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IL-4 Promotes the Migration of Circulating B Cells to the Spleen and Increases Splenic B Cell Survival

Masaaki Mori,2‡ Suzanne C. Morris,*† Tatyana Orekhova,*,‡ Mariarosaria Marinaro,‡ Edward Giannini,§ and Fred D. Finkelman3*†

We report that IL-4 causes a redistribution of B cells and modestly increases B cell life span. Intravenous injection of a long-acting formulation of IL-4 induces increases in both spleen cell number and the percentage of splenic B cells. These effects are observed within 1 day of IL-4 administration and plateau after ~3 days if IL-4 treatment is continued. The increase in splenic B cell number is IL-4 dose dependent, CD4+ T cell independent, FcyRII/FcyRIII independent, and Stat6 independent. Decreases in the number of B cells in the blood and the percentage of mature B cells in the bone marrow, concomitant with the increase in splenic B cell number, suggest that redistribution of circulating B cells to the spleen is partially responsible for IL-4 induction of splenic B cell hyperplasia. Considerable reduction in the effect of 5 days of IL-4 treatment on splenic B cell number when B lymphopoiesis is blocked with anti-IL-7 mAb suggests that generation of new B cells is also involved in IL-4-induced splenic B cell hyperplasia. 5-Bromo-2'-deoxyuridine labeling experiments demonstrate that IL-4 modestly prolongs the life span of newly generated splenic B cells, and experiments that measure B cell HSA (CD24) expression as an indicator of B cell age suggest that IL-4 may also prolong the life span of mature splenic B cells. Thus, IL-4 increases splenic B cell number through two Stat6-independent effects: increased net migration of circulating B cells to the spleen and increased B cell life span. Both effects may promote Ab responses to a systemic Ag challenge. The Journal of Immunology, 2000, 164: 5704–5712.

Materials and Methods

Animals

Female BALB/c mice were purchased from the Small Animals Division of the National Cancer Institute (Frederick, MD) and were used at age 8–14 wk. Wild-type mice and mice deficient for the Stat6 gene (both on a mixed C57BL/6 and 129 genetic background) were bred and typed by PCR at the Cincinnati Veterans Affairs Medical Center from mice heterozygous for the nonfunctional Stat6 gene (18) that were provided by Dr. James Ihle (St. Jude’s Children’s Research Hospital, Memphis, TN). BALB/c IL-4Rα-deficient mice (19) were bred at the Cincinnati Veterans Affairs Medical Center from mice given to us by Dr. Nancy Noben-Trauth (National Institute of Allergy and Infectious Disease, National Institutes of Health, Bethesda, MD). IL-4

Murine rIL-4 was a gift from Dr. Robert Coffman (DNAX Research Institute, Palo Alto, CA).

Immunological reagents

The following Abs were produced and tested for specificity as previously described: BVD4.1D11.2 (20) (a neutralizing rat IgG2a anti-mouse IL-4 mAb), BVD6-24G2.3 (20) (a nonneutralizing rat IgG2a anti-mouse IL-4 mAb), m25 (21) (a mouse IgG2b mAb that neutralizes both human and mouse IL-7), GK1.5 (22) (a rat IgG2b mAb that kills CD4+ T cells and blocks Th cell function), 24G2 (23) (a rat IgG2b mAb that binds to mouse FcγRII and blocks its ability to bind IgG), HB66 (24) (a rat IgG2a anti-monocyte IgG1 mAb, also known as LO-MD-6), FF1-4D5 (25) (a mouse IgG2a mAb of the b allotype specific for IgD of the α allotype that is not blocked by HB66), DS-1 (26) (a mouse IgG1 mAb of the b allotype that binds to monocyte IgD of the α allotype), 6B2 (27) (a rat IgG2b mAb specific for mouse B20, the B cell form of CD45), 1D3 (28) (a rat IgG2b mAb that binds to the B cell marker CD19), B3B4 (29) (a rat IgG2b mAb that binds to mouse FcεRII), and MKD6 (30) (a mouse IgG2a alloantibody specific for I-Aα. Some of these Abs were labeled with FITC (31), biotin-N-hydroxysuccinimide (32), or the fluorochrome Cy5 (Research Organics, Cleveland, OH). M1/69 (33) (a rat IgG2b mAb that binds to heat-stable Ag (HSA; CD24)) was purchased from PharMingen (San Diego, CA).

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Preparation of IL-4-anti-IL-4 Ab complexes

IL-4-anti-IL-4 Ab complexes, which greatly extend the in vivo half-life of IL-4 (34), were prepared by mixing IL-4 with the neutralizing anti-IL-4 Ab BVD4-1D11.2 at a 2:1 molar (1/5, w/w) ratio. After 5 min at room temperature, complexes were diluted with 1% BALB/c serum in PBS to a concentration that would allow injection of a 0.2-ml volume. Complexes were always prepared freshly before use.

Preparation of lymphocytes from peripheral blood

Mice were tail-bleed 1 min after i.v. injection of 100 IU of heparin sodium. Heparinized blood (0.5 ml) was pelleted by centrifugation, after which the cell pellet was resuspended in 2.5 ml of distilled water for 10 s to lyse erythrocytes. NaCl saline (0.28 ml of 1.5 M) was added, after which nucleated cells were pelleted, resuspended in HBSS supplemented with 10% newborn bovine serum and 0.2% sodium azide (HNA), counted with a Coulter counter (Hialeah, FL), and adjusted to a concentration of 20 × 10⁶ cells/ml.

