

SHIP1 Is a Repressor of Mast Cell Hyperplasia, Cytokine Production, and Allergic Inflammation In Vivo¹

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SHIP1 inhibits immune receptor signaling through hydrolysis of the PI3K product phosphatidylinositol 3,4,5-trisphosphate, forming phosphatidylinositol 3,4-bisphosphate. In mast cells, SHIP1 represses FcεRI- and cytokine-mediated activation in vitro, but little is known regarding the function of SHIP1 in mast cells in vivo or the susceptibility of *Ship1*^{-/-} mice to mast cell-associated diseases. In this study, we found that *Ship1*^{-/-} mice have systemic mast cell hyperplasia, increased serum levels of IL-6, TNF, and IL-5, and heightened anaphylactic response. Further, by reconstituting mast cell-deficient mice with *Ship1*^{+/+} or *Ship1*^{-/-} mast cells, we found that the above defects were due to loss of SHIP1 in mast cells. Additionally, we found that mice reconstituted with *Ship1*^{-/-} mast cells suffered worse allergic asthma pathology than those reconstituted with *Ship1*^{+/+} mast cells. In summary, our data show that SHIP1 represses allergic inflammation and mast cell hyperplasia in vivo and exerts these effects specifically in mast cells. *The Journal of Immunology*, 2009, 183: 228–236.

The Src homology 2-containing inositol 5'-phosphatase SHIP1 inhibits immune receptor signaling by hydrolysis of the PI3K product phosphatidylinositol 3,4,5-trisphosphate, forming phosphatidylinositol 3,4-bisphosphate. SHIP1 represses the activation, survival, or proliferation of T cells, B cells, macrophages, NK cells, neutrophils, and mast cells (1–6). *Ship1*^{-/-} mice are viable and fertile but suffer from excessive granulocyte and macrophage numbers, profound splenomegaly, extramedullary hematopoiesis, massive myeloid infiltration of the lungs, osteoporosis, wasting, and a shortened lifespan (7, 8). Because SHIP1 is expressed in all hematopoietic cell lineages, it has been difficult to determine which cell types are responsible for given phenotypes.

Mast cells are immune effector cells known for releasing their cytoplasmic granules when IgE Abs, bound to FcεRI receptors, are crosslinked by multivalent exogenous Ag. During degranulation, mast cells release inflammatory and immunomodulatory molecules, including cytokines, chemokines, lipid-derived mediators,

histamine, proteoglycans, and neutral proteases (reviewed in Ref. 9). Although SHIP1 is not required for mast cell differentiation, it functions as a repressor of mast cell degranulation and cytokine secretion in vitro. Specifically, *Ship1*^{-/-} mast cells exhibit increased degranulation and cytokine secretion compared with *Ship1*^{+/+} mast cells in response to IgE crosslinking (6, 10). Further, stimulation with stem cell factor (SCF)⁴ or IgE without crosslinking Ag induces aberrant degranulation in *Ship1*^{-/-} but not *Ship1*^{+/+} mast cells (6, 11).

Mast cells contribute to a wide variety of inflammatory diseases, including anaphylaxis and allergic asthma (reviewed in Refs. 12 and 13). Anaphylaxis is a type I hypersensitivity reaction primarily provoked by allergen-induced mast cell degranulation. Symptoms of anaphylaxis include vascular permeability, tachycardia, hypothermia, and mortality. Allergic asthma is a complex, chronic inflammatory disease of the airways and lungs. Naive *Ship1*^{-/-} mice have symptoms of allergic asthma under steady-state conditions, including severe airway inflammation, mucus hyperproduction, and airway remodeling, but the contribution of *Ship1*^{-/-} mast cells to these symptoms is unknown (14).

Mast cell physiology can be studied in vivo by using mast cell-deficient *Kit*^{W-sh/W-sh} mice, which lack mast cells due to a chromosomal inversion that disrupts the 5' regulatory sequences of *Kit* (15, 16). Reconstituting *Kit*^{W-sh/W-sh} mice with *Kit*^{+/+} bone marrow derived mast cells (BMMCs) selectively repairs their mast cell deficiency (17). Furthermore, if *Kit*^{W-sh/W-sh} mice are reconstituted with BMMCs from knockout mice they can be used to study gene function in mast cells in vivo (18–20).

Although the role of SHIP1 in immune receptor signaling has been established in mast cells in vitro, little is known about its function in mast cells in vivo or about the susceptibility of *Ship1*^{-/-} mice to mast cell-associated diseases. In this study, we found that *Ship1*^{-/-} mice have systemic mast cell hyperplasia, increased serum levels of IL-6, TNF, and IL-5, and heightened

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Received for publication February 9, 2009. Accepted for publication April 23, 2009.

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¹ This study was supported by operating grants from the Canadian Institutes for Health Research (CIHR) and AllerGen Network Centre of Excellence. D.J.H. was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) and the University of British Columbia (UBC) Faculty of Medicine. F.A. was supported by NSERC, the UBC Faculty of Medicine, and the Michael Smith Foundation for Health Research (MSFHR). M.R.H. was supported by the UBC Centre for Blood Research CIHR/Heart and Stroke Foundation of Canada Strategic Training Program in Transfusion Science. M.-R.B. was supported through the CIHR/Canadian Asthma, Allergy and Immunology Foundation Training Program. K.M.M. is a MSFHR Senior Scholar.

D.J.H. designed and performed research, analyzed and interpreted data, and wrote the manuscript. F.A. and M.R.H. designed and performed research, analyzed and interpreted data, and edited the manuscript. M.-R.B. and L.Z. performed research. G.K. and K.M.M. analyzed and interpreted data and edited the manuscript.

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⁴ Abbreviations used in this paper: SCF, stem cell factor; BAL, bronchoalveolar lavage; BMMC, bone marrow cultured mast cells; HSA, human serum albumin; PSA, passive systemic anaphylaxis.

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susceptibility to anaphylaxis. Furthermore, by reconstituting mast cell-deficient *Kit^{W-sh/W-sh}* mice with *Ship1^{+/+}* or *Ship1^{-/-}* BMMCs, we established that loss of SHIP1 in the mast cell lineage was responsible for these defects (with the exception of increased IL-5). In addition, we found that mice reconstituted with *Ship1^{-/-}* mast cells suffered worse allergic asthma pathology than those reconstituted with *Ship1^{+/+}* mast cells. In summary, our data show that SHIP1 is a repressor of allergic inflammation and mast cell hyperplasia in vivo and that it exerts these effects specifically in mast cells.

Materials and Methods

Mice

All mice were housed in specific pathogen-free mouse facilities at The Biomedical Research Centre or the British Columbia Cancer Research Centre, with approval from the University of British Columbia Animal Care Committee and according to the Canadian Council on Animal Care guidelines. C57BL/6 (B6) and B6.Cg-*Kit^{W-sh/HNhrJaeBsmJ}* (*Kit^{W-sh/W-sh}*) mouse strains were acquired from The Jackson Laboratory, and each were maintained as homozygous colonies by sibling breeding. B6-congenic *Ship1^{-/-}* mice were a kind gift from Dr. F. R. Jirik (University of Calgary, Calgary, Alberta, Canada) and were backcrossed at least 12 times. *F₂ Ship1^{-/-}* mice (*F₂-Ship1^{-/-}*) were maintained on a B6 × 129/Sv mixed background. All mice used were at least 4-wk old and controls were littermates or were age- and sex-matched from the same colony.

Cell culture

Bone marrow cells were flushed from the femurs of 6-wk-old *Ship1^{+/+}* and *Ship1^{-/-}* mice and placed in IMDM supplemented with 15% FCS (Stem-Cell Technologies), 150 μ M monothio glycerol (Sigma-Aldrich), 100 U/ml penicillin, 100 μ g/ml streptomycin (Sigma-Aldrich), and 30 ng/ml recombinant mouse IL-3 (R&D Systems). Cells were maintained at 37°C in a 5% CO₂ humidified atmosphere, selected for nonadherence, and considered to be BMMCs after 6 wk in culture as verified by Wright-Giemsa staining and flow cytometry (data not shown).

