# Role of IL-10 in the distribution of B cell subsets in the mouse B-1 cell population

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**ABSTRACT. The B lymphocyte compartment is comprised of B-1 and B-2 cells. The former is divided into B-1a, which express CD5, and B-1b cells which do not: both are self-renewing, although the mechanisms are yet to be identified. IL-10$^{-/-}$ mice were used to delineate the role of the B cell activator IL-10 in this process. Its absence had no effect on the total number of B-1 cells, but decreased that of B-1a cells ($0.8 \pm 0.1$ *versus* $1.7 \pm 0.2 \times 10^6$, $p < 0.002$), while increasing that of B-1b cells ($1.9 \pm 0.4$ *versus* $0.8 \pm 0.1 \times 10^6$, $p < 0.03$). The number of B-1a cells remained low in IL-10-injected IL-10$^{-/-}$ mice, whereas the excess of B-1b cells further increased ($2.8 \pm 0.2$ *versus* $1.6 \pm 0.4 \times 10^6$, $p < 0.03$). On the basis that Bax and Bad were augmented in B-1a cells, and Bcl-2 and Bcl-$x_L$ reduced, we conclude that the disappearance of B-1a cells, but not B-1b, in IL-10$^{-/-}$ mice results from their enhanced susceptibility to apoptosis. In addition, culture of IL-10$^{-/-}$ B-1a and B-1b cells in the presence of IL-10 drives more of the latter than of the former into cycle ($p < 0.02$). Therefore, IL-10 exerts two, complementary effects on the distribution of B-1 cell sub-populations, rescuing B-1a cells from apoptosis and encouraging B-1b cell proliferation.**

Keywords: lymphocyte, self-renewal, apoptosis, peritoneal cells

## INTRODUCTION

B-lymphocytes have been classified into B-1 and B-2 [1] cells. The latter population resides in the peritoneal cavity (PerC) and comprises B-1a, which express CD5, and CD5- B-1b, which do not, but which show all other characteristics of B-1 cells. The frequency of B-1 cells [2] and the relative proportions of B-1a and B-1b cells [3] differ between mouse strains. Most studies of B-1 cells have, however, been limited by the fact that they have not distinguished between B-1a and B-1b cells, although B-1a cells can be distinguished from other B cell sub-populations on the basis of CD5 expression, and B-1b cells identified through their expression of Mac-1 in the PerC.

It is unclear whether CD5 is induced by activation [5], or signifies a separate lineage [6]. The activation concept implies that B-1a cells could be generated from CD5-negative B cells, i.e. B-1b or non-B-1 cells [7]. Acquired expression of CD5 might thus be due to the cross-linking of surface immunoglobulin of B-2 cells, but such an acquisition of the B-1b phenotype by B-2 cells has never been established. Alternatively, the lineage paradigm postulates the existence of separate progenitors for each of the three main B cell subsets, B-1a, B-1b and B-2 [8]. The inability to reconstitute B-1a cells after transfer of adult bone-marrow or PerC cells into irradiated mice [9] indicates that progenitors for mature B-1a cells do not persist throughout life. According to this concept, the only way for mature B-1 cells to survive is self-replenishment. In contrast to B-1a cells, significant progenitor B-1b cell activity is retained in adult mice [10].

The triggering stimuli for self-renewal have never been identified. Chronic autoantigen-stimulation of B-1a cells might be amplified by cytokines and/or chemokines, such as stromal cell-derived factor 1. This has indeed been recently revealed as a cofactor for the persistence of human B-1a cells [11]. IL-10 is another candidate, as suggested by four sets of experiments. Firstly, B-1a cells are the main source of B cell-derived IL-10 [12]; secondly, continuous treatment of normal mice with anti-IL-10 antibody (Ab) depletes B-1a but not B-2 cells [13]; thirdly, antisense oligodesoxynucleotides specific for IL-10 mRNA, inhibit the growth of murine leukemic B-1 cells *in vitro* [14]; and fourthly, IL-10 is involved in autoimmune B lymphocyte hyperactivity [15].

Our goal was to delineate the role of IL-10 in the reciprocal regulation of B-1a and B-1b cells, by using IL-10-deficient (IL-10$^{-/-}$) mice. We measured the levels of B-1a, B-1b and non-B-1 cells in the PerC of wild-type (WT), IL-10$^{-/-}$, and recombinant (r) IL-10-injected IL-10$^{-/-}$ mice.

## METHODS

### *Mice*

CBA/J (H-2$^k$) mice were purchased from Iffa-Credo (L'Abresle, France) and allowed to adapt to their environ-

ment for one week. IL-$10^{-/-}$ 129/Sv/Ev (H-$2^b$) mice were provided by Dr R. Müller (Cologne, Germany). Given the equal number of B-1a and B-1b cells in the PerC, CBA mice are more suitable than 129/Sv/Ev mice to assess modest variations in the distribution of B-1a and B-1b cells. CBA IL-$10^{-/-}$ mice were, therefore, established by crossing the IL-$10^{-/-}$ 129/Sv/Ev mice with the CBA/J H-$2^k$ background for five generations, and experiments were performed in 9-week-old male mice. WT male CBA/J mice of the same age served as controls.

### *Cell preparation*

Unless otherwise indicated, all Abs were purchased from BD Pharmingen (Le Pont de Claix, France). PerC cells were collected from mice after intraperitoneal injection of HBSS, incubated with a cocktail of unconjugated anti-Thy 1-2, anti-CD4 and anti-CD8 monoclonal Abs (mAb), washed twice in PBS, and lysed with guinea-pig complement (Tebu, Le Perray-en-Yvelines, France). Monocytes were removed by plastic adhesion at 37°C for 90 min. FCM analysis of the resulting cell population, with FITC-anti-IgM and phyco-erythrin (PE)- anti-CD3 mAbs, showed that more than 90% of the cells were positive for membrane IgM, and 96-98% were CD3-negative.

