The Journal of Immunology
RESEARCH ARTICLE | MAY 15 2004

The Role of IL-5 for Mature B-1 Cells in Homeostatic Proliferation, Cell Survival, and Ig Production [1] FREE

Byoung-gon Moon; ... et. al

J Immunol (2004) 172 (10): 6020–6029.
https://doi.org/10.4049/jimmunol.172.10.6020

The Role of IL-5 for Mature B-1 Cells in Homeostatic Proliferation, Cell Survival, and Ig Production[1]

Byoung-gon Moon,* Satoshi Takaki,* Kensuke Miyake,† and Kiyoshi Takatsu[2]*

B-1 cells, distinguishable from conventional B-2 cells by their cell surface marker, anatomical location, and self-replenishing activity, play an important role in innate immune responses. B-1 cells constitutively express the IL-5R α-chain (IL-5Rα) and give rise to Ab-producing cells in response to various stimuli, including IL-5 and LPS. Here we report that the IL-5/IL-5R system plays an important role in maintaining the number and the cell size as well as the functions of mature B-1 cells. The administration of anti-IL-5 mAb into wild-type mice, T cell-depleted mice, or mast cell-depleted mice resulted in reduction in the total number and cell size of B-1 cells to an extent similar to that of IL-5Rα-deficient (IL-5R$\alpha^{-/-}$) mice. Cell transfer experiments have demonstrated that B-1 cell survival in wild-type mice and homeostatic proliferation in recombination-activating gene 2-deficient mice are impaired in the absence of IL-5Rα. IL-5 stimulation of wild-type B-1 cells, but not IL-5R$\alpha^{-/-}$ B-1 cells, enhances CD40 expression and augments IgM and IgG production after stimulation with anti-CD40 mAb. Enhanced IgA production in feces induced by the oral administration of LPS was not observed in IL-5R$\alpha^{-/-}$ mice. Our results illuminate the role of IL-5 in the homeostatic proliferation and survival of mature B-1 cells and in IgA production in the mucosal tissues. ***The Journal of Immunology*****, 2004, 172: 6020–6029.**

B-1 cells differ from conventional B-2 cells in their surface phenotype, anatomical localization, self-replenishing activity, and V_H usage of IgM (1, 2). B-1 cells constitutively express three different markers, namely Mac-1 (CD11b/CD18), FcϵR (CD23), and the IL-5R α-chain (IL-5Rα).[3] Mac-1 is present in peritoneal and pleural cavity B-1 cells but is not expressed on B-2 cells, whereas FcϵR is preferentially expressed on B-2 cells in the peritoneal cavity and in the spleen (3, 4). IL-5Rα is constitutively expressed on all B-1 cells, but is expressed on a small proportion (2–4%) of resting B-2 cells in the spleen (5).

The progenitors of B-1 cells are abundant in the fetal omentum and liver but are missing in the bone morrow of adult animals (6, 7). In contrast with B-2 cells, which are supplied from progenitors in the bone marrow throughout life, B-1 cells maintain their number in adult animals by their self-replenishing capacity (3, 7). In the adult, these self-replenishing B-1 cells are clearly enriched in the peritoneal and pleural cavities, and a low frequency is seen in the spleen, but B-1 cells are virtually absent from the lymph nodes, Peyer's patches (PP), and peripheral blood, where most conventional B-2 cells are localized (3). B-1 cells are categorized into B-1a cells that express CD5 and B-1b cells that express cell surface markers similar to those of B-1a cells, except for the low expression, if any, of CD5 (2, 3).

B-1 cells are believed to be the primary source of natural IgM Ab, although they can become Ig-producing cells for all isotypes. Consistent with a major role of B-1 cells in natural IgM production, a number of specificities of natural IgM Ab have been identified in the B-1 repertoire. These include specificities for LPS, phosphorylcholine, undefined determinants on *Escherichia coli* and *Salmonella* spp., phosphatidylcholine, and complement-binding Abs (3). Furthermore, B-1 cells in the peritoneal cavity serve as an important source of IgA-producing plasma cells at mucosal sites. These findings are largely supported by transfer experiments of peritoneal B-1 cells in irradiated mice or otherwise B cell-depleted mice and by analysis of genetically altered immune-deficient mice (8). The role of B-1 cells in IgA production in the gut is further supported by evidence that mice with a selective B-1 cell reduction in number showed decreased frequencies of IgA-producing cells in the lamina propria (LP) (9). The helper T cell dependency of B-1 cells on gut-associated IgA production is still controversial (9, 10).

Studies of gene-targeted and transgenic mice have revealed that B cell receptor (BCR) signaling is critical for B-1 cell development or maintenance. Mutant mice that lack Bruton's tyrosine kinase protein kinase C β, CD19, the p85α subunit of phosphatidylinositol-3 kinase, p95^{vav}, CD21/CD35, and CD81, which are strongly associated with BCR signaling, have substantial depletion of B-1 cells but largely spare B-2 cells (11–18). Conversely, mutation or overexpression of Src homology protein-1, CD22, or CD72 that induces enhanced BCR signaling results in an expanded B-1 cell compartment (19–21).

IL-5, mainly produced by activated Th2 cells and mast cells, acts on B-1 and B-2 cells to induce proliferation and differentiation into Ig-producing cells (22–25). IL-5 also controls the production and functions of eosinophils and basophils. The IL-5R consists of two distinct membrane proteins, IL-5Rα and βc, each of which is a member of the cytokine receptor superfamily (5). The binding of IL-5 occurs through the IL-5Rα, and the βc forms a high-affinity IL-5R in combination with the IL-5Rα, transducing signals into nuclei. Although the molecular mechanisms for IL-5 signal

Divisions of *Immunology and †Infectious Genetics, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, Tokyo, Japan

Received for publication October 30, 2003. Accepted for publication March 3, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by a research grant from the Human Frontier Science Program (to K.T.), by Special Coordination Funds for Promoting Science and Technology (to K.T.), by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science, Sports and Culture (Japan), and by an international postdoctoral fellowship from the Japan Society for the Promotion of Science (to B.M.).

[2] Address correspondence and reprint requests to Dr. Kiyoshi Takatsu, Division of Immunology, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai Minato-ku, Tokyo 108-8639, Japan. E-mail address: takatsuk@ims.u-tokyo.ac.jp

[3] Abbreviations used in this paper: IL-5Rα, IL-5R α-chain; PP, Peyer's patch; LP, lamina propria; BCR, B cell receptor; RAG, recombination-activating gene; PEC, peritoneal exudate cell; MLN, mesenteric lymph node; s, surface; TLR, Toll-like receptor 4; HPRT, hypoxanthine phosphoribosyltransferase; m, murine; CD40L, CD40 ligand.

0022-1767/04/$02.00

transduction are not fully characterized, the activation of Bruton's tyrosine kinase and Janus kinase 2 kinases, rapid tyrosine phosphorylation of βc and Src homology 2/Src homology 3-containing cellular proteins, and the induction of the transcription of several nuclear proto-oncogenes are essential for signal transduction (26–32).

Transgenic mice expressing the *IL-5* gene exhibit elevated levels of serum IgM, IgA, and IgE, and an increase in the number of B-1 cells and autoantibody production and show persistent eosinophilia (33, 34). IL-5Rα$^{-/-}$ mice and IL-5$^{-/-}$ mice show a decrease in B-1 cells in the peritoneal cavity and in B-1 cell-derived surface (s)IgA^{+} cells in the LP (35–39). Although these results suggest that IL-5 is an important cytokine for B-1 cell development, maintenance, or triggering, the role of IL-5 in mature B-1 cell maintenance and activation in vivo remains to be properly evaluated.

This study examines whether the IL-5/IL-5R system plays an important role in the homeostatic proliferation and survival of mature B-1 cells. We show that IL-5 regulates the cell number and cell size of B-1 cells in the absence of T cells or mast cells. We also demonstrate the role of IL-5 in gut-associated B-1 cell response to CD40 and LPS.

Materials and Methods

Mice

C57BL/6J (IL-5Rα$^{+/+}$) and W/W^{V} mice were purchased from Japan SLC (Hamamatsu, Japan). Recombination-activating gene (RAG)2-deficient (RAG-2$^{-/-}$) mice and TCRβ δ double null mutant (TCRβ$^{-/-}$ δ$^{-/-}$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The IL-5Rα null mutant (IL-5Rα$^{-/-}$) mice (35) used in this study were backcrossed with C57BL/6J mice for >10 generations. IL-5 null mutant (IL-5$^{-/-}$) mice on a C57BL/6 background (38) were donated by M. Kopf (University of Freiburg, Germany). All of the mice were bred and maintained in animal facilities under specific pathogen-free conditions using ventilated microisolater cages in the experimental animal facility at the Institute of Medical Science, University of Tokyo. All experiments were conducted according to our institution's guidelines for the care and treatment of experimental animals.

Administration of anti-IL-5 mAb and LPS

A single i.p. administration of anti-IL-5 mAb (clone NC17) or isotype-matched control IgG into 6- to 8-wk-old mice (1 mg in a volume of 250 μl per mouse) was performed (40, 41). Six days after treatment, peritoneal washouts were obtained and analyzed. LPS (*E. coli* serotype O55: B5; Sigma-Aldrich, St. Louis, MO) dissolved in PBS was orally administered (0.1 mg in a volume of 200 μl per mouse per week) for 3 wk into the gut of 8-wk-old mice through a 1-mm diameter polyethylene tube. Seven days after the last administration, the mice were anesthetized with ether, sacrificed, and analyzed.

Cell preparation

Single cell suspensions were prepared from the lymphoid organs of 6- to 8-wk-old mice. A standard procedure was used to prepare single cell suspensions from the peritoneal exudate cells (PECs), mesenteric lymph nodes (MLNs), PP, SP, lung, and the LP of the small intestine. Briefly, PECs were obtained by washing the peritoneal cavity with HBSS (Life Technologies, Grand Island, NY) containing 3% FCS. Mononuclear cells from MLNs or PP were isolated by a mechanical method using a stainless steel screen. Mononuclear cells from the lung and LP were isolated by a procedure of shaking in an RPMI 1640 medium (Life Technologies) containing 5 mM EDTA and by enzymatic dissociation procedures with collagenase type VIII (Sigma-Aldrich) (37).

Purification of B-1 cells

PECs were collected from >10 mice and were mixed together. After washing twice with PBS containing 1% BSA, the cells were incubated with anti-FcγR (2.4G2; American Type Culture Collection, Rockville, MD) to prevent the nonspecific binding of the labeled Abs. After another washing, macrophages, B-2 cells, and T cells were depleted from the cells using a MACS system (Miltenyi Biotec, Cologne, Germany) after incubation with a mixture of biotinylated Abs (anti-F4/80, anti-CD23, and anti-CD3) and streptavidine-coupled microbeads (Miltenyi Biotec). In B-1 cell transfer experiments, we took another purification step of B-1 cells using a FACSVantage (BD Biosciences, San Jose, CA) to obtain B-1 cells with a higher degree of purity. In addition to using the MACS system, the resulting F4/80^{-}/CD23^{-}/CD3^{-} cells were stained with FITC-labeled anti-CD23 and PE-labeled F(ab′)$_2$ of anti-IgM, and the CD23^{-}sIgM^{+} cells were sorted using a FACSVantage.

