_{internal\ reference}$. Values shown were derived from the average of 3 or more replicate pairs of PCR reactions (target and internal reference) for each of 2 to 6 independently generated cDNA preparations.

To present data in Section 3, values were first normalized to HPRT as described above. After, expression values shown for wt early pro-B, *Justy* pre-pro B and *Justy* early proB cells were all normalized to the value obtained from wild-type pre-pro B cells, which was set to 1.

Cloning

Overlapping DNA fragments spanning the entire Gon4l coding sequence were obtained by PCR amplification of cDNA from wild-type bone marrow. Fragments were cloned into pCR2.1 (Invitrogen) and sequenced. Standard methods were used to assemble a full length Gon4l cDNA from that was fused in frame at the 5' end to sequences encoding the HA epitope. HA-Gon4l cDNA was inserted into the retroviral vector pMIG immediately upstream of an IRES and GFP coding sequences, allowing for co-expression of HA-Gon4l and GFP from a bicistronic mRNA. HA-Gon4l cDNA was also inserted into the mammalian expression plasmid pcDNA 3.1 HisB- (Invitrogen). Effectene (Qiagen) was used to transiently transfect human embryonic kidney (HEK) 293T cells with pMIG-HA-Gon4l.

Indirect Immunofluorescent Confocal Microscopy

After transfection, cells were trypsinized and transferred to glass coverslips coated with poly-L-lysine (Sigma). 48 hr after transfection, cells were washed and fixed with 4% paraformaldehyde for 30 min at RT. After fixation, cells were washed 4 times for 10 minutes, permeabilized and blocked by incubation in PBS containing 2% FBS and 0.1% Triton X-100 for 30 min at RT. Cells were incubated overnight with anti-HA antibody (clone 3F10, Roche) diluted 1 to 500 in Stain buffer (PBS containing 2% FBS and 0.05% Tween 20). Bound anti-HA antibody was detected using biotinylated goat anti-rat IgG (Jackson ImmunoResearch) diluted 1 to 200 in Stain buffer followed by

Alexa Fluor 568-streptavidin (Molecular Probes) diluted in Stain buffer. Cells were incubated with each reagent for 30 min at RT and then washed 4 times for 10 min. Cells were then incubated for 5 min at RT with TOPRO3 (Invitrogen) diluted 1 to 2000 in Stain buffer and rinsed. Vectashield (Vector Laboratories) was used to mount cover slips onto microscopy slides. Data images were collected using a Radience 2100MP Confocal Microscope (Bio-Rad).

Identification of Putative Protein Domains

Putative protein structural domains were identified using the SMART (http://smart.emblheidelberg.de/smart/set_mode.cgi?NORMAL=1) and Pfam (http://www.sanger.ac.uk/Software/Pfam/) databases.

Cell culture

Each cell types were grown in a 37°C humidified, 5% CO_2 gassed incubator.

S17 culture - S17 stromal cells were grown in 10% FBS/RPMI 1640 medium (supplemented with 1 x glutamine, 10 mM Hepes, 50uM β-ME, and 1% PS). For maintaining the cell line, cells were passaged before reaching confluence. For experiments, cells were passaged into 24-well plates in the same medium and allowed to reach confluence before use.

OP9 culture - OP9-control and OP9-DL1 cell lines were obtained from Dr. Juan Carlos Zúñiga-Pflücker (University of Toronto, Canada). OP9-control cells and OP9-DL1 cells were cultured as a monolayer in OP9 media (MEM supplemented with 20% FCS, 1% penicillin and streptomycin and 2.2 g/liter sodium bicarbonate). For experiments, cells were passaged into 24-well plates in the same medium and were used before reaching confluence.

Whitlock-Witte culture - 2 x 10^6/ml total bone marrow of 8-12 week-old wild-type and *Justy* mice was plated onto confluent layer of S17 stromal cell layer in medium (RPMI-1640, 5% heat inactivated FCS, 50μM β-mercaptoethanol (β-ME) and 1% PS) in the presence of 12.5 ng/ml IL-7. After 10-14 days, cells in the supernatant were analyzed by flow cytometry using B220, CD19, CD43, Gr-1, Ly6c and CD49b.