Reconstitution of mast cell-deficient *Kit^{W-sh/W-sh}* mice

Kit^{W-sh/W-sh} mice were injected with 1×10^7 *Ship1^{+/+}* or *Ship1^{-/-}* BMMCs in 200 μ l of HBSS via the tail vein (designated *Ship1^{+/+}*-BMMC and *Ship1^{-/-}*-BMMC mice). After 12 wk the mice were bled for serum collection, subjected to passive systemic anaphylaxis, or sensitized for allergic asthma and then euthanized via CO₂ inhalation. After euthanizing the mice, peritoneal washes were taken for subsequent analysis by flow cytometry. Samples of back skin, spleen, lung, mesentery, ileum, and colon were fixed in 10% neutral buffered formalin. Fixed samples were paraffin embedded, sectioned, and stained by Wax-it Histology Services. All tissues were stained with toluidine blue. Mast cells were enumerated by counting the number of toluidine blue-positive cells in each section at $\times 40$ or $\times 100$ objective on a Zeiss Axioplan 2 microscope. The area of the section was then determined using the polygon area tool with OpenLab 4.0.4 software (Improvision) at $\times 5$ objective.

Cytokine assay

Serum (50 μ l) was collected from the saphenous vein and assayed using BD mouse inflammation and mouse Th1/Th2 cytometric bead array kits according to manufacturer's instructions (BD Biosciences).

Passive systemic anaphylaxis

Mice were sensitized by i.v. injection with 20 μ g of IgE SPE-7 (Sigma-Aldrich) in 100 μ l of HBSS plus 0.1% BSA. The following day, anaphylaxis was induced by i.v. injection with 1 mg of DNP-human serum albumin (HSA) in 100 μ l of PBS. Rectal temperature was taken 5 min before and immediately preceding injection and then monitored every 5 min for 40 min and again at 75 and 90 min, at which time all mice were sacrificed. Temperature measurements were performed with a traceable expanded range metric digital thermometer (VWR International).

Asthma disease course

Female mice were sensitized with 100 μ l of 2 mg/ml OVA in alum (Al(OH)₃; Sigma-Aldrich) via i.p. injection on days 1 and 8. Following sensitization, asthma was induced by introducing 50 μ l of 20 mg/ml OVA in HBSS intranasally on days 22, 23, 24, 27, and 28. During intranasal

sensitizations, mice were anesthetized via inhalation of 3% isoflurane in 0.8 liters of oxygen per minute. Mice were sacrificed by CO₂ inhalation on day 29. Bronchoalveolar lavages (BAL) were performed by completing three separate additions and removals of 1 ml of PBS. Total CD45.2⁺ BAL cells were enumerated using flow cytometry, and differentials were performed on Wright-Giemsa stained cytopins.

A portion of the right lower lobe of the lung was taken from each mouse for histopathological grading and mast cell enumeration. Lung portions were fixed in 10% neutral buffered formalin, paraffin-embedded, sectioned, and H&E stained by Wax-it Histology Services. Samples were blinded and clinically scored from 0 (no infiltration) to 4 (profound infiltration) on each of the following parameters: perivascular infiltration, peribronchial infiltration, parenchymal infiltration, and epithelial damage (maximum score of 16).

Flow cytometry

Samples were blocked with anti-CD16/32 in 10% goat serum and then stained with anti-c-kit-PE (BD Pharmingen), anti-CD45.2-PE (eBioscience), or anti-Fc ϵ R1-biotin (eBioscience) followed by streptavidin-FITC (BD Pharmingen) or anti-CD45.2-PE (eBioscience). Latex beads (10 μ m; Invitrogen) were added to HBSS with 0.1% BSA before peritoneal washes and to BAL to calculate total cell recovery via flow cytometry. All flow cytometry experiments were performed on a BD FACSCalibur with CellQuest software and postcollection analyses were performed with FlowJo (Tree Star).

Statistics

The unpaired *t* test (two-tailed) was used for analysis of tissue mast cell numbers, asthma BAL cellular infiltrate, BAL differentials, lung clinical scores, serum cytokine levels, and passive systemic anaphylaxis (PSA) assays. A Kruskal-Wallis test was used for the mesentery of BMMC-reconstituted mice (due to all 0 values in the *Ship1^{+/+}* condition). A log-rank (Mantel-Cox) test was used to compare the PSA survival rates. Separate allergic asthma experiments were normalized based on their OVA-treated B6 levels before analysis. All of the above tests were performed using GraphPad Prism 5 or Microsoft Excel X software. Missing samples from some tissues used in the enumeration of mast cells in *Ship1^{-/-}* and BMMC-reconstituted mice were due to errors during sample processing.

Results

Ship1^{-/-} mice have mast cell hyperplasia in multiple tissues

We suspected that the chronic inflammatory phenotype of *Ship1^{-/-}* mice could be due in part to increased tissue mast cell numbers. With this in mind, we enumerated the tissue mast cells of *Ship1^{+/+}* and *Ship1^{-/-}* mice and found significantly higher numbers in the spleen, lung, ileum, mesentery, and back skin of *Ship1^{-/-}* mice (Fig. 1A). We also found that *Ship1^{-/-}* mice had higher numbers of colon mast cells, although the counts were more variable than those for other tissues assessed. Similarly, *Ship1^{-/-}* mice had over two times more peritoneal mast cells than wild-type controls (Fig. 1B). The frequency of peritoneal mast cells was similar between *Ship1^{-/-}* and wild-type mice, reflecting a greater number of total peritoneal cells in *Ship1^{-/-}* mice than in *Ship1^{+/+}* mice (6.8, SD 2.8 vs 2.0, SD 0.13 cells ($\times 10^6$); $p < 0.0114$). Thus, *Ship1^{-/-}* mice have mast cell hyperplasia in the spleen, lungs, colon, ileum, mesentery, peritoneal cavity, and back skin.

Mast cell hyperplasia in Ship1^{-/-} mice is mast cell autonomous

Because SHIP1 is expressed in many hematopoietic cell types, it was unclear whether the mast cell hyperplasia we observed in ubiquitous *Ship1^{-/-}* mice was mast cell autonomous. To answer this question, we i.v. reconstituted *Kit^{W-sh/W-sh}* mast cell-deficient mice with *Ship1^{+/+}* or *Ship1^{-/-}* BMMC (designated *Ship1^{+/+}*-BMMC and *Ship1^{-/-}*-BMMC mice) and, following 12 wk of engraftment, found mast cell hyperplasia in the spleen, lung, colon, ileum, mesentery, and back skin of *Ship1^{-/-}*-BMMC mice (Fig. 2A). Likewise, *Ship1^{-/-}*-BMMC mice also had a greater number and frequency (0.92, SD 0.52% vs 0.10, SD 0.13%; $p < 0.0015$) of peritoneal mast cells than *Ship1^{+/+}*-BMMC controls (Fig. 2B).

