

Continuous Anti-Interleukin 10 Antibody Administration Depletes Mice of Ly-1 B Cells but Not Conventional B Cells

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Summary

Ly-1 B cells have the distinctive property of continuous self-replenishment and, as we have shown previously, can be further distinguished from conventional B cells on the basis of greatly elevated constitutive and inducible production of the recently described cytokine interleukin 10 (IL-10). To test the possibility that IL-10 acts as either an autocrine or paracrine growth factor for Ly-1 B cells, we treated mice continuously from birth to 8 wk of age with a monoclonal rat IgM antibody that specifically neutralizes mouse IL-10. Mice treated in this way lacked peritoneal-resident Ly-1 B cells, contained greatly reduced serum immunoglobulin M levels, and were unable to generate significant *in vivo* antibody responses to intraperitoneal injections of α 1,3-dextran or phosphorylcholine, antigens for which specific B cells reside in the Ly-1 B cell subset. In contrast, conventional splenic B cells of anti-IL-10-treated mice were normal with respect to total numbers, phenotype, and *in vitro* responsiveness to B cell mitogens and the thymus-dependent antigen trinitrophenyl-keyhole limpet hemocyanin (TNP-KLH). The mechanism of Ly-1 B cell depletion appeared to be related to elevation of endogenous interferon γ (IFN- γ) levels in anti-IL-10-treated mice, since coadministration of neutralizing anti-IFN- γ antibodies substantially restored the number of peritoneal-resident Ly-1 B cells in these mice. These results implicate IL-10 as a regulator of Ly-1 B cell development, and identify a procedure to specifically deplete Ly-1 B cells, thereby allowing further evaluation of the role of these cells in the immune system.

Ly-1 B cells (1–5) comprise \sim 2% of the total B cells of an adult mouse and exhibit several intriguing properties that distinguish them from conventional B cells: although barely detectable in most primary and secondary lymphoid tissues (6), they are greatly enriched in the peritoneal and pleural cavities, as are their progeny in gut-associated lymphoid tissue (7, 8); they develop and predominate in early ontogeny (7), and are then self-replenishing for the life of the animal (1, 9); they produce a restricted repertoire (10) of low-affinity antibodies that are highly cross-reactive with self-determinants (11), and do not appear to mature by somatic mutation (10, 12); they generate most of the IgM antibody found in serum (6, 9, 11), and produce the entire antibody response elicited by several bacterial determinants, such as phosphorylcholine and α 1,3-dextran (9, 13). Although their precise role in the immune system is unclear, the various models that have been advanced, based on the specificities of antibodies produced by Ly-1 B cells, include roles in antibacterial immunity (14, 15), in clearance of host cellular debris such as senescent erythrocytes (16, 17), and in modulation of the antibody repertoire during development (18–21). Our recent finding that Ly-1 B cells are potent producers of IL-10 (22, 23), an im-

munosuppressive cytokine that downregulates production of several monokines (24, 25) and T cell-derived cytokines (26, 27), raises the possibility of a broader immunoregulatory role of Ly-1 B cells. Many of these distinguishing features are difficult to evaluate in humans, but a population of Ly1-bearing human B lymphocytes with related properties has been identified (5, 28–32). In this manuscript we test the possibility that constitutive IL-10 production by Ly-1 B cells (22, 23) contributes either directly or indirectly to their demonstrated ability for self-replenishment.

Materials and Methods

Mice. Mid-term pregnant BALB/c mice and C3H/HeJ mice were obtained from Simonsen Laboratory (Gilroy, CA).

Anti-IL-10 Treatment. 5–10 age-matched BALB/c mice were injected intraperitoneally three times per week from birth until 8 wk of age with the neutralizing rat IgM anti-mouse IL-10 antibody designated SXC.1 (33) (0.2 mg/injection for week one, 0.5 mg/injection for week two, 1.0 mg/injection for weeks three to eight), equivalent amounts of an isotype control designated J5/D, or equivalent volumes (100 or 200 μ l) of PBS. Untreated age-matched BALB/c mice were included in all experiments for com-

parison. The SXC.1 and J5/D antibodies were obtained from serum-free hybridoma supernatants, and purified by two sequential 35% ammonium sulfate precipitation steps. In some experiments, mice received similar amounts of a separate rat IgG1 anti-mouse IL-10 antibody designated 2A5 or its isotype, control GL113. After this treatment regimen, pooled spleens, thymuses, lymph nodes, or peritoneal wash cells were collected from each of the four groups of mice and analyzed by flow cytometry and functional assays. In some experiments, mice additionally received the neutralizing rat IgG1 anti-mouse IFN- γ antibody designated XMG1.2 (34), or its isotype control GL113. These latter antibodies were administered intraperitoneally at 0.5 mg/injection for week one, 1 mg/injection for week two, 2 mg/injection for weeks three to eight, according to the previously established regimen of Coffman et al. (35).