Preparation of Peyer’s patch lymphocytes

Peyer’s patches were carefully excised from the intestinal wall and dissociated using the neutral protease dispase (Roche, Indianapolis, IN) in Joklik-modified medium (Life Technologies, Gaithersburg, MD) to obtain single-cell preparations (35).

In vitro culture conditions

Spleen cells and nucleated peripheral blood cells were cultured in 1 ml of RPMI medium 1640 supplemented with 10% FBS, 10 μM of nonessential amino acid solution, 1% 2-methyl-2-glutamine, 1 mM sodium pyruvate, 25 mM 2-ME, 10 mM HEPES, penicillin, streptomycin, fungazone, and gentamycin, with or without 1 ng of IL-4, for 24 h at 37°C in an atmosphere containing 5.5% CO₂ in 24-well flat-bottom Costar culture dishes (Cambridge, MA) at a density of 4 × 10⁶ cells/ml/well. At the end of the culture period, cells were washed once in HNA, counted with a Coulter counter, and resuspended in ice-cold HNA at a concentration of 20 × 10⁶ cells/ml.

Immunofluorescence staining

Single-cell suspensions of spleen, peripheral lymph node (axillary, supraclavicular, inguinal, and popliteal lymph nodes), and bone marrow were depleted of erythrocytes by treatment with a buffered ammonium chloride solution, resuspended in HNA, filtered through nylon gauze, counted with a Coulter counter, and brought to a concentration of 20 × 10⁶ cells/ml. One hundred microliters of cell suspensions from the above organs and blood were stained for 30 min on ice with 1 μg each of an FITC-labeled Ab and a biotin-labeled Ab. Cells were washed twice with HNA and then stained for 30 min on ice with streptavidin-R-PE (Life Technologies). All staining was performed in the presence of 10 μg/ml of unlabeled anti-FcRII mAb (24G2) to block the binding of IgG staining reagents to FcγRII. After washing once more with HNA, cells were washed once with HBSS/0.2% sodium azide, then fixed in PBS/2% paraformaldehyde. Cells were analyzed with a FACSscan (Becton Dickinson, Mountain View, CA) and CellQuest software. Light scatter gates were set to exclude cells that had died before fixation as well as nonlymphoid cells, except that light scatter gates for analysis of bone marrow cells were set to include all living nucleated cells. Spleen cells that had been stained with a single fluorochrome-labeled Ab were used to determine compensation for overlap between FITC and PE emission spectra. Data were analyzed to determine the percentages of specifically stained cells and the mean and/or median fluorescence intensities of specifically stained cells. In some experiments cells stained with FITC-PE-, and Cy5-labeled reagents were analyzed by flow cytometry with a FACSScaliber flow cytometer (Becton Dickinson), and PE fluorescence histograms of Cy5FITC and Cy5FITCbright cells were prepared.

Staining for 5-bromo-2'-deoxyuridine (BrdU)-labeled cells

BrdU (Sigma, St. Louis, MO) was added at a concentration of 0.8 mg/ml to the drinking water. Water bottles were covered with aluminum foil, and water was changed daily. Spleen cell suspensions were stained for surface markers as described above, then washed in PBS and resuspended in 0.5 ml of ice-cold 0.15 M sodium chloride, to which 1.2 ml of ice-cold 95% ethanol was added dropwise as cells were being gently vortex mixed. Cells were incubated on ice for 30 min, pelleted by centrifugation at 1600 rpm for 15 min, and washed with PBS, after which they were incubated for 30 min at room temperature in 1 ml of PBS/1% paraformaldehyde/0.01% Tween 20. Cells were kept at 4°C overnight, after which they were pelleted by centrifugation at 1600 rpm for 15 min and resuspended in 1 ml of 0.15 M sodium chloride/0.0042 M magnesium chloride/50,000 Kunitz units of DNase I (Sigma). Cells were then incubated for 10 min at room temperature, washed with PBS, stained with 20 μl of FITC-anti-BrdU (Becton Dickinson) for 30 min at room temperature, washed twice in PBS, and analyzed for surface markers and BrdU by flow cytometry.

Determination of cell numbers

Cells were counted with a Coulter counter, using settings that excluded dead cells. Total cell counts were multiplied by the percentages of cells that stained specifically for a particular marker to determine the number of cells that expressed that marker.

Statistics

Hypotheses that IL-4 causes an increase in splenic B cell number were tested with a one-tailed t test. All other experimental results were tested with a two-tailed t test. The hypothesis that IL-4 treatment causes a larger increase in splenic B cell number than in splenic CD4⁺ T cell number was tested with a two-tailed t test comparison of the ratios of splenic B cell numbers in untreated vs IL-4-treated mice with the ratios of splenic CD4⁺ T cell numbers in untreated vs IL-4-treated mice (see Fig. 5). Results were considered significant at p < 0.05, to be highly significant at p < 0.01, and to lack significance at p > 0.05. The results of tests for significance are provided in the figure legends.

Results

IL-4 induces increases in spleen cell number and percentage and number of splenic B cells

A previous study showed that IL-4 treatment causes an increase in the percentage of B cells in the spleen (34). We performed additional studies to determine whether this reflects an increase in the absolute number of splenic B cells or a decrease in splenic non-B cell number. Treatment of BALB/c mice 5 and 2 days before sacrifice with IL-4 that contained 1 μg of IL-4 caused both a 2-fold increase in spleen cell number and a nearly 40% increase in the percentage of B220⁺mlgM⁺ spleen cells (Fig. 1). Thus, systemic IL-4 treatment causes splenic B cell number to increase 2- to 3-fold and the number of splenic non-B cells to increase to less of an extent.