B/myeoid cell inducing condition - Wild-type and *Justy* pre-pro-B and early pro-B cells were sort-purified from bone marrow of 8-12 week-old mice and 2x10^3 cells were plated directly into wells on a 24 well plate containing a confluent monolayer of S17

stromal cells. All cells were grown in α-MEM medium supplemented with 5% FBS, 50μM β-ME and 1% PS in the presence of IL-7, SCF and Flt3L (each at 10 ng/ml). After 7-14 days, cells were harvested by pipetting, and then stained with antibody to CD19, Ly6c, Gr-1 and CD11b and analyzed by flow cytometry.

T cell inducing condition - Pre-pro-B and early pro-B cells from 8-12 week-old wild-type and *Justy* mice were sort-purified and $2x10^3$ cells were plated into wells on a 24-well plate containing a sub-confluent layer of either OP9-GFP or OP9-DL1 cells. All cells were grown in α-MEM medium supplemented with 20% FBS, 2.2g/l sodium bicarbonate and 1% PS in the presence of IL-7 and Flt3L (each at 10 ng/ml), IL-6 (ng/ml) and IL-15 (25ng/ml) to enhance the growth of B, T and NK cells. After 7-10 days, wells were scored for growth. Cells from individual wells were harvested and stained for expression of B220, CD19, CD90/Thy-1, CD25 and CD49b. $CD90^+CD25^+$ cells were considered as early T cells.

Mast cell culture - Mouse bone marrow derived mast cells (BMMCs) were cultured from marrow cells of the femur and tibia of 129/ Bl6 $Socs7^{+/-}$ or $Socs7^{-/-}$ mice. These cells were maintained in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 25 mM HEPES, 1.0 mM sodium pyruvate, nonessential amino acids (BioSource International, Camarillo, CA), 0.0035% β-ME and 300 ng/ml recombinant mouse IL-3 (PeproTech, Rocky Hill, NJ) as described. BMMCs (>95% purity as determined by toluidine blue staining) were used at 4–6 weeks of culture. For sensitization, BMMCs were seeded at 2×105 cells/well and sensitized for with 100 ng/ml IgE anti-DNP (Sigma-Aldrich) for 30 min, 2h and 4h.

Western analysis and immunoprecipitation

Cellular lysates were homogenized in NP-40 lysis buffer containing 0.5% NP-40, 50 mM Tris pH 8.0, 10% glycerol, 0.1 mM EDTA, 150 mM NaCl, 0.5 mM Na_3VO_4, 4.5 mM $Na_2P_4O_7$, 50 mM NaF, 1 mM DTT, 0.4 mM PMSF, 3 μg/ml aprotinin, 1 μg/ml leupeptin, and 2 μg/ml pepstatin. The homogenates were solubilized for 1 hour at 4°C and clarified by centrifugation at 16,000 *g* for 30 minutes. Supernatants containing equal amounts of protein (2–3 mg) were immunoprecipitated (≥ 2 hours or overnight) with 2 μg of α-X-press (Invitrogen Corp.) or α-HA (Roche) antibodies. Immune complexes were collected with 60 μl of a 50% slurry of protein A agarose (Santa Cruz) resolved on 12%

SDS–PAGE and transferred to nitrocellulose. The blots were probed with antibodies against X-press epitope (Invitrogen Corp.) or HA epitope (Roche).

Statistical analyses

All the statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, Sand Diego, CA, USA). P values below 0.5 were considered significant.

RESULTS AND DISCUSSION

The Justy mutation disrupts RNA splicing

Sequence analysis identified a T to A transversion roughly in the center of an 876 nucleotide intron between exons 24 and 25 of the predicted *Gon4l* gene. The T to A transversion increases homology to the consensus 5' splice site, suggesting that the mutation could affect pre-mRNA splicing. Primers complementary to exons 24 and 26 in *Gon4l* were used to amplify cDNA derived from different organs by PCR. Amplification of cDNA generated from wild-type organs gave rise to a 446 bp product that consisted of the expected sequences. However, amplification of cDNA derived from *Justy* organs generated a 446 bp product as well as a prominent 527 bp product and a minor 638 bp product. Sequence analysis showed that the 446 bp product is identical to that amplified from wild-type cDNA. In contrast, in addition to the expected sequences, the 527 bp and 638 bp products contained cryptic exons between exons 24 and 25: an 81 nt cryptic exon in the case of the 527 bp fragment and a 192 bp cryptic exon in the case of the 638 bp product. The 192 nt cryptic exon consists of the 81 nt exon fused at the 5' end to an additional 111 nt. The sequence common to both cryptic exons is located immediately upstream of the T to A mutation in the *Justy* genome. Inclusion of either cryptic exon into Gon4l mRNA requires the use of sequences surrounding the T to A mutation as a 5' splice site in concert with the 3' splice site that defines the beginning of exon 25. The T to A mutation also activates the use of splice sites that mediate fusion of the cryptic exons to the end of exon 24. Strikingly, inclusion of either the 81 nt or 192 nt cryptic exon places termination codons in frame with the coding sequence of Gon4l mRNA; thus mRNA containing either the 81 nt or 192 nt cryptic exon encodes a truncated form of Gon4l lacking the last 626 C-terminal amino acids of the native protein.

