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

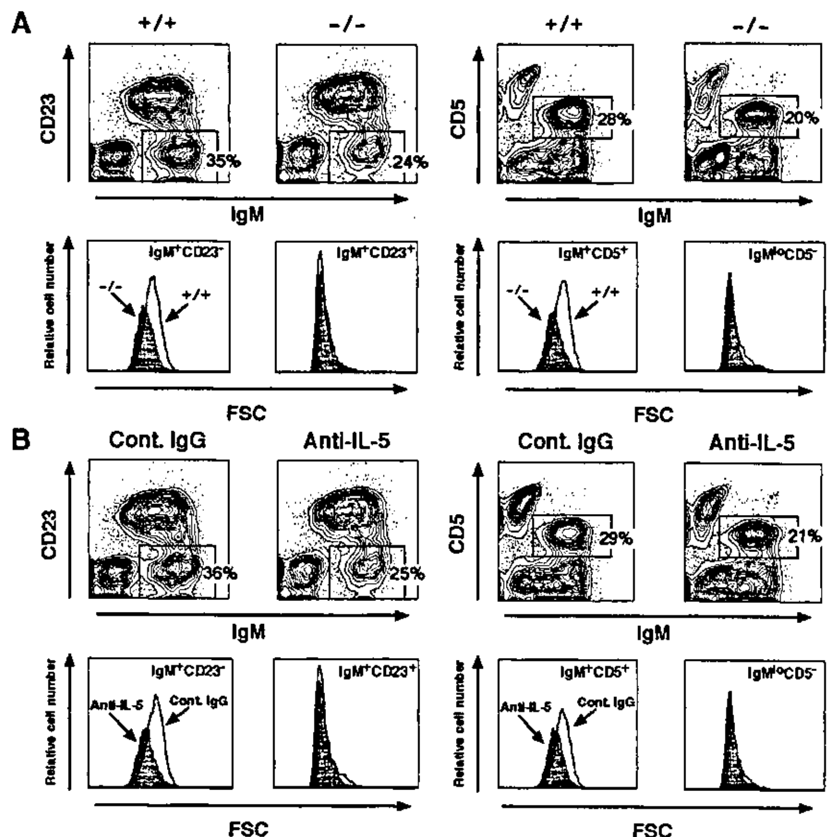

FIGURE 1. Decreased cell number and size of B-1 cells by blocking IL-5 and IL-5R interaction. Representative two-color contour plots show the expression of IgM/CD23 and IgM/CD5 on PECs from 8-wk-old IL-5R$\alpha^{+/+}$ or IL-5R$\alpha^{-/-}$ mice (*A*) and from control IgG- or anti-IL-5-treated IL-5R$\alpha^{+/+}$ 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 (× 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*

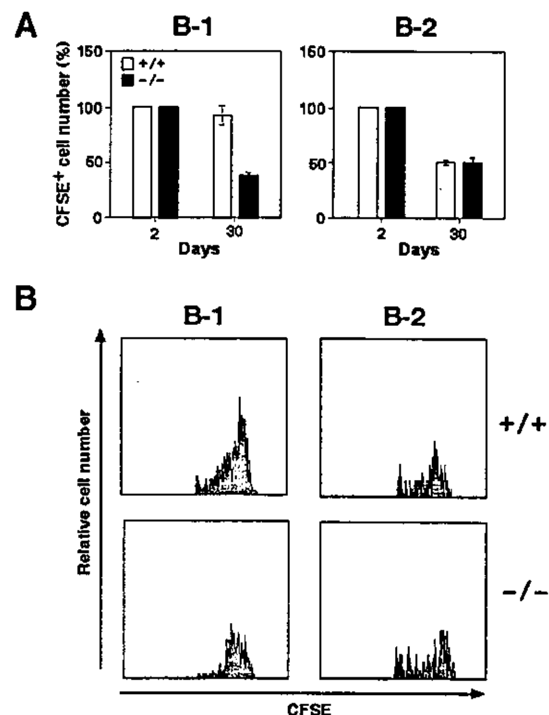

FIGURE 2. Impaired survival of mature IL-5R$\alpha^{-/-}$ B-1 cells. CFSE-labeled PECs from IL-5R$\alpha^{+/+}$ or IL-5R$\alpha^{-/-}$ mice were transferred (1 × 10^6 cells per head) into the peritoneal cavity of IL-5R$\alpha^{+/+}$ mice. On days 2 and 30 after cell transfer, the recipient mice were 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.

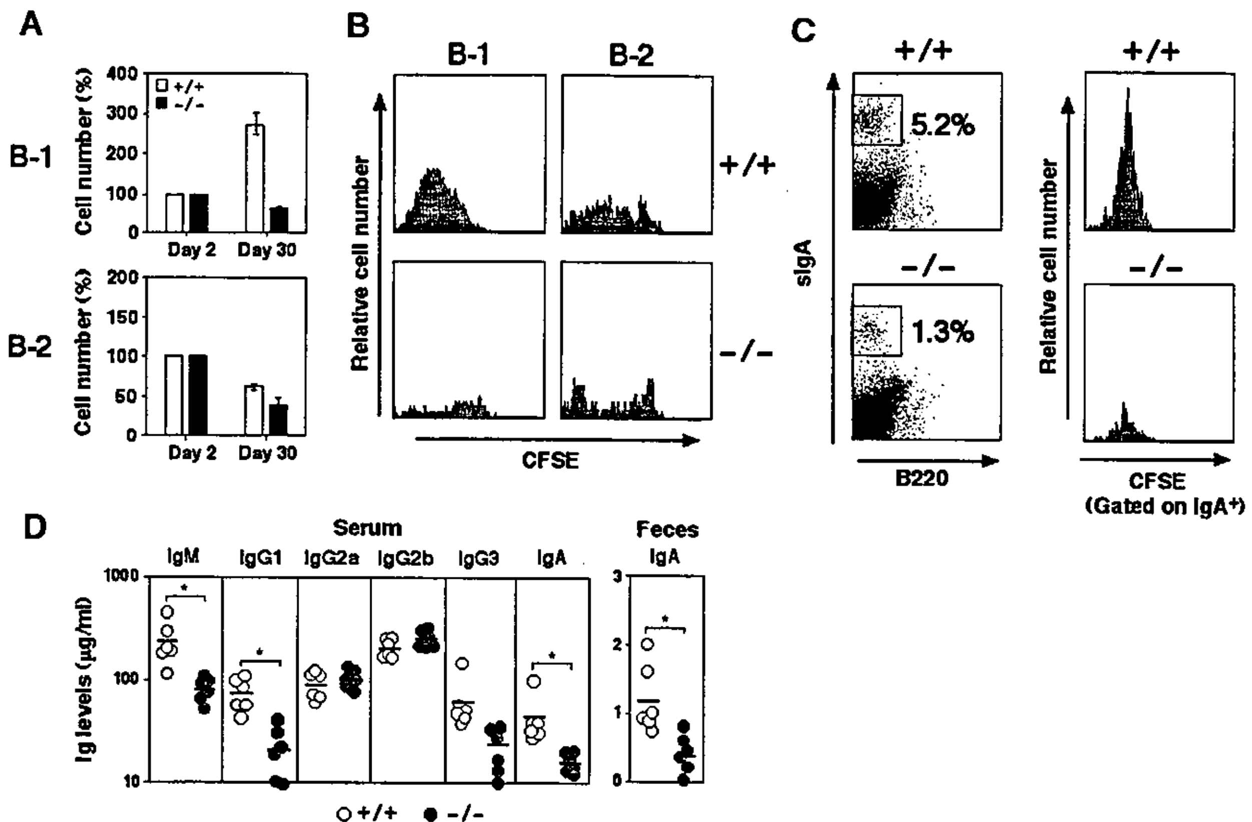

FIGURE 3. Impaired homeostatic B-1 cell proliferation and Ig production from IL-5Rα$^{-/-}$ mice. *A*, IL-5Rα$^{-/-}$ B-1 cells have a defect in homeostatic proliferation. CFSE-labeled peritoneal cells were injected i.p. into RAG-2$^{-/-}$ mice. The recipient mice of each group were analyzed as described in Fig. 2. *B*, IL-5Rα$^{-/-}$ 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-5Rα$^{-/-}$ 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-5Rα$^{-/-}$ 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-5Rα$^{-/-}$ 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-5Rα$^{-/-}$ B-1a cells decreased to ~71% on day 30 and on day 60, whereas IL-5Rα$^{-/-}$ 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-5Rα$^{+/+}$ B-1 cells compared with those of IL-5Rα$^{-/-}$ 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 αβT cells in the peritoneal cavity and intestinal intraepithelial lymphocytes (8, 45), but also freshly isolated γδT cells in the intraepithelial lymphocytes are capable of producing IL-5 (45). We injected anti-IL-5 mAb into TCRβ$^{-/-}$δ$^{-/-}$ mice and examined B-1 cell survival in T cell-deficient conditions. Anti-IL-5-treated TCRβ$^{-/-}$δ$^{-/-}$ 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 TCRβ$^{-/-}$δ$^{-/-}$ 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$^{-/-}$, TCRβ$^{-/-}$δ$^{-/-}$, 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, TCRβ$^{-/-}$δ$^{-/-}$, 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

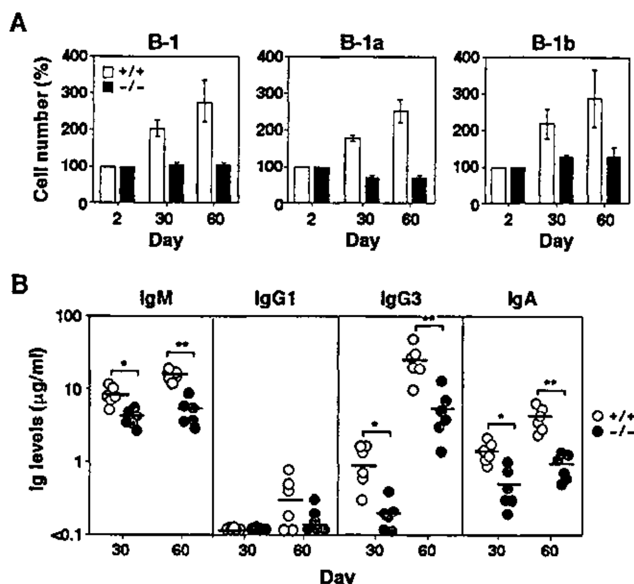

FIGURE 4. T cell-independent homeostatic proliferation of B-1 cells. *A*, B-1 cells from IL-5Rα$^{+/+}$ mice but not from IL-5Rα$^{-/-}$ mice show homeostatic proliferation even in T cell-deficient conditions. Purified B-1 cells were transferred (1 × 10^5 per head) i.p. into RAG-2$^{-/-}$ mice. The proportion 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-5Rα$^{-/-}$ 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-5Rα$^{-/-}$ B-1 cells to anti-CD40 mAb and LPS

