METHODS

Chitinase assays

Chitinase activity was determined by using a fluorogenic substrate, as described previously.[E1] Briefly, 50 μL of sample was mixed with 30 μL of citrate/phosphate buffer (0.1 mol/L/0.2 mol/L), pH 5.2, and 20 μL of 0.5 mg/mL substrate 4-methylumbelliferyl-D-N,N′-diacetylchito-bioside (Sigma, St Louis, Mo) in a final concentration of 0.17 mmol/L in excess. The samples were incubated at 37°C for varying amounts of time, and the reaction was stopped by adding 1 mL of Stop Solution (0.3 mol/L glycine/NaOH buffer, pH 10.6). The fluorescence of released 4-methy-lumbelliferone was measured with a fluorometer at excitation of 350 nm and emission of 450 nm. A standard curve was generated by using 4-methylumbelliferone with serial dilutions (Sigma). Chitinase extracted from *Serratia marcescens* (Sigma) was used as a positive control.

Measurement of histamine in tissues

Single-cell suspensions were prepared from spleens and lungs. Splenocytes were disaggregated by squeezing the cells from the splenic capsule through the 100-μm nylon mesh of a Cell Strainer (BD Falcon) into PBS to obtain a single-cell suspension. Erythrocytes were lysed, and the washed splenocytes were resuspended in 50×10^6/mL in PAG buffer [Piperazine-1,4-bis(2-ethanesulfonic acid) buffer with 0.003% human serum albumin and 0.1% dextrose]. Perfused lungs were resected, minced to a fine slurry, and incubated in 300 U/mL type IV collagenase (Worthington) and 100 U/mL DNase I (Roche Diagnostics GmbH, Mannheim, Germany) in RPMI 1640 (Invitrogen, Carlsbad, Calif) at 37°C for 1 hour. Lung cells were obtained by gently mashing through the 100-μm nylon Cell Strainer. Erythrocytes were lysed, and the washed cells were resuspended at 25×10^6/mL in PAG buffer. The total content of histamine and spontaneous release of histamine of the isolated cells from spleens and lungs were determined as previously described.[E2] Briefly, single-cell suspensions and reagents were prewarmed at 37°C for 30 minutes. Cells (10 or 20×10^6 cells from lungs or spleens, respectively) in a volume of 0.4 mL were mixed with 0.1 mL of 8% $HClO_4$ to lyse the cells for total histamine or with 0.1 mL of PAG CM (PAG plus Ca and Mg) buffer for spontaneous release of histamine. All the samples were tested in duplicates. The cells were incubated at 37°C for 45 minutes. PAG buffer (0.5 mL) was added to stop the reaction, and the cells or debris were microcentrifuged at 1000 rpm for 10 minutes. The supernatant was analyzed for histamine by using a RFA 300 rapid flow analyzer (Astoria-Pacific Int, Clackamas, Ore). The percentage of spontaneous release was calculated, as previously described,[E2] as follows:

Percentage of spontaneous release = (*Histamine released in PAG CM alone/Total histamine content*) ×100%.

RESULTS

Chitinase upregulation

We observed in the BAL fluid and lungs of *SHIP-1*$^{-/-}$ mice numerous crystal particles (Fig E1, *A*), which had been reported in IL-13–transgenic mice and in other models of allergic asthma.[E3] These crystals were later identified as YM-1 and YM-2 proteins.[E4] Analysis of mRNA expression demonstrated that AMCase, YM-1, and YM-2 were upregulated in the lungs of *SHIP-1*$^{-/-}$ mice, whereas chitotriosidase, a newly identified mouse chitinase,[E5] appeared to be downregulated (Fig E1, *B*). Furthermore, significantly increased chitin degradation activity was seen in the BAL fluid of *SHIP-1*$^{-/-}$ mice (Fig E1, *C*).

Increased IgE levels in the lung

It was reported that *SHIP-1*$^{-/-}$ mice had enhanced B-cell functions and increased levels of several types of Igs in the serum, including IgG1, IgG2a, and IgG2b.[E6] However, the levels of IgE in the serum or in local tissues, particularly in the lung, have not been determined. Because no apparent allergen could be attributed to the immune response, we only measured total IgE levels in the BAL fluid. The concentration of total IgE was significantly increased in the BAL fluid of *SHIP-1*$^{-/-}$ mice compared with that of WT mice (Fig E2).

