Transfer of Small Resting B Cells into Immunodeficient Hosts Results in the Selection of a Self-renewing Activated B Cell Population

By Fabien Agenès and António A. Freitas

From the Laboratoire des Dynamiques Lymphocytaires, Centre National de la Recherche Scientifique, Unité de Recherche Associée 1961, Institut Pasteur, 75724 Paris, cedex 15, France

Summary

We studied the role of bone marrow B cell production in the renewal of peripheral B cells and the feedback mechanisms that control the entry of newly formed B cells into the peripheral B cell pools. When resting lymph node B cells are injected into B cell–deficient hosts, a fraction of the transferred cells expands and constitutes a highly selected population that survives for prolonged periods of time by continuous cell renewal at the periphery. Although the number of donor B cells recovered is low, a significant fraction shows an activated phenotype, and the serum immunoglobulin (Ig)M levels are as in normal mice. This population of activated B cells is resistant to replacement by a new cohort of B cells and is able to feedback regulate both the entry of newly formed B cells into the peripheral pool and terminal differentiation. These findings suggest that peripheral B cell selection follows the first come, first served rule and that IgM-secreting cells are generated from a pool of stable activated B cells with an independent homeostasis.

Key words: homeostasis • B cell renewal • lymphocyte competition • B cell lifespans • natural antibodies

In adult mice, the presence of a homeostatic control of B cell numbers implies a kinetic steady state where cell production equals cell loss (1). According to current estimates, the rate of B cell production ($1–2 \times 10^7$ B cells generated daily) could replenish the peripheral B cell pool in 5–10 d (2–6). It was also shown that the number of peripheral B cells is not limited by the rate of B cell production in the bone marrow (BM)[1] (7, 8); mice with a threefold reduced BM B cell production still had normal numbers of peripheral B cells and the B cell production of one mouse sufficed to replenish the peripheral B cell pools of three mice (7). In an immune system where there is a continuous excess of B cell production and the total number of B cells is kept constant, each newly produced B cell can only establish itself upon loss of other cells (9), i.e., the rate of peripheral B cell renewal depends on the life span of peripheral B cells. However, the life expectancy of a B lymphocyte is not an intrinsic property of the cell, but is determined by the environment (10) and by the presence or absence of other competing cell populations (9, 11). Therefore, the persistence of B cells at the periphery could be modified by the continuous arrival of the newly formed BM migrants, while the fate of the latter at the periphery could also be altered by the presence or absence of resident B cell populations.

In this study, we investigated the possible mutual influences between established resident populations of peripheral B cells and newly formed B cell migrants. The questions addressed by these studies are: What is the role of B cell production in peripheral B cell renewal? Are there feedback mechanisms that control new B cell entry into the peripheral B cell pools? To answer these questions we compared (a) the persistence of peripheral B cells in the presence or absence of new BM B cell production; for this purpose we studied the fate of mature resting B cells transferred alone or simultaneously with a pool of precursor cells into immunodeficient mice ($Rag2^{-/-}$); and (b) the fate of a population of B cells (recent BM migrants or mature B cells) in the presence of a previously established peripheral B cell population; in this case we followed the seeding and the persistence of a second population of B cells in B cell–deficient mice previously injected with a cohort of mature B cells.

These studies provide a novel insight into the origin of IgM-secreting cells and serum IgMs, and into the independent homeostatic control of resting and activated peripheral

[1] *Abbreviations used in this paper:* BM, bone marrow; BrdU, bromodeoxyuridine; LN, lymph node; SPL, spleen; Tg, transgenic.

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B cell pools. They also contribute to our understanding of B cell physiology during peripheral lymphocyte reconstitution of immunodeficient hosts.

Materials and Methods

Mice. The inbred strains of mice C57Bl/6 (IgH^b, $Ly5^2$), Ly5 congenic C57Bl/6 (IgH^b, $Ly5^1$), IgH congenic C57Bl/6 (IgH^a, $Ly5^2$), SP6 transgenic (Tg) C57Bl/6 (transgene IgM^a, $Ly5^2$) (12), MD4 Tg C57Bl/6 (transgene IgM^a, $Ly5^2$) (13), B cell–deficient IgM homologous recombinant mutant μMT C57Bl/6 (Ig^-, $Ly5^2$) (14), the alymphopenic Rag2-deficient mutant $Rag2^{-/-}$ C57Bl/6 (Ig^-, $Ly5^2$) (15), the T cell–deficient CD3 homologous recombinant mutant $CD3\epsilon^{-/-}$ C57Bl/6 (IgH^b, $Ly5^2$) (16), and the B6-Eμ-bcl-2-22 Tgs (17) were obtained from Iffa-Credo and the Centre de Développement des Techniques Avancées, Centre National de la Recherche Scientifique, France.

Cell Transfers. Sterile suspensions of pooled mesenteric and inguinal lymph nodes (LN) collected free of blood were prepared. Host 8-wk-old $Rag2^{-/-}$ mice were exposed to irradiation given by a Cesium source. A 300 rad dose of irradiation was given to the animals when they were reconstituted with a single injection of LN cells. The irradiation dose was increased up to 900 rad when BM cells were added to the inoculum, to allow seeding of the precursor cells. In this case $Rag2^{-/-}$ and μMT hosts were used indifferently with similar results. In experiments involving sequential injections (two injections of LN suspensions separated by a time interval of 4 wk) the recipient mice (in this case only $Rag2^{-/-}$ were used) were not irradiated. We must note that the dose of irradiation did not modify the fate of transferred mature B cells. A total of $\sim 1–2 \times 10^7$ LN cells and/or 10^6 BM cells were injected intravenously (retro-orbital vein) into the immunodeficient hosts. Injected populations differ by allotypic markers ($Ly5^{1 \text{ or } 2}$ or $IgH^{a \text{ or } b}$ allotype).

Flow Cytometry Analysis. The following mAbs were used: anti-$Ly5^1$ (A20), anti-$Ly5^2$ (104.2), anti-B220 (RA3-6B2), anti-IgM (R6-60.2), anti-IgM^a (RS3.1), anti-IgM^b (MB86), anti-HSA (J11d or M1/69), anti-IgD, anti-CD23 (B3B4), anti-Mel14 (CD62L), anti-CD5 (53-7.3), and anti-CD21 (7G6). Cells were triple stained with appropriate combinations of FITC-, PE-, and biotin-labeled antibodies, followed by streptavidin TRI-Color (Caltag). Dead cells were excluded from the analysis by light-scatter and/or propidium iodide. All analyses were performed on a FACScan® (Becton Dickinson) interfaced to a Macintosh computer with the Becton Dickinson Cell Quest software.

