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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

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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 (Gimap)5 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+ T cells and B cells appear to undergo normal development, they fail to proliferate upon Ag-receptor stimulation although NF-kB, MAP kinase and Akt activation occur normally. In addition, in Gimap5-deficient mice, CD4+ T cells adopt a CD44highCD62LlowCD69low phenotype and show reduced IL-7rα 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.

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+ T cells and have reduced CD4+ T cell and NKR-P1+ NK 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 Iam4, Iam5, or Iodh) (25).

The Gimap genes (previously known as immune-associated nucleotide binding proteins [Iun]) 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 Gi(27). In mammals, evidence suggests that Gimap3 and Gimap5 both which was originally identified in a gene involved in plant immunity

Gimap5 maintains lymphocyte quiescence

oGal-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’)2–anti-IgM (Jackson ImmunoResearch), anti-CD40 (eBioscience), rIL-4 (R&D Systems, Minneapolis, MN), LPS (Axoressa, 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+ T cells or splenic B cells in 5 μM CFSE 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+ T cell function

We immunized G3 ENU mice with 1 × 108 γ-irradiated (1500 rad) act-mOVA splenocytes. Seven days later, we injected 1 × 107 CFSE-labeled cells i.v., consisting of a mixture of three different splenocyte populations (C57BL/6J, β2m–/–, and SINFEKL-peptide pulsed C57BL/6J splenocytes) into 3–4-week-old mice. 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 Y3Ag.12.3 (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 CO2, 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-μm cell strainer. Total liver cells were washed with 40 ml ice-cold RPMI 1640/5% FBS and centrifuged for 10 min at 300 × 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 × g and 4°C. Supernatants containing lymphocyte-enriched cell populations were collected and centrifuged at 300 × 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+ 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 μg LPS (Alexis) and 5 μg NP50-Ficoll (Biosearch Technologies). Serum NP-specific Abs were detected by ELISA. Briefly, 96-well round-bottom plates (Fisher Scientific) were coated with 5 μg/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 Biotec). 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% PS) and left untreated or treated with 0.1 μg/ml LPS at 37°C, 5% CO2. After 48 h, 1 × 106 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 μl propidium iodide (PI) solution (50 μg/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

Gimap5sph/sph 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, Gimap5sph/sph mice at 25–35 d old were injected i.v. with 1 × 106 spleenocytes 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 germ line mutation causing severe lymphopenia

We identified sphinx in a forward genetic screen designed to detect defective in vivo cytotoxic NK cell and CD8+ T cell responses in G3-G6 ENU mutagenized mice (38). The original sphinx founder, previously immunized with OVA-expressing cells, was unable to reject adoptively transferred NK cell or CD8+ T cell target cells (Fig. 1A). Necropsy revealed a near absence of CD8+ T cells and NK cells in mice that became moribund.

Gimap5sph/sph mice were depleted of RBCs (RBC lysis buffer; Sigma-Aldrich). Mice were monitored and euthanized according to veterinarian recommendations when they became moribund.

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+ 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 × 103 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 G15V, 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 Protozoa (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 disulfide 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−/− 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 Gimap5sph/sph) 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 Gimap5sph/sph 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 Gimap5sph/sph 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 Gimap5sph/sph mice and their heterozygous littermates appeared similar (Fig. 2C). However, more hematopoietic cells remained in...
Gimap5sph/sph 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 Gimap52/2 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 Gimap5sph/sph mice (data not shown), suggesting that the metabolic functions of the liver remained largely intact.

Cell-intrinsic hematopoietic defects in Gimap5sph/sph mice

We next considered that aberrant hematopoiesis rather than liver failure might contribute to wasting disease and mortality in Gimap5sph/sph mice. Despite the liver abnormalities, the bone marrow of 5-wk-old Gimap5sph/sph mice contained normal numbers of Lin-Scal1c-Kit+ HSCs and hematopoietic progenitor cells (Supplemental Fig. 4A, B). However, the numbers of megakaryocyte/erythroid progenitors (MEPs) declined with age, in correlation with the onset of anemia and thrombocytopenia in adult Gimap5sph/sph 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 Gimap5sph/sph 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 Gimap5sph/sph liver (Fig. 3A). Compared to normal hematopoiesis in the bone marrow, extramedullary liver hematopoiesis in Gimap5sph/sph mice was skewed toward generation of...
Lin-"Sca1"c-Kit-IL-7ra+ CLPs, and it is noteworthy that IL-7ra expression was increased in this population (Fig. 3A).