Dose response, kinetic characteristics, and specificity of the IL-4-induced increase in spleen B cell number

To determine the dose of IL-4 required to induce a maximal increase in splenic B cell number, BALB/c mice were left untreated.
or were injected i.v. 5 and 2 days before sacrifice with IL-4C that contained 125-1000 ng of IL-4. Spleen cells were counted and stained for B220 and Ia, and percentages of B220^Ia\textsuperscript{+} cells and the intensity of their Ia staining were determined by flow cytometry (Fig. 2). Both splenic B cell number and Ia mean fluorescence intensity were increased at the lowest dose of IL-4 tested, while splenic B cell number peaked at a dose of 500 ng of IL-4, while B cell Ia median fluorescence intensity continued to increase as the dose of IL-4 was increased to 1000 ng.

To determine the timing of IL-4 induction of splenic B cell hyperplasia and whether IL-4 treatment induced increases in mature and/or immature splenic B cells, mice were untreated or were given a single i.v. injection of IL-4C that contained 1 μg of IL-4 and were sacrificed 1-20 days later. Spleen cells from individual mice were counted, and percentages or absolute numbers of mature (B220\textsuperscript{HSA\textsuperscript{bright}}) and immature (B220\textsuperscript{HSA\textsuperscript{dull}}) splenic B cells were determined by immunofluorescence staining and flow cytometry (Figs. 3 and 4A). Because mature splenic marginal zone B cells stain more brightly than HSA for most mature splenic B cells, although not as brightly as immature B cells (33, 36, 37), a preliminary experiment was performed to determine the degree of HSA staining required to discriminate immature from mature splenic B cells. This was done by comparing the HSA staining profiles of splenic B cells from untreated mice and anti-IL-7 mAb-treated mice (Fig. 3), because anti-IL-7 mAb treatment prevents lymphopenia and thus depletes most immature B cells from the spleen (21, 33, 36). B220\textsuperscript{+} spleen cells that stained more brightly than the channel used to demarcate HSA\textsuperscript{bright} from HSA\textsuperscript{dull} decreased by >76% after 12 days of anti-IL-7 mAb treatment, while this treatment caused no appreciable change in the percentage of B220\textsuperscript{+} spleen cells that stained less brightly than this channel (Fig. 3).

Based on this method for identifying mature and immature splenic B cells, >60% increases in the numbers of both splenic B cell populations were observed 1 day after IL-4C injection (Fig. 4A). Numbers of mature, but not immature, splenic B cells increased by an additional 80% during the next 2 days. Numbers of mature splenic B cells decreased to day 1 levels by 6 days after IL-4C injection and changed little during the subsequent 14 days. Additional experiments demonstrated that splenic B cell number continued to increase between 2 and 3 days after IL-4C injection and remained elevated, but did not increase further, when mice were reinfected with IL-4C on day 3 and sacrificed on day 5 (Fig. 4B) or reinjected every 3 days for 14 days (data not shown).

**The IL-4C-induced increase in splenic B cell number is CD4^+ T cell and FcγRII/III independent**

Because IL-4 can stimulate CD4^+ T cell proliferation and helper activity (6) and can suppress B cell FcyRII expression and function (38), it was possible that the IL-4-induced increase in splenic B cell number was CD4^+ T cell and/or FcγRII dependent. To examine these possibilities, the effect of treatment for 5 days with IL-4C was studied in mice treated with anti-CD4 and anti-FcγRII/III mAbs. IL-4C-induced similar increases in splenic B cell number in mice treated with anti-CD4 and anti-FcγRII/III mAbs and in mice that did not receive these mAbs (Fig. 5). Treatment with IL-4C for 5 days also caused a doubling of the number of splenic CD4^+ T cells in mice that received neither anti-CD4 nor anti-FcγRII/III mAbs.
The IL-4C-induced increase in splenic B cell number is Stat6 independent

IL-4 stimulates cells through at least two molecular pathways, one of which involves activation of Stat6 (18, 39). In general, IL-4 effects on cell differentiation are Stat6 dependent, while IL-4 effects of cell survival and proliferation are Stat6 independent (40).

To determine the Stat6 dependence of IL-4-induced migration of B cells to the spleen and increased B cell survival, we examined the effects of treatment with IL-4C for 1 or 5 days on spleen cell number in wild-type and Stat6-deficient mice. Although untreated Stat6-deficient mice had fewer splenic B cells than untreated wild-type mice, IL-4C treatment caused similar percent increases in splenic B cell number in both sets of mice; these were larger after 5 days of IL-4C treatment than after 1 day of IL-4C treatment (Fig. 6).