Quantification of B Cell Subpopulations in the Host Mice. Recipient mice were killed at different times after transfer. A standard procedure was used to prepare single cell suspensions from spleen (SPL), BM, and a pool of mesenteric and inguinal LN. The total number of B cells present in each organ was calculated from the frequency estimated by immunofluorescence analysis and the total number of cells recovered in each organ.

BrdU Labeling. Mouse chimeras received 1 mg of BrdU intraperitoneally every 4 h the day before killing (four injections). SPL cells from BrdU injected mice were triple stained with PI and the appropriate anti-B220 and anti-IgM antibody combinations. The $B220^+IgM^+$ labeled B cells sorted using a FACStar$^{+®}$ (Becton Dickinson). Purified populations were fixed, their DNA denatured and labeled with a mouse γ1 anti-BrdU monoclonal (a gift of Dr. T. Ternynck, Institut Pasteur, Paris, France) followed by a FITC-labeled goat anti–mouse γ1 Ab (Southern Biotechnology Associates). The fraction of $BrdU^+$ cells in each purified B cell population was evaluated on a FACScan®.

ELISA. Sera Ig concentrations were quantified by ELISA. Plates were coated either with antibodies to total IgM, IgM^a, or IgM^b and saturated with PBS-1% gelatin. Dilutions of sera were added. After incubation (1 h, 37°C) and washing, peroxidase-labeled goat anti–mouse IgM antibodies were added. After incubation and washing, bound antibodies were revealed with the substrate *O*-phenylenediamine and H_2O_2. The reaction was stopped after 10 min by addition of 10% SDS and the absorbance read at 450 nm in a Titertek Multiscan Spectrometer (Flow Laboratories). Titration of serum IgM was performed using as standards purified mouse IgM (Southern Biotechnology, Inc.). IgM concentrations were determined by comparing the displacement of the dilution curves in the linear interval between standards at a concentration of 10 μg/ml and the serum samples.

ELISA Spot Assay. The quantification of IgM secreting cells was assayed by a modification of the Sedgwick and Holt ELISA Spot Assay technique (7). In brief, plates were coated with goat anti–mouse IgM antibodies or antiallotypic IgM antibodies. After saturating, the cells were distributed into the microwells in RPMI 1640–2% FCS. The plates were incubated for 4–6 h at 37°C, 5% CO_2 atmosphere. After an extensive wash, plates were incubated with goat anti–mouse IgM labeled with alkaline phosphatase. After washing, the revealing substrate was added (2.3 mM 5-bromo-4-chloro-3-indolyl phosphate diluted in 2-amino-2-methyl-1-proprenolol buffer).

RT-PCR. Populations of LN-derived total ($B220^+IgM^+$), SPL resting ($IgM^{low}IgD^{high}$), or SPL activated B cells (IgM^{high} IgD^{low}) were purified by cell sorting after staining with the appropriate antibody combinations. Total RNA was isolated from the sorted B cells (>99% pure). RNA was reverse transcribed into cDNA. The primers used for the detection of HPRT, IL-2, IL-4, IL-6, IL-10, IL-12, and IFN-γ cDNA were as described previously (18). The 40 cycles of amplification were: 10 s at 94°C, 30 s at 60°C, 30 s at 72°C, followed by 10 min of elongation at 72°C. Samples were separated in 2% agarose gels and stained with ethidium bromide.

Results

Fate of Resting LN B Cells Transferred into Immunodeficient Host Mice in the Absence of BM B Cell Production. To study the fate of a peripheral population of resting B cells in absence of de novo BM B cell production, we transferred 10^7 LN cells from B6.$Ly5^1$ donors into 300 rad irradiated B cell–deficient B6.$Ly5^2$ hosts (B6.$Rag2^{-/-}$). These LN B cells are highly enriched for $IgM^{low}IgD^{high}$ small resting cells (>98%) which were HSA^{low}, $CD21^{int}$, $CD23^{bright}$, Mel14 $(CD62L)^{bright}$, $CD44^{int}$, $CD5^-$, and $CD69^-$ (see below and data not shown). The transferred cell cohort lacked hematopoietic precursors, as their injection into lethally irradiated (900 rad) mice failed to rescue these recipient mice, which all died between days 10 and 14 after transfer.

We quantified the number of donor LN B cells recovered from the spleen of the host mice during the 16-wk period after transfer (Fig. 1 A and data not shown). Soon after injection and homing the number of donor B cells decreased: of the $4–5 \times 10^6$ B cells transferred only 10^5 B lymphocytes survived at day 2. From day 2 onwards the number of donor B cells increased to reach a maximum of $1–2 \times 10^6$ by day 14, corresponding to ~30% of the number of B cells injected. This increase in donor B cell num-