B-1 cells that were smaller in size in IL-5Rα$^{-/-}$ 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-5Rα$^{+/+}$ and IL-5Rα$^{-/-}$ mice and stimulated them with anti-CD40, LPS, IL-4, or combinations of these. Interestingly, IL-5Rα$^{-/-}$ B-1 cells showed lower proliferation than did IL-5Rα$^{+/+}$ B-1 cells in response to anti-CD40 (58% of IL-5Rα$^{+/+}$ cells) and LPS (49% of IL-5Rα$^{+/+}$ 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-5Rα$^{-/-}$ 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-5Rα$^{+/+}$ B-1 cells (Fig. 6*B*, *upper panel*). Although IL-5Rα$^{-/-}$ 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-5Rα$^{+/+}$ B-1 cells (Fig. 6*B*, *middle panel*). IgG3 production induced by LPS was also impaired in IL-5Rα$^{-/-}$ B-1 cells (Fig. 6*B*, *lower panel*). In contrast with B-1 cells, IL-5Rα$^{+/+}$ and IL-5Rα$^{-/-}$ 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-5Rα$^{-/-}$ B-1 cells to anti-CD40 may be the impaired expression of CD40. We compared the expression levels of CD40 on IL-5Rα$^{-/-}$ B-1 cells with those on IL-5Rα$^{+/+}$ B-1 cells and found that IL-5Rα$^{-/-}$ B-1 cells showed a significantly lower expression of CD40 than did IL-5Rα$^{+/+}$ B-1 cells (Fig. 7*A*). In contrast with B-1 cells, IL-5Rα$^{-/-}$ B-2 cells showed CD40 expression comparable with that of IL-5Rα$^{+/+}$ 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-5Rα$^{-/-}$ mice

As described previously, IL-5Rα$^{-/-}$ 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-5Rα$^{+/+}$ 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-5Rα$^{+/+}$ B-1 and IL-5Rα$^{-/-}$ 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-5Rα$^{+/+}$ and IL-5Rα$^{-/-}$ mice and examined Ig levels in serum and fecal extracts by ELISA. IgA levels in serum and fecal extracts in LPS-administered IL-5Rα$^{+/+}$ 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-5Rα$^{+/+}$ mice, the IgA levels in serum and fecal extracts in the IL-5Rα$^{-/-}$ 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-5Rα$^{-/-}$ 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-5Rα$^{+/+}$ and IL-5Rα$^{-/-}$ mice. Importantly, the number of IgA-producing cells in the LP and PP was significantly lower in LPS-administered IL-5Rα$^{-/-}$ mice than in LPS-administered IL-5Rα$^{+/+}$ mice. The number of IgM-producing cells in PECs in IL-5Rα$^{-/-}$ mice was also lower than in IL-5Rα$^{+/+}$ mice, whereas their splenocytes showed a number of IgM-producing cells comparable with that in IL-5Rα$^{+/+}$ 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-5Rα$^{-/-}$ 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

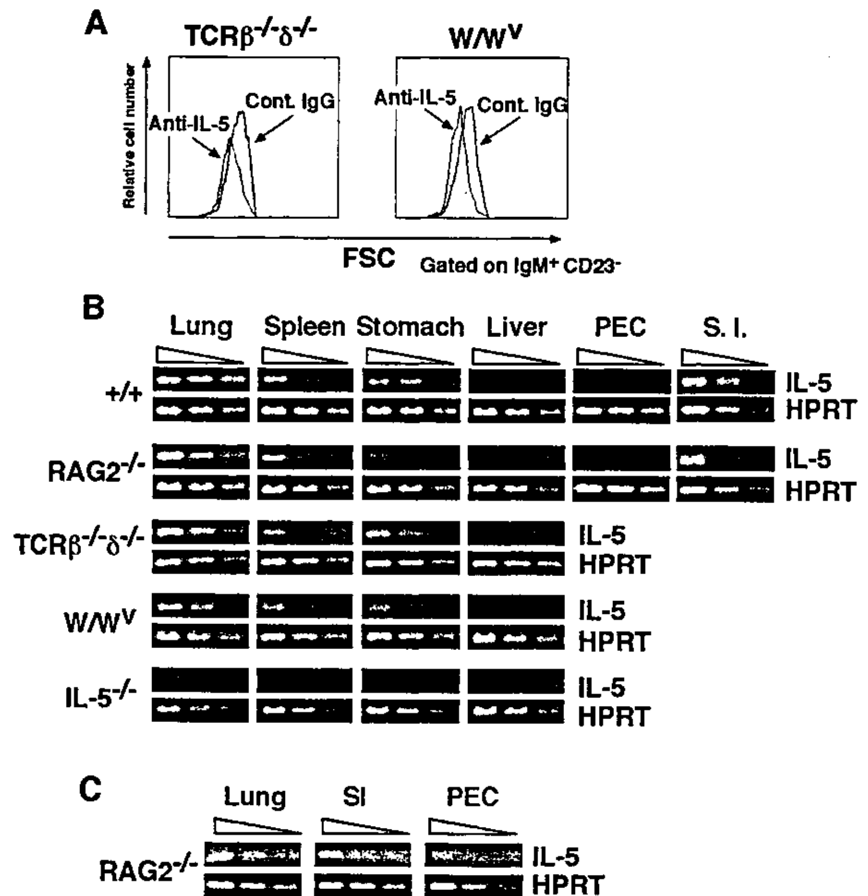

FIGURE 5. IL-5 production by cells other than T cells, mast cells, and eosinophils. *A*, Anti-IL-5 Ab treatment affects B-1 cell maintenance in T cell- or mast cell-deficient mice. A single i.p. administration of anti-IL-5 mAbs or control IgG (1 mg/250 μl) into TCR$\beta^{-/-}\delta^{-/-}$ or W/W^V mice 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

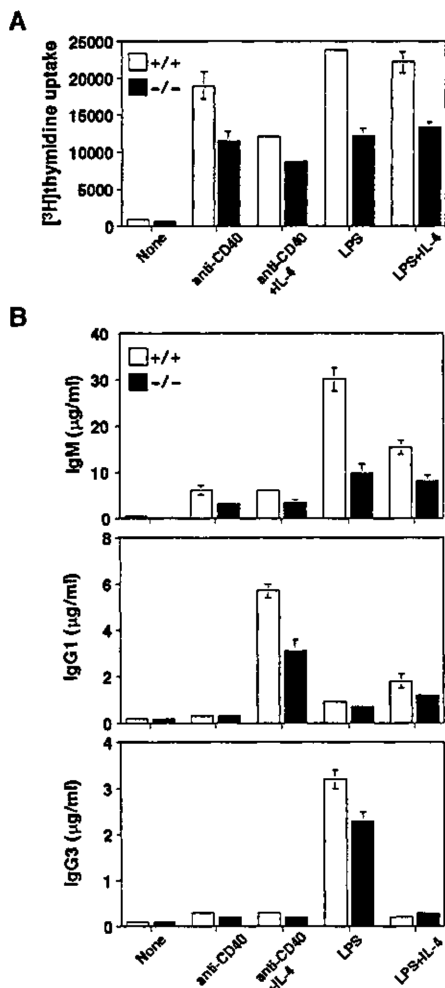

FIGURE 6. Defective activation of IL-5Rα$^{-/-}$ B-1 cells to anti-CD40 mAb or LPS. *A*, IL-5Rα$^{-/-}$ B-1 cells have defective proliferative responses to anti-CD40 mAb or LPS. B-1 cells purified by the MACS system were cultured (1 × 10^5 cells in a 200-μl culture) for 3 days with anti-CD40 mAb (1 μg/ml), 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-5Rα$^{-/-}$ 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*).

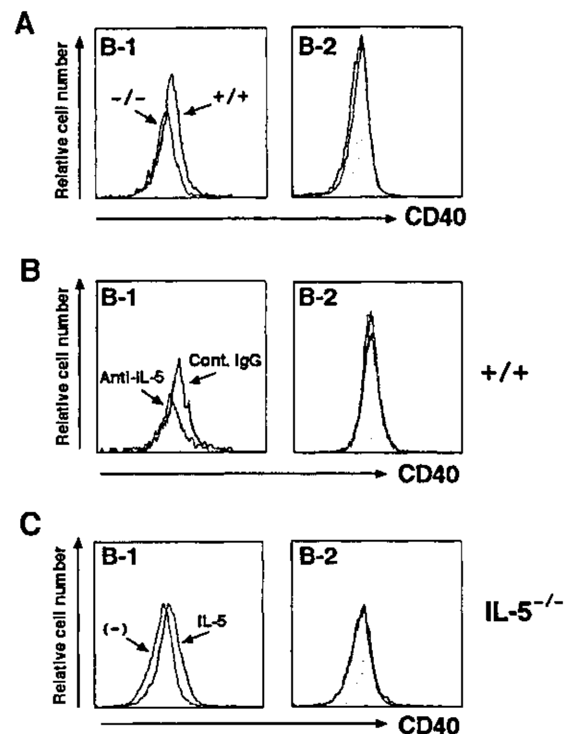

FIGURE 7. IL-5-dependent CD40 expression on B-1 cells. *A*, IL-5Rα$^{-/-}$ B-1 cells express low levels of CD40. *B*, Decreased CD40 expression on B-1 cells from anti-IL-5-treated mice is reduced. *C*, CD40 expression on IL-5$^{-/-}$ B-1 cells is recovered by IL-5 stimulation. Peritoneal cells from IL-5$^{-/-}$ 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 TCRβ$^{-/-}$δ$^{-/-}$ 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

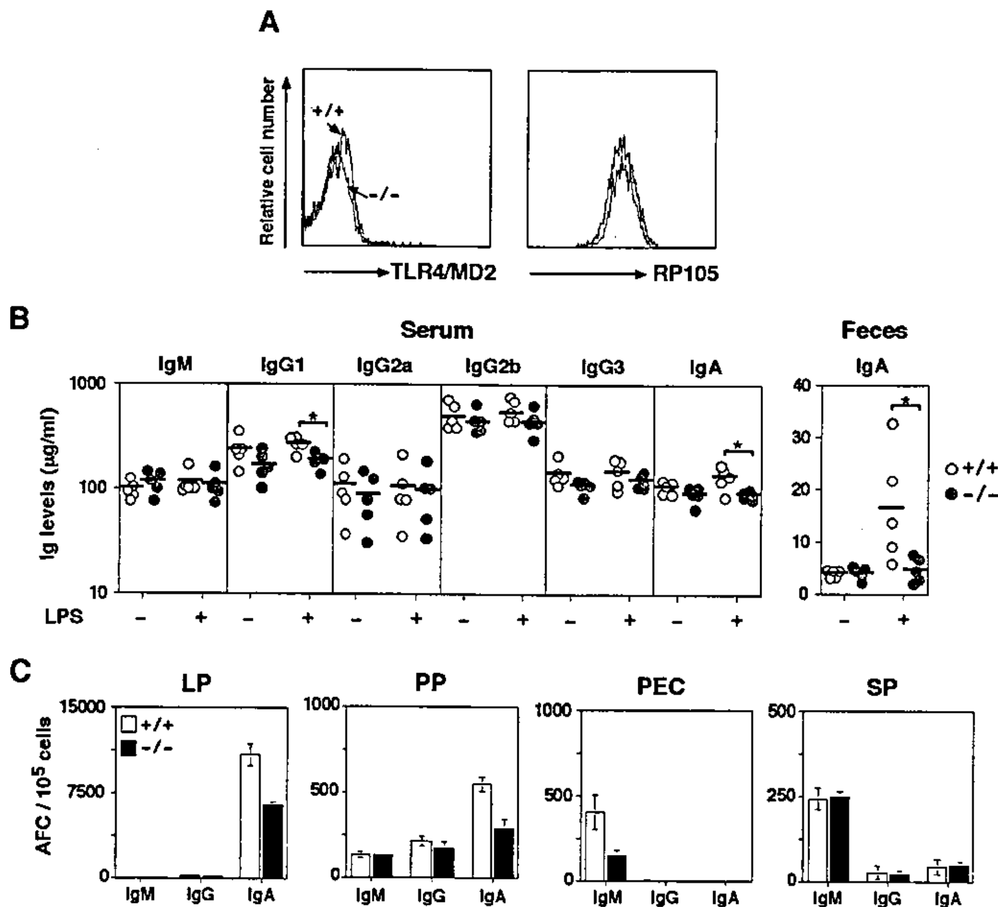

FIGURE 8. Ab production in IL-5R$\alpha^{-/-}$ mice that were administered LPS orally. *A*, IL-5R$\alpha^{-/-}$ B-1 cells express normal levels of TLR4/MD2 and RP105. Representative histograms show TLR/MD2 or RP105 expression on B-1 cells from IL-5R$\alpha^{+/+}$ or IL-5R$\alpha^{-/-}$ mice. *B*, IgA 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 ± 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.