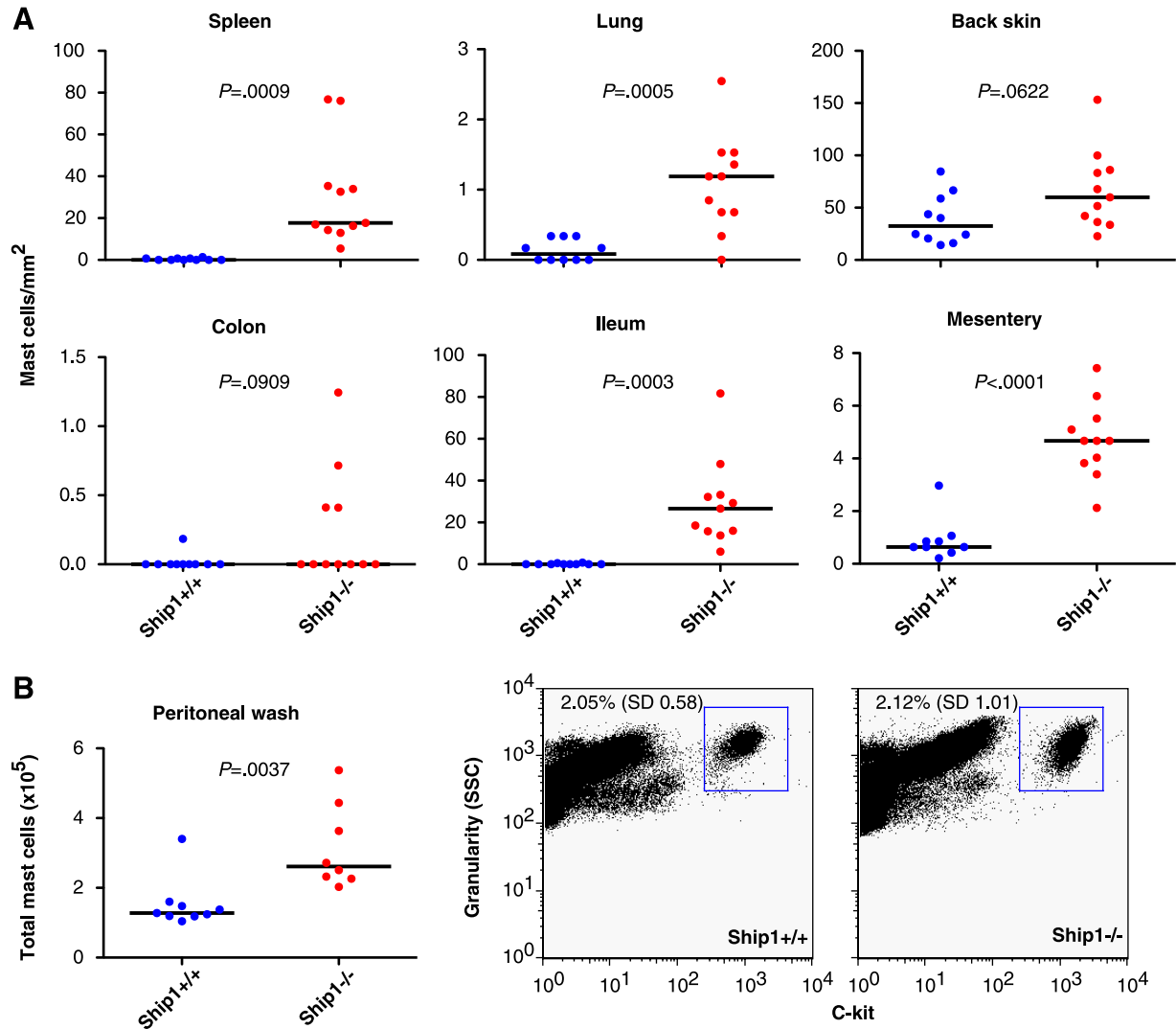


FIGURE 1. Mast cell hyperplasia in multiple tissues of *Ship1*^{-/-} mice. **A**, Toluidine blue histological staining revealed that *Ship1*^{-/-} mice ($n = 11$) had significantly higher numbers of metachromatic tissue mast cells than *Ship1*^{+/+} controls ($n = 10$). **B**, *Ship1*^{-/-} mice ($n = 10$) had a higher total number (graph) but similar frequency (gate statistics) of SSC^{high}/c-kit^{high} peritoneal wash mast cells compared to *Ship1*^{+/+} mice ($n = 9$), as determined by flow cytometry. Data represent pooled results from two independent experiments.

Kit^{W-sh/W-sh} mice were mast cell deficient in all tissues assessed, and the mast cell numbers in the tissues of B6 and *Kit*^{W-sh/W-sh} mice reconstituted with wild-type mast cells were similar to those of previous reports (17). Thus, the mast cell hyperplasia observed in *Ship1*^{-/-} mice is a mast cell autonomous defect.

Ship1^{-/-} mice have mast cell-dependent systemic inflammation

Because *Ship1*^{-/-} mast cells exhibit enhanced degranulation and cytokine secretion in response to FcεRI stimulation and react aberrantly to c-kit stimulation in vitro, we investigated the levels of inflammatory cytokines in the serum of adult *Ship1*^{-/-} mice (6, 10, 11). *Ship1*^{-/-} mouse serum contained significantly increased levels of IL-5, IL-6, and TNF (and a small increase in CCL2) compared with *Ship1*^{+/+} controls (Fig. 3A). To determine whether *Ship1*^{-/-} mast cells were responsible for the increased levels of inflammatory serum cytokines observed in *Ship1*^{-/-} mice, we evaluated the serum of *Ship1*^{-/-}-BMMC-reconstituted mice. We detected significantly higher levels of IL-6 and TNF in the serum of *Ship1*^{-/-}-BMMC mice, whereas the levels of IL-5 and CCL2 were only marginally increased compared with those of controls (Fig. 3B). We concluded that *Ship1*^{-/-} mast cells induce increased serum levels of the inflammatory cytokines IL-6 and TNF in vivo.

Ship1^{-/-} mice are hyperresponsive to passive systemic anaphylaxis

Because *Ship1*^{-/-} mice possess mast cell hyperplasia in multiple tissues and *Ship1*^{-/-} mast cells exhibit increased degranulation in response to IgE crosslinking in vitro, we suspected that *Ship1*^{-/-} mice would be more susceptible to anaphylaxis (6). To test this hypothesis, we subjected *Ship1*^{-/-} mice and littermate controls to PSA by sensitizing them with anti-DNP IgE, challenging them with DNP-HSA the next day, and monitoring their rectal temperature for 90 min (Fig. 4). Interestingly, the body temperature of *Ship1*^{-/-} mice (before challenge) was on average 1°C lower than that of wild-type controls. This may reflect low-level degranulation induced by the IgE sensitization, as *Ship1*^{-/-} mast cells aberrantly degranulate in response to IgE without crosslinking Ag in vitro (6). More importantly, we found that *Ship1*^{-/-} mice suffered increased mortality, more severe hypothermia (at $t = 20$ min; 5°C, SD 0.6°C vs 3°C, SD 0.7°C; $p < 0.0001$), and no body temperature recovery compared with wild-type controls (Figs. 4, A and B).

It was unclear whether the increased severity of anaphylaxis suffered by *Ship1*^{-/-} mice was due to increased mast cell activation and mast cell numbers or to the reactivity of other cell