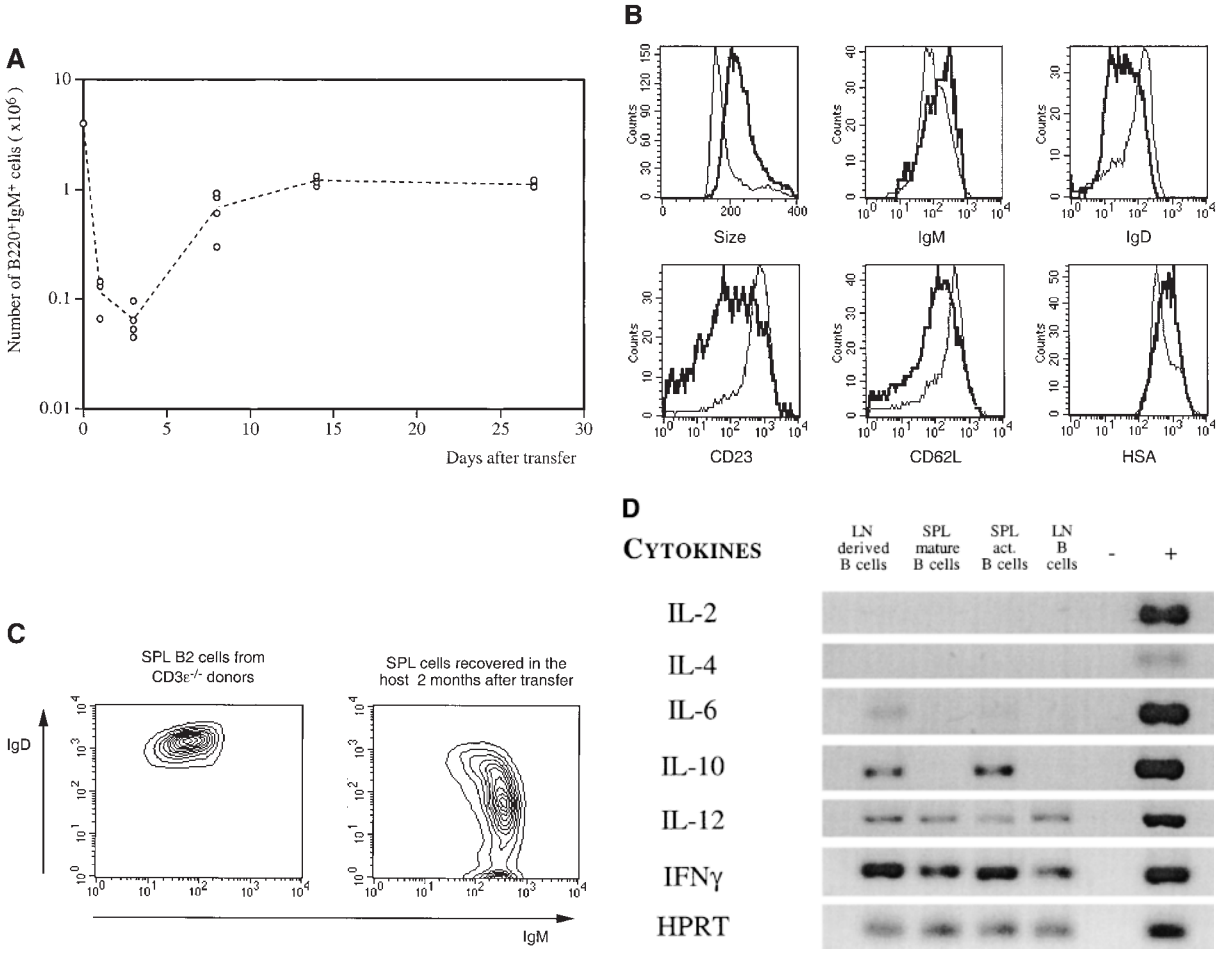

Figure 1. (A) Fate of LN B cells transferred into immunodeficient Rag2$^{-/-}$ mice. Each host mouse was injected with 10^7 cells (4×10^6 B cells). Each point represents the number of donor LN-derived B cells (B220$^+$ μ^+) recovered in the SPL of three to six host mice as a function of time. The curve joins the mean of the values obtained at each time point. (B) Phenotype of in vivo activated B cells. Rag2$^{-/-}$ mice were injected with 4×10^7 LN cells (1.8×10^7 B cells). The hosts were killed 2 mo later and the SPL cells analyzed by flow cytometry. Histograms are gated for the B220$^+$ B cell population. Bold line, phenotypic analysis of LN-derived B cells. Thin line, phenotypic analysis of splenic B cells from C57Bl/6 control mice. (C) Contour plot showing the sorted B2 cells from CD3$\epsilon^{-/-}$ donors before and after in vivo activation in Rag2$^{-/-}$ hosts. Similar observations were made when LN cells from the same donors were transferred. (D) Cytokine profile of in vivo activated B cells. RT-PCR was performed on sorted B cells (>99% pure). Lanes from the left to the right: splenic cells from Rag2$^{-/-}$ mice injected 1 mo before with LN cells from CD3$\epsilon^{-/-}$ donors (pool of 10 mice); mature B cells (IgM^{low}IgD^{high}) from the SPL of CD3$\epsilon^{-/-}$ mice (pool of 5 mice); activated B cells (IgM^{high}IgD^{low}) from the SPL of CD3$\epsilon^{-/-}$ mice (pool of 5 mice); B cells from the LN of CD3$\epsilon^{-/-}$ mice (pool of 5 mice). (E) Number of IgM-secreting cells in the SPL of immunodeficient hosts injected with 10^7 cells (4×10^6 B cells) as a function of time. Each point represents one individual mouse and the curve joins the mean values obtained in each time point.

bers was due to the peripheral expansion of donor cells, since after a 24-h pulse of BrdU, given at day 5 after transfer, a significant fraction of the donor B cells was cycling (20% of the B cells were BrdU$^+$) (Table I).

The population of donor B cells recovered at 2 wk after injection was able to persist at constant numbers up to 4 mo. A significant fraction of these cells incorporated BrdU (Table I), suggesting that self-renewal contributed to their prolonged survival. In spite of the continuous division, transferred B cells were unable to reconstitute normal peripheral B cell numbers.

When the B cell populations recovered 1 mo after transfer were characterized, a significant fraction of these B cells exhibited an activated phenotype (19); their size was in-

Table I. *Fraction of BrdU-labeled B Cells in Normal and Immunodeficient Hosts*

	% $BrdU^+$ B cells
LN-derived B cells at day 6	19.0 ± 4.0
LN-derived B cells at day 30	5.8 ± 1.7
B cells from normal C57Bl/6 mice	1.4 ± 0.2

BrdU staining of sorted SPL cells from C57Bl/6 control mice and from immunodeficient chimeras injected with LN cells. Each mouse was injected with 4 i.p. injections of 1 mg of BrdU. The day after, the mice were killed, the SPL $B220^+\mu^+$ cells sorted, and $BrdU^+$ cells identified as described in the Materials and Methods section (mean ± SD of three to five mice). Chimeras were injected with 10^7 total LN cells and killed either 6 d (phase of exponential growth) or 4 wk (steady state) after transfer.

creased, they were IgM^{bright}, IgD^{dull}, HSA^{bright}, $CD23^{dull}$, Mel14 $(CD62L)^{dull}$, $CD44^{int}$, and, in contrast to recent BM migrants, they were $CD21^{bright}$ (Fig. 1 B and data not shown) (20). They did not express the CD5 marker, the CD69 early activation marker, and they were $Mac1^-$, B7-1^-, B7-2^-, and $Syndecan^-$. We must note that the transferred LN B remained $CD5^-$ throughout the duration of the kinetics. These findings suggest that a fraction of the donor B cells differentiates from a resting to an activated phenotype or that rare activated cells present in the cell inoculum can expand substantially after in vivo transfer. This latter possibility is unlikely since 2 mo after transfer of highly purified splenic $IgM^{dull}IgD^{bright}$ B cells from $CD3\epsilon^{-/-}$ donors into $Rag2^{-/-}$ mice again, we recovered predominantly cells bearing an activated phenotype (Fig. 1 C).

