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## Loss of T Cell and B Cell Quiescence Precedes the Onset of Microbial Flora-Dependent Wasting Disease and Intestinal Inflammation in Gimap5-Deficient Mice

Michael J. Barnes<sup>\*1</sup>, Halil Aksoylar<sup>†1</sup>, Philippe Krebs<sup>\*</sup>, Tristan Bourdeau<sup>†</sup>, Carrie N. Arnold<sup>\*</sup>, Yu Xia<sup>\*</sup>, Kevin Khovananth<sup>\*</sup>, Isaac Engel<sup>‡</sup>, Sosathya Sovath<sup>\*</sup>, Kristin Lampe<sup>†</sup>, Eleana Laws<sup>†</sup>, Amy Saunders<sup>§</sup>, Geoffrey W. Butcher<sup>§</sup>, Mitchell Kronenberg<sup>‡</sup>, Kris Steinbrecher<sup>†</sup>, David Hildeman<sup>†</sup>, H. Leighton Grimes<sup>†</sup>, Bruce Beutler<sup>\*</sup>, and Kasper Hoebe<sup>†</sup>

<sup>\*</sup>Department of Genetics, Scripps Research Institute, La Jolla

<sup>†</sup>Cincinnati Children's Hospital Research Foundation, Cincinnati, OH 45229

<sup>‡</sup>La Jolla Institute for Allergy and Immunology, San Diego, CA 92037

<sup>§</sup>The Babraham Institute, Babraham Research Campus, Cambridge, United Kingdom

### Abstract

Homeostatic control of the immune system involves mechanisms that ensure the self-tolerance, survival and quiescence of hematopoietic-derived cells. In this study, we demonstrate that the GTPase of immunity associated protein (Gimap5) regulates these processes in lymphocytes and hematopoietic progenitor cells. As a consequence of a recessive *N*-ethyl-*N*-nitrosourea-induced germline mutation in the P-loop of Gimap5, lymphopenia, hepatic extramedullary hematopoiesis, weight loss, and intestinal inflammation occur in homozygous mutant mice. Irradiated fetal liver chimeric mice reconstituted with Gimap5-deficient cells lose weight and become lymphopenic, demonstrating a hematopoietic cell-intrinsic function for Gimap5. Although Gimap5-deficient CD4<sup>+</sup> T cells and B cells appear to undergo normal development, they fail to proliferate upon Ag-receptor stimulation although NF- $\kappa$ B, MAP kinase and Akt activation occur normally. In addition, in Gimap5-deficient mice, CD4<sup>+</sup> T cells adopt a CD44<sup>high</sup> CD62L<sup>low</sup> CD69<sup>low</sup> phenotype and show reduced IL-7 $\alpha$  expression, and T-dependent and T-independent B cell responses are abrogated. Thus, Gimap5-deficiency affects a noncanonical signaling pathway required for Ag-receptor-induced proliferation and lymphocyte quiescence. Antibiotic-treatment or the adoptive transfer of Rag-sufficient splenocytes ameliorates intestinal inflammation and weight loss, suggesting that immune responses triggered by microbial flora causes the morbidity in Gimap5-deficient mice. These data establish Gimap5 as a key regulator of hematopoietic integrity and lymphocyte homeostasis.

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Address correspondence and reprint requests to Kasper Hoebe, Cincinnati Children's Hospital Research Foundation, MLC7021, Room S5.421, 3333 Burnet Avenue, Cincinnati, OH 45229. kasper.hoebe@cchmc.org.

<sup>1</sup>M.J.B. and H.A. contributed equally to this article.

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### Disclosures

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Many layers of regulation ensure homeostatic control of the immune system during development and throughout life. In fetal and neonatal mice, hematopoietic stem cells (HSCs) and precursor cells migrate from the fetal liver to the bone marrow and thymus (1). Thereafter, maintenance of the HSC niche involves both cell-extrinsic and cell-intrinsic mechanisms in which responsiveness to growth factors, cell cycle control, and breakdown of metabolic by-products are essential (2). When these processes occur normally, the many diverse lineages of hematopoietic cells are continually generated in the bone marrow and thymus. Given their potential to undergo clonal expansion and long-term survival, additional checkpoints are needed to limit the survival of self-reactive T cells and B cells (3). In T cells, these checkpoints include the induction of apoptosis in thymocytes that recognize self-Ags with high affinity (negative selection) (4) and the mitigation of inappropriate immune responses by regulatory T cells (5). The availability of common- $\gamma$ -chain cytokines governs the size and composition of the T cell niche (6–8). Notably, IL-2, IL-7, and IL-15 promote T cell survival by modulating the expression of Bcl-2-family member proteins.

Somewhat paradoxically, mutations or conditions that partially impair T cell survival can be associated with the development of immunopathologies (9). One potential explanation for this association is that excess common- $\gamma$ -chain cytokines available in lymphopenic conditions allow the survival and proliferation of self-reactive T cell clones. For example, when naive CD4<sup>+</sup> T cells are transferred into Rag-deficient recipient mice, they undergo lymphopenia-induced proliferation (LIP) and adopt a characteristic phenotype similar to memory CD4<sup>+</sup> T cells that includes the expression of increased amounts of CD44 (CD44<sup>high</sup>) and reduced amounts of CD62L (CD62L<sup>low</sup>), whereas CD69 expression remains mostly unaffected (CD69<sup>low</sup>) (8, 10). In some genetic backgrounds, intestinal microbial flora promote the expansion of colitogenic IFN- $\gamma$ -producing CD4<sup>+</sup> T cells undergoing LIP, resulting in intestinal inflammation (11). Congenitally lymphopenic *Tcr $\alpha$* <sup>-/-</sup> mice also develop intestinal inflammation owing to excessive IL-4-dependent Th2 cell and  $\gamma\delta$  T cell responses (12–14). Lymphopenia has also been linked to the precipitation of diabetes in animal models (15, 16). Increased rates of T cell turnover, altered ratios of regulatory to conventional T cells, and acquisition of effector function by T cells undergoing LIP are all aspects of the lymphopenic environment that might favor the onset of immunopathology.

Homozygosity for the *lyp* mutation causes severe T cell lymphopenia and has been shown to predispose rats to the development of autoimmune diabetes (16) and intestinal inflammation (17). However, lymphopenia caused by the *lyp* mutation alone is insufficient to trigger immunopathology, which requires the presence of additional modifier alleles at other loci in the genome (17–21). In both susceptible and disease-free genetic backgrounds, homozygous *lyp* rats largely lack CD8<sup>+</sup> T cells and have reduced CD4<sup>+</sup> T cell and NKR-P1<sup>+</sup> NKT cell numbers (21–23), whereas deficiencies in conventional B cells have not been reported (24). Positional cloning efforts identified the *lyp* mutation as a frameshift mutation in the gene *Gimap5* (also known as *Ian4*, *Ian5*, or *Irod*) (25).

The *Gimap* genes (previously known as immune-associated nucleotide binding proteins [*Ian*]) comprise a family of genes that are physically clustered in the genome and predominantly expressed in lymphocytes (26). The cellular localization and functions of the GTPase of immunity-associated proteins (*Gimap*) remain nebulous. All *Gimap* proteins

share a GTP-binding AIG1 homology domain, which was originally identified in a gene involved in plant immunity (27). In mammals, evidence suggests that Gimap3 and Gimap5 both promote thymocyte and T cell survival (26, 28–30), whereas Gimap4 can limit T cell viability (31, 32). More recently, *Gimap5*<sup>-/-</sup> mice were reported to have decreased numbers of peripheral T cells, similar to the *lyp* rat, and also showed disrupted development of NK cells, reduced survival of Vα14 TCR-expressing invariant NKT (*iNKT*) cells, granulocyte accumulation, increased hepatocyte apoptosis and early mortality (33). Thus, the effect of complete Gimap5-deficiency in mice is much more severe than the truncation of Gimap5 by the *lyp* mutation in rats.

These discrepancies open several new avenues for investigation into the function of Gimap5 in vivo. First, although liver failure caused by hepatocyte apoptosis in *Gimap5*<sup>-/-</sup> mice was suggested (33), the cause of the early morbidity in these mice remained unclear. In addition, despite the report of decreased B1 cell accumulation in the peritoneum of *Gimap5*<sup>-/-</sup> mice (33), the effect of Gimap5-deficiency on B cell development was not examined, nor was the role of Gimap5 in thymocyte selection or peripheral T cell and B cell function explored. In this study, we describe a recessive *N*-ethyl-*N*-nitrosourea (ENU)-induced mutation in *Gimap5*, which we termed sphinx. Like *Gimap5*<sup>-/-</sup> mice, *sphinx* homozygotes are lymphopenic, show granulocyte accumulation, exhibit liver abnormalities, and die by 14 weeks old. Using the *sphinx* mouse, we demonstrate that *Gimap5* serves previously uncharacterized cell-intrinsic roles in lymphocyte survival, quiescence, and Ag-receptor induced proliferation, and link the early morbidity in *sphinx* mice to suppressible, microbial flora-dependent, intestinal inflammation and wasting disease.

## Materials and Methods

### Mice and reagents

All experiments were performed according to the U.S. National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute (TSRI) and The Cincinnati Children's Hospital. *BALB/cJ*, *Bim*<sup>-/-</sup>, *C57BL/6J*, *Cd4*<sup>-/-</sup>, *Cd8*<sup>-/-</sup>, *DBA/2J*, *FVB/NJ*, and *NODShiLtJ* mice were obtained from The Jackson Laboratory (Bar Harbor, ME). *β2m*<sup>-/-</sup>, *C3H/HeN*, *H-Y TCR* transgenic, *Rag2*<sup>-/-</sup>, and *Rag2*<sup>-/-</sup> *Il2rγ*<sup>-/-</sup> mice were obtained from Taconic (Germantown, NY). *Gimap5*<sup>sph/sph</sup> mice were generated at TSRI using ENU mutagenesis. *Ja18*<sup>-/-</sup> mice were housed at the La Jolla Institute for Allergy and Immunology and *act-mOVA* transgenic mice were housed at TSRI. All mice were maintained under specific pathogen-free conditions.

αGal-Cer-mouse CD1d tetramers labeled with PE or APC were generated as described previously (34), and all Abs used for flow cytometry were purchased from eBioscience (San Diego, CA) or BioLegend (San Diego, CA). Abs for immunoblotting were purchased from Cell Signaling Technology (Beverly, MA). Purified CD3ε (145-2C11) and CD28 (37.51) abs (eBioscience) were used for T cell activation. F(ab')<sub>2</sub>-anti-IgM (Jackson ImmunoResearch), anti-CD40 (eBioscience), rIL-4 (R&D Systems, Minneapolis, MN), LPS (Axxora, San Diego, CA) and PMA/ionomycin (Sigma-Aldrich, St. Louis, MO) were used for B cell activation. T cells and B cells were labeled with CFSE by incubating MACS (Miltenyi Biotech, Auburn, CA) purified CD4<sup>+</sup> T cells or splenic B cells in 5 μMCFSE in

PBS with 0.1% FCS for 10 min. ELISAs were used to measure serum cytokine concentrations (eBioscience).