Gon4l mRNA is broadly expressed in various tissues

Q RT-PCR analysis was used to define how the *Justy* mutation affects Gon4l RNA expression. With the exception of brain, total *Gon4l* RNA levels were modestly reduced in organs from *Justy* mice relative to those from wild type. The wild-type splice was detected in cDNA generated from wild-type or *Justy* organs. The aberrant splice was undetectable in cDNA derived from wild-type organs but was abundant in that generated from *Justy* organs, indicating that the effect of the mutation is widespread. Moreover, the levels of the wild-type and aberrant splice detected in cDNA from *Justy* organs were inversely related. For example, in *Justy* brain, low levels of the aberrant splice and normal levels of the wild-type splice were observed, while in *Justy* thymus, spleen or bone marrow, high levels of the aberrant splice and low levels of the wild-type splice were seen. These results suggest that tissue and cell-specific factors influence the effect of the *Justy* mutation at the level of pre-RNA splicing. Furthermore, the mutation has a profound effect on the splicing of Gon4l RNA in pre-pro-B and early pro-B cells, which correlates well with the nature of the developmental block.

Gon4l mRNA is expressed in B cell progenitors and upregulated during B cell maturation

Quantitative (Q) RT-PCR analysis was performed to measure the levels of Gon4l RNA expressed in wild-type cells at various stages of B lymphopoiesis. Consistent with the phenotype of *Justy* mice, Gon4l RNA was detected in pre-pro-B and early and late pro-B cells. Relative to pre-pro-B cells, Gon4l RNA levels were somewhat lower in pro-B cell fractions as well as in pre-B cells. In contrast, Gon4l RNA levels in immature B cells, bone marrow fraction F and splenic follicular and marginal zone (MZ) B cells were equal to or greater than that detected in pre-pro-B cells, suggesting that Gon4l expression is upregulated with B cell maturation.

Justy mutation abolishes synthesis of wild-type Gon4l mRNA in pre-pro-B and early pro-B cells

As Gon4l mRNA is expressed during B lymphopoiesis, we asked how the mutation affects Gon4l mRNA levels in wild-type and Justy pre-pro-B and early pro-B cells. Similar levels of total Gon4l RNA were detected in *Justy* and wild type pre-pro-B and early pro-B cells. The aberrant splice was undetectable in cDNA derived from wild-type pre-pro-B and early pro-B cells, as expected, but was clearly present in *Justy* cells. The inverse relationship was observed for the wild-type splice: this was present at robust levels in wild-type pre-pro-B and early pro-B cells but was nearly undetectable in *Justy* cells. These results indicate that the *Justy* mutation abolishes synthesis of wild-

type Gon4l mRNA in pre-pro-B and early pro-B cells, which is consistent with the observed developmental block.

The observation that Gon4l is widely expressed seems to argue against the idea that Gon4l expression is important only in the B lineage. A more likely explanation for how the *Justy* mutation specifically disrupts B lymphopoiesis comes from our data demonstrating that the mutation has a profound effect on Gon4l pre-mRNA splicing. These observations suggest the *Justy* mutation has created a conditional allele of *Gon4l* rather than one with complete and constitutive loss of function.

