Interleukin 10 (IL-10) Upregulates Functional High Affinity IL-2 Receptors on Normal and Leukemic B Lymphocytes

By Anne-Catherine Fluckiger, Pierre Garrone, Isabelle Durand, Jean-Pierre Galizzi, and Jacques Banchereau

From the Laboratory for Immunological Research, Schering-Plough, 69571 Dardilly, France

Summary

Interleukin 10 (IL-10) has recently been shown to induce normal human B lymphocytes to proliferate and differentiate into immunoglobulin (Ig)-secreting cells. Herein, we show that IL-10 also promotes DNA synthesis and IgM production by anti-CD40 activated B cell chronic lymphocytic leukemia (B-CLL). Most strikingly, IL-2 and IL-10 were found to synergize to induce the proliferation and differentiation of B-CLL cells. This synergy between IL-2 and IL-10 was also observed with normal B cells which proliferated strongly and secreted large amounts of IgM, IgG, and IgA. The observed synergy is likely to be due to the IL-10–induced increase of high affinity IL-2 receptors on both normal and leukemic B cells. This increase of high affinity receptor is associated to an increase of Tac/CD25 expression that can be detected by flow cytometric analysis. Taken together, these results indicate that IL-10 permits anti-CD40 activated B cells to respond to IL-2 through an induction of high affinity IL-2 receptors. This effect of IL-10 may partly explain how T cells, which activate B cells in a CD40-dependent fashion, induce B cell proliferation and differentiation mostly through IL-2.

The maturation of B cells into efficient producers of high affinity and high specificity antibodies is regulated by various cell types through cell surface molecules and soluble cytokines. In vitro studies have demonstrated the important role of IL-2, IL-4, and IL-10 on B cell maturation (1). IL-10 originally isolated for its ability to inhibit the production of IFN-γ (2), acts as a growth and, most notably, a differentiation factor for normal B lymphocytes activated through their CD40 antigen or their antigen receptor (3, 4). IL-2 was found to be a potent proliferation- and differentiation-inducing agent of B lymphocytes after crosslinking of their surface Igs (5, 6). However, the effects of IL-2 on the growth and differentiation of anti-CD40 activated B cells were constantly minor (7). The reactivity of B lymphocytes to IL-2 is mediated by high affinity receptors (K_d = 10^{-11}M), which display at least three polypeptides: the α chain of 55 kD (also named Tac or CD25, K_d = 10^{-8} M); the β chain of 70–75 kD (K_d = 10^{-9} M); and a γ chain of 56 kD which does not bind IL-2 (8, 9). IL-2 was found to upregulate its own receptor (10, 11), whereas IL-4 was described to increase the p55 component (12, 13), though it inhibited the expression of high affinity receptors (14, 15).

B chronic lymphocytic leukemias (B-CLL)[1] are commonly defined as the accumulation in blood of slow-dividing and long-lived B lymphocytes closely resembling small resting B lymphocytes (16). Although B-CLL cells were first considered to be frozen at an early stage of maturation, it was demonstrated subsequently that they could differentiate into Ig-secreting cells after activation with phorbol esters (17). Half of the cases express the CD25 molecule and spontaneously react to IL-2 (18, 19). As with normal B cells, IL-2 also enhances proliferation of B-CLL cells after triggering through their antigen receptor (20) and through their CD40 antigen to a lesser extent (21). B-CLL cells differentiate into Ig-secreting cells upon stimulation by IL-2 and PMA (22, 23) or by IL-2 and SAC (21).

In the present study, we demonstrate that IL-10 acts as a growth and differentiation factor for anti-CD40 activated B-CLL cells. Furthermore it is shown that IL-10 and IL-2 act in synergy to induce the growth and differentiation of anti-CD40 activated B lymphocytes of both normal and leukemic origin.

Materials and Methods

Antibodies. The monoclonal and polyclonal antibodies used for the phenotyping of the leukemic B cell preparations were purchased from the following sources: Becton Dickinson Monoclonal Center (Mountain View, CA): FITC-conjugated anti-CD5 (Leu 1), anti-CD19 (Leu 12), anti-CD20 (Leu 16), anti-CD14 (Leu M3), anti-CD10 (Calla), anti-HLA DR, and anti-CD25 (IL-2R) mAbs; Im-

[1] *Abbreviation used in this paper:* B-CLL, B chronic lymphocytic leukemia.

A. C. Fluckiger and P. Garrone have contributed equally to these studies.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/93/11/1473/09 $2.00
Volume 178 November 1993 1473–1481

munotech (Marseille, France): FITC-conjugated anti-CD3 (IOT3) and anti-CD2 (IOT11) mAbs; Dako (Glostrup, Denmark): FITC-conjugated F(ab)'2 fragments of rabbit anti-human IgM, IgD, IgA, and IgG antibodies; Kallestad (Austin, TX): FITC-conjugated F(ab)'$_2$ fragments of donkey anti-human λ or κ light chains antibodies. The anti-CD40 mAb 89 was produced in the laboratory as described previously (24). The mAbs used for the negative selection of B cells with magnetic beads were purchased from the following manufacturers: Aster Laboratories (La Gaude, France): anti-CD2 and anti-CD3 mAbs; Immunotech: anti-CD14 mAb.

Flow Cytometric Analysis. Cell staining was performed as described previously (25). Briefly, 10^5 cells were incubated 15 min at 4°C with the appropriate dilution of antibody in PBS-BSA-azide. Samples were then washed twice in the same buffer before analysis on a FACScan® (Becton Dickinson, Sunnyvale, CA). Propidium iodide (2 μg/ml) was added in each sample before flow cytometry analysis in order to gate-out nonviable cells. The negative control was performed with an isotype-matched unrelated mAb. The phenotype of proliferating cells was determined by double fluorescence analysis. Briefly, 10^6 cells were incubated during 45 min at 37°C with 10 μM of Bisbenzimide Hoechst 33342 fluorochrome (Behring Diagnostics, La Jolla, CA). Direct immunofluorescence staining was then performed using anti-CD5, anti-κ and anti-λ antibodies. Samples were analyzed with a FACStar Plus® (Becton Dickinson) equipped with a dual laser.

Factors. Purified recombinant IL-2 (3×10^6 U/mg; Amgen Biologicals, Thousand Oaks, CA) and highly purified recombinant human IL-10 from CHO transfected cells (Schering-Plough Research Institute, Kenilworth, NJ) were routinely used at the final concentration of 20 U/ml (6.7 ng/ml) and 100 ng/ml, respectively.