### **In vivo cytotoxicity assay measuring NK cell and CD8<sup>+</sup> T cell function**

We immunized G3 ENU mice with  $1 \times 10^7$   $\gamma$ -irradiated (1500 rad) act-mOVA splenocytes. Seven days later, we injected  $1 \times 10^7$  CFSE-labeled cells i.v., consisting of a mixture of three different splenocyte populations (C57BL/6J,  $\beta 2m^{-/-}$ , and SIINFEKL-peptide pulsed C57BL/6J splenocytes) at a ratio of 1:1:1. After 48 h, blood was drawn from the retro-orbital plexus, RBCs were lysed, and the presence or absence of each CFSE-labeled cell population was determined by flow cytometry.

### **mAb MAC421 against mouse Gimap5**

A glutathione-S-transferase fusion protein containing residues 1–270 of mouse Gimap5 was produced using *Escherichia coli* RosettaDE3 bacteria (Novagen, Madison, WI). Purification was performed by elution from glutathione beads and used to immunize a LEW rat. The rat was euthanized 3 d after a final boost with this immunogen, isolated splenocytes were fused with the plasmacytoma cell line Y3Ag1.2.3 (35), and hybridomas were derived by standard methods. The selected clone MAC421 secretes a rat IgG2a Ab specific for Gimap5—that is, it shows no cross-reactivity on other members of the mouse Gimap family. Separate studies have shown that the epitope recognized by MAC421 is dependent on residues 1–10 at the NH2 terminus of mouse Gimap5 (C. Carter, G.W. Butcher, T. Nitta, K. Yano, and Y. Takahama, unpublished observations).

### **Complete blood counts**

Blood samples were taken from the retro-orbital plexus of mice at indicated ages and analyzed using a Hemavet 950 veterinary hematology system Fisher Scientific (Pittsburgh, PA).

### **Hepatic lymphoid cell isolation**

Mice were euthanized using CO<sub>2</sub>, and livers were flushed with ice cold PBS via the hepatic portal vein using a 25-gauge needle and syringe. After excision of the liver, the gall bladder was removed and livers were diced using scissors. Further homogenization was achieved using a sieve and plunger, and liver fragments were gently passed through a 100- $\mu$ M strainer and collected in a 50-ml centrifuge tube. Total liver cells were washed with 40 ml ice-cold RPMI 1640/5% FBS and centrifuged for 10 min at  $300 \times g$ . The resulting pellets were resuspended in digestion medium (RPMI 1640 containing 0.02% [w/v] collagenase IV [Sigma-Aldrich] and 0.002% [w/v] DNase I [Sigma-Aldrich]) and left at 37°C for 40 min under gentle shaking. Ice cold serum free medium was added to each tube, and cells were centrifuged for 3 min at  $30 \times g$  and 4°C. Supernatants containing lymphocyte-enriched cell populations were collected and centrifuged at  $300 \times g$  for 10 min and 4°C. Subsequently, cell pellets were resuspended in PBS containing 0.5% FBS and labeled with the mouse Lineage Cell Depletion Kit (Miltenyi Biotec). Lin<sup>-</sup> cells were then separated using an AutoMacs magnetic sorter (Miltenyi Biotec), and various HSCs or precursor populations were identified by flow cytometry as described before (36).

## Ab responses

For T-dependent Ab responses, age- and sex-matched mice were immunized i.p. with 5  $\mu$ g LPS (Axxora) and 50  $\mu$ g of NP36-CGG (Biosearch Technologies, Novato, CA) mixed 1:1 with alum (Pierce, Rockford, IL). For T-independent Ab responses, mice were immunized i.p. with 50  $\mu$ g NP50-Ficoll (Biosearch Technologies). Serum NP-specific Abs were detected by ELISA. Briefly, 96-well round-bottom plates (Fisher Scientific) were coated with 5  $\mu$ g/ml NP30-BSA (Biosearch Technologies) for detection of NP-specific Ig. Serum samples from immunized mice were serially diluted in 1% milk and added to coated or uncoated (to determine background) wells. Plates were incubated with HRP-conjugated goat anti-mouse IgM or IgG1 (Southern Biotechnology Associates, Birmingham, AL), developed with Peroxidase Substrate and Stop Solution (KPL), and measured for absorbance at 450 nm.

## Immunoblotting

Immunoblotting was performed using total spleen, thymus, or bone marrow lysates, or splenic B cells that were isolated using MACS purification with anti-CD19 microbeads (Miltenyi Biotech). Cell lysis and immunoblotting were performed as described previously (37).

## Cell cycle analysis of splenic B cells

Splenic B cells were cultured with complete IMDM (containing 10% FCS and 1% P/S) and left untreated or treated with 0.1  $\mu$ g/ml LPS at 37°C, 5% CO<sub>2</sub>. After 48 h,  $1 \times 10^6$  cells were collected in 1 ml PBS and subsequently were added dropwise to 2.5 ml absolute ethyl alcohol. After 15 min fixation on ice, cells were washed with PBS and incubated in 500  $\mu$ l propidium iodide (PI) solution (50  $\mu$ g/ml PI, 0.1 mg/ml RNase A, and 0.05% Triton X-100 in PBS) for 40 min at 37°C. Cells were washed and resuspended in PBS and subsequently analyzed by flow cytometry.

## Antibiotic treatment and survival assays

*Gimap5<sup>+/sph</sup>* breeding pairs received antibiotic treatment (1 g/l ampicillin, 500 mg/l vancomycin, 1 g/l neomycin sulfate, and 1 g/l metronidazole) administered orally via drinking water. Subsequent litters remained on antibiotic treatment until analysis at 9-wk-old. For adoptive transfer studies, *Gimap5<sup>sph/sph</sup>* mice at 25235 d old were injected i.v. with  $1 \times 10^7$  splenocytes that had been depleted of RBCs (RBC lysis buffer; Sigma-Aldrich). Mice were monitored and euthanized according to veterinarian recommendations when they became moribund.

## Statistical analysis

Data were analyzed using the GraphPad Prism4 software (GraphPad Software, San Diego, CA). Unless indicated otherwise, statistical significance of the differences among groups was determined from the mean and SD by Student's two-tailed test or by ANOVA followed by Dunnett's test for three or more groups. All data were considered statistically significant if  $p < 0.05$ .

## Results

### *sphinx*: an ENU-induced germline mutation causing severe lymphopenia

We identified *sphinx* in a forward genetic screen designed to detect defective in vivo cytotoxic NK cell and CD8<sup>+</sup> T cell responses in G3-ENU mutagenized mice (38). The original *sphinx* founder, previously immunized with OVA-expressing cells, was unable to reject adoptively transferred NK cell or CD8<sup>+</sup> T cell target cells (Fig. 1A). Necropsy revealed a near absence of CD8<sup>+</sup> T cells and NK cells in the peripheral lymphoid organs (Fig. 1B, 1C) and a marked reduction in total T cell numbers. However, relatively normal thymocyte development occurred, including the CD4<sup>+</sup> T cell, CD8<sup>+</sup> T cell,  $\gamma\delta$  T cell, Foxp3<sup>+</sup> regulatory T cell, and *i*NKT cell lineages (data not shown), whereas the bone marrow contained a high frequency of NK cells expressing the activation marker CD69 (Supplemental Fig. 1). Most strikingly, *sphinx* livers exhibited an abnormal morphology that presented itself as extramedullary hematopoiesis with associated foci of hematopoietic cells (Fig. 1D, 1E). Hyperplastic nodules developed in adult livers (Fig. 1D) that contained well-differentiated hepatocytes bordered by clusters of hematopoietic cells (Fig. 1E). Hepatocytes from *sphinx* mice showed no growth advantage in cell culture and no dysplastic liver cells were observed throughout this study (M. Barnes and K. Hoebe, unpublished observations). In addition, when *sphinx* mice (C57BL/6J genetic background) were crossed with mice of one of five different genetic backgrounds (BALB/cJ, C3H/HeN, DBA/2J, FVB/NJ, or NOD/ShiLtJ) for two generations and then intercrossed, the liver phenotype remained consistent and dysplastic cells were not observed in any other organs (data not shown). Thus, it appeared that *sphinx* livers exhibited an abnormal morphology owing to extramedullary hematopoiesis and reactive hyperplasia of hepatocytes, rather than a hepatic neoplasia. In addition, *sphinx* mice progressively developed normocytic anemia (Fig. 1F, Supplemental Fig. 2A), with increased variation in RBC width (Supplemental Fig. 2B) and thrombocytopenia (Supplemental Fig. 2C). This was correlated with a rapid reduction in circulating blood lymphocytes (Fig. 1G) and concomitant neutrophilia (Supplemental Fig. 2D). These observations indicated that the *sphinx* mutation affected a gene essential for lymphocyte maintenance and normal hematopoiesis.

### Positional cloning of the *sphinx* mutation

To identify the causative mutation, we used a positional cloning strategy (39). Using 13 meioses from *sphinx* mice backcrossed to the C3H/HeN strain, we linked the NK cell and CD8<sup>+</sup> T cell deficiency to chromosome 6 with a peak LOD score of 4.0 (Supplemental Fig. 3A). Further fine mapping with 572 meioses derived from F1 intercrosses confined the mutation to a 2.9-megabase critical region, bounded by the microsatellite markers D6mit315 and D6mit276. We amplified and sequenced all annotated and predicted coding base pairs in the critical region, obtaining high-quality sequence for 89.9% of total coding base pairs and 100% of coding base pairs for genes in the *Gimap* cluster. Among  $1.74 \times 10^5$  bp sequenced, a single G→T point mutation in the *Gimap5* coding region differed between the *sphinx* and C57BL/6J genomes (Supplemental Fig. 3B, 3C). *Gimap5* is predominantly transcribed in lymphocytes (26), but is also expressed in HSCs (40). The *sphinx* mutation appeared to destabilize the *Gimap5* protein in hematopoietic tissues, including total bone marrow cells

and splenic B cells, resulting in a lack of detectable protein (Fig. 1H), whereas mRNA expression of *Gimap5* remained unaffected (Fig. 1I).

The *sphinx* mutation resulted in the amino acid substitution G38C, which changed an amino acid that is highly conserved within all mouse *Gimap* homologs and within all annotated orthologous genes containing an AIG1 domain, in kingdoms as distant as Planta and Protista (Supplemental Fig. 3D). This residue is located in the predicted P-loop of the AIG1 domain of *Gimap5*, in the pocket that binds and hydrolyzes GTP. Previous studies of the p21Ras GTPase have shown that mutation of the analogous Gly residue to Val (G15V) resulted in a protein that was unable to hydrolyze GTP or to transform NIH 3T3 cells when overexpressed (41). The mutation of this residue to Cys might destabilize *Gimap5* by altering the disulphide bonding patterns of the mutant *Gimap5* protein. Whereas no *Gimap5* protein was detected by immunoblotting, any residual intact protein below the limit of detection would be predicted to be nonfunctional because of the mutation of the conserved Gly residue. Overall, the *sphinx* phenotype mirrors the phenotype of previously described *Gimap5*<sup>-/-</sup> mice (33), indicating that the *sphinx* mutation effectively results in a *Gimap5*-null animal.