### *Identification of B cell subsets*

The proportion of B cell subsets was evaluated in IL-$10^{-/-}$ and WT mice by FCM. Firstly, $10^7$ cells were incubated with 1mg of anti-CD16/CD32 Abs (Fc Block™, BD Pharmingen) at 4°C for 30 min, washed twice in PBS, resuspended in HBSS, and incubated with staining Abs for 30 min. They were analyzed on a EPICS Elite flow cytometer (Beckman-Coulter, Villepinte, France). Apoptotic and necrotic cells were excluded, on the basis that they have low forward scatter (FS) and high side scatter (SS) properties [16]; a minimum of 100,000 living cells were analyzed.

To identify PerC B cell subsets, the staining mAb combination consisted of biotin (biot)-anti-IgM plus Cy-chrome-SA, FITC-anti-CD5, and PE-anti-Mac-1. B-1a cells were defined as $IgM^+/CD5^+/Mac\text{-}1^+$, B-1b cells as $IgM^+/CD5^-/Mac\text{-}1^+$, and non-B-1 cells as $IgM^+/CD5^-/Mac\text{-}1^-$ (Fig. 1). The percentages of B-1a and B-1b were measured separately, and the percentages of total B-1 cells obtained by adding up those of B-1a and B-1b cells. The remaining B cells were regarded as non-B-1 cells. The relative percentages of the B-1a and B-1b subpopulations within the B-1 population were then calculated, and converted into absolute numbers.

### *Detection of IL-10 receptors (R) and IL-2Rs*

IL-10Rs and IL-2Rs levels were determined in B cell subsets using a 30-min incubation with four Abs at 4°C. For the detection of IL-10R, biot-anti-IgM plus enhanced coupled dye (ECD)-SA (Beckman-Coulter), Cy-chrome-anti-CD5 and PE-anti-Mac-1 mAbs were added together with unconjugated goat anti-IL-10R Ab (R & D System, Abington, UK) plus FITC-rabbit $F(ab')_2$ anti-goat IgG (Jackson Immuno-Research Laboratories, West Grove, PA, USA). For the detection of IL-2-R, we used FITC-anti-IgM, Cy-chrome-anti-CD5, PE-anti-Mac, and biot-anti-IL-2R mAbs plus ECD-SA.

### *Measurement of apoptotic cells*

Apoptotic cells were recognized through their FS and SS properties. They were also identified using FITC- annexin V (Beckman-Coulter), combined with propidium iodide (PI) to exclude necrotic cells. The percentages of apoptotic cells (i.e. annexin V-positive cells within the PI-negative population) were calculated.

### *Evaluation of Bcl-2, Bcl-$x_L$ and Bax transcripts by RT-PCR*

One million FCM-sorted B-1a, B-1b and non-B-1 cells were washed twice with HBSS, and each cell suspension divided into four aliquots. Total RNA was isolated from each using guanidine isothiocyanate (Tri-Reagent, MRC, Cincinnati, OH, USA). One µg of total RNA was reverse transcribed, with a mixture of oligo (dT) nucleotides, along with 200U of reverse transcriptase (Life Technologies, Cergy Pontoise, France). After a 2-h incubation at 42°C, 4U of RNasine were added, and the mixture further incubated at 37°C for 20 min. Two µl of this mixture were used for each of the subsequent PCR amplification steps. Four pairs of primers (Sigma-Genosys, London, UK) were used: 5'-C-AAAGTAGAAGAGGCAACC-3' plus 5'-TGCTACAGGGTTTCATCAG-3' for Bax; 5'-CAT-AAGGCAACCACACCATC-3' plus 5'-CGAGAAGAA-GGGAG-AATCAC-3' for Bcl-2; 5'-CAACACCCA-AGGCAAA-GATG-3' plus 5'-GCCATTGAGTGAGGT-GCTTT-3' for Bcl-$x_L$; and 5'-TGGAATCCTGTGGCAT-CCATGAAAC-3' plus 5'-TAAAACGCAG-CTCAGT-

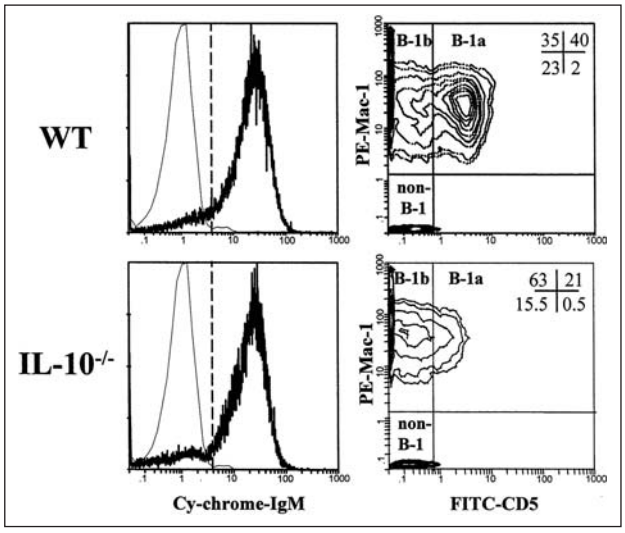


**Figure 1**
Representative example of an FCM analysis of B cell subsets in the peritoneum of IL-$10^{-/-}$ and WT mice. B cells were stained with biotin-anti-IgM in association with Cy-chrome-streptavidin, FITC-anti-CD5 and PE-anti-Mac-1 mAbs. Bi-parametric analysis gated on $IgM^+$ cells resolves B-1a cells as $IgM^+/CD5^+/Mac\text{-}1^+$, B-1b cells as $IgM^+/CD5^-/Mac\text{-}1^+$, and non-B-1 cells as $IgM^+/CD5^-/Mac\text{-}1^-$ (the results are expressed as percentages).