To investigate the functionality of Gimap5\textsuperscript{ph/ph} HSCs, we made radiation bone marrow and fetal liver chimeras. Similar to what was reported for Gimap5\textsuperscript{ph/ph} males (33), Gimap5\textsuperscript{ph/ph} 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\textsuperscript{ph/ph} mice, we reconstituted lethally irradiated Rag2\textsuperscript{-/-}Il2ry\textsuperscript{-/-} recipients with Gimap5\textsuperscript{ph/ph} or Gimap5\textsuperscript{ph/+} 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\textsuperscript{ph/ph} fetal liver cells were severely lymphopenic and had substantially fewer thymocytes than mice reconstituted with Gimap5\textsuperscript{ph/+} fetal liver cells. In the spleen, CD8\textsuperscript{+} T cell, B cell, and NK cell numbers were markedly reduced compared with recipients of control fetal liver cells, whereas CD4\textsuperscript{+} T cells were present at normal numbers (Fig. 3B). Gimap5\textsuperscript{ph/ph} fetal liver cell recipients exhibited weight loss and wasting, whereas control fetal liver cell recipients appeared healthy (data not shown). In Gimap5\textsuperscript{ph/ph} fetal liver cell recipients, CD11b-Gri1\textsuperscript{+} 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\textsuperscript{ph/ph} fetal liver cells suggested that the early mortality observed in Gimap5\textsuperscript{ph/ph} mice is at least partially caused by defective hematopoiesis. Furthermore, the Gimap5\textsuperscript{ph/ph} fetal liver cells exhibited an intrinsic defect in lymphopoiesis, affecting T cells, B cells, and NK cells.

**Reduced survival and IL-7ra expression in Gimap5\textsuperscript{ph/ph} 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\textsuperscript{ph/ph} 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\textsuperscript{ph/ph} rats and Gimap5\textsuperscript{-/-} mice, 6-wk-old Gimap5\textsuperscript{ph/ph} 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\textsuperscript{ph/ph} 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\textsuperscript{+} thymocytes are normally deleted by negative selection in male mice, whereas they develop into mature CD8\textsuperscript{+} T cells in female mice. In Gimap5\textsuperscript{ph/ph} males, H-Y-reactive thymocytes were deleted, implying that the mutation did not impede negative selection (Fig. 4A). In contrast, the thymus of Gimap5\textsuperscript{ph/ph} females contained normal numbers of H-Y TCR\textsuperscript{+} DP thymocytes, but significantly fewer H-Y TCR\textsuperscript{+} 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\textsuperscript{+} CD8\textsuperscript{+} T cells were found at reduced numbers and frequencies in the spleen of both male and female Gimap5\textsuperscript{ph/ph} 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 TCDB\textsuperscript{+} CD4 SP and CD8 SP thymocytes upregulate CD5 and IL-7ra (CD127) and downregulate CD24 (HSA) and CD69 (4, 42). Gimap5\textsuperscript{ph/ph} 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\textsuperscript{ph/ph} mice. In addition, as IL-7 promotes naïve T cell survival by modulating expression of anti-apoptotic molecules (44), reduced IL-7ra expression in Gimap5\textsuperscript{ph/ph} CD8 SP thymocytes likely contributes to the failure of CD8\textsuperscript{+} T cells to survive and accumulate in the periphery.

CD4\textsuperscript{+} T cells isolated from the spleen of 6-wk-old Gimap5\textsuperscript{ph/ph} 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\textsuperscript{+} T cell lymphopenia in Gimap5\textsuperscript{ph/ph} mice. As the lymphopenia became more pronounced with age (Fig. 1G), CD4\textsuperscript{+} 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\textsuperscript{ph/ph} iNKT 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 iNKT 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 nNKT 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 nNKT cells and aberrant nNKT cell cytokine response that occurs in Gimap5 sph/sph mice suggests roles for Gimap5 in both lymphocyte survival and function.

**Functional defects in Gimap5 sph/sph B cells**

Before the onset of wasting disease, splenic CD19+ B cells in Gimap5 sph/sph mice, unlike T cells and NK cells, were less reduced in number compared with C57BL/6J littersmates. 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 littersmates. Between 6–10 wk, splenic B cell numbers declined in Gimap5 sph/sph 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 sph/sph mice contained ~75% less IgM and 50% less total IgG than heterozygous littersmates (Supplemental Fig. 5B). The reduced total IgG reflected significant reductions in concentrations of the isotypes IgG1, IgG2b, and IgG3 and a trend toward reduced IgG2a (Supplemental Fig. 5B). These abnormalities suggested the possibility that B cell function was impaired in Gimap5 sph/sph mice. To assess B cell function, we immunized 6-wk-old mice with T-dependent (NP-CGG + alum + LPS) or T-independent (NP-ficol) Ags. Gimap5 sph/sph 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 sph/sph B cells might have an intrinsically functional defect. To investigate B cell-intrinsic signaling, we isolated B cells from 6-wk-old Gimap5 sph/sph and C57BL/6J mice and determined their capacity to proliferate upon activation in vitro. Gimap5 sph/sph B cells failed to proliferate after BCR stimulation, or treatment with the diacylglycerol mimetic, PMA, and the Ca++ mobilizing agent ionomycin (Fig. 6F). However, alternative pathways triggered by LPS or CD40 ligation induced normal Gimap5 sph/sph B cell proliferation (Fig. 6F).