The IL-4-induced increase in spleen cell number raised the possibility that this effect was nonspecific and did not involve stimulation of the IL-4R. Two experiments were performed to investigate this possibility. First, the effect on spleen cell number of IL-4C prepared with IL-4 and the neutralizing anti-IL-4 mAb, BVD4-1D11, which increase and prolong in vivo stimulation by IL-4, was compared with the effect of IL-4C prepared with IL-4 and the nonneutralizing anti-IL-4 mAb, BVD6-24G2.3, which do not enhance in vivo IL-4 stimulation (34). As before, treatment of mice with IL-4C made with BVD4-1D11 caused an increase in splenic B cell number, while no significant increase

FIGURE 4. Kinetics of IL-4C effects on splenic B cells. A, Effects of a single dose of IL-4C. BALB/c mice (three per group) were untreated or were injected i.v. with IL-4C that contained 1 μg of IL-4 on day 0 and were sacrificed on day 1, 3, 6, 12, or 20. Splenic cells from individual mice were counted and stained with FITC-anti-B220 and biotin-anti-HSA mAbs, followed by streptavidin-R-PE. Stained cells were analyzed for percentages of mature (B220<sup>+</sup> HSA<sup>bright</sup>) and immature (B220<sup>+</sup> HSA<sup>dull</sup>) cells by flow cytometry. Means and SEs of numbers of mature and immature B cells per spleen are shown. Increases in the number of B220<sup>+</sup> HSA<sup>bright</sup> splenic B cells from days 0 to 1 and from days 1 to 3 and decreases in the number of B220<sup>+</sup> HSA<sup>dull</sup> splenic B cells from days 3 to 6 and from days 6 to 12 were highly significant. Changes in the number of B220<sup>+</sup> HSA<sup>bright</sup> splenic B cells from days 12 to 20 and changes in the number of B220<sup>+</sup> HSA<sup>dull</sup> splenic B cells lacked statistical significance. B, Effects of continuing IL-4C treatment. BALB/c mice (three per group) were left untreated or were injected i.v. on days 0 and 3 with IL-4C that contained 1 μg of IL-4 and were sacrificed on the day shown after the initial IL-4C treatment. Splenic cells from individual mice were counted, stained with FITC-anti-B220 and biotin-anti-IgM mAbs followed by streptavidin-R-PE, and analyzed for percentages of B220<sup>+</sup> IgM<sup>+</sup> cells with a FACScan. Means and SEs are shown. No further increase in splenic B cell number was observed when IL-4C treatment was continued every 3 days for 14 days (data not shown). Increases in splenic B cell number from days 0 to 1 and from days 2 to 3 were highly significant and significant, respectively. Changes from days 1 to 2 and from days 3 to 5 lacked statistical significance.

FIGURE 5. The IL-4C-induced increase in spleen cell number is CD4<sup>+</sup> T cell and FcγRII/III independent. BALB/c mice (three per group) were treated i.v. with saline or anti-CD4 mAb (1 mg/wk). One week after the initiation of treatment saline-treated mice were injected with saline or IL-4C that contained 1 μg of IL-4, and anti-CD4 mAb-treated mice were injected with 0.5 μg of 24G2 anti-FcγRII/III mAb i.p. or IL-4C and anti-FcγRII/III mAb. Saline or IL-4C injection was repeated 3 days later, and mice were sacrificed 5 days after the first IL-4C injection. Splenic cells were counted and stained for B220 and IgM or CD4 and were analyzed with a FACScan for percentages of cells that expressed these markers. Means and SEs are shown. The IL-4C-induced increases in splenic B cell number in otherwise untreated and anti-CD4- plus anti-FcγRII/III mAb-treated mice and the IL-4C-induced increase in splenic CD4<sup>+</sup> T cell number in otherwise untreated mice were highly significant. In mice that did not receive anti-CD4 mAb, the IL-4C-induced increase in splenic B cell number was highly significantly greater than the IL-4C-induced increase in splenic CD4<sup>+</sup> T cell number.

FIGURE 6. The IL-4-induced increase in spleen cell number is Stat6 independent. Wild-type and Stat6-deficient mice (three per group) were untreated or were injected i.v. with IL-4C that contained 1 μg of IL-4 on day 0 and sacrificed on day 1 (upper panel) or were injected with IL-4C on days 0 and 3 and sacrificed on day 5 (lower panel). Splenic cells from individual mice were counted and stained with FITC-anti-IgM and biotin-anti-B220 mAbs followed by streptavidin-R-PE and analyzed with a FACScan. Means and SEs of numbers of B220<sup>+</sup> mIgM<sup>+</sup> spleen cells per mouse are shown. Numbers to the right of the bars are percent increases in splenic B cell number induced by IL-4C treatment. IL-4-induced increases in splenic B cell numbers on days 1 and 5 in wild-type and Stat6-deficient mice were all highly significant. Two similar experiments gave comparable results.
Ia decrees in the number of Ia cell expression or splenic B cell number in IL-4R aity in wild-type mice, but did not significantly increase either splenic B injected i.v. with saline or with IL-4C that contained 1 g of IL-4. BALB/c mice were untreated or were treated with IL-4C for 1 day selectively increases the number IL-4-induced splenic B cell hyperplasia could result from the redistribution of mature B cells, although a marginal decrease was observed in the number of CD4 T cells in blood.

When mice were treated with IL-4C for 5 days before sacrifice, we observed a nearly 3-fold increase in splenic B cell number; no significant effect on the number of B cells in mesenteric lymph node, percentages of B cells in peripheral lymph node or Peyer’s patch, or percentages of pre-B cells or immature B cells in bone marrow; and an ~60–70% decreases in blood B cell number and the percentage of mature bone marrow B cells (Fig. 9); (IL-4 had no significant effect on the numbers of cells recovered from bone marrow, Peyer’s patches, or peripheral lymph nodes in this experiment, but results were reported as percentages of B cells only because of concerns about the completeness of bone marrow and Peyer’s patch cell recovery and because of the considerable variability in peripheral lymph node cell number within groups). Microscopic examination of sections of small intestine that had been stained with anti-B220 Ab showed no noticeable effect of IL-4 on B cell number (3.0 ± 0.7 B cells/villus for untreated mice; 2.7 ± 0.9 for IL-4-treated mice). The increases in mesenteric lymph node B cell number that were seen 1 day after the initiation of IL-4C treatment were no longer observed on day 5. Taken together, these observations suggest that IL-4 induces a redistribution of mature circulating B cells to the spleen, but that this redistribution does not deplete B cells from other peripheral lymphoid organs.

