The RAG1 Ubiquitin Ligase Domain Enhances T Cell Receptor Gene Assembly and Thymic Selection

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Abstract

RAG1/RAG2 (RAG) endonuclease-mediated assembly of diverse lymphocyte antigen receptor genes by V(D)J recombination is critical for the development and immune function of T and B cells. However, this process creates highly self-reactive cells that must be negatively selected to suppress autoimmunity. The RAG1 protein contains a ubiquitin ligase domain that stabilizes RAG1 and stimulates RAG endonuclease activity. We report here that mice lacking RAG1 ubiquitin ligase activity exhibit diminished recombination of T cell receptor (TCR) β and α loci, and impaired thymocyte developmental transitions that require the assembly of these genes and signaling by their proteins. The mice also have reduced expression of TCR signaling proteins within thymocytes, less efficient negative selection of highly self-reactive thymocytes, and mature αβ T cells of elevated autoimmune potential. Thus, we propose that the RAG1 ubiquitin ligase domain provides αβ T cell developmental stage-specific means to augment TCR signaling and thereby enhance selection for beneficial TCR genes and against αβ TCRs possessing high autoimmune potential.
Introduction

The ability of T and B lymphocyte populations to express antigen receptors able to recognize a potentially unlimited number of distinct pathogens is the fundamental basis of adaptive immunity. RAG1/RAG2 (RAG) endonuclease-mediated assembly of T cell receptor (TCR) and immunoglobulin (Ig) genes through V(D)J recombination establishes this vast pool of diverse receptors. Germline TCR and Ig loci consist of variable (V), joining (J), and sometimes diversity (D), gene segments residing upstream of constant (C) region exons. During T and B cell development, the lymphocyte-specific RAG complex cooperates with lineage- and developmental stage-specific activation of TCR and Ig loci to perform V(D)J recombination (1). RAG induces DNA double strand breaks (DSBs) between coding sequences and flanking recombination signal sequences of two participating gene segments, liberating hairpin-sealed coding ends and blunt signal ends (2). In a subsequent reaction phase guided by RAG, DNA repair factors ligate signal ends to generate a signal join and open, process, and ligate coding ends to create a V(D)J coding join, which comprises the second exon of an assembled V(D)J-C antigen receptor gene (2). The high number of V(D)J joining events, broad utilization of gene segments, and inherent imprecision in opening and processing coding ends all cooperate to yield a vast population and diversity of receptors. Due to imprecise means of opening and processing coding ends, the outcomes of V(D)J recombination include out-of-frame genes unable to make protein and receptors that recognize self-antigens and have potential for autoimmunity. Accordingly, developing T and B lymphocytes engage quality control checkpoints to select for potentially beneficial antigen receptor proteins and against hazardous highly self-reactive receptors.

Both immature T and B cells employ developmental programs that link functional protein expression from in-frame V(D)J rearrangements to signal either survival and continued differentiation or apoptosis (1). The maturation of αβ T cells in the thymus provides a paradigm for how V(D)J recombination and antigen receptor protein quality control checkpoints cooperate to create a large pool of lymphocytes with diverse
receptors that provides immunity from pathogens without causing overt autoimmunity. After entering the thymus, early thymocyte progenitor cells differentiate into CD44+/CD25- stage 2 and then into CD44+/CD25+ stage 3 CD4+CD8- double negative (DN) thymocytes (3, 4). Both DN2 and DN3 cells express RAG and conduct V(D)J recombination of Tcra, Tcra, and/or Tcrg loci (3, 4). Although DN2-to-DN3 thymocyte maturation can occur independent of RAG and V(D)J recombination, an in-frame Tcra rearrangement is necessary for development beyond the DN3 stage (3, 4). The resultant TCRα protein associates with invariant pTα protein to produce pre-TCR complexes that signal antigen-independent survival, proliferation, and differentiation of DN3 cells (β-selection) into CD44+/CD25- stage 4 DN thymocytes and then CD4+CD8+ double-positive (DP) thymocytes (3, 4). DP cells express RAG and conduct V(D)J recombination of Tcra loci, which typically proceeds through multiple successive rounds of V-to-J rearrangement on both alleles (5). After an in-frame VJ rearrangement, the resulting TCRα protein can pair with TCRβ protein and yield surface αβ TCRs, which must signal to prevent death by neglect (6-9). This signal activation requires appropriate physical interactions between αβ TCRs and self-peptide/MHC (pMHC) complexes displayed on thymic epithelial cells or dendritic cells, meaning that a functional αβ TCR is inherently self-reactive (6-9). Interactions below a particular low threshold of affinity (or avidity) cannot activate signalling to block apoptosis (6-9). At the other extreme, contacts above a higher threshold trigger strong TCR signalling that causes apoptosis (negative selection) to delete highly self-reactive cells with substantial autoimmune potential (6-9). However, interactions between these two thresholds activate TCR signalling of a strength within a range that promotes survival and differentiation of DP cells (positive selection) into CD4+ or CD8+ single-positive (SP) thymocytes, which lack RAG expression and exit the thymus as mature αβ T cells (6-9). A notable aspect of this αβ TCR quality control checkpoint is that thymocytes are more sensitive than mature αβ T cells as positively-selecting pMHC activates TCR signaling only in the former (9-14). This differential is thought to enable positive selection from low affinity/avidity interactions, prevent the same contacts from activating mature...
αβ T cells, and provide a “safety net” for negative selection to more effectively suppress autoimmunity (6-9). Although the T lineage-specific Themis protein contributes to this differential in TCR sensitivity between immature and mature αβ T cells (9, 15, 16), the precise mechanisms by which this protein and possibly additional factors “tune” TCR signaling and control αβ TCR selection remain to be elucidated.