The interleukin mRNA expression of $B220^+IgM^+$ sorted LN $CD3\epsilon^{-/-}$ donor B cells before and 2 mo after in vivo transfer, and of resting ($IgM^{low}IgD^{high}$) and activated B cells ($IgM^{high}IgD^{low}$) sorted from the SPL of normal $CD3\epsilon^{-/-}$ mice was studied. As shown in Fig. 1 D, all B cell populations transcribed IFN-γ and IL-12 mRNA, but we could not detect IL-2 or IL-4 mRNA transcription. Transferred B cells, as well as sorted activated splenic B cells, were unique in their transcription of IL-10 and IL-6 mRNA (Fig. 1 D).

We studied the splenic IgM-secreting cells and serum IgM levels of the immunodeficient mice hosting transferred B cells. We found that by 2 wk after injection, the number of IgM-secreting cells (~3×10^5) (Fig. 1 E) as well as the serum IgM levels (200–250 mg/ml) in transferred hosts reached the normal levels found in a B6 mouse. Whereas IgM-secreting cells in normal mice generally represent ~0.2–0.6% of the total number of splenic B cells, in injected immunodeficient hosts the IgM-secreting cells represent 7–30% of all splenic B cells. This finding confirms previous observations about the presence of normal numbers of activated IgM-secreting B cells and serum IgM levels in mice with reduced peripheral B cell numbers (7, 21). We conclude that, after transfer, donor B cells were rapidly activated to establish the normal number of plasmocytes and serum levels of IgM. The results reported were obtained regardless of the presence of T cells or when donor and host mice were devoid of T cells, i.e., when $B6.Rag2^{-/-}$ were injected with resting B cells from $CD3\epsilon^{-/-}$ mice (see results shown in Fig. 1, C and D). We concluded that the full reconstitution of the persistent activated IgM-secreting B cell compartment is T cell independent.

Fate of B Cells as a Function of the Number of Lymphocytes Injected. Immunodeficient mice were injected with varying numbers of LN B cells (from 0.1 to 40×10^6) and the number and phenotype of the B cells recovered in different host tissues studied 2 mo after transfer. Comparing different groups of hosts, we observed that in mice injected with 10^6 and 30×10^6 cells (a 30-fold difference) the difference in the number of B cells recovered 4 mo after transfer was only sixfold, suggesting that after the initial burst of expansion, the transferred B cells tend to slowly reach a stable equilibrium number (Fig. 2 A). Independent of the initial number of B cells transferred, the number of IgM-secreting cells recovered in all recipient mice was identical and similar to the physiological number of IgM-secreting cells of normal C57Bl/6 (B6) mice (Fig. 2 B).

Studying the homing of the transferred cells, we found that in mice injected with $<10^6$ B cells we were not able to recover any donor B cells outside the host SPL (Fig. 2 C). The vast majority of splenic donor–derived cells showed an activated $IgM^{bright}IgD^{dull}$ phenotype. With the transfer of progressively higher numbers of B cells, we recovered a similar number of B cells bearing an activated phenotype which localized mainly in the host SPL as well as increasing numbers of resting B cells that accumulate first in the SPL and subsequently in the LN and BM (Fig. 2, C and D). Donor B cells were not detected in the peritoneal cavity of the different chimeras. These results suggest that when low numbers of B cells are available there is a preferential generation of cells with an activated phenotype that ensures normal levels of IgM antibodies. Only after replenishment of the activated B cell compartment do resting B cells accumulate at the periphery.

Replacement of Transferred Peripheral B Cells by a Second Population of LN B Cells: Feedback Regulation of Terminal Differentiation of the Second B Cell Population. We showed that after transfer into immunodeficient hosts and in absence of other B cell populations, a fraction of the injected mature resting B cells persists at the periphery. To study whether the survival of these B cells was modified by presence of other B lymphocyte populations, $Rag2^{-/-}$ recipients were injected with 2×10^7 $Ly5^1$ IgH^a LN B cells alone, or coinjected with the same number of $Ly5^2$ IgH^b LN B cells 28 d later. The fate of the different donor B cell populations in host mice was followed for 2 mo.

We found that the fate of the first donor B cell population was not modified by: the injection of a second inoculum; the total number of the B cells recovered (Table II); the fraction of these cells bearing an activated phenotype, or the fact that the total number of IgM plasma cells and the serum IgM secreted by the first donor cell population