In vivo IL-4C treatment has distinct effects on B cells and CD4 T cells

The ability of 1-day treatment with IL-4C to increase splenic B cell, but not splenic CD4 T cell, number (Fig. 7) suggested that IL-4-induced migration of lymphocytes to the spleen might be B

**FIGURE 7.** IL-4C induction of an increase in splenic B cell number requires sustained IL-4R stimulation by IL-4. A. Five BALB/c mice were untreated, while groups of three BALB/c mice were injected i.v. with IL-4C composed of 1 µg of IL-4 and 5 µg of the neutralizing anti-IL-4 mAb, BVD4-1D11 or 1 µg of IL-4 and 5 µg of the nonneutralizing anti-IL-4 mAb, BVD6-24G2.3. Mice were sacrificed 3 days after IL-4C injection. Splenocytes were counted, stained for B220, and analyzed with a FACScan. Means and SEs of numbers of B220+ splenic cells per mouse are shown. Treatment with IL-4C made with BVD6-1D11 induced a significant increase in splenic B cell number, while treatment with IL-4C made with BVD6-24G2.3 had no significant effect on splenic B cell number. B, BALB/c wild-type and IL-4Rα-deficient mice (three per group) were injected i.v. with saline or with IL-4C that contained 1 µg of IL-4 on days 0 and 3 and were sacrificed on day 5. Spleen cells were stained with FITC-anti-Ia and analyzed for percentages and mean fluorescence intensities of Ia+ cells. Means and SEs are shown. IL-4 caused highly significant increases in the number of Ia+ spleen cells and Ia mean fluorescence intensity in wild-type mice, but did not significantly increase either splenic B cell Ia expression or splenic B cell number in IL-4Rα-deficient mice.

**FIGURE 8.** IL-4C induces redistribution of mature B cells to spleen and bone marrow within 1 day of injection. BALB/c mice (five per group) were treated i.v. on day 0 with saline or with IL-4C that contained 1 µg of IL-4 and were sacrificed on day 1. Numbers of nucleated spleen, mesenteric lymph node, peripheral lymph node, bone marrow, and blood cells (in 0.5 ml of heparinized blood) were counted and stained for B220 and IgM or for CD4, then analyzed with a FACScan. Numbers shown are means and SEs of B cells per organ (spleen and lymph nodes), per two femurs and tibiae (bone marrow), or per milliliter of blood. IL-4 treatment induced a highly significant increase in splenic B cell number, a significant decrease in blood B cell number, and a highly significant decrease in the number of mature bone marrow B cells. IL-4 had no significant effect on mesenteric lymph node or peripheral lymph node B cell number or on bone marrow immature B cell number. IL-4 had no significant effect on the number of CD4 T cells in any organ tested. A second experiment (not shown) produced similar results.

Treatment with IL-4C for 1 day selectively increases the number of splenic B cells while decreasing the numbers of blood B cells and mature bone marrow B cells

IL-4-induced splenic B cell hyperplasia could result from the redistribution of mature B cells, increased B lymphopoiesis, increased migration of recently generated B cells from bone marrow to spleen, increased splenic B cell survival, mature splenic B cell proliferation, or a combination of these effects. To determine whether redistribution of B cells might contribute to IL-4-induced splenic B cell hyperplasia, we examined whether the increase in splenic B cell number is accompanied by the loss of B cells from any other organ. BALB/c mice were untreated or were treated with IL-4C that contained 1 µg of IL-4 1 day before sacrifice. IL-4C treatment caused splenic and mesenteric lymph node number to increase by 60–100%, while blood B cell number decreased by ~35%, and most mature B cells disappeared from the bone marrow (Fig. 8 and data not shown). No significant change was seen in the number of immature bone marrow B cells or on the number of spleen or lymph node CD4 T cells, although a marginal decrease was observed in the number of CD4 T cells in blood.
Increased splenic B cell survival contributes to the IL-4-induced increase in splenic B cell number

Because the number of B cells lost from the blood and bone marrow was smaller than the number gained by spleen (Figs. 8 and 9), it seemed possible that changes in B cell survival might also contribute to the increase in splenic B cell number. Alternatively, large numbers of B cells from an additional source that we could not evaluate, such as lymph, may have migrated to the spleen. Experiments were performed to evaluate the possibility that IL-4 contributes to splenic B cell number by enhancing new B cell production or B cell survival. One experiment examined the effect of 5 days of in vivo IL-4C treatment on B cell expression of HSA, a surface marker that decreases in expression as B cells age (36). If IL-4 primarily contributed to splenic B cell hyperplasia by increasing B cell production and enhancing the migration of newly generated (HSAbright) B cells to the spleen, we would expect it to cause an increase in mean HSA expression by splenic B cells. In fact, IL-4C treatment induced significant decreases in mean HSA expression by both splenic and blood B cells, but had no effect on mean HSA expression by lymph node B cells (Fig. 11A). This effect is consistent with IL-4 induction of accelerated splenic B cell maturation and/or increased splenic B cell longevity. It cannot be explained solely by migration of HSAbright blood B cells to the spleen, because the mean HSA staining intensity of splenic B cells from IL-4C-treated mice is similar to or lower than that of blood B cell specific. In contrast, treatment of mice with IL-4C for 5 days significantly increased splenic CD4+ T cell number, although less than this treatment increased splenic B cell number (Fig. 5). An additional, kinetic study was performed to evaluate whether these different effects of IL-4C on B and T cells reflected true kinetic differences or were due to experimental variability. As was observed in our two previous studies that evaluated single time points, treatment with IL-4C until day 3, and, as observed previously, the increase at that time point and on day 5 was smaller than the increase in spleen B cell number. Thus, the effects of IL-4C on splenic B cell and CD4+ T cell number differ in both kinetics and magnitude.