Immunofluorescence. Washed cells were stained with combinations of the following reagents: fluoresceinated anti-mouse IgM antibody (DS-1; Pharmingen, San Diego, CA); biotinylated rat anti-mouse IgD antibody (11-26c, produced by J. Kearney); fluoresceinated anti-mouse CD3 antibody (145-2C11, Boehringer Mannheim Corp., Indianapolis, IN); and biotinylated anti-mouse B220 antibody (RA3-6B2; Caltag Labs., South San Francisco, CA). Biotinylated reagents were used in conjunction with phycoerythrin-conjugated streptavidin (Becton Dickinson & Co., Mountain View, CA). Cells were analyzed using a FACScan[®], and dead cells were excluded on the basis of forward angle and side scatter. Results show the fluorescence intensities of 5,000 live cells counted from each experimental group.

Antibody ELISAs. Serum samples collected after 8 wk of treatment were assayed for the presence of mouse IgM using a sandwich ELISA where rat anti-mouse IgM (R8-103, Pharmingen) was coated at 5 μ g/ml on PVC microtiter plates, dilutions of serum samples, or a mixture of several purified myeloma mouse IgM proteins (provided by Dr. Coffman, DNAX) as standard were added, and immune complexes were subsequently detected using biotinylated rat anti-mouse IgM (R19-15, Pharmingen) and avidin-conjugated horseradish peroxidase (CalBiochem Corp., La Jolla, CA), plus 1 μ g/ml substrate (2,2'-azinobis [3-ethylbenzthiazolin sulfonic acid; Sigma Chemical Corp.).

Specific antibody responses against phosphorylcholine and α 1,3-dextran were determined after challenging anti-IL-10 or control mice intraperitoneally with 0.5 ml saline, 50 μ g α 1,3-dextran derived from *Leuconostoc mesenteroides* provided by Dr. Slodki (U.S. Department of Agriculture Agricultural Research Service), or 2×10^8 heat-killed *Streptococcus pneumoniae* as a source of phosphorylcholine, as previously described (36, 37). Sera were collected from all mice 7 d later, and analyzed for specific antibody to α 1,3-dextran or phosphorylcholine using ELISAs described in detail elsewhere (36, 37). Specific antibody responses against TNP-KLH were determined after challenging mice intraperitoneally with 10 μ g TNP-KLH, and collecting sera 7 d later for IgM analysis, and 10 and 14 d later for IgG analysis. TNP-specific antibodies were quantitated using an ELISA described in detail elsewhere (38). In all cases, anti-IL-10 treatment was continued between antigen challenge and sera collection.

Proliferation Assays. Pooled spleen cells at 2×10^6 /ml obtained from three mice in each group were cultured for 3 d in medium alone, or medium supplemented with LPS (50 μ g/ml), goat anti-mouse-IgM antibodies (0611-0201; Cappel Laboratories, Cochranville, PA) (50 μ g/ml), or hamster anti-mouse CD3 antibodies (a gift from Dr. J. Bluestone, University of Chicago, Chicago, IL) (5 μ g/ml). For anti-CD3 stimulation, the antibody was coated onto the microtiter plate before addition of spleen cells. Proliferation was evaluated via incorporation of [³H]thymidine, after a 16-h

pulse with 1 μ Ci/well [³H]thymidine (NET 027; New England Nuclear, Boston, MA).

IFN- γ ELISA. Serum samples collected from anti-IL-10-treated or control mice were assayed for murine IFN- γ using a cytokine-specific ELISA described in detail elsewhere (34, 35).