Source and Purification of B Lymphocytes. Normal B lymphocytes were isolated from tonsils. Mononuclear cells were separated by standard Ficoll-Hypaque gradient method and were next submitted to E-rosetting with SRBC. Nonrosetting cells (E-fraction) were labeled with anti-T cell (anti-CD2 and anti-CD3) and anti-monocyte (anti-CD14) mAbs and subsequently incubated with magnetic beads coated with anti-mouse IgG antibodies (Dynal, Oslo, Norway). Residual non B cells were removed by applying a magnetic field for 10 min. B cell populations expressed ≥99% $CD19^+$, $CD20^+$. Leukemic B cells were isolated from seven patients showing the clinical and immunophenotype criteria for B-CLL and were processed as described for the normal B cells. All pathological samples displayed the typical phenotype of B-CLL cells: $CD5^+$, $CD19^+$, $CD20^+$, $CD40^+$, HLA class II^+, $CD10^-$, with low intensity surface IgM coexpressed or not with IgD. Monoclonality can be demonstrated on the basis of monotypic L chain expression. Two patients were classified as early clinical stages (Binet's stage A, Rai's stage I and II) and five patients were classified as advanced clinical stages (Binet's stage B and C, Rai's stage III and IV) (26, 27). Patient FLA was treated by chemotherapy at the time of the study. All other patients had not received any chemotherapy in the 3 mo preceding this study. Six samples were obtained from peripheral blood and one (PAS) was obtained from spleen.

B Cell Cultures. Purified leukemic and normal B cells were cultured in Iscove's medium (Flow Laboratories, Irvine, CA) enriched with 50 μg/ml human transferrin (Sigma Chemical Co., St. Louis, MO), 0.5% BSA, 5 μg/ml bovine insulin, 5% selected heat-inactivated FCS (all from Sigma Chemical Co.), 100 U/ml penicillin and 100 μg/ml streptomycin (both from Flow Laboratories, McLean, VA). For activation through the CD40 antigen, B cells were cultured in the presence of the anti-CD40 mAb 89 presented by a mouse Ltk^- cell line stably expressing CDw32 (CDw32 L cells) according to the experimental procedure described previously (28). Briefly, 2×10^4 tonsillar B cells or 10^5 B-CLL cells were cultured in round-bottomed microtiter trays under a final volume of 100 μl, together with 5×10^3 irradiated (7,000 rad) CDw32 L cells and with the anti-CD40 mAb (0.5 μg/ml). Cytokines were added at the onset of the culture. DNA synthesis was determined by pulsing cells with tritiated thymidine ([^{3}H]TdR) for the final 16 h of the culture period. Each culture point was performed in triplicate. Results are expressed as the mean ± 1 SD.

To evaluate differentiation of B cells into Ig-secreting cells, 2×10^4 tonsillar B cells or 10^5 B-CLL cells were cultured for 12 d in round-bottomed microtiter plates under a final volume of 200 μl in the enriched medium described above. Ig levels were measured in culture supernatants by standard ELISA techniques as described elsewhere (6). Each culture point was performed in triplicate. Results are expressed as mean ± 1 SD.

^{125}I-IL-2 Binding Assay. Equilibrium binding of ^{125}I-IL-2 was carried out as described elsewhere (29). Cell aliquots ($2–5 \times 10^6$) were incubated for 3 h at 4°C in the presence of various concentrations of ^{125}I-IL-2 (New England Nuclear, Boston, MA) in 250 μl of RPMI 1640 medium supplemented with 2% BSA and 20 nM Hepes, pH 7.2 (binding medium). Free ^{125}I-IL-2 was separated from surface-bound ^{125}I-IL-2 by rapid centrifugation on binding oil (dibutyl phtalate [Sigma Chemical Co.]-di[2-ethylhexyl] phtalate [Prolabo, Paris, France] 1–1). Nonspecific binding was determined in the presence of 100-fold excess of cold IL-2 (Amgen Biologicals).

Results

IL-10 Enhances the DNA Synthesis of Anti-CD40 Activated B-CLL Cells and Synergizes with IL-2. As IL-4 was recently found able to enhance the proliferation of anti-CD40 activated B-CLL cells (21), we wondered whether IL-10 would also act on these cells as it does on normal B cells (3). Thus, 10^5 highly purified B-CLL cells from seven different patients were cultured together with 5×10^3 irradiated CDw32 L cells and 0.5 μg/ml of anti-CD40 mAb 89, with or without cytokines. DNA synthesis was determined as measured by [^{3}H]TdR incorporation after 7 d of culture. As shown in Table 1, IL-10 enhanced by two- to fivefold the anti-CD40–induced DNA synthesis in six of seven samples. The inhibition by IL-10 of the anti-CD40–induced DNA synthesis of sample PAS was reproducible. The concentrations of IL-10 stimulating the DNA synthesis of anti-CD40 activated B-CLL cells were comparable with those inducing proliferation of anti-CD40 activated normal B cells with a peak response being obtained for 30–100 ng/ml of IL-10 (data not shown). The DNA synthesis of anti-CD40 activated B-CLL cells was significantly stimulated with IL-2 ($p = 0.02$). Interestingly, combinations of IL-10 and IL-2 synergized to induce strong DNA synthesis of all tested B-CLL samples (Table 1). This synergy was dose dependent, increasing with the concentrations of IL-10 or IL-2 (data not shown).

The cooperation between IL-10 and IL-2 was further analyzed in cell cycle studies using DNA labeling with the Hoechst 33342 fluorochrome and cell surface staining with FITC-conjugated anti-CD5. Fig. 1 illustrates one experiment performed with B cells from patient PIG. Only 0.3% of cells entered into S/G_2/M phases when activated in the CD40 system. Addition of IL-2 or IL-10 to cultures increased the

Table 1. *IL-10 Enhances the DNA Synthesis of Anti-CD40 Activated B-CLL Cells*

	[^{3}H]TdR uptake (cpm × 10^{-3})				
B-CLL	Medium	anti-CD40	anti-CD40 + IL-10	anti-CD40 + IL-2	anti-CD40 + IL-10 + IL-2
BAE	0.8	1.0	2.5	1.6	14.6
GER	0.9	2.8	6.4	5.2	25.0
FLA	0.4	6.3	27.0	28.1	110.4
LES	0.7	1.6	3.8	2.3	8.5
MAG	0.2	4.1	21.4	6.7	42.9
PIG	0.1	2.3	5.9	20.2	88.8
PAS	0.7	6.3	3.5	13.8	31.7

10^5 B-CLL cells were cultured in the absence or in the presence of 0.5 μg/ml of anti-CD40 mAb 89 and 5,000 irradiated CDw32 L cells (referred as CD40 system or àCD40) without or with 100 ng/ml of IL-10, 20 U/ml of IL-2, or a combination of IL-10 and IL-2. Results are expressed as the mean of triplicate determination. [^{3}H]TdR uptake was measured at day 7. SD never exceeded 10% of the mean value.

percentage of cells in S/G_2/M to 1–2%. More strikingly, in response to the combination of IL-2 and IL-10, as many as 10.9% of the B-CLL cells were found to be in S/G_2/M. All cells in cycle expressed either CD5 (Fig. 1) or a single L chain (data not shown).