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Because the number of B cells lost from the blood and bone marrow was smaller than the number gained by spleen (Figs. 8 and 9), it seemed possible that changes in B cell survival might also contribute to the increase in splenic B cell number. Alternatively, large numbers of B cells from an additional source that we could not evaluate, such as lymph, may have migrated to the spleen. Experiments were performed to evaluate the possibility that IL-4 contributes to splenic B cell number by enhancing new B cell production or B cell survival. One experiment examined the effect of 5 days of in vivo IL-4C treatment on B cell expression of HSA, a surface marker that decreases in expression as B cells age (36). If IL-4 primarily contributed to splenic B cell hyperplasia by increasing B cell production and enhancing the migration of newly generated (HSAbright) B cells to the spleen, we would expect it to cause an increase in mean HSA expression by splenic B cells. In fact, IL-4C treatment induced significant decreases in mean HSA expression by both splenic and blood B cells, but had no effect on mean HSA expression by lymph node B cells (Fig. 11A). This effect is consistent with IL-4 induction of accelerated splenic B cell maturation and/or increased splenic B cell longevity. It cannot be explained solely by migration of HSAbright blood B cells to the spleen, because the mean HSA staining intensity of splenic B cells from IL-4C-treated mice is similar to or lower than that of blood B cell specific. In contrast, treatment of mice with IL-4C for 5 days significantly increased splenic CD4+ T cell number, although less than this treatment increased splenic B cell number (Fig. 5). An additional, kinetic study was performed to evaluate whether these different effects of IL-4C on B and T cells reflected true kinetic differences or were due to experimental variability. As was observed in our two previous studies that evaluated single time points, treatment with IL-4C until day 3, and, as observed previously, the increase at that time point and on day 5 was smaller than the increase in spleen B cell number. Thus, the effects of IL-4C on splenic B cell and CD4+ T cell number differ in both kinetics and magnitude.
cells from untreated mice (Fig. 11A). An alternate possibility, that IL-4 directly down-regulates B cell HSA expression, appears unlikely because in vitro IL-4 treatment, at a dose (1 ng/ml) that substantially up-regulates B cell 1a expression, had no effect on splenic B cell HSA expression (Fig. 11B).

These observations suggested that IL-4 may in part increase splenic B cell number by prolonging the survival of B cells or inducing them to proliferate after they had entered the spleen. To examine these possibilities, we labeled newly generated B cells by providing mice for 4 days (days 0–4) with water that contained BrdU, and then examined the fate of these cells in the presence or the absence of IL-4. Some BrdU-treated mice were injected with IL-4C on days 0 and 3, then sacrificed on day 5; a second set of mice was injected with IL-4C on days 5 and 8, then sacrificed on day 10 (also 5 days after initiation of IL-4C treatment); and a third set was injected with IL-4C on days 5, 8, 11, 14, and 17 and sacrificed on day 19 (14 days after initiation of IL-4C treatment). Spleen cells from individual mice were counted and stained for B220, HSA, and BrdU. Stained cells were analyzed by flow cytometry for percentages of B220+HSA dull (mature) and B220+HSA bright (immature) B cells and for percentages of these cells that were BrdU+ and had thus synthesized DNA during the first 4 days of the experiment (Fig. 12). IL-4C treatment induced 2- to 3-fold increases in HSA dull B cells at each time point studied, but did not consistently affect the number of HSA bright splenic B cells. Mice treated simultaneously with IL-4C and BrdU (day 5 point) had similar percentages of BrdU+ splenic B cells as mice that received BrdU alone. This suggests that IL-4C did not stimulate B cells to proliferate and is consistent with previous evidence that IL-4 is not a B cell mitogen (6). Percentages of HSA bright splenic B cells that were BrdU+ were similar in untreated and IL-4-treated mice on days 5 and 19 and declined from ~40% on day 5 to ~8% on day 19. However, a significantly larger percentage of HSA bright splenic B cells were BrdU+ in the IL-4-treated than in untreated mice on day 10 (5 days after the initiation of IL-4 treatment), although the absolute number of splenic HSA bright B cells was only slightly increased at this time. Both the absolute numbers of BrdU+HSA dull splenic B cells and percentages of HSA dull splenic B cells that were BrdU+ peaked on day 10, at which time they were substantially greater in IL-4-treated mice than in control mice. By day 19, the percentage of HSA dull splenic B cells that were BrdU+ had declined by nearly 50% in the IL-4-treated mice and was only slightly greater than that in mice that had not received IL-4. Taken together, these observations suggest that IL-4 has relatively little effect on B cell production or on the migration of immature B cells to the spleen, but modestly prolongs their survival, and possibly promotes their maturation, after they reach the spleen. These effects appear to be sufficient for some of the newly produced B cells to acquire a mature (HSA dull) phenotype.