Results

Continuous IL-10 Neutralization Depletes Mice of Ly-1 B Cells. Male and female BALB/c mice were injected three times per week from birth to 8 wk of age with graded doses of a neutralizing rat IgM anti-mouse IL-10 mAb designated SXC.1 (33), and subsequently analyzed for Ly-1 B cell number and function. Control groups of age-matched BALB/c mice receiving no treatment or equivalent injections of either PBS or an irrelevant rat IgM isotype control (designated J5/D) were included for comparison. Antibodies were administered either intraperitoneally or subcutaneously without significant alteration of the outcome. The antibody injection regimen used yielded an average serum rat IgM level at 8 wk of 50 μ g/ml as measured by a rat IgM-specific ELISA in the case of both SXC.1 and J5/D antibodies (data not shown). After the 8 wk of treatment, the anti-IL-10-treated mice were indistinguishable from the three control groups of mice in terms of the following criteria: total body weight, gross histological examination of liver, spleen, thymus, lymph nodes, intestines, and lungs; hematocrits; total numbers of white blood cells in spleen, thymus, lymph nodes, and peritoneum; and proportions of B cells, T cells, and non-B/T cells in spleen, lymph nodes, and thymus (data not shown). In contrast, immunofluorescent phenotyping of cells obtained in the pooled peritoneal washes collected from the 5–10 mice comprising each of the four experimental groups described revealed a striking depletion of IgM⁺ and IgD⁺ cells in the anti-IL-10 (SXC.1)-treated mice, but not in any of the control animals (Fig. 1). Identical data have been obtained in 24 independent experiments including two using C3H/HeJ mice, and several using a separate rat IgG1 anti-IL-10 neutralizing antibody. Anti-IL-10-treated animals were also depleted of B220-bearing peritoneal cells, as evaluated by immunofluorescence (data not shown), and of LPS-responsive peritoneal cells, as evaluated by the ability of these cells to incorporate [³H]thymidine after 3 d of coculture with 50 μ g/ml LPS (not shown). These findings suggest that anti-IL-10-treated BALB/c mice contain no B cells in their peritoneal cavities, in contrast to the $1\text{--}5 \times 10^6$ B cells that can normally be recovered from this site. It is significant that we show elsewhere (23) by three-color immunofluorescence that the peritoneal cavities of 8-wk-old BALB/c mice in our animal facility contain very few (<5%) conventional B cells. The results shown in Fig. 1 therefore represent a depletion of Ly-1 B cells, predominantly. Despite this striking depletion of peritoneal B cells, the total cellularity of the peritoneal cavities of anti-IL-10-treated mice did not differ significantly from those of the control groups (data not shown). Further immunofluorescence analyses, together with differential hemopoietic cell counts, showed that the loss of B cells was compensated by an increase in peritoneal CD4⁺ T cells and granulocytes in

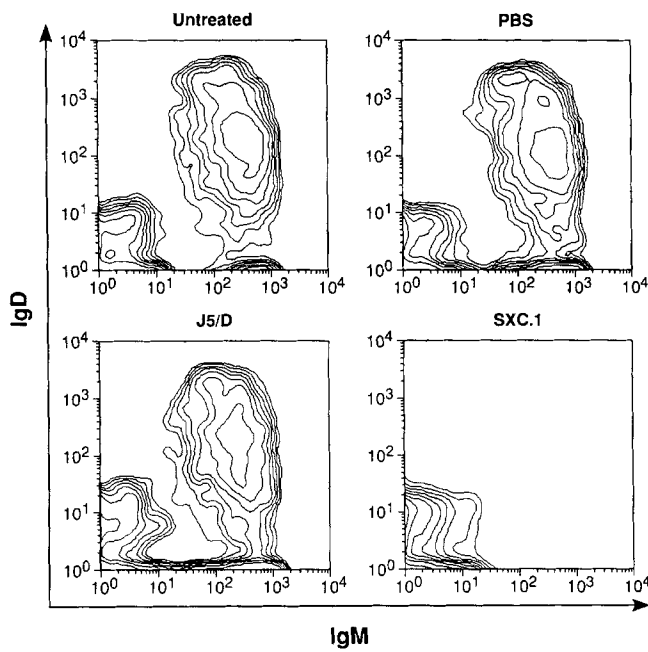


Figure 1. Immunofluorescence analysis of surface IgM and IgD expression by total live peritoneal wash cells obtained from anti-IL-10-treated and control mice. BALB/c mice were injected from birth until 8 wk with SXC-1 anti-IL-10 antibody, J5/D isotype control antibody, PBS, or nothing, as described in Materials and Methods. Results show the fluorescence intensities of 5,000 live cells counted from each experimental group.