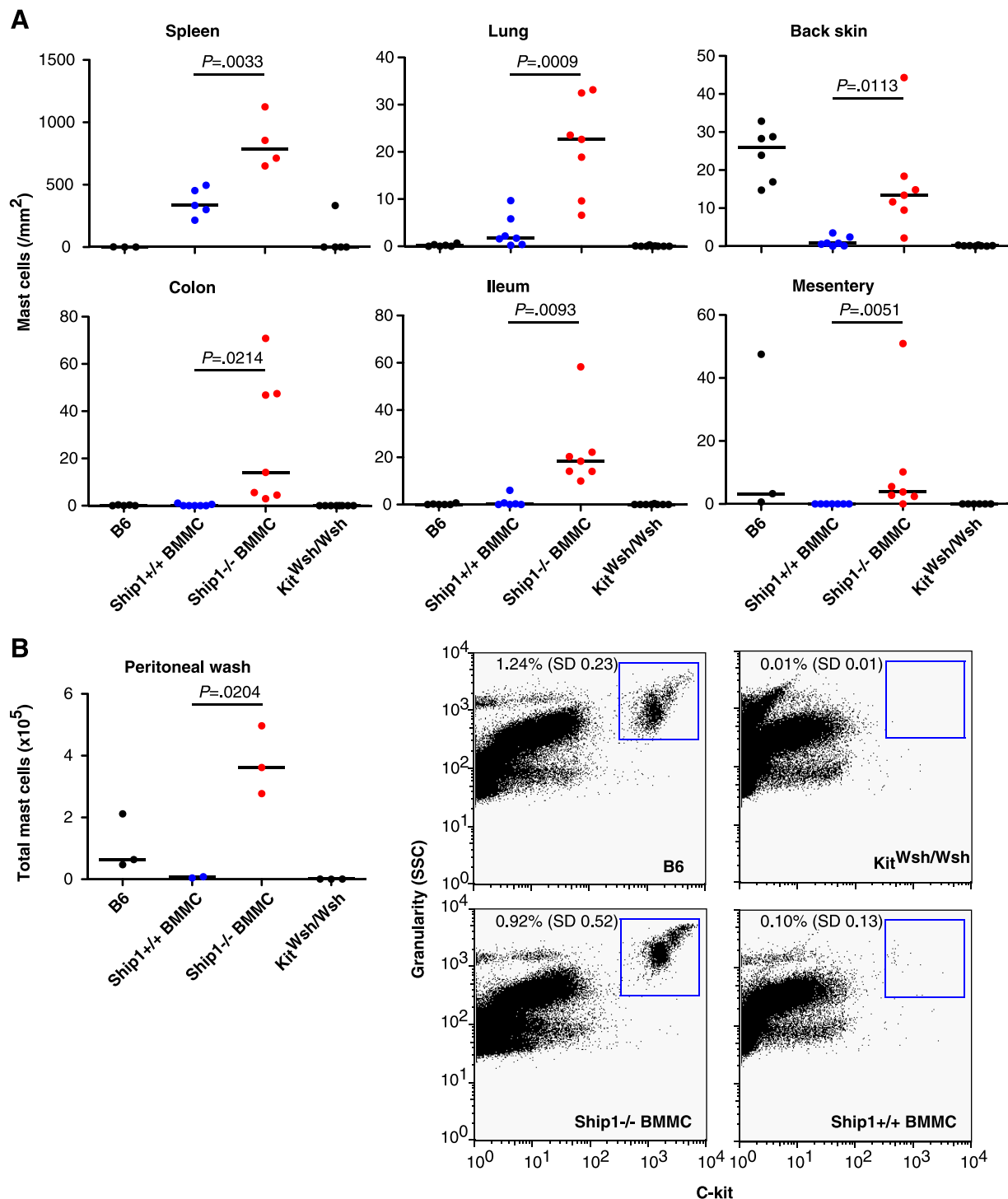


FIGURE 2. Mast cell hyperplasia in *Ship1*^{-/-} mice is mast cell autonomous. *Kit*^{W-sh/W-sh} mice were injected with 1×10^7 *Ship1*^{+/+} or *Ship1*^{-/-} BMMC via the tail vein and, after 12 wk of reconstitution, the mice were sacrificed and their tissue mast cells were enumerated. **A**, The number of metachromatic tissue mast cells was significantly higher in *Ship1*^{-/-}-BMMC mice ($n = 7$) than in *Ship1*^{+/+}-BMMC mice ($n = 7$) and, in most cases, B6 mice ($n = 6$), whereas *Kit*^{W-sh/W-sh} mice ($n = 9$) were mast cell deficient as determined by toluidine blue histological staining. **B**, Similar results were observed for the total number (graph) and frequency (gate statistics) of SSC^{high}/c-kit^{high} (where SSC is side scatter) peritoneal mast cells from B6 ($n = 3$), *Ship1*^{-/-}-BMMC ($n = 3$), *Ship1*^{+/+}-BMMC ($n = 2$), and *Kit*^{W-sh/W-sh} ($n = 3$) mice by flow cytometry. Data represent pooled results from two independent experiments.

types to the mediators released during PSA challenge (reviewed in Ref. 21). Therefore we subjected reconstituted *Ship1*^{-/-}-BMMC mice to PSA and found that they also suffered a much larger temperature drop than *Ship1*^{+/+}-BMMC mice (at $t = 35$; 7°C, SD 2.2°C vs 1°C, SD 2.5°C; $p = 0.0037$) (Fig. 4C). Notably, unlike ubiquitous *Ship1*^{-/-} mice, *Ship1*^{-/-}-BMMC mice

exhibited body temperature recovery 40 min postchallenge. Although *Kit*^{W-sh/W-sh} mice were resistant to PSA, B6 mice suffered a similar temperature drop as that of *Ship1*^{-/-}-BMMC mice (Fig. 4D). In summary, we find that the increased severity of anaphylaxis experienced by *Ship1*^{-/-} mice is largely mast cell autonomous.

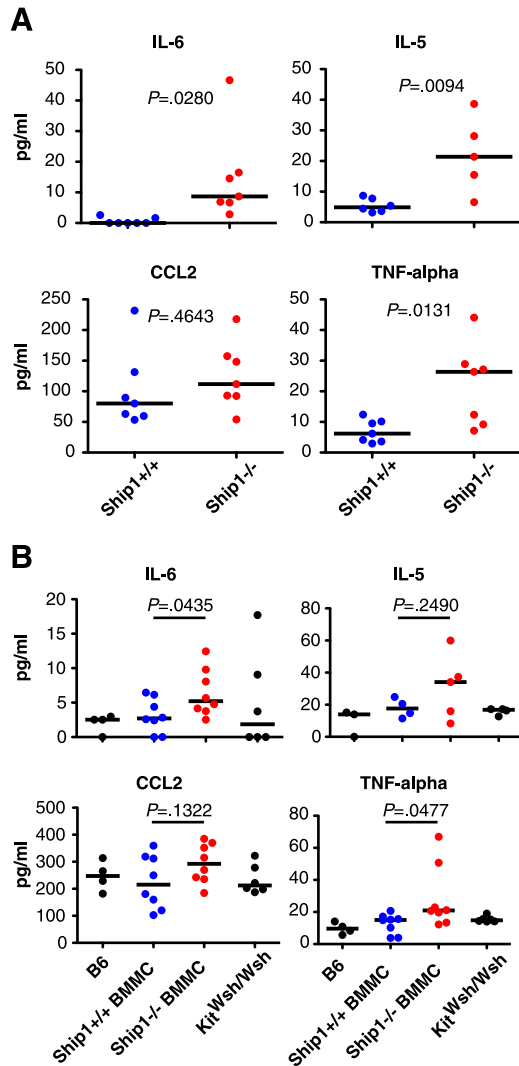


FIGURE 3. Increased inflammatory cytokine levels in the serum of *Ship1*^{-/-} mice and mice reconstituted with *Ship1*^{-/-} mast cells. **A**, Cytometric bead arrays of mouse cytokines were used to assay the serum of both F2-*Ship1*^{-/-} ($n = 7$) and F2-*Ship1*^{+/+} ($n = 7$) mice. Data represent pooled results from two independent experiments. **B**, Cytometric bead arrays were also used to assay the serum of B6 ($n = 4$), *Ship1*^{-/-}-BMMC ($n = 8$), *Ship1*^{+/+}-BMMC ($n = 8$), and *Kit*^{W-sh/W-sh} ($n = 6$) mice. Data represent pooled results from two independent experiments.