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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.*

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The role of antigenic peptide in CD4+ T helper phenotype development in a T cell receptor transgenic model

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Abstract

CD4+ Th1 cells play a critical role in the induction of cell-mediated immune responses that are important for the eradication of intracellular pathogens. Peptide-25 is the major Th1 epitope for Ag85B of *Mycobacterium tuberculosis* and is immunogenic in I-A^b mice. To elucidate the role of the TCR and IFN-γ/IL-12 signals in Th1 induction, we generated TCR transgenic mice (P25 TCR-Tg) expressing TCR α- and β-chains of Peptide-25-reactive cloned T cells and analyzed Th1 development of CD4+ T cells from P25 TCR-Tg. Naive CD4+ T cells from P25 TCR-Tg differentiate into both Th1 and Th2 cells upon stimulation with anti-CD3. Naive CD4+ T cells from P25 TCR-Tg preferentially develop Th1 cells upon Peptide-25 stimulation in the presence of I-A^b splenic antigen-presenting cells under neutral conditions. In contrast, a mutant of Peptide-25 can induce solely Th2 differentiation. Peptide-25-induced Th1 differentiation is observed even in the presence of anti-IFN-γ and anti-IL-12. Furthermore, naive CD4+ T cells from STAT1 deficient P25 TCR-Tg also differentiate into Th1 cells upon Peptide-25 stimulation. Moreover, Peptide-25-loaded I-A^b-transfected Chinese hamster ovary cells induce Th1 differentiation of naive CD4+ T cells from P25 TCR-Tg in the absence of IFN-γ or IL-12. These results imply that interaction between Peptide-25/I-A^b and TCR may primarily influence determination of the fate of naive CD4+ T cells in their differentiation towards the Th1 subset.

Introduction

Naive CD4+ Th cells recognize an antigenic peptide through their TCR in the context of MHC class II molecules on antigen-presenting cells (APC) and undergo differentiation to effector cells that can produce cytokines and chemokines. During this process, naive CD4+ T cells can differentiate to at least two functionally distinct subsets of cells, represented by Th1 and Th2 (1). Th1 cells produce IFN-γ and lymphotoxin (TNF-β) in addition to IL-2 and are responsible for directing cell-mediated immune responses leading to the eradication of intracellular pathogens such as *Mycobacterium*, viruses and parasites (1–4). Th1 cells also regulate IgG2a and IgG3 antibody production via IFN-γ production, which is involved in the opsonization and phagocytosis of particulate microbes. Th2 cells secrete IL-4, IL-5 and IL-13 as effector cytokines and are responsible for humoral immune responses for the eradication of helminths. Th2 cells also cause inflammatory damage during allergic diseases, such as asthma and atopic dermatitis. The process by which an uncommitted Th cell develops into a mature Th1 or Th2 subset is a matter of fact for regulating the immune response to various antigens.

Considerable progress has been made in identifying the factors that govern the progression of cell differentiation during the generation of Th subsets (2–4). Using T cells stimulated with polyclonal activators or T cells from mice expressing transgenic antigen receptors of known specificities, it has become clear that Th1 and Th2 subsets develop

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from the same T cell precursor (5–7), which is a naive CD4⁺ T cell. There is a body of evidence to indicate that the cytokines IL-12 and IL-4 are key determinants of the Th1 and Th2 response, respectively (4). For example, IL-12 directs Th1 development from antigen-stimulated naive CD4⁺ T cells and activates STAT4 in Th1 cells (8,9). In terminally differentiated Th1 cells, successive IFN-γ production can occur through TCR ligation or IL-12 and IL-18 stimulation. Using mice deficient in either cytokines or STAT, it has been shown that activation of the IFN-γR/STAT1 is also important for the differentiation of CD4⁺ T cells into Th1 cells (10,11). The IL-4R/STAT6 signaling pathway plays a central role in the differentiation of naive CD4⁺ T cells into Th2 cells (12–14). The balance of IFN-γ and IL-4 levels present during T cell activation is considered to be the major influence on Th1 versus Th2 differentiation. Although the strength of the interaction mediated through TCR and MHC/peptide complex is suspected to affect the lineage commitment of Th cells to Th1 cells and clonal expansion (15–17), it remains unclear whether Th1 cells can develop from naive CD4⁺ T cells upon antigenic peptide stimulation in the presence of APC under neutral conditions.

Ag85B (also known as α antigen or MPT59) is the most potent antigen species yet purified for both humans and mice (18). Ag85B can elicit strong Th1 response *in vitro* from PPD⁺ asymptomatic individuals (19–21). We have shown that *in vitro* stimulation of lymph node cells from *Mycobacterium tuberculosis*-primed C57BL/6 mice with Ag85B induces the production of IFN-γ and IL-2 and expansion of CD4⁺ T cells expressing Vβ11 of TCR (TCRVβ11) in an I-A^b-restricted manner (22,23). We identified the 15-mer peptide (Peptide-25), covering amino acids residues 240–254 (FQDAYNAAGGHNAVF) of Ag85B, as the major epitope for Ag85B-specific TCRVβ11⁺ T cells (22). Using Peptide-25-reactive Vβ11⁺ T cell clones (BP1, BP4, BM5, BM7 and BM12) and substituted Peptide-25 mutants, we determined which amino acid residues within Peptide-25 were critical for TCR recognition (23,24). Peptide-25 contains the motif that is conserved for I-A^b binding and requires processing by APC to trigger Ag85B-specific TCRVβ11⁺ T cells (22). Active immunization of C57BL/6 mice with Peptide-25 can induce the differentiation of CD4⁺ TCR Vβ11⁺ Th1 that produce IFN-γ and TNF-α and protect against subsequent infection with live *M. tuberculosis* H37Rv (23).

Here we generate transgenic mice (P25 TCR-Tg) expressing functional TCR that interacts with Peptide-25 in conjunction with I-A^b. We report that naive CD4⁺ T cells in the spleen of P25 TCR-Tg mice respond specifically to Peptide-25 in the presence of APC from I-A^b mice and differentiate to Th1 cells in the absence of IFN-γ or IL-12 under neutral conditions.

Methods

Mice

C57BL/6 mice were purchased from Japan SLC Inc. (Hamamatsu, Japan). STAT1 deficient mice were kindly provided by Dr R. D. Schreiber, Center for Immunology, Washington University School of Medicine. These mice were maintained under specific pathogen-free conditions in our animal facility according to our Institute's guidelines, and used at 8–15 weeks of age.

Cell lines

Five different Peptide-25-reactive CD4⁺ Th1 clones (BP1, BP4, BM5, BM7 and BM12) were established *in vitro* by culturing lymph node cells from C57BL/6 mice immunized with heat-killed *M. tuberculosis* H37Rv as described (23). TG40 is a variant T cell hybridoma cell line lacking the expression of surface TCR-α and -β chains that has been used a recipient cells for TCR (25). PLAT-E is a packaging cell line that produces retroviruses (26). Chinese hamster ovary cells expressing I-A^b (I-A^b-CHO) (27) were kindly provided by Dr Y. Fukui (Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan).

Reagents and antibodies

All peptides including Peptide-25 and its substituted mutants were synthesized by Funakoshi Co. Ltd (Tokyo, Japan). Anti-IFN-γ–FITC (XMG1.2), anti-IL-4–allophycocyaninn (11B11), anti-Vβ11–PE (RR3-15), anti-CD4–FITC or -PE (GK1.5), anti-CD8–PE (53.6.72), anti-CD25–FITC (7D4), anti-CD28–FITC (37.51), anti-CD69–FITC (H1.2F3), anti-CD44–FITC (IM7), anti-CD45RB–PE (16A) and anti-LFA1–FITC (2D7) were purchased from BD Biosciences PharMingen (San Diego, CA). Purified anti-CD3 ε (2C11), anti-IFN-γ (R4-6A2) and anti-IL-12 (C17.8) were purchased from BD Biosciences PharMingen.

Subcloning of TCR

Total cellular RNA was isolated from BP1 by using acid guanidinium–phenol–chloroform method. cDNA was synthesized with random hexamer primers and superscript II cDNA kit (GIBCO BRL, Grand Island, NY). 5′-Rapid amplification of cDNA end (5′-RACE) was performed using the 5′-RACE System Ver.2.0 (Life Technologies, Rockville, MD) according to the manufacturer's instructions. The first strand of cDNA was synthesized with gene-specific primer 1 (5′-ATCCATAGCTTTCATGTCCA for TCR α-chain and 5′-GCCATTCACCCACCAGCTCA for TCR β-chain). The first PCR amplification was carried out by using gene-specific primer 2 (5′-GCGAATTCTGAGACCGAGGATCTTTTAACTGGTAC for TCR α-chain and 5′-GCGTCGACTCTGCTTTTGATGGCTCAAAC for TCR β-chain). The second PCR amplification was carried out with nested gene-specific primer (5′-GCGTCGACACAGCAGGTTCTGGGTTCTGGAT for TCR α-chain and 5′-GCGTCGACAAGGAGACCTTGGGTGGAGTCAC for TCR β-chain). The PCR fragment was subcloned in Bluescript SK⁺ and sequences by automatic DNA sequencer (ABI PRISM 3700 DNA analyzer, Applied Biosystems, Foster City, CA).

Retrovirus-mediated gene transfer

Full length cDNAs genes encoding the TCR α- and β-chains of BP1 were inserted into a retroviral vector pMX-IRES-GFP vector, pMX-BP1-α and pMX-BP1-β, respectively, and were transfected into a retroviral packaging cell line, PLAT-E with LipofectAMINE Plus Reagent (GIBCO BRL) (28) The cultured supernatant of PLAT-E after 24 h culture was collected, and added to TG40 cells together with DOTAP Liposomal Transfection Reagent (Roche Diagnostics GmbH, Mannheim, Germany) (29). Transfection was monitored by the cell surface expression of TCR by FACS analysis. TG40 cells were transfected with each of plasmids or in their combinations

and selected TG40 cells expressing TCR-αβ (TG40-BP1). TG40-BP1 cell line for expression of CD4 (TG40-BP1/CD4) was established by electroporation of the expressible constructs of full length CD4 cDNA into TG40-BP1 cells by Gene Pulser (Bio-Rad laboratories, Hercules, CA).