Flow cytometry

The cells (1–10 × 10^5) were stained with predetermined optimal concentrations of the respective Abs together with 2.4G2 (10 μg/ml). After washing, the cells were analyzed on FACScan or a FACSCaliber instrument (BD Biosciences). The following mAbs were used: biotinylated anti-IL-5Rα (T21) (42); FITC-labeled, PE-labeled, or biotinylated anti-CD23 (B3B4), PE-labeled or biotinylated anti-CD5 (53-7.3) and biotinylated anti-CD3 (145-2C11) (all purchased from BD PharMingen, San Diego, CA); FITC-labeled, PE-labeled, or biotinylated anti-B220 (RA3-6B2), PE-labeled F(ab′)$_2$ of anti-mouse IgM, and FITC-labeled or biotinylated anti-Mac-1 (M1/70) (all obtained from Caltag Laboratories, Burlingame, CA); PE-labeled anti-Toll-like receptor 4 (TLR4)/MD2 (MTS 510) (43) and PE-labeled anti-RP105 (RP/14) (44); biotinylated anti-CD40 (1C10; R&D Systems, Minneapolis, MN), biotinylated anti-F4/80 (A3-1; Serotec, Oxford, U.K.); and biotinylated anti-IgA (Southern Biotechnology, Birmingham, AL). PE-labeled streptavidin (Ancell, Bayport, MN) or allophycocyanin-conjugated streptavidine (BD PharMingen) were also used. In some stainings, 2 μg/ml 7-amino-actinomycin D (Sigma-Aldrich) was used to gate out dead cells.

Cell transfer and homeostatic proliferation assay

PECs obtained from IL-5Rα$^{+/+}$ or IL-5Rα$^{-/-}$ mice were washed with PBS and suspended in PBS at 1 × 10^7 cells/ml. CFSE (Molecular Probes, Eugene, OR) was then added to the cell suspensions at a final concentration of 1 μM. The cell suspensions were incubated at 37°C for 10 min and washed three times with cold sterile PBS. The resulting CFSE-labeled cells (1 × 10^6) were injected i.p. into IL-5Rα$^{+/+}$ or RAG-2$^{-/-}$ mice. In some experiments, sorted B-1 cells (1 × 10^5) were injected i.p. into RAG-2$^{-/-}$ mice. The PECs of recipient mice were recovered on days 2, 30, and 60 after the cell transfer, and their cellularities in the B-1 cell compartment were analyzed.

RNA isolation and semiquantitative RT-PCR

Total RNA was isolated from various mouse tissues using the SV Total RNA Isolation System (Promega, Madison, WI), according to the manufacturer's instructions, and first strand cDNA templates synthesized by Superscript II reverse transcriptase (Life Technologies) using random primers (TaKaRa, Kyoto, Japan). Serial dilutions of cDNA templates were subjected to PCR amplification by using primer sets encompassing several introns for IL-5 (forward primer, 5′-ATGGAGATTCCCATGAGCAC; reverse primer, 5′-GCACAGTTTTGTGGGGTTTT) or hypoxanthine phosphoribosyltransferase (HPRT; forward primer, 5′-TGCTCGAGATGTCATGAAGG; reverse primer, 5′-TTGCGCTCATCTTAGGCTTT). The cycling parameters were 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C for 35 cycles to detect *IL-5* mRNA or 27 cycles for HPRT. The PCR products were separated through 1.0% agarose gel and were stained with ethidium bromide.

Assay for B-1 cell proliferation and differentiation

MACS-sorted PECs were cultured in an RPMI 1640 medium supplemented with 8% heat-inactivated FCS, 2 mM glutamine, 5 μM 2-ME, penicillin (100 U/ml), and streptomycin (100 μg/ml) in 96-well flat-bottom microtiter plates (1 × 10^5/well in 200 μl of medium) with or without stimulants. Anti-CD40 mAb (1C10; R&D Systems; 1 μg/ml), LPS (40 μg/ml), IL-4 (1000 U/ml), or a selected combination of these agents was added at the onset of cell culture. For the proliferation assay, cells were pulse-labeled with [^{3}H]thymidine (0.2 μCi per well) during the last 8 h of the 72-h culture period, and the incorporated [^{3}H]thymidine was measured using a MATRIX 96 Direct Beta Counter (Packard, Meriden, CT). The results were expressed as the mean cpm and the SD of the duplicate cultures. For determining IgM, IgG1, and IgG3 secretion, cells (1 × 10^5 in a 200-μl culture) were cultured for 7 days. The cultured supernatants were used for ELISA to determine the amounts of IgM, IgG1, and IgG3. Each experiment was repeated at least three times.

Enumeration of Ig-producing cells using ELISPOT

An ELISPOT assay was conducted according to the procedures previously described (37). The 96-well filtration plates with a nitrocellulose base (Millipore, Bedford, MA) were coated with 5 μg/ml anti-Ig (Southern Biotechnology) overnight and were blocked with a culture medium. The mononuclear cells suspended in the culture medium were added at various concentrations and were incubated for 6 h. After washing, 1 μg/ml HRP-conjugated anti-IgM, anti-IgG, or anti-IgA Ab (all obtained from Southern Biotechnology) was added, and the plates were incubated for 10 h at 4°C.

After the incubation, the spots were developed with 2-amino-9-ethylcarbazole containing hydrogen peroxide (Polysciences, Warrington, PA). Reddish-brown-colored spots were counted as Ab-forming cells using the KS ELISPOT compact system (Carl Zeiss, Jena, Germany).

ELISA

Freshly collected fecal samples were weighed, dissolved in PBS (0.1 g/ml), and centrifuged at 15,000 rpm for 5 min. The supernatants were used as fecal extract. The amount of each Ig isotype in sera and in fecal extract was measured by sandwich ELISA with Abs specific for each murine (m)Ig isotype according to the procedures previously described (36). In brief, 96-well trays (Greiner, Frickenhausen, Germany) were coated with 10 μg/ml isotype-specific goat anti-mIg polyclonal Abs for total Igs. Samples were added to the wells and the trays were incubated for 2 h. After washing with PBS containing 0.05% Tween 20 (washing buffer), biotinylated isotype-specific goat anti-mIg polyclonal Abs were added to each well. After washing, HRP-streptavidine was added to each well, and the incubation continued for 1 h. Finally, the trays were washed with the buffer, and 100-μl aliquots of substrate, *o*-phenylene-diamine (final 0.4 mg/ml), and hydrogen peroxide (final 0.015%), dissolved in 0.1 M citrate buffer (pH 5.0), were added to each well. Enzyme reaction was terminated by adding 2 M sulfuric acid, and OD at 495 nm was measured with a V-max kinetic Micro Plate Reader (Molecular Devices, Sunnyvale, CA). Using myeloma proteins (BD PharMingen), standard curves were generated for each isotype and the concentration of mIg was determined.

Results

Decrease in cell number and cell size of mature B-1 cells by administration of anti-IL-5 mAb in vivo

As we reported previously, IL-5R$\alpha^{-/-}$ mice have a significant reduction in cell number and cell size of IgM$^+$ CD5$^+$ B-1a cells in the PECs (36). When we analyzed the entire IgM$^+$ CD23$^-$ B-1 cell populations, a significant reduction in percentage and smaller cell size of B-1 cells was also observed (Fig. 1*A*). These results were confirmed by the analysis of IgM$^+$ CD5$^+$ B-1 cell populations. We also found that the total number and size of B-1 cells in the PECs decreased in IL-5$^{-/-}$ mice (data not shown). In contrast with B-1 cells, the total percentage and size of IgM$^+$CD23$^+$ (or IgM^{low}CD5$^-$) B-2 cells in IL-5R$\alpha^{-/-}$ mice were similar to those of wild-type mice (Fig. 1*A*). The total numbers of B-1 cells in IL-5R$\alpha^{+/+}$ and IL-5R$\alpha^{-/-}$ mice were 4.2×10^5 and 3.1×10^5 on average, respectively, (statistically significant, $p < 0.05$), whereas those of B-2 cells were 4.0×10^5 and 4.3×10^5, respectively (Table I).

Our first question is whether the abnormalities observed in B-1 cells from IL-5R$\alpha^{-/-}$ and IL-5$^{-/-}$ mice originated in the developmental process or in fully developed mature B-1 cells. We administered anti-IL-5 mAb i.p. into a group of 8-wk-old wild-type mice, which developed mature B-1 cells. As a control, isotype-matched rat IgG was injected in another group of mice. A smaller B-1 cell size was observed in anti-IL-5-treated mice 3 days after treatment (data not shown). Six days after anti-IL-5 treatment, not only cell size but also the total percentage of B-1 cells significantly decreased (Fig. 1*B*). The total numbers of B-1 cells on average in the control and anti-IL-5-treated mice were 4×10^5 and 3.2×10^5, respectively (statistically significant, $p < 0.05$), whereas those of B-2 cells were 3.8×10^5 and 3.7×10^5, respectively (Table I). The levels of reduction in B-1 cell number and size in anti-IL-5-treated mice were similar to those of B-1 cells in IL-5R$\alpha^{-/-}$ mice. Anti-IL-5 treatment did not cause significant changes in total number or cell size in the IgM$^+$CD23$^+$ (or IgM^{low}CD5$^-$) B-2 cell compartment (Fig. 1*B*). We infer from these results that the abnormality of B-1 cells observed in IL-5R$\alpha^{-/-}$ mice is reproduced in mature B-1 cells in wild-type mice by blocking IL-5 signals.