### Lethal wasting disease, colitis, and hepatic extramedullary hematopoiesis in *sphinx* mice

From birth until weaning, conventionally housed *sphinx* (hereafter *Gimap5*<sup>sph/sph</sup>) mice appeared outwardly healthy. After 4 wk, males showed a reduced growth rate (Fig. 2A) and developed diarrhea. Although significant, weight loss in females was less dramatic and only occurred after 6 wk (data not shown). In male and female *Gimap5*<sup>sph/sph</sup> mice, cell infiltration and inflammation were observed in the colon by 6 wk. Severe colitis, exemplified by goblet cell depletion, leukocyte infiltration into the lamina propria, epithelial cell hyperplasia, and crypt loss, developed by 10 wk (Fig. 2B). Wasting disease and intestinal inflammation likely contributed to the early morbidity of *Gimap5*<sup>sph/sph</sup> mice, which generally occurred by 14 wk.

Liver dysfunction remained another possible factor contributing to early morbidity. To better understand the onset of liver abnormalities, we investigated the development of the embryonic and neonatal liver. At the embryonic day (ED) 16 developmental stage and at birth, livers from *Gimap5*<sup>sph/sph</sup> mice and their heterozygous littermates appeared similar (Fig. 2C). However, more hematopoietic cells remained in *Gimap5*<sup>sph/sph</sup> livers at day 8. By day 14, extramedullary hematopoiesis could be observed before the initiation of nodule formation. Extramedullary hematopoiesis, which persisted in adult mice, was limited to the liver (Fig. 3A), although a disrupted splenic architecture that included reduced follicle size and number and granulocyte accumulation was also observed (data not shown). Schulteis et al. (33) argued that *Gimap5*<sup>-/-</sup> mice die as a consequence of liver dysfunction and hepatocyte apoptosis. Despite the abnormal liver morphology, we found normal concentrations of serum bilirubin and albumin in 8-wk-old *Gimap5*<sup>sph/sph</sup> mice (data not shown), suggesting that the metabolic functions of the liver remained largely intact.

### Cell-intrinsic hematopoietic defects in *Gimap5<sup>sph/sph</sup>* mice

We next considered that aberrant hematopoiesis rather than liver failure might contribute to wasting disease and mortality in *Gimap5<sup>sph/sph</sup>* mice. Despite the liver abnormalities, the bone marrow of 5-wk-old *Gimap5<sup>sph/sph</sup>* mice contained normal numbers of Lin<sup>-</sup> Sca1<sup>+</sup> c-Kit<sup>+</sup> HSCs and hematopoietic progenitor cells (Supplemental Fig. 4 A, B). However, the numbers of megakaryocyte/erythroid progenitors (MEPs) declined with age, in correlation with the onset of anemia and thrombocytopenia in adult *Gimap5<sup>sph/sph</sup>* mice (Fig. 1F, Supplemental Fig. 2C). In addition, despite normal numbers of common lymphoid progenitors (CLPs) in the bone marrow, rapid atrophy of the thymus occurred with age (data not shown). More dramatic differences were observed in the *Gimap5<sup>sph/sph</sup>* liver. Examination of hematopoietic progenitor cell populations revealed that a significant number of HSCs and hematopoietic precursors, representing all hematopoietic lineages, remained in the adult *Gimap5<sup>sph/sph</sup>* liver (Fig. 3A). Compared to normal hematopoiesis in the bone marrow, extramedullary liver hematopoiesis in *Gimap5<sup>sph/sph</sup>* mice was skewed toward generation of Lin<sup>-</sup> Sca1<sup>+</sup> c-Kit<sup>-</sup> IL-7 $\alpha$ <sup>+</sup> CLPs, and it is noteworthy that IL-7 $\alpha$  expression was increased in this population (Fig. 3A).

To investigate the functionality of *Gimap5<sup>sph/sph</sup>* HSCs, we made radiation bone marrow and fetal liver chimeras. Similar to what was reported for *Gimap5<sup>-/-</sup>* mice (33), *Gimap5<sup>sph/sph</sup>* bone marrow cells poorly reconstituted the hematopoietic compartment of lethally irradiated congenic recipient mice (data not shown), suggesting functional impairment. To exclude the possibility that defective HSC function was a secondary consequence of the inflammation and wasting disease that occurs in *Gimap5<sup>sph/sph</sup>* mice, we reconstituted lethally irradiated *Rag2<sup>-/-</sup> Il2r $\gamma$ <sup>-/-</sup>* recipients with *Gimap5<sup>sph/sph</sup>* or *Gimap5<sup>sph/+</sup>* littermate control ED 19 fetal liver cells. The recipients were chosen because they lacked endogenous T cells, B cells, and NK cells, but could support the development of a normal lymphocyte compartment from wild type HSCs. Six weeks after transfer, mice reconstituted with *Gimap5<sup>sph/sph</sup>* fetal liver cells were severely lymphopenic and had substantially fewer thymocytes than mice reconstituted with *Gimap5<sup>sph/+</sup>* fetal liver cells. In the spleen, CD8<sup>+</sup> T cell, B cell, and NK cell numbers were markedly reduced compared with recipients of control fetal liver cells, whereas CD4<sup>+</sup> T cells were present at normal numbers (Fig. 3B). *Gimap5<sup>sph/sph</sup>* fetal liver cell recipients exhibited weight loss and wasting, whereas control fetal liver cell recipients appeared healthy (data not shown). In *Gimap5<sup>sph/sph</sup>* fetal liver cell recipients, CD11b<sup>+</sup> Gr1<sup>+</sup> myeloid cells accumulated in the spleen (Fig. 3B), whereas liver abnormalities were not observed. The transfer of weight loss and wasting disease, but not liver abnormalities, by *Gimap5<sup>sph/sph</sup>* fetal liver cells suggested that the early mortality observed in *Gimap5<sup>sph/sph</sup>* mice is at least partially caused by defective hematopoiesis. Furthermore, the *Gimap5<sup>sph/sph</sup>* fetal liver cells exhibited an intrinsic defect in lymphopoiesis, affecting T cells, B cells, and NK cells.

### Reduced survival and IL-7 $\alpha$ expression in *Gimap5<sup>sph/sph</sup>* T cells

Several mouse models have linked impaired lymphocyte function and lymphopenia with the development of various manifestations of immunopathology (9, 15). To better understand the contribution of lymphocytes to the hematopoietically transferrable abnormalities observed in *Gimap5<sup>sph/sph</sup>* mice, we investigated lymphocyte development, survival and



function. Roles for *Gimap5* in T cells have been proposed for thymic selection, thymic export, and survival in the periphery (16). Like *Gimap5<sup>lyp/lyp</sup>* rats and *Gimap5<sup>-/-</sup>* mice, 6-wk-old *Gimap5<sup>sph/sph</sup>* mice exhibited normal thymus cellularity and frequencies of DN1–4, DP, CD4 SP, and CD8 SP thymocyte subpopulations (data not shown). To examine thymic selection in *Gimap5<sup>sph/sph</sup>* thymocytes, we bred the mutation onto the H-Y TCR transgenic background. The H-Y TCR recognizes an endogenous peptide presented by MHC class I molecules that is derived from the H-Y Ag, which is expressed only in male mice. Consequently, H-Y TCR<sup>+</sup> thymocytes are normally deleted by negative selection in male mice, whereas they develop into mature CD8<sup>+</sup> T cells in female mice. In *Gimap5<sup>sph/sph</sup>* males, H-Y-reactive thymocytes were deleted, implying that the mutation did not impede negative selection (Fig. 4A). In contrast, the thymus of *Gimap5<sup>sph/sph</sup>* females contained normal numbers of H-Y TCR<sup>+</sup> DP thymocytes, but significantly fewer H-Y TCR<sup>+</sup> CD8 SP thymocytes (Fig. 4A), suggesting that positively selected thymocytes became more sensitive to proapoptotic negative selection cues in the absence of *Gimap5*. H-Y TCR<sup>+</sup> CD8<sup>+</sup> T cells were found at reduced numbers and frequencies in the spleen of both male and female *Gimap5<sup>sph/sph</sup>* mice (Fig. 4B), indicating that lymphopenia occurred regardless of TCR specificity for self-Ag.

We next examined the expression of molecules important for thymic egress and peripheral accumulation of T cells. Normally, terminally differentiated TCRβ<sup>+</sup> CD4 SP and CD8 SP thymocytes upregulate CD5 and IL-7ra (CD127) and downregulate CD24 (HSA) and CD69 (4, 42). *Gimap5<sup>sph/sph</sup>* CD8 SP, but not CD4 SP thymocytes, failed to modulate CD24, CD69, or IL-7ra expression (Fig. 4C), suggesting that the mutation either blocked terminal CD8 SP thymocyte maturation or impaired survival of CD8 SP cells. Downregulation of CD69 expression has been associated with the ability of thymocytes to exit the thymus (43), and it is possible that export of CD8 SP thymocytes is impaired in *Gimap5<sup>sph/sph</sup>* mice. In addition, as IL-7 promotes naive T cell survival by modulating expression of anti-apoptotic molecules (44), reduced IL-7ra expression in *Gimap5<sup>sph/sph</sup>* CD8 SP thymocytes likely contributes to the failure of CD8<sup>+</sup> T cells to survive and accumulate in the periphery.

CD4<sup>+</sup> T cells isolated from the spleen of 6-wk-old *Gimap5<sup>sph/sph</sup>* mice showed a significant reduction in IL-7ra surface expression (Fig. 4D) and were unable to proliferate upon TCR stimulation (Fig. 4E). Thus, impaired IL-7 signaling and TCR-induced proliferation represent two contributing factors to the CD4<sup>+</sup> T cell lymphopenia in *Gimap5<sup>sph/sph</sup>* mice. As the lymphopenia became more pronounced with age (Fig. 1G), CD4<sup>+</sup> T cells began to express cell-surface markers characteristic of cells undergoing LIP, including reduced amounts of CD62L but normal, low expression of CD69 (Fig. 4F).

We also noted that peripheral *Gimap5<sup>sph/sph</sup>* *i*NKT cells expressed reduced amounts of NK1.1 (Fig. 5A) and rapidly declined in number with age. NK1.1 expression is acquired at a late step in *i*NKT cell differentiation, most typically after export from the thymus, and evidence suggests that it requires engagement of the invariant TCR with the CD1d Ag-presenting molecule (45). Upon *in vivo* activation of *i*NKT cells with the agonist αGal-Cer, we detected little intracellular IFN-γ or TNF-α, reduced amounts of IL-4 and normal amounts of IL-13 on a per cell basis (Fig. 5B). We confirmed these results by ELISA (Fig. 5C). The impaired survival of *i*NKT cells and aberrant *i*NKT cell cytokine response that

occurs in *Gimap5<sup>sph/sph</sup>* mice suggests roles for *Gimap5* in both lymphocyte survival and function.