Abbreviations: Ab: antibody / Ag: antigen / Biot: biotin / FCM: flow cytometry / FITC: fluorescein isothiocyanate / FS: forward scatter / Ig: immunoglobulin / IL: interleukin / IL-$10^{-/-}$: interleukin-10-deficient / mAb: monoclonal antibody / PE: phyco-erythrin / PerC: peritoneal cavity / PI: propidium iodide / R: receptor / rIL-10: recombinant IL-10 / SA: strepavidin / SDF: stromal cell-derived factor / SS: side scatter / WT: wild-type

AACAGTCCG-3' for β-actin. DNA fragments of 272 bp (Bax), 289 bp (Bcl-2), 382 bp (Bcl-$x_L$) and 348 bp (β-actin) were obtained. After the initial template denaturation step (5 min at 95°C), 5U of *Taq* polymerase (Genaxis Biotechnology, Saint Cloud, France) were added, and 35 rounds of PCR amplification carried out (30 for β-actin). The resulting products were run on a 3% agarose gel containing 0.5 µg/ml ethidium bromide. For each sample, β-actin-encoding transcripts were measured at the same points as those selected for the study of apoptosis regulators. PCR band densities were determined using the Molecular Analyst software (BioRad, Hercules, CA, USA) and the mean density of each PCR product expressed as a ratio to that of β-actin PCR product.

### *Detection of Bcl-2, Bcl-$x_L$, Bax and Bad proteins in B cell subsets*

The expression of apoptosis proteins within each B cell subset was evaluated by membrane and intracytoplasmic staining. Firstly, B-1a, B-1b and non-B-1 cells were identified using biot-anti-CD5 plus Cy-chrome-SA, and PE-anti-Mac-1 mAbs, washed twice in staining buffer, incubated in Cytofix-Cytoperm solution (BD Pharmingen) for 20 min at 4°C, washed twice in Perm/Wash solution, and stained with FITC-anti-Bcl-2 Ab (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA), or unconjugated rabbit anti-Bcl-$x_L$ Ab, rabbit anti-Bax Ab or hamster anti-Bad Ab, plus FITC-goat F(ab')$_2$ anti-rabbit or anti-hamster IgG (both from Jackson Immuno-Research Laboratories).

### *Treatment of IL-10$^{-/-}$ mice with rIL-10*

Murine rIL-10 (Sanofi, Paris, France) at doses of 0, 6, 60 or 600 ng in 200 µl saline was injected intraperitoneally every day for 20 days into IL-10$^{-/-}$ mice (six mice per group), and their PerC cells analyzed thereafter.

### *Cell cycle analysis of B cell subsets cultured with rIL-10*

In the presence of 10 ng rIL-10 for up to 72 h, $5 \times 10^5$ FCM-sorted PerC B-1a, B-1b and non-B-1 cells from IL-10$^{-/-}$ mice were incubated in RPMI-1640 containing 10% FCS, 1% *L*-glutamine, 100U/ml penicillin, 100mg/ml streptomycin, 10mM Hepes, and $5 \times 10^{-5}$M β-2 mercaptoethanol. An aliquot of cells was taken at 12, 24, 36, 48 and 72h, and incubated in 0.1M sodium citrate buffer containing 0.1% Triton X-100 and 10µg/ml PI. Increased PI-staining intensity (i.e. G2/M cells, relative to G0/G1 cells) reflects proliferation of the cells.

## RESULTS

### *Distribution of B-1 cells into B-1a and B-1b in IL-10$^{-/-}$ mice*

The numbers of B-1a within the B-1 cells were reduced in IL-10$^{-/-}$ (Table 1), compared with WT mice ($0.8 \pm 0.1$ *versus* $1.7 \pm 0.2$ x $10^6$, $p < 0.002$). Those of B-1b cells were proportionally increased in IL-10$^{-/-}$ mice ($1.9 \pm 0.4$ *versus* $0.8 \pm 0.1 \times 10^6$, $p < 0.03$). As a net result, the total number of B-1 cells was similar in both strains of mice ($2.7 \pm 0.5$ *versus* $2.6 \pm 0.3 \times 10^6$). These data establish that, in IL-10$^{-/-}$ mice, a reduction of B-1a cells is compensated for by an increased number of B-1b cells within the B-1 population.

**Table 1**
**Peritoneal B cell subpopulation in wild-type and IL-10-deficient mice**

| Strain of mice | Absolute numbers ($\times 10^6$) | | | | |
|---|---|---|---|---|---|
| | Total B cells | Non-B-1 | Total B-1 | B-1a | B-1b |
| Wild-type | $3.7 \pm 0.4$ | $1.2 \pm 0.2$ | $2.6 \pm 0.3$ | $1.7 \pm 0.2$ | $0.8 \pm 0.1$ |
| IL-10-deficient | $3.8 \pm 0.5$ | $1.1 \pm 0.1$ | $2.7 \pm 0.5$ | $0.8 \pm 0.1$ | $1.9 \pm 0.4$ |
| *p* | 0.9 | 0.8 | 0.8 | < 0.002 | < 0.03 |

Mean ± SEM of ten 9-week-old male mice per strain. Data were compared using Student's t-test

### *Susceptibility of IL-10$^{-/-}$ B-1 cells to apoptosis*

Given that the reduced number of IL-10$^{-/-}$ PerC B-1a cells could result from exagerated apoptosis, the number of apoptosing cells were enumerated among PerC B cells. Two populations were separated by FS and SS analyses of PerC B cells from IL-10$^{-/-}$ and WT mice (Fig. 2). Gate 1 contained cells with normal size and granularity, which constitute resting B cells ($1.3 \pm 0.3 \times 10^6$ in IL-10$^{-/-}$, and $1.5 \pm 0.3 \times 10^6$ in WT mice), whereas gate 2 contained B cells with reduced size and marked granularity, which represent apoptosing B cells ($0.8 \pm 0.2 \times 10^6$ B cells in IL-10$^{-/-}$, and $0.6 \pm 0.2 \times 10^6$ in WT mice). This interpretation was confirmed by annexin V and PI staining. While there was hardly any apoptosing B cells in gate 1, we found $81.5 \pm 2.6$% in gate 2. A further $14.0 \pm 1.6$% of the B cells within this gate were necrotic. Gate 2 contained similar numbers of total B cells in IL-10$^{-/-}$ and WT mice, yet surface marker analysis revealed an excess of B-1a cells in gate 2 of IL-10$^{-/-}$, compared with WT mice: $6.4 \pm 0.2$ x $10^5$ apoptotic B cells are B-1a in IL-10$^{-/-}$ mice, compared with $4.4 \pm 0.2 \times 10^5$ in WT ($p < 0.02$). These findings suggest that IL-10 is involved in the prevention of apoptosis of B-1a cells, but not of B-1b cells.