IL-10 Cooperates with IL-2 for the Proliferation of Anti-CD40 Activated Normal and Leukemic B Cells. In view of the above findings, we tested whether IL-10 and IL-2 would cooperate to induce the proliferation of normal B cells cultured in the CD40 system. Thus, 2 × 10^4 B cells were cultured over 5 × 10^3 irradiated CDw32 L cells with 0.5 μg/ml of mAb 89 and [^{3}H]TdR uptake was measured after 5 d of incubation. As previously described, IL-10 elicited [^{3}H]TdR uptake of anti-CD40 activated B cells (Fig. 2 *A*) whereas IL-2 was virtually inactive whatever its concentration, from 0.06 to 50 U/ml (Fig. 2, *A* and *C*). Combination of IL-2 and IL-10 resulted in a strong enhancement of the DNA synthesis. As illustrated on Fig. 2, *B* and *C*, the cooperation between IL-2 and IL-10 was dose dependent, varying with the concentrations of IL-10 and IL-2. 100 ng/ml of IL-10 gave the

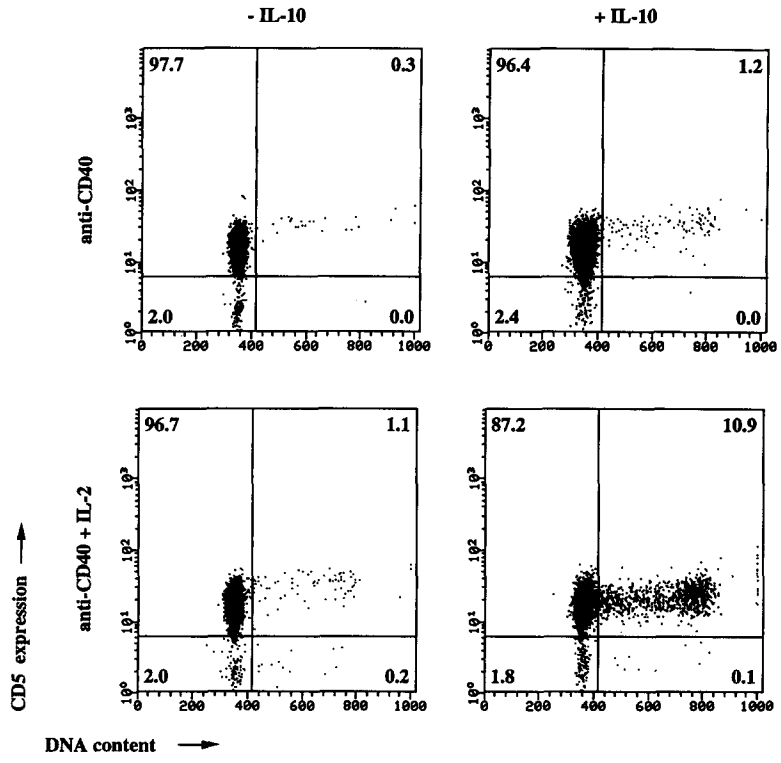


Figure 1. IL-2 and IL-10 synergize to induce anti-CD40 activated B-CLL cells to enter into cycle. 5 × 10^5 B-CLL cells from patient PIG were cultured together with 5 × 10^4 irradiated CDw32 L cells and 0.5 μg/ml of mAb 89 with or without IL-2 (20 U/ml), IL-10 (100 ng/ml), or IL-2 and IL-10. At day 7, cells were incubated with Hoechst 33342 before cell surface staining with anti-CD5 mAb. Samples were analyzed with dual laser FACStar®. Numbers indicate the percentage of positive cells in each quadrant. (*y*-axis) CD5 expression; (*x*-axis) DNA content.

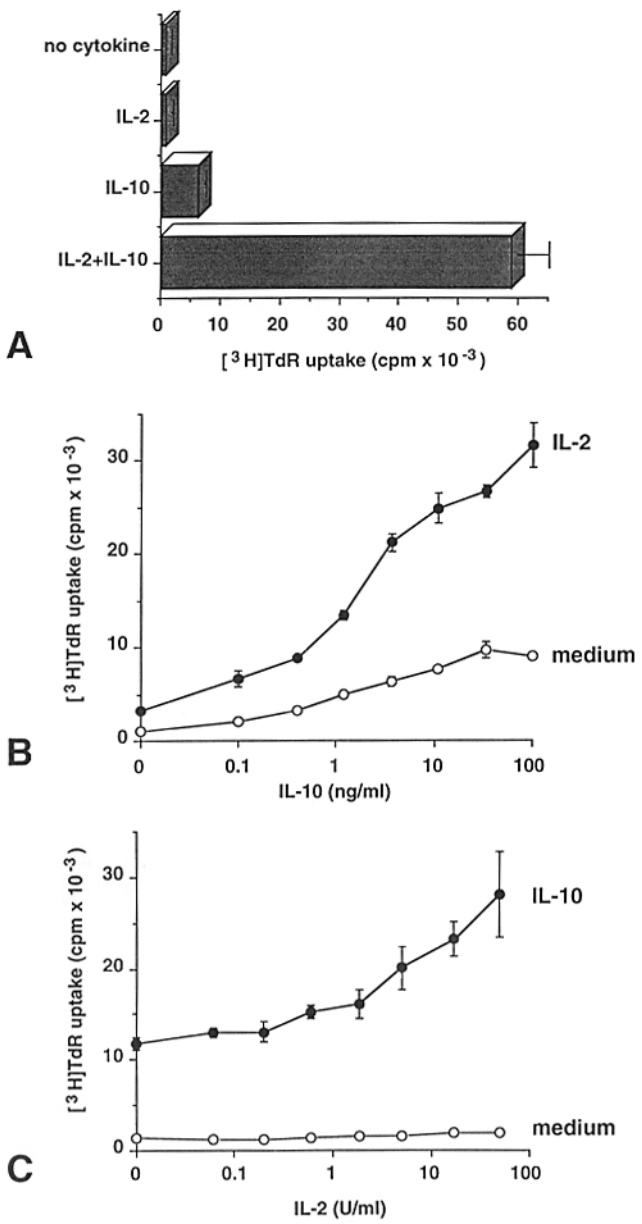


Figure 2. IL-10 and IL-2 synergize for the proliferation of anti-CD40 activated tonsillar B cells. 2 × 10^4 highly purified B cells were cultured together with 5,000 irradiated CDw32 L cells and 0.5 μg/ml of mAb 89. (*A*) 20 U/ml of IL-2, or 100 ng/ml of IL-10 or a combination of IL-2 and IL-10 were added. (*B*) Serial dilutions of IL-10 were added without (○) or with (●) IL-2 (20 U/ml). (*C*) Serial dilutions of IL-2 were added without (○) or with (●) IL-10 (100 ng/ml). [^{3}H]TdR uptake was measured at day 5. Results are expressed as mean ± SD of triplicates. Representative of at least three experiments. B and C were obtained with a different B cell sample than A, explaining the variation of [^{3}H]TdR uptake.