Increased histamine content and release

The main source of histamine content in the tissues is mast cells, and spontaneous release of histamine from cells in culture is an indication of the activation status of the cells. We measured the total cellular histamine level and spontaneous release of histamine in single-cell suspensions obtained from the spleens and lungs of WT and *SHIP-1*$^{-/-}$ mice. As shown in Fig E3, compared with those of WT mice, cells isolated from the spleens and lungs of *SHIP-1*$^{-/-}$ mice had higher amounts of total histamine and increased spontaneous release of histamine in culture.

REFERENCES

E1. Guo L, Johnson RS, Schuh JC. Biochemical characterization of endogenously formed eosinophilic crystals in the lungs of mice. J Biol Chem 2000;275:8032-7.

E2. Schroeder JT, Saini S. Assay methods for measurement of mediators and markers of allergic inflammation. In: Detrick B, Hamilton RG, Folds JD, editors. Manual of molecular and clinical laboratory immunology. 7th ed. Washington (DC): ASM; 2006. p. 964-74.

E3. Zhu Z, Homer RJ, Wang Z, Chen Q, Geba GP, Wang J, et al. Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. J Clin Invest 1999;103:779-88.

E4. Zhu Z, Zheng T, Homer RJ, Kim YK, Chen NY, Cohn L, et al. Acidic mammalian chitinase in asthmatic Th2 inflammation and IL-13 pathway activation. Science 2004;304:1678-82.

E5. Zheng T, Rabach M, Chen NY, Rabach L, Hu X, Elias JA, et al. Molecular cloning and functional characterization of mouse chitotriosidase. Gene 2005;357:37-46.

E6. Helgason CD, Kalberer CP, Damen JE, Chappel SM, Pineault N, Krystal G, et al. A dual role for Src homology 2 domain-containing inositol-5-phosphatase (SHIP) in immunity: aberrant development and enhanced function of b lymphocytes in ship -/- mice. J Exp Med 2000;191:781-94.

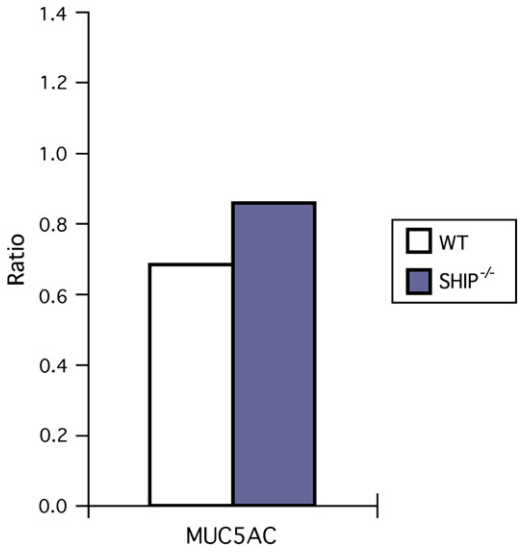

FIG E1. Densitometric analysis of MUC5AC mRNA expression. Shown is the average ratio of MUC5AC mRNA to that of β-actin from RT-PCR analysis of total RNA from lung tissues of 2 mice each of the WT and *SHIP-1*$^{-/-}$ groups (Fig 3, *B*).

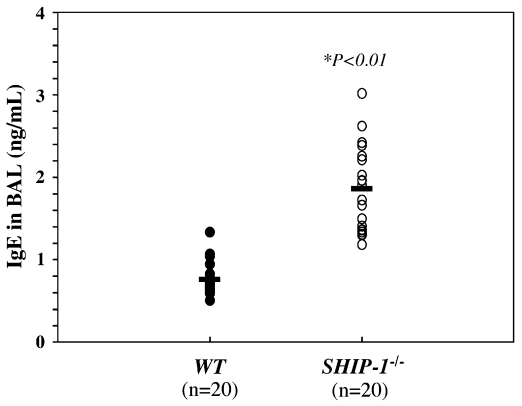

FIG E2. IgE levels in the BAL fluid. Total IgE levels in the BAL fluid of WT and *SHIP-1*$^{-/-}$ mice were determined by means of ELISA (*$P <$ .01 for comparison between WT and *SHIP-1*$^{-/-}$ mice, n = 20 for each group).