To further address this issue, we examined apoptosis regulatory factors in PerC B cells. Transcripts for Bcl-2 were expressed at lower levels (Fig. 3A) in B1-a cells from IL-10$^{-/-}$ than from WT mice (Bcl-2/β-actin: 0.63 *versus* 0.82). The level of Bcl-$x_L$ mRNA was low in IL-10$^{-/-}$, as well as in WT B-1a cells, and mRNA for Bax more intensively expressed in B-1a cells from IL-10$^{-/-}$ than from WT mice (Bax/β-actin: 0.99 *versus* 0.80). Unlike B-1a, B-1b cells contained equal levels of transcripts for anti- and pro-apoptotic factors in IL-10$^{-/-}$ and WT mice. These values were expressed as ratios of Bcl-2/β-actin to Bax/β-actin and Bcl-$x_L$/β-actin to Bax/β-actin (Fig. 3B). The ratios Bcl-2/Bax (0.63 *versus* 1.02) and Bcl-$x_L$/Bax (0.51 *versus* 0.68) were reduced in IL-10$^{-/-}$ B-1a cells. The Bcl-2/Bax and the Bcl-$x_L$/Bax ratios were similar in B-1b cells from both strains of mice.

To confirm these observations at the protein level, we used intracytoplasmic staining rather than Western blotting, because apoptotic factors were expressed in a minority of cells. In the IL-10$^{-/-}$, compared with the WT mice, we detected (Fig. 3C) lower percentages of B-1a cells containing Bcl-2 ($18.9 \pm 1.9$ *versus* $29.9 \pm 1.3$%, $p < 0.002$), higher percentages containing Bax ($46.0 \pm 3.6$ *versus* $34.8 \pm 3.4$%, $p < 0.003$) and higher percentages containing

Bad (15.2 ± 2.7 *versus* 8.1 ± 1.5%, $p < 0.003$). Those B-1b cells containing Bax and Bad were negligible, and B-1b cells containing Bcl-2 and Bcl-$x_L$ comparable in both strains. Clearly, peritoneal B-1a cells, but not B-1b cells, contain fewer anti-apoptotic proteins, and more pro-apoptotic proteins in the IL-$10^{-/-}$ than in the WT mice.

### *Effects of injecting of rIL-10 into IL-$10^{-/-}$ mice on B-1a and B-1b cells*

As a prerequisite for injecting rIL-10, the presence of IL-10R on IL-$10^{-/-}$ B cell subsets was determined, since the absence of IL-10 could have down-regulated the related receptors. Percentages of IL-10R-bearing B-1a and B-1b cells were similarly high in IL-$10^{-/-}$ and WT mice (86.2 ± 3.2 *versus* 94.2 ± 0.3%, and 84.7 ± 3.1 *versus* 87.9 ± 0.7%). Therefore, rIL-10 was injected daily for 20 days into 9-week-old IL-$10^{-/-}$ mice, and the frequency of B-1a and B-1b cells within the B-1 cell population assessed at the age of 13 weeks (Table 2). Depletion of B-1a cells was not reversed, but the original excess in B-1b cells was further increased (from 1.4 ± 0.5 up to 2.8 ± 0.3 × $10^6$, $p < 0.02$). These data were confirmed by dose-effect analysis (Fig. 4) showing that the increase in the number of B-1b cells plateaued at the dose of 60 ng.

As indicated by FS and SS analyses (Fig. 5, Table 3), a third gate of PerC B cells emerged in IL-$10^{-/-}$ mice injected with 60 ng of rIL-10, but not in those injected with saline. The cells in this gate 3 (2.0 ± 1.0% of the peritoneal B cells in saline-injected mice *versus* 25.9 ± 7.3% in rIL-10-injected mice, $p < 10^{-3}$) derived from those in gate 1 (35.4 ± 3.7% *versus* 30.2 ± 2.8), which was fed by cells rescued from apoptosis by IL-10 in gate 2 (21.0 ± 0.3 *versus* 2.0 ± 0.3%, $p < 0.002$). The size and granularity of the B cells in gate 3 were elevated, suggesting proliferation. Phenotyping of their subsets revealed that as many as 93.5 ± 2.3% of these cells were B-1b in rIL-10-injected IL-$10^{-/-}$ mice.

### In vitro *effects of rIL-10 on B-1a, B-1b and non-B-1 cells*

To ensure that this selective proliferation of B-1b cells was directly generated by rIL-10, B-1a, B-1b and non-B-1 cells