The RAG1 protein N-terminus has a Zinc-binding RING finger E3 ubiquitin ligase domain of undetermined relevance for antigen receptor gene assembly. RAG1 autoubiquitylates on lysine 233 (17), which enhances RAG endonuclease activity as evidenced by impaired cleavage upon mutation of this residue (18). RAG1 also ubiquitylates other proteins including histone H3 within chromatin (19, 20). RAG1 RING domain mutations that disrupt ubiquitin ligase activity impair RAG-mediated cleavage and joining of chromatin substrates, which could be due to loss of histone H3 ubiquitylation and/or alteration in RAG1 protein structure (20-22). The C328Y mutation of a critical structural zinc-binding cysteine of human RAG1 impairs V(D)J recombination and causes severe T and B cell immunodeficiency associated with uncontrolled proliferation of activated oligoclonal αβ T cells (23). The analogous C325Y mutation in mouse Rag1 destabilizes Rag1 tertiary structure, abrogates ubiquitin ligase activity, reduces V(D)J recombination in vitro, and causes a near complete block of αβ T cell development at the DN3 thymocyte stage due to impaired Tcrb recombination (21, 22). RING domains have a conserved proline (proline 326 in mouse Rag1) that is critical for ubiquitin ligase activity by permitting functional interaction with ubiquitin-conjugating enzymes (24, 25). Relative to C325Y mutation, P326G mutation abrogates RAG1 ubiquitin ligase activity equivalently, but has substantially less severe effects on destabilizing RAG1 protein and reducing RAG endonuclease activity (22). Accordingly, P326 mutation of endogenous mouse RAG1 protein allows an approach to elucidate potential physiologic roles of the RAG1 ubiquitin ligase domain beyond promoting V(D)J recombination by stabilizing RAG1 protein structure.
To determine potential in vivo functions of RAG1 ubiquitin ligase activity, we created and analyzed mice carrying a homozygous Rag1 P326G mutation. We show that these mice exhibit reduced recombination of Tcrb and Tcra loci and lower efficiencies of β-selection, positive selection, and negative selection. We find that inactivation of the RAG1 ubiquitin ligase lowers expression of the pre-TCR signaling protein Syk in DN3 thymocytes and of the αβ TCR signaling protein Zap70 in DP thymocytes. Finally, we demonstrate that mature αβ T cells of these mice exhibit normal Zap70 expression and immune responses to activation, yet nevertheless confer greater potential for autoimmunity. Although reduced V(D)J recombination alone could underlie impaired β-selection and positive selection, this does not provide a plausible explanation for less efficient negative selection and greater autoimmunity hazard of αβ T cells. Thus, we propose that the RAG1 ubiquitin ligase domain might provide developmental stage-specific means to increase TCR signaling sensitivities in thymocytes and thereby enhance selection for beneficial Tcrb genes and against highly self-reactive αβ TCRs with autoimmune potential.
Materials and Methods

Mice

C57BL/6 background PG mice were made through gene-targeting as performed by InGenious Targeting Laboratory (2200 Smithtown Avenue, Ronkonkoma, NY). The 9.5 kb genomic DNA used to construct the targeting vector was sub-cloned from a 129Svev BAC clone. The CC>GG double point mutation was shuttled from pJM029[P326G](22) into the targeting vector using unique restriction sites, then confirmed by sequencing. Linearized targeting vector was electroporated into BA1 (C57BL/6 x 129/SvEv) embryonic stem (ES) cells. After selection with G418 antibiotic, clones were expanded for PCR analysis to identify recombinant ES clones. Positive clones were screened for short arm integration and then confirmation of the point mutation was performed by PCR and sequencing. Positive clones were analyzed by Southern blot to confirm long arm integration. Injected blastocysts were grown in foster mothers and chimeras were backcrossed against C57BL/6 mice to generate heterozygotes. These were crossed with CMV-cre mice (JAX #006054)(26) to remove the neo reporter cassette and generate the $\text{Rag1}^{P326G}$ allele. After confirmation of neo cassette removal, the strain was backcrossed against C57BL/6, and removal of CMV-cre was confirmed by PCR. Sequencing of the entire Rag1 locus in progeny confirmed the intended CC>GG mutation and an additional T>C point mutation that generates a V238A substitution outside of the RING finger domain. The strain was deposited at the Mutant Mouse Regional Resource Center at the University of North Caroline (RAG1-tm1, MMRRC #: 37105). Before initiating studies described herein, the $\text{Rag1}^{P326G}$ allele was backcrossed for nine generations onto the C57BL/6 background. To generate NOD background PG mice, CRISPR/Cas9-mediated gene editing was conducted in zygotes from in vitro fertilization of NOD eggs with NOD sperm. A guide RNA (5′-AAGCACGGATATCGGCAAGA-3′) that hybridizes near Rag1 sequences that encode proline 326 was generated as described (27). The following mix was electroporated into zygotes: 4μM of purified guide RNA, 4μM of Cas9-NLS nuclease (qb3 MacroLab Berkeley), 10μM of ssDNA 5′-
C*A*G*AAAGGACTTCAGTGGCAGTCTCCAGGGATAGGCAGGATATCGGCAGGAT
CCACAATAGCTGCCCATGACTTTTGAAGACATCTGAGAATGCAGATCCTACAGAATAGATGCT
T-3' (IDT) that contains nucleotides encoding glycine in place of proline at amino acid 326 and a silent point mutation of the next codon which introduce a cleavage site for BamHI restriction enzyme. Mice were screened for introduction of the mutation by PCR with primers 5’-CGTCGCAAGAGAACTCAGGCT-3' and 5’-TCTCGTCAGTGACAGGAGATGCT-3’, followed by BamHI digestion of the PCR product. Sanger sequence of PCR products confirmed incorporation of the desired mutation and no other mutations in the Rag1 coding sequence. Founders with mutations were mated with NOD mice to establish NOD Rag1P326G/+ mice, which were bred with each other to generate NOD PG mice and WT littermate controls. NOD mice, C57BL/6 background wild-type and Rag1-/- mice and BALB/c background Rag1-/- mice were purchased from Jackson Laboratories. All experimental mice were littermates or age-matched with control wild-type animals. All experiments were conducted in accordance with national guidelines and approved by the Institutional Animal Care and Use Committee (IACUC) of the Children’s Hospital of Philadelphia.

Flow Cytometry

Single cell suspensions of all organs were ACK lysed to remove erythrocytes before being stained with LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit (Invitrogen) and antibodies against respective surface antigens (BD Biosciences, eBioscience, and Biolegend). Cells were stained for respective intracellular proteins using Cytofix/Cytoperm Fixation/Permeabilization solution kit (BD Biosciences). For staining of intracellular cytokines, cells (1 x10^6) were cultured in the absence or presence of 50 ng/ml PMA (Sigma) and 1µg/ml Ionomycin (Cell Signaling Technology), with 2 µg/ml brefeldin A (Sigma) and 2 µM monensin (eBioscience) for 4 hours at 37°C. After staining for LIVE/DEAD and surface antigens, cells were stained for respective cytokines using the Cytofix/Cytoperm kit (BD Bioscience) or transcription factors using the FoxP3/Transcription Factor staining kit (eBiosciences). Data were acquired on a
MACSQuant flow cytometer (Miltenyi Biotec) or LSRII Fortessa (BD Biosciences) and analyzed using Flowjo software version 10.5.3 (Tree Star).