### Functional defects in *Gimap5<sup>sph/sph</sup>* B cells

Before the onset of wasting disease, splenic CD19<sup>+</sup> B cells in *Gimap5<sup>sph/sph</sup>* mice, unlike T cells and NK cells, were less reduced in number compared with C57BL/6J littermates. However, the B cell compartment contained fewer mature B cells (Fig. 6A), whereas the percentages of marginal zone B cells (Fig. 6B) and follicular B cells (Fig. 6C) were similar to C57BL/6J littermates. Between 6–10 wk, splenic B cell numbers declined in *Gimap5<sup>sph/sph</sup>* mice (Supplemental Fig. 5A). In the peritoneal cavity, few B1 cells were found (M. Barnes and K. Hoebe, unpublished data), suggesting that B1 cells either fail to develop or survive. In addition, the serum of 8-wk-old *Gimap5<sup>sph/sph</sup>* mice contained ~75% less IgM and 50% less total IgG than heterozygous littermates (Supplemental Fig. 5B). The reduced total IgG reflected significant reductions in concentrations of the isotypes IgG1, IgG2b, and IgG3 and a trend toward reduced Ig-G2a (Supplemental Fig. 5B). These abnormalities suggested the possibility that B cell function was impaired in *Gimap5<sup>sph/sph</sup>* mice. To assess B cell function, we immunized 6-wk-old mice with T-dependent (NP-CGG + alum + LPS) or T-independent (NP-ficoll) Ags. *Gimap5<sup>sph/sph</sup>* mice failed to produce either T-dependent Ag-specific IgG1 Abs (Fig. 6D) or T-independent Ag-specific IgM responses (Fig. 6E), suggesting that *Gimap5<sup>sph/sph</sup>* B cells might have an intrinsic functional defect. To investigate B cell-intrinsic signaling, we isolated B cells from 6-wk-old *Gimap5<sup>sph/sph</sup>* and C57BL/6J mice and determined their capacity to proliferate upon activation in vitro. *Gimap5<sup>sph/sph</sup>* B cells failed to proliferate after BCR stimulation, or treatment with the diacylglycerol mimetic, PMA, and the Ca<sup>++</sup> mobilizing agent ionomycin (Fig. 6F). However, alternative pathways triggered by LPS or CD40 ligation induced normal *Gimap5<sup>sph/sph</sup>* B cell proliferation (Fig. 6F).

Activation of the NF- $\kappa$ B, MAP kinase, and Akt pathways is essential for either BCR or PMA and ionomycin induced proliferation. We examined the activation of these pathways in *Gimap5<sup>sph/sph</sup>* B cells stimulated with PMA and ionomycin. Normal degradation of the NF- $\kappa$ B inhibitor I $\kappa$ B and phosphorylation of the Erk, Jnk, and p38 MAP kinases occurred (Supplemental Fig. 5C). In addition, normal activation of Akt, marked by phosphorylation of serine and threonine residues, and other proximal molecules in the Akt pathway was observed (Supplemental Fig. 5D). Nonetheless, *Gimap5<sup>sph/sph</sup>* B cells failed to proliferate upon PMA and ionomycin activation. Ex vivo cell cycle analysis of *Gimap5<sup>sph/sph</sup>* B cells detected an increased percentage of B cells in S phase and a reduced number of B cells in G2 phase. When proliferation was induced with LPS, an equivalent number of *Gimap5<sup>sph/sph</sup>* B cells entered G2 phase, yet an increased percentage of cells remained in S phase (Fig. 6G), suggesting that defective cycling might cause the decline in peripheral B cell numbers in *Gimap5<sup>sph/sph</sup>* mice (Supplemental Fig. 5A). Overall, we found no major defects in proximal mitogenic signaling or cell cycle entry, but we did find aberrant cell cycle progression in *Gimap5<sup>sph/sph</sup>* B cells.

## Prevention of wasting disease and intestinal inflammation, but not lymphopenia, by antibiotic treatment

Microbial flora in the intestine represents the major reservoir of foreign Ags and exogenous innate immune stimuli in specific pathogen-free housed mice. Localized inflammation in the gastrointestinal tract of *Gimap5<sup>sph/sph</sup>* mice suggested the possibility that aberrant responses triggered by microbial flora contributed to systemic CD4<sup>+</sup> T cell LIP and subsequent cell death, ultimately resulting in lymphopenia. Thus, we sought to address the role of microbial flora in *Gimap5<sup>sph/sph</sup>* mice. Homozygotes and hetero-zygote littermates were put on a continuous antibiotic regimen from birth until 9 wk, when they were analyzed. Interestingly, antibiotic treatment did not prevent lymphopenia (Fig. 7A), the expression of CD44<sup>high</sup> CD62L<sup>low</sup> markers by CD4<sup>+</sup> T cells (Fig. 7B) or the occurrence of hepatic extramedullary hematopoiesis and hepatocyte hyperplasia (Fig. 7C). Antibiotic-treatment completely blocked the accumulation of CD11b<sup>+</sup> myeloid cells (Fig. 7A), ameliorated intestinal inflammation (Fig. 7C), and prevented weight loss (data not shown) in *Gimap5<sup>sph/sph</sup>* mice. These data suggest that the *Gimap5*-dependent hematopoietic cell-intrinsic phenotypes, including hepatic extramedullary hematopoiesis, lymphopenia, and acquisition of a CD44<sup>high</sup> CD62L<sup>low</sup> phenotype by CD4<sup>+</sup> T cells, can occur in the absence of intestinal inflammation, whereas microbial florae promote granulocyte accumulation, colitis, and wasting disease.

## Adoptively transferred lymphocytes reduce extramedullary hematopoiesis and early mortality in *Gimap5<sup>sph/sph</sup>* mice

To further assess the involvement of lymphocytes in the immunopathology observed in *Gimap5<sup>sph/sph</sup>* mice, we adoptively transferred  $1 \times 10^7$  congenically labeled C57BL/6J splenocytes into young *Gimap5<sup>sph/sph</sup>* mice before the onset of wasting disease. Strikingly, recipient *Gimap5<sup>sph/sph</sup>* mice did not succumb to early death (Fig. 8A) or develop wasting disease (Fig. 8B). Adoptively transferred splenocytes filled the CD8<sup>+</sup> T cell niche (Fig. 8C) and promoted the maintenance of endogenous *Gimap5<sup>sph/sph</sup>* CD4<sup>+</sup> T cells (Fig. 8D). In the CD4<sup>+</sup> T cell compartment, cells of *Gimap5<sup>sph/sph</sup>* origin retained a CD44<sup>high</sup> CD62L<sup>low</sup> phenotype (Fig. 8E). Thus, adoptively transferred splenocytes promoted the accumulation of endogenous *Gimap5<sup>sph/sph</sup>* CD4<sup>+</sup> T cells, but not CD8<sup>+</sup> T cells.

We also examined whether adoptively transferred C57BL/6J splenocytes affected the extramedullary hematopoiesis in recipient *Gimap5<sup>sph/sph</sup>* mice. In the liver of untreated 15-wk-old *Gimap5<sup>sph/sph</sup>* mice, an abundance of common myeloid progenitors were present, including both granulocyte/macrophage progenitors (GMPs) and MEPs. In the livers of age-matched *Gimap5<sup>sph/sph</sup>* mice that received adoptively transferred C57BL/6J splenocytes, clusters of hematopoietic cells, nodules, and extramedullary hematopoiesis were still observed, but at a reduced frequency (Supplemental Fig. 6A, 6B) and fewer common myeloid progenitor, MEP, and CLP cells were found (Fig. 8F, Supplemental Fig. 6B). In addition, the bone marrow of aged *Gimap5<sup>sph/sph</sup>* mice showed enhanced granulopoiesis and reduced erythropoiesis (Fig. 8G). These changes in medullary hematopoiesis likely contributed to the neutrophilia (Supplemental Fig. 2D) and anemia (Fig. 1G) observed in adult *Gimap5<sup>sph/sph</sup>* mice. Recipients of adoptively transferred splenocytes maintained normal numbers and frequencies of GMP and MEP in the bone marrow (Fig. 8G), indicating

that transferred cells also affected medullary hematopoiesis. Accordingly, recipient *Gimap5<sup>sph/sph</sup>* mice did not become anemic or exhibit thymic atrophy (data not shown). Enhanced granulopoiesis and thymic atrophy are often observed in conditions of chronic inflammation, and their amelioration by either antibiotics or the adoptive transfer of splenocytes suggests that they are most likely triggered by the inflammatory environment present in *Gimap5<sup>sph/sph</sup>* mice.

Finally, we observed no reduction in mortality or wasting disease when *Gimap5<sup>sph/sph</sup>* mice received *Rag2<sup>-/-</sup>* splenocytes, indicating that a lymphocyte population is required to prevent wasting disease (Fig. 8A). Because adoptively transferred *Cd4<sup>-/-</sup>*, *Cd8<sup>-/-</sup>*, or *Ja18<sup>-/-</sup>* (*iNKT* cell-deficient) splenocytes, or anti-NK1.1 Ab depleted splenocytes could each prevent weight loss and early mortality (data not shown), multiple lymphocyte populations might contribute to the suppression of wasting disease.

## Discussion

Our identification and subsequent characterization of the *sphinx* mutation confirms the nonredundant role of *Gimap5* in lymphocyte survival and normal hematopoiesis (33). The *Gimap5<sup>sph/sph</sup>* mouse exhibits a number of hematologic abnormalities. From birth, peripheral CD8<sup>+</sup> T cells and NK cells fail to accumulate, whereas HSCs accumulate in the liver, suggesting that *Gimap5* has a direct role in controlling these processes. After weaning, intestinal inflammation, weight loss, severe lymphopenia, and acquisition of a CD44<sup>high</sup> CD62L<sup>low</sup> CD69<sup>low</sup> phenotype characteristic of cells undergoing LIP by CD4<sup>+</sup> T cells all occur before premature death at ~14-wk-old. We used several approaches to examine the relationships between these phenotypes. Fetal liver HSCs, which were not exposed to the inflammatory environment present in adult *Gimap5<sup>sph/sph</sup>* mice, could transfer wasting disease and lymphopenia to lethally irradiated recipients, demonstrating an HSC intrinsic function for *Gimap5*. Wasting disease and colitis were mitigated by adoptively transferred lymphocytes, indicating that either defective immune regulation or a lymphopenic environment, or both, contributed to the onset of intestinal inflammation. Antibiotic-sensitive microbes were also required to trigger granulocyte accumulation, wasting disease and intestinal inflammation, but not lymphopenia or accumulation of CD44<sup>high</sup> CD62L<sup>low</sup> CD69<sup>low</sup> CD4<sup>+</sup> T cells.