Activation of the NF-κB, 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 sph/sph B cells stimulated with PMA and ionomycin. Normal degradation of the NF-κB inhibitor IkB 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 sph/sph B cells failed to proliferate upon PMA and ionomycin activation. Ex vivo cell cycle analysis of Gimap5 sph/sph 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 sph/sph 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 sph/sph 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 sph/sph 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 sph/sph mice suggested the possibility that aberrant responses triggered by microbial flora contributed to systemic CD4+ T cell LIP and subsequent cell death, ultimately resulting in lymphopenia. Thus, we sought to address the role of microbial flora in Gimap5 sph/sph mice. Homozygotes and heterozygote 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 sph/CD62L sph markers by CD4+ T cells (Fig. 7B) or the occurrence of hepatic extramedullary hematopoiesis and hepatocyte hyperplasia (Fig. 7C). Antibiotic-treatment completely blocked the accumulation of CD11b+ myeloid cells (Fig. 7A), ameliorated intestinal inflammation (Fig. 7C), and prevented weight loss (data not shown) in Gimap5 sph/sph mice. These data suggest that the Gimap5-dependent hematopoietic cell-intrinsic phenotypes, including hepatic extramedullary hematopoiesis, lymphopenia, and acquisition of a CD44 sph/CD62L sph phenotype by CD4+ T cells, can occur in the absence of intestinal inflammation, whereas microbial flora promote granulocyte accumulation, colitis, and wasting disease.

**Adoptively transferred lymphocytes reduce extramedullary hematopoiesis and early mortality in Gimap5 sph/sph mice**

To further assess the involvement of lymphocytes in the immunopathology observed in Gimap5 sph/sph mice, we adoptively transferred 1 × 107 congenically labeled C57BL/6J splenocytes into young Gimap5 sph/sph mice before the onset of wasting disease. Strikingly,
We also examined whether adoptively transferred C57BL/6J splenocytes affected the extramedullary hematopoiesis in recipient Gimap5sph/sph mice. In the liver of untreated 15-wk-old Gimap5sph/sph mice, an abundance of common myeloid progenitors were present, including both granulocyte/macrophage progenitors (GMPs) and MEPs. In the livers of age-matched Gimap5sph/sph 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 Gimap5sph/sph 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 Gimap5sph/sph 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 Gimap5sph/sph 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 Gimap5sph/sph mice.

Finally, we observed no reduction in mortality or wasting disease when Gimap5sph/sph mice received Rag2−/− splenocytes, indicating that a lymphocyte population is required to prevent wasting disease (Fig. 8A). Because adoptively transferred Cd4−/−, Cd8−/−, or Jα18−/− (iNK1 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 Gimap5sph/sph mouse exhibits a number of hematologic abnormalities. From birth, peripheral CD8+ 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 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 Gimap5sph/sph 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 CD44highCD62LlowCD69low phenotype CD4+ T cells.

Before the onset of postweaning abnormalities, extramedullary hematopoiesis occurred in the Gimap5sph/sph neonatal liver. Defective HSC egress or migration from the neonatal liver could underlie this phenomenon. One possibility that we favor is that Gimap5sph/sph 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 Gimap5sph/sph 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 Gimap5sph/sph bone marrow HSCs have reduced reconstituting capacity because of the chronically inflamed Gimap5sph/sph environment or because of a cell-intrinsic requirement for Gimap5 that becomes more pronounced with age.

FIGURE 5. Reduced iNKT cell survival and aberrant αGal-Cer-induced cytokine responses in Gimap5sph/sph mice. A, iNKT cells in the spleen of 6-wk-old Gimap5sph/sph mice were identified by αGal-Cer-loaded CD1d-tetramer binding and quantified by flow cytometry. B, Cytokine production was measured 90 min after injection of the iNKT cell agonist αGal-Cer on a per cell basis by intracellular staining for IFN-γ, TNF-α, IL-4, or IL-13 expression among αGal-Cer-loaded CD1d-tetramer+ iNKT cells (blue, unstimulated C57BL/6J; red, stimulated C57BL/6J; green, stimulated Gimap5sph/sph). C, Serum cytokine concentrations of IFN-γ and IL-4 were also measured by ELISA 90 min after injection of αGal-Cer. SD is shown. n = 3; ****p < 0.001.