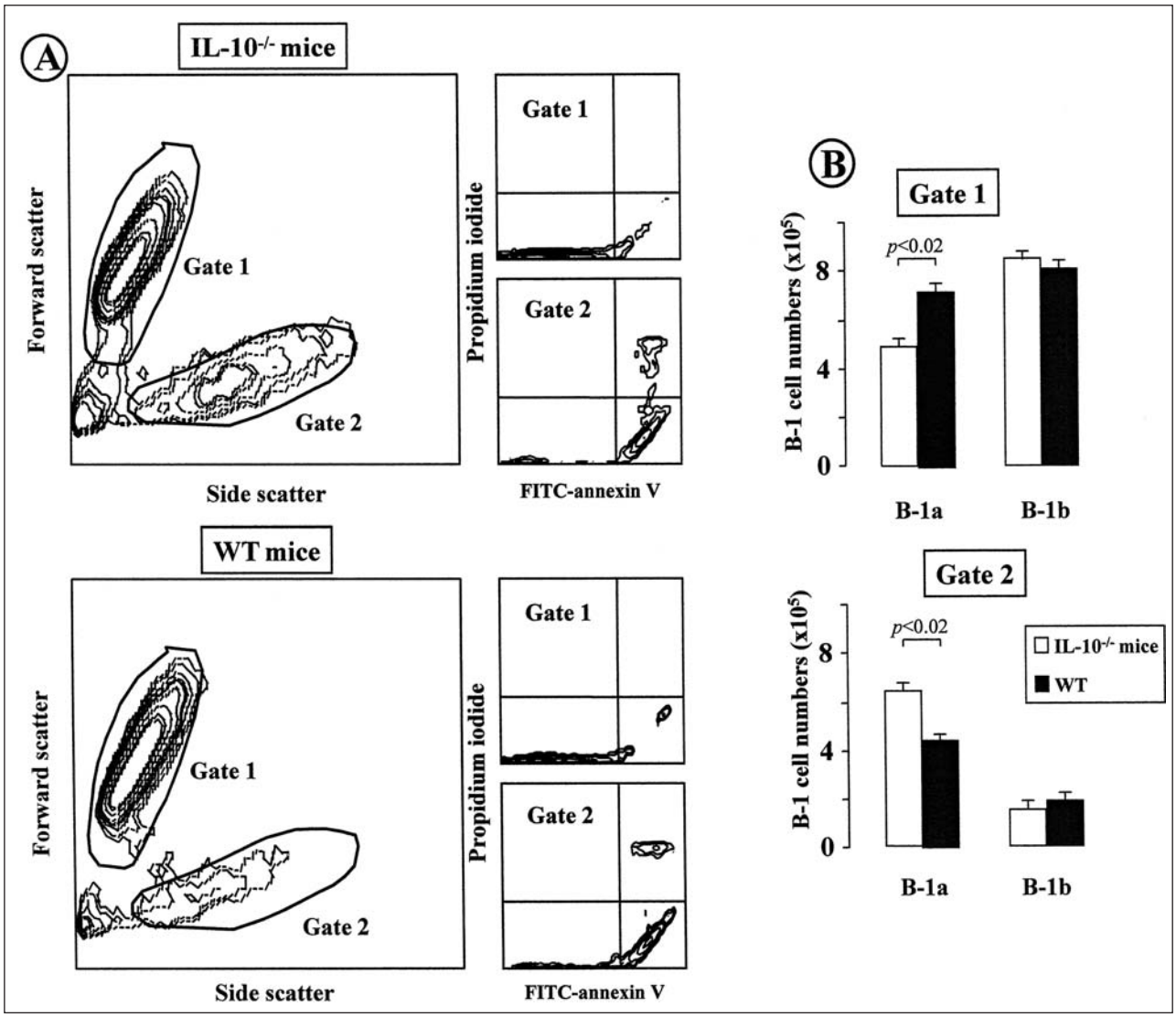


**Figure 2**

Spontaneous apoptosis of fresh peritoneal IL-$10^{-/-}$ and WT B cells. FS- and SS parameters of fresh unstained B cells distribute the cells into two gates: in gate 1, the size and the granularity of the cells are normal; in gate 2, their size is reduced and their granularity augmented. Peritoneal IL-$10^{-/-}$ B cells were then stained with FITC-annexin V and PI to distinguish apoptotic (annexin $V^+/PI^-$) from necrotic cells (annexin $V^+/PI^+$).