Before the onset of post weaning abnormalities, extramedullary hematopoiesis occurred in the *Gimap5<sup>sph/sph</sup>* neonatal liver. Defective HSC egress or migration from the neonatal liver could underlie this phenomenon. One possibility that we favor is that *Gimap5<sup>sph/sph</sup>* HSCs become activated in the neonatal liver, thereby halting their migration and inducing extramedullary hematopoiesis. For example, HSC express Toll-like receptors and can undergo myelopoiesis at extramedullary sites in response to LPS-induced inflammation (46, 47). *Gimap5* might act as a negative regulator of HSC activation, initiated by TLR agonists or other stimuli, that maintains quiescence of the HSC niche. In young *Gimap5<sup>sph/sph</sup>* mice, these abnormalities appear to be limited to the liver, and hematopoietic abnormalities in the bone marrow were observed only in older mice, concurrent with granulocyte accumulation and chronic inflammation. Further studies will be needed to determine whether *Gimap5<sup>sph/sph</sup>* bone marrow HSCs have reduced reconstituting capacity because of the

chronically inflamed *Gimap5<sup>sph/sph</sup>* environment or because of a cell-intrinsic requirement for Gimap5 that becomes more pronounced with age.

In addition to aberrant hematopoiesis, intestinal inflammation contributed to the weight loss and early morbidity in *Gimap5<sup>sph/sph</sup>* mice. Spontaneous mouse models of colitis have proven valuable in identifying molecules and mechanisms involved in the pathogenesis of inflammatory bowel disease (48). An abundance of IL-13-producing NKT cells are found in the intestinal lamina propria of human ulcerative colitis patients (49), and IL-13-producing *i*NKT cells can drive colitis in a mouse model induced by the chemical oxazolone (50). Our data suggest that the *Gimap5<sup>sph/sph</sup>* environment favors the polarization of *i*NKT cells toward the production of IL-13. Although the cellular and molecular basis of intestinal inflammation in *Gimap5<sup>sph/sph</sup>* mice demands further dissection, IL-13-producing *i*NKT cells may represent one contributing factor. Another potential influence predisposing *Gimap5<sup>sph/sph</sup>* mice to developing colitis is CD4<sup>+</sup> T cells undergoing LIP. In the naive CD4<sup>+</sup> T cell transfer model of colitis, using C57BL/6 Rag-deficient recipient strains, the lymphopenic environment drives CD4<sup>+</sup> T cells in to LIP. These CD4<sup>+</sup> T cells undergoing LIP acquire effector function and cause both intestinal inflammation and wasting disease (11). Of note, intestinal inflammation is significantly reduced when naive T cells are transferred into germ-free mice, suggesting that microbial flora are necessary for colitogenic immune responses in this model (51). The presence of microbial flora might similarly promote the acquisition of effector function among intestinal CD4<sup>+</sup> T cells undergoing LIP in *Gimap5<sup>sph/sph</sup>* mice. Interestingly, in partially lymphopenic settings such as in sublethally  $\gamma$ -irradiated mice, colitis is not observed after naive T cell transfer (8). Residual radioresistant intestinal lymphocytes that enhance intestinal barrier function, such as IgA-secreting plasma cells or  $\gamma\delta$  T cells, might limit colitogenic CD4<sup>+</sup> T cell responses in this setting (8, 52). In *Gimap5<sup>sph/sph</sup>* mice, the loss of non-CD4<sup>+</sup> lymphocytes with age might generate conditions that promote CD4<sup>+</sup> T cells to undergo LIP, and thereby contribute to the onset of intestinal inflammation.

Our findings that *Gimap5<sup>sph/sph</sup>* HSCs were unable to reconstitute the B cell compartment of irradiated recipients, and that *Gimap5<sup>sph/sph</sup>* B cells were unable to undergo Ag-receptor induced proliferation or generate Ag-specific IgG or IgM responses were especially surprising. In the *lyp* rat model, *lyp* bone marrow cells are able to normally reconstitute the B cell compartment of irradiated recipients (30) and gross B cell functional defects are not observed (24). This discrepancy might reflect different requirements for Gimap proteins in rats and mice. The *Gimap* gene cluster has undergone divergent evolution between rodent species, including changes in the number of *Gimap* genes, amino acid sequence variation between homologous Gimap proteins, and differential Gimap gene expression patterns in lymphocytes (26, 53). To characterize better the consequences of Gimap5-deficiency in mouse B cells, we first considered that Gimap5 might be involved in the activation of the NF- $\kappa$ B and MAP kinase signaling pathways by BCR stimulation, because others had reported that these pathways are dysregulated in *lyp* T cells (54). However, *Gimap5<sup>sph/sph</sup>* mice have normal percentages of marginal zone B cells, which require NF- $\kappa$ B signaling to develop (55), and display normal I $\kappa$ B degradation and MAP kinase phosphorylation upon activation. Normal activation of the PI3K/Akt pathway, which is similarly required for

lymphocyte proliferation, also occurs in *Gimap5<sup>sph/sph</sup>* B cells. One interesting attribute of *Gimap5<sup>sph/sph</sup>* B cells is their accumulation in S phase in vivo. The role for Gimap5 in cell cycle regulation appears to be especially important in the bone marrow or fetal liver cell reconstitution setting, in which donor cells must undergo rapid proliferation to reconstitute the B cell niche. In summary, Gimap5 impairs BCR-induced proliferation and Ig responses independently of activation of the canonical BCR signaling pathways and may link BCR triggering to cell cycle progression.

Although the requirement for Gimap5 in TCR-dependent T cell proliferation and survival has been known for some time, our study identifies several important mechanisms that fail to operate in *Gimap5<sup>sph/sph</sup>* T cells. In the thymus, developing *Gimap5<sup>sph/sph</sup>* CD8 SP thymocytes show impaired survival after positive selection and reduced expression of IL-7 $\alpha$ , suggesting that Gimap5 modulates survival thresholds during thymic selection as well as the expression of a critical cytokine receptor for T cell survival. Although CD4 SP thymocytes express normal amounts of IL-7 $\alpha$ , expression is reduced in the periphery among CD4<sup>+</sup> T cells. These findings provide a partial molecular basis for previous observations made in the lyp rat (16) and *Gimap5<sup>-/-</sup>* mouse (33). In these strains, and in *Gimap5<sup>sph/sph</sup>* mice, CD8<sup>+</sup> T cells and naive CD4<sup>+</sup> T cells, which are more dependent on IL-7 for their survival than Ag-experienced or regulatory CD4<sup>+</sup> T cells (56), are most affected by Gimap5-deficiency. As discussed below, IL-7-independent mechanisms regulating T cell survival also appear to require Gimap5. The net effect of Gimap5 deficiency in CD4<sup>+</sup> T cells is to promote the accumulation of cells with a CD44<sup>high</sup> CD62L<sup>low</sup> LIP-like phenotype with the potential to cause immunopathology. Importantly, other factors such as MHC haplotype and modifier alleles are needed to cause diabetes (18–20) and, as we have shown in this study, microbial flora are required for the onset of colitis. Finally, although peripheral CD4<sup>+</sup> Foxp3<sup>+</sup> regulatory T cell numbers are minimally affected in *Gimap5<sup>sph/sph</sup>* mice, it is not known whether they function and express a normal TCR repertoire, or whether the lymphopenic environment inhibits their ability to control aberrant immune responses. Alternatively, a Foxp3<sup>-</sup> regulatory lymphocyte population might be absent or defective in *Gimap5<sup>sph/sph</sup>* mice.

Our data identify a critical role for Gimap5 in T cell, B cell, NK cell, *i*NKT cell and HSC survival and function. A previous study reported that Gimap5 might mediate its effects in part by interacting directly with Bcl-2 and a related anti-apoptotic protein Bcl-x<sub>L</sub> (26). IL-7 $\alpha$  signaling promotes expression of Bcl-2, which can directly inhibit the proapoptotic activity of Bim (7), and indeed genetic loss of *Bim* restores the T cell compartment in IL-7 $\alpha$ -deficient mice (57). Therefore, we considered that *Gimap5<sup>sph/sph</sup> Bim<sup>-/-</sup>* mice might exhibit less lymphopenia if reduced IL-7 $\alpha$  expression alone prevented T cell accumulation. However, combined Gimap5 and Bim deficiency did not prevent lymphopenia (H. Aksoylar and K. Hoebe, unpublished observations), suggesting that additional survival pathways are perturbed in the absence of Gimap5. Gimap5 may be more than just a prosurvival protein, as it appears to also maintain quiescence in lymphoid cells and HSCs. In support of this possibility, we have found that negative selection thresholds are reduced in thymocytes, HSCs become aberrantly activated in the neonatal liver, and bone marrow NK cells show an activated phenotype. Among B cells, Gimap5 deficiency might in some ways mimic B cell

energy, which can be induced by constant low-affinity BCR stimulation (58), and might favor the accumulation of B cells in cell cycle arrest. Thus, *Gimap5<sup>sph/sph</sup>* B cells can proliferate in response to LPS or CD40 stimulation, but, like anergic B cells, fail to respond to BCR stimulation alone. In vivo, activation by weak stimuli that are normally ignored could abrogate quiescence, promoting the accumulation of lymphocytes in cell cycle arrest and driving HSCs to undergo extramedullary hematopoiesis. Future studies will explore the role of pathways important for setting activation thresholds and enforcing quiescence in *Gimap5<sup>sph/sph</sup>* mice.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations used in this paper

<b>CLP</b>	common lymphoid progenitor
<b>ED</b>	embryonic day
<b>ENU</b>	<i>N</i> -ethyl- <i>N</i> -nitrosourea
<b>Gimap</b>	GTPase of immunity associated protein
<b>GMP</b>	granulocyte/macrophage progenitor
<b>HSC</b>	hematopoietic stem cell
<b><i>i</i>NKT</b>	invariant NKT
<b>LIP</b>	lymphopenia-induced proliferation
<b>MEP</b>	megakaryocyte-throid progenitor
<b>PI</b>	propidium iodide
<b>TSRI</b>	The Scripps Research Institute