**Cell Sorting**

To isolate DN3 cells, thymocytes were stained with PE-labelled CD4, CD8, CD11b, CD11c, NK1.1, Gr1, and Ter119. Non-labelled cells were enriched by MACS depletion using anti-PE microbeads and LS columns (Miltenyi). Enriched cells were then stained with CD4, CD8, CD44, CD25 and anti-lineage/stage markers (TCRβ, B220, CD19, CD11b, CD11c, TCRδ, NK1.1, Ter119) antibodies. DN3 thymocytes were sorted on Lin-CD4-CD8-CD44-CD25+ phenotype. To isolate DP cells, thymocytes were stained with CD4, CD8, and anti-lineage (B220, CD19, CD11b, CD11c, TCRδ, NK1.1, Ter119) antibodies. DP thymocytes were sorted on Lin-CD4+CD8+ phenotype. The cells of interest were isolated using a FACSAria Fusion sorter (BD Biosciences).

**Analyzing Tcrb and Tcra Rearrangements**

Genomic DNA was extracted from DN3 or DP cells using the DNeasy Blood and Tissue kit (Qiagen). To measure Tcrb rearrangements in DN3 cells, a Taqman PCR assay was used to quantify Vβ-to-DJβ1.1 and Vβ-to-DJβ2.1 rearrangement levels with a primer specific for each Vβ paired and a probe, FAM or HEX, specific for Jβ1.1 or Jβ2.1, respectively. Taqman PCR was performed with conditions according to the manufacturer’s instructions (IDT DNA) on the ViiA 7 system (Applied Biosystems). PCR of CD19 was used for normalization. Primers, probes, and reaction conditions are as described (28). To assay Tcrb repertoire, DNA from DN3 thymocytes were sent to Adaptive Biotechnologies, who used multiplex PCR to amplify and deep sequence Vβ-Dβ-Jβ rearrangements. Gene segment usage was analyzed by ImmunoSEQ Analyzer software (Adaptive Biotechnologies). To assess Tcra rearrangements in DP cells,
representative Vα-Jα rearrangements were quantified using a QuantiFast SYBR Green PCR kit (Qiagen) on the ViiA 7 system (Applied Biosystems) as described (29, 30). PCR of β2M was used for normalization.

**RT-qPCR for mRNA Expression**

Respective cell populations were lysed in RLT buffer (Qiagen) containing 2-mercaptoethanol or TRIzol (Life Technologies) immediately after cell sorting. Total RNA was isolated using RNeasy Mini kit (Qiagen), treated with DNase (RNase-Free DNase Set, Qiagen), and reverse transcribed to generate cDNA with High-Capacity RNA-to-cDNA™ Kit (Thermo-Fisher Scientific) according to manufacturer’s directions. The cDNA was subjected to qPCR using Power SYBR Green kit (Applied Biosystems) and actin, HPRT, ZAP70, and SYK primers (Qiagen). Relative expression was calculated using the ddCt method, using actin or HPRT as a housekeeping gene, and relevant calibrator sample (explained in figure legends for respective samples).

**In vitro T Cell Differentiation**

Splenocytes from C57BL/6 background Rag1−/− mice were irradiated with 2,500 Rads (X-rad irradiator) and 3 x 10^5 cells were seeded into each well of a 96 well plate. Naïve T cells (CD90.2+, CD4+, CD62L+, CD44+, CD25+) were sorted from C57BL/6 background WT or Rag1−/− mice using a FACSaria Fusion cell sorter (BD Biosciences), and 3 x 10^4 sorted cells were added to each well containing irradiated feeder cells. All cells were cultured with 10 µg/ml anti-CD3 (Biolegend) and 3 µg/ml anti-CD28 (Biolegend) in RPMI with 10% FBS, 1% PSG, and 1x NEAA (Invitrogen), 1x Sodium Pyruvate (Invitrogen), and 0.001% 2-mercaptoethanol. rhIL-2 was used at 30 IU/mL, rmIL-12 and rmIL-4 at 10 ng/mL, rmIL-6 at 20 ng/mL, mTGFβ1 at 1 ng/mL, anti-mIL-4, anti-mIFNγ and anti-mIL-12 at 10 µg/mL (from Biolegend except rhIL-2, rmIL-12, and rmIL-6 from Prepotech). The following cytokines/blocking antibodies were used to skew towards respective Th-subsets: Th0 condition: rhIL-2; Th1 condition: rhIL-2, rmIL-12 and anti-mIL-4;
Th2 condition: rhIL-2, rmIL-4, anti-mIFNγ and anti-mIL-12; Th17 condition: rhIL-2, mIL-6, mTGFβ1, anti-mIL-4 and anti-mIL-12; Treg condition: rhIL-2, mTGFβ1, anti-mIL-4 and anti-mIL-12.

**LCMV Infections**

Mice were infected intraperitoneally with $2 \times 10^5$ plaque-forming units of LCMV-Armstrong and euthanized at indicated timepoints. Virus and gp33-tetramer were kindly provided by E. John Wherry (University of Pennsylvania).

**T Cell Transfer Model of Colitis**

This model was performed as described (31). Briefly, naïve CD4$^+$ T cells were sorted from respective donor mice (CD90.2$^+$, CD4$^+$, CD62L$^+$, CD44$^+$, CD25$^-$) on a FACSaria Fusion cell sorter. 0.5 $\times 10^6$ cells were transferred in 100 µl of PBS into each of the donor mice via intraperitoneal injection. Non-transferred mice were injected with PBS alone. Mice were weighed weekly for the first 4 weeks, and then twice weekly for 4 weeks. Mice were euthanized 8 weeks post-transfer unless severe morbidity enforced premature euthanasia (in accordance with CHOP IACUC approved guidelines) or spontaneous death occurred.

**Diabetes Onset in NOD Background Mice**

NOD background WT and PG mice were aged and assessed for spontaneous development of diabetes. Mice were aged and monitored for disease to a maximum of 30 weeks of age. Mice older than 10 weeks of age were weekly tested for glycosuria (Uristix®, Siemens). For positive mice, glycosuria was assayed by a second test 24 hours later. Capillary blood glucose of mice testing positive twice for glycosuria was tested via facial vein bleeding. Mice with blood glucose $\geq 250$ mg/dl (13.9 mmol/l) were considered as diabetic and euthanized.
Statistical analysis

All data was analyzed in Graphpad Prism 8 using statistical tests indicated in the figure legends. Error bars indicate mean +/- SEM unless otherwise stated. n.s.=p>0.05, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
Results

**Homozygous Rag1 P326G mutation diminishes TCR gene assembly and subsequent thymocyte developmental stage transitions.**