Establishment of transgenic mice

The transgenic TCR-α and -β genes were isolated from BP-1 as described in the previous session. The DNA sequences of the PCR products revealed that BP1-TCR-α was composed of Vα5, Jα15 and Cα1, and the TCR-β chain of Vβ11, Jβ2.3 and Cβ2. The pHSE3′ plasmid contains the H-2K^b promoter (provided by H. Pircher), a poly(A) signal from β-globin and the immunoglobulin heavy chain enhancer (30). The full-length BP1 TCR α and β cDNAs were subcloned into the *SalI* and *BamHI* sites of the expression vector pHSE3′ plasmid under control of the H-2K^b promoter. The constructs were excised from these plasmids by *XhoI* cleavage for TCR-α chain and *ApaLI* cleavage for TCR-β, and purified by using QIAEX II gel extraction system (Qiagen Inc., Valencia, CA). The purified expression constructs for TCR α and β cDNAs were co-injected into fertilized eggs of C57BL/6 mice. We finally obtained a TCR-Tg line of mice expressing TCR-Vα5-Vβ11 (P25 TCR-Tg). P25 TCR-Tg mice were bred to STAT1 deficient mice (STAT1 deficient P25 TCR-Tg) on a C57BL/6 background in our animal faculty under specific pathogen-free conditions.

Preparation of naive CD4$^+$ T cells and APC

Splenic T cells from either P25 TCR-Tg or littermate C57BL/6 mice were enriched by passing splenocytes through a nylon wool column. To further purify primary CD4$^+$ T cells, the splenic T cells were incubated with a mixture of Microbead-bound monoclonal antibodies that were specific for CD8 (53-6.72), CD49b (DX5), B220 (RA3-6B2) and I-A^b (M5/114.15.2) (Miltenyi Biotec, Bergisch Gladbach, Germany). MEL-14^{high} T cells were purified from splenic CD4$^+$ T cells by positive sorting using MACS after treatment with anti-CD62L (MEL-14)-Microbeads (Miltenyi Biotec) and were used as naive CD4$^+$ T cells. The purity of CD4$^+$ naive T cells was >98%. Splenocytes from wild-type (WT) C57BL/6 mice were incubated with a mixture of anti-Thy1 (30-H12)-Microbeads and anti-CD49b-Microbeads (Miltenyi Biotec) to deplete T cells and NK cells. Cells were then recovered by passage through a MACS column according to the manufacturer's instructions. Recovered cells were irradiated with a total of 3500 Rad, and used as APC. I-A^b-CHO was incubated with 10 μg/ml Peptide-25 for 12 h and extensively washed and incubated with 50 μg/ml mitomycin C for 15 min in 37°C and used as APC in some experiments.

Cell culture

Stably transfected TG40-BP1 or TG40-BP1/CD4 cells (1 × 10^4/culture) were stimulated with various concentrations of peptide in the presence of irradiated spleen cells (5 × 10^5/culture) from various strains of mouse in 96-well flat-bottom microtiter plates (Nunc, Roskilde, Denmark). The cultured supernatants were collected and subjected to ELISA.

To examine Th differentiation *in vitro*, two-step cultures were employed. For the first culture, purified splenic naive CD4$^+$ T cells (5 × 10^5/culture) were activated for 6 days with 10 μg/ml of anti-CD3 or 10 μg/ml Peptide-25 or its substituted mutant in the presence of T- and NK cell-depleted C57BL/6 splenic APC (2.5 × 10^6/culture) in a 48-well plate. In some experiments, we used Peptide-25 loaded I-A^b-CHO (2.5 × 10^5/culture) as APC. For the second culture, the cells collected from the first culture were extensively washed and dead cells were removed by centrifugation through Ficol–Hypaque gradients. The viable primed CD4$^+$ T cells were re-stimulated with 10 μg/ml of anti-CD3 or 10 μg/ml of Peptide-25 in the presence of splenic APC or 1 μg/well of immobilized anti-CD3.

Intracellular cytokine staining and FACS analysis

We identified cytokine-producing cells by cytoplasmic staining with anti-cytokine antibody as previously described (24). First, 2 μM of Monensin (BD Biosciences PharMingen) was added to the secondary culture for the last 4 h of each stimulation. The cells were harvested at 24 h of the secondary culture and stained with 7-amino-actinomycin D and with anti-Vβ11–PE or anti-CD4–PE. The cells were fixed with 4% formaldehyde after washing with 0.05% azide–1% FCS–PBS, permeabilized with 0.1% saponin, and stained with both anti-IFN-γ–FITC and anti-IL-4–allophycocyanin. Isotype-matched control antibodies were also used. The cells stained were gated on live Vβ11- or CD4-positive cells and analyzed on a FACSCalibur instrument (Becton Dickinson, Mountain View, CA).

ELISA

Amounts of IL-2, IL-4 and IFN-γ in the culture supernatant were measured by ELISA. All monoclonal antibodies specific for mouse IL-2, IL-4 and IFN-γ used for capture and detection of cytokines were purchased from BD Biosciences PharMingen. ELISA was performed following the instruction of BD Biosciences PharMingen.

ELISPOT assay

Cytokine producing cells were identified by ELISPOT assay, using the IFN-γ and IL-4 ELISPOT assay kits (R&D Systems, Minneapolis, MN). After naive CD4$^+$ T cells from P25 TCR-Tg mice were cultured with Peptide-25-loaded I-A^b-CHO for 20 h in a 96-well plate coated with capture antibodies, ELISPOT assay was performed following the manufacturer's instructions. Spots were analyzed by KS ELISPOT compact (Carl Zeiss, Oberkochen, Germany).

Results

Analysis of Peptide-25 recognition by reconstituted TCR-αβ pairs

To investigate the functional TCR able to bind a Peptide-25/MHC complex at the clonal level, we first determined the usage of TCR-α and -β chains of Peptide-25-reactive Vβ11$^+$ Th1 clone (BP1) that was of C57BL/6 (I-A^b) mouse origin (23) with the use of 5′-RACE. BP1-TCR α-chain was found to be composed of Vα5 and Jα15 and Cα (Accession No.: AB183189). BP1-TCR β-chain was also identified to be Vβ11, Jβ2.3 and Cβ2 (Accession No.: AB183190).

In order to analyze Peptide-25-recognition by TCR dimers composed of the TCR α- and β-chains of BP1, TCR α- and

β-chain were subcloned into a retrovirus vector and then transfected by retrovirus-mediated gene transfer into a TCR-αβ- and CD4-deficient recipient T cell hybridoma cell line, TG40 (28), and the reconstruction and functional specificity of the TCR was assessed by measuring IL-2 production (Fig. 1). TG40-BP1 produced substantial amounts of IL-2 in response to Peptide-25 plus APC in a dose dependent manner. Enforced expression of CD4 molecules on TG40-BP1 (TG40-BP1/CD4) augmented IL-2 production even upon a lower dose of Peptide-25 stimulation (0.3 μg/ml). TG40-BP1/CD4 produced much more IL-2 than TG40-BP1 when stimulated with higher concentrations of Peptide-25 (10 μg/ml) (Fig. 1A). TG40 transfectants of TCR-α alone or TCR-β alone did not respond to Peptide-25 in the presence of splenic APC (data not shown). These results indicate that recombinant TCR α- and β-chains can reconstruct functional TCR and recognize Peptide-25/I-A^b complex to become IL-2-producing cells.

The specificity of BP1 TCR for Peptide-25 and splenic APC from C57BL/6 mice was examined by culturing TG40-BP1/CD4 with various I-A^b-binding peptides in the presence of APC from different strains of mice. Although we do not show data here, among the various peptides only Peptide-25 could induce IL-2 production by TG40-BP1/CD4 in the presence of splenic APC from C57BL/6 (I-A^b) mice. The 11-mer from Peptide-25 was stimulatory while the 8-mer from Peptide-25 was ineffective. We then stimulated TG40-BP1/CD4 cells with a mutant of Peptide-25 as an altered peptide ligand (APL). The APL preserves those amino acid residues within Peptide-25 essential for I-A^b binding, while one of TCR-binding amino acid residues, glutamic acid at position 248 of Peptide-25, was substituted to alanine, G248A. The APL stimulation at 10 μg/ml of TG40-BP1/CD4 induced marginal IL-2 production, and the stimulatory activity was much lower than with Peptide-25 (Fig. 1B).

We then determined the amino acid sequences for the TCR-α and -β chains of four other Peptide-25-reactive Th1 clones (BP4, BM5, BM7 and BM12). All these Th1 clones responded to Peptide-25 for proliferation and IFN-γ production (23). Analysis of the TCR-α and -β chain amino acid sequences for

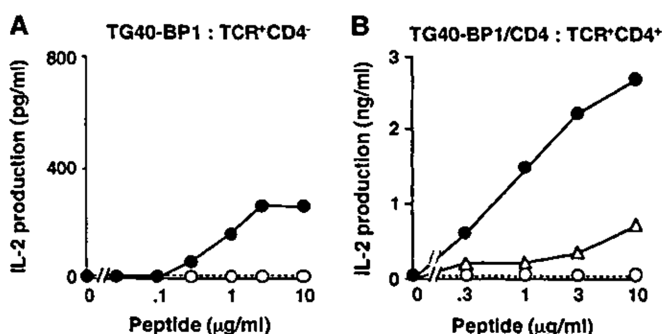

Fig. 1. IL-2 production of TG40 transfectants upon stimulation with Peptide-25. TG40 transfectants retrovirally introduced TCR-αβ of BP1, TG40-BP1 (TCR^+ $CD4^-$) and TG40-BP1 transfectants of CD4, TG40-BP1/CD4 (TCR^+ $CD4^+$) (1 × 10^4 cells/culture) were stimulated with various concentrations of Peptide-25 in the presence (closed circles) or absence (open circles) of irradiated C57BL/6 spleen cells (5 × 10^5 cells/culture) as APC in 96-well microplates. We also stimulated TG40-BP1/CD4 with APL (triangles) in the presence of C57BL/6 spleen cells (5 × 10^5 cells/culture) as APC. After incubation for 24 h, IL-2 in the cultured supernatants were titrated by ELISA.

each clone revealed no obvious differences from BP1 except in the sequence and in the length of CDR3 regions of TCR α- and β-chain (Supplementary table 1, available at *International Immunology* Online). Taking all these results together, the TCR-α5 and -β11 can reconstitute a functional TCR complex that is able to recognize and respond to Peptide-25 when presented in the context of I-A^b. As BP1 is the best Peptide-25-reactive Th1 clone with respect to IFN-γ production in response to Peptide-25, we chose BP1 TCR cDNAs for generating P25 TCR-Tg mice.