the anti-IL-10 treated animals (data not shown). Two other observations indicated that depletion of Ly-1 B cells in anti-IL-10-treated mice occurred throughout the immune system and was not restricted to the peritoneal cavity. First, anti-IL-10-treated mice exhibited a striking 90–100% reduction in serum IgM levels compared with the three control groups, as monitored by a mouse IgM-specific ELISA (Fig. 2). Second, anti-IL-10-treated mice were profoundly deficient in their abil-

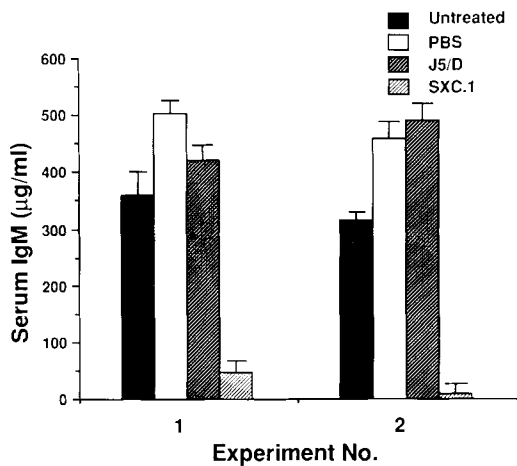


Figure 2. Serum IgM levels of anti-IL-10-treated and control mice tested after 8 wk of treatment. The results show the geometric mean \pm SEM of five individual sera in each group, and include data from two different experiments. Three additional experiments gave identical results.

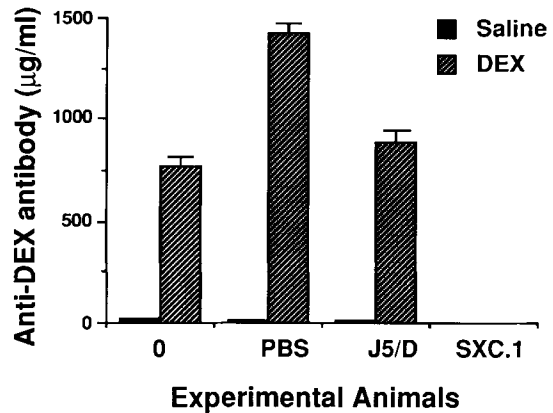
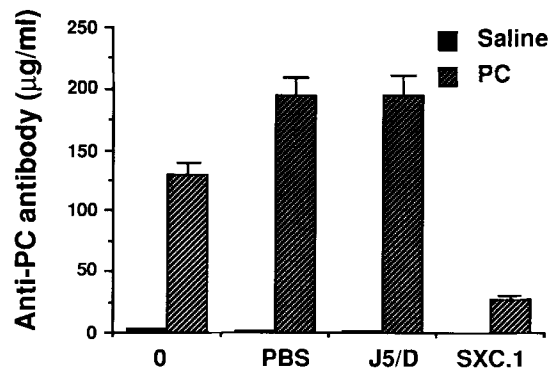


Figure 3. In vivo antibody responses of anti-IL-10-treated and control mice to α 1,3 dextran or phosphorylcholine. BALB/c mice were injected from birth to 9 wk with SXC.1 anti-IL-10 antibody, J5/D isotype control antibody, PBS, or left untreated. At 8 wk, mice were challenged with α 1,3-dextran, or heat killed *Streptococcus pneumoniae* as a source of phosphorylcholine, as described in Materials and Methods. Results show the geometric mean \pm SEM of specific antibody levels detected in five individual sera.

ities to generate in vivo antibody responses to α 1,3-dextran or phosphorylcholine (36, 37) (Fig. 3), two thymus-independent antigens for which functionally responsive B cells reside entirely within the Ly-1 B cell subset (9, 13).