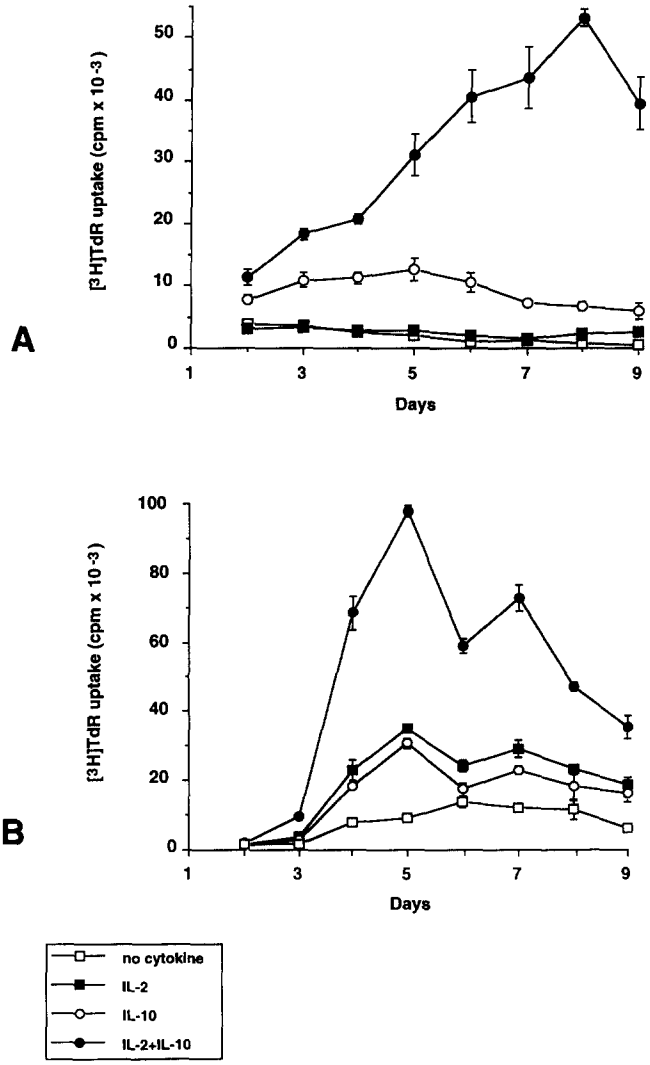


Figure 3. Anti-CD40 activated normal and leukemic B cells show different growth kinetics in response to IL-10 and IL-2. 2 × 10^4 normal B cells (*A*) or 10^5 B-CLL (*B*) were cultured in the presence of 5,000 irradiated CDw32 L cells and 0.5 μg/ml of mAb89 (□). 100 ng/ml of IL-10 (○), 20 U/ml of IL-2 (■), or IL-2 and IL-10 (●) were added at the onset of the culture. [^{3}H]TdR uptake was measured daily by 16-h pulses from days 2 to 9. Results are expressed as mean ± SD of triplicates. Two other experiments performed with distinct samples yielded similar results.

maximal growth-promoting effect with 20 U/ml of IL-2. Furthermore, the dose–response curves illustrating the [^{3}H]TdR uptake in B cells cultured with IL-2 and serial dilutions of IL-10 show a greater slope than the curve illustrating the [^{3}H]TdR uptake in B cells cultured without IL-2 (Fig. 2 *B*). This indicates a real synergy between IL-10 and IL-2.

Kinetic studies showed that the peak response of the DNA synthesis induced by the combination of IL-2 and IL-10 was delayed on normal B cells (Fig. 3 *A*) when compared with B-CLL cells (Fig. 3 *B*). For normal B cells, the peak DNA synthesis induced by IL-2 and IL-10 was reached at day 8, whereas with B-CLL cells (Fig. 3 *B*) the DNA synthesis rapidly increased to peak at day 5. The decrease of DNA synthesis was not due to an increased cell death since Trypan blue dye exclusion still showed a 80% cell viability at day 7.

The cytokine-dependent increase of [^{3}H]TdR uptake actually reflected cell growth as shown by the increase of viable

Table 2. *IL-10 and IL-2 Induce the Growth of Anti-CD40 Activated B Cells*

B cells	Viable cell recovery ($\times 10^{-5}$)			
	No CK	IL-2	IL-10	IL-2 + IL-10
Tonsil day 5	0.5	0.7	3.8	4.6
day 7	1.7	1.9	4.1	6.6
CLL day 5	2.1	2.6	2.5	3.5
day 10	1.3	2.4	1.9	5.3

10^5 normal tonsillar B cells or 2×10^5 B-CLL cells were cultured together with 5,000 irradiated CDw32 L cells and 0.5 μg/ml of anti-CD40 mAb 89, without or with IL-2 (20 U/ml), IL-10 (100 ng/ml), or a combination of IL-2 and IL-10. Viable cell recovery was determined by Trypan blue dye exclusion at days 5 and 7 for normal B cells and at days 5 and 10 for B-CLL cells. Representative of four experiments.

Table 3. *IL-10 and IL-2 Synergize to Induce Anti-CD40 Activated B-CLL Cells to Produce IgM*

CLL	IgM production			
	No cytokine	IL-2	IL-10	IL-2 + IL-10
		μg/ml		
FLA	0.1	1.2	4.3	50
GER	0.03	0.2	0.1	1.1
LES	0.2	0.4	9.1	19.9
MAG	0.3	0.2	6.3	13.7
PAS	0.3	0.3	14.8	24.1
PIG	0.4	0.5	3.3	7.1

10^5 B-CLL cells were cultured during 12 d in the CD40 system without or with IL-10 (100 ng/ml), IL-2 (20 U/ml), or a combination of IL-10 and IL-2. IgM levels were measured by ELISA. Results are expressed as mean of quadruplicate determination. SD never exceeded 10% of the mean value.

cell recovery (Table 2). Addition of IL-10 increased the recovery of viable normal B cells cultured for 5 d in the CD40 system whereas IL-2 was inefficient. Then, the IL-10 induced cellular replication sloped. At day 7, the combination of IL-2 and IL-10 was the most efficient, resulting in a sixfold increase of input-viable B cells. In contrast with normal B cells, at day 5, the number of viable anti-CD40 activated B-CLL cells was not increased by addition of IL-10 alone. However, the combination of IL-2 and IL-10 led to a fourfold increase of B-CLL cell counts after 10 d of culture in the CD40 system.