To determine potential in vivo functions of the RAG1 ubiquitin ligase, we created and analyzed C57BL/6 strain mice homozygous for the Rag1 P326G mutation (Rag1\(^{P326G/P326G}\) mice; see Materials and Methods) that abrogates ubiquitin ligase activity but minimally destabilizes RAG1 protein (22). We initially analyzed αβ T cell development and TCR gene rearrangements in Rag1\(^{P326G/P326G}\) (PG) mice and wild-type (WT) mice. As compared to WT mice, PG mice have a ~2-fold higher percentage and number of DN3 thymocytes and correspondingly lower percentage and number of DN4 thymocytes (Fig. 1, A and B). PG mice also exhibit ~10% reductions in the percentage and number of DP thymocytes and the ratio of CD4\(^+\) SP thymocytes to DP thymocytes (Fig. 1, C-E). Despite these minor impairments of the DN-to-DN4 and DP-to-SP thymocyte developmental transitions, PG mice have normal numbers of total thymocytes and splenic αβ T cells (Fig. 1 F). We observed ~50% lower levels of rearrangement for nearly all individual V\(\beta\) segments in DN3 thymocytes of PG mice (Fig. 2, A and B). Rag1 or Rag2 mutations that substantially impair RAG endonuclease activity, thymocyte development, and thymic cellularity also alter the usage of individual V\(\beta\) segments (32, 33). Yet, reflecting the minor effects of Rag1 P326G mutation, unbiased high-throughput sequencing of Tcrb rearrangements in DN3 cells shows similar usage of each V\(\beta\) segment in PG and WT mice (Fig. 2 C). Finally, we detected ~50% lower levels of rearrangements for many of the V\(\alpha\)/J\(\alpha\) combinations assayed in DP thymocytes of PG mice (Fig. 2 D). Together, these data show that homozygous Rag1\(^{P326G}\) mutation produces RAG endonuclease complexes that catalyze decreased levels of Tcrb and Tcra rearrangements and support less efficient thymocyte developmental transitions that depend on the functional assembly of a Tcrb or Tcra gene. Notably, the degrees to which TCR gene rearrangements and αβ T cell development are reduced in PG mice are dramatically less than reported for homozygous Rag1 C235Y mutation (21). For example, Rag1\(^{P236G}\) mice generate 90% of the normal...
number of DP thymocytes, whereas homozygous Rag1<sup>C235Y</sup> mice produce only 1% of the normal number of DP thymocytes. The markedly different severities of these phenotypes indicate that abrogation of RAG1 ubiquitin ligase activity without substantial disruption of RAG1 protein structure only slightly impairs V(D)J recombination and αβ T cell development.

**Inactivation of the RAG1 ubiquitin ligase decreases expression of the signaling proteins Syk and Zap70 in thymocytes.**

The greater than normal number of DN3 thymocytes in *PG* mice is not typical of a mutation that impairs V(D)J recombination. Instead, this phenotype is consistent with diminished pre-TCR signaling where DN3 cells experiencing β-selection exhibit increased survival but are less efficient than normal at transitioning into DN4 thymocytes. It is notable that thymocytes lacking the Syk pre-TCR signaling protein display this phenotype (34, 35), and that RAG DSBs induced in pre-B cells trigger a genetic program that transcriptionally represses expression of Syk to dampen pre-BCR signaling (36). Thus, we determined whether inactivation of the RAG1 ubiquitin ligase reduces Syk expression in DN3 thymocytes.

We detected a slightly lower than normal level of Syk protein in *PG* mice (Fig. 3, A and B); however, unexpectedly, this correlated with an increase in the level of Syk mRNA (Fig. 3 C). These results suggest that RAG DSBs downregulate Syk gene transcription in DN thymocytes, as in pre-B cells, and fewer RAG DSBs from less *Tcrb* recombination in *PG* DN cells leads to higher than normal Syk transcript levels. Despite this, the lower Syk protein expression in *PG* DN thymocytes implies that the RAG1 ubiquitin ligase normally increases translation of Syk mRNA and/or stability of Syk protein during αβ T cell development. Regardless of the underlying mechanisms, these data open the possibility that Syk-mediated pre-TCR signaling is impaired, underlies the accumulation of DN3 thymocytes, and contributes to impaired DN3-to-DN4 thymocyte progression in mice lacking RAG1 ubiquitin ligase activity.
Given that Syk is related to the Zap70 protein that transduces αβ TCR signals, we determined whether inactivation of the RAG1 ubiquitin ligase also reduces Zap70 expression. To this end, we quantified levels of Zap70 protein and mRNA in DP cells at each of four stages of αβ TCR-signaled positive selection as defined by expression of CD69 and CCR7 proteins (37)(Fig. 3 D). In PG mice, we observed lower than normal Zap70 protein levels, but normal levels of TCRβ protein, at each stage of selection (Fig. 3, E-H). We detected normal levels of Zap70 mRNA in cells at each stage assayed in PG mice (Fig. 3 I), implying that lower Zap70 mRNA translation and/or Zap70 protein stability is the basis for lower Zap70 expression.

RAG DSBs induced during lymphocyte development signal gene expression changes that persist in mature NK cells lacking RAG1 and RAG2 expression (38). We therefore considered whether the Zap70 alterations caused by mutant RAG1 during development would also persist in mature T cells after RAG1 is no longer expressed. However, we detected normal Zap70 protein levels in splenic CD4+ and CD8+ αβ T cells of PG mice (Fig. 3, J and K), indicating that Rag1P326G mutation only reduces Zap70 expression in immature αβ T cells. These data imply that Zap70-mediated TCR signaling might be weaker than normal in developing αβ T cells and thereby contribute to the impaired DP-to-SP thymocyte progression of mice lacking RAG1 ubiquitin ligase activity.

**Homzygous Rag1 P326G mutation decreases efficiencies of positive and negative selection.** Considering the critical role of Zap70 for positive and negative selection, we investigated whether either of these αβ TCR quality control processes is impaired by the Rag1P326G mutation. As a proxy to establish whether positive selection is impaired in PG mice, we determined the frequency of αβ TCR expressing (TCRβ+) Stage 1 DP thymocytes that initiate positive selection as indicated by progression to Stage 2 in PG and WT mice (Fig. 4 A). This frequency is lower in PG mice (Fig. 4 B). We note that this is an imperfect approach to quantify positive selection because it cannot measure differences in progression.
through subsequent stages nor cellular fate decisions from specific TCR/pMHC contacts. Nevertheless, our data are consistent with less efficient positive selection in mice lacking RAG1 ubiquitin ligase activity.