Impaired survival and homeostatic proliferation of B-1 cells in IL-5R$\alpha^{-/-}$ mice

To examine the role of IL-5 in maintaining the mature B-1 cell compartment in more detail, PECs from IL-5R$\alpha^{+/+}$ or IL-5R$\alpha^{-/-}$ mice were labeled with CFSE and transferred into the peritoneal cavity of unirradiated IL-5R$\alpha^{+/+}$ mice, where the normal number

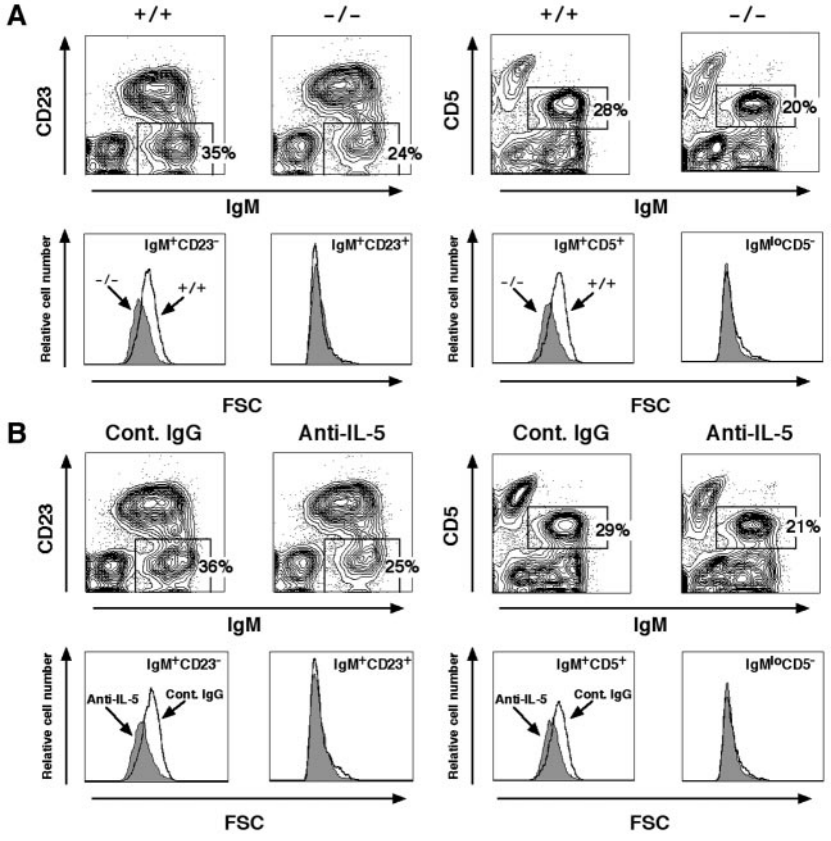

FIGURE 1. Decreased cell number and size of B-1 cells by blocking IL-5 and IL-5R interaction. Representative two-color contour plots show the expression of IgM/CD23 and IgM/CD5 on PECs from 8-wk-old IL-5R$\alpha^{+/+}$ or IL-5R$\alpha^{-/-}$ mice (*A*) and from control IgG- or anti-IL-5-treated IL-5R$\alpha^{+/+}$ mice (*B*). A single i.p. administration of anti-IL-5 mAbs or control IgG (1 mg/250 μl) into IL-5R$\alpha^{+/+}$ mice was performed. PECs were obtained and analyzed on day 6 after treatment (*B*). The percentages represent the fractions of the lymphocyte-gated live cells that fall into the indicated boxes. Representative histograms depict the relative cell number and sizes of B-1 cells (IgM$^+$CD23$^-$), B-1a cells (IgM$^+$CD5$^+$), and B-2 cells (IgM$^+$CD23$^+$ or IgM^{low}CD5$^-$). The representative results of three independent experiments are shown.

Table I. *Reduced absolute cell number of B-1 cells by blocking IL-5 and IL-5R interaction*[a]

	IL-5R$\alpha^{+/+}$	IL-5R$\alpha^{-/-}$	Control IgG	Anti-IL-5
B-1 (IgM^+CD23^-)	4.2 ± 0.36	3.1 ± 0.34*	4.4 ± 0.32	3.2 ± 0.17*
B-1a (IgM^+CD5^+)	3.8 ± 0.28	2.8 ± 0.39*	4.0 ± 0.41	3.0 ± 0.23*
B-2 (IgM^+CD23^+)	4.0 ± 0.42	4.3 ± 0.52	3.8 ± 0.21	3.7 ± 0.33

[a] The results indicate the mean cell numbers ± SEM ($\times 10^5$) of indicated groups of five mice.
*, $p < 0.05$ compared with IL-5R$\alpha^{+/+}$ or control IgG-administered mice.

of B-1 cells resides. The $CFSE^+$ B-1 and $CFSE^+$ B-2 cells in the PECs of the recipient mice were examined by FACS analysis on day 2 or on day 30. As shown in Fig. 2*A* (*left panel*), the proportion of $CFSE^+$IL-5R$\alpha^{+/+}$ B-1 cells on day 30 (94%) was close to that on day 2, suggesting the long-term survival of CFSE-labeled B-1 cells in the recipient. In contrast, the proportion of $CFSE^+$ IL-5R$\alpha^{-/-}$ B-1 cells was reduced to 39% on day 30 compared with that on day 2. The intensity of CFSE labeling showed a broad distribution in both IL-5R$\alpha^{+/+}$ B-1 and IL-5R$\alpha^{-/-}$ B-1 cells on day 30 (Fig. 2*B*), indicating that cells have divided slightly. However, we could not estimate how many times the B-1 cells divided, because of faint intensities of CFSE labeling. $CFSE^+$ IL-5R$\alpha^{+/+}$ B-2 cells in the recipient on day 30 were reduced to 55%, which was comparable with $CFSE^+$IL-5R$\alpha^{-/-}$ B-2 cells (52%) (Fig. 2*A*, *right panel*). We examined the distribution of CFSE-positive B cells from IL-5R$\alpha^{+/+}$ or IL-5R$\alpha^{-/-}$ mice in the LP, PP, and MLNs of recipient mice 30 days after cell transfer. CFSE-positive B cells were rarely detected in the LP (data not shown). We observed some CFSE-positive B cells (0.02–0.03% of the total cells) in MLNs and PP (data not shown). However, there was no significant difference between recipient mice transferred IL-5R$\alpha^{+/+}$B-1 and IL-5R$\alpha^{-/-}$B-1 cells. Thus, the survival of mature B-1 cells, but not B-2 cells, in the peritoneal cavity was severely impaired in the absence of IL-5Rα.

Next, the role of IL-5 in the self-replenishing activity of B-1 cells was examined. Peritoneal exudate cells from IL-5R$\alpha^{+/+}$ or IL-5R$\alpha^{-/-}$ mice were CFSE-labeled and transferred into RAG-$2^{-/-}$ mice. On day 30, the proportion of $CFSE^+$ IL-5R$\alpha^{+/+}$ B-1 cells in the recipient RAG-$2^{-/-}$ mice increased ~3-fold (276%) compared with that on day 2, whereas the proportion of $CFSE^+$ IL-5R$\alpha^{-/-}$ B-1 cells did not increase (70%) (Fig. 3*A*, *upper panel*). The CFSE intensity of IL-5R$\alpha^{-/-}$ B-1 cells somewhat decreased, but maintained higher intensities than did IL-5R$\alpha^{+/+}$ B-1 cells (Fig. 3*B*, *lower left panel*). The difference in B-2 cell number on day 30 in the RAG-$2^{-/-}$ recipient mice between IL-5R$\alpha^{+/+}$ B-2 cells and IL-5R$\alpha^{-/-}$ B-2 cells (62% and 48%, respectively) was not obvious compared with that of B-1 cells (Fig. 3*A*, *lower panel*). The CFSE intensity of IL-5R$\alpha^{-/-}$ B-2 cells was comparable with that of IL-5R$\alpha^{+/+}$ B-2 cells (Fig. 3*B*, *lower right panel*). In the LP 30 days after cell transfer, the proportion of $CFSE^+B220^-sIgA^+$ cells in RAG-$2^{-/-}$ mice transferred with IL-5R$\alpha^{+/+}$ cells was significantly higher than that transferred with IL-5R$\alpha^{-/-}$ cells (Fig. 3*C*, *left panels*). We examined the levels of CFSE in $sIgA^+$ cells in the LP in recipients transferred IL-5R$\alpha^{+/+}$ PECs or IL-5R$\alpha^{-/-}$ PECs and found that the levels of CFSE of $sIgA^+$ cells were very low and were similar between the two experimental groups (Fig. 3*C*, *right panels*). Intriguingly, ~3-fold higher levels of IgM, IgG1, IgG3, and IgA in serum were observed in RAG-$2^{-/-}$ mice transferred IL-5R$\alpha^{+/+}$ cells compared with those transferred with IL-5R$\alpha^{-/-}$ cells, whereas the serum levels of IgG2a and IgG2b were comparable between the two groups (Fig. 3*D*, *left panel*). The amount of IgA in fecal extracts was also ~3-fold higher in the recipients of IL-5R$\alpha^{+/+}$ cells compared with that in IL-5R$\alpha^{-/-}$ cells (Fig. 3*D*, *right panel*). These results imply that IL-5 plays a critical role in the homeostatic proliferation of mature B-1 cells and leads to the maintenance of optimal levels of Ig production, although these processes may occur inefficiently even in the absence of IL-5.

Homeostatic proliferation and Ig production of B-1 cells in the absence of T cells

We examined the effect of T cell dependency in the IL-5-mediated homeostatic proliferation of mature B-1 cells. IgM^+CD23^- B-1 cells were purified (>98% purity) from the PECs of IL-5R$\alpha^{+/+}$ or IL-5R$\alpha^{-/-}$ mice by cell sorting and were transferred into RAG-$2^{-/-}$ mice. As shown in Fig. 4*A*, the proportion of IL-5R$\alpha^{+/+}$ B-1 cells in PECs increased ~2-fold (198%) on day 30 and ~3-fold (276%) on day 60 in the RAG-$2^{-/-}$ recipient mice. An increase in both the $IgM^+CD23^-CD5^+$B-1a and $IgM^+CD23^-CD5^-$B-1b cell populations was also observed (Fig. 4*A*, *center* and *right*

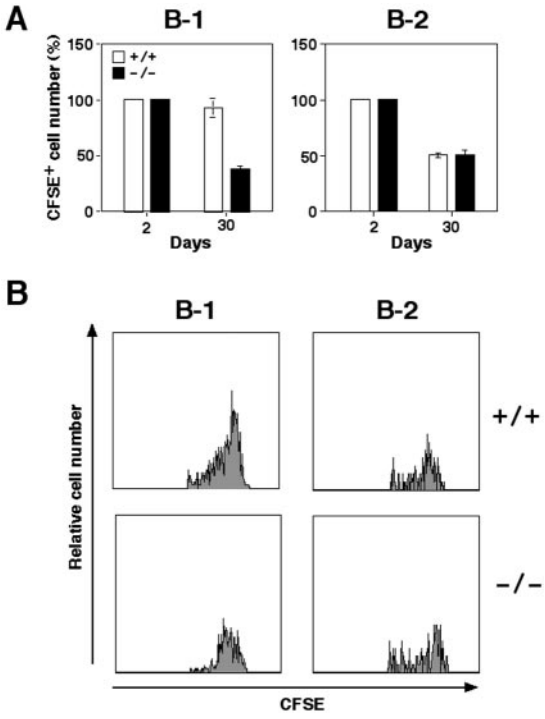

FIGURE 2. Impaired survival of mature IL-5R$\alpha^{-/-}$ B-1 cells. CFSE-labeled PECs from IL-5R$\alpha^{+/+}$ or IL-5R$\alpha^{-/-}$ mice were transferred (1×10^6 cells per head) into the peritoneal cavity of IL-5R$\alpha^{+/+}$ mice. On days 2 and 30 after cell transfer, the recipient mice were killed and the numbers of $CFSE^+$ B-1 (IgM^+CD23^-) and B-2 (IgM^+CD23^+) cells in PECs were analyzed by flow cytometry. Two and three recipient mice in each group were analyzed and the mean ± SEM is shown (*A*). The mean cell number of $CFSE^+$ peritoneal cells from two mice on day 2 was set as 100% (*A*). Representative histograms show the intensity of CFSE in B-1 cells ($CFSE^{+IgM^+}CD23^-$) and B-2 cells ($CFSE^+IgM^+CD23^+$) in the peritoneal cavity of the recipients (*B*). The data shown are representative results from three independent experiments.