**Early and late effects of IL-4 on spleen cell number differ in their IL-7 dependence**

An IL-4-induced increase in spleen cell number that results from the net migration of mature B cells from blood and bone marrow to the spleen should not depend on the generation of new B cells. In contrast, an IL-4-induced increase in splenic B cell number that results from enhanced survival of newly produced B cells will depend on new B cell production. In addition, increases in B cell number that result from population shifts might occur relatively rapidly, while the accumulation and maturation of newly produced B cells would occur over a longer period of time. Based on these considerations, we examined the dependence of the IL-4-induced increase in splenic B cell number on B lymphopoiesis by determining whether anti-IL-7 mAb inhibition of B lymphopoiesis would inhibit the IL-4-induced increase. All mice were treated with anti-CD4 and anti-FcγRII/III mAbs in these experiments to block any effects that the injection of a large quantity of IgG might have on Th cell activation or on B cell FcγR-dependent interactions.

Treatment of BALB/c mice for 1 day with IL-4C increased splenic B cell number to at least the same extent in mice in which B lymphopoiesis had been suppressed with anti-IL-7 mAb as in mice with normal B lymphopoiesis (Fig. 13, left panels). In contrast, the increase in splenic B cell number induced by 5 days of

![Day of Sacrifice](http://www.jimmunol.org/)

**FIGURE 12.** IL-4C modestly prolongs the life span of newly generated B cells. BALB/c mice (three per group) were provided with BrdU-containing drinking water (0.8 mg/ml of BrdU/ml) from days 0–4. Some of these mice were injected with IL-4C that contained 1 μg of IL-4 on days 0 and 3, then sacrificed on day 5; a second set was injected with IL-4C on days 5 and 8, then sacrificed on day 10 (5 days after the start of IL-4C treatment); a third set was injected with IL-4C on days 5, 8, 11, 14, and 17 and sacrificed on day 19 (14 days after the start of IL-4C treatment). Spleen cells from individual mice were counted and stained with FITC-anti-BrdU, biotin-anti-HSA followed by streptavidin-R-PE, and Cy5-anti-B220 mAbs. Stained cells were analyzed with a FACScalibur flow cytometer for percentages of B220+HSA dull, B220+HSA bright, B220+HSA bright, and B220+HSA bright cells. Means and SEs are shown. The numbers of HSA dull and HSA bright splenic B cells from untreated and IL-4C-treated mice are shown in the upper and middle panels, respectively. Total B cells = total number of HSA dull or HSA bright B cells per spleen; BrdU+ B cells = number of BrdU+ HSA dull or BrdU+ HSA bright B cells per spleen. The lower panel shows the percentages of HSA dull or HSA bright B cells that are BrdU+ 5, 10, or 19 days after the initiation of BrdU treatment (1, 6, or 15 days after the cessation of BrdU treatment). IL-4 treatment induced highly significant increases in the numbers of splenic HSA dull B cells and HSA bright B cells on days 5, 10, and 19. The numbers of HSA dull splenic B cells on days 5, 10, and 19 in IL-4-treated mice were not significantly different from each other. IL-4 treatment caused a significant increase in the number of HSA bright splenic B cells on day 5 only and did not cause a significant increase in the number of BrdU+ HSA bright splenic B cells at any time point studied. IL-4 treatment caused a highly significant increase in the percentage of HSA dull splenic B cells that were BrdU+ and a significant increase in the percentage of HSA bright splenic B cells that were BrdU+ on day 10, but did not have a significant effect on the percentage of splenic B cells that were BrdU+ on day 5 or day 19.
FIGURE 13. Effects of anti-IL-7 mAb on splenic B cell numbers in mice injected with IL-4C. BALB/c mice (three per group) were injected i.v. with 1 mg of anti-CD4 mAb/wk and i.p. with 0.5 mg of anti-FcγRII/III mAb or with these mAbs plus 3 mg of anti-IL-7 mAb, injected i.p. three times per week. One week after initiation of these treatments, some mice were also injected with IL-4C that contained 1 μg of IL-4. Mice were sacrificed 1 day later (day 1) or were reinfected with IL-4C 3 days after the initial IL-4C injection and sacrificed 2 days after the second IL-4C injection (day 5). Nucleated spleen and bone marrow cells from individual mice were counted, stained with FITC-anti-IgM and biotin-anti-B220 Abs followed by streptavidin-R-PE, and analyzed with a FACScan. Means and SEs are shown. Anti-IL-7 mAb treatment induced a highly significant decrease in numbers of bone marrow pre-B cells and immature B cells. This treatment had a variable effect on the number of mature bone marrow B cells, inducing a significant decrease in some, but not all, experiments. IL-4 treatment, 1 day after injection, induced highly significant increases in splenic B cell number in mice treated with either anti-CD4 mAb or anti-CD4 plus anti-IL-7 mAbs. IL-4 treatment for 5 days induced a highly significant increase in splenic B cell number in anti-CD4 mAb-treated mice, but did not induce a significant increase in splenic B cell number in mice treated with anti-CD4 and anti-IL-7 mAbs. The number of splenic B cells in mice treated for 5 days with IL-4 was highly significantly greater in mice treated with anti-CD4 mAb than in mice treated with anti-CD4 and anti-IL-7 mAbs. Two similar experiments gave comparable results.

IL-4C treatment was twice as large in mice with normal B lymphopoiesis as in anti-IL-7 mAb-treated mice (Fig. 13, right panels). Thus, the initial IL-4-induced increase in splenic B cell number is independent of B lymphopoiesis, while the additional increase in splenic B cell number that results from further treatment with IL-4C requires lymphopoiesis.