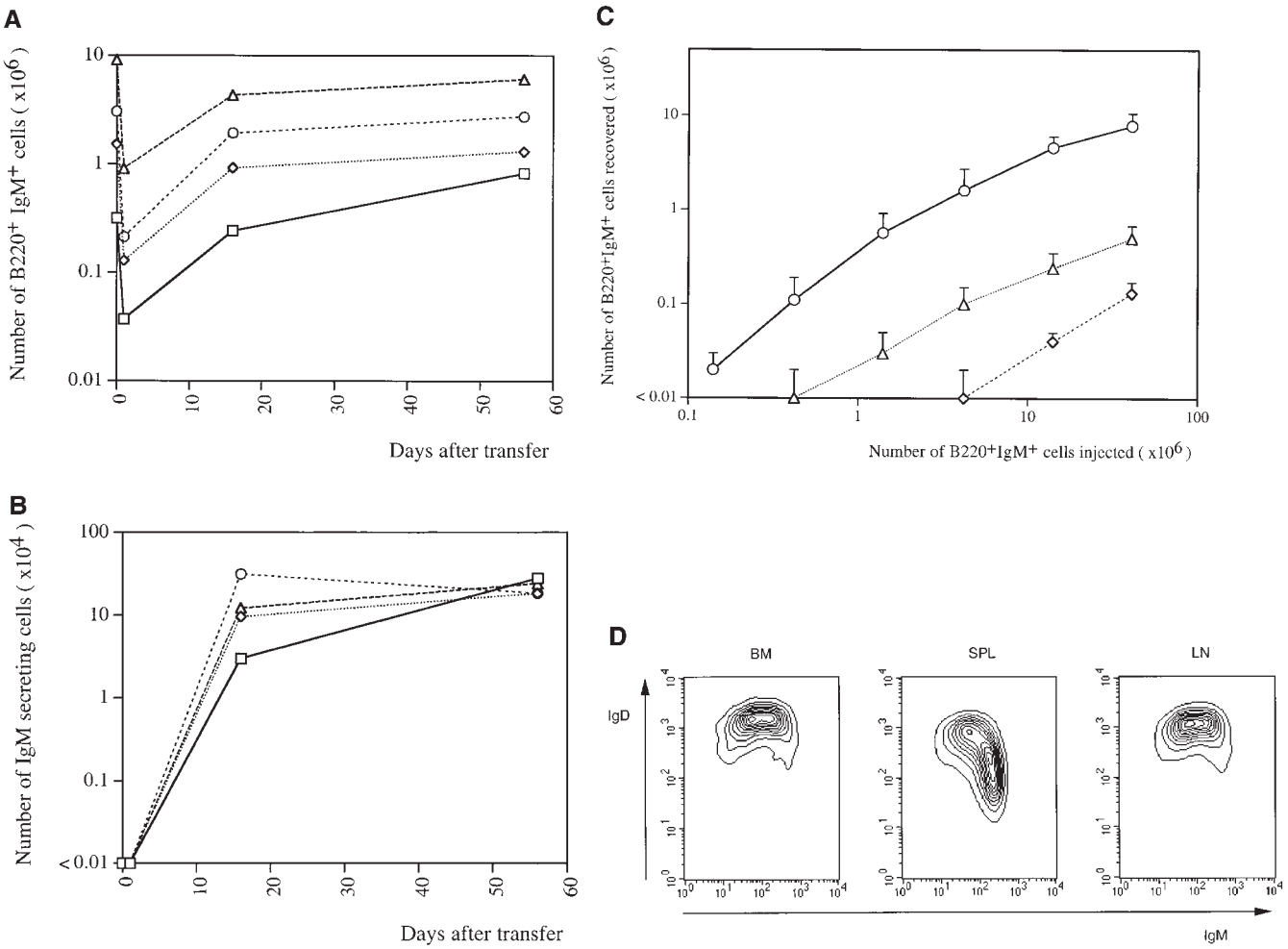

Figure 2. (A) Number of B cells recovered in the SPL of immunodeficient Rag2$^{-/-}$ mice as a function of time. Each curve corresponds to a group of mice (three to six individuals) injected with a particular number of cells: □, 10^6 LN cells (0.3×10^6 B); ◇, 5×10^6 LN cells (1.5×10^6 B cells); ○, 10×10^6 LN cells (3×10^6 B cells); and △, 30×10^6 LN cells (9×10^6 B cells). (B) Number of IgM-secreting cells recovered in the SPL of immunodeficient mice as a function of time. Each curve corresponds to a group of mice (three to six individuals) injected with a particular number of cells: □, 10^6 LN cells (0.3×10^6 B cells); ◇, 5×10^6 LN cells (1.5×10^6 B cells); ○, 10×10^6 LN cells (3×10^6 B cells); △, 30×10^6 LN cells (9×10^6 B cells). (C) Number of B cells recovered in the SPL (○), the LN (△), and the BM (◇) as a function of the number of LN B cells injected into immunodeficient mice two mo after transfer (mean ± SD of three to six mice). (D) Phenotype of B cells recovered in the BM, SPL, and LN of the same immunodeficient mouse reconstituted with 4×10^7 LN B cells. In the SPL the majority of B cells show an activated phenotype (IgM^{high}IgD^{low}), whereas resting B cells (IgM^{low}IgD^{high}) accumulate in the SPL, LN, and BM.

remained the same after the transfer of a second B cell population (not shown). With respect to the second population, a fraction of these new B cells survived in host mice, adding to the numbers of the first B cells already present in the host (Table II). The kinetics and cell recovery of this second population was not affected by the presence of a first B cell population (not shown). However, the majority of the persisting new B cells retained a resting phenotype. Thus, in host mice injected sequentially with two sets of resting LN B cells we found that the majority of the activated B cells (Fig. 3 A) and most of the serum IgMs (>80%) are derived from the first group of B cells. Directly comparing the amount of IgM produced by the same B cells transferred alone or into mice hosting an established population of B cells, we found that the level of serum IgM produced by the second B cell population was diminished (Fig. 3 B). These findings suggest that once the compartment of peripheral activated B cells is replenished and normal serum IgM levels are attained, there exists a feedback mechanism which inhibits the differentiation and IgM secretion of a second population of transferred B cells. However, this feedback regulation did not prevent in these experimental conditions the accumulation of the newly injected B cells in a resting state.

Replacement of Transferred Peripheral B Cells by a Second Population of BM-derived B Cells in Immunodeficient Hosts. Next we studied the possible mutual influences between a resident B cell population and BM newly formed B cell migrants. For this purpose, 10^7 LN cells and 10^6 BM cells were coinjected intravenously into 900 rad irradiated immunodeficient host mice (B6μMT or B6.Rag2$^{-/-}$). Using this approach, we allow the establishment of a peripheral B cell

Table II. *Number of B Cells Recovered in the Spleen of Mice Injected with One or Two LN Cell Populations*

Number of LN B cells injected ($\times 10^6$)		Number of B cells recovered/host spleen from ($\times 10^6$)	
First population ($Ly5^2IgH^a$)	Second population ($Ly5^1IgH^b$)	First population ($Ly5^2IgH^a$)	Second population ($Ly5^1IgH^b$)
7	7	2.36 ± 0.30	2.15 ± 0.20
7	–	2.23 ± 0.49	–
–	7	–	2.46 ± 0.68

$Rag2^{-/-}$ mice received one or two injections of 7×10^6 LN B cells within a time interval of 4 wk. Control mice received just one injection of either LN population. The values represent the number of LN-derived B cells recovered in the spleen of the different hosts 4 wk after the transfer of the second LN population (i.e., 8 wk after the first population). Mean ± SD of five to seven mice per group.

population which occurs 2 wk after mature LN B cell transfer (Fig. 1), i.e., before the appearance of newly formed BM-derived cell migrants. Therefore B lymphocytes of BM origin enter a peripheral compartment which is already seeded with activated LN-derived B cells. Since irradiation of the hosts was required to allow BM reconstitution, we first studied the fate of LN B cells in lethally irradiated hosts coinjected with BM cells from μMT donors (unable to generate B cells). We found that the kinetics of recovery of the transferred LN B cell population was identical to that described in nonirradiated or 300 rad irradiated mice reconstituted with LN B cells alone (see Fig. 1 A). Thus, the fate of the transferred B cells was independent of host irradiation.