Generation of Peptide-25-reactive TCR-Tg mice

We then analyzed the clonal basis of preferential Th1 development by single TCR-Tg mice line expressing TCR-α5 and -β11. We constructed transgenes for TCR α5- and β11-chains under the control of the H-$2K^b$ promoter, the poly(A) signal from human β-globin gene and the immunoglobulin heavy chain enhancer. The transgenes were excised from the vector sequences and co-microinjected into fertilized eggs from C57BL/6 mice. Transgenic mice were screened by Southern blot analysis of tail DNA and by staining peripheral blood T cells with anti-Vβ11, followed by FACS analysis. We obtained founder mice expressing $V\alpha5^+$-$V\beta11^-$, $V\alpha5^-$-$V\beta11^+$ and $V\alpha5^+$-$V\beta11^+$ T cells. In the present study, we have mainly analyzed TCR transgenic (P25 TCR-Tg) mice expressing both TCR-Vα5 and -Vβ11.

FACS analysis revealed that >85% of splenic $CD4^+$ T cells from the P25 TCR-Tg mice expressed TCR β11-chain, while 5–7% of splenic $CD4^+$ T cells were $V\beta11^+$ in WT mice (22). Over 98% of splenic $CD4^+$ T cells from the RAG-2 deficient P25 TCR-Tg mice expressed TCR Vβ11-chain. Similar results were obtained by staining splenic $CD4^+$ T cells from P25 TCR-Tg mice with anti-idiotypic antibody (KN7) for the recombinant TCR αβ (A.K. and K.T., unpublished observation). We did not observe any significant $KN7^+$ lymph node cells from transgenic mice expressing TCR α-chain alone or β-chain alone. We compared the expression patterns of LFA-1, CD25, CD28, CD44, CD45RB and CD69 on splenic $CD4^+$ T cells from P25 TCR-Tg mice with those from WT mice. There were no significant differences in the expression pattern or mean fluorescence intensity of these cell surface molecules between the two groups. RT–PCR analysis revealed that T-bet and IFN-γ mRNA expressions were not detected in freshly prepared splenic $CD4^+$ cells of P25 TCR-Tg mice. Taking these results together, $CD4^+$ T cells from P25 TCR-Tg mice are not pre-activated *in vivo*.

Naive $CD4^+$ T cells from P25 TCR-Tg mice are able to differentiate into both Th1 and Th2

Naive $CD4^+$ T cells from P25 TCR-Tg and WT mice were purified from the spleen and stimulated *in vitro* with anti-CD3 in the presence of exhaustively T- and NK cell-depleted irradiated C57BL/6 splenocytes as APC. After 6 days in culture, the proliferated cells were harvested and re-stimulated for another day with anti-CD3 in the presence of APC. After culturing, IFN-γ- and IL-4-producing cells were analyzed by intracellular staining. The cultured supernatants were subjected to ELISA assay for cytokine titration. The results revealed that *in vitro* stimulation of naive $CD4^+$ T cells from

P25 TCR-Tg mice with anti-CD3 induced the propagation of both IFN-γ- and IL-4-producing cells to a similar extent as from WT mice (Fig. 2A). The IFN-γ and IL-4 production were confirmed by ELISA (Fig. 2B). It is also evident from Fig. 2 that P25 TCR-Tg T cells has a higher proportion of IFN-γ-producing cells and IFN-γ production upon anti-CD3 stimulation compared with T cells from WT mice. These results indicate that naive CD4$^+$ T cells from P25 TCR-Tg mice can differentiate into both Th1 and Th2 upon TCR cross-linking.

Induction of naive CD4$^+$ T cells from P25 TCR-Tg mice to Th1 differentiation upon Peptide-25 stimulation

To examine the differentiation of naive CD4$^+$ T cells from P25 TCR-Tg mice upon *in vitro* Peptide-25 stimulation, naive CD4$^+$ splenic T cells were purified from P25 TCR-Tg mice and stimulated *in vitro* for 6 days with Peptide-25 in the presence of T and NK cell-depleted irradiated C57BL/6 splenocytes as APC. The activated cells produced IL-2 and proliferated upon Peptide-25 stimulation in a dose dependent manner in the presence of APC, but they did not produce IL-2 in the absence of Peptide-25 or in the presence of APC from strains of mice other than C57BL/6 mice (data not shown).

In another set of cultures, we stimulated naive CD4$^+$ T cells from P25 TCR-Tg mice *in vitro* with Peptide-25. After 6 days in culture, the proliferated cells were re-stimulated for another day with immobilized anti-CD3. After culturing, IFN-γ- and IL-4-producing cells were analyzed by cytoplasmic staining, followed by FACS analysis. The cultured supernatants were subjected to ELISA for titration of cytokine levels. As a control, we also cultured the cells with APL or medium alone. Naive CD4$^+$ T cells stimulated with Peptide-25 in the presence of splenic APC became solely IFN-γ-producing cells under neutral conditions (Fig. 3A). IFN-γ production was detected on the first day of culture and increased for the rest of the culture period at day 5 (data not shown). IL-4 secretion was not detected even after 5 days of culture. Importantly, stimulation of the cells with APL, in place of Peptide-25, solely induced IL-4-producing cells (Fig. 3B). When we cultured naive CD4$^+$ T cells and splenic APC in the absence of Peptide-25 or APL in the primary culture, cells did not proliferate well (data not shown). These results indicate that naive CD4$^+$ T cells from P25 TCR-Tg mice can be activated leading to proliferation and differentiate solely into Th1 cells upon stimulation with Peptide-25 under neutral conditions.

Roles of IFN-γ/STAT1 and IL-12 signaling in the Th1 differentiation of naive CD4$^+$ T cells from P25 TCR-Tg mice

It is well known that in addition to the TCR signals IFN-γ and IL-12 play an important role in the Th1 development. To examine whether IFN-γ and IL-12 are required for Th1 development, we

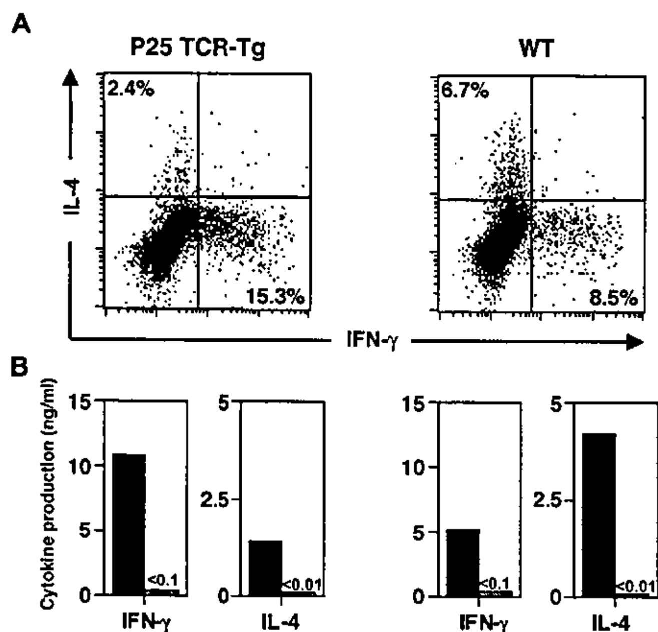

Fig. 2. Induction of Th1 and Th2 differentiation of naive CD4$^+$ T cells from P25 TCR-Tg mice upon stimulation with anti-CD3. Naive CD4$^+$ T cells from P25 TCR-Tg and WT mice were purified and cultured with 10 μg of anti-CD3 in the presence of T- and NK-cell depleted C57BL/6 splenic APC for 6 days. (A) After the culture, the cells were washed extensively and re-stimulated with 10 μg/ml of anti-CD3 in the presence of T- and NK-cell depleted C57BL/6 splenic APC for another day. IFN-γ- and IL-4-producing cells were assessed by intracellular staining and FACS analysis. The percentages of IL-4- and IFN-γ-producing cells are presented in the upper left and the lower right regions, respectively. (B) After the culture, the cells were washed extensively and re-stimulated with (black bar) or without (hatched bar) 10 μg/ml of anti-CD3 in the presence of T- and NK-cell depleted C57BL/6 splenic APC for another day. IFN-γ and IL-4 in the cultured supernatants were titrated by ELISA.

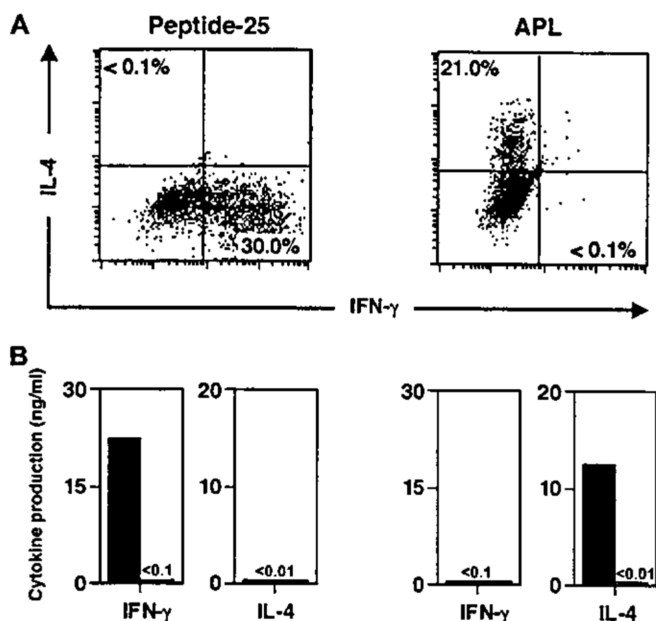

Fig. 3. Induction of Th1 and Th2 differentiation of naive CD4$^+$ T cells from P25 TCR-Tg mice upon stimulation with Peptide-25 and APL, respectively. Naive CD4$^+$ T cells from P25 TCR-Tg mice were stimulated with 10 μg/ml of Peptide-25 or APL for 6 days. (A) On day 6, the cells were washed and re-stimulated with 1 μg/well of immobilized anti-CD3 for another day. IFN-γ- and IL-4-producing cells were assessed by intracellular staining and FACS analysis. The percentages of IL-4- and IFN-γ-producing cells are presented in the upper left and the lower right regions, respectively. (B) On day 6, the cells were washed and re-stimulated with (black bar) or without (hatched bar) 1 μg/well of immobilized anti-CD3 for another day. IFN-γ and IL-4 in the cultured supernatants were titrated by ELISA.

cultured naive CD4$^+$ T cells from P25 TCR-Tg mice with Peptide-25 and splenic APC in the presence of anti-IFN-γ, anti-IL-12 or anti-IFN-γ and anti-IL-12 for 6 days. Results revealed that IFN-γ-producing cells were predominantly observed even when cultured in the presence of anti-IFN-γ and anti-IL-12 (Fig. 4). It was also evident that addition of anti-IL-12 partially reduced the proportion of IFN-γ-producing cells without enhancing IL-4-producing cells, while the addition of anti-IFN-γ treatment slightly increased the frequencies of both IFN-γ- and IL-4-producing cells. These results imply that IFN-γ and IL-12 are not essential for Th1 development of CD4$^+$ T cells from P25 TCR-Tg mice in response to Peptide-25. To evaluate further the role of IFN-γ in the Th1 development, we examined the differentiation fate of naive CD4$^+$ T cells from STAT1 deficient P25 TCR-Tg mice upon Peptide-25 stimulation. This result revealed that Peptide-25-stimulated naive CD4$^+$ T cells from STAT1 deficient P25 TCR-Tg mice became solely IFN-γ-producing cells after 6 days of culture under neutral conditions (Fig. 5).