To determine whether the Rag1P326G mutation impairs negative selection, we utilized a naturally occurring system of superantigen-mediated negative selection (39). Laboratory mice express remnant mouse mammary tumor virus (MMTV) retroviral products. Some of these products can bind the MHCII I-E^d protein and certain Vβ peptides, including Vβ5.1-5.2 and Vβ12, to trigger exceptionally strong αβ TCR signaling that promotes negative selection (39, 40). BALB/c, but not C57BL/6, strain mice express I-E^d protein. As a result, thymocytes expressing Vβ5.1-5.2 or Vβ12 are efficiently deleted in BALB/c, but not B6, genetic backgrounds. We bred BALB/c background Rag1^−/−_mice with C57BL/6 background Rag1^PG_ or WT mice to generate and analyze Rag1^PG/−_ and Rag1^WT/−_ mice expressing I-E^d protein. To ascertain whether these mice exhibit a difference in negative selection, we measured frequencies of pre-selected DP thymocytes and post-selection SP thymocytes expressing I-E^d:MMTV-reactive (Vβ5^+ or Vβ12^+) or -unreactive (Vβ6^+, Vβ8^+, or Vβ14^+) αβ TCRs (example staining shown in Fig. 4 C). We quantified the fractions of thymocytes expressing each Vβ that progresses through development (are positively selected) and normalized these to the frequencies of total pre- and post-selected cells. For each experiment, we normalized values to the fraction for Rag1^WT/−_ mice so that we could combine data from independent experiments (Fig. 4 D). Our analysis indicates that greater fractions of I-E^d:MMTV-reactive thymocytes proceed through DP-to-SP development in Rag1^PG/−_ mice as compared to Rag1^WT/−_ mice (Fig. 4 D), consistent with impaired negative selection of highly self-reactive thymocytes. In contrast, we found that the types of un-reactive thymocytes assayed develop equivalently in each genotype (Fig. 4 D). While the latter finding does not reflect impaired positive selection, we note that this experiment monitors a small subset of thymocytes expressing specific Vβ peptides and thus is not as sensitive as determining the
frequencies of DP cells that initiate positive selection. Regardless, we conclude that inactivation of the RAG1 ubiquitin ligase renders negative selection less efficient.

Mature αβ T cells that develop without RAG1 ubiquitin ligase activity exhibit normal responses to activation.

RAG endonuclease activity during lymphocyte ontogeny triggers permanent changes in gene expression that correlate with impaired immune responses of mature lymphocytes (38). Although we did not observe any differences in Zap70 protein levels between splenic αβ T cells of PG and WT mice, we sought to elucidate whether mature αβ T cells that develop without RAG1 ubiquitin ligase activity have normal or altered response to activation. We first conducted in vitro analyses. Upon stimulation with PMA and ionomycin, splenic CD44hi αβ T cells from PG mice produce normal levels of IFNγ, TNFα, IL-2, and IL-17A (Fig. 5 A). These cells also show normal proliferation upon anti-CD3/anti-CD28 stimulation (Fig. 5 B). Moreover, stimulated CD4+ and CD8+ αβ T cells of PG mice normally express their canonical transcription factors (Fig. 5, C and D) and cytokines (Fig. 5, E and F) when cultured in respective skewing conditions. We next monitored αβ T cell responses in vivo following infection of mice with an acute strain of lymphocytic choriomeningitis virus (LCMV-Armstrong). We analyzed mice either seven days later to monitor at the immune response peak or 35 days later to monitor memory cells. At each timepoint, we detected similar frequencies and numbers of antigen-specific CD8+ αβ T cells that stain with gp33:H-2Db tetramer in PG and WT mice (Fig. 5 G), indicating that PG mice display normal numbers and dynamic responses of αβ T cells that recognize LCMV-Armstrong. We also observed equivalent dynamic expression of Tbet, Eomes, KLRG1, and CD127 proteins on αβ T cells from PG and WT mice (Fig. 5 H), indicating normal contraction of short-lived effector cells (SLECs), and sustained memory/memory precursor effector cells (MPECs) in PG mice. Finally, we detected similar numbers of antigen specific αβ T cells able to respond to the gp33, NP396, or gp61 viral peptides within a peptide re-stimulation assay
Together, these data support the notion that the development of αβ T cells in the absence of RAG1 ubiquitin ligase activity does not substantially alter responses of these mature immune cells to activation.

Mature αβ T cells that develop in the absence of RAG1 ubiquitin ligase activity exhibit increased predisposition to autoimmunity.

In a setting of less efficient negative selection as with homozygous Rag1P326G mutation, an increased predisposition to αβ T cell mediated autoimmunity would be expected. However, we have not observed overt autoimmune symptoms within PG mice, possibly because mice of the C57BL/6 background are resistant to spontaneous autoimmune diseases (41, 42). Thus, to investigate whether the Rag1P326G mutation can predispose to autoimmunity, we utilized an induced model of autoimmune colitis in C57BL/6 mice and a spontaneous model of autoimmune diabetes in NOD mice.

The colitis model involves transferring into Rag1−/− mice naïve CD4+ effector αβ T cells, which become activated and trigger cytokine-driven colitis and associated systemic pathologies including weight loss due to the absence of Tregs (31, 43, 44). We observed accelerated wasting of Rag1−/− mice receiving PG cells relative to WT cells or no transfer (Fig. 6 A). We analyzed mice at eight weeks after transfer. As compared to WT recipients, we detected greater numbers of total splenic CD4+ T cells and of CD4+ T cells producing IFNγ, TNFα, and/or GM-CSF cytokines (Fig. 6, B and C). Colon length is used as a measure of disease severity in this model, and mice receiving transferred PG cells had shorter colons relative to mice receiving WT cells, consistent with worse disease caused by PG cells (Fig. 6 D). Histologic assessment and scoring in this colitis model has been formalized (44), and can be grossly broken down into scores related to inflammatory cell infiltration and scores related to injury of tissue architecture. There was no statistical difference in inflammation scores between mice receiving WT and PG cells. However,
tissue damage scores were higher in mice receiving PG cells, suggesting that, on a per cell basis, PG cells induce greater tissue injury in this model (Fig. 6, E-F). Notably, specifically in PG-transferred animals, we observed autoimmune pathologies in addition to colitis, including dermatitis, chylous ascites, severe small intestine enlargement, and neurological stress (Fig. 6 H). Dermatitis was most common and involved massive inflammatory infiltration and tissue damage (Fig. 6 I). Consistent with more widespread autoimmune conditions in PG-transferred mice, we significant splenomegaly in these mice (Fig. 6 J). Finally, while not statistically significant, we observed an increased rate of premature death in the mice transferred with PG cells compared to WT cells (Fig. 6K), which was especially surprising because death is an unexpected outcome in this model. Collectively, the data from this colitis model indicate that CD4+ effector T cells from PG mice have greater than normal intrinsic autoimmune potential.