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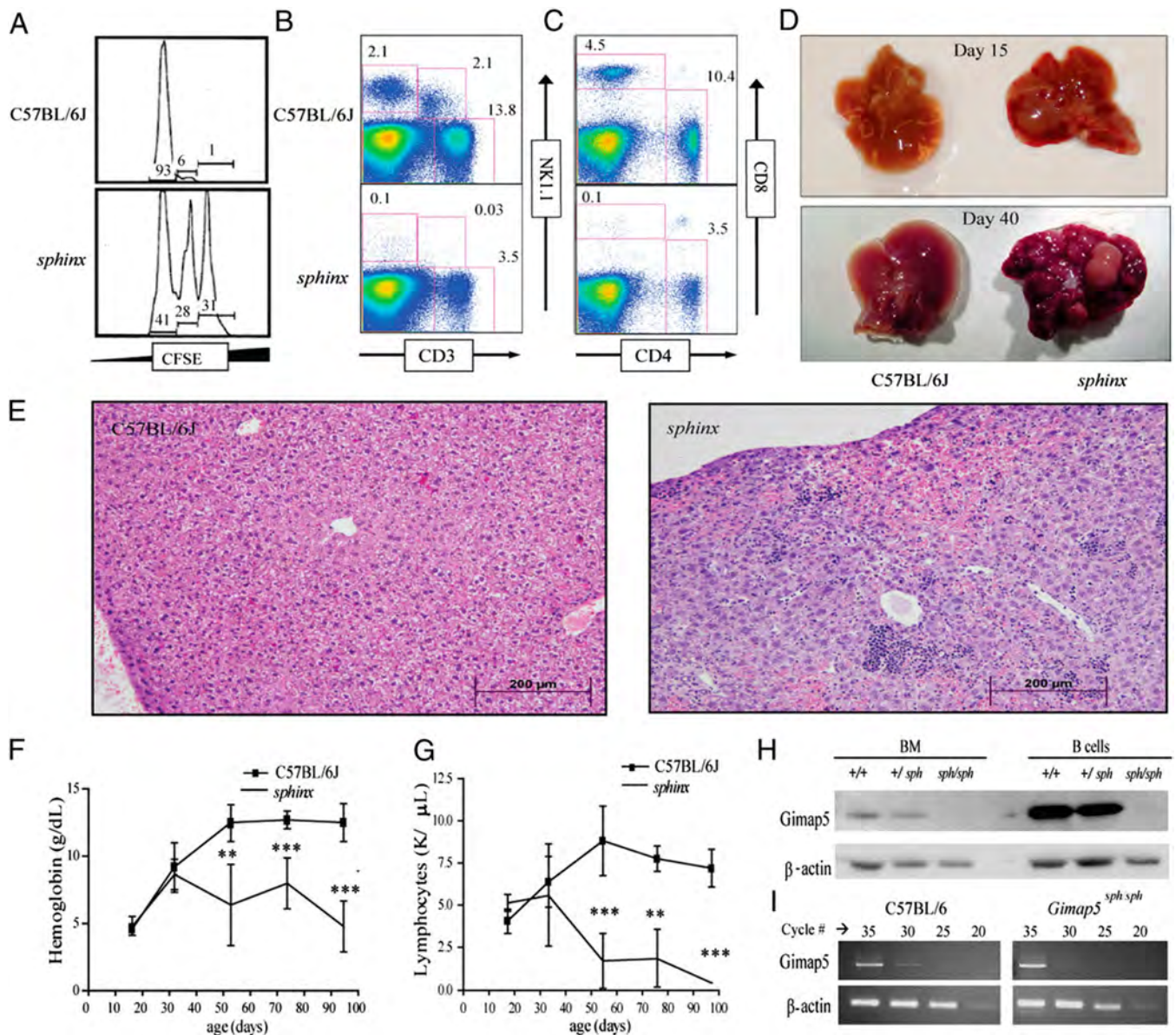
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**FIGURE 1.**

Identification of *sphinx*, an ENU germline mutant exhibiting severe lymphopenia and hepatic extramedullary hematopoiesis. A, CD8<sup>+</sup> T cell and NK cell cytotoxicity were tested in ENU mutagenized mice that had been previously immunized with irradiated act-mOVA cells. Cytotoxic function was assessed by the ability to remove  $\beta 2m^{-/-}$  (CFSE<sup>int</sup>) NK cell or SIINFEKL-loaded (CFSE<sup>high</sup>) CD8<sup>+</sup> T cell target cells. C57BL/6J control cells (CFSE<sup>low</sup>) were used as a reference population. The percentage of CD3<sup>+</sup> T cells and NK1.1<sup>+</sup> NK cells (B), and CD4<sup>+</sup> and CD8<sup>+</sup> T cells (C) were quantified among splenocytes from 6-wk-old mice. D, Gross liver morphology in 15-d-old (upper) and 40-d-old (lower) mice. E, H&E staining of liver sections from 40-d-old mice (original magnification ×100). Circulating concentrations of hemoglobin (F) and lymphocytes (G) were measured in the blood of mice of various ages. SD is shown and statistical analyses were performed for each time point. *n*

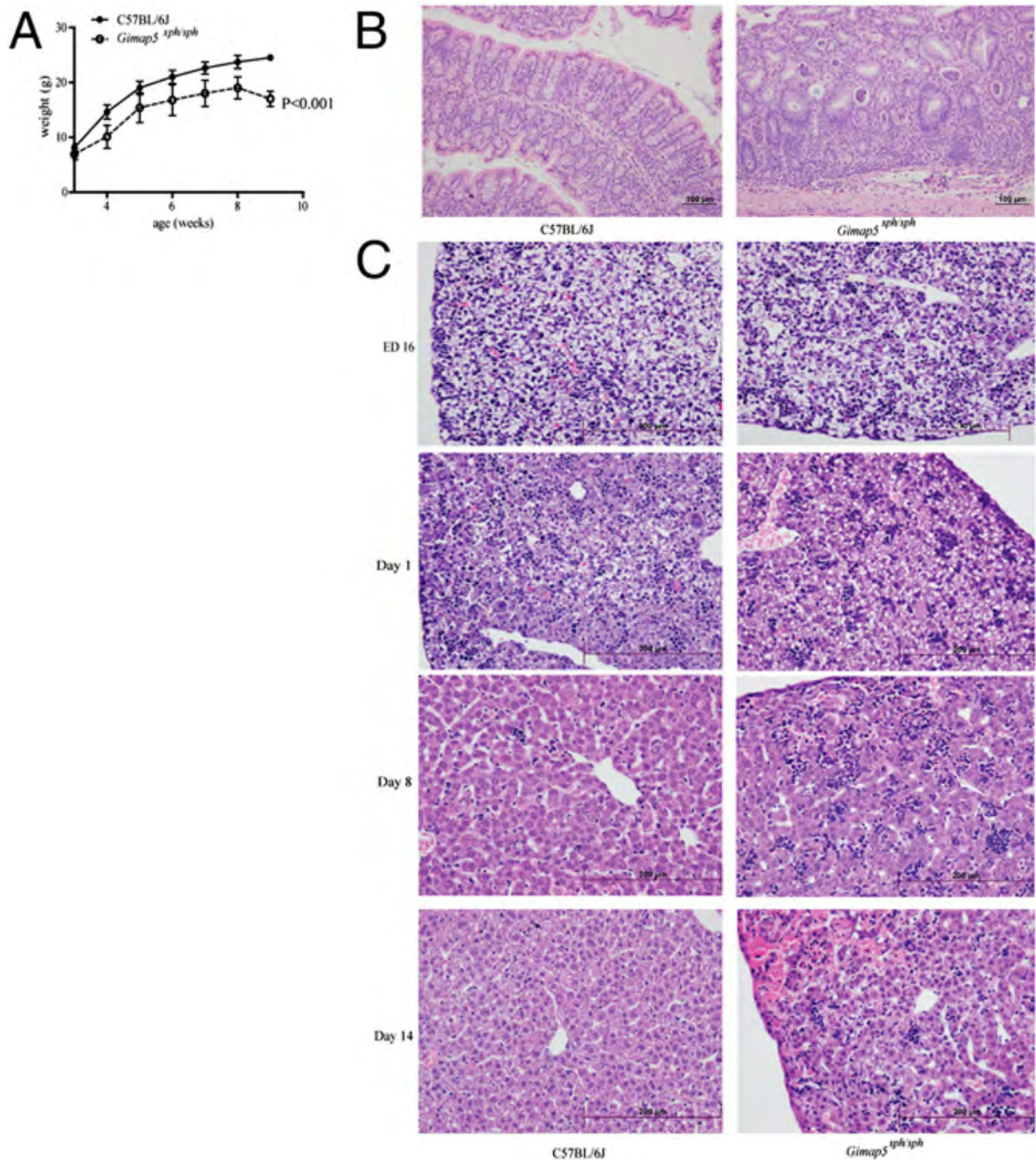
= 5; \*\*p < 0.01; \*\*\*p < 0.001. *H*, Gimap5 protein expression was measured in lysates of total bone marrow and purified splenic CD19<sup>+</sup> B cells. *I*, *Gimap5* RNA levels in purified splenic CD19<sup>+</sup> B cells isolated from C57BL/6J and *Gimap5*<sup>sph/sph</sup> mice were measured by RT-PCR. All analyses were performed at least three times.

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**FIGURE 2.**

*Gimap5<sup>sp/sp</sup>* mice develop wasting disease, colitis and aberrant hematopoiesis. **A**, Cohoused C57BL/6J and *Gimap5<sup>sp/sp</sup>* males were weighed at indicated ages. SEMs shown for each time point ( $n = 6$ ) and statistical analysis was performed using a two-tailed paired  $t$  test. **B**, H&E-stained sections from the colon of 10-wk-old cohoused mice were assessed for intestinal inflammation (original magnification  $\times 100$ ). **C**, Hematopoietic cell egress from the neonatal liver was monitored by examining H&E-stained sections from

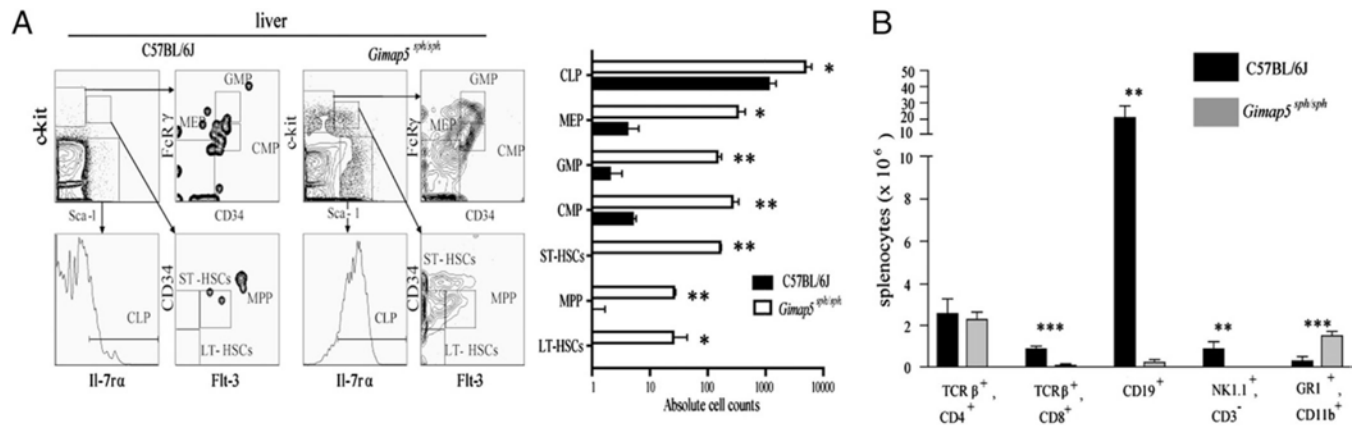
livers obtained at indicated developmental time-points (original magnification  $\times 200$ ). At least three livers were analyzed for each time point, and representative sections are shown.