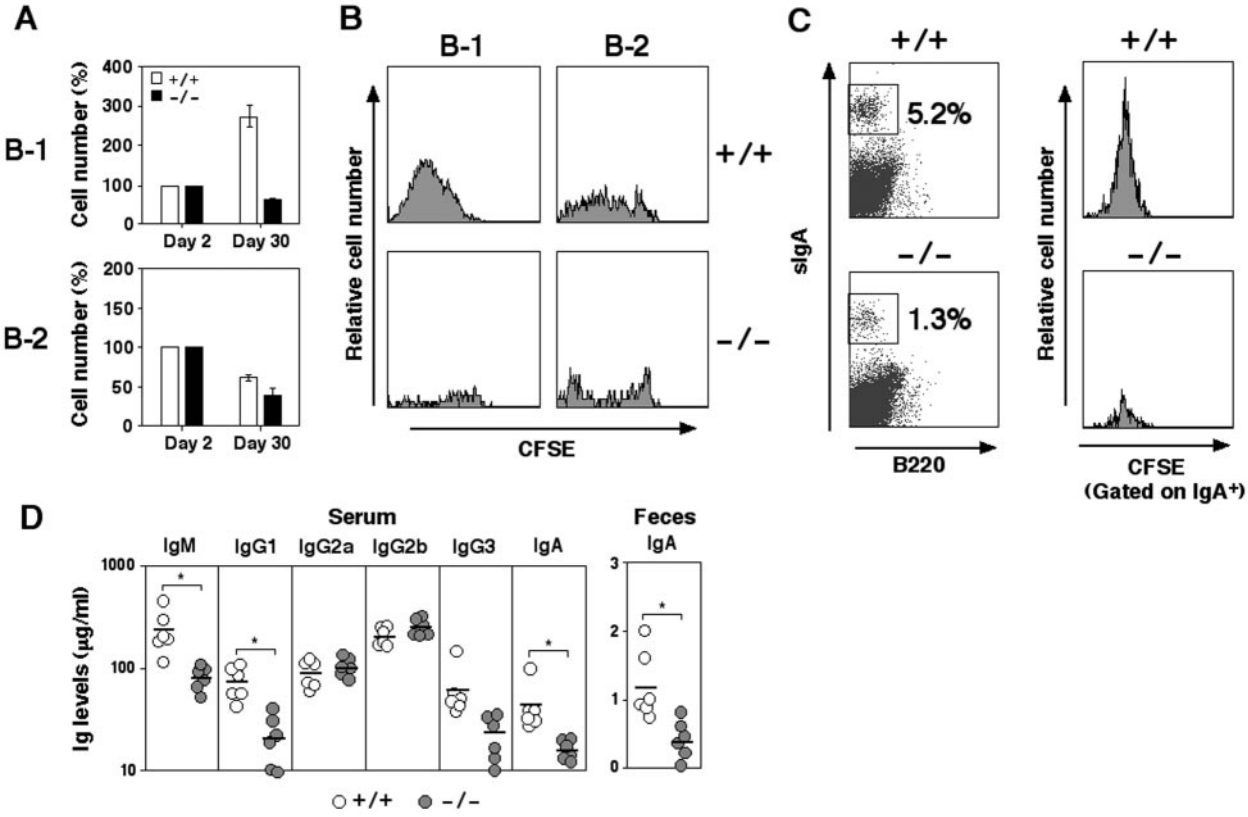

FIGURE 3. Impaired homeostatic B-1 cell proliferation and Ig production from IL-5R$\alpha^{-/-}$ mice. *A*, IL-5R$\alpha^{-/-}$ B-1 cells have a defect in homeostatic proliferation. CFSE-labeled peritoneal cells were injected i.p. into RAG-2$^{-/-}$ mice. The recipient mice of each group were analyzed as described in Fig. 2. *B*, IL-5R$\alpha^{-/-}$ B-1 cells maintain high CFSE intensity. Representative histograms depict CFSE intensity in B-1 cells and B-2 cells in PECs of RAG-2$^{-/-}$ recipient mice. *C*, IL-5R$\alpha^{-/-}$ B-1 cells migrate to the LP, but the number of sIgA$^+$ cells is decreased. Cells in the LP were purified as in the method described and were stained with anti-B220 and anti-IgA. Representative two-color fluorescence plots show B220$^+$ and sIgA$^+$ cells in the LP of the recipient mice on day 30. Representative histograms depict CFSE intensity in sIgA$^+$ cells in LP of RAG-2$^{-/-}$ recipient mice. The percentages represent the fractions of the lymphocyte gated live cells that fall into the indicated box. Representative results of three independent experiments are shown (*A–C*). *D*, IL-5R$\alpha^{-/-}$ peritoneal cells transferred into RAG-2$^{-/-}$ mice produce low levels of Igs. The concentration of Ig subclasses in serum or in fecal extracts in the RAG-2$^{-/-}$ recipient mice on day 30 was determined by isotype-specific ELISA. The mean values of Igs in the indicated groups of recipient mice are represented as a bar. *, $p < 0.05$ by Student's *t* test.

panels), whereas CD5^{high} T cells or CD23$^+$ B-2 cells were not detected even 60 days after cell transfer (data not shown). In contrast, IL-5R$\alpha^{-/-}$ B-1 cells did not show a significant increase on day 30 (114%) or on day 60 (103%) compared with that 2 days after cell transfer (Fig. 4*A*, *left panel*). IL-5R$\alpha^{-/-}$ B-1a cells decreased to ~71% on day 30 and on day 60, whereas IL-5R$\alpha^{-/-}$ B-1b cells showed a small but significant increase up to 129% on day 30 (Fig. 4*A*, *center* and *right panels*). These results indicate that mature B-1 cells undergo homeostatic proliferation in T cell-deficient conditions. The serum levels of IgM, IgG3, and IgA were elevated to ~3- to 5-fold in the recipient transferred IL-5R$\alpha^{+/+}$ B-1 cells compared with those of IL-5R$\alpha^{-/-}$ B-1 cells (Fig. 4*B*). The Ig levels of IgG1 and IgG2 were virtually undetectable in both groups of recipient mice on day 30 (Fig. 4*B* and data not shown).

Production of IL-5 in T cell- and mast cell-deficient mice

IL-5 is produced by T cells, mast cells, and eosinophils once they are activated (22). In particular, not only $\alpha\beta$T cells in the peritoneal cavity and intestinal intraepithelial lymphocytes (8, 45), but also freshly isolated $\gamma\delta$T cells in the intraepithelial lymphocytes are capable of producing IL-5 (45). We injected anti-IL-5 mAb into TCR$\beta^{-/-}\delta^{-/-}$ mice and examined B-1 cell survival in T cell-deficient conditions. Anti-IL-5-treated TCR$\beta^{-/-}\delta^{-/-}$ mice showed a decrease in B-1 cell number and cell size 6 days after treatment, compared with the control group of mice (Fig. 5*A*, *left panel*). Anti-IL-5 injection into W/W^V mice also caused a decrease in B-1 cell size (Fig. 5*A*, *right panel*), although the total B-1 cell number did not change significantly. The total number and size of B-2 cells did not change in either the TCR$\beta^{-/-}\delta^{-/-}$ mice or the W/W^V mice as a result of anti-IL-5 treatment (data not shown).

To evaluate *IL-5* mRNA expression in tissues, total RNA was isolated from the various tissues of RAG-2$^{-/-}$, TCR$\beta^{-/-}\delta^{-/-}$, and W/W^V mice and was used for *IL-5* mRNA expression analysis. As controls, wild-type mice and IL-5$^{-/-}$ mice were also used. RT-PCR analysis revealed significant *IL-5* mRNA expression in the lungs, spleen, small intestine, and stomach of wild-type mice, RAG-2$^{-/-}$ mice, TCR$\beta^{-/-}\delta^{-/-}$, and W/W^V mice (Fig. 5*B*). A lesser extent of *IL-5* mRNA expression was observed in PECs. We did not detect any *IL-5* mRNA expression in the tissues of the IL-5$^{-/-}$ mice. *IL-5* mRNA expression was not observed in the liver. To examine *IL-5* mRNA expression in cells other than T cells, mast cells, and eosinophils, we purified c-*kit*$^-$IL-5Rα^- cells by sorting (>99% purity) from the tissues of RAG-2$^{-/-}$ mice. The RNA from these cells was isolated and used for RT-PCR analysis. As shown in Fig. 5*C*, high levels of *IL-5* mRNA expression were observed in c-*kit*$^-$IL-5Rα^- cells in the lungs and small intestine of RAG-2$^{-/-}$ mice. The c-*kit*$^-$IL-5Rα^- PECs also expressed *IL-5* mRNA, although the expression levels were low. These results

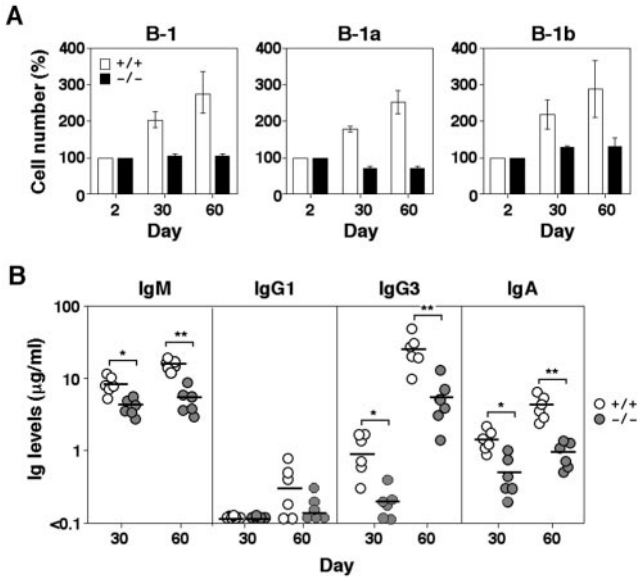

FIGURE 4. T cell-independent homeostatic proliferation of B-1 cells. *A*, B-1 cells from IL-5R$\alpha^{+/+}$ mice but not from IL-5R$\alpha^{-/-}$ mice show homeostatic proliferation even in T cell-deficient conditions. Purified B-1 cells were transferred (1×10^5 per head) i.p. into RAG-$2^{-/-}$ mice. The proportion of B-1 (IgM^+CD23^-), B-1a ($IgM^+CD23^-CD5^+$), or B-1b ($IgM^+CD23^-CD5^-$) cells in the PECs of RAG-$2^{-/-}$ mice was analyzed by flow cytometry. The recipient mice of each group were analyzed as described in Fig. 2 on days 2, 30, and 60. The data shown are representative results from two independent experiments. *B*, RAG-$2^{-/-}$ mice that have received IL-5R$\alpha^{-/-}$ B-1 cell transfer show low levels of Igs. On days 30 and 60, the concentrations of Igs in the serum of the recipient mice were determined by isotype-specific ELISA. The mean values of the indicated groups of mice are represented as a bar. **, $p < 0.01$; *, $p < 0.05$; by Student's *t* test.

suggest that IL-5 is also produced by non-T/non-mast/non-eosinophil cells and may support the maintenance and Ab production of mature B-1 cells in vivo.