To determine whether inactivation of the Rag1 ubiquitin ligase enhances autoimmunity in another genetic background, we utilized CRISPR/Cas9-mediated gene editing to establish NOD mice with the Rag1P326G mutation. NOD mice develop spontaneous αβ T cell driven diabetes at an incidence of 60–80% in females and 20–30% in males (45, 46). We bred together NOD:Rag1P326G/WT mice to create cohorts of NOD PG and WT females, which we aged and screened for diabetes by monitoring glycosuria and blood glucose levels. We observed accelerated onset of diabetes in PG mice compared to WT mice (Fig. 6 L). Collectively, our analyses of the colitis and diabetes models on mice of two different genetic backgrounds indicate that homozygous Rag1P326G mutation increases predisposition to αβ T cell mediated autoimmunity.
Discussion

Our analysis of homozygous Rag1\textsuperscript{P326G} mice reveals roles for the RAG1 RING domain ubiquitin ligase in stimulating TCR gene assembly, shaping thymocyte gene expression, and enhancing TCR quality control checkpoints. As expected from \textit{in vitro} analyses, the Rag1\textsuperscript{P326G} mutant produces RAG endonuclease complexes that catalyze modestly decreased V(D)J recombination of Tcrb and Tcra loci in thymocytes. While a less efficient RAG endonuclease could account entirely for the reduced assembly of Tcrb and Tcra genes, additional experiments are necessary to investigate potential functions of the RAG1 ubiquitin ligase in modulating chromatin accessibility and/or RAG binding of these loci. The reduced assembly of Tcrb genes most likely contributes to the impaired DN3-to-DN4 thymocyte development of PG mice, as is the case in mice homozygous for other Rag1 or Rag2 mutations that lower RAG endonuclease activity (21, 33). To our knowledge, reduced V(D)J recombinase function has not been demonstrated to increase DN3 thymocyte numbers; however, Syk inactivation has because it impairs pre-TCR signals that drive differentiation of DN3 cells (34, 35). Accordingly, it is plausible that reduced Syk protein expression in DN3 thymocytes contributes to their accumulation in PG mice, highlighting a potential role of the RAG1 ubiquitin ligase in promoting early thymocyte development beyond stimulating Tcrb gene assembly. Yet, unequivocal conclusions require elucidating how Rag1\textsuperscript{P326G} mutation impairs Syk expression and specifically disrupting these mechanisms.

We next considered effects of Rag1 RING domain ubiquitin ligase inactivation on the DP-to-SP thymocyte developmental transition. Given that deletion of most J\textalpha segments in mice impairs DP-to-SP thymocyte development due to fewer chances for each DP cell to produce a positively-selected TCR (47), it is possible that reduced Tcra recombination contributes to less efficient positive selection in PG mice. Accordingly, we cannot definitively attribute a non-V(D)J recombination effect of Rag1 ubiquitin ligase inactivation to positive selection. In contrast, our data indeed support a non-V(D)J recombination effect
of reduced Rag1 ubiquitin ligase activity on negative selection. In DP thymocytes with normal V(D)J recombinase function, the expression of a highly self-reactive αβ TCR signals apoptosis before ongoing Tcra recombination can generate a new αβ TCR (48). Consequently, it is hard to conceive how reduced RAG endonuclease activity would impair negative selection. However, as reduced Zap70 activity impairs αβ TCR signaling and negative selection (49-51), attenuated αβ TCR signaling from lower Zap70 protein expression is a plausible explanation for less efficient superantigen-mediated negative selection in PG mice. However, definitive conclusions necessitate determining how Rag1P326G mutation impairs Zap70 expression and specifically disrupting these mechanisms.

Based on our findings, we propose that RAG1 ubiquitin ligase activity upregulates expression of Syk and Zap70 protein respectively, to enhance β-selection of positive and negative selection of thymocytes. We acknowledge that Syk and Zap70 protein levels are only slightly lower than normal in PG mice and that we have not proven that these differences underlie impaired β-selection, positive selection, and negative selection. We did not observe differences in Syk or Zap70 activation or other aspects of TCR signaling between WT and PG thymocytes following anti-CD3 stimulation (data not shown). However, this methods of activating TCR signaling is not as sensitive for detecting differences as using MHC to present different peptides to a fixed transgenic αβ TCR (9, 10, 16), which is beyond the scope of this study. Moreover, we note that small reductions in Zap70 activity render negative selection less efficient (49-51). In addition, we consider it likely that Rag1P326G mutation also might alter expression of other proteins, with the cumulative changes having synergistic effects on TCR signaling and thymocyte selection. To rigorously test our model, it will be necessary to elucidate the extent and mechanisms by which the RAG1 ubiquitin ligase modulates gene expression, and then disrupt these pathways, preferably without also reducing RAG endonuclease activity. Likewise, it will be important to determine whether Rag1P326G mutation has any effects on the thymic microenvironment (i.e. defective maturation of thymic epithelial cells and/or
distribution of dendritic cells) as is observed in mouse models with impaired V(D)J recombination and a
resulting dramatically reduced thymic cellularity (52). However, unlike these models, \( PG \) mice have
relatively normal numbers of thymocytes, which should facilitate distinction of thymocyte intrinsic versus
extrinsic effects on \( \alpha \beta \) TCR selection.

There are multiple not mutually exclusive mechanisms by which the RAG1 ubiquitin ligase domain could
increase Syk and Zap70 protein levels within thymocytes. In pre-B cells, RAG DSBs signal transcriptional
activation of a broad multifunctional genetic program, with one component being decreased Syk protein
expression to attenuate pre-BCR signaling and facilitate assembly of Ig light chain genes (36, 53). RAG
DSBs in thymocytes could trigger transcriptional and post-transcriptional up-regulation of Syk and Zap70
expression, where fewer RAG DSBs from \( \text{Rag}1^{P326G} \) mutation specifically impairs post-transcriptional
mechanisms. DSB-induced covalent modifications of histones in chromatin around breakage sites are
critical for amplifying DNA damage response (54). The inability of \( \text{Rag}1^{P326G} \) to ubiquitylate histone H3
and other potential target proteins at RAG DSBs might dampen or prevent signals that mediate post-
transcriptional upregulation of Syk and Zap70. Alternatively, the RAG1 ubiquitin ligase domain might
elevate Syk and Zap70 protein expression independent of RAG endonuclease activity, such as by binding
and/or ubiquitylating factors that regulate translation or turnover of these TCR signaling proteins.
Whatever mechanisms an intact RAG1 ubiquitin ligase domain employs to stimulate Syk and Zap70
protein expression, their effects would be temporary and terminated after cessation of RAG1 expression
following positive selection as evidenced by normal Zap70 protein levels in mature \( \alpha \beta \) T cells as well as
their normal function to infectious challenges.