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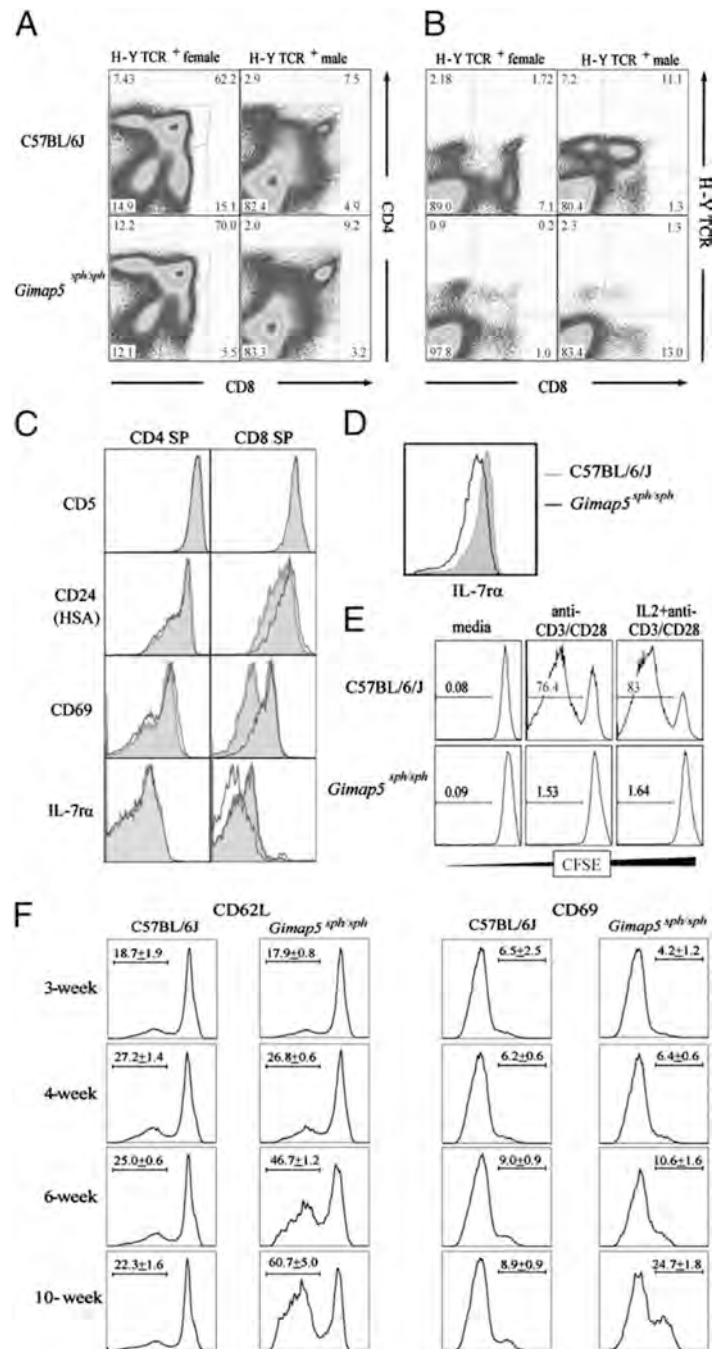
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**FIGURE 3.**

Hematopoietic defects in the adult and fetal liver of *Gimap5<sup>sph/sph</sup>* mice. **A**, HSCs and hematopoietic precursor cells in the liver of 6-wk-old mice were analyzed by flow cytometry. The mean number of cells for each subset is indicated along with SEM.  $n = 3$ ; \* $p < 0.05$ ; \*\* $p < 0.01$ . **B**, Irradiated (1000 rad  $\gamma$ -radiation) *Rag2<sup>-/-</sup> Il2r $\gamma$ <sup>-/-</sup>* recipient mice were reconstituted with ED 19 fetal liver cells. Six weeks later, splenocyte populations in chimeric mice were quantified by flow cytometry. The mean total number of each cell type in the spleen is shown along with SD.  $n = 3$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

**FIGURE 4.**

T cell development and function in *Gimap5<sup>sph/sph</sup>* mice. To assess positive and negative selection, thymocyte (A) and splenocyte (B) populations from H-Y TCR<sup>+</sup> transgenic mice of both genders were quantified and the average percentage of cells in each quadrant is shown. C, Expression of terminal thymocyte maturation markers—CD5, CD24, CD69, and IL-7Rα—was compared between C57BL/6J (shaded, gray) and *Gimap5<sup>sph/sph</sup>* (open, dark line) TCRβ<sup>+</sup> CD4 SP and CD8 SP thymocytes. D, Surface expression of IL-7Rα was measured in splenic CD4<sup>+</sup> T cells. E, Proliferation of CFSE-labeled CD4<sup>+</sup> T cells isolated from 8-wk-old



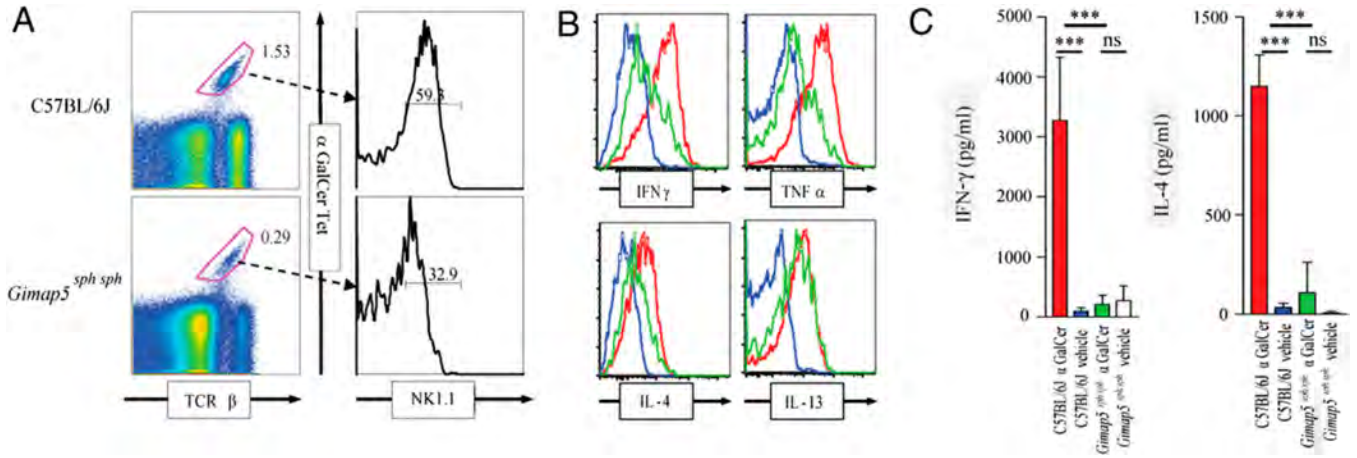
mice was assessed 96 h after stimulation with anti-CD3/CD28, with or without exogenous IL-2. *F*, Expression of CD62L and CD69 was measured on splenic CD4<sup>+</sup> T cells from 3-, 4-, 6- and 10-wk-old mice. Numbers represent mean  $\pm$  SD. All analyses were performed at least twice.