Defective responses of IL-5R$\alpha^{-/-}$ B-1 cells to anti-CD40 mAb and LPS

B-1 cells that were smaller in size in IL-5R$\alpha^{-/-}$ mice and anti-IL-5-treated mice led us to address the possibility that these small cells might show an impaired response to various activation signals. We purified B-1 cells in PECs from IL-5R$\alpha^{+/+}$ and IL-5R$\alpha^{-/-}$ mice and stimulated them with anti-CD40, LPS, IL-4, or combinations of these. Interestingly, IL-5R$\alpha^{-/-}$ B-1 cells showed lower proliferation than did IL-5R$\alpha^{+/+}$ B-1 cells in response to anti-CD40 (58% of IL-5R$\alpha^{+/+}$ cells) and LPS (49% of IL-5R$\alpha^{+/+}$ cells) (Fig. 6*A*). Similar results were obtained when the cells were stimulated with anti-CD40 plus IL-4 and LPS plus IL-4. IL-5R$\alpha^{-/-}$ B-1 cells secreted significantly lower levels of IgM when they were cultured with anti-CD40, LPS, anti-CD40 plus IL-4, or LPS plus IL-4 than they did IgM secreted from IL-5R$\alpha^{+/+}$ B-1 cells (Fig. 6*B*, *upper panel*). Although IL-5R$\alpha^{-/-}$ B-1 cells were capable of producing IgG1 upon stimulation with anti-CD40 plus IL-4 or LPS plus IL-4, the amount of IgG1 produced by these cells was significantly lower (~55% and ~70%, respectively) than the amount of IgG1 produced by IL-5R$\alpha^{+/+}$ B-1 cells (Fig. 6*B*, *middle panel*). IgG3 production induced by LPS was also impaired in IL-5R$\alpha^{-/-}$ B-1 cells (Fig. 6*B*, *lower panel*). In contrast with B-1 cells, IL-5R$\alpha^{+/+}$ and IL-5R$\alpha^{-/-}$ B-2 cells in the spleen responded comparably upon anti-CD40 or LPS stimulation (data not shown).

Regulation of CD40 expression in B-1 cells by IL-5

One possible reason for the impaired response of IL-5R$\alpha^{-/-}$ B-1 cells to anti-CD40 may be the impaired expression of CD40. We compared the expression levels of CD40 on IL-5R$\alpha^{-/-}$ B-1 cells with those on IL-5R$\alpha^{+/+}$ B-1 cells and found that IL-5R$\alpha^{-/-}$ B-1 cells showed a significantly lower expression of CD40 than did IL-5R$\alpha^{+/+}$ B-1 cells (Fig. 7*A*). In contrast with B-1 cells, IL-5R$\alpha^{-/-}$ B-2 cells showed CD40 expression comparable with that of IL-5R$\alpha^{+/+}$ B-2 cells. CD40 expression on IL-$5^{-/-}$ B-1 cells was also lower than on wild-type B-1 cells (data not shown). B-1 cells from the anti-IL-5-treated mice showed reduced CD40 expression 6 days after treatment, whereas CD40 expression on B-2 cells was not affected (Fig. 7*B*). Conversely, IL-5 stimulation of IL-$5^{-/-}$ B-1 cells enhanced CD40 expression, whereas CD40 expression on B-2 cells was unaltered (Fig. 7*C*). These results strongly suggest that the IL-5 signal is important for CD40 expression and CD40-related activation in B-1 cells.

Defective IgA production in LPS-administered IL-5R$\alpha^{-/-}$ mice

As described previously, IL-5R$\alpha^{-/-}$ B-1 cells respond poorly to LPS stimulation (Fig. 6). Because TLR4/MD2 and RP105 expressed on B cells play an essential role in LPS-mediated B cell activation (43, 44), we examined TLR4/MD2 and RP105 expression on B-1 cells. IL-5R$\alpha^{+/+}$ B-1 cells showed very low levels of TLR4/MD2 expression and significant levels of RP105 expression. The expression levels of TLR4/MD2 and RP105 on B-1 cells were comparable between IL-5R$\alpha^{+/+}$ B-1 and IL-5R$\alpha^{-/-}$ B-1 cells (Fig. 8*A*). IL-5 might not be involved in the regulation of TLR4/MD2 or RP105 expression, but rather might participate in modulating LPS-induced intracellular signaling.

LPS is capable of inducing differentiation of B-1 cells in gut-associated lymphoid tissue (46). We orally administered LPS to IL-5R$\alpha^{+/+}$ and IL-5R$\alpha^{-/-}$ mice and examined Ig levels in serum and fecal extracts by ELISA. IgA levels in serum and fecal extracts in LPS-administered IL-5R$\alpha^{+/+}$ mice were elevated ~40% and ~400%, respectively, compared with those in the PBS-treated control group of mice. The levels of other Ig isotypes were comparable with those in the PBS-treated control mice (Fig. 8*B*). In contrast with the IL-5R$\alpha^{+/+}$ mice, the IgA levels in serum and fecal extracts in the IL-5R$\alpha^{-/-}$ mice did not increase as a result of oral LPS administration (Fig. 8*B*). To determine whether impaired IgA production due to LPS administration in IL-5R$\alpha^{-/-}$ mice was because of a decrease in IgA-producing cells, mononuclear cells were isolated from different tissues in LPS-administered mice and isotype-specific ELISPOT assays were performed. As shown in Fig. 8*C*, we detected a significant number of Ig-producing cells in the LP and PP in both IL-5R$\alpha^{+/+}$ and IL-5R$\alpha^{-/-}$ mice. Importantly, the number of IgA-producing cells in the LP and PP was significantly lower in LPS-administered IL-5R$\alpha^{-/-}$ mice than in LPS-administered IL-5R$\alpha^{+/+}$ mice. The number of IgM-producing cells in PECs in IL-5R$\alpha^{-/-}$ mice was also lower than in IL-5R$\alpha^{+/+}$ mice, whereas their splenocytes showed a number of IgM-producing cells comparable with that in IL-5R$\alpha^{+/+}$ mice. These results indicate that IL-5 signals are required for cells in the LP and PP to induce the optimal LPS response for terminal differentiation into Ab-producing cells in vivo.

Discussion

IL-5 and B-1 cell maintenance

A significant reduction in B-1 cells has been shown in IL-5R$\alpha^{-/-}$ and IL-$5^{-/-}$ mice (35, 38) and in 129 mice whose B cells show impaired response to IL-5 (47). These results imply that IL-5 is a

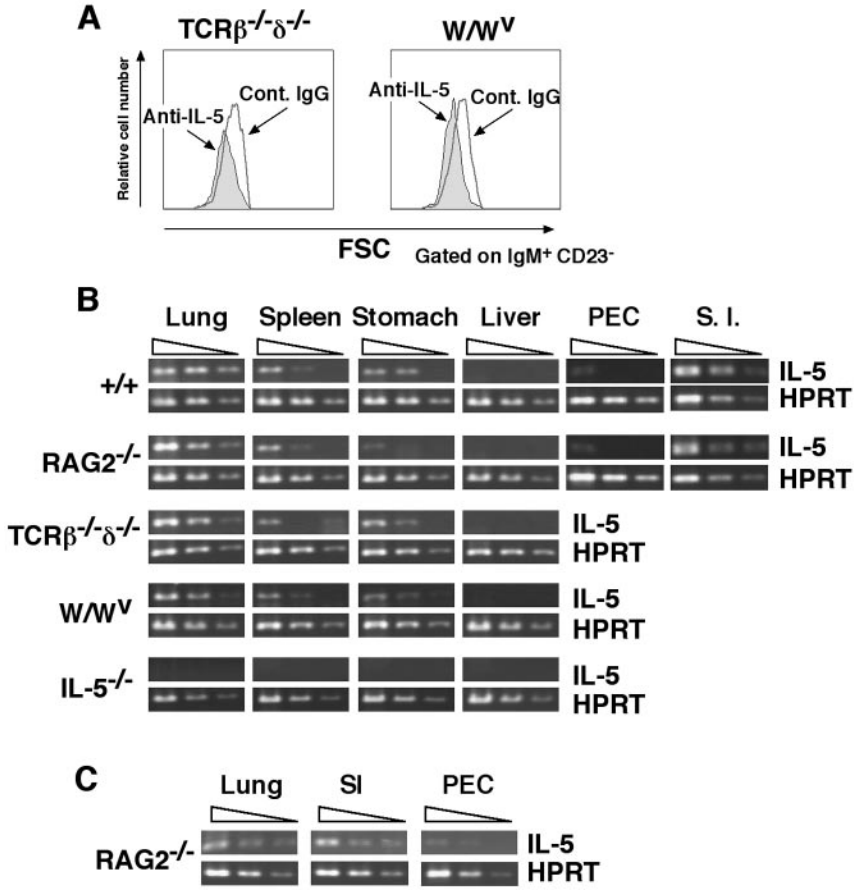

FIGURE 5. IL-5 production by cells other than T cells, mast cells, and eosinophils. *A*, Anti-IL-5 Ab treatment affects B-1 cell maintenance in T cell- or mast cell-deficient mice. A single i.p. administration of anti-IL-5 mAbs or control IgG (1 mg/250 μl) into TCR$\beta^{-/-}\delta^{-/-}$ or W/W^{V} mice was performed. On day 6 after treatment, peritoneal cells were obtained and analyzed. Representative histograms depict the relative cell number and size of the B-1 cells. Representative results of three independent experiments are shown. *B*, Tissues from T cell- or mast cell-deficient mice show *IL-5* mRNA expression. Various tissues were freshly isolated from IL-5R$\alpha^{+/+}$, RAG-2$^{-/-}$, TCR$\beta^{-/-}\delta^{-/-}$, W/W^{V}, and IL-5$^{-/-}$ mice. *C*, Non-T/non-mast/non-eosinophil cells express *IL-5* mRNA. Single cell suspensions were prepared from the lungs, small intestine (SI), and PECs of RAG-2$^{-/-}$ mice and c-*kit*$^{+}$ and IL-5Rα^{+} cells purified by negative sorting using MACS (>99% purity). Serial dilutions (4-fold) of cDNA templates were prepared and subjected to RT-PCR analysis using primer sets designed to amplify *IL-5* or HPRT cDNA fragments (*B* and *C*).

crucial cytokine for B-1 cell development or maintenance. Supporting this notion, we showed B-1 cells that were fully restored in number and function in IL-5R$\alpha^{-/-}$ mice due to the enforced expression of IL-5Rα by crossing with IL-5Rα transgenic mice (36). It should be noted that the decrease in B-1 cell proportion and number is more obvious in young IL-5R$\alpha^{-/-}$ mice than in older ones. This suggests that the IL-5 signal is at least required to facilitate B-1 cell development. However, it is not clear whether IL-5 is required for the maintenance of mature B-1 cells. Thus, the key question is to what extent IL-5 is involved in mature B-1 cell survival and homeostatic proliferation.