Anti-IL-10-treated Mice Contain Phenotypically and Functionally Normal Conventional B Cells. In view of the striking effect of anti-IL-10 treatment on Ly-1 B cells, it was important to carefully evaluate the status of conventional B cells in these animals. As stated above, the total number of white blood cells in spleens of anti-IL-10-treated or control animals did not differ significantly in 24 independent experiments. Fig. 4 shows that the proportions of B220⁺ B cells, CD3⁺ T cells, and non-B/T cells (B220⁻CD3⁻) did not differ in any of the four experimental groups of mice. Equivalent data were obtained when Ig⁺ B cells or CD4⁺ T cells were compared (data not shown). These data indicate that the total number and phenotype of splenic B cells in anti-IL-10-treated mice are the same as that of each of the control groups. The immunocompetence of conventional B cells in anti-IL-10-treated mice was tested in two ways. First, anti-IL-10-treated mice developed normal IgM and IgG antibodies in response

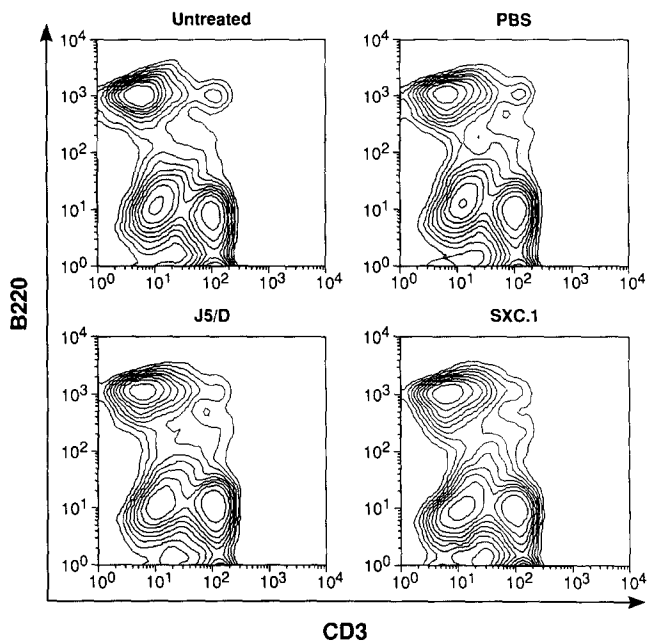


Figure 4. Immunofluorescence analysis of surface B220 and CD3 expression of live splenic lymphoid cells obtained from SXC.1 anti-IL-10-treated and control mice. For further details, see Fig. 1 legend.

to injection with the thymus-dependent antigen TNP-KLH (Fig. 5). Secondary responses to TNP-KLH were also normal in anti-IL-10-treated mice (data not shown). Second, splenic B cells from anti-IL-10-treated mice developed normal *in vitro* proliferative responses to 50 $\mu\text{g/ml}$ LPS or anti-IgM antibody (Table 1). The background proliferative responses of spleen cells from anti-IL-10-treated mice were frequently three- to fivefold higher than that of controls, however, the significance and explanation for this is not yet clear (Table 1). Collectively, these data suggest that conventional B cells in anti-IL-10-treated mice are quantitatively and functionally indistinguishable from those in control mice.

Mechanism of Ly-1 B Cell Depletion. Several possible mechanisms were considered as explanations for the depletion of Ly-1 B cells in anti-IL-10-treated mice. This effect did not appear to involve selective cytotoxicity of Ly-1 B cells by the anti-IL-10 antibodies, as injection of the same antibodies into adult mice had no effect on subsequent recoveries of total peritoneal wash cells (not shown), or total peritoneal B cells (Fig. 6) 1, 2, or 3 d later. As an alternative explanation, we considered the possibility that Ly-1 B cell depletion reflected a secondary consequence of some other endogenous cytokine perturbation. Indeed, anti-IL-10-treated mice were generally found to have elevated serum IFN- γ levels (Fig. 7), an observation that was consistent with the previously reported ability of IL-10 to suppress IFN- γ production by Th1 and NK cells *in vitro* (26, 27, 39). To test the possibility that this anti-IL-10-induced elevation of IFN- γ was either directly or indirectly responsible for depletion of Ly-1 B cells, mice were injected from birth to adulthood with a combination of anti-IL-10 and anti-IFN- γ -neutralizing antibodies, or anti-IL-10 and an appropriate isotype control antibody. The results showed that