Discussion

The data presented in this paper demonstrate that IL-4 has previously undescribed effects on B cells that may promote humoral immune responses by substantially increasing the number of B cells in the spleen. These effects of IL-4 appear to be at least partially selective (no increase is seen in splenic CD4+ T cell number after IL-4C treatment for 1 day, and treatment with IL-4C for 5 days was associated with a larger increase in splenic B cell than in CD4+ T cell number); occur rapidly; are IL-4Rα dependent and CD4+ T cell, Stat-6, and FcγRII/III independent; and can be observed at doses of IL-4 low enough to induce only small increases in B cell class II MHC expression (Fig. 2A). Endogenously produced IL-4 may well induce similar effects on splenic B cells; greater than 100-fold increases in total body IL-4 secretion and large, IL-4-dependent increases in splenic B cell class II MHC expression and splenic B cell number are observed in mice infected with the nematode parasite Nippostrongylus brasiliensis; the latter effects are blocked when infected mice are treated with anti-IL-4 mAb (41) (J. F. Urban and F. D. Finkelman, unpublished observations).

Five different mechanisms could account for IL-4-induced splenic B cell hyperplasia: increased B cell production, increased migration of recently produced B cells from bone marrow to spleen, increased peripheral B cell proliferation, increased B cell survival, and redistribution of mature B cells to the spleen from other organs. No evidence has been found for increased B cell production; no increase was observed in IL-4C-treated mice in the percentage or number of immature B cells in bone marrow or in the percentage of B cells in spleen that have an immature phenotype. Both of these changes, in contrast, are observed in mice treated with IL-7, an established stimulus of B lymphopoiesis (34). Furthermore, anti-IL-7 treatment, which blocks B lymphopoiesis (21), does not inhibit the initial (day 1) increase in B cell number in IL-4C-treated mice. These same observations are also incompatible with the possibility that IL-4-induced splenic B cell hyperplasia is caused by increased migration of recently produced B cells from bone marrow to spleen. In addition, our observations make it unlikely that IL-4-induced splenic B cell hyperplasia results from increased peripheral B cell proliferation; no increase in the percentage of BrdU+ splenic B cells was observed in mice that were treated with IL-4C while receiving BrdU.

IL-4 does appear, however, to contribute to the increase in splenic B cell number by causing a redistribution of circulating B lymphocytes to the spleen and by increasing the life span of recently generated B cells. Evidence in favor of redistribution is provided by observations that numbers of mature blood and bone marrow B cells decline at the same time that the number of splenic B cells increases, and that the initial (day 1) IL-4-induced increase in splenic B cell number does not depend on the production of new B cells (it is not inhibited by anti-IL-7 mAb treatment). Because the IL-4C-induced increase in mature splenic B cell number is considerably greater than the loss in mature blood or bone marrow B cells (Figs. 8 and 9), and IL-4C treatment does not cause a net loss in B cells from lymph nodes, Peyer’s patch, or intestinal lamina propria, it is necessary to hypothesize that IL-4 induces B cells from an additional source to redistribute to the spleen. The most likely source, which we have not been able to directly evaluate, is lymph; thoracic duct drainage of an adult mouse may yield 2 × 107 B cells in 24 h (42). Thus, we propose that IL-4 has little effect on the net flux of parenchymal B cells from lymph node, Peyer’s patch, or lamina propria but induces actively circulating bone marrow, blood, and lymph B cells to redistribute to the spleen. Because most B lymphocytes rapidly circulate to the spleen under normal conditions (43), this net migratory effect most likely results from decreased exit of B cells from the spleen, perhaps as a result of increased adhesion molecule expression or loss of a molecule require for exit from the spleen, rather than from increased entry of B cells into the spleen.

Our experiments also support the possibility that IL-4C treatment increases splenic B cell number by increasing the survival of newly generated B cells: 1) IL-4C treatment causes a decrease in splenic B cell expression of HSA, consistent with an increase in average B cell age (36); 2) BrdU labeling studies indicate that IL-4 extends the normally short life span of recently generated splenic B cells (44) (the percentage of BrdU-labeled splenic B cells is increased 10 days after the start of a 5-day pulse with BrdU); and 3) the additional increase in splenic B cell number when mice are treated for >1 day with IL-4C, unlike the initial (day 1) increase, is IL-7 dependent and thus probably dependent on new B cell production. It is unlikely that this IL-7 dependence reflects IL-7 stimulation of T cell help, because our anti-IL-7 experiments were performed in mice treated with anti-CD4 mAb and because anti-CD4 mAb treatment did not affect IL-4 induction of splenic B cell hyperplasia. The IL-7 dependence of the increase in splenic B cell number that occurs from 1–3 days after the initiation of IL-4C treatment taken together with the failure of splenic B cell number to increase further when IL-4C treatment is continued for >3 days.
suggests that IL-4 increases the survival of newly generated B cells by ~2 days. Small, but persistent, increases in the number of splenic B cells that have a mature phenotype after IL-4 treatment is discontinued (Fig. 4A), however, suggest that IL-4 may also contribute to increased splenic B cell number to a slight extent by accelerating B cell maturation, inducing some immature splenic B cells to survive long enough to mature into long-lived B cells, and/or enhancing the survival of mature B cells. Thus, our observations provide evidence that IL-4 increases splenic B cell number by increasing the net migration of circulating B cells to the spleen and by increasing splenic B cell survival. These effects of IL-4 may contribute to both the large, IL-4-dependent Ab responses that are made to nematode parasites (45) and to the humoral autoimmunity that is induced in mice by chronic IL-4 overproduction (46).

References