This study demonstrates the marked impairment of the maintenance of mature B-1 cell survival and its homeostatic proliferation by blocking IL-5 signals. Intriguingly, the administration of anti-IL-5 mAb into IL-5R$\alpha^{+/+}$ mice could induce a rapid reduction in the total number and size of B-1 cells within 6 days to a degree comparable with that observed in IL-5R$\alpha^{-/-}$ B-1 cells (Fig. 1*B*). Cell transfer experiments of CFSE-labeled B-1 cells to wild-type mice revealed that CFSE^{+} B-1 cells from wild-type mice survived longer in the peritoneal cavity than did those from IL-5R$\alpha^{-/-}$ B-1 cells (Fig. 2). This may not be due to the impairment of the migratory activity of IL-5R$\alpha^{-/-}$ B-1 cells, because the distribution pattern of CFSE-positive IL-5R$\alpha^{-/-}$ B cells in the LP, PP, and MLNs in the recipient mice 30 days after cell transfer was similar to that of CFSE-positive IL-5R$\alpha^{+/+}$ B cells (data not shown). We were surprised to see CFSE-positive B-2 cells (>50% more than starting cells) in the recipients 30 days after cell transfer, because B-2 cells are thought to be recirculating cells that do not reside in the peritoneal cavity. A proportion of B-2 cells in the peritoneal cavity tend to reside in or to migrate to the peritoneal cavity.

CFSE-labeled B-1 cells of wild-type mice expanded in the peritoneal cavity on day 30 of cell transfer in the RAG-2$^{-/-}$ mice, whereas IL-5R$\alpha^{-/-}$ B-1 cells did not (Figs. 3, *A* and *B*, and 4*A*). This again may not be due to the enhanced migration of IL-5R$\alpha^{-/-}$ B-1 cells in RAG-2$^{-/-}$ mice to the B cell compartment other than in the peritoneal cavity, because IL-5R$\alpha^{-/-}$sIgA^{+} B cells resided in the LP to lesser extent than did IL-5R$\alpha^{+/+}$sIgA^{+} B cells (Fig. 3*C*). The CFSE-labeled B-2 cells in RAG-2$^{-/-}$ mice expressed a wide range of CFSE labeling intensities (Fig. 3*B*). It is likely that cotransferred T cells may expand in the peritoneal cavity of RAG-2$^{-/-}$ recipient mice because of their ability to homeostatically proliferate, during which they may produce cytokines that induce the proliferation of B-2 cells. Alternatively, the B-2 cells that we detected may have been contaminated B-1 cell progenitors with long-lived and self-replenishing activity.

We were surprised to observe that both IL-5R$\alpha^{+/+}$sIgA^{+} B cells and IL-5R$\alpha^{-/-}$sIgA^{+} B cells showed relatively low CFSE labeling (Fig. 3*C*), suggesting extensive cell divisions before differentiation to sIgA^{+} B cells. Although CFSE intensities of B cells were similar, a reduced proportion of sIgA^{+} B cells in the LP of RAG-2$^{-/-}$ mice transferred with IL-5R$\alpha^{-/-}$ B cells was observed compared with mice transferred with IL-5R$\alpha^{+/+}$ B cells. This may be due to the impairment of cell survival and expansion of IL-5R$\alpha^{-/-}$ B-1 cells, although a small proportion of IL-5R$\alpha^{-/-}$ B-1 cells may be sufficient for proliferation and differentiation to sIgA^{+} B cells. Our results imply that the IL-5/IL-5R system plays an important role in maintaining mature B-1 cell survival and homeostatic proliferation in our short-term cell transfer assay.

IL-5-dependent B-1 cell maintenance in T cell- and mast cell-deficient mice

Although it is well known that T cells are a major IL-5 producer, we observed IL-5-mediated homeostatic proliferation of purified B-1 cells in recipient RAG-2$^{-/-}$ mice (Fig. 4). It was possible that

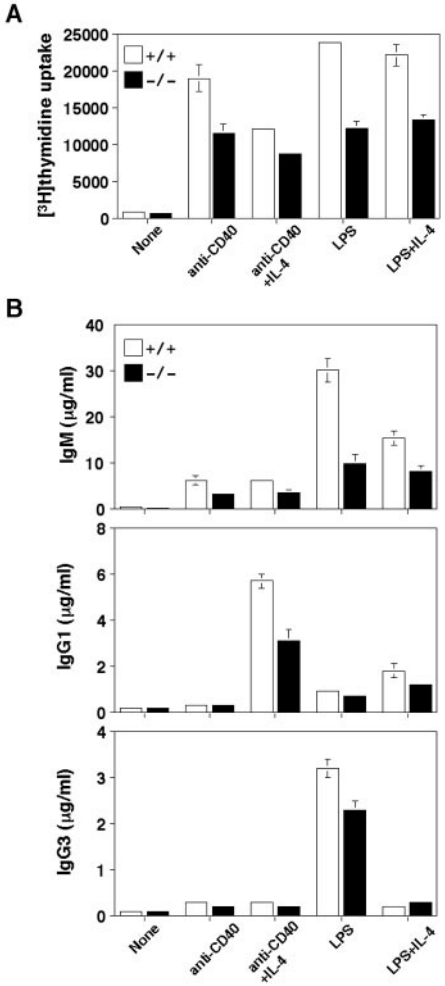

FIGURE 6. Defective activation of IL-5R$\alpha^{-/-}$ B-1 cells to anti-CD40 mAb or LPS. *A*, IL-5R$\alpha^{-/-}$ B-1 cells have defective proliferative responses to anti-CD40 mAb or LPS. B-1 cells purified by the MACS system were cultured (1×10^5 cells in a 200-μl culture) for 3 days with anti-CD40 mAb (1 μg/ml), LPS (40 μg/ml), IL-4 (1000 U/ml), or a selected combination of these agents. The cells were pulse-labeled with [^{3}H]thymidine (0.2 μCi/well) for the last 8 h of the culture. The results represent the mean cpm ± SD of the duplicate determinations. *B*, IL-5R$\alpha^{-/-}$ B-1 cells produce a small amount of Igs in response to anti-CD40 mAb or LPS. Purified B-1 cells were cultured (1×10^5 cells in a 200-μl culture) for 7 days with each stimulant as described in *A*. The IgM, IgG1, and IgG3 concentrations in the cultured supernatants were determined by ELISA. The values represent the mean and SD of the duplicate wells. The data shown are representative results from three independent experiments (*A* and *B*).

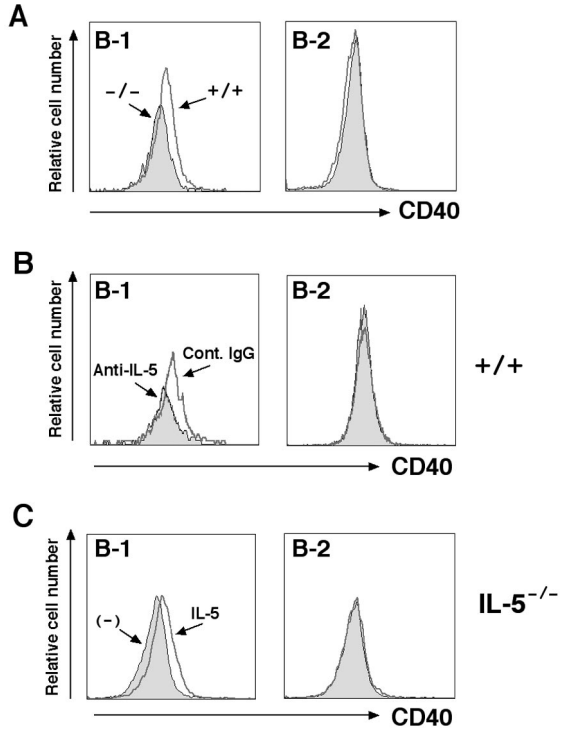

FIGURE 7. IL-5-dependent CD40 expression on B-1 cells. *A*, IL-5R$\alpha^{-/-}$ B-1 cells express low levels of CD40. *B*, Decreased CD40 expression on B-1 cells from anti-IL-5-treated mice is reduced. *C*, CD40 expression on IL-5$^{-/-}$ B-1 cells is recovered by IL-5 stimulation. Peritoneal cells from IL-5$^{-/-}$ mice were cultured (1×10^6 cells in a 2-ml culture) for 2 days with or without IL-5 (500 U/ml). The cultured cells were stained and analyzed by flow cytometry. Representative histograms show the CD40 expression of B-1 or B-2 cells from IL-5$^{-/-}$ mice, which were cultured for 2 days. Representative results from three different experimental sets are shown.

extremely low numbers of T cells in RAG-2$^{-/-}$ mice may provide T cell help, as described by Kushnir et al. (48), but we found no significant T cell population when we examined the PECs from RAG-2$^{-/-}$ mice 30 days after purified B-1 cell transfer (data not shown). Moreover, the results in which anti-IL-5-treated T cell-deficient mice show a reduction in number and cell size of B-1 cells in the peritoneal cavity also support our conclusion that non-T cells produce the IL-5 that supports maintenance and Ab production by B-1 cells (Fig. 5*A*). In fact, cells in various tissues including the lungs, stomach, and spleen of RAG-2$^{-/-}$ mice or TCR$\beta^{-/-}\delta^{-/-}$ mice showed *IL-5* mRNA expression (Fig. 5*B*). Moreover, small intestine and peritoneal washouts also expressed *IL-5* mRNA. Fort et al. (49) demonstrated using RAG-2$^{-/-}$ splenocytes that non-T/non-B cells produce IL-5 in response to IL-25 and that cells responding to IL-25 are accessory cells, which belong to the MHC class II^{high}, CD11c^{dull}, F4/80^{low}, CD8α^-, and CD4$^-$ populations. In vitro stimulation of mouse mast cells by FcϵRI cross-linking induces increased levels of mRNA expression or secretion of various inflammatory cytokines including IL-5 (50). W/W^V mice showed *IL-5* mRNA expression and IL-5-dependent B-1 cell maintenance (Fig. 5, *A* and *B*). In addition to T cells and mast cells, eosinophils and NK cells have also been shown to possess IL-5-producing ability (51, 52). This study shows that c-*kit*$^-$IL-5Rα^- cells purified from the lungs and small intestine of RAG-2$^{-/-}$ mice expressed *IL-5* mRNA (Fig. 5*C*). Our results support the notion that IL-5 can be produced even in T cell-, mast cell-, and eosinophil-deficient conditions, possibly by nonhemopoietic cells, leading to the support of B-1 cell maintenance.