Gon4l encodes a putative regulator of chromatin

The *Gon4l* gene contains 33 exons with a predicted open reading frame spanning exons 2-33. The encoded protein is 2260 amino acids with a predicted molecular weight of ~250 kDa. Computer analysis identified several regions in Gon4l with homology to other proteins that regulate transcription and chromatin. The N-terminal of the Gon4l protein contains a predicted nuclear localization signal (NLS), suggesting that Gon4l is a nuclear protein. 293T cells were transiently cotransfected with a plasmid expressing HA-epitope-tagged Gon4l; immunoblot confirmed that Gon4l was expressed. Fluorescent confocal microscopy demonstrated that Gon4l accumulated mainly in the nucleus, although in some cells it was also found in the cytoplasm, but was excluded from the nucleolus. In contrast, a Gon4l derivative lacking 318 amino acids spanning the putative NLS near the N-terminus failed to localize to the nucleus. These results demonstrate that Gon4l encodes a nuclear protein. The predicted structural domains and the demonstration that Gon4l localizes in the nucleus suggest that Gon4l functions to regulate gene transcription.

Regulation of gene expression is disrupted in Justy B cell progenitors

As Gon4l protein is predicted to form domains associated with regulation of transcription, we evaluated gene expression in sort-purified pre-pro-B and early pro-B cells via Q RT-PCR analysis. We observed a significant decrease in IL7Rα expression in *Justy* early pro-B cells, which might result in decreased survival and expansion of these cells. Early pre-pro-B cells from *Justy* mice expressed significantly more PU.1 RNA compared to their wild-type counterpart. This may be important in the light of the in vitro experiments where increased PU.1 expression was shown to suppress B cell development. We also measured levels of RNA encoding key B cell transcription factors including E2A (E12 and E47) and Ebf1, which are absolutely required for pre-pro-B to early pro-B transition. RNA levels in *Justy* cells were not considerably different relative

to that detected in wild-type cells. In case of Pax5 RNA, there is a modest increase in both pre-pro-B and early pro-B cells relative to wild type. These data suggest that the changes in E2A, EBF or Pax5 expression are not responsible for the *Justy* phenotype and that mechanisms required for regulation of lymphoid and B cell-associated genes during B lineage commitment are largely intact in *Justy* cells.

Analysis of RNA encoding components of the immunoglobulin gene rearrangement machinery (RAG1 & 2, TdT) showed slight decrease of RAG1 and RAG2 expression in both pre-pro-B and early pro-B cells relative to wild type, however, the differences were not significant. On the other hand, there was a significant decrease in the expression of the TdT RNA in Justy cells. Tdt is a key player in influencing the outcome of V(D)J recombination during lymphocyte development.

We have also observed significant differences in the expression of some genes important for the formation of the pre-BCR complex. Both VpreB1 and λ5, which are important for the formation of surrogate light chain of the preBCR, were downregulated in *Justy* early pro-B cells relative to wild type cells. Igα forms heterodimer with Igβ and are together responsible for signal transduction of the preBCR. Levels of Igβ significantly increased in *Justy* early pro-B cells compared to wild type.

We also tested whether RNA expression from alternative lineage genes (i.e. T cell or myeloid-associated genes) was altered in sort-purified pre-pro-B and early pro-B cells. During development of pre-pro-B cells into early pro-B cells, T and myeloid-associated genes are repressed. Levels of RNA encoding the T lineage factors Notch1 or GATA3 were increased in *Justy* pre-pro-B cells, although the difference did not reach significance. On the other hand, Notch1 RNA level was significantly increased in early pro-B cells relative to wild type. This result is interesting as Notch signaling has been suggested to have a role in determining lineage choice in developing T cells and can suppress B cell development.

Levels of RNA encoding the myeloid factors C/EBPα or Csf1r were also elevated in *Justy* pre-pro-B cells compared to wild type. Strikingly, in *Justy* early pro-B cells C/EBPα and Csf1r RNA levels were increased 17-fold and 77-fold, respectively, compared to wild-type cells. *Csf1r* and *Cebpα* are myeloid specific genes and their RNA expression are normally low or absent in B cell progenitors. Our data indicate that the mechanisms required for repression of at least some alternative (non-B) lineage genes is disrupted in *Justy* cells. Repression of alternative lineage genes coincides with the pre-pro-B cell transition, resulting in complete commitment to the B cell fate and

restriction of alternative lineage potential. Thus, our data suggest that the inability to completely repress alternative lineage genes is in part responsible for the developmental block observed in *Justy* mice.