Induction of IFN-γ-producing cells upon stimulation of naive CD4$^+$ T cells from P25 TCR-Tg mice with Peptide-25-loaded I-A^b-CHO

To elucidate the mechanism that ensures Th1 differentiation upon TCR stimulation with peptide/MHC, naive CD4$^+$ T cells were stimulated *in vitro* with Peptide-25-loaded I-A^b-CHO for 20 h and assayed for IFN-γ and IL-4 production by ELISPOT assay. IFN-γ-producing cells were induced upon treatment with Peptide-25-loaded I-A^b-CHO stimulation in a dose-dependent manner; however, IL-4-producing spots were not detected. Neither IFN-γ nor IL-4 spots were detected when naive CD4$^+$ T cells from P25 TCR-Tg mice were cultured *in vitro* without Peptide-25-loaded I-A^b-CHO for 20 h. These results indicate that activated CD4$^+$ T cells stimulated with Peptide-25/I-A^b produced IFN-γ in primary culture within 24 h.

To evaluate the role of IFN-γ and IL-12 in Th1 development, naive CD4$^+$ T cells from P25 TCR-Tg mice were stimulated for 6 days *in vitro* with Peptide-25-loaded I-A^b-CHO in the presence of anti-IFN-γ and anti-IL-12. At 24 h after the re-stimulation with immobilized anti-CD3, the frequency of IFN-γ producing cells was 14.5% for the live CD4$^+$ T cells (13% for the live TCRVβ11$^+$ T cells) (Fig. 6), indicating that naive CD4$^+$ T cells can differentiate into Th1 by TCR activation with Peptide-25/I-A^b stimulation even in the absence of IFN-γ and IL-12. In separate experiments, we confirmed IFN-γ-producing cells when CD4$^+$ naive T cells from RAG-2$^{-/-}$ P25 TCR-Tg mice were cultured with Peptide-25-loaded I-A^b-CHO even in the presence of anti-IFN-γ or anti-IL-12. Therefore, direct interaction between Peptide-25/I-A^b and TCR may determine the fate of naive CD4$^+$ T cells for differentiating into Th1 subsets.

Discussion

Peptide-25 is the major antigenic epitope for Ag85B of *M. tuberculosis*, is immunogenic in C57BL/6 (I-A^b) mice, and preferentially induces Vβ11$^+$ Th1 cells. It remains unclear why Peptide-25 can preferentially induce Th1 immune responses in C57BL/6 mice. We approached this question by analyzing naive CD4$^+$ T cells from transgenic mice, whose T cells express functional TCR capable of recognizing Peptide-25 in the context with I-A^b molecules. In the present study we generated TCR-Tg mice for the Th1-inducing peptide, Peptide-25, to elucidate the role of TCR signals in the decision of CD4$^+$ T cells to development into either a Th1 or Th2 cell. Our data support the notion that TCR signals may play a role in the determination of Th1 development under neutral conditions in the absence of IFN-γ or IL-12.

We determined usage of TCR α-chain in five different Peptide-25-reactive Vβ11$^+$ Th1 clones. All Peptide-25-reactive Vβ11$^+$ Th1 clones expressed Vα5, while each clone showed slightly different amino acid sequences in CDR3 regions of both Vα5 and Vβ11 chains (Supplementary table 1). Although each Th1 clone responds to Peptide-25 to a similar extent with regard to proliferation and IFN-γ production, it responds differently to a mutant of Peptide-25 where an amino acid required for TCR-binding had been substituted to alanine (data not shown). However, this may be due to the heterogeneity of the CDR3 regions of both Vα5 and Vβ11 chain. TG40 transfectants (TG40-BP1) expressing α and β chains from the BP1 clone constructed functional TCRs that recognize Peptide-25 in the context of I-A^b on APC resulting in IL-2 production even in the absence of CD4 expression (Fig. 1A). Enforced expression of CD4 in TG40-BP1 enhanced IL-2 production along with a low dose of Peptide-25 stimulation (Fig. 1B), suggesting that the avidity of the TCR and Peptide-25/I-A^b complex is potent enough to trigger TG40-BP1

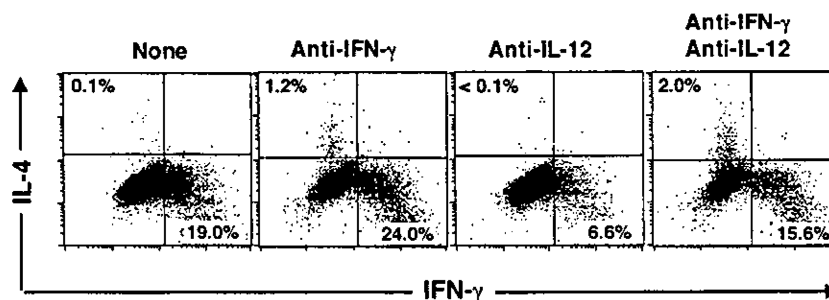

Fig. 4. Effect of anti-IFN-γ and anti-IL-12 on Th1 differentiation of naive CD4$^+$ T cells from P25 TCR-Tg mice upon stimulation with Peptide-25. Naive CD4$^+$ T cells in the spleen of P25 TCR-Tg mice were stimulated with 10 μg/ml of Peptide-25 for 6 days. Anti-IFN-γ (10 μg/ml), anti-IL-12 (10 μg/ml) or anti-IFN-γ (10 μg/ml) plus anti-IL-12 (10 μg/ml) were added at the onset of culture. On day 6, the cells were washed and re-stimulated with 1 μg/well of immobilized anti-CD3 for another day in the absence of antibodies. IFN-γ- and IL-4-producing cells were assessed by intracellular staining and FACS analysis. The percentages of IL-4- and IFN-γ-producing cells are presented in the upper left and the lower right regions, respectively.

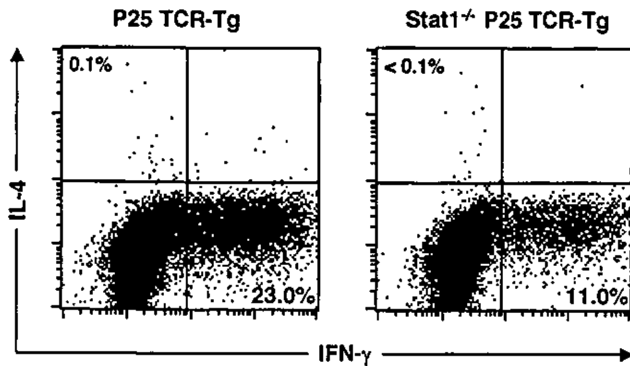

Fig. 5. Induction of Th1 differentiation of naive CD4+ T cells from STAT1 deficient P25 TCR-Tg mice upon stimulation with Peptide-25. Naive CD4+ T cells in the spleen from STAT1 deficient P25 TCR-Tg mice were stimulated with 10 μg/ml of Peptide-25 for 6 days. On day 6, the cells were washed and re-stimulated with 1 μg/well of immobilized anti-CD3 for 24 h. IFN-γ- and IL-4-producing cells were assessed by intracellular staining and FACS analysis. The percentages of IL-4- and IFN-γ-producing cells are presented in the upper left and the lower right regions, respectively.

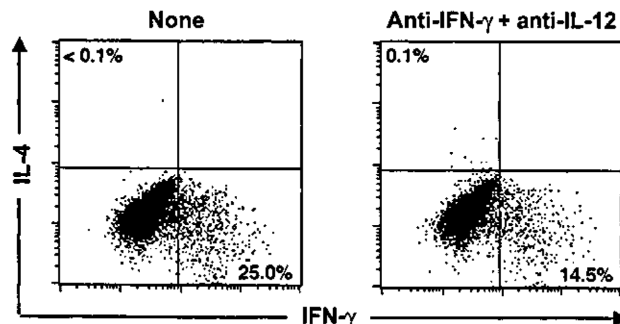

Fig. 6. Induction of Th1 differentiation of naive CD4+ T cells from P25 TCR-Tg mice upon stimulation with Peptide-25-loaded I-A^b-CHO. Naive CD4+ T cells from P25 TCR-Tg mice were stimulated for 6 days *in vitro* with Peptide-25-loaded I-A^b-CHO in the presence or absence of anti-IFN-γ and anti-IL-12. Six days after the culture, the proliferated cells were harvested and re-stimulated with 1 μg/well of immobilized anti-CD3 for 24 h and subjected to cytoplasmic staining for IFN-γ and IL-4. The percentages of IL-4- and IFN-γ-producing cells are presented in the upper left and the lower right regions, respectively.

transfectants. CD4 expression may facilitate the interaction between TG40-BP1 and APC, resulting in augmented IL-2 production. Intriguingly, the APL could stimulate TG40-BP1/CD4 IL-2 production to a much lesser extent even at higher peptide concentrations (Fig. 1B). As APL fully preserves the I-A^b-binding amino acids of Peptide-25, the APL/I-A^b complex may have lower avidity for the TCR compared with Peptide-25.

Expression profiles of cell surface activation markers on splenic T cells from P25 TCR-Tg mice were similar to these from WT mice, and mRNA expression of neither T-bet nor IFN-γ was observed, suggesting that CD4+ T cells in P25 TCR-Tg mice are not pre-activated. Naive CD4+ T cells from P25 TCR-Tg mice could differentiate into IFN-γ- and IL-4-producing cells upon anti-CD3 stimulation (Fig. 2), indicating that they keep their potential to differentiate into either Th1- or Th2-lineage cells upon TCR ligation. Interestingly, naive CD4+ T cells differentiated solely to IFN-γ-producing cells, but not to IL-4-producing cells upon Peptide-25 stimulation (Fig. 3). This preferential Th1 differentiation induced by Peptide-25 stimulation was also dependent on APC from C57BL/6 mice. As we described, stimulation of naive CD4+ T cells from P25 TCR-Tg mice with Peptide-25 at 10 μg/ml (6.0 μM) preferentially induces Th1 development. In contrast, when we stimulated the T cells with Peptide-25 at 0.1 μg/ml (0.06 μM), we observed a Th2-dominant response (data not shown). These observations are consistent with the published data (31) addressing that IFN-γ production is preferentially induced at 1.6–6.2 μM of OVA peptide in the OVA TCR-Tg mouse model. These results further support the notion that the Peptide-25 has an intrinsically highly potential to induce Th1. Intriguingly, stimulation with APL in place of Peptide-25 induced solely IL-4-producing cells (Fig. 3). When we analyzed APC cell surface marker expression after stimulation with either Peptide-25 or APL, we did not observe an activation-dependent alteration of cell surface marker expression such as CD80, CD86, or CD40 (data not shown). The differences between Peptide-25 and APL regarding Th1 and Th2 differentiation may be due to differences in avidity between Peptide-25/I-A^b and APL/I-A^b to TCR.