The requirement for \( \alpha \beta \) TCR selection on pMHC presents major challenges for establishing an \( \alpha \beta \) T cell
population that recognizes and responds to diverse foreign peptides, but not self-peptides. An inability to
positively-select \( \alpha \beta \) TCRs with very low affinity/avidity for pMHC could eliminate T cells capable of
binding foreign peptides with no similarities to self-peptides, generating holes in adaptive immunity. At the same time, any positively-selected TCR is able to recognize a self-peptide and thus could cause autoimmunity. To meet these challenges, cell intrinsic mechanisms have evolved to render thymocytes more sensitive to TCR stimulation than their mature αβ T cell counterparts (10-14). This “TCR signal tuning” elevates the affinity/avidity thresholds for both positive and negative selection because any functional TCR/pMHC interaction triggers a stronger TCR signal in thymocytes versus αβ T cells (6-9). This lower sensitivity of mature αβ T cells prevents their activation when encountering an pMHC identical to that on which they were positively-selected. Although several factors have been implicated in regulating TCR signal tuning, each is expressed in both thymocytes and mature αβ T cells (16, 55-59). Our observations of reduced thymic Zap70 expression and less efficient positive and negative selection in PG mice suggest that the RAG1 protein might serve as a “TCR signal tuning” factor. The thymocyte specific developmental timing of RAG1 expression makes it an excellent candidate to serve as one such tuning factor. In this scenario, the ability of the RAG1 ubiquitin ligase domain to upregulate Zap70, and perhaps modulate expression of additional factors, would raise TCR/pMHC affinity/avidity thresholds for positive selection and negative selection. Therefore, we propose that the RAG1 ubiquitin ligase domain provides developmental stage-specific means to stimulate positive selection of potentially beneficial TCRs and negative selection of highly self-reactive TCRs with greatest autoimmune potential. Critically, our study provides a phenotypic framework from which to elucidate the exact molecular mechanisms by which the RAG1 ubiquitin ligase promotes TCR gene assembly and transcends this process to govern the proper selection of developing αβ T cells.
We thank Kyutae Lee for designing the gene-editing strategy and Adele Harman of the CHOP Transgenic Core to generate the NOD PG mice. The authors have no completing financial interests to declare.
References


Ginnis, G. K. Mahowald, on, not receptor editing, is a physiological response of


This work was supported by the Fondation ARC pour la recherche sur le cancer (C.M.), La Ligue contre le cancer (C.M.), Fondation Pasteur Mutualité (C.M.), Nancy Taylor Foundation for Chronic Disease (E.M.B.) and NIH grants R03 AI097611 (J.M.J.), R01 HL112836 (E.M.B.), R01 AI121250-A1 (E.M.B.), R21 A1 135435 (C.H.B.), and RO1 AI 112621 (C.H.B.).
Figure Legends

**Figure 1.** *PG* mice exhibit impaired thymocyte developmental transitions that require TCR gene assembly. (A) Representative flow cytometry plots of DN stages of thymocyte development showing the frequency of cells at each stage. (B) Quantification of numbers of cells in each DN stage. (C) Representative flow cytometry plots of DN, DP, CD4+ SP, and CD8+ SP thymocytes. (D) Quantification of numbers of cells in each stage. (E) Quantification of the ratio of CD4+ SP or CD8+ SP thymocytes to DP thymocytes. (F-G) Quantification of the numbers of total thymocytes (F) and splenic αβ T cells (G). (A-G) All data were collected from 6-10 weeks old mice and (B, D, E, F, and G) are combined from four independent experiments including a total of 14-16 mice per genotype. Bars indicate mean +/- SEM. Stats: 2-way ANOVA with Sidak multiple comparison test. n.s. p>0.05; * p<0.05, **p<0.01, *** p<0.001, **** p<0.0001.

**Figure 2.** Thymocytes of *PG* mice support diminished levels of *Tcrb* and *Tcra* recombination. (A-B) Taqman qPCR quantification of rearrangements of each Vβ segment to DJβ1.1 (A) or DJβ2.1 (B) complexes conducted on genomic DNA from DN3 thymocytes. Signals from each assay were normalized to values from an assay for the invariant CD19 gene. Shown are average values +/- SEM from three independent DN cell preparations. (C) Adaptive Immunoseq quantification of the percentage usage of each Vβ in *Tcrb* rearrangements of DN3 thymocytes. Shown are average values from two independent DN cell preparations. (D) qPCR quantification of specific Va/Jα rearrangements performed on genomic DNA from DP thymocytes. Signals from each assay were normalized to values from an assay for the invariant β2m gene. Shown are the average values +/- SEM from three independent DP cell preparations. For all graphs in figure: #, not detected, n.s. p>0.05; * p<0.05, **p<0.01, *** p<0.001, **** p<0.0001.
Figure 3. Thymocytes of PG mice have reduced Syk and Zap70 protein levels. (A-B) Representative flow cytometry histogram plots (A) and quantification (B) of Syk protein in DN3 thymocytes. (B) Shown are all data points and average values +/- SEM from one of two independent experiments (n = 3-4 mice per genotype, unpaired t-test). (C) qRT-PCR quantification of Syk mRNA in DN3 cells. Relative mRNA levels were calculated using ddCt method with signals from each assay normalized to values from an assay for the Hprt gene. Shown are all data points and average values +/- SEM from two independent experiments (n = 8-9 mice per genotype, 2-way ANOVA with experiment an independent variable, stats indicate difference as a result of genotype). (D) Gating strategy for Stages 1-4 of DP thymocyte selection. Pre-gated on live singlets and CD8$^+$ and/or CD4$^+$. (E-H) Representative flow cytometry histogram plots (E, G) and quantification (F, H) of Zap70 (E, F) and TCR$\beta$ (G, H) protein in Stages 1-4 of DP thymocyte selection. (F, H) Shown are all data points and average values +/- SEM from one of three independent experiments (n = 4-5 mice per genotype, 2-way ANOVA with Sidak’s multiple comparison post-test) (I) qRT-PCR quantification of Zap70 mRNA in DN3 cells or Stage 2 or 4 DP cells. Relative mRNA levels were calculated using ddCt method with signals from each assay normalized to values from an assay for the Hprt gene. Shown are all data points and average values +/- SEM from one experiment (n = 4-5 mice per genotype, 2-way ANOVA with sidak multiple comparison post-test. (J-K) Representative flow cytometry histogram plots (J) and quantification (K) of Zap70 protein in naïve CD4$^+$ or CD8$^+$ T cells. Shown are all data points and average values +/- SEM from two independent experiments (n = 3 mice per genotype, 2-way ANOVA with Sidak multiple comparison post-test). For all graphs in figure: n.s. p>0.05; * p<0.05, **p<0.01, *** p<0.001, **** p<0.0001.