IL-10 with or without IL-2 Elicits Differentiation of Anti-CD40 Activated B-CLL Cells. We next investigated whether anti-CD40 activated B-CLL cells were able to differentiate in response to IL-10 and/or IL-2. As previously reported (21), the CD40 system alone ($p = 1$) or with IL-2 ($p = 0.3$) did not induce B-CLL cells to secrete significant amounts of IgM (Table 3). IL-10 induced five of six B-CLL samples to secrete fairly high levels of IgM (3.3–14.8 μg/ml) and this secretion was further enhanced by addition of IL-2. Actually the combination of IL-2 and IL-10 permitted the differentiation of the GER sample which was nonreactive to either IL-2 or IL-10. Finally, PAS secreted large amounts of IgM (14.8 μg/ml) in response to IL-10 in the CD40 system whereas its DNA synthesis measured at day 7 was inhibited by IL-10 (Table 1).

As shown in Fig. 4, normal B lymphocytes cultured for 12 d in the CD40 system alone or with IL-2, did not produce a significant amount of Ig. In contrast, upon culturing with IL-10, B lymphocytes secreted large amounts of IgM, IgG, and IgA. Addition of IL-2 further stimulated IL-10–induced Ig production. The most striking enhancement was that of IgA, production of which was increased 15-fold whereas that of IgM and IgG was enhanced six- and threefold, respectively (means of three distinct experiments).

IL-10 Upregulates CD25 Expression on Normal and Leukemic B Lymphocytes. To further document the mechanism of the synergy between IL-10 and IL-2, we studied the influence of IL-10 on the expression of the IL-2 receptor on anti-CD40 activated B cells. For this purpose, the expression of CD25/Tac was first analyzed by flow cytometry at different times of the culture period. Under all experimental conditions the pres-

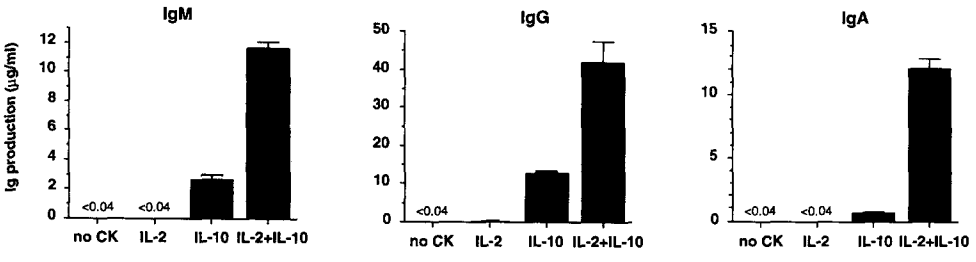


Figure 4. IL-10 and IL-2 synergize for the Ig production of anti-CD40 activated normal B cells. 2×10^4 highly purified tonsillar B cells were cultured together with 5×10^3 irradiated CDw32 L cells and 0.5 μg/ml of mAb 89 without or with IL-2, IL-10, or a combination of IL-2 and IL-10. Supernatants were harvested at day 12 and IgM, IgG, and IgA levels were determined by ELISA. Results, expressed in μg/ml, are the means ± SD of triplicates. No IgE was ever detected.

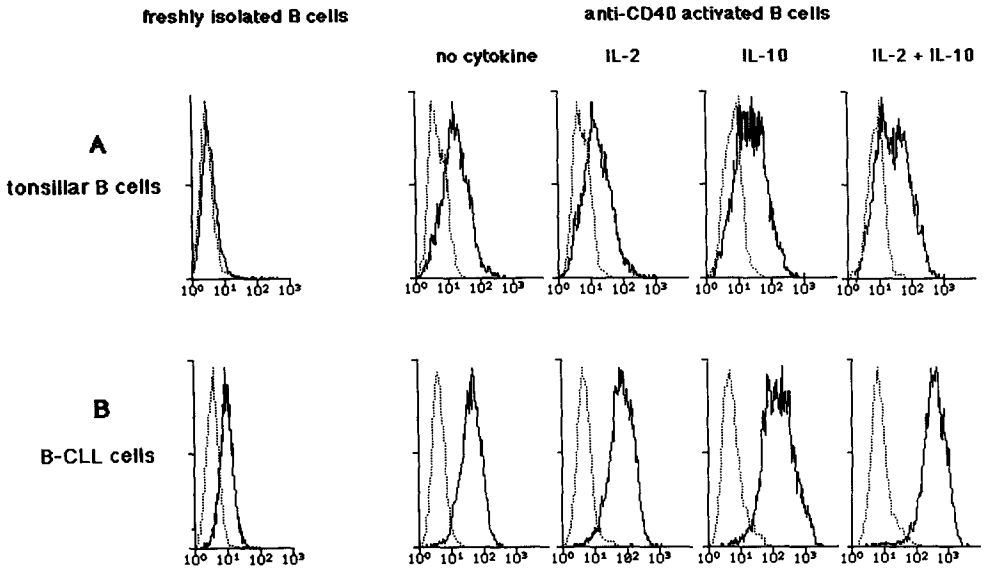
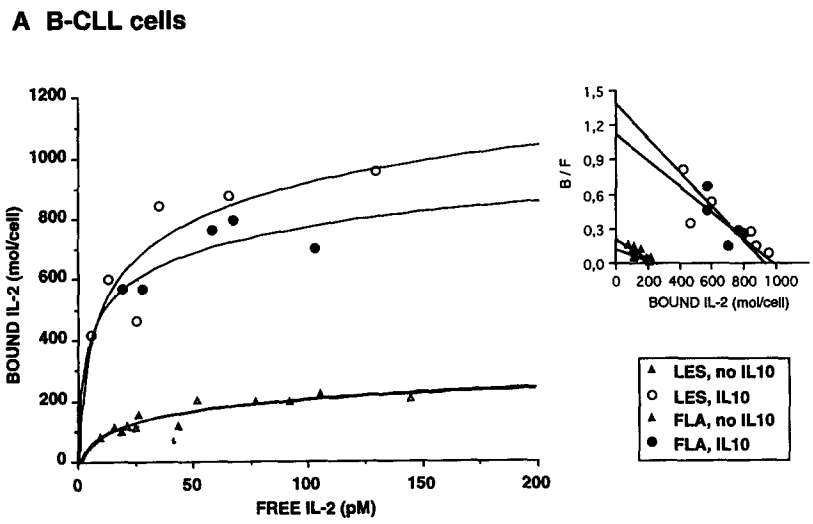