Allergic airway inflammation is greater in *Ship1*^{-/-}-BMMC mice than in *Ship1*^{+/+}-BMMC controls

Naive *Ship1*^{-/-} mice have allergic asthma-like symptoms, including severe airway inflammation, mucus hyperproduction, and symptoms of airway remodeling (14). To determine the role of *Ship1*^{-/-} mast cells in allergic asthma pathology, we induced an acute form of the disease in *Ship1*^{-/-}-BMMC- and *Ship1*^{+/+}-BMMC-reconstituted mice (Fig. 5A). We found that asthmatic *Ship1*^{-/-}-BMMC mice had over two times more infiltrating cells in their airways than *Ship1*^{+/+}-BMMC mice, including increased eosinophils, which are the primary effector cells in asthma (Figs. 5, B and C). Perivascular, peribronchial, and parenchymal infiltration, as well as epithelial damage, were most prominent in the lungs of asthmatic *Ship1*^{-/-}-BMMC mice, and the clinical scores of asthmatic *Ship1*^{-/-}-BMMC lung sections were significantly higher than those of *Ship1*^{+/+}-BMMC mice (Fig. 5, D and E). *Kit*^{W-sh/W-sh} mice suffered comparable symptoms as those of B6

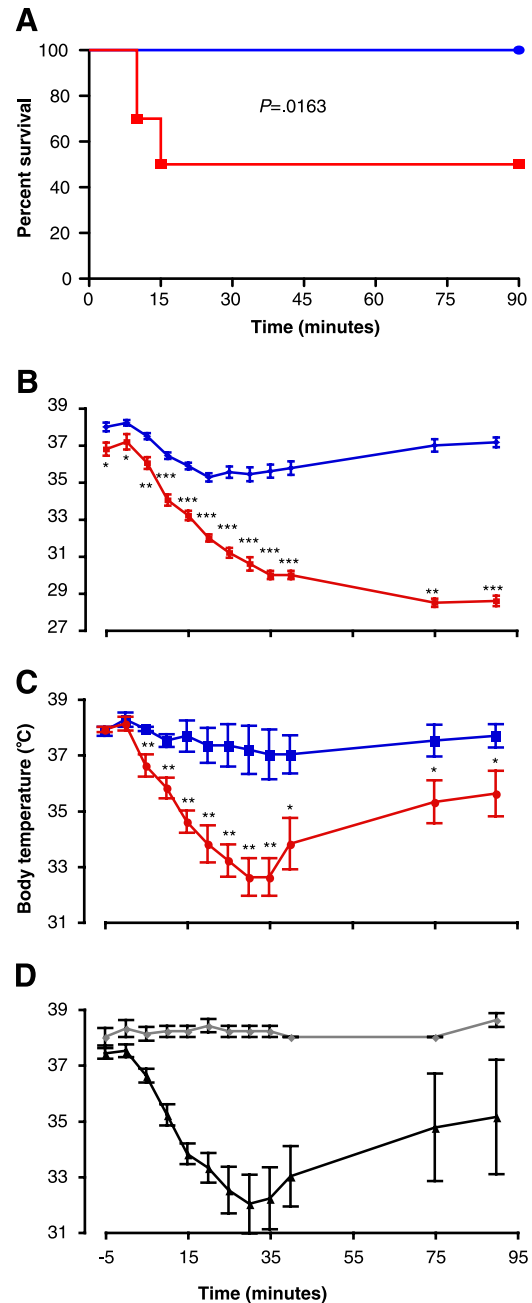


FIGURE 4. Hyperresponsive anaphylaxis in *Ship1*^{-/-} mice and in mice reconstituted with *Ship1*^{-/-} mast cells. To induce passive systemic anaphylaxis, *Ship1*^{-/-} ($n = 10$, red line) and *Ship1*^{+/+} ($n = 9$, blue line) mice were sensitized with anti-DNP IgE and challenged the following day with DNP-HSA. Increased mortality (**A**) and hypothermia (**B**), measured rectally, were observed in *Ship1*^{-/-} mice. Anaphylaxis was induced and monitored in *Ship1*^{-/-}-BMMC ($n = 5$, red line) and *Ship1*^{+/+}-BMMC ($n = 6$, blue line) mice (**C**), as well as B6 ($n = 5$, black line) and *Kit*^{W-sh/W-sh} ($n = 5$, gray line) controls (**D**) as described above. Data for **C** and **D** were measured concurrently but separated for clarity. Data in each panel represent pooled results from two separate experiments. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Bars represent SE.

mice, similar to the findings of a study published after we completed our asthma course that also used an OVA-induced (with alum adjuvant) model of allergic asthma (see Fig. E1 in Ref. 20). *Ship1*^{+/+}-BMMC mice had slightly reduced number of infiltrating BAL cells compared with *Kit*^{W-sh/W-sh} mice, highlighting the importance of wild-type reconstituted controls. We concluded that

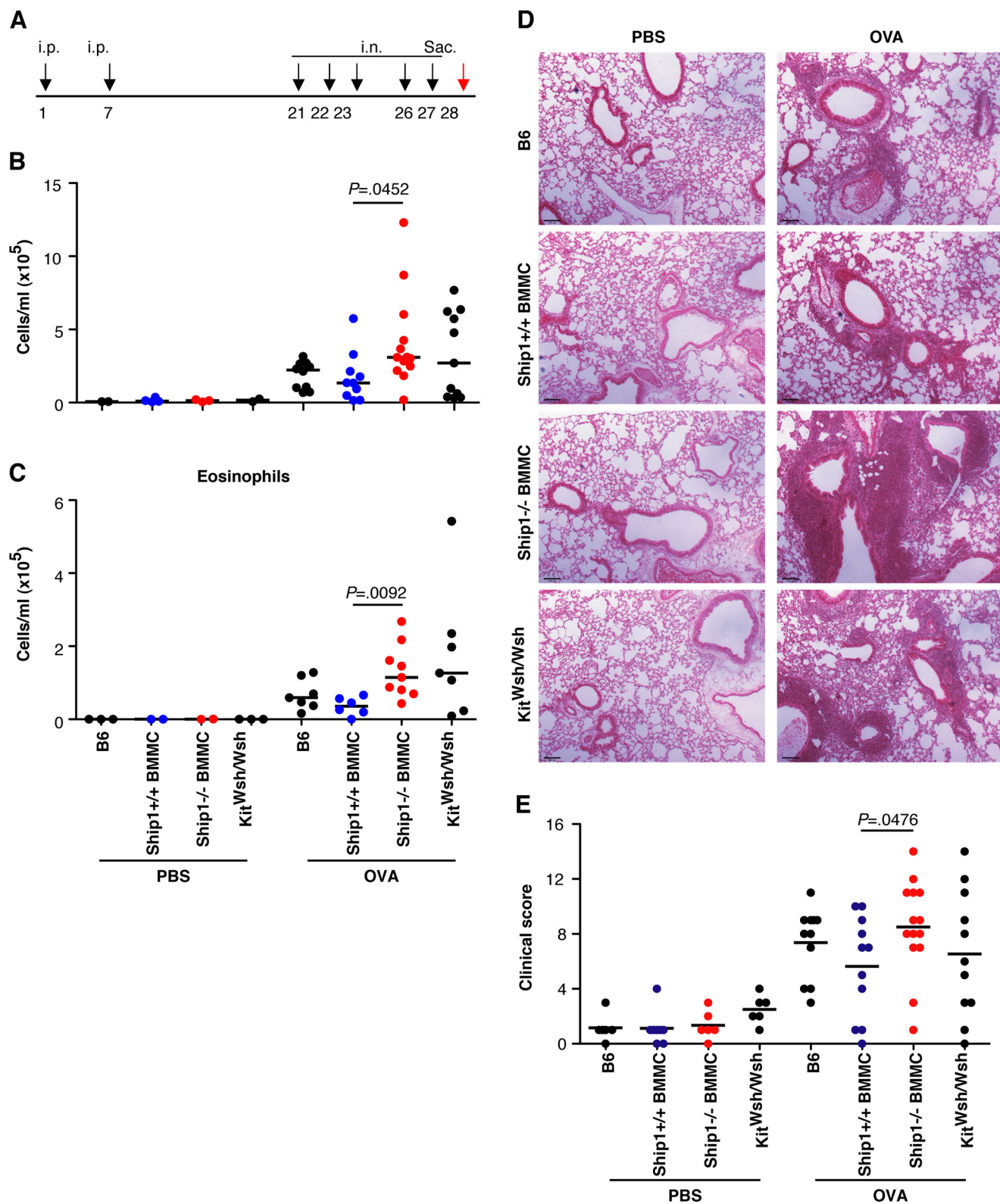


FIGURE 5. Increased allergic asthma pathology in mice reconstituted with *Ship1*^{-/-} mast cells. **A**, Asthma was induced in B6 ($n = 10$), *Ship1*^{-/-}-BMMC ($n = 13$), *Ship1*^{+/+}-BMMC ($n = 10$), and *Kit*^{Wsh/Wsh} ($n = 11$) mice via two i.p. sensitizations (with alum adjuvant; days 1 and 7) and five intranasal (i.n.) challenges (days 21, 22, 23, 26, and 27) of OVA before sacrificing (Sac.) the mice (day 28). **B**, Inflammation was assessed by enumerating the cellular infiltrate in the BAL using flow cytometry. **C**, Differentials were performed with a modified Wright-Giemsa stain; B6 ($n = 7$), *Ship1*^{-/-}-BMMC ($n = 9$), *Ship1*^{+/+}-BMMC ($n = 6$), and *Kit*^{Wsh/Wsh} ($n = 7$). BAL data represent pooled results from three independent experiments, and differentials from two independent experiments. **D**, Selected representative micrographs of H&E-stained lungs from PBS- or OVA-treated *Ship1*^{-/-}-BMMC-reconstituted mice and controls (bars = 100 μ m). **E**, Clinical scores were assigned to each lung based on the following parameters: perivascular infiltration, peribronchial infiltration, parenchymal infiltration, and epithelial damage (maximum score of 16). Data represent pooled results from three independent experiments. Each circle represents a mouse.