Differentiation of naive CD4+ Th precursors to Th1 and Th2 is affected by the manner and environment that they encounter (2,32,33). The strength of interaction between the TCR and MHC/peptide complex affects the lineage commitment of Th cells (15,17,31,34). It is well known that Th1 cell development involves IFN-γ signaling through STAT1 and IL-12 signaling through STAT4 activation (35,36). Peptide-25-induced Th1 differentiation of naive CD4+ T cells from P25 TCR-Tg mice was observed even in the presence of anti-IFN-γ and anti-IL-12 (Fig. 4). We obtained similar results using T cells of STAT1 deficient P25 TCR-Tg mice (Fig. 5). This indicates that both IFN-γ/STAT1 and IL-12 signals are not essential for preferential induction of P25 TCR-Tg naive CD4+ T cells to Th1.

The activation and differentiation of naive CD4+ T cells appears to require at least three separate signals. The first signal is delivered through the TCR/CD3 complex after its interaction with MHC/peptide complex on APC. The second signal is provided by a number of co-stimulatory or accessory molecules on the APC that interact with their ligands on T cells such as CD28/CD80/86, CTLA-4/CD80/86, LFA-1/ICAM-1, OX40/OX40L or ICOS/B7h (37–43). The dose or antigen concentration is also important in determining the Th1-dominated immune response. Third, cytokines such as IFN-γ, IL-12 or IL-18 play a role in the expansion of the committed Th1 cells (10,11,44–46). Stimulation of naive CD4+ T cells from P25 TCR-Tg mice with Peptide-25-loaded I-A^b-CHO in primary culture lead to lower proliferation and cell recovery after culturing compared to stimulation with Peptide-25-loaded splenic APC (data not shown). Interestingly, anti-CD3 stimulation of the T cells, recovered from culture with Peptide-25-loaded I-A^b-CHO, could induce Th1 development preferentially as shown in T cells stimulated with Peptide-25 and splenic APC in primary culture (Fig. 6). As Chinese hamster ovary cells do not express detectable levels of CD80, CD86, ICAM-1, OX40L or B7h, we are in favor of the hypothesis that preferential induction of Th1 development in P25 TCR-Tg naive CD4+ T cells may be independent of these well-known co-stimulating signals from APC.

A complex network of gene transcription events is likely to be involved in establishing an environment that promotes Th1 development. T-bet, a recently discovered member of T-box transcription factor is expressed selectively in thymocytes and Th1 cells, and controls the expression of the hallmark Th1 cytokine, IFN-γ (47). T-bet expression correlates with IFN-γ expression in Th1 and NK cells. Ectopic expression of T-bet both transactivates the IFN-γ gene and induces endogenous IFN-γ production (47). T-bet appears to initiate Th1 lineage development from naive Th cells both by activating Th1 genetic programs and by repressing the opposing Th2 programs (47). It has been reported that T-bet is regulated by IFN-γ signaling through STAT1 activation in the context of TCR ligation (10,11) and induces chromatin remodeling of the *ifn-γ* locus (48). As naive $CD4^+$ T cells are capable of differentiating into IFN-γ producing cells even in the presence of anti-IFN-γ, the interaction between Peptide-25/I-A^b and TCR may directly induce T-bet that leads to Th1 differentiation. We are currently investigating T-bet expression during Th1 differentiation in P25 TCR-Tg naive $CD4^+$ T cells in response to Peptide-25-loaded I-A^b-CHO.

There are several possibilities to account for the immunogenicity and adjuvant activity of Peptide-25 for Th1 development. First, Peptide-25 may activate DCs directly or indirectly through Th cells to enhance expression of co-stimulatory molecules leading to activate Th1 precursors by enhancing well-known transcription factors such as T-bet or unidentified 'master cytokine' for Th1 development. Second, the avidity of Peptide-25 to its specific TCR would be potent enough leading to Th1 development preferentially. Third, Peptide-25 might enhance activation or selection of unidentified T cell subpopulations that suppress GATA-3 leading to Th2 development.

In conclusion, we have presented data showing that naive $CD4^+$ T cells from P25 TCR-Tg mice stimulated with Peptide-25/I-A^b that polarize to Th1 differentiation preferentially in the absence of IFN-γ or IL-12. We propose the hypothesis that direct interaction of the specific antigenic peptide/MHC class II complex and TCR may primarily influence the determination of naive $CD4^+$ T cell fate in development towards the Th1 subset. Therefore, P25 TCR-Tg mice may provide us with new insights and help us understand how Th cell fate is determined.

Supplementary data

Supplementary data are available at *International Immunology* Online.

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Abbreviations

APL	altered peptide ligand of Peptide-25
I-A^b-CHO	Chinese hamster ovary cells expressing I-A^b
P25 TCR-Tg	TCR-Tg line of mice expressing TCR-Vα5-Vβ11
TCRVβ11	Vβ11 of TCR
TG40-BP1	TG40 cells expressing TCR-αβ
TG40-BP1/CD4	TG40-BP1 cell line for expression of CD4

References

1 Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A. and Coffman, R. L. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* 136:2348.
2 O'Shea, J. J. and Paul, W. E. 2002. Regulation of TH1 differentiation—controlling the controllers. *Nat. Immunol.* 3:506.
3 Murphy, K. M. and Reiner, S. L. 2002. The lineage decisions of helper T cells. *Nat. Rev. Immunol.* 2:933.
4 Szabo, S. J., Sullivan, B. M., Peng, S. L. and Glimcher, L. H. 2003. Molecular mechanisms regulating Th1 immune responses. *Annu. Rev. Immunol.* 21:713.
5 Rocken, M., Muller, K. M., Saurat, J. H., Muller, I., Louis, J. A., Cerottini, J. C. and Hauser, C. 1992. Central role for TCR/CD3 ligation in the differentiation of $CD4^+$ T cells toward A Th1 or Th2 functional phenotype. *J. Immunol.* 148:47.
6 Kamogawa, Y., Minasi, L. A., Carding, S. R., Bottomly, K. and Flavell, R. A. 1993. The relationship of IL-4- and IFN γ-producing T cells studied by lineage ablation of IL-4-producing cells. *Cell* 75:985.
7 Seder, R. A. and Paul, W. E. 1994. Acquisition of lymphokine-producing phenotype by $CD4^+$ T cells. *Annu. Rev. Immunol.* 12:635.
8 Hsieh, C. S., Macatonia, S. E., Tripp, C. S., Wolf, S. F., O'Garra, A. and Murphy, K. M. 1993. Development of TH1 $CD4^+$ T cells through IL-12 produced by Listeria-induced macrophages. *Science* 260:547.
9 Manetti, R., Parronchi, P., Giudizi, M. G., Piccinni, M. P., Maggi, E., Trinchieri, G. and Romagnani, S. 1993. Natural killer cell stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4-producing Th cells. *J. Exp. Med.* 177:1199.
10 Lighvani, A. A., Frucht, D. M., Jankovic, D. *et al.* 2001. T-bet is rapidly induced by interferon-γ in lymphoid and myeloid cells. *Proc. Natl Acad. Sci. USA* 98:15137.
11 Afkarian, M., Sedy, J. R., Yang, J., Jacobson, N. G., Cereb, N., Yang, S. Y., Murphy, T. L. and Murphy, K. M. 2002. T-bet is a STAT1-induced regulator of IL-12R expression in naive $CD4^+$ T cells. *Nat. Immunol.* 3:549.
12 Takeda, K., Tanaka, T., Shi, W., Matsumoto, M., Minami, M., Kashiwamura, S., Nakanishi, K., Yoshida, N., Kishimoto, T. and Akira, S. 1996. Essential role of Stat6 in IL-4 signalling. *Nature* 380:627.
13 Shimoda, K., van Deursen, J., Sangster, M. Y. *et al.* 1996. Lack of IL-4-induced Th2 response and IgE class switching in mice with disrupted Stat6 gene. *Nature* 380:630.
14 Kaplan, M. H., Schindler, U., Smiley, S. T. and Grusby, M. J. 1996. Stat6 is required for mediating responses to IL-4 and for development of Th2 cells. *Immunity* 4:313.
15 Constant, S., Pfeiffer, C., Woodard, A., Pasqualini, T. and Bottomly, K. 1995. Extent of T cell receptor ligation can determine the functional differentiation of naive $CD4^+$ T cells. *J. Exp. Med.* 182:1591.
16 Constant, S. L. and Bottomly, K. 1997. Induction of Th1 and Th2 $CD4^+$ T cell responses: the alternative approaches. *Annu. Rev. Immunol.* 15:297.
17 Tao, X., Grant, C., Constant, S. and Bottomly, K. 1997. Induction of IL-4-producing $CD4^+$ T cells by antigenic peptides altered for TCR binding. *J. Immunol.* 158:4237.
18 Huygen, K., Content, J., Denis, O. *et al.* 1996. Immunogenicity and protective efficacy of a tuberculosis DNA vaccine. *Nat. Med.* 2:893.
19 Silver, R. F., Wallis, R. S. and Ellner, J. J. 1995. Mapping of T cell epitopes of the 30-kDa alpha antigen of *Mycobacterium bovis* strain bacillus Calmette–Guerin in purified protein derivative (PPD)-positive individuals. *J. Immunol.* 154:4665.

20 Kamath, A. T., Feng, C. G., Macdonald, M., Briscoe, H. and Britton, W. J. 1999. Differential protective efficacy of DNA vaccines expressing secreted proteins of *Mycobacterium tuberculosis*. *Infect. Immun.* 67:1702.

21 Mustafa, A. S., Shaban, F. A., Abal, A. T., Al-Attiyah, R., Wiker, H. G., Lundin, K. E., Oftung, F. and Huygen, K. 2000. Identification and HLA restriction of naturally derived Th1-cell epitopes from the secreted *Mycobacterium tuberculosis* antigen 85B recognized by antigen-specific human $CD4^+$ T-cell lines. *Infect. Immun.* 68:3933.

22 Yanagisawa, S., Koike, M., Kariyone, A., Nagai, S. and Takatsu, K. 1997. Mapping of $V\beta11^+$ helper T cell epitopes on mycobacterial antigen in mouse primed with *Mycobacterium tuberculosis*. *Int. Immunol.* 9:227.

23 Kariyone, A., Higuchi, K., Yamamoto, S., Nagasaka-Kametaka, A., Harada, M., Takahashi, A., Harada, N., Ogasawara, K. and Takatsu, K. 1999. Identification of amino acid residues of the T-cell epitope of *Mycobacterium tuberculosis* α antigen critical for $V\beta11^+$ Th1 cells. *Infect. Immun.* 67:4312.

24 Kariyone, A., Tamura, T., Kano, H., Iwakura, Y., Takeda, K., Akira, S. and Takatsu, K. 2003. Immunogenicity of Peptide-25 of Ag85B in Th1 development: role of IFN-γ. *Int. Immunol.* 15:1183.

25 Ohno, H., Ushiyama, C., Taniguchi, M., Germain, R. N. and Saito, T. 1991. CD2 can mediate TCR/CD3-independent T cell activation. *J. Immunol.* 146:3742.

26 Morita, S., Kojima, T. and Kitamura, T. 2000. Plat-E: an efficient and stable system for transient packaging of retroviruses. *Gene Ther.* 7:1063.