Figure 5. IL-10 upregulates CD25 expression of anti-CD40 activated normal or leukemic B cells. Normal (*A*) and CLL (*B*) B cells were cultured together with irradiated CDw32 L cells and mAb 89 without or with IL-2 or IL-10 or IL-2 plus IL-10. After 3 d, cells were washed twice and stained with a FITC-labeled anti-CD25 or a FITC-labeled nonrelated Ab. Fluorescence was analyzed with a FACScan® using the same settings as for the freshly isolated B cells. Histograms show fluorescence intensity of the CD25 staining (*solid lines*) superimposed with that of the negative control (*dashed lines*).



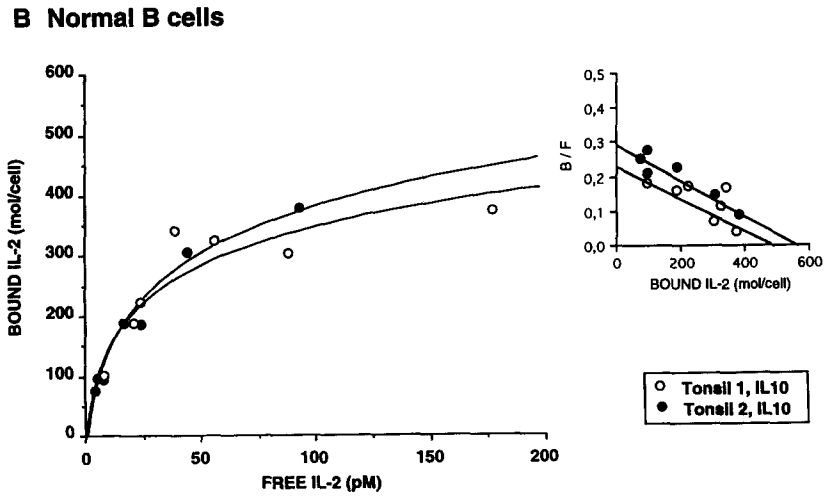


Figure 6. IL-10 enhances the expression of high affinity IL-2 receptors on anti-CD40 activated normal and leukemic B cells. Two different samples of both normal and leukemic B lymphocytes were cultured together with irradiated CDw32 L cells and mAb 89 without or with IL-10. After 48 h, cells were washed twice before performing the ^{125}I-IL-2 binding assays described in Materials and Methods. Large panels illustrate the equilibrium binding data for ^{125}I-IL-2 binding and insets represent the Scatchard analysis of the specific binding data. (*A*) Leukemic B cells, samples LES and FLA; (*B*) normal B cells.

ence of contaminating T cells was ruled out by staining with anti-CD3 mAb. The histograms of Fig. 5 show the expression of CD25 on anti-CD40 activated B cells in response to IL-2, IL-10, or combination of IL-2 and IL-10. As previously described, CD25 expression was undetectable on freshly isolated tonsillar B cells (Fig. 5 *A*). Anti-CD40 activation slightly increased the expression of CD25/Tac on B cells measured after 3 d. In the presence of IL-10 but not IL-2, a distinct population of $CD25^{bright}$ cells emerged. Combination of IL-2 and IL-10 induced CD25 expression as did IL-10 alone. This pattern of response was observed at various times of culture, 45% of cells being $CD25^{bright}$ between days 3 and 4. In contrast to normal B lymphocytes, freshly isolated B-CLL cells expressed low levels of CD25 which were slightly upregulated by IL-10 alone in the seven tested cases (data not shown). After culture in the CD40 system, B-CLL cells expressed higher levels of CD25. IL-10 strongly upregulated CD25 expression on anti-CD40 activated B-CLL cells (Fig. 5 *B*) whereas IL-2 was much less efficient. Combination of IL-2 and IL-10 resulted in an induction of CD25 which was higher than that obtained with IL-10 alone. The kinetic of CD25 upregulation was comparable with that obtained with normal B cells. The IL-10–induced CD25 upregulation was dose dependent, with the half maximal effect obtained around 2 ng/ml and the plateau reached with ≈10 ng/ml (data not shown).

IL-10 Increases the Expression of High Affinity IL-2 Receptors on Anti-CD40 Activated B Cells. The expression of IL-2 receptors was analyzed by measuring the binding of ^{125}I–IL-2 on anti-CD40 activated normal and leukemic B cells, costimulated or not with IL-10. Fig. 6 shows typical binding experiments performed with two samples of normal and two samples of leukemic B cells and Scatchard plots of the binding data. B-CLL cells cultured in the CD40 system for 3 d expressed 296 and 229 receptors per cell with a K_d of 38 and 30 pM, respectively, (Fig. 6 *A*). In the presence of IL-10, the number of binding sites increased to 960 and 942 receptors per cell with a K_d of 8 and 15 pM, respectively. On normal B cells (Fig. 6 *B*), high affinity IL-2 receptors were undetectable after culture in the CD40 system. When IL-10 was added to culture, anti-CD40 activated normal B cells showed 496 and 582 IL-2 receptors per cell with a K_d of 40 and 46 pM.

Discussion

The present study demonstrates that IL-10 enhances the DNA synthesis and Ig secretion of anti-CD40 activated chronic lymphocytic leukemia B cells. The proliferating cells were derived from the leukemic cell clone rather than from contaminating normal B lymphocytes since the proliferating cells expressed a single L chain and CD5 antigen, which is not upregulated on normal B cells cultured in the CD40 system (data not shown). In contrast with the long-lasting effect of IL-4 on B cell proliferation (21, 28), the growth activity of IL-10 was short-term and did not result in an increase of viable cell counts. Indeed, this limited growth potential of IL-10 on leukemic cells was also observed on normal B cells (Rousset, F., and J. Banchereau, manuscript in preparation) and is best explained by the IL-10–induced differentiation of B lymphocytes into plasma cells with a relatively short life. The present study also indicates that IL-10 acts as a potent inducer of IgM production by anti-CD40 activated B-CLL cells. Furthermore, numerous plasma cells were observed on Giemsa-stained smears (data not shown). This confirms the great efficiency of IL-10 as a B cell differentiating factor and further extends the previous studies which demonstrated that B-CLL cells could differentiate into Ig-secreting cells (17, 22).