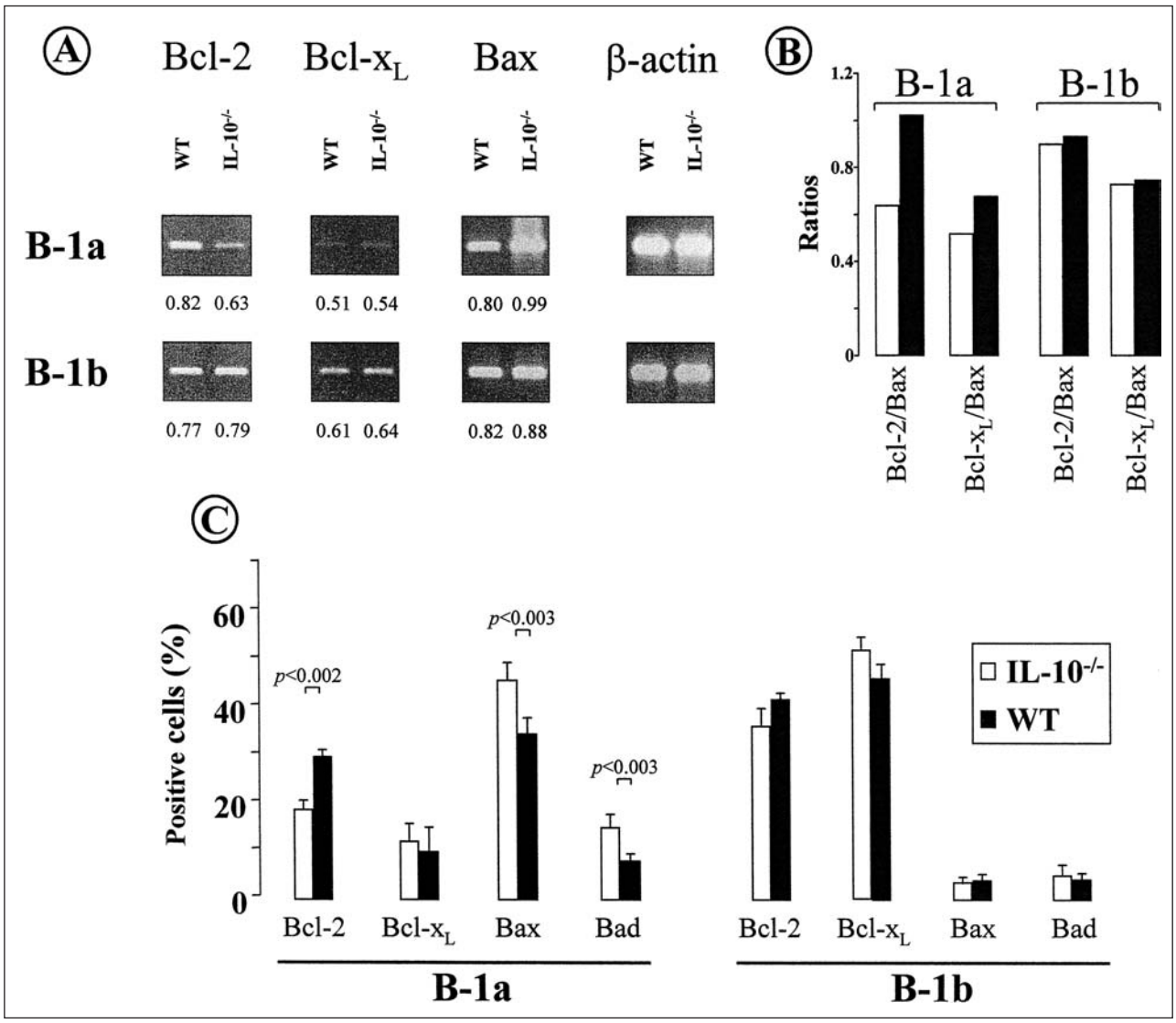


**Figure 3**

mRNA and protein level assessment of apoptosis regulators in peritoneal B cells from IL-10–/– and WT mice. (A) mRNA expression of Bcl-2, Bcl-$x_L$, Bax and β-actin after RT-PCR and agarose gel electrophoresis. Densitometry results of each mRNA are expressed as ratios of the density of apoptosis regulators to that of β-actin (figures below the lanes). A representative example of two experiments is shown. (B) Bcl-2/β-actin relative to Bax/β-actin and Bcl-$x_L$/β-actin relative to Bax/β-actin in B-1a and B-1b cells of IL-$10^{-/-}$ and WT mice. (C) Expression of anti-apoptotic (Bcl-2 and Bcl-$x_L$), and pro-apoptotic (Bax and Bad) proteins in IL-$10^{-/-}$ and WT peritoneal B cell subsets. Mean ± SEM of six experiments.

sorted from the PerC of non-injected IL-$10^{-/-}$ mice were cultured in the presence of 10 ng of rIL-10 for 24 h, the cell cycle analyzed using PI, and the percentages of G2/M cells calculated (Fig. 6A). More B-1b than B-1a and non-B-1 cells (Fig. 6B) were in cycle: 27.1 ± 1.6% of B-1b cells compared with 6.0 ± 4.2% of B-1a cells ($p < 0.008$), and 0.8 ± 0.6% of non-B-1 cells ($p < 0.0001$). Similar results were obtained with WT cells incubated with IL-10.

**Table 2**
**Peritoneal B cell subpopulations in IL-10-deficient mice injected with rIL-10**

| Treatment | Absolute numbers (× $10^6$) | | | | |
|---|---|---|---|---|---|
| | Total B cells | Non-B-1 | Total B-1 | B-1a | B-1b |
| Saline | 3.9 ± 0.7 | 1.5 ± 0.5 | 2.4 ± 0.4 | 1.0 ± 0.2 | 1.4 ± 0.5 |
| rIL-10 | 5.5 ± 0.5 | 1.7 ± 0.2 | 3.9 ± 0.7 | 1.1 ± 0.5 | 2.8 ± 0.3 |
| *P* | 0.08 | 0.4 | 0.08 | 0.6 | < 0.02 |

200 µl of saline or 60 ng of murine rIL-10 in 200 µl saline were injected intraperitoneally, daily for 20 days into 9-week-old male, IL-10-deficient mice (six 13-week-old mice per group were investigated at the termination of this treatment). Mean ± SEM. Data were compared using the Student's t-test.

**Table 3**
**FS and SS analysis distributes peritoneal B cells into three gates following injection of saline or rIL-10 into IL-$10^{-/-}$ mice (a representative example is show in Fig. 5A)**

| | Percentages (mean ± SEM) | | |
|---|---|---|---|
| | Saline | rIL-10 | *P* |
| Gate 1 (resting cells) | 35.4 ± 3.7 | 30.2 ± 2.8 | 0.3 |
| Gate 2 (apoptotic and necrotic cells) | 21.0 ± 1.0 | 2.0 ± 0.3 | 0.002 |
| Gate 3 (proliferating cells) | 2.0 ± 1.0 | 25.9 ± 3.3 | < $10^{-3}$ |

A time-lag could, however, be necessary before B-1a and non-B-1 cells proliferate. To address this issue, cultures were prolonged until 72h (Fig. 6C). Again, significantly more B-1b than B-1a cells from untreated IL-$10^{-/-}$ mice underwent *in vitro* proliferation in the presence of IL-10: 12.1 ± 3.1 *versus* 6.0 ± 2.1% at 36 h, ($p < 0.008$), and 19.0 ± 1.7 *versus* 7.3 ± 1.2% at 72 h ($p < 0.0004$). These data indicate that IL-10 triggers the B-1b but not the B-1a, or the non-B-1, to proliferate.