27 Gyotoku, T., Fukui, Y. and Sasazuki, T. 1998. An endogenously processed self peptide and the corresponding exogenous peptide bound to the same MHC class II molecule could be distinct ligands for TCR with different kinetic stability. *Eur. J. Immunol.* 28:4050.

28 Kitamura, T., Koshino, Y., Shibata, F., Nakajima, H., Nosaka, T. and Kumagai, H. 2003. Retrovirus-mediated gene transfer and expression cloning, powerful tools in functional genomics. *Exp. Hematol.* 31:1007.

29 Yokosuka, T., Takase, K., Suzuki, M., Nakagawa, Y., Taki, S., Takahashi, H., Fujisawa, T., Arase, H. and Saito, T. 2002. Predominant role of T cell receptor (TCR)-α chain in forming preimmune TCR repertoire revealed by clonal TCR reconstitution system. *J. Exp. Med.* 195:991.

30 Pircher, H., Mak, T. W., Lang, R., Ballhausen, W., Ruedi, E., Hengartner, H., Zinkernagel, R. M. and Burki, K. 1989. T cell tolerance to Mlsa encoded antigens in T cell receptor Vβ8.1 chain transgenic mice. *EMBO J.* 8:719.

31 Hosken, N. A., Shibuya, K., Heath, A. W., Murphy, K. M. and O'Garra, A. 1995. The effect of antigen dose on $CD4^+$ T helper cell phenotype development in a T cell receptor-αβ-transgenic model. *J. Exp. Med.* 182:1579.

32 Abbas, A. K., Murphy, K. M. and Sher, A. 1996. Functional diversity of helper T lymphocytes. *Nature* 383:787.

33 O'Garra, A. 1998. Cytokines induce the development of functionally heterogeneous T helper cell subsets. *Immunity* 8:275.

34 Pfeiffer, C., Stein, J., Southwood, S., Ketelaar, H., Sette, A. and Bottomly, K. 1995. Altered peptide ligands can control CD4 T lymphocyte differentiation *in vivo*. *J. Exp. Med.* 181:1569.

35 Pernis, A., Gupta, S., Gollob, K. J., Garfein, E., Coffman, R. L., Schindler, C. and Rothman, P. 1995. Lack of interferon γ receptor beta chain and the prevention of interferon γ signaling in TH1 cells. *Science* 269:245.

36 Brombacher, F., Kastelein, R. A. and Alber, G. 2003. Novel IL-12 family members shed light on the orchestration of Th1 responses. *Trends Immunol.* 24:207.

37 Rulifson, I. C., Sperling, A. I., Fields, P. E., Fitch, F. W. and Bluestone, J. A. 1997. CD28 costimulation promotes the production of Th2 cytokines. *J. Immunol.* 158:658.

38 Kato, T. and Nariuchi, H. 2000. Polarization of naive $CD4^+$ T cells toward the Th1 subset by CTLA-4 costimulation. *J. Immunol.* 164:3554.

39 Salomon, B. and Bluestone, J. A. 1998. LFA-1 interaction with ICAM-1 and ICAM-2 regulates Th2 cytokine production. *J. Immunol.* 161:5138.

40 Smits, H. H., de Jong, E. C., Schuitemaker, J. H., Geijtenbeek, T. B., van Kooyk, Y., Kapsenberg, M. L. and Wierenga, E. A. 2002. Intercellular adhesion molecule-1/LFA-1 ligation favors human Th1 development. *J. Immunol.* 168:1710.

41 Ohshima, Y., Yang, L. P., Uchiyama, T., Tanaka, Y., Baum, P., Sergerie, M., Hermann, P. and Delespesse, G. 1998. OX40 costimulation enhances interleukin-4 (IL-4) expression at priming and promotes the differentiation of naive human $CD4^+$ T cells into high IL-4-producing effectors. *Blood* 92:3338.

42 Akiba, H., Miyahira, Y., Atsuta, M. *et al.* 2000. Critical contribution of OX40 ligand to T helper cell type 2 differentiation in experimental leishmaniasis. *J. Exp. Med.* 191:375.

43 Dong, C., Juedes, A. E., Temann, U. A., Shresta, S., Allison, J. P., Ruddle, N. H. and Flavell, R. A. 2001. ICOS co-stimulatory receptor is essential for T-cell activation and function. *Nature* 409:97.

44 Thierfelder, W. E., van Deursen, J. M., Yamamoto, K. *et al.* 1996. Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells. *Nature* 382:171.

45 Kaplan, M. H., Sun, Y. L., Hoey, T. and Grusby, M. J. 1996. Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice. *Nature* 382:174.

46 Nakanishi, K., Yoshimoto, T., Tsutsui, H. and Okamura, H. 2001. Interleukin-18 regulates both Th1 and Th2 responses. *Annu. Rev. Immunol.* 19:423.

47 Szabo, S. J., Kim, S. T., Costa, G. L., Zhang, X., Fathman, C. G. and Glimcher, L. H. 2000. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 100:655.

48 Mullen, A. C., High, F. A., Hutchins, A. S. *et al.* 2001. Role of T-bet in commitment of TH1 cells before IL-12-dependent selection. *Science* 292:1907.

特集　粘膜免疫

経粘膜ワクチン

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要旨　生体防御の最前線である粘膜局所においては，物理・化学的および生物学的バリアーの形成とともに，病原微生物と常在細菌叢の認識・識別，あるいは粘膜を介して侵襲する様々な抗原に対する正の免疫応答と負の免疫寛容という相反する対応が厳格に行われている。しかし，その制御機構をつかさどる細胞や分子は，全身免疫システムとは随分異なっていることがしだいに明らかになってきた。ヒトの生命を脅かす病原微生物感染症の多くは，皮膚を介して感染するマラリアや新生児破傷風などを除いて，消化器，呼吸器，生殖器などの粘膜を介して感染するものである。このことからも粘膜という水際で致死的な病原細菌やウイルスによる感染症と対峙している粘膜免疫システムの重要性をうかがい知ることができる。したがって，全身系の免疫システムとは似て非なる粘膜免疫システムの特徴を生かした微生物感染症やアレルギー・免疫疾患の新たな制御法の開発が大いに期待されている。

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Mucosal Vaccine

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Summary

The mucosal immune system consists of molecules, cells, and organized lymphoid structures intended to provide immunity to pathogens that impinge upon mucosal surfaces. Mucosal infection by pathogens, such as bacteria, virus, and protozoa, results in the induction of cell mediated immunity, as manifested by T helper, as well as cytotoxic T lymphocytes. These responses are normally accompanied by the synthesis of secretory immunoglobulin A antibodies, which provide an important first line of defense against invasion of mucosal surfaces by these pathogens. A new generation of live, attenuated mucosal vaccines, such as the cold-adapted, recombinant nasal influenza, can optimize this form of mucosal immune protection. Despite these advances, emerging and re-emerging infectious diseases are tipping the balance in favor of the parasite; continued mucosal vaccine development will be needed to effectively combat these new threats.

Key words: Mucosal vaccine, Mucosal immune system, Gut-associated lymphoid tissue, Secretory immunoglobulin A, Emerging and re-emerging infectious diseases

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はじめに

わが国では戦後の高度経済成長期を経て，医療・保健・福祉を取り巻く社会・経済・行政的環境が格段に整備・改善された。したがって，結核やはしかなど，過去に猖獗を極めた感染症はもはや克服された病気として，これらに対する対応を軽視する風潮が産・官・学・民のいずれの次元においても見受けられた。しかし眼を世界に転ずると，エイズ（AIDS），サーズ（SARS），エボラ出血熱，病原性大腸菌O157など，感染症が原因となる新たな疾病の脅威は枚挙に暇がない。また，もはや地球上から消滅したと考えられた天然痘が，昨今バイオ・テロリズムの手段として悪用されるという新たな脅威が生じている。このようにウイルスや細菌などの微生物による感染症は，グローバルな視点に立てば未だに深刻な問題を投げかけている人類の脅威なのである。本稿ではこのような疾病に対する予防や治療の有効な手段として注目されている体にやさしい経粘膜ワクチン研究の動向をわれわれの研究成果を踏まえて紹介したい。

I. ワクチンとは

人類の英知により病の本質に対する理解が深まるにつれ，疾病にかからない手段としての予防医学の重要性が近年とみに増している。肉体的，精神的，経済的な負担など，多くの点で予防は治療に勝る疾病対策であり，とりわけ感染症対策としてのワクチン開発は優れた科学的予防法といえる。ワクチンとは病原微生物に対する能動的あるいは受動的な特異免疫を付与する目的で投与される製剤であり，1786年に英国のJennerが天然痘を予防する目的で牛痘の病原ウイルスをPhipps少年に接種したのが予防接種のはじまりであった。この予防接種，すなわち種痘に使用した牛痘の病原体はvacca（ウシ）から採取したことにちなんでvaccine（ワクチン）と呼ばれたが，後にPasteurはこの功績を顕彰してワクチンを予防接種に利用される製剤に対して広義に用いた。世界保健機関は1979年の天然痘の根絶に引き続き，ワクチンによるポリオ，麻疹（はしか）の根絶をめざしている。このようにワクチンは予防医学のもたらした優れた生物製剤の一つといえる。また，最近はバイオ・テロリズムに対する予防・治療法としてのワクチンの重要性が注目されている。

II. 現行のワクチン

わが国で予防接種として利用されている現行のワクチンは，大別すれば弱毒生ワクチン，あるいは不活化ワクチンのいずれかであり，ポリオの経口弱毒生ワクチンを除くすべてのワクチンにおいて経皮注射による接種が実施されている。弱毒生ワクチンは自然宿主以外の宿主（鶏卵やマウスなどの動物や培養細胞）において継代を重ねて選択した弱毒変異株を用いることが一般的で，比較的自然感染（野生株の感染）に近い特異感染防御免疫を引きだすことができる。したがって，血清IgGを中心とした体液性免疫応答のみならず，ウイルス感染防御に重要な細胞傷害性T細胞（CTL）を中心とした細胞性免疫応答や結核などの細胞内寄生性細菌の防御に重要なIFN-γ産生性Tヘルパー1型（Th1）細胞の誘導が期待できる。しかしながら，自然罹患に比べて軽いが症状を引き起こすこと，さらに弱毒生ワクチン株の病原性の復帰の問題，また免疫不全者への使用が禁忌であることなどの副作用が懸念される。一方，不活化ワクチンはホルマリンなどの化学物質で処理して感染力を消失させた，不活化した形で投与するワクチン製剤であり，細菌毒素を無毒化（トキソイド）したものや病原微生物の成分を精製分離したもの，また遺伝子工学の手法を応用した組換え型ワクチンなども不活化ワクチンとみなすことができる。弱毒生ワクチンとは異なり，症状の発現や毒力を復帰することはない。しかし，十分な感染防御能を有する特異免疫応答を誘導するためには，ある程度以上の抗原量の接種が必要であり，時に免疫応答強化のための水酸化アルミニウムゲルに代表されるアジュバント（後述）の併用が要求され，細胞性免疫の誘導は期待し難い。望ましくない免疫応答を避けるためには不純物を取り除き，かつ強い免疫原性が維持されているワクチンの精製が必要な場合がある。

III. 粘膜ワクチン

前項において紹介した実用化済みのワクチンのほとんどは急性の経過をとり，かつ，終生免疫の