Ship1^{-/-}-BMMC mice suffered worse allergic asthma pathology than *Ship1*^{+/+}-BMMC mice.

Discussion

Our results show the following: 1) *Ship1*^{-/-} mice have systemic mast cell hyperplasia, increased serum levels of the inflammatory cytokines IL-6, TNF, and IL-5, and increased susceptibility to anaphylaxis; 2) the above phenotypes are mast cell autonomous (with the exception of increased serum IL-5); and 3) mice reconstituted with *Ship1*^{-/-} mast cells suffered worse allergic asthma pathology than *Ship1*^{+/+} controls. Previous studies have focused on the function of SHIP1 in mast cells in vitro. In this study we demonstrate that SHIP1 is also a repressor of mast cells in vivo and that the loss of SHIP1 increases the symptoms of two mast cell-associated diseases. Furthermore, we demonstrate a cell-autonomous role for SHIP1 in mast cell homeostasis.

Mast cell hyperplasia is observed in chronic inflammatory processes, fibrotic disorders, wound healing, and neoplastic tissue transformation, but little is known about normal mast cell homeostasis (reviewed in Ref. 22). Our results demonstrate that SHIP1 is a cell-autonomous repressor of mast cell hyperplasia and a key player in mast cell homeostasis. *Ship1*^{-/-} mice have increased numbers of granulocyte-macrophage progenitors in their bone marrow that exhibit enhanced sensitivity to multiple cytokines (7). In our studies, because the reconstituted BMDCs were composed exclusively of mast cells and their committed precursors and the numbers of mast cells observed in the tissues of *Ship1*^{-/-}-BMMC mice were comparable to or greater than those in ubiquitous *Ship1*^{-/-} mice, we concluded that the mast cell hyperplasia observed in *Ship1*^{-/-} mice is not due to the increased number or sensitivity of granulocyte-macrophage progenitors. Furthermore, we found that the loss of SHIP1 specifically in mast cells was not sufficient to induce the excessive granulocyte and macrophage numbers previously observed in the blood, spleen, and lungs of *Ship1*^{-/-} mice (data not shown) (7).

Interestingly, we found that *Ship1*^{-/-} mice have elevated serum levels of the inflammatory cytokines IL-5, IL-6, and TNF compared with those of *Ship1*^{+/+} controls. This is in agreement with a previous report that *Ship1*^{-/-} mice have increased levels of serum IL-6 (8). We demonstrated that the increased levels of IL-6 and TNF were due to loss of SHIP1 specifically in mast cells. Because *Ship1*^{-/-} mast cells produce more IL-6 and TNF than wild-type mast cells upon FcεRI-mediated stimulation in vitro, we suspect that the increased serum levels of these cytokines arose directly from *Ship1*^{-/-} mast cells via stimulation with endogenous IgE (10). Although the high level of IL-6 in the serum of *Ship1*^{-/-} mice was previously attributed to macrophages, in this study we demonstrate that *Ship1*^{-/-} mast cells also contribute to the increased IL-6 levels in vivo (8). Furthermore, the increased IL-6 was previously proposed to inhibit B cells and enhance myeloid cell development, but we found the frequency of B cells and myeloid cells in the bone marrow, peripheral blood, and spleen of *Ship1*^{-/-}-BMMC-reconstituted mice were similar to that in controls (data not shown) (23). Thus, our data suggest that increased serum IL-6 is not sufficient to cause the hematopoietic lineage skewing observed in *Ship1*^{-/-} mice, and a local increase of IL-6 in the bone marrow may be required.

Our studies also suggest that the systemic mast cell hyperplasia observed in *Ship1*^{-/-} mice is due to the high levels of IL-6 and TNF produced by *Ship1*^{-/-} mast cells. TNF is required for mast cell development and stimulates mast cell colony formation in vitro (24, 25). In addition, IL-6 promotes mast cell development

from mixed cultures, likely via a secondary mediator such as PGE (24). We suspect that the systemic mast cell hyperplasia observed in *Ship1*^{-/-} mice is also due to the hypersensitivity of *Ship1*^{-/-} mast cells to stimulation with SCF or IgE (without crosslinking Ag) (6, 11). SCF and IgE induce mast cell proliferation and survival, respectively, and endogenous levels of these factors may be capable of sustaining higher levels of hypersensitive *Ship1*^{-/-} mast cells in vivo (26, 27).

We also found that *Ship1*^{-/-} mice suffer considerably more severe anaphylaxis than controls. Although mast cells are the primary effector cells in the PSA model of anaphylaxis, other cell types also play a role in its pathology. For example, IL-4 or IL-13 treatment increases the severity of anaphylaxis by enhancing the responsiveness of target cells to the vasoactive mediators released during anaphylaxis (reviewed in Ref. 21). We found that *Ship1*^{-/-}-BMMC mice also suffered more severe anaphylaxis than controls, suggesting that this effect is largely mast cell autonomous. We suspect that the increased severity of anaphylaxis observed in *Ship1*^{-/-} (and *Ship1*^{-/-}-BMMC) mice is due to their systemic mast cell hyperplasia and the enhanced degranulation of the *Ship1*^{-/-} mast cells, but we have not ruled out the possibility that the increased levels of IL-6 and TNF in their serum could have also contributed to this effect (6).

In our studies, we also found that *Ship1*^{-/-}-BMMC mice suffered worse allergic asthma pathology than *Ship1*^{+/+}-BMMC mice in an acute, OVA-induced (with alum adjuvant) model of allergic asthma (28). Because *Kit*^{W-sh/W-sh} mice also developed symptoms of asthma, it made it difficult to determine to what extent *Ship1*^{-/-} mast cells were proinflammatory or whether they instead lacked the anti-inflammatory activity present in *Ship1*^{+/+} mast cells. Indeed, asthmatic *Ship1*^{+/+}-BMMC mice had lower numbers of infiltrating cells and clinical scores than *Kit*^{W-sh/W-sh} controls, suggesting an anti-inflammatory role for mast cells in this model. With this in mind, we believe that *Ship1*^{-/-} mast cells also contributed to inflammation for the following reasons. First, mast cells amplify allergic asthma symptoms in a similar model of asthma (without alum adjuvant) (29). Second, mast cells are hyperplastic in the lungs of asthmatic mice, and the pre-existing mast cell hyperplasia of the lungs in *Ship1*^{-/-}-BMMC mice would likely predispose them to heightened allergic asthma pathology (30). Third, IL-6 and TNF, inflammatory cytokines associated with increased asthma pathology, are elevated in the serum of *Ship1*^{-/-}-BMMC mice (20, 31). Finally, *Ship1*^{-/-} mast cells intensified the severity of anaphylaxis, another mast cell-dependent inflammatory disease model. The fact that *Ship1*^{-/-}-BMMC mice exhibit greater allergic asthma pathology than *Ship1*^{+/+}-BMMC mice strongly suggests a mast cell-autonomous role for SHIP1 in allergic asthma response.