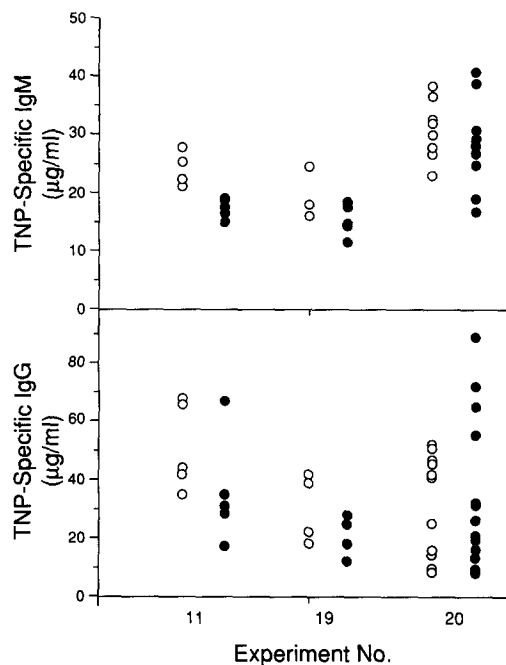


Figure 5. *In vivo* antibody response of anti-IL-10-treated or control mice to TNP-KLH. Mice were injected from birth until 10 wk of age with anti-IL-10 antibody (●), or an isotype control (○). At 8 wk, the animals were challenged intraperitoneally with 10 μg of TNP-KLH. Serum levels of TNP-specific IgM and IgG were determined 7 and 14 d after immunization, respectively. The results show three separate experiments, with each circle representing an individual mouse.

coadministration of anti-IFN- γ antibodies (Fig. 8), but not its isotype control (data not shown), substantially reduced the ability of anti-IL-10 treatment to deplete mice of peritoneal Ly-1 B cells. It is important to note that continued ad-

Table 1. *In Vitro* Proliferative Response of Spleen Cells from Anti-IL-10-treated and Control Mice to LPS, Anti-IgM, and Anti-CD3 Stimulation

Animal group*	[³ H]thymidine			
	+ 0	+ LPS	+ Anti-IgM	+ Anti-CD3
			<i>cpm</i>	
Untreated	224	22,570	3,346	90,093
PBS	320	42,298	3,821	115,692
J5/D	547	46,748	2,897	135,172
SXC.1	2,779	61,609	7,218	96,389

* Animals were treated from birth to 8 wk of age as described in Fig. 1. Pooled spleen cells at $2 \times 10^6/\text{ml}$ obtained from three mice in each group were cultured for 3 d in medium alone, or medium supplemented with LPS (50 $\mu\text{g/ml}$), goat anti-mouse IgM antibodies (50 $\mu\text{g/ml}$), or hamster anti-mouse CD3 antibodies (5 $\mu\text{g/ml}$). For anti-CD3 stimulation, the antibody was coated onto the microtiter plate before addition of spleen cells. Proliferation was evaluated via incorporation of [³H]thymidine, after a 16 h pulse with 1 $\mu\text{Ci/well}$ [³H]thymidine (NET 027; New England Nuclear, Boston, MA).