We compared the fate of the different B cell populations in mice coinjected with LN B cells and BM cells from immunocompetent donors. In these mice, the total number of B cells of LN origin was comparable to those recovered in absence of BM derived B cells, i.e., was unchanged by the continuous BM B cell generation and B cell output (Fig. 4 and Table III). The rate of reconstitution of peripheral B cell numbers by the BM-derived B cells was also similar in mice reconstituted with BM alone or mixtures of LN and BM cells. In contrast to the population of persisting LN-derived B cells the vast majority of the BM-derived B cells showed a resting $IgM^{dull}IgD^{bright}$ phenotype (not shown). From 2 wk onwards the number of BM-derived B cells present at the periphery was 10–100 times higher than the number of LN-derived B cells (Fig. 4).

The total serum IgM concentrations were similar to those in normal mice. The number of IgM-secreting cells of BM origin represented <1% of the total number of BM-derived B cells, whereas the number of IgM-secreting B cells of LN origin was ~6% of the number of LN-derived B cells. However, the levels of IgM of LN origin were diminished compared to mice injected with LN B cells alone, suggesting that a small fraction of the LN-derived IgM-secreting cells had been successfully replaced from the bulk of BM-derived cells in excess.

We conclude that a fraction of the LN-derived B cells upon activation can persist as a stable self-renewing population which is only very slowly replaced by newly formed B cells generated in the BM. In the presence of this resident activated population newly produced BM B cells accumulate to fully reconstitute the physiological size of the resting B cell pool.

Increased Peripheral B Cell Survival Modifies the Rate of Entry of Newly Formed B Cells from the BM into the Peripheral Pool. Constitutive expression of the protein encoded by the protooncogene bcl-2 can enhance the survival of both immature and mature B cells (17, 22). To investigate whether an increased cell survival and accumulation of B cells at the periphery could modify the persistence of the transferred LN populations or the rate of entry of new BM B cell migrants we used cells from control and bcl-2 Tg donors to reconstitute immunodeficient host mice.

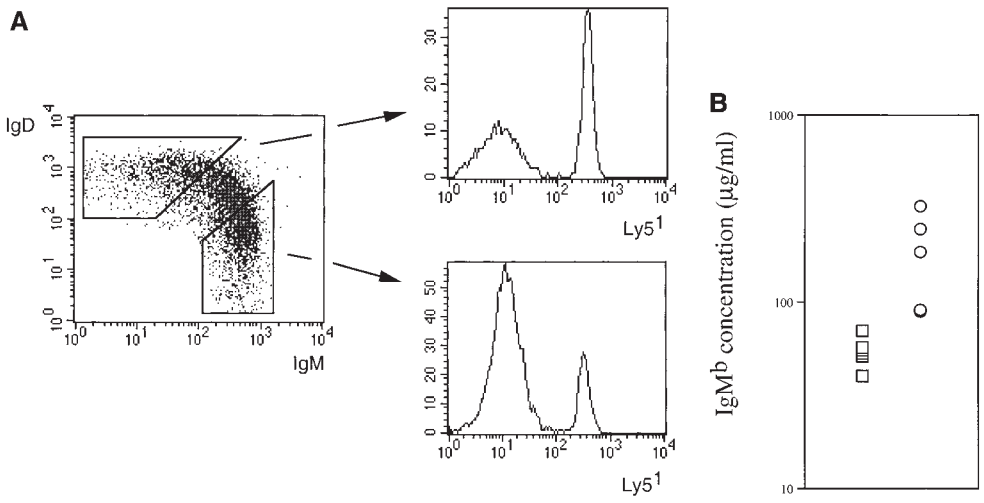

Figure 3. (A) IgM/IgD staining on SPL B cells recovered in a $Rag2^{-/-}$ mouse injected with two LN populations sequentially. (Top) Relative proportion of $Ly5^1$ cells in $IgM^{low}IgD^{high}$ subpopulation (mature B cells). (Bottom) Relative proportion of $Ly5^1$ cells in $IgM^{high}IgD^{low}$ subpopulation (activated B cells). Note that activated B cells ($IgM^{high}IgD^{low}$) are primarily $Ly5^{1-}$, thus derived from the first population injected. (B) IgM^b allotype concentrations in mice injected with $B6Ly5^1$ LN population ($Ly5^1$, IgH^b) either alone (○) or that also received B6IgHa LN population ($Ly5^2$, IgH^a) 1 mo before (□). Mice were killed 1 mo after the transfer of the $B6Ly5^1$ LN population.

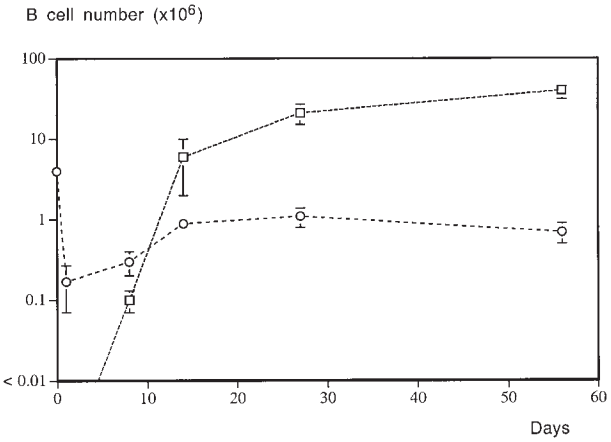

Figure 4. (A) Fate of LN-derived and BM-derived B cells in immunodeficient mice. Each host mouse was injected simultaneously with 4×10^6 LN B cells and 10^6 BM cells. Each point represents the number of donor LN-derived B cells (○) and BM-derived B cells (□) recovered in the SPL of three to six host mice as a function of time. The curves join the mean of the values obtained at each time point for each B cell population.

We compared the persistence of the LN-derived B cells from normal B6.Ly5^1 donors in chimeras simultaneously receiving BM cells from either B6 control or B6.bcl-2 Tg Ly5^2 donors. 2 mo after transfer we recovered an identical number of LN-derived B cells in the two groups of host mice (Table III, Exp. 1). This finding suggests that the established B cells represent a highly selected population which persists even when in the presence of newly formed B cells with an increased life span.