Figure 4. PG mice exhibit impaired positive and negative selection. (A) Gating strategy for measuring positive selection of thymocytes showing Stage 1 and 2 gates. Pre-gated on live singlets and CD4$^+$ and/or CD8$^+$. (B) Quantification of the ratio of the number of Stage 2 cells to the total number of Stage 1 and 2 cells. Shown are all data points and global mean from three independent experiments (n = 10-11 mice per experiment). For all graphs in figure: n.s. p>0.05; * p<0.05, **p<0.01, *** p<0.001, **** p<0.0001.
genotype, 2-way ANOVA indicating effect of genotype). (C) Gating strategy form measuring negative selection of Vβ6+ and Vβ8+ thymocytes in F1 progeny of C57BL/6 background PG mice bred with BALB/c background Rag1−/− mice. Pre-gated on live singlets. (D) Quantification of ratios of indicated Vβ+ cells that progress to post-selection versus those Vβ+ cells that are produced at the pre-selection stage. Normalized to total thymocytes in pre or post-selection population and WT controls. Shown are all data points and average +/- SEM from two independent experiments (n = 9 mice per genotype, 2-way ANOVA with Sidak multiple comparison post-test. For all graphs in figure: n.s. p>0.05; * p<0.05, **p<0.01, *** p<0.001, **** p<0.0001.

Figure 5. Mature αβ T cells of PG mice exhibit normal immune response. (A) Representative plot of cytokine expression on CD4+CD44hi αβ T cells following ex vivo stimulation of splenocytes with PMA and ionomycin. (B-F) Representative analyses of naïve splenic CD4+ or CD8+ T cells after ex vivo simulation with anti-CD3/anti-CD28 antibodies and additional antibodies to skew towards indicated lineages. Shown is CFSE incorporation (B) or expression of relevant transcription factors (C, D) or cytokines (E, F). (G, H) Representative flow data showing (G) frequencies of splenic αβ T cells that stain with the gp33:H-2Db tetramer or (H) express indicated cytokines after LCMV infection of mice. Data are representative of two independent experiments with at least four mice of each genotype. (I, J) Representative plots (J) and (I) quantification of antigen-specific αβ T cells able to respond to indicated viral peptides within a peptide re-stimulation assay. (A-F) Data are representative of two independent experiments. (G-J) Data are combined from four independent experiments (two D7 and two D35 timepoints) with 8-11 mice of each genotype per time-point.
Figure 6. Mature αβ T cells of PG mice possess higher than normal autoimmune hazard. (A) Graph of body weights of Rag1−/− hosts over time after transfer of WT or PG CD4+ T cells or no cells (NT). Data combined from three independent experiments with a total of 13-17 mice of each genotype. Lines indicate fitted segmented-linear regression lines +/- 95% confidence interval. Day 15 was fixed as the inflection point. Difference between gradients of second slope was determined by linear regression analysis. (B) Numbers of CD4+ T cells in spleen eight weeks post-transfer. (C) Total number of IFNγ, TNFα, and/or GM-CSF producing cells per spleen. Shown are all data points from three independent experiments with 12-14 mice per group. Bar indicates global mean. Pre-gating on live singlets, CD90.2+, CD4+, and CD44+. (D) Colon length of mice sacrificed at eight weeks post-infection. (E) Representative hemotoxylin and eosin stained colon sections from respective mice at eight weeks post-transfer. 10x magnification. (F-G) Total colitis score as assessed by blinded pathologist. Scored as per previously described guidelines for the five phenotypes (44): (F) Colon inflammation score (contribution of scores for crypt abcesses and inflammatory cell infiltrate). (G) Colon tissue damage score (contribition of scores for crypt architecture, tissue damage, goblet cell loss). (H) Non-colitis pathologies described for mice transferred with WT versus PG cells. (I) Hemotoxylin and eosin stained skin section from PG-transferred mouse with severe dermatitis at eight weeks post-transfer. 10x magnification. (J) Spleen weight at eight weeks post-transfer normalized to total mouse bodyweight prior to transfer. (K) Survival curves of mice transferred with WT or PG cells. Data combined from three independent experiments. Statistical analysis = Log-rank Mantel-Cox test. Overall: 0/13 NT mice died, 1/15 WT-transferred mice died, and 4/17 PG-transferred mice died. (B, C, F, G, and J) Shown are all data points from three independent experiments, 12-14 mice per group. Experiment indicated by shape of data point. Bar indicates global mean. Analyzed by 2-way ANOVA with Tukey HSD post-test. n.s. p>0.05; * p<0.05, **p<0.01, *** p<0.001, **** p<0.0001 (L) Curves showing diabetes free survival of female NOD WT and PG mice. Mice were generated by mating together NOD Rag1WT/PG mice and monitored for their first 30 weeks of life. The percent diabetes survival curves...
include mice that have passed 20 weeks of age or gotten diabetes before 20 weeks of age. Analyzed by Gehan-Breslow-Wilcoxon test. p=0.049.
Figure 1
Figure 2

A. Sorted DN3: TCRβ-VDJ1

B. Sorted DN3: TCRβ-VDJ2

C. Sorted DN3

D. Sorted DP: TCRα
Figure 3

(A) DN3

(B) SYK MFI

(C) Relative Syk mRNA

(D) CD4^+ AND/OR CD8^+

(E) Stage 1, Stage 2, Stage 3, Stage 4

(F) ZAP70 MFI

(G) TCRβ

(H) TCRβ MFI

(I) ZAP70 mRNA

(J) Naive CD4^+ T cells, Naive CD8^+ T cells

(K) ZAP70 MFI
Figure 4

A

RAG1\textsuperscript{WT}  
\begin{itemize}
  \item Stage 2: 16.1%  
  \item Stage 1: 75.9%  
\end{itemize}

RAG1\textsuperscript{PG}  
\begin{itemize}
  \item Stage 2: 11.8%  
  \item Stage 1: 80.4%  
\end{itemize}

Stage 1 TCRβ\textsuperscript{+}  
\begin{itemize}
  \item 42.6%  
  \item 41.1%  
\end{itemize}

B

Stage 1 Cells + Stage 2 Cells

C

CD4\textsuperscript{+} OR CD8\textsuperscript{+}

DP Pre-selection

CD4\textsuperscript{+} OR CD8\textsuperscript{+}

Post-selection

C

\begin{itemize}
  \item\textit{vβ6 pre-selection}  
  \item\textit{vβ12 pre-selection}  
\end{itemize}

\begin{itemize}
  \item\textit{vβ6 post-selection}  
  \item\textit{vβ12 post-selection}  
\end{itemize}

D

\textit{Normalized to WT}  
\begin{itemize}
  \item \textit{vβ}\textsuperscript{+} Post-selection cells  
  \item \textit{vβ}\textsuperscript{+} Pre-selection cells  
  \item Total Post-selection cells  
  \item Total Pre-selection cells  
\end{itemize}
Figure 5
Figure 6