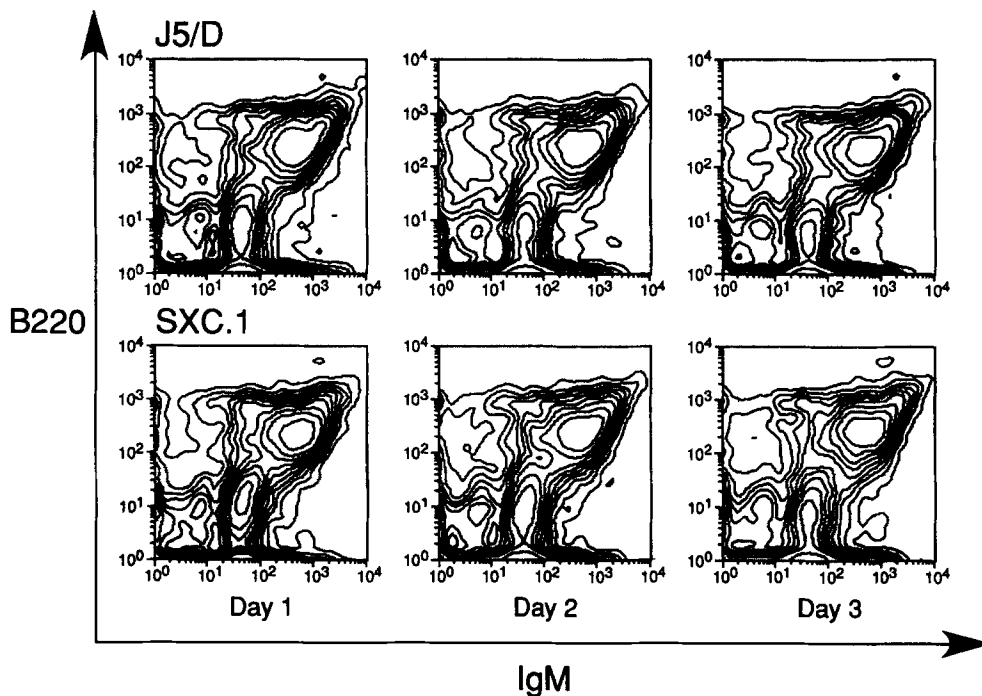


Figure 6. Anti-IL-10 antibodies are not directly cytotoxic for peritoneal wash B cells. Unprimed 8-wk-old BALB/c mice were injected intraperitoneally with 1 mg anti-IL-10 antibody (SXC.1), or 1 mg of an isotype control (J5/D). Peritoneal wash cells were collected from different animals 1, 2, or 3 d later, and analyzed for coexpression of surface IgD and IgM. Results show the fluorescence intensities of 5,000 live cells counted from each experimental group.

ministration of anti-IFN- γ antibodies alone, or the combination of anti-IL-10 plus anti-IFN- γ antibodies, did not alter the recovery of total peritoneal wash cells from mice treated with just anti-IL-10 or its isotype control. We interpret these data as evidence in support of the concept that Ly-1 B cell depletion is at least in part a consequence of IFN- γ elevation in anti-IL-10-treated mice.

Discussion

The data outlined in this study indicate that continuous treatment of mice from birth to adulthood with anti-IL-10 antibodies drastically reduces total Ly-1 B cell number and function, without altering the number, phenotype, or immunocompetence of conventional B cells located in spleens of these same animals. Several observations support the pro-

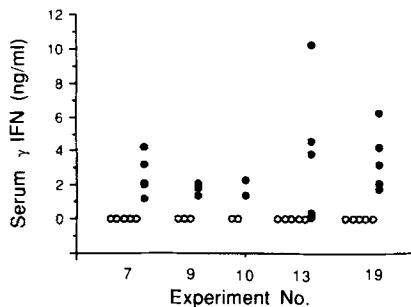


Figure 7. Serum IFN- γ levels in anti-IL-10-treated or control mice. BALB/c mice were injected from birth until 8 wk with anti-IL-10 antibody (●) or an isotype control (○). Sera collected at 8 wk were analyzed for IFN- γ by immunoassay (34, 35). The results show five separate experiments, with each circle representing an individual mouse.

posed depletion of Ly-1 B cells in anti-IL-10-treated mice: (a) anti-IL-10-treated mice contain few or no B cells in their peritoneal cavities, a site of Ly-1 B cell enrichment in normal mice. It is significant that we show elsewhere that peritoneal wash cells of 8-wk-old BALB/c mice in our animal facility contain fewer than 5% conventional B cells by phenotypic analysis (22, 23); (b) anti-IL-10-treated mice contain 0–10% of normal serum IgM levels, which is consistent with previous reconstitution experiments identifying Ly-1 B cells as the predominant source of circulating IgM (7, 9, and 11); and (c) anti-IL-10-treated mice generate little or no antibody in response to injection with phosphorylcholine and α 1,3-dextran, antigens for which specific B cells reside in the Ly-1 B cell subset (9, 13). The data suggesting an unaltered conventional B cell compartment in anti-IL-10-treated mice are equally compelling, with unchanged numbers of splenic B cells displaying normal cell surface marker phenotypes, and responding normally to a thymus-dependent antigen and B cell mitogens. The selective depletion of Ly-1 B cells in anti-IL-10-treated mice was found to be transient, as Ly-1 B cells reappeared in the peritoneal cavities of these animals several weeks after anti-IL-10 treatment was discontinued (H. Ishida, et al., manuscript in preparation).