### *IL-2Rs are constitutively expressed in B-1b, but not in B-1a cells*

Their activated status could have induced B-1b cells to proliferate. We examined whether B-1 cells expressed IL-2R in non-injected IL-$10^{-/-}$ and WT mice. High percentages of IL-2R-bearing B-1b cells were found in the PerC (70.8 ± 2.3% in IL-$10^{-/-}$ and 61.5 ± 4.3% in WT mice). As opposed to B-1b cells, the expression of IL-2R was marginal in B-1a and non-B-1 cells in non-injected IL-$10^{-/-}$ and WT mice. Thus, in contrast to B-1a cells, and consistent with their high sensitivity to rIL-10, B-1b cells are constitutively activated.

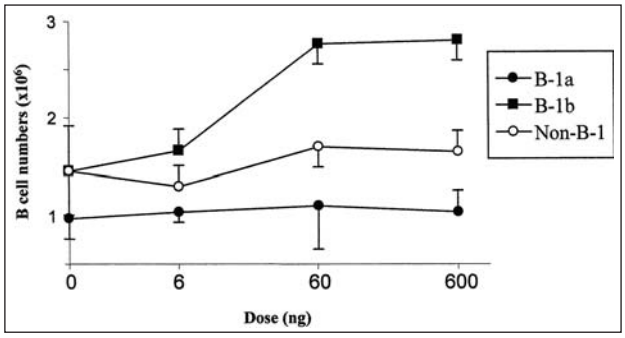


**Figure 4**
Dose-effect analysis of the distribution of peritoneal B-1a, B-1b and non-B-1 cells after recombinant IL-10 administration to IL-$10^{-/-}$ mice. Mean ± SEM of six experiments.

## DISCUSSION

We present evidence that IL-10 is central to the interrelationship between B-1a and B-1b cells within the B-1 cell compartment. In spite of differences in the mechanisms involved in regulating B-1a cells, which are dependent on IL-5 [17], and B-1b, cells which are dependent on IL-9 [18], there appears to be balancing feedback mechanisms between B-1a and B-1b cells. These are possibly related to cytokines, among which IL-10 is a credible candidate. Like others (Emilie *et al.*, personal communication), we observed a reduction in the number of B-1a cells in the IL-$10^{-/-}$ model, which was compensated for by an expansion of B-1b cells, such that the overall number of B-1 cells remained constant.

The absence of B-1a cells may thus generate a compensatory production of genuine B-1b. It is, however, difficult to distinguish whether the lack of IL-10 hinders the initial outgrowth of B-1a cells, or whether it prevents their ensuing self-renewal. The first possibility is consistent with the expression of IL-10R in primitive progenitor cells [19]. A role for IL-10 as a developmental factor is more satisfactory than that as an outgrowth factor, as it offers an explanation for the discrepancy between the normal B-1a cell development in IL-$10^{-/-}$ mice until six weeks [20], and their reduction three weeks later (this study). Because IL-10-deficiency induces enterocolitis, it could be argued that the disease, rather than the lack of IL-10 itself, alters the B cell phenotype. Although unlikely, since enterocolitis is a T-cell-mediated disease [21], this mechanism cannot be entirely excluded.

Such a defect in the self-replenishment of B-1a cells cannot be explained by a slowing down in their division,

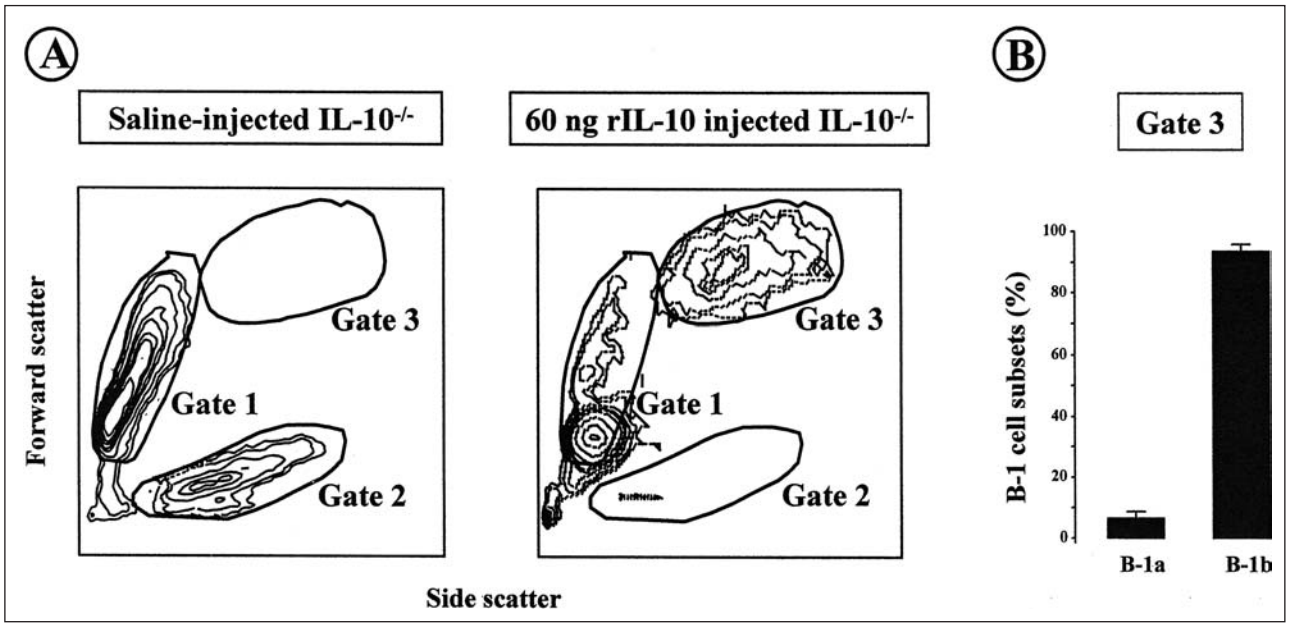


**Figure 5**
IL-10-induced changes in the FS and SS profiles of B cell subsets. IL-$10^{-/-}$ mice were given saline, or 60 ng rIL-10 for 20 days. (A) Peritoneal B cells were collected, and their FS and SS profile determined. A third population, segregated into gate 3, became apparent. (B) Triple-staining allows evaluation, among the gate 3 cells, of those exhibiting the B-1a and the B-1b phenotypes. Mean ± SEM of six experiments.