The PI3K pathway is involved in cytokine production and the amplification of FcεRI-mediated degranulation in mast cells in vitro and enhances anaphylaxis in vivo (reviewed in Ref. 32). In mast cells, FcεRI and c-kit receptors activate heterodimeric class IA PI3Ks, whereas G protein-coupled receptors, including the adenosine A3 receptor, activate class IB PI3Ks. SHIP1 negatively regulates PI3K activation via its phosphatase activity and can also act as a signaling adaptor (33). Other investigators have reported that the loss of the p85α subunit of class IA PI3K causes selective loss of gastrointestinal mast cells but does not affect mast cell degranulation in vitro or PSA in vivo (34). Loss of the p110δ subunit of PI3K (class IA) results in reductions in numbers of some dermal mast cells, degranulation, cutaneous anaphylaxis, and TNF and IL-6 production (35). PI3Kγ^{-/-} (class IB)-cultured mast cells, in contrast, have reduced degranulation, and PI3Kγ^{-/-} mice experience less severe PSA (36).

The PI3K inhibitor LY294002 has also been reported to reduce asthma symptoms in vivo (37). In combination with our results, the studies outlined above indicate that SHIP1 may repress p85 α subunit-dependent PI3K activity (class IA) during the formation or maintenance of gastrointestinal mast cells, p110 δ -dependent PI3K activity (class IA) in the formation or maintenance of some dermal mast cells and TNF and IL-6 production, and both PI3K $\gamma^{-/-}$ (class IB) and p110 δ -dependent (class IA) PI3K activity in degranulation and anaphylaxis. This suggests that SHIP1 phosphatase activity (rather than adaptor function) is central to negative regulation of these processes. It is interesting to note the breadth of SHIP1 activity compared with the functional specificity of the PI3K family potentially making SHIP1 a more extensive target for therapy.

We propose that *Ship1* $^{-/-}$ -BMMC-reconstituted mice can serve as an in vivo model of hyperactive and hyperplastic mast cells for the investigation of mast cell-associated diseases and immune reactions. In this study we investigated the harmful roles that *Ship1* $^{-/-}$ mast cells play in inflammatory diseases. It would be interesting to test whether *Ship1* $^{-/-}$ mast cells also have an enhanced function in the beneficial roles of mast cells, such as clearing peritoneal and helminth infections (38, 39).

SHIP1 repressor activity in mast cells is conserved in humans, and agonists and antagonists of SHIP1 function have recently been identified and tested in vivo (40, 41). Our study suggests that SHIP1 agonists could reduce the severity of anaphylaxis, allergic asthma, and other mast cell-associated diseases in humans. Given that mast cells also have a role in such diverse diseases as rheumatoid arthritis, inflammatory bowel disease, and tumor progression and conversely aid in clearing peritoneal and helminth infections, modulators of SHIP1 function in mast cells may offer a variety of therapeutic strategies against key human diseases (25, 38, 39, 42, 43).

Acknowledgments

We thank Drs. Jami Bennett and Colby Zaph for thoughtful reviews of the manuscript.

Disclosures

G.K. is a founder and Chief Science Officer of Aquinox Pharmaceuticals Inc., a company dedicated to developing small molecule activators and inhibitors of SHIP1. Aquinox did not support this work in any way. All other authors have no conflicting financial interests.