IL-5 and CD40-related response of B-1 cells

T cell-dependent activation of B cells requires CD40-CD40 ligand (CD40L) interaction and a defined set of cytokines. Although B-1 cells are classified as B cells responding to T cell-independent Ags, T cells can influence other aspects of B-1 cell activation and differentiation. In fact, B-1 cells exhibit a strong proliferation and IgG1 production when cocultured with activated T cells plus IL-4 or with recombinant CD40L plus IL-4 (53). T cells enhance Ig production by B-1 cells and induce switching from IgM to IgG1 in B-1 cell-transferred SCID mice (10). Moreover, Erickson et al. (53) have demonstrated that B-1 cells require IL-5 in conjunction with CD40-CD40L interaction for maximal T cell-dependent responses. We showed that IL-5 regulates CD40 expression solely

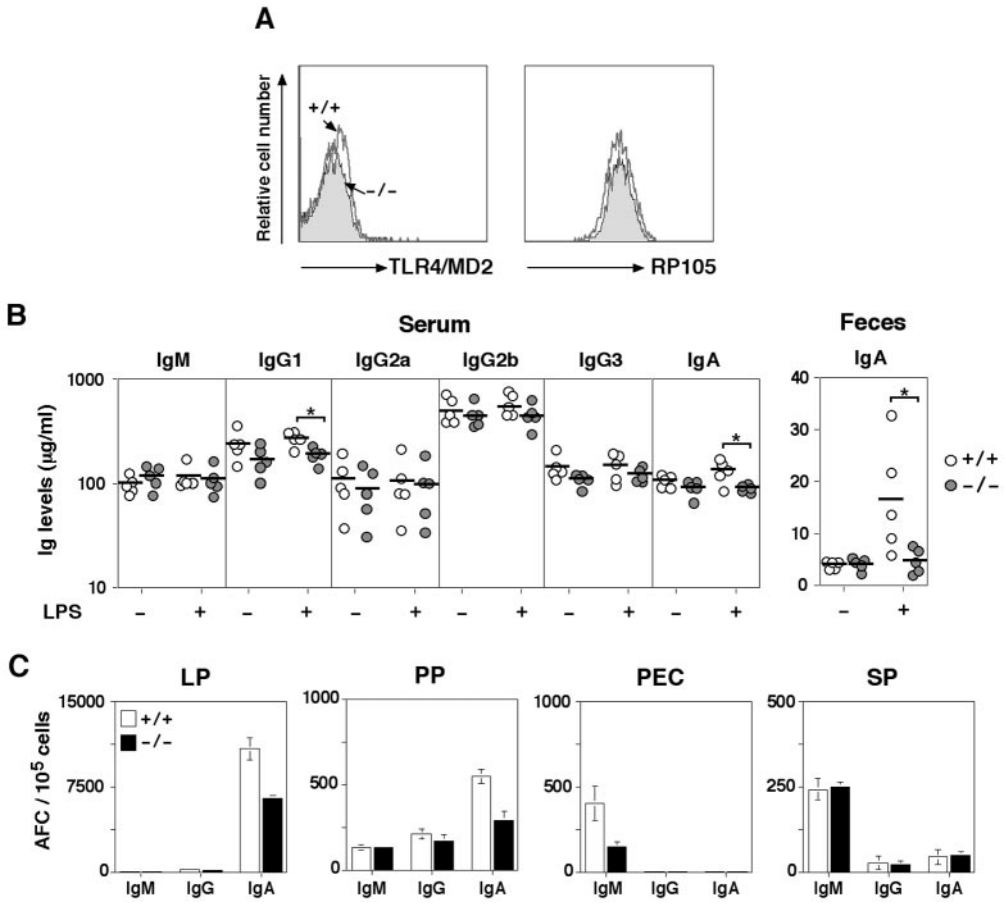

FIGURE 8. Ab production in IL-5R$\alpha^{-/-}$ mice that were administered LPS orally. *A*, IL-5R$\alpha^{-/-}$ B-1 cells express normal levels of TLR4/MD2 and RP105. Representative histograms show TLR/MD2 or RP105 expression on B-1 cells from IL-5R$\alpha^{+/+}$ or IL-5R$\alpha^{-/-}$ mice. *B*, IgA and IgG1 levels are not elevated in IL-5R$\alpha^{-/-}$ mice by oral injection of LPS. LPS (0.1 mg/200 μl/week) was injected orally into the gut of IL-5R$\alpha^{+/+}$ or IL-5R$\alpha^{-/-}$ mice for 3 wk. On day 7 after the last injection, the serum and fecal Ig levels of the mice were analyzed by isotype-specific ELISA. The mean Ig levels of the indicated group of mice are represented as a bar. $*$, $p < 0.05$. *C*, The numbers of Ab-producing cells are reduced in LPS-injected IL-5R$\alpha^{-/-}$ mice. IgM-, IgG-, or IgA-producing cells were examined in the LP, PP, peritoneal cavity, and spleen (SP) from LPS-injected mice by isotype-specific ELISPOT assay. The results represent the mean $\pm$ SD of the duplicate wells. Representative results of three independent experiments are shown (*A* and *C*).

on B-1 cells, not on B-2 cells (Fig. 7). Moreover, IL-5R$\alpha^{-/-}$ B-1 cells showed a defective response to anti-CD40 or anti-CD40 plus IL-4 (Fig. 6*A*). Taking these results together, we propose that constitutive stimulation by IL-5 is important for the full activation of B-1 cells in T cell-dependent response as well as LPS-dependent response in mucosal tissues as described below.

IL-5 and B-1 cell-derived IgA

IL-5 is an important cytokine for the mucosal immune system, which distinguishes it from the systemic immune compartment (54). IL-5 is postulated to be a major cytokine that induces sIgA$^+$ B-2 cells to differentiate into IgA-producing plasma cells in PP and to a lesser extent in the spleen (25, 54). Approximately one-half of IgA plasma cells in the LP of the intestine appear to be derived from B-1 cells in the peritoneal cavity, and B-1 cell-derived IgA is specific for commensal bacteria (55). Hiroi et al. (37) have reported the critical role of IL-5 in IgA secretion in mucosal tissues using IL-5R$\alpha^{-/-}$ mice. In IL-5R$\alpha^{-/-}$, the number of sIgA$^+$ B-1 cells from the effector site are significantly reduced, and IgA levels in mucosal secretions are reduced (37). Interestingly, there were significant differences in serum and fecal IgA levels in LPS-treated IL-5R$\alpha^{+/+}$ and IL-5R$\alpha^{-/-}$ mice (Fig. 8*B*). Although the B-1 cells of IL-5R$\alpha^{-/-}$ mice showed defective proliferation and Ig production upon LPS stimulation in vitro (Fig. 6), the expression levels of TLR4/MD2 and RP105 and the sensor of LPS signals on IL-5R$\alpha^{+/+}$ B-1 cells were comparable with those on IL-5R$\alpha^{-/-}$ B-1 cells (Fig. 8*A*). The IL-5-mediated signaling pathway may couple or cross-talk with the LPS-induced signaling pathway. Because LPS, CD40, and BCR triggering of B cells results in the activation of NF-κB factors (56), NF-κB activation induced by LPS or CD40 may be influenced by IL-5 in B-1 cells.

In summary, the present study provides new insight for an understanding of the important role of IL-5 in homeostatic proliferation and survival of mature B-1 cells. Furthermore, constant IL-5 stimulation may be required for optimal B-1 cell activation in response to CD40 or LPS requirements.

Acknowledgments

We are grateful to A. Kariyone, C. Kubo-Akashi, Y. Tezuka, and R. Shiraishi for their technical assistance. We thank T. Hiroi, H. Kaku, K. Horikawa, Y. Oe-Kikuchi, and T. Tamura for helpful discussion and our colleagues for critical reading of the manuscript.

References

1. Kantor, A. B. 1991. The development and repertoire of B-1 cells (CD5 B cells). *Immunol. Today 12:389.*
2. Hardy, R. R., and K. Hayakawa. 1994. CD5 B cells, a fetal B cell lineage. *Adv. Immunol. 55:297.*
3. Kantor, A. B., and L. A. Herzenberg. 1993. Origin of murine B cell lineages. *Annu. Rev. Immunol. 11:501.*