Normal CLPs but increased numbers of CMPs present in Justy bone marrow cells

Although the block in *Justy* mice occurs during the transition from pre-pro-B to pro-B cells we sought to determine whether Gon4l has a role earlier during lineage commitment and might affect the development of other lineages. During hemopoiesis, multipotent progenitors differentiate into common myeloid progenitors (CMPs) that give rise to erythrocytes and myeloid cells, and the common lymphoid progenitors (CLPs) that are the immediate precursors of pre-pro-B cells. The frequency and total number of CLPs in *Justy* bone marrow was normal. These data indicate that CLPs are not grossly affected by the *Justy* mutation. On the other hand, CMPs showed a clear increase in *Justy* bone marrow compared to wild type. Although the difference was not significant, we observed this difference each time when analyzing bone marrow population, even in samples from old (10 month-old) mice.

Block in B cell development is caused by the Justy mutation

In order to better understand the mechanism underlying the developmental block caused by the *Justy* mutation, we tested the potential of bone marrow cells to develop into B cells using Witlock-Witte in vitro culture. We co-cultured total *Justy* bone marrow with S17 stromal cells and IL-7. Wild-type cultures proliferated and expansion was clearly visible by eyes under the microscope, while in *Justy* culture, very few cells could be collected from the supernatant. FACS analysis of the cells collected from wild type culture revealed that the majority of the cells are $B220^{+}CD19^{+}$ B cells, while *Justy* cultures failed to generate cells expressing these markers. These results indicate that exogenous stromal cells and IL-7 cannot overcome the block in B cell development caused by the *Justy* mutation.

The number of cells that develop into non-B cells are very similar in wild type and *Justy* cultures, suggesting that cells in the *Justy* culture do not have higher tendency to develop into $B220^{+}CD19^{-}$ and $B220^{-}CD19^{-}$ cells. However, we cannot rule out the possibility that in a certain cytokine milieu they take on an alternative fate as a result of their retained developmental plasticity.

Alternative lineage potential of purified Justy pre-pro-B and early pro-B cells: fail to differentiate in B/myeloid inducing cultures

We tested the alternative lineage potential of sort-purified *Justy* pre-pro-B and early pro-B cells. Stromal cell co-culture experiments have provided important insights regarding the stages at which developmental potential is restricted during B cell development. We hypothesize that *Gon4l* functions to establish a gene expression pattern that is necessary for the pre-pro-B to early pro-B cell transition. More specifically, our preliminary gene expression analysis suggests that *Gon4l* is required to fully repress transcription from non-B lineage genes. A prediction stemming from this hypothesis is that loss of *Gon4l* function will increase the developmental plasticity of pre-pro-B cells due to their inability to shut down alternative lineage genes.

Wild-type and *Justy* pre-pro-B cells were sort-purified and populations plated directly into wells on a 24 well plate containing a confluent monolayer of S17 stromal cells and in the presence of IL-7, SCF and Flt3 ligand. After 7-14 days, wells were scored for growth. Wild type pre-pro-B and early pro-B cells expanded well, while *Justy* cultures had very few cells present in the culture. Wild-type pre-pro-B and early pro-B cells produced almost exclusively $B220^{+}CD19^{+}$ B cells. In *Justy* cultures, pre-pro-B cells were able to produce some $B220^{+}CD19^{+}$ B cells although their number was very low. Instead, these cells proliferated into $Ly6c^{+}$ myeloid cells, which corresponds with the fact that pre-pro-B cells retain some residual myeloid potential. However, early pro-B cells from the *Justy* culture hardly produced any B or myeloid cells. These results suggest that, although *Justy* pre-pro-B cells are capable of producing some $B220^{+}CD19^{+}$ B cells in vitro, they do not expand even in the presence of IL-7, suggesting that there is a block downstream of the IL-7 signaling pathway.

Justy pre-pro-B cells differentiate under T/NK cell inducing condition

To further test the developmental potential of *Justy* cells, we performed a preliminary experiment where pre-pro-B and early pro-B cells from wild-type and *Justy* mice were sort-purified and populations plated into wells on a 24-well plate containing a confluent layer of either OP9-GFP or OP9-DL1 cells. After 7-10 days, wells were scored for growth. The OP9-GFP culture provides a negative control system where no Notch signal is present, thus the cells are unable to differentiate into T/NK cells. In the OP9-DL1 system, where the Notch-1 ligand (DL1) is expressed, a Notch signal is provided, which results in the development of T/NK cell ($CD25^{+}Thy1.2^{+}CD49b^{+}$) precursors. Both wild type and *Justy* pre-pro-B cell cultures gave rise to similar number of cells.