References

- Tarasenko, T., H. K. Kole, A. W. Chi, M. M. Mentink-Kane, T. A. Wynn, and S. Bolland. 2007. T cell-specific deletion of the inositol phosphatase SHIP reveals its role in regulating Th1/Th2 and cytotoxic responses. *Proc. Natl. Acad. Sci. USA* 104: 11382–11387.
- Liu, Q., A. J. Oliveira-Dos-Santos, S. Mariathasan, D. Bouchard, J. Jones, R. Sarao, I. Kozieradzki, P. S. Ohashi, J. M. Penninger, and D. J. Dumont. 1998. The inositol polyphosphate 5-phosphatase SHIP is a crucial negative regulator of B cell antigen receptor signaling. *J. Exp. Med.* 188: 1333–1342.
- Rauh, M. J., V. Ho, C. Pereira, A. Sham, L. M. Sly, V. Lam, L. Huxham, A. I. Minchinton, A. Mui, and G. Krystal. 2005. SHIP represses the generation of alternatively activated macrophages. *Immunity* 23: 361–374.
- Trotta, R., R. Parihar, J. Yu, B. Becknell, J. Allard II, J. Wen, W. Ding, H. Mao, S. Tridandapani, W. E. Carson, and M. A. Caligiuri. 2005. Differential expression of SHIP1 in CD56^{bright} and CD56^{dim} NK cells provides a molecular basis for distinct functional responses to monokine costimulation. *Blood* 105: 3011–3018.
- Gardai, S., B. B. Whitlock, C. Helgason, D. Ambruso, V. Fadok, D. Bratton, and P. M. Henson. 2002. Activation of SHIP by NADPH oxidase-stimulated Lyn leads to enhanced apoptosis in neutrophils. *J. Biol. Chem.* 277: 5236–5246.
- Huber, M., C. D. Helgason, J. E. Damen, L. Liu, R. K. Humphries, and G. Krystal. 1998. The src homology 2-containing inositol phosphatase (SHIP) is the gatekeeper of mast cell degranulation. *Proc. Natl. Acad. Sci. USA* 95: 11330–11335.
- Helgason, C. D., J. E. Damen, P. Rosten, R. Grewal, P. Sorensen, S. M. Chappel, A. Borowski, F. Jirik, G. Krystal, and R. K. Humphries. 1998. Targeted disruption of SHIP leads to hemopoietic perturbations, lung pathology, and a shortened life span. *Genes Dev.* 12: 1610–1620.
- Takeshita, S., N. Namba, J. J. Zhao, Y. Jiang, H. K. Genant, M. J. Silva, M. D. Brodt, C. D. Helgason, J. Kalesnikoff, M. J. Rauh, et al. 2002. SHIP-deficient mice are severely osteoporotic due to increased numbers of hyper-resorptive osteoclasts. *Nat. Med.* 8: 943–949.
- Galli, S. J., M. Grimaldeston, and M. Tsai. 2008. Immunomodulatory mast cells: negative, as well as positive, regulators of immunity. *Nat. Rev. Immunol.* 8: 478–486.
- Kalesnikoff, J., N. Baur, M. Leitges, M. R. Hughes, J. E. Damen, M. Huber, and G. Krystal. 2002. SHIP negatively regulates IgE⁺ antigen-induced IL-6 production in mast cells by inhibiting NF- κ B activity. *J. Immunol.* 168: 4737–4746.
- Huber, M., C. D. Helgason, M. P. Scheid, V. Duronio, R. K. Humphries, and G. Krystal. 1998. Targeted disruption of SHIP leads to Steel factor-induced degranulation of mast cells. *EMBO J.* 17: 7311–7319.
- Okayama, Y., C. Ra, and H. Saito. 2007. Role of mast cells in airway remodeling. *Curr. Opin. Immunol.* 19: 687–693.
- Grimaldeston, M. A., M. Metz, M. Yu, M. Tsai, and S. J. Galli. 2006. Effector and potential immunoregulatory roles of mast cells in IgE-associated acquired immune responses. *Curr. Opin. Immunol.* 18: 751–760.
- Oh, S. Y., T. Zheng, M. L. Bailey, D. L. Barber, J. T. Schroeder, Y. K. Kim, and Z. Zhu. 2007. Src homology 2 domain-containing inositol 5-phosphatase 1 deficiency leads to a spontaneous allergic inflammation in the murine lung. *J. Allergy Clin. Immunol.* 119: 123–131.
- Nagle, D. L., C. A. Kozak, H. Mano, V. M. Chapman, and M. Bucan. 1995. Physical mapping of the Tec and Gabr1 loci reveals that the Wsh mutation on mouse chromosome 5 is associated with an inversion. *Hum. Mol. Genet.* 4: 2073–2079.
- Nigrovic, P. A., D. H. Gray, T. Jones, J. Hallgren, F. C. Kuo, B. Chaletzky, M. Gurish, D. Mathis, C. Benoist, and D. M. Lee. 2008. Genetic inversion in mast cell-deficient (W^{sh}) mice interrupts corin and manifests as hematopoietic and cardiac aberrancy. *Am. J. Pathol.* 173: 1693–1701.
- Grimaldeston, M. A., C. C. Chen, A. M. Piliponsky, M. Tsai, S. Y. Tam, and S. J. Galli. 2005. Mast cell-deficient W^{sh}-sash c-kit mutant *Kit*^{W^{sh}/W^{sh} mice as a model for investigating mast cell biology in vivo. *Am. J. Pathol.* 167: 835–848.}
- Hua, X., M. Kovarova, K. D. Chason, M. Nguyen, B. H. Koller, and S. L. Tilley. 2007. Enhanced mast cell activation in mice deficient in the A2b adenosine receptor. *J. Exp. Med.* 204: 117–128.
- Mallen-St Clair, J., C. T. Pham, S. A. Villalta, G. H. Caughey, and P. J. Wolters. 2004. Mast cell dipeptidyl peptidase I mediates survival from sepsis. *J. Clin. Invest.* 113: 628–634.
- Nakae, S., L. H. Ho, M. Yu, R. Monteforte, M. Iikura, H. Suto, and S. J. Galli. 2007. Mast cell-derived TNF contributes to airway hyperreactivity, inflammation, and TH2 cytokine production in an asthma model in mice. *J. Allergy Clin. Immunol.* 120: 48–55.
- Finkelman, F. D. 2007. Anaphylaxis: lessons from mouse models. *J. Allergy Clin. Immunol.* 120: 506–515; quiz 516–517.
- Bischoff, S. C., and G. Sellge. 2002. Mast cell hyperplasia: role of cytokines. *Int. Arch. Allergy Immunol.* 127: 118–122.
- Nakamura, K., T. Kouro, P. W. Kincade, A. Malykhin, K. Maeda, and K. M. Coggeshall. 2004. Src homology 2-containing 5-inositol phosphatase (SHIP) suppresses an early stage of lymphoid cell development through elevated interleukin-6 production by myeloid cells in bone marrow. *J. Exp. Med.* 199: 243–254.
- Hu, Z. Q., W. H. Zhao, and T. Shimamura. 2007. Regulation of mast cell development by inflammatory factors. *Curr. Med. Chem.* 14: 3044–3050.
- Gounaris, E., S. E. Erdman, C. Restaino, M. F. Gurish, D. S. Friend, F. Gounari, D. M. Lee, G. Zhang, J. N. Glickman, K. Shin, et al. 2007. Mast cells are an essential hematopoietic component for polyp development. *Proc. Natl. Acad. Sci. USA* 104: 19977–19982.
- Tsai, M., T. Takeishi, H. Thompson, K. E. Langley, K. M. Zsebo, D. D. Metcalfe, E. N. Geissler, and S. J. Galli. 1991. Induction of mast cell proliferation, maturation, and heparin synthesis by the rat c-kit ligand, stem cell factor. *Proc. Natl. Acad. Sci. USA* 88: 6382–6386.
- Kalesnikoff, J., M. Huber, V. Lam, J. E. Damen, J. Zhang, R. P. Siraganian, and G. Krystal. 2001. Monomeric IgE stimulates signaling pathways in mast cells that lead to cytokine production and cell survival. *Immunity* 14: 801–811.
- Blanchet, M. R., S. Maltby, D. J. Haddon, H. Merckens, L. Zhytniuk, and K. M. McNagny. 2007. CD34 facilitates the development of allergic asthma. *Blood* 110: 2005–2012.
- Williams, C. M., and S. J. Galli. 2000. Mast cells can amplify airway reactivity and features of chronic inflammation in an asthma model in mice. *J. Exp. Med.* 192: 455–462.
- Yu, M., M. Tsai, S. Y. Tam, C. Jones, J. Zehnder, and S. J. Galli. 2006. Mast cells can promote the development of multiple features of chronic asthma in mice. *J. Clin. Invest.* 116: 1633–1641.
- Doganci, A., T. Eigenbrod, N. Krug, G. T. De Sanctis, M. Hausding, V. J. Erpenbeck, E. Haddad, H. A. Lehr, E. Schmitt, T. Bopp, et al. 2005. The IL-6R α chain controls lung CD4⁺CD25⁺ Treg development and function during allergic airway inflammation in vivo. *J. Clin. Invest.* 115: 313–325.
- Gillfillan, A. M., and C. Tkaczky. 2006. Integrated signalling pathways for mast-cell activation. *Nat. Rev. Immunol.* 6: 218–230.
- Tamir, I., J. C. Stolpa, C. D. Helgason, K. Nakamura, P. Bruhns, M. Daeron, and J. C. Cambier. 2000. The RasGAP-binding protein p62^{delok} is a mediator of inhibitory Fc γ RIIB signals in B cells. *Immunity* 12: 347–358.

34. Fukao, T., T. Yamada, M. Tanabe, Y. Terauchi, T. Ota, T. Takayama, T. Asano, T. Takeuchi, T. Kadowaki, J. Hata Ji, and S. Koyasu. 2002. Selective loss of gastrointestinal mast cells and impaired immunity in PI3K-deficient mice. *Nat. Immunol.* 3: 295–304.
35. Ali, K., A. Bilancio, M. Thomas, W. Pearce, A. M. Gilfillan, C. Tkaczyk, N. Kuehn, A. Gray, J. Giddings, E. Peskett, et al. 2004. Essential role for the p110 δ phosphoinositide 3-kinase in the allergic response. *Nature* 431: 1007–1011.
36. Laffargue, M., R. Calvez, P. Finan, A. Trifilieff, M. Barbier, F. Altruda, E. Hirsch, and M. P. Wymann. 2002. Phosphoinositide 3-kinase γ is an essential amplifier of mast cell function. *Immunity* 16: 441–451.
37. Duan, W., A. M. Aguinaldo Datiles, B. P. Leung, C. J. Vlahos, and W. S. Wong. 2005. An anti-inflammatory role for a phosphoinositide 3-kinase inhibitor LY294002 in a mouse asthma model. *Int. Immunopharmacol.* 5: 495–502.
38. Echtenacher, B., D. N. Mannel, and L. Hultner. 1996. Critical protective role of mast cells in a model of acute septic peritonitis. *Nature* 381: 75–77.
39. Ierna, M. X., H. E. Scales, K. L. Saunders, and C. E. Lawrence. 2008. Mast cell production of IL-4 and TNF may be required for protective and pathological responses in gastrointestinal helminth infection. *Mucosal Immunol.* 1: 147–155.
40. Langdon, J. M., J. T. Schroeder, B. M. Vonakis, A. P. Bieneman, K. Chichester, and S. M. Macdonald. 2008. Histamine-releasing factor/translationally controlled tumor protein (HRF/TCTP)-induced histamine release is enhanced with SHIP-1 knockdown in cultured human mast cell and basophil models. *J. Leukocyte Biol.* 84: 1151–1158.
41. Ong, C. J., A. Ming-Lum, M. Nodwell, A. Ghanipour, L. Yang, D. E. Williams, J. Kim, L. Demirjian, P. Qasimi, J. Ruschmann, et al. 2007. Small-molecule agonists of SHIP1 inhibit the phosphoinositide 3-kinase pathway in hematopoietic cells. *Blood* 110: 1942–1949.
42. Lee, D. M., D. S. Friend, M. F. Gurish, C. Benoist, D. Mathis, and M. B. Brenner. 2002. Mast cells: a cellular link between autoantibodies and inflammatory arthritis. *Science* 297: 1689–1692.
43. Rijnierse, A., A. S. Koster, F. P. Nijkamp, and A. D. Kraneveld. 2006. Critical role for mast cells in the pathogenesis of 2,4-dinitrobenzene-induced murine colonic hypersensitivity reaction. *J. Immunol.* 176: 4375–4384.