Most importantly, upon CD40 triggering, IL-10 was found to synergize with IL-2 for proliferation and differentiation of normal and leukemic B cells. The combination of IL-10 and IL-2 has been, in our hands, the most efficient cytokine combination allowing differentiation of normal and leukemic B cells into Ig-secreting cells. This contrasts with the combination of IL-10 and IL-4 which leads to an increased cellular replication but results in a lower Ig production (3).

The synergy between IL-2 and IL-10 was explained by the property of IL-10 to upregulate the IL-2 receptors. IL-10 induced an increased expression of CD25/Tac on normal and leukemic B cells cultured in the CD40 system. In the absence of cytokine, the CD40 system slightly increased the expression of CD25/Tac on normal B cells, but in contrast to anti-IgM mAb or SAC (14, 30), it did not induce high affinity receptors for IL-2. These results are in accordance with the fact that normal B cells did not proliferate in response to IL-2 in the CD40 system. On the other hand, B-CLL cells, which spontaneously express CD25 and synthesize DNA in response to IL-2, were found to express an average of 200 high affinity IL-2 receptors with a K_d of 19 pM (data not shown). Accordingly, these cells incorporate [^{3}H]TdR in response to IL-2 in the CD40 system. IL-10 induced high affinity IL-2 receptors on anti-CD40 activated normal B cells and increased the spontaneous expression of high affinity IL-2 receptors on anti-CD40 activated B-CLL cells. These data suggest that IL-10 sensitizes anti-CD40 activated B lymphocytes to IL-2 by inducing their high affinity IL-2 receptors. Very low expression of the β chain of the IL-2 receptor (p75) was detected by flow cytometric analysis on four unstimulated B-CLL populations. After 24–48 h of incubation with IL-10, β chain expression significantly increased but in contrast with the persistent CD25 upregulation, this phenomenon was transient and no more IL-2 receptor β chain was detected after 5 d of culture (our unpublished data). The surface levels of the IL-2 receptor β chain on normal B cells were undetectable by flow cytometry.

The synergy described herein between IL-2 and IL-10 on anti-CD40 activated B cells was also observed, although to a lesser extent, on B cells activated through their antigen receptors. This was most conspicuous on the Ig production of B cells stimulated by particles of SAC (our unpublished results). The synergy between IL-2 and IL-10 may not be specific to B cells as preliminary experiments have shown that the two cytokines synergize for the proliferation of preactivated T cells (our unpublished results). In keeping with this, IL-10 was found earlier to synergize with IL-2 for the proliferation of mature and immature T cells (31) and their differentiation into cytotoxic effectors (32).

The present results may thus explain how T cells, which activate B cells in a CD40-dependent fashion (33, 34, and Blanchard, D., and J. Banchereau, manuscript in preparation) can induce a B cell growth and differentiation which is essentially dependent on IL-2 (35, 36). Whether IL-10 originates from T cells (37, 38) or from B cells (39) during these cell interactions remains to be established.

The authors wish to thank Dr. J. Chiller for support and critical reading of the manuscript.

A. C. Fluckiger is the recipient of a grant from the Foundation Mérieux (Lyon, France).

Address correspondence to A. C. Fluckiger, Schering-Plough, Laboratory for Immunological Research, 27, chemin des Peupliers, B.P. 11, 69571 Dardilly Cedex, France.

Received for publication 12 April 1993 and in revised form 21 July 1993.

References

1. Banchereau, J., and F. Rousset. 1992. Human B lymphocytes: phenotype, proliferation and differentiation. *Adv. Immunol.* 52:125.
2. Fiorentino, D.F., M.W. Bond, and T.R. Mosmann. 1989. Two types of mouse T helper cell. IV Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J. Exp. Med.* 170:2081.
3. Rousset, F., E. Garcia, T. Defrance, C. Péronne, N. Vezzio, D.H. Hsu, R. Kastelein, K.W. Moore, and J. Banchereau. 1992. Human and viral IL-10 are potent growth and differentiation factors for activated human B lymphocytes. *Proc. Natl. Acad. Sci. USA.* 89:1890.
4. Defrance, T., B. Vanbervliet, F. Brière, I. Durand, F. Rousset, and J. Banchereau. 1992. IL-10 and TGF-β cooperate to induce anti-CD40-activated naive human B cells to secrete IgA. *J. Exp. Med.* 175:671.
5. Nakagawa, T., T. Hirano, N. Nakagawa, K. Yoshizaki, and T. Kishimoto. 1985. Effect of recombinant IL-2 and IFN-γ on proliferation and differentiation of human B cells. *J. Immunol.* 134:959.
6. Defrance, T., B. Vanbervliet, J.P. Aubry, and J. Banchereau. 1988. Interleukin 4 inhibits the proliferation but not the differentiation of activated human B cell in response to interleukin 2. *J. Exp. Med.* 168:1321.
7. Rousset, F., E. Garcia, and J. Banchereau. 1991. Cytokine-induced proliferation and immunoglobulin production of human B lymphocytes triggered through their CD40 antigen. *J. Exp. Med.* 173:705.
8. Waldmann, T.A. 1991. The interleukin-2 receptor. *J. Biol. Chem.* 266:2681.
9. Takeshita, T., H. Asao, K. Ohtani, N. Ishii, S. Kumaki, N. Tanaka, H. Munakata, M. Nakamura, and K. Sugamura. 1992. Cloning of the gamma chain of the IL-2 receptor. *Science (Wash. DC).* 257:379.
10. Saiki, O., T. Tanaka, S. Doi, and S. Kishimoto. 1988. Expression and the functional role of a p70/75 interleukin 2-binding molecule in human B cells. *J. Immunol.* 140:853.
11. Tanaka, T., O. Saiki, S. Doi, M. Hatakeyama, T. Doi, T. Kono, H. Mori, M. Fuji, S. Sugamura, S. Negoro, et al. 1987. Functional interleukin 2 receptors in cells lacking Tac antigen. *Eur. J. Immunol.* 17:1379.
12. Tomizawa, K., A. Ishizaka, K. Kojima, M. Nakanishi, Y. Sakiyama, and S. Matsumoto. 1991. Interleukin-4 regulates the interleukin-2 receptors on human peripheral blood B lymphocytes. *Clin. Exp. Immunol.* 83:492.
13. Butcher, R.D., and W. Cushley. 1991. Flow cytometric studies of IL-4–stimulated expression of the CD25 antigen by quiescent human B lymphocyte subpopulations. *Immunology.* 74:511.
14. Karray, S., A. Dautry-Varsat, M. Tsudo, H. Merle-Beral, P. Debre, and P. Galanaud. 1990. IL-4 inhibits the expression of high affinity IL-2 receptors on monoclonal human B cells. *J. Immunol.* 145:1152.
15. Lee, H.K., X. Xia, and Y.S. Choi. 1990. IL-4 blocks the upregulation of IL-2 receptors induced by IL-2 in normal human B cells. *J. Immunol.* 144:3431.
16. Dighiero, G., P. Travade, S. Chevret, S. Fenaux, C. Chastang, J.-L. Binet, and The French Cooperative Group on CLL. 1991. B-cell chronic lymphocytic leukemia: present status and future directions. *Blood.* 78:1901.
17. Tötterman, T.H., K. Nilsson, and C. Sundström. 1980. Phorbol ester–induced differentiation of chronic lymphocytic leukemia cells. *Nature (Lond.).* 288:176.
18. Touw, I., L. Dorssers, and B. Löwenberg. 1987. The proliferative response of B cell chronic lymphocytic leukemia to interleukin 2: functional characterization of the interleukin 2 membrane receptors. *Blood.* 69:1667.
19. Hivroz, C., C. Grillot-Courvalin, J.C. Brouet, and M. Seligman. 1986. Heterogeneity of responsiveness of chronic lymphocytic leukemic B cells to B cell growth factor or IL-2. *Eur. J. Immunol.* 16:1001.
20. Lantz, O., C. Grillot-Courvallin, C. Schmitt, J.P. Fermand, and J.C. Brouet. 1985. Interleukin 2–induced proliferation of leukemic human B cells. *J. Exp. Med.* 161:1225.
21. Fluckiger, A.C., J.F. Rossi, A. Bussel, P. Bryon, J. Banchereau, and T. Defrance. 1992. Responsiveness of chronic lymphocytic leukemia B cells activated via surface Igs or CD40 to B cell tropic factors. *Blood.* 80:3173.
22. Carlsson, M., T.H. Tötterman, A. Rosèn, and K. Nilsson. 1989. IL-2 and a T cell hybridoma (MP6) derived B cell stimulatory factor act synergistically to induce proliferation and differentiation of chronic lymphocytic leukemia cells. *Leukemia.* 3:593.
23. Carlsson, M., C. Sundström, M. Bengtsson, T.H. Tötterman, A. Rosén, and K. Nilsson. 1989. Interleukin 4 strongly augments or inhibits DNA synthesis and differentiation of B-type chronic lymphocytic leukemia cells depending on the costimulatory activation and progression signals. *Eur. J. Immunol.* 19:913.
24. Vallé, A., C.E. Zuber, T. Defrance, O. Djossou, M. De Rie,