Furthermore, the number of $CD25^{+}Thy1.2^{+}CD49b^{+}$ T/NK cells was also similar, suggesting that the survival and proliferation of Justy pre-pro-B cells are not entirely defective. Furthermore, it provides evidence, that pre-pro-B cells are capable of giving rise to lineages other than B cells. In either early pro-B cell cultures, however, the cells did not expand which corresponds with the commitment of early-pro-B cells into the B lineage.

Gon4l paralog is widely expressed in various tissues

Interestingly, the *Gon4l* locus has undergone duplication in mice at some point after speciation from other rodents, resulting in a paralog that is immediately upstream of *Gon4l* and which lacks exons 4-11 and 26-33 from *Gon4l* but contains all other exons. VISTA Browser (Berkeley National Laboratory) analysis showed that Gon4l homologues exist in all mammals examined as well as in frog (*Xenopus tropicalis*) and zebrafish. A *Gon4l* gene homologue was not found in the chicken genome in its present form, but a cDNA (XM_422861) with homology to *Gon4l* has been isolated from these species. Results from the VISTA website indicate that a *Gon4l paralog* homologue does not exist in other rodent species. One explanation for this is that *Gon4l paralog* arose relatively recently in mouse following its divergence from other rodent species. The putative protein encoded by this paralog would lack the C-terminal PAH and SANT domains as well as the putative nuclear localization signal (NLS), suggesting that this protein, if expressed, would be excluded from the nucleus.

While it is clear that *Gon4l paralog* was generated via partial duplication of *Gon4l*, it is not known whether *Gon4l paralog* expresses an mRNA or protein or is a pseudogene. Thus, we ought to determine the expression pattern of Gon4l paralog mRNA, if present, in different tissues. We developed a *Gon4l paralog* specific Q RT-PCR method. The difficulty of differentiating *Gon4l paralo*g from *Justy* is coming from the almost 100% similarity among exons shared by both mRNA. We took advantage of single nucleotide differences spanning across exon 22 and designed primers that are specific for *Gon4l paralog* but do not amplify *Gon4l*. We ordered HPLC purified oligos to increase specificity and using plasmids encoding region from exon 22 of either *Gon4l paralog* or *Gon4l* DNA. We developed an assay where the amplification is specific for *Gon4l paralog* but not for *Gon4l*. The Q RT-PCR analysis indicated that this gene is widely expressed in different tissues. Based on our prediction that it lacks the NLS it cannot substitute for *Gon4l*, at least in the nucleus. Interestingly, we have found that the mRNA level decreases during transition from pre-pro-B to early pro-B cells in both wild-

type and *Justy* cells. This suggests that *Gon4l paralog* may have a role during B cell development; however, whether its role in the cytoplasm is similar to *Gon4l* will need to be further defined.

Socs7 negatively regulates IL-Rα expression

IL-7 and TSLP are both key cytokines during B cell development. IL-7Rα is the common receptor for both IL-7 and Tslp. As I observed decreased IL-7Rα expression in Justy early pro-B cells, I wanted to learn more about the underlying mechanism regulating IL-7Rα expression. It has been reported that Tslp signaling involves the activation of Stat5 in a pre-B cell line. Furthermore, Stat5 has been shown to be a key player entraining B cell development downstream of the IL-7R pathway. As Socs7 has been shown to interact with Stat5 in vitro I hypothesised that Socs7 might be a suppressor of Tslp/IL-7 signaling.

Socs7 deficient mice exhibit a severe skin disease that resembles human atopy. These mice showed increased mast cell degranulation in the affected skin. As mast cells both express and respond to Tslp, I decided to analyze bone marrow derived mast cells (BMMCs) from both wild-type and Socs7-/- mice. BMMCs were stimulated by FcεRI aggregation. mRNA levels of Tslp and its heterodimeric receptor consisting of IL-7rα and Tslpr were determined. There was no change in Tslpr mRNA levels upon stimulation of either Socs7$^{-/-}$ or control mast cells. IL-7rα mRNA levels, however, were increased in a time-dependent manner in both Socs7$^{-/-}$ and wild type mast cells. There was a greater increase in IL-7rα mRNA expression in the absence of Socs7 ($p=0.04$ after 2 hours of treatment). Although there was almost no detectable Tslp mRNA in control BMMCs after 30 minutes stimulation, Tslp mRNA was detected in BMMCs obtained from Socs7$^{-/-}$ mice as early as 30 minutes and increased with prolonged stimulation. After 2 and 4 hours of stimulation, Socs7$^{-/-}$ mast cells produced approximately 4.5 fold more Tslp transcript than control cells. In control BMMCs, the level of Socs7 transcript was increased upon stimulation (0 vs 2h: $p=0.003$; 0 vs 4h: $p=0.002$).

