

Interleukin 10, a Novel B Cell Stimulatory Factor: Unresponsiveness of X Chromosome-linked Immunodeficiency B Cells

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Summary

Highly purified, small dense splenic B cells from unstimulated mice showed increased expression of class II major histocompatibility complex (MHC) antigens and enhanced viability when cultured with affinity-purified recombinant interleukin 10 (rIL-10), compared with B cells cultured in medium alone. These responses were blocked by a monoclonal antibody (mAb) specific for IL-10, but not by an isotype-matched control antibody. IL-10 did not upregulate the expression of Fcε receptors (CD23) or class I MHC antigens on small dense B cells or induce their replication as monitored by [³H]thymidine incorporation. While these B cell-stimulatory properties of IL-10 are also mediated by IL-4, the two cytokines appear to act independently in these assays; anti-IL-10 antibodies blocked IL-10 but not IL-4-mediated B cell viability enhancement, and vice versa. Similarly, since IL-4 upregulates CD23 on small dense B cells, the inability of IL-10 to do so argues against its acting via endogenously generated IL-4. Finally, IL-10 did not upregulate class II MHC antigens on B cells from X chromosome-linked immunodeficiency (XID) mice, while the same cells showed normal upregulation of class II antigens in response to IL-4. This report also extends our understanding of the relationship between IL-10 and the highly homologous Epstein-Barr virus (EBV)-encoded Bam HI fragment C rightward reading frame no. 1 (BCRF1) protein. It has previously been shown that BCRF1 protein exhibits the cytokine synthesis inhibitory activity of IL-10. This report indicates that BCRF1 protein also enhances *in vitro* B cell viability, but does not upregulate class II MHC antigens on B cells. One explanation for these data is that IL-10 contains at least two functional epitopes, only one of which has been conserved by EBV.

B lymphocytes contribute to the immune response by their production of specific antibodies in response to antigenic stimuli. This process is regulated by a subset of soluble glycoproteins collectively termed cytokines (1-3). The precise number of cytokines involved and their modes of action require further clarification. IL-10 (originally designated cytokine synthesis inhibitory factor [CSIF]¹; 4) is a cytokine produced by activated type 2 T helper (Th2) cells (4, 5) and B cells (6, 7), which has the property of suppressing cytokine production by type 1 T helper (Th1) cells (4). Isolation of the cDNA for mouse IL-10 (8) revealed a striking homology between

this cytokine and a previously uncharacterized open reading frame, designated BamHI fragment C rightward reading frame no. 1 (BCRF1), in the EBV genome. Subsequent expression and testing of the protein encoded by BCRF1 revealed that this molecule also mediated suppression of Th1 cytokine production (9). Isolation of IL-10 genomic clones (8) confirmed the mammalian origin of this cytokine, and suggested viral capture of a cellular gene that presumably conferred some survival advantage on the virus.

In this report, we show that IL-10 is also a novel B cell stimulatory factor, exerting at least two effects on small dense splenic B cells from unstimulated mice, namely upregulation of their class II MHC antigens and enhancement of their *in vitro* viability. X chromosome-linked immunodeficiency (XID) B cells are unresponsive to the class II inducing activity of IL-10, suggesting a possible role for this cytokine in the immunodeficiency of these animals.

¹ Abbreviations used in this paper: BCRF1, BamHI fragment C rightward reading frame no. 1; CSIF, cytokine synthesis inhibitory factor; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; SA, streptavidin; Th1/Th2, type 1/type 2 T helper cells; XID, X chromosome-linked immunodeficiency.

Materials and Methods

Mice. 8–12-wk-old female BALB/cByJ, DBA/2J, and CBA/J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The XID gene-bearing (CBA/N × DBA/2J) male and their normal female littermates were bred at the DNAX animal facility (DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA) from breeding pairs obtained from The Jackson Laboratory.

Anticytokine Antibodies. Hybridoma cell lines producing rat IgM anti-IL-10 antibody (SXC1) (10) or an irrelevant rat IgM isotype control designated J5/D were expanded in serum-free medium, and the accumulated antibody was purified to a single band on a Coomassie blue-stained SDS-PAGE gel by two sequential 50% ammonium sulfate precipitates. Both antibodies were used at 10–50 µg/ml. Anti-IL-4 antibody (11B11) (11) and an IgG1 isotype control (GL113) were used at 10 µg/ml.

Cytokines. Mouse rIL-10 was expressed in *Escherichia coli* and affinity purified using the SXC1 anti-IL-10 antibody (Castle, B. E., W. Dang, R. Kastelein, and M. Howard, manuscript in preparation). Alternatively, mouse rIL-10 (8), human rIL-10 (12), and BCRFI (9) were expressed in COS7 cells. Mock supernatants from COS7 cells transfected with no cDNA or irrelevant cDNA were used as negative controls. Natural IL-10 derived from Con A-stimulated D10 Th cells was purified as described elsewhere (4) and generously provided by M. W. Bond (DNAX). For both IL-10 and BCRFI, a unit of activity was defined as the amount of protein per milliliter that produced 50% of saturating activity in the cytokine synthesis inhibitory assay (4). Murine rIL-4 was expressed in *E. coli* as described by van Kimmenade et al. (13) and was affinity purified as described elsewhere (14). A unit of IL-4 activity was defined as the amount of IL-4 per milliliter that produced 50% of saturating activity in the HT-2 cell proliferation assay (5).