We also injected immunodeficient mice with BM precursors from normal B6.Ly5^1 donors and LN cells from B6.Ly5^2 normal or bcl-2 Tg mice. In the chimeras receiving bcl-2 Tg cells we recovered a significantly higher number of persisting LN-derived B cells than in mice injected with LN cells from non-Tg donors (Table III, Exp. 2). More interestingly, we found that in the chimeras injected with LN cells from bcl-2 Tg donors there was a modest reduction in the number of BM-derived B cells compared to the chimeras receiving LN cells from non-Tg mice (Table III, Exp. 2). To further elucidate these results we performed a third experiment in which we transferred BM precursors from double congenic B6.Ly5^{1}IgH^a donors alone or together with LN B cells from bcl-2 Tg Ly5^{2}IgH^b donors. By transferring a higher number of LN cells (5×10^7) we were able to recover an increased number of LN-derived B cells. In this case we observed that the reduction of the number of BM-derived B cells in the hosts coinjected with LN cells was more marked than in the previous experiment (Table III, Exp. 3). Considering that the actual number of resident peripheral B cells is minor ($\sim 9 \times 10^6$) compared with the total number of BM-derived B cells these findings may suggest that the presence of an increased number of long-lived B cells can reduce or delay the entry of new B cells from the BM into the peripheral B cell pools. In these latter chimeras using double congenic Ly5 and IgH B6 donors we were also able to test the differential origin of the serum IgMs. We found that the serum IgM^b levels produced by the LN-derived bcl2 Tg B cells were increased (518.4 ± 51.8 μg/ml) compared to those in mice hosting normal B cells. In contrast the IgM^a secretion by BM-derived B cells was considerably reduced in the presence of the LN-derived bcl2 Tg population (IgM^a serum levels of BM cells alone: 193.6 ± 83.9 μg/ml; in the presence of LN cells: 9.3 ± 4.8 μg/ml). We concluded that the feedback regulation capacity seems to correlate with the number and the increased life span of resident peripheral B cells.

Discussion

In this study we have investigated whether the persistence of peripheral B cells could be modified by de novo BM B cell production and whether the entry of BM migrants

Table III. *Number of BM-derived and LN-derived B Cells in Chimeras Injected Simultaneously with LN and BM Cells from Different Donor Mice*

	Number of B cells recovered per host spleen ($\times 10^6$)		
	Injected cells	BM-derived	LN-derived
Exp. 1	LN Ly5^1 alone	—	1.05 ± 0.22
	LN Ly5^1 + BM Ly5^2	37.4 ± 9.2	0.97 ± 0.34
	LN Ly5^1 + BM Ly5^2.bcl2Tg	103.6 ± 26.8	1.11 ± 0.36
Exp. 2	LN Ly5^2 + BM Ly5^1	50.1 ± 20.5	1.56 ± 0.84
	LN Ly5^{2}bcl2Tg + BM Ly5^1	30.2 ± 13.6	4.88 ± 3.6
Exp. 3	BM Ly5^1.IgH^a alone	49.4 ± 2.6	—
	LN Ly5^2.IgH^b bcl2Tg + BM Ly5^1.IgH^a	17.8 ± 9.2	8.51 ± 0.9

B cell–deficient Rag2$^{-/-}$ mice were lethally irradiated and injected simultaneously with mixtures of LN (10^7 in Exp. 1; 1.5×10^7 for Exp. 2, and 5×10^7 in Exp. 3) and BM cells ($1–2 \times 10^6$) from different donor mice. 1 mo after reconstitution host mice were killed and the number of LN-derived and BM-derived B cells quantified in the spleen. Each value represents the mean ± SD of five to eight mice. In Exp. 3, where donor mice also differed by IgH allotype the IgM levels produced by either LN- or BM-derived B cells were quantified (see Results).

could be altered by the presence of a population of resident B cells in the periphery. This novel approach could clarify contradictory results obtained in studies on lymphocyte life spans and renewal rates (for review see reference 23).

We studied the fate of mature LN B cells injected into immunodeficient hosts unable to produce B cells. We found that a fraction of the transferred population of LN B cells expands and persists for prolonged periods of time. These results contrast with the fate of B cells injected into fully immunocompetent adult hosts, where it was reported that most of the injected cells were lost shortly after transfer (1). However, the same donor B cells persisted when transferred into newborn or 1-wk-old hosts (10). These experiments confirm that B cell survival is not predetermined by intrinsic properties of the cell but rather is dependent on the host environment and the presence of other competitor populations (9).

By following the kinetics of transferred B cells we found that 48 h after the injection of a total of 4×10^6 LN B cells we recovered only 10^5 donor B cells in the host SPL, i.e., less than the 20% predicted by homing (24). A fraction of these cells divides extensively and at day 15 the number of B cells recovered reaches $1–2 \times 10^6$. By extrapolation of the exponential growth curve observed between days 2 and 10 after transfer, we estimate that the persisting B cells represent the progeny of $\sim 3–4 \times 10^4$ cells. This is $\sim$1% of the B cells present in the initial LN inoculum. 2 wk after transfer the number of B cells reached a steady state that remains constant for at least 4 mo. An increased frequency of cycling cells, as well as the continuous production of IgM-secreting cells, suggests that the constant size of the surviving B cell population is maintained by self-renewal. Therefore, our findings do not support previous reports claiming the survival of most of the naive B cells transferred into immunodeficient SCID hosts (25). We show that the B cells persisting after 2 wk of transfer constitute a highly selected population which has undergone at least a 30-fold expansion through five to six rounds of division and which persists by continuous self-renewal in the periphery. Moreover, a significant fraction of the surviving B cells expresses an activated phenotype and $\sim$7–30% of the cells are actively engaged in IgM secretion.