4. Berland, R., and H. H. Wortis. 2002. Origins and functions of B-1 cells with notes on the role of CD5. *Annu. Rev. Immunol. 20:253*.
5. Takatsu, K., S. Takaki, and Y. Hitoshi. 1994. Interleukin-5 and its receptor system: implications in the immune system and inflammation. *Adv. Immunol. 57:145*.
6. Hayakawa, K., R. R. Hardy, and L. A. Herzenberg. 1985. Progenitors for Ly-1 B cells are distinct from progenitors for other B cells. *J. Exp. Med. 161:1554*.
7. Hardy, R. R. 1992. Variable gene usage, physiology and development of Ly-1$^+$ (CD5$^+$) B cells. *Curr. Opin. Immunol. 4:181*.
8. Kroese, F. G., and N. A. Bos. 1999. Peritoneal B-1 cells switch in vivo to IgA and these IgA antibodies can bind to bacteria of the normal intestinal microflora. *Curr. Top. Microbiol. Immunol. 246:343*.
9. Macpherson, A. J., D. Gatto, E. Sainsbury, G. R. Harriman, H. Hengartner, and R. M. Zinkernagel. 2000. A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria. *Science 288:2222*.
10. Taki, S., M. Schmitt, D. Tarlinton, I. Forster, and K. Rajewsky. 1992. T cell-dependent antibody production by Ly-1 B cells. *Ann. NY Acad. Sci. 651:328*.
11. Khan, W. N., F. W. Alt, R. M. Gerstein, B. A. Malynn, I. Larsson, G. Rathbun, L. Davidson, S. Muller, A. B. Kantor, L. A. Herzenberg, et al. 1995. Defective B cell development and function in Btk-deficient mice. *Immunity 3:283*.
12. Kerner, J. D., M. W. Appleby, R. N. Mohr, S. Chien, D. J. Rawlings, C. R. Maliszewski, O. N. Witte, and R. M. Perlmutter. 1995. Impaired expansion of mouse B cell progenitors lacking Btk. *Immunity 3:301*.
13. Leitges, M., C. Schmedt, R. Guinamard, J. Davoust, S. Schaal, S. Stabel, and A. Tarakhovsky. 1996. Immunodeficiency in protein kinase cβ-deficient mice. *Science 273:788*.
14. Engel, P., L. J. Zhou, D. C. Ord, S. Sato, B. Koller, and T. F. Tedder. 1995. Abnormal B lymphocyte development, activation, and differentiation in mice that lack or overexpress the CD19 signal transduction molecule. *Immunity 3:39*.
15. Fruman, D. A., S. B. Snapper, C. M. Yballe, L. Davidson, J. Y. Yu, F. W. Alt, and L. C. Cantley. 1999. Impaired B cell development and proliferation in absence of phosphoinositide 3-kinase p85α. *Science 283:393*.
16. Tarakhovsky, A., M. Turner, S. Schaal, P. J. Mee, L. P. Duddy, K. Rajewsky, and V. L. Tybulewicz. 1995. Defective antigen receptor-mediated proliferation of B and T cells in the absence of Vav. *Nature 374:467*.
17. Ahearn, J. M., M. B. Fischer, D. Croix, S. Goerg, M. Ma, J. Xia, X. Zhou, R. G. Howard, T. L. Rothstein, and M. C. Carroll. 1996. Disruption of the Cr2 locus results in a reduction in B-1a cells and in an impaired B cell response to T-dependent antigen. *Immunity 4:251*.
18. Maecker, H. T., and S. Levy. 1997. Normal lymphocyte development but delayed humoral immune response in CD81-null mice. *J. Exp. Med. 185:1505*.
19. Cyster, J. G., and C. C. Goodnow. 1995. Protein tyrosine phosphatase 1C negatively regulates antigen receptor signaling in B lymphocytes and determines thresholds for negative selection. *Immunity 2:13*.
20. O'Keefe, T. L., G. T. Williams, S. L. Davies, and M. S. Neuberger. 1996. Hyperresponsive B cells in CD22-deficient mice. *Science 274:798*.
21. Pan, C., N. Baumgarth, and J. R. Parnes. 1999. CD72-deficient mice reveal nonredundant roles of CD72 in B cell development and activation. *Immunity 11:495*.
22. Takatsu, K., A. Tominaga, N. Harada, S. Mita, M. Matsumoto, T. Takahashi, Y. Kikuchi, and N. Yamaguchi. 1988. T cell-replacing factor (TRF)/interleukin 5 (IL-5): molecular and functional properties. *Immunol. Rev. 102:107*.
23. Mita, S., Y. Hosoya, I. Kubota, T. Nishihara, T. Honjo, T. Takahashi, and K. Takatsu. 1989. Rapid methods for purification of human recombinant interleukin-5 (IL-5) using the anti-murine IL-5 antibody-coupled immunoaffinity column. *J. Immunol. Methods 125:233*.
24. Mita, S., A. Tominaga, Y. Hitoshi, K. Sakamoto, T. Honjo, M. Akagi, Y. Kikuchi, N. Yamaguchi, and K. Takatsu. 1989. Characterization of high-affinity receptors for interleukin 5 on interleukin 5-dependent cell lines. *Proc. Natl. Acad. Sci. USA 86:2311*.
25. Sonoda, E., R. Matsumoto, Y. Hitoshi, T. Ishii, M. Sugimoto, S. Araki, A. Tominaga, N. Yamaguchi, and K. Takatsu. 1989. Transforming growth factor β induces IgA production and acts additively with interleukin 5 for IgA production. *J. Exp. Med. 170:1415*.
26. Ogata, N., T. Kouro, A. Yamada, M. Koike, N. Hanai, T. Ishikawa, and K. Takatsu. 1998. JAK2 and JAK1 constitutively associate with an interleukin-5 (IL-5) receptor α and βc subunit, respectively, and are activated upon IL-5 stimulation. *Blood 91:2264*.
27. Sato, S., T. Katagiri, S. Takaki, Y. Kikuchi, Y. Hitoshi, S. Yonehara, S. Tsukada, D. Kitamura, T. Watanabe, O. Witte, et al. 1994. IL-5 receptor-mediated tyrosine phosphorylation of SH2/SH3-containing proteins and activation of Bruton's tyrosine and Janus 2 kinases. *J. Exp. Med. 180:2101*.
28. Alam, R., K. Pazdrak, S. Stafford, and P. Forsythe. 1995. The interleukin-5/receptor interaction activates Lyn and Jak2 tyrosine kinases and propagates signals via the Ras-Raf-1-MAP kinase and the Jak-STAT pathways in eosinophils. *Int. Arch. Allergy Immunol. 107:226*.
29. Takaki, S., H. Kanazawa, M. Shiiba, and K. Takatsu. 1994. A critical cytoplasmic domain of the interleukin-5 (IL-5) receptor α chain and its function in IL-5-mediated growth signal transduction. *Mol. Cell. Biol. 14:7404*.
30. Li, T., S. Tsukada, A. Satterthwaite, M. H. Havlik, H. Park, K. Takatsu, and O. N. Witte. 1995. Activation of Bruton's tyrosine kinase (BTK) by a point mutation in its pleckstrin homology (PH) domain. *Immunity 2:451*.
31. Kikuchi, Y., M. Hirano, M. Seto, and K. Takatsu. 2000. Identification and characterization of a molecule, BAM11, that associates with the pleckstrin homology domain of mouse Btk. *Int. Immunol. 12:1397*.
32. Kouro, T., Y. Kikuchi, H. Kanazawa, K. Hirokawa, N. Harada, M. Shiiba, H. Wakao, S. Takaki, and K. Takatsu. 1996. Critical proline residues of the cytoplasmic domain of the IL-5 receptor α chain and its function in IL-5-mediated activation of JAK kinase and STAT5. *Int. Immunol. 8:237*.
33. Tominaga, A., S. Takaki, N. Koyama, S. Katoh, R. Matsumoto, M. Migita, Y. Hitoshi, Y. Hosoya, S. Yamauchi, Y. Kanai, et al. 1991. Transgenic mice expressing a B cell growth and differentiation factor gene (interleukin-5) develop eosinophilia and autoantibody production. *J. Exp. Med. 173:429*.
34. Katoh, S., M. M. Bendig, Y. Kanai, L. D. Shultz, Y. Hitoshi, K. Takatsu, and A. Tominaga. 1993. Maintenance of CD5$^+$ B cells at an early developmental stage by interleukin-5: evidence from immunoglobulin gene usage in interleukin-5 transgenic mice. *DNA Cell Biol. 12:481*.
35. Yoshida, T., K. Ikuta, H. Sugaya, K. Maki, M. Takagi, H. Kanazawa, S. Sunaga, T. Kinashi, K. Yoshimura, J. Miyazaki, S. Takaki, and K. Takatsu. 1996. Defective B-1 cell development and impaired immunity against *Angiostrongylus cantonensis* in IL-5Rα-deficient mice. *Immunity 4:483*.
36. Moon, B. G., T. Yoshida, M. Shiiba, K. Nakao, M. Katsuki, S. Takaki, and K. Takatsu. 2001. Functional dissection of the cytoplasmic subregions of the interleukin-5 receptor α chain in growth and immunoglobulin G_1 switch recombination of B cells. *Immunology 102:289*.
37. Hiroi, T., M. Yanagita, H. Iijima, K. Iwatani, T. Yoshida, K. Takatsu, and H. Kiyono. 1999. Deficiency of IL-5 receptor α-chain selectively influences the development of the common mucosal immune system independent IgA-producing B-1 cell in mucosa-associated tissues. *J. Immunol. 162:821*.
38. Kopf, M., F. Brombacher, P. D. Hodgkin, A. J. Ramsay, E. A. Milbourne, W. J. Dai, K. S. Ovington, C. A. Behm, G. Kohler, I. G. Young, and K. I. Matthaei. 1996. IL-5-deficient mice have a developmental defect in CD5$^+$ B-1 cells and lack eosinophilia but have normal antibody and cytotoxic T cell responses. *Immunity 4:15*.
39. Bao, S., K. W. Beagley, A. M. Murray, V. Caristo, K. I. Matthaei, I. G. Young, and A. J. Husband. 1998. Intestinal IgA plasma cells of the B1 lineage are IL-5 dependent. *Immunology 94:181*.
40. Harada, N., T. Takahashi, M. Matsumoto, T. Kinashi, J. Ohara, Y. Kikuchi, N. Koyama, E. Severinson, Y. Yaoita, T. Honjo, et al. 1987. Production of a monoclonal antibody useful in the molecular characterization of murine T-cell-replacing factor/B-cell growth factor II. *Proc. Natl. Acad. Sci. USA 84:4581*.
41. Hitoshi, Y., N. Yamaguchi, M. Korenaga, S. Mita, A. Tominaga, and K. Takatsu. 1991. In vivo administration of antibody to murine IL-5 receptor inhibits eosinophilia of IL-5 transgenic mice. *Int. Immunol. 3:135*.
42. Hitoshi, Y., N. Yamaguchi, S. Mita, E. Sonoda, S. Takaki, A. Tominaga, and K. Takatsu. 1990. Distribution of IL-5 receptor-positive B cells: expression of IL-5 receptor on Ly-1(CD5)$^+$ B cells. *J. Immunol. 144:4218*.
43. Nagai, Y., S. Akashi, M. Nagafuku, M. Ogata, Y. Iwakura, S. Akira, T. Kitamura, A. Kosugi, M. Kimoto, and K. Miyake. 2002. Essential role of MD-2 in LPS responsiveness and TLR4 distribution. *Nat. Immunol. 3:667*.
44. Miyake, K., Y. Yamashita, Y. Hitoshi, K. Takatsu, and M. Kimoto. 1994. Murine B cell proliferation and protection from apoptosis with an antibody against a 105-kD molecule: unresponsiveness of X-linked immunodeficient B cells. *J. Exp. Med. 180:1217*.
45. Yamamoto, M., K. Fujihashi, K. W. Beagley, J. R. McGhee, and H. Kiyono. 1993. Cytokine synthesis by intestinal intraepithelial lymphocytes: both $\gamma\delta$ T cell receptor-positive and $\alpha\beta$ T cell receptor-positive T cells in the G_1 phase of cell cycle produce IFN-γ and IL-5. *J. Immunol. 150:106*.
46. Xu-Amano, J., H. Kiyono, R. J. Jackson, H. F. Staats, K. Fujihashi, P. D. Burrows, C. O. Elson, S. Pillai, and J. R. McGhee. 1993. Helper T cell subsets for immunoglobulin A responses: oral immunization with tetanus toxoid and cholera toxin as adjuvant selectively induces Th2 cells in mucosa associated tissues. *J. Exp. Med. 178:1309*.
47. Corcoran, L. M., and D. Metcalf. 1999. IL-5 and Rp105 signaling defects in B cells from commonly used 129 mouse substrains. *J. Immunol. 163:5836*.
48. Kushnir, N., N. A. Bos, A. W. Zuercher, S. E. Coffin, C. A. Moser, P. A. Offit, and J. J. Cebra. 2001. B2 but not B1 cells can contribute to CD4$^+$ T-cell-mediated clearance of rotavirus in SCID mice. *J. Virol. 75:5482*.
49. Fort, M. M., J. Cheung, D. Yen, J. Li, S. M. Zurawski, S. Lo, S. Menon, T. Clifford, B. Hunte, R. Lesley, et al. 2001. IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies in vivo. *Immunity 15:985*.
50. Gordon, J. R., P. R. Burd, and S. J. Galli. 1990. Mast cells as a source of multifunctional cytokines. *Immunol. Today 11:458*.
51. Broide, D. H., M. M. Paine, and G. S. Firestein. 1992. Eosinophils express interleukin 5 and granulocyte macrophage-colony-stimulating factor mRNA at sites of allergic inflammation in asthmatics. *J. Clin. Invest. 90:1414*.
52. Walker, C., J. Checkel, S. Cammisuli, P. J. Leibson, and G. J. Gleich. 1998. IL-5 production by NK cells contributes to eosinophil infiltration in a mouse model of allergic inflammation. *J. Immunol. 161:1962*.
53. Erickson, L. D., T. M. Foy, and T. J. Waldschmidt. 2001. Murine B1 B cells require IL-5 for optimal T cell-dependent activation. *J. Immunol. 166:1531*.
54. McGhee, J. R., J. Mestecky, C. O. Elson, and H. Kiyono. 1989. Regulation of IgA synthesis and immune response by T cells and interleukins. *J. Clin. Immunol. 9:175*.
55. Kroese, F. G., E. C. Butcher, A. M. Stall, P. A. Lalor, S. Adams, and L. A. Herzenberg. 1989. Many of the IgA producing plasma cells in murine gut are derived from self-replenishing precursors in the peritoneal cavity. *Int. Immunol. 1:75*.
56. Baeuerle, P. A., and T. Henkel. 1994. Function and activation of NF-κB in the immune system. *Annu. Rev. Immunol. 12:141*.