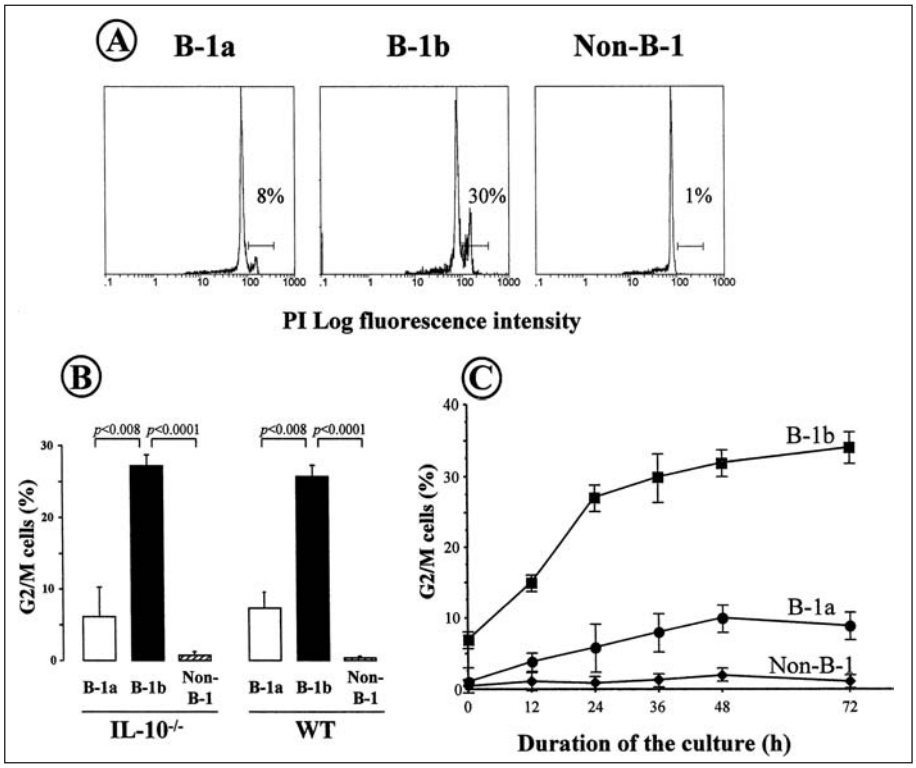


**Figure 6**

*In vitro* rIL-10-induced proliferation of peritoneal B cells from IL-$10^{-/-}$ and WT mice. $5 \times 10^6$ FCM-sorted B-1a, B-1b and non-B-1 cells were cultured in the presence of 10 ng of rIL-10 for up to 72 h. Aliquots were collected after 12, 24, 48 and 72 h in culture for cycle analysis, using PI. (A) One of six representative cell cycle profile of B-1a, B-1b and non-B-1 cells from an IL-$10^{-/-}$ mouse after a 24-h culture with rIL-10. (B) Percentages of IL-$10^{-/-}$ and WT B-1a, B-1b and non-B-1 in cycle after a 24-h culture. Increased PI-staining characterizes G2/M cells. (C) Time-course analysis of rIL-10-induced proliferation of B-1b cells from IL-$10^{-/-}$ mice (mean ± SEM of six experiments), and WT mice (mean ± SEM of three experiments).

because rIL-10 fails to drive them into cycle. In contrast, the fact that they apoptose in IL-$10^{-/-}$ mice suggests that a unique feature of IL-10 is to protect these cells from apoptosis. Inasmuch as the daily renewal rate is 1.3% for B-1a cells in the PerC [22], apoptosis of B-1a cells would result in a daily substitution of 1.3% of B-1a by B-1b cells. This is consistent with our finding of an enhanced expression of Bcl-$x_L$ in IL-$10^{-/-}$ B-1b cells, which confers upon them resistance to apoptosis. The few B-1a cells left behind might represent the B-1a cell subset described as $CD5^{high}$ [11], and postulated to be bone-marrow-derived [23].

A previous report suggested that B-1a cells might be activated [24]. This was suggested by the constitutive activation of the signal transducer and activator of transcription 3 in B-1a cells [25], with a role for IL-10 signaling or a combination of IL-10 and IL-6 [26]. Our results do not confirm that B-1a cells are constitutively activated, but rather the opposite with IL-2R expressed on B-1b cells, but not on B-1a cells. Whether it explains why B-1a cells are less sensitive than B-1b to the apoptosis protective effect of IL-10 remains to be determined.

Ishida *et al.* [13] have shown that the anti-IL-10 Ab-induced reduction in the level of B-1a cells proceeds from the excessive production of IFN-γ in response to the blockade of IL-10. Accordingly, we injected rIL-10 into IL-$10^{-/-}$ mice, to ascertain that the effects of IL-10 gene disruption were directly due to the absence of IL-10, rather than an excess in IFN-γ. The number of B-1a cells did not return to normal, because of the absence of progenitors for B-1a cells, and of the no-return evolution of these cells along the apoptosis pathway. In contrast, the excess of B-1b cells was further amplified. These data are reinforced by our finding that the percentages of IL-10R-expressing B-1b cells augment in a dose-dependent manner when these cells are cultured in the presence of rIL-10.

The role of IL-10 in the interplay between B-1a and B-1b cells requires further investigation to elucidate its mechanism of action. It is, therefore, interesting that synergy between IL-10 and stromal cell-derived factor 1 for the survival of peritoneal B cells has been demonstrated [27]. IL-5 may also be involved in this cooperation, since IL-10-induced differentiation of B cells is severely impaired in IL-$5R^{-/-}$ mice [17]. Other candidates, such as IL-3 [28], IL-7 [29], IL-9 [18] and IL-12 [30], warrant further investigation.

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