Other Socs proteins have been shown to target proteins for proteasomal degradation, thus I determined whether Socs7 interact with the Elongin B/C complex. Xpress epitope tagged full-length and ΔSox Box (ΔSB) Socs7 cDNAs were transfected into HEK293 cells together with HA epitope tagged Elongin C. Lysates from these cells were subjected to immunoprecipitation and blotted with an antibody recognizing Elongin C (a-HA). While full-length Socs7 bound to, the ΔSB mutant failed to interact with

Elongin C. These results demonstrate that Socs7 can interact with Elongin C and suggest that Socs7 targets IL-7Rα for proteosomal degradation.

SUMMARY

B cell development occurs through several stages and regulated by the combinatorial activity of growth factors and their receptors as well as transcriptional regulators. In this work I have presented the work I performed to understand more about the complex mechanism underlying B cell development. I have analyzed a mouse model named Justy (for just T cells) generated by ENU induced mutation. The causal recessive mutation results in a profound, cell-intrinsic block in B lymphopoiesis and in the almost complete absence of B220+CD19+ pre-B cells in the bone marrow, as well as mature B cells in the periphery, but has no apparent effect on the development of other hematopoietic cell lineages. The Justy mutation was mapped to an intron within a poorly characterized gene named Gon4l. Molecular analysis demonstrated that the mutation disrupts splicing of Gon4l RNA, resulting in the absence of wild-type Gon4l mRNA expression in Justy pre-pro-B and early pro-B cells. I have also demonstrated that the Gon4l protein localizes to the nucleus and predicted to form domains found in other proteins that function as transcriptional co-repressors. QRT-PCR experiments, consistent with the gene expression profiling on pre-pro-B and early pro-B cells from Justy mice, demonstrated that Gon4l is important for reprogramming gene expression during lineage commitment phase of B lymphopoiesis. Further analyses depicting the role of Gon4l in the potential of pre-pro-B and early pro-B cell to develop into alternative lineages suggest that Justy cells are able to differentiate into other then B cells, thus the mutation results in a defect specific for B cell development. Furthermore, I suggested a mechanism for the regulation of IL-7/TSLP signaling. These cytokines play important roles during B cell development and learning more about the mechanisms that regulate them will deepen our understanding about hematopoiesis. I have shown that Socs7 negatively regulates IL-7Rα expression and TSLP production by mast cells and proposed that Socs7 targets IL-7Rα for proteosomal degradation.

All together, my analyses provide new insights into the regulation of B lineage commitment.

PUBLICATIONS

Publications related to this thesis

Lu P*****, Hankel IL*****, **Knisz J*,** Marquardt A, Chiang M-Y, Grosse J, Constien R, Meyer T, Schroeder A, Zeitlmann L, Al-Alem U, Friedman A, Meyerholz DK, Waldschmidt TJ, Rothman PB and Colgan JD: The *Justy* Mutation Identifies the Transcriptional repressor Gon4l as a critical regulator of B Lineage Commitment. In Press. Journal of Experimental Medicine ***IF : 15.46***

*****These authors made equal contributions to this work.

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Presentations, posters related to this thesis

Presentations

Knisz J. *Justy is required for the regulation of linage-specific genes during B cell development* **- Autumn Immunology Conference** – Chicago, IL, USA, 2007.

Posters

Knisz J.,Chiang MY., Hankel I.,Rothman PB., Colgan J. *Justy is required for the regulation of linage-specific genes during B cell development* **- Autumn Immunology Conference** – Chicago, IL, USA, 2007.

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Mostecki J, Klimecki WT, Yu L, **Knisz J**, Graves P, Miller RL, Showalter BM, van Peer M, Russo J, Halonen M, Martinez FD, Vercelli D, Rothman PB: A SOCS-1 Promoter Variant is Associated with Total Serum IgE Levels. (Submitted)

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