and J. Banchereau. 1989. Activation of human B lymphocytes through CD40 and interleukin 4. *Eur. J. Immunol.* 19:1463.
25. Defrance, T., J.P. Aubry, F. Rousset, B. Vanbervliet, J.Y. Bonnefoy, N. Arai, Y. Takebe, T. Yokota, F. Lee, K. Arai, et al. 1987. Human recombinant IL-4 induces Fcε receptors (CD23) on normal human B lymphocytes. *J. Exp. Med.* 165:1459.
26. Binet, J.L., M. Leporrier, G. Dighiero, D. Charron, P. D'Athis, G. Vaugier, H. Merle-Beral, J.C. Natali, M. Raphael, B. Nizet, and J.Y. Follezou. 1977. A clinical staging system for chronic lymphocytic leukemia. *Cancer.* 40:855.
27. Rai, K.R., A. Sawitsky, E.P. Cronkite, A.D. Chanana, R.N. Levy, and B.S. Pasternack. 1975. Clinical staging of chronic lymphocytic leukemia. *Blood.* 46:219.
28. Banchereau, J., P. de Paoli, A. Vallé, E. Garcia, and F. Rousset. 1991. Long term human B cell lines dependent on interleukin 4 and anti-CD40. *Science (Wash. DC).* 251:70.
29. Robb, R.J., W.C. Greene, and C.M. Rusk. 1984. Low and high affinity cellular receptors for interleukin 2. Implications for the level of Tac antigen. *J. Exp. Med.* 160:1126.
30. Begley, C.G., J.D. Burton, M. Tsudo, B.H. Brownstein, J.L. Ambrus, and T.A. Waldmann. 1990. Human B lymphocytes express the p75 component of the interleukin 2 receptor. *Leukemia Res.* 14:263.
31. MacNeil, I.A., T. Suda, K.W. Moore, T.R. Mosmann, and A. Zlotnik. 1990. IL-10, a novel growth cofactor for mature and immature T cells. *J. Immunol.* 145:4167.
32. Chen, W.F., and A. Zlotnik. 1991. IL-10: a novel cytotoxic T cell differentiation factor. *J. Immunol.* 147:528.
33. Fanslow, W.C., D.M. Anderson, K.H. Grabstein, E.A. Clark, D. Cosman, and R.A. Armitage. 1992. Soluble forms of CD40 inhibit biologic responses of human B cells. *J. Immunol.* 149:655.
34. Noelle, R.J., M. Roy, D.M. Shepherd, I. Stamenkovic, J.A. Ledbetter, and A. Aruffo. 1992. A 39-kDa protein on activated helper T cells binds CD40 and transduces the signal for cognate activation of B cells. *Proc. Natl. Acad. Sci. USA.* 89:6550.
35. Forman, M.S., and E. Puré. 1991. T-independent and T-dependent B lymphoblasts: helper T cells prime for interleukin 2-induced growth and secretion of immunoglobulins that utilize downstream heavy chains. *J. Exp. Med.* 173:687.
36. Lipsky, P.E. 1990. The induction of human B-cell activation, proliferation and differentiation by anti-CD3-stimulated T cells—a model of T cell/B cell collaboration. *Res. Immunol.* 141:424.
37. Yssel, H., R. de Waal Malefyt, M.G. Roncarolo, J.S. Abrams, R. Lahesmaa, H. Spits, and J.E. de Vries. 1992. IL-10 is produced by subsets of human $CD4^+$ T cell clones and peripheral blood T cells. *J. Immunol.* 149:2378.
38. Del Prete, G., M. De Carli, F. Almerigogna, M.G. Guidizi, R. Biagiotti, and S. Romagnani. 1993. Human IL-10 is produced by both type 1 helper (Th1) and type 2 helper (Th2) T cell clones and inhibits their antigen-specific proliferation and cytokine production. *J. Immunol.* 150:353.
39. Burdin, N., C. Péronne, J. Banchereau, and F. Rousset. 1993. Epstein-Barr virus transformation induces B lymphocytes to produce human interleukin 10. *J. Exp. Med.* 177:295.