Our findings suggest that the first priority of the transferred cells is the maintenance of normal serum IgM levels to ensure a first barrier of protection. By injecting different numbers of B cells, we found that when low numbers of B cells are available there is a preferential generation of activated IgM-secreting cells which localize almost exclusively in the host's SPL. With the transfer of increasing numbers of B cells there is an augmented recovery of resting B cells which accumulate progressively first in the SPL, then in the LN and later in the BM. Serum concentrations of IgM identical to those of control mice readily were reached in the presence of a reduced B cell number. Resting B cells accumulate at the periphery only when the activated B cell compartment is complete. These results support the notion that the homeostatic regulation of the resting and activated B cell compartments is autonomous, indicating that the two B cell populations belong to different immunological niches. They may differ in survival requirements, interact with different cell types, and follow different patterns of homing and localization, e.g., the diminished expression of L-selectin at the surface of the activated cells may prevent them from homing to the LN (26). Moreover, it has been suggested that after immunization two different subsets of B cells would be responsible for primary (IgM unmutated antibodies) and secondary antibody responses (mutated antibodies) (27, 28). Hence it is possible that the resting B cells generated continuously in the BM are mainly available for immune response towards new antigens, whereas the activated B cells may be committed to exclusively produce natural antibodies. The ability of each subpopulation of resting and activated B cells to respond to different antigens is currently under study.

Once established, the population of activated B cells is rather resistant to replacement and persists even in the presence of new emigrating cells generated from BM precursors or contained in a second inoculum of LN cells. This is not surprising considering that these activated B cells represent a highly selected population particularly adapted to the host environment. The study of the repopulation of immunodeficient hosts by transferred B cells compares the persistence of two populations across the different steps of growth, differentiation, and migration. In contrast, parabiosis studies the relative substitution of two preestablished populations when mixed together. We have found that after parabiosis between B6.Ly5^{2}IgH^a/B6.Ly5^1.IgH^b congenic mice or between Ig-Tg (SP6 or MD4) and non-Tg mice (B6.Ly5^1) the chimerism among the IgM-secreting cells was lower than the chimerism observed among resting B cells (Agenès, F., unpublished observations). These results suggest that resident activated B cells can more easily persist and avoid replacement by incoming new B cells.

The selection of the persisting B cells and the production of IgM in the immunodeficient hosts may partially mimic the early development of the immune system. We would like to extrapolate the origin of the natural IgM-secreting cells from the present results. In newborn and young mice, during the expansion of the immune system and when the mature B cells are scarce, the percentage of activated B lymphocytes is increased (29). In the adult mouse, a proportion of the natural IgM antibodies may be produced by plasma cells derived from a pool of activated B cells selected early during ontogeny which resist replacement (30). Substitution of the initially selected population may be achieved by cellular competition based on B cell receptor diversity and antigenic environment (9, 11, 31). Thus, as observed here, a population of BM-derived B cells would be more likely to enter the pool of IgM-secreting cells than a second population of LN B cells expressing a less diverse repertoire. Studies using Tg animals clearly show that although Tg B cells are in excess and are the first to be produced, most of the circulating IgMs originate from the more diverse endogenous cell pool (32, 33). The immune system seems to be organized to ensure several alternative sources for the production of the natural antibodies which

constitute the first barrier of protection. Every newly formed B cell has the ability to differentiate into a plasma cell, but this process is dependent on the nature and the number of cells already present at the periphery. We propose that early during the development and expansion of the immune system an initial pool of activated B cells is selected, which can eventually be competed out by new specificities formed in the BM (34).

It has also been suggested that B1 B cells are the source of natural IgMs (35). The present findings may be interpreted to suggest that natural IgM production cannot be attributed solely to peritoneal cavity B1 B cells. The transferred LN B cells were $CD5^-$ and remained $CD5^-$ during the expansion phase and at the plateau. In absence of the B1 cells, other B cell populations might be called to exert similar functions. The fact that injected B2 LN B cells acquire a B1b phenotype (36), and migrate first to the spleen and not to the host's LN, shows that these B cells were not irreversibly committed to a particular B cell subset, but that they can undergo a process of adaptive differentiation and ecological succession. We also know that the selection of the persisting B cells and the production of the IgM antibodies are T cell independent, since identical results were obtained in alymphopenic Rag2-deficient mice injected with LN B cells from CD3ε-deficient donors.

Can an established B cell population alter the fate of recent new B cell migrants at the periphery? The answer is yes. As it has been shown that the rates of BM B cell production are not modified by the size of the peripheral B cell pools (7, 8), the finding of a reduced number of BM-derived B cells in chimeras hosting an increased number of resident long-lived B cells from bcl2 Tg donors suggests that the entry of B cells into the peripheral B cell compartments may be conditioned by the number of established B cells. We also demonstrate that in the presence of an established population of activated B cells there is a diminution in IgM production by a subsequently introduced B cell population. These findings indicate that there are mechanisms of feedback regulation controlling terminal B cell differentiation which could explain former results on allotype suppression (37) and on B1 cell development (38, 39). Feedback regulation could be exerted at the level of terminal B cell differentiation and/or B cell proliferation. The similar rate of growth of the second transferred LN B cell population in the absence or the presence of resident B cells (not shown) suggests that it might operate mainly at the level of differentiation. Nevertheless, a complete study of the rate of B cell proliferation in the different experimental conditions is required. Feedback regulation may be also exerted either through direct suppression of new cell subsets, or indirectly, by the preconsumption of common resources necessary for the growth and/or the differentiation of the B cells (40). In the latter case we must assume that the highly selected resident B cells will be more efficient in the usage of the common resources. The different possible mechanisms and the role of the final IgM product in feedback regulation are currently under investigation.

We have a final comment on the constitutive expression of IL-10 mRNA by the activated B cells. This lymphokine downregulates the expression of costimulatory molecules in antigen-presenting cells and affects T cell proliferation by blocking both IL-2Rα expression and IL-2 production (41–43). Activated B cells may have major regulatory functions by preventing inflammation and inducing a Th2 immune response and IgG1 production in their close microenvironment.

In conclusion, we show that B cells remaining after 2 wk of transfer in immunodeficient hosts represent a stable B cell population which persists for prolonged periods of time by self-renewing, resists replacement, and can modify the fate of newly arriving B cells. The present findings, which have profound implications in the mechanisms controlling the origin of the IgM-secreting cells, suggest that the priority of the immune system is the production of circulating IgM levels to ensure a first barrier of protection against infection and that once a particular B cell is selected it is kept by the system. Thus, peripheral B cell selection into the activated B cell compartment follows the rule first come, first served.

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Address correspondence to Fabien Agenès, Laboratoire des Dynamiques Lymphocytaires, CNRS URA1961, Institut Pasteur, 25-28 rue du Dr. Roux, 75724 Paris cedex 15, France. Phone: 33-1-45-68-85-82; Fax: 33-1-45-68-86-39; E-mail: fagenes@pasteur.fr

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