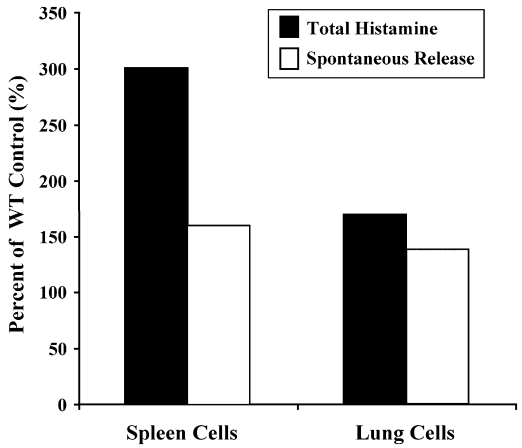

FIG E3. Histamine levels. Single-cell suspensions from spleens and lungs of WT (n = 3) and *SHIP-1*$^{-/-}$ mice (n = 3) were prepared and pooled. The values of total and spontaneously released histamine were expressed as a percentage of WT control values.

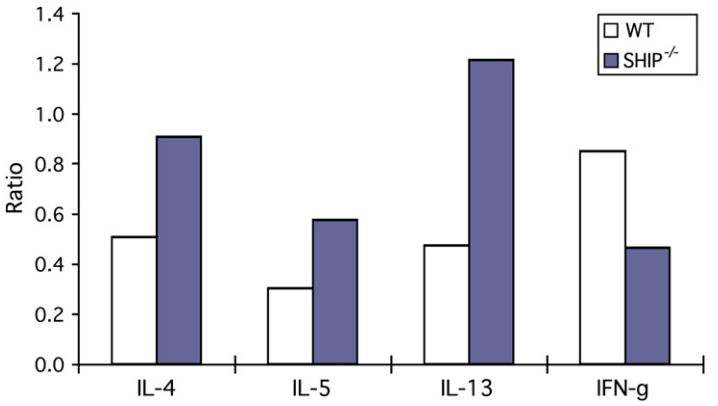

FIG E4. Densitometric analysis of cytokine mRNA expression. Shown is the average ratio of cytokine mRNA to that of β-actin from RT-PCR analysis of total RNA from lung tissues of 2 mice each of the WT and *SHIP-1*$^{-/-}$ groups (Fig 6, *A*).

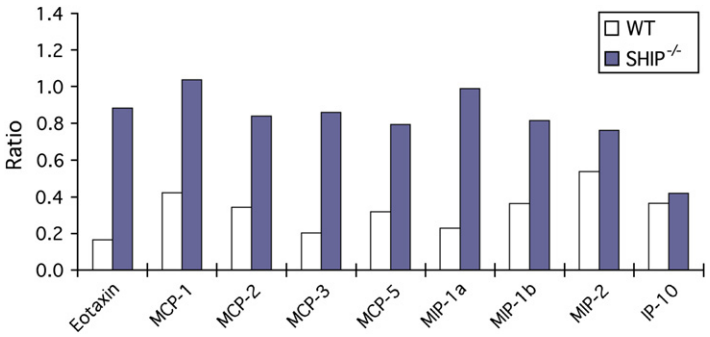

FIG E5. Densitometric analysis of chemokine mRNA expression. Shown is the average ratio of chemokine mRNA to that of β-actin from RT-PCR analysis of total RNA from lung tissues of 2 mice each of the WT and *SHIP-1*$^{-/-}$ groups (Fig 7, *A*).