B Cell Preparation and Culture. Small dense B cells from unstimulated mouse spleens were purified as described previously (15). Briefly, spleen cells were teased into complete RPMI (cRPMI) containing 5% FCS (J.R. Scientific, Woodland, CA), 5×10^{-5} M 2-ME (Polysciences, Inc., Warrington, PA), 100 U/ml penicillin and 100 µg/ml streptomycin (Irvine Scientific, Santa Ana, CA), 2 mM glutamine (J.R. Scientific), and 25 mM Hepes buffer (Irvine Scientific). RBC were lysed using 0.83% ammonium chloride, pH 7.4. In some experiments adherent cells were removed by incubating cells on petri dishes (Falcon Labware, Oxnard, CA) at 2×10^7 cells/ml, 10 ml/dish at 37°C for 1 h. T cells were subsequently removed using two successive treatments of anti-mouse Thy-1.2 mAb (New England Nuclear, Boston, MA) and anti-L3T4 antibody (RL172.4 hybridoma, a gift from Dr. H. R. MacDonald, Ludwig Institute, Epalinges, Switzerland) for 20 min at 4°C, followed by complement (1:10 dilution of Low-Tox-Rabbit complement; Cedarlane Laboratory, Ontario, Canada) for 30 min at 37°C. Small dense B cells were then isolated by density centrifugation using a discontinuous gradient composed of 75, 65, and 50% Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) at $2,500 \times g$ for 25 min, at 4°C. Cells collected from the interface between 65 and 75% Percoll solutions were shown to be small dense B cells by several criteria (15, 16) and used in all subsequent experiments. Small B cells were cultured with various cytokines at $1-2 \times 10^5$ cells/well in 100 µl cRPMI in 96-well flat-bottomed culture plates (3072; Falcon Labware).

Immunofluorescence Determination of Surface Markers. Cultured B cells were harvested from microtiter plates, washed, then incubated at $1-2 \times 10^5$ cells/well for 30 min at 4°C with one of the following antibodies: biotinylated anti-I-A^d (mouse IgG2a) at 2.5 µg/ml, biotinylated anti-H-2K^k (mouse IgG2a) at 2.5 µg/ml, biotinylated anti-huHLA-DR (mouse IgG2a) at 5 µg/ml, or bio-

tinylated anti-rat κ chain (MAR 18.5, mouse IgG2a) at 5 µg/ml, biotinylated anti-CD23 antibody (B3B4, rat IgG2a) at 5 µg/ml. With the exception of anti-CD23 (17), which was obtained as a gift from Dr. D. H. Conrad at Medical College of Virginia, these antibodies were purchased from Becton Dickinson & Co. (Mountain View, CA). All antibodies were biotinylated in our laboratory by standard methods. Cells were washed in 1–2 ml HBSS (Gibco Laboratories, Grand Island, NY) + 3% BSA (Sigma Chemical Co., St. Louis, MO) and subsequently incubated with streptavidin-PE (SA-PE) (Becton Dickinson & Co.) at 1:10 in HBSS + 3% BSA for 30 min at 4°C. Stained cells were fixed in HBSS (Gibco Laboratories) + 1% formaldehyde (J.T. Baker Chemical Co., Phillipsburg, NJ) and analyzed within 3 d. The relative fluorescence intensities of individual cells were measured using a FACScan analyzer (Becton Dickinson & Co.). Dead cells and aggregated cells were excluded based on their forward angle light scatter. In some experiments, cells were not fixed and dead cells were discriminated based on their forward angle light scatter and propidium iodide uptake.

Measurement of B Cell Viability or Proliferation. Cell viability was estimated by either trypan blue exclusion (0.4% trypan blue stain in 0.85% saline; Gibco Laboratories) or MTT [3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay (18). The latter assay involved culturing cells at 2×10^5 B cells/well for 3 d followed by a 4-h pulse with 50 ng/ml MTT (Sigma Chemical Co.). The colorimetric change, as measured on ELISA reader at 570–650 nm, is an index of total viable cells. DNA synthesis was measured by the incorporation of tritiated thymidine after 4-h pulse with 1 µCi/well [³H]thymidine (NET027; New England Nuclear, Boston, MA).

Results

IL-10 Upregulates Expression of Class II MHC Antigens on Small Dense B Cells. Highly purified small dense B cells from unstimulated BALB/c mouse spleens were cultured for 20–24 h either with various dilutions of affinity-purified rIL-10 expressed in *E. coli*, 100 U/ml affinity-purified rIL-4 as a positive control, or medium alone. Fig. 1A shows that both IL-4 and a saturating dose (200 U/ml) of IL-10 caused substantial upregulation of B cell class II MHC antigen expression compared with B cells cultured in medium alone. B cells cultured under the same three conditions showed no difference in background staining using any of several biotinylated isotype-matched negative control antibodies [e.g., anti-huHLA-DR, anti-H-2K^k, and MAR 18.5 (anti-rat κ chain)] or after preincubation of the cells with anti-Fc receptor antibody (2.4G2) (data not shown). These data indicate that IL-10 does not increase nonspecific binding to B cells. Identical results of increased class II antigen expression were obtained using rIL-10 expressed in COS7 cells or highly purified natural IL-10 produced by activated D10 Th cells (data not shown). IL-10-induced upregulation of class II antigen expression could be totally inhibited by anti-IL-10 mAb (SXC1) but not by an irrelevant isotype-matched control antibody (J5/D) (Fig. 1B). The amount of murine IL-10 required for optimal induction of class II MHC antigens on B cells was comparable with that needed for optimal suppression of cytokine synthesis by Th1 cells; $\sim 10-100$ U/ml produced an optimal response in each assay. Affinity-purified rIL-10 did not upregulate expression of class I MHC antigens or CD23 on small