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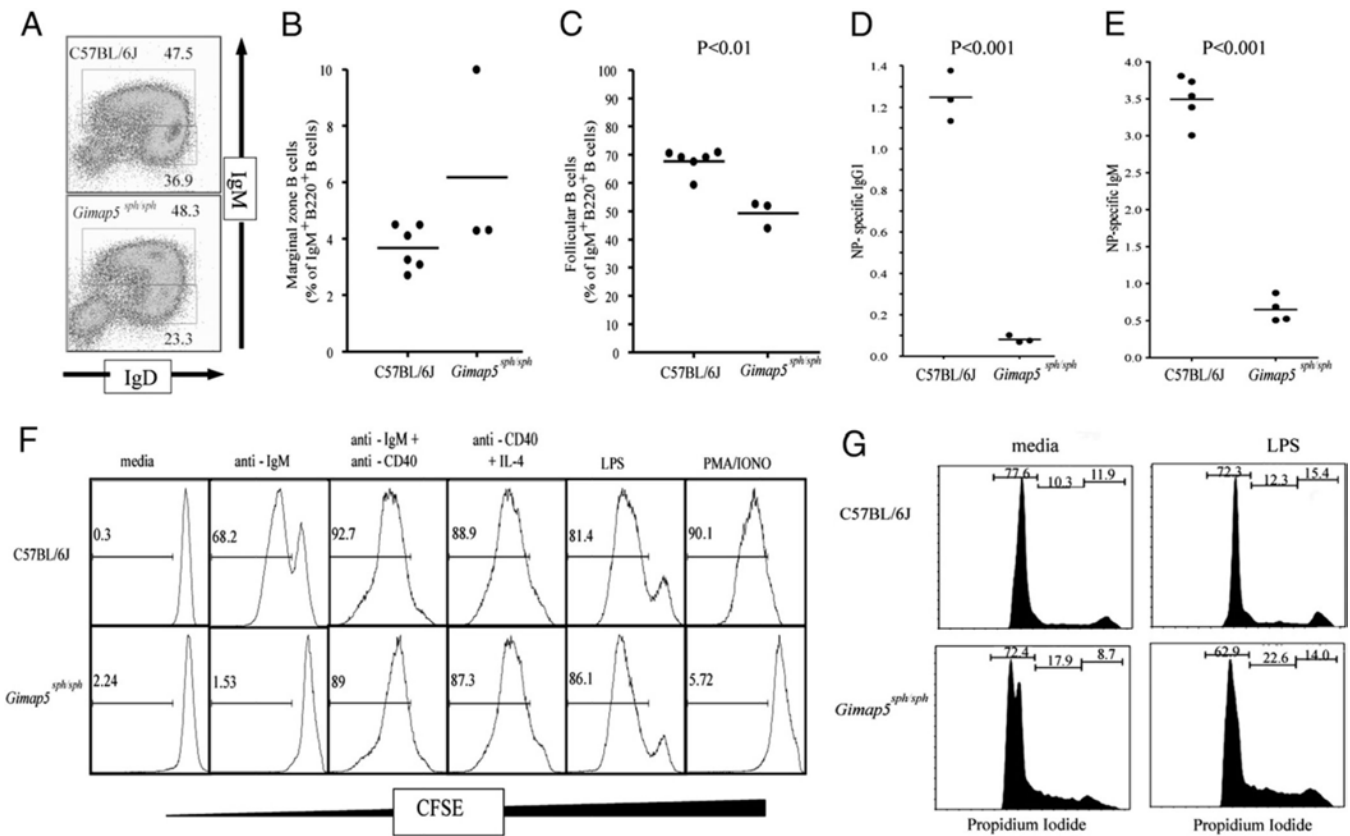
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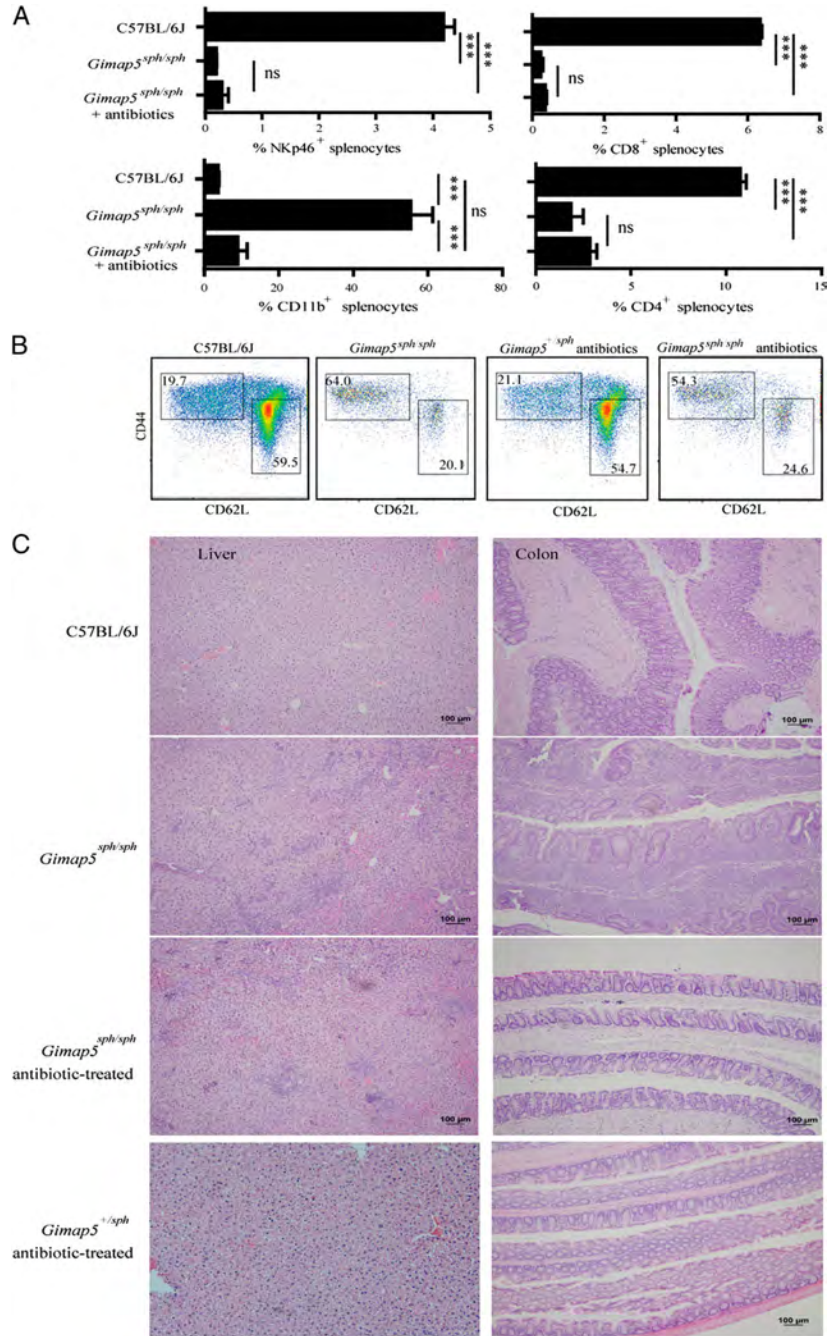
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**FIGURE 5.** Reduced *i*NKT cell survival and aberrant  $\alpha$ Gal-Cer-induced cytokine responses in *Gimap5<sup>sph/sph</sup>* mice. **A**, *i*NKT cells in the spleen of 6-wk-old *Gimap5<sup>sph/sph</sup>* mice were identified by  $\alpha$ Gal-Cer-loaded CD1d-tetramer binding and quantified by flow cytometry. **B**, Cytokine production was measured 90 min after injection of the *i*NKT cell agonist  $\alpha$ Gal-Cer on a per cell basis by intracellular staining for IFN- $\gamma$ , TNF- $\alpha$ , IL-4, or IL-13 expression among  $\alpha$ Gal-Cer-loaded CD1d-tetramer<sup>+</sup> *i*NKT cells (blue, unstimulated C57BL/6J; red, stimulated C57BL/6J; green, stimulated *Gimap5<sup>sph/sph</sup>*). **C**, Serum cytokine concentrations of IFN- $\gamma$  and IL-4 were also measured by ELISA 90 min after injection of  $\alpha$ Gal-Cer. SD is shown.  $n = 3$ ; \*\*\* $p < 0.001$ .

**FIGURE 6.**

Impaired BCR-dependent proliferation in *Gimap5<sup>sph/sph</sup>* B cells. *A–C*, Among CD19<sup>+</sup> splenocytes from 6-wk-old mice, the percentage of IgM<sup>high</sup> IgD<sup>int</sup> naive and IgM<sup>low</sup> IgD<sup>high</sup> mature B cells (*A*) and the percentage of CD21<sup>high</sup> CD23<sup>low</sup> marginal zone (*B*) and CD21<sup>high</sup> CD23<sup>high</sup> follicular B cells (*C*) were determined. Six-week-old mice were immunized with 50  $\mu$ g NP36-CGG with alum and LPS or 50  $\mu$ g of NP50-Ficoll to assess T-dependent (*D*) or T-independent (*E*) Ab responses. NP-specific serum Abs were measured 14 d after immunization by ELISA. *F*, To assess B cell proliferation, CFSE-labeled CD19<sup>+</sup> splenocytes were cultured in vitro for 90 h with media alone or with anti-IgM-Fab (10  $\mu$ g/ml), LPS (2  $\mu$ g/ml), anti-CD40 (10  $\mu$ g/ml) plus IL-4 (10 ng/ml), or PMA (50 ng/ml) and ionomycin (500 ng/ml). *G*, Cell cycle progression of B cells cultured in the presence or absence of LPS was measured by propidium iodide staining after 48 h of incubation.



**FIGURE 7.** Antibiotic treatment abrogates colitis but not lymphopenia and hepatic extramedullary hematopoiesis in *Gimap5<sup>sph/sph</sup>* mice. The percentage of NKp46<sup>+</sup>, CD8<sup>+</sup>, CD4<sup>+</sup>, and CD11b<sup>+</sup> splenocytes (A) and splenic CD44<sup>high</sup> CD62L<sup>low</sup> CD4<sup>+</sup> T cells (B) in 9-wk-old C57BL/6J, *Gimap5<sup>sph/sph</sup>*, and antibiotic-treated *Gimap5<sup>sph/sph</sup>* mice was determined. Bars represent mean values ± SEM. n = 3 per group; \*\*\*p < 0.001. C, H&E-stained sections from the colons and livers of 9-wk-old cohoused mice were assessed for extramedullary

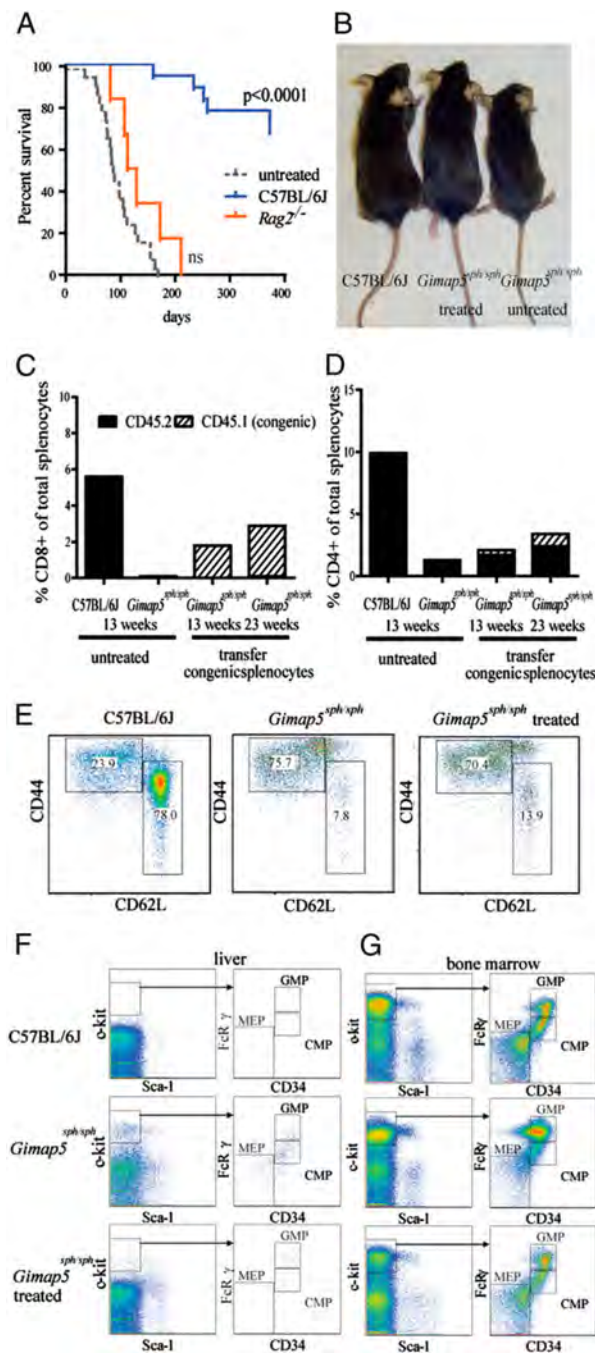
hematopoiesis and intestinal inflammation (original magnification  $\times 100$ ). Representative histology from each group is shown.  $n = 3$ .

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**FIGURE 8.**

Prevention of wasting disease by adoptive transfer of splenocytes. **A**, Twenty-five- to-35-d-old  $Gimap5^{spH/spH}$  mice were injected with  $1 \times 10^7$  splenocytes from C57BL/6J or  $Rag2^{-/-}$  donors. The survival of recipient mice was monitored for up to 9 mo ( $n = 6$  per group) and differences were statistically analyzed using the Gehan-Breslow-Wilcoxon test. **B**,  $Gimap5^{spH/spH}$  mice injected with C57BL/6J splenocytes did not develop wasting disease. Representative 15-wk-old mice are shown. **C** and **D**, In 30-d-old  $Gimap5^{spH/spH}$  mice injected with congenically marked splenocytes, the percentages of transferred (CD45.1) and

endogenous (CD45.2) CD8<sup>+</sup> T cells (*C*) and CD4<sup>+</sup> T cells (*D*) were determined at the indicated time points after transfer. *E*, Expression of CD62L and CD44 on splenic CD4<sup>+</sup> T cells from 13-wk-old C57BL/6 mice, *Gimap5<sup>sph/sph</sup>* mice, and *Gimap5<sup>sph/sph</sup>* mice injected with CD45.1<sup>+</sup> congenic splenocytes (only cells of *Gimap5<sup>sph/sph</sup>* origin are shown). Lin myeloid precursor cells were quantified in the liver (*F*) and bone marrow (*G*) of 15-wk-old mice.

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