Several possible mechanisms were considered as explanation for the selective depletion of Ly-1 B cells in anti-IL-10-treated mice. The data presented indicate that this is at least partially the consequence of IFN- γ elevation after anti-IL-10 treatment, since coadministration of neutralizing anti-IL-10 and anti-IFN- γ antibodies substantially prevented the depletion of peritoneal B cells in these studies. The implication that IFN- γ either directly or indirectly inhibits Ly-1 B cell development is reminiscent of our previous observations that IFN- γ causes slight suppression of IL-5 induced *in vitro*

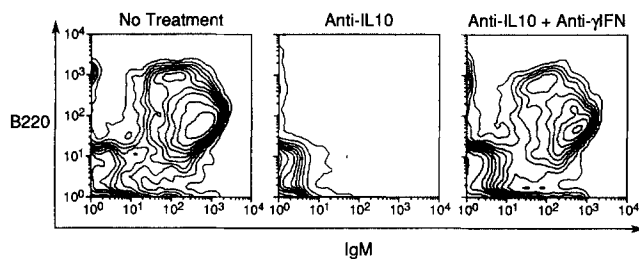


Figure 8. Coadministration of anti-IFN- γ antibodies reduces the ability of anti-IL-10 treatment to deplete mice of peritoneal B cells. BALB/c mice were treated from birth to 8 wk with anti-IL-10 antibodies, anti-IL-10 plus anti-IFN- γ antibodies, or were untreated. Peritoneal wash cells were then analyzed for coexpression of B220 and of IgM. Results show the fluorescence intensities of 5,000 live cells counted from each experimental group.

proliferation of the Ly-1⁺ B lymphoma BCL1 (40). To extend these studies, we have recently observed IFN- γ -mediated suppression of LPS-induced proliferation of peritoneal cells, but not spleen cells from normal BALB/c mice (O'Garra and Howard, unpublished observations). Whether the elevation of IFN- γ in anti-IL-10-treated mice totally accounts for Ly-1 B cell depletion, and whether this reflects a direct action of IFN- γ on Ly-1 B cells or some IFN- γ -mediated indirect effect, awaits further study. It is possible to imagine other changes in anti-IL-10-treated mice that may additionally contribute to the depletion of Ly-1 B cells. Based on previously published *in vitro* data (24, 25), it is likely that anti-IL-10 treatment will lead to elevation of endogenous monokine levels. Indeed, we show elsewhere (H. Ishida et al., manuscript in preparation) that anti-IL-10-treated mice are \sim 50-fold more susceptible to death by LPS-induced shock, an event that is known to be mediated by monokines (41–44), and that 5 of 32 individual anti-IL-10-treated mice contained substantial levels of serum IL-6, a monokine that is generally not found in the circulation of normal animals and that could

not be detected in the sera of any of 10 control mice from these experiments. On this point however, it is worth considering that IL-6 transgenic mice or animals with greatly elevated serum monokine levels resulting from *in vivo* administration of LPS appear to have normal serum IgM levels (45, 46), suggesting unchanged numbers of Ly-1 B cells. Our initial premise that IL-10 acts as an autocrine growth factor for Ly-1 B cells but not conventional B cells, a notion derived from our previous findings that Ly-1 B cells, but not conventional B cells generate constitutive and inducible IL-10 (22, 23), now seems unlikely in light of the substantial numbers of peritoneal Ly-1 B cells recovered from mice treated with both anti-IL-10 and anti-IFN- γ antibodies.

The Ly-1 B cell-depleted mouse we have created by continuous anti-IL-10 treatment bears considerable similarity to the previously described immunodeficient *xid* mouse, a spontaneous mutant strain derived from CBA/CaH mice which lacks Ly-1 B cells, and is unresponsive to a subset of thymus-independent antigens (7, 47). Despite these similarities, our preliminary investigations have revealed that *xid* mice produce IL-10 normally, and contain functional IL-10 receptors (N. Go and M. Howard, manuscript in preparation), thus distinguishing *xid* mice and anti-IL-10-treated mice mechanistically. Furthermore, one property that distinguishes anti-IL-10-treated mice from *xid* mice is the *in vitro* responsiveness of spleen cells to anti-IgM stimulation, exhibited by the former (Table 1), but not the latter animals (48). Further efforts to characterize the responsiveness of anti-IL-10-treated mice to the variety of thymus-independent type II antigens and microorganisms that are not recognized by *xid* mice are in progress. Whatever the relationship between *xid* mice and anti-IL-10-treated mice, it is hoped that the latter procedure will allow us to generate Ly-1 B cell-depleted mice of any haplotype, thereby allowing us to explore the role of this numerically small population of B cells within the context of the various genetic restrictions that underlie responsiveness to different antigens and microorganisms.

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