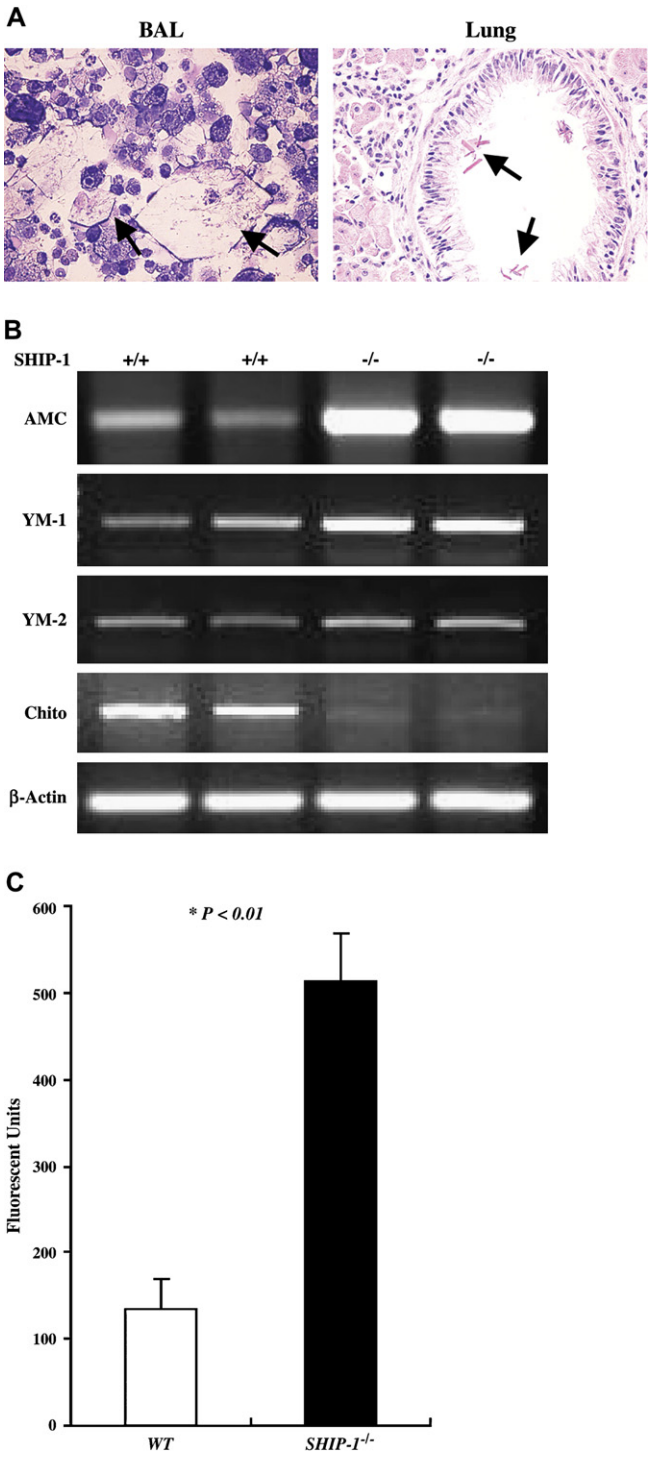

FIG E6. Upregulation of chitinases in the lungs of *SHIP-1*$^{-/-}$ mice. **A**, Crystal materials *(arrows)* were found in the BAL fluid, airways lumen, and lung parenchyma of *SHIP-1*$^{-/-}$ mice but not in WT mice. **B**, RT-PCR analysis of AMCase, YM-1, YM-2, and chitotriosidase *(Chito)* expression in the lung. **C**, Chitinase activity in BAL fluid expressed as mean ± SEM fluorescent units (n = 6 for each group, **P* < .01).

TABLE E1. Severity of pulmonary inflammation in *SHIP-1*$^{-/-}$ mice

Degree of severity	No./total	Percentage	Lung histology (H&E)	BAL cell counts
None	2/12	16.6	Not detected	Normal
Minimum	2/12	16.6	Not detected	Increased
Mild	2/12	16.6	Focal areas	Increased
Moderate	2/12	16.6	>2 Lobes	Increased
Severe	4/12	33.3	All lobes	Increased

A set of 12 *SHIP-1*$^{-/-}$ mice was examined. Both lung histology and BAL fluid cell counts were compared with those of WT mice.
H&E, Hematoxylin and eosin.