FIGURE 6. Impaired BCR-dependent proliferation in Gimap5sph/sph B cells. A–C, Among CD19+ splenocytes from 6-wk-old mice, the percentage of IgMhighIgDint naive and IgMlowIgDhigh mature B cells (A) and the percentage of CD21highCD23low marginal zone (B) and CD21highCD23high follicular B cells (C) were determined. Six-week-old mice were immunized with 50 μg NP36-CGG with alum and LPS or 50 μg 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+ splenocytes were cultured in vitro for 90 h with media alone or with anti-IgM-Fab (10 μg/ml), LPS (2 μg/ml), anti-CD40 (10 μg/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 CD4<sup>+</sup>CD62L<sup>high</sup>CD44<sup>high</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 ×100). Representative histology from each group is shown. n = 3.
In addition to aberrant hematopoiesis, intestinal inflammation contributed to the weight loss and early morbidity in Gimap5sph/sph 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 NKT cells can drive colitis in a mouse model induced by the chemical oxazolone (50). Our data suggest that the Gimap5sph/sph environment favors the polarization of NKT cells toward the production of IL-13. Although the cellular and molecular basis of intestinal inflammation in Gimap5sph/sph mice demands further dissection, IL-13–producing NKT cells may represent one contributing factor. Another potential influence predisposing Gimap5sph/sph mice to developing colitis is CD4+ T cells undergoing LIP. In the naïve CD4+ T cell transfer model of colitis, using C57BL/6 Rag-deficient recipient strains, the lymphopenic environment drives CD4+ T cells into LIP. These CD4+ T cells undergoing LIP acquire effector function and cause both intestinal inflammation and wasting disease (11). Of note, intestinal inflammation is significantly reduced when naïve 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+ T cells undergoing LIP in Gimap5sph/sph mice. Interestingly, in partially lymphopenic settings such as in sublethally γ-irradiated mice, colitis is not observed after naïve T cell transfer (8). Residual radioresistant intestinal lymphocytes that enhance intestinal barrier function, such as IgA-secreting plasma cells or γ6 T cells, might limit colitogenic CD4+ T cell responses in this setting (8, 52). In Gimap5sph/sph mice, the loss of non-CD4+ lymphocytes with age might generate conditions that promote CD4+ T cells to undergo LIP, and thereby contribute to the onset of intestinal inflammation.

Our findings that Gimap5sph/sph HSCs were unable to reconstitute the B cell compartment of irradiated recipients, and that Gimap5sph/sph 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-κB and MAP kinase signaling pathways by BCR stimulation, because others had reported that these pathways are dysregulated in lyP T cells (54). However, Gimap5sph/sph mice have normal percentages of marginal zone B cells, which require NF-κB signaling to develop (55), and display normal IκB degradation and MAP kinase phosphorylation upon activation. Normal activation of the PI3K/Akt pathway, which is similarly required for lymphocyte proliferation, also occurs in Gimap5sph/sph B cells. One interesting attribute of Gimap5sph/sph 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.

**FIGURE 8.** Prevention of wasting disease by adoptive transfer of splenocytes. A, Twenty-five- to 35-d-old Gimap5sph/sph mice were injected with 1 × 107 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, Gimap5sph/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 Gimap5sph/sph mice injected with congenically marked splenocytes, the percentages of transferred (CD45.1) and endogenous (CD45.2) CD4+ T cells (C) and CD4+ T cells (D) were determined at the indicated time points after transfer. E, Expression of CD62L and CD44 on splenic CD4+ T cells from 13-wk-old C57BL/6 mice, Gimap5sph/sph mice, and Gimap5sph/sph mice injected with CD45.1+ congenic splenocytes (only cells of Gimap5sph/sph origin are shown). Lin− myeloid precursor cells were quantified in the liver (F) and bone marrow (G) of 15-wk-old mice.
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>−/−</sup> T cells. In the thymus, developing Gimap5<sup>−/−</sup> CD8 SP thymocytes show impaired survival after positive selection and reduced expression of IL-7Rα, 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-7Rα, 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>−/−</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>hi</sup>/CD62L<sup>lo</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 pediatric diabetes (18–20) and, as we have shown in this study, microbial flora are required for the onset of colitis (18–20), and, as we have shown in this study, microbial flora are required for the onset of colitis. Finally, although pediatric diabetes (18–20) and, as we have shown in this study, microbial flora are required for the onset of colitis. Finally, although pediatric diabetes (18–20) and, as we have shown in this study, microbial flora are required for the onset of colitis. Finally, although pediatric diabetes (18–20) and, as we have shown in this study, microbial flora are required for the onset of colitis. Finally, although pediatric diabetes (18–20) and, as we have shown in this study, microbial flora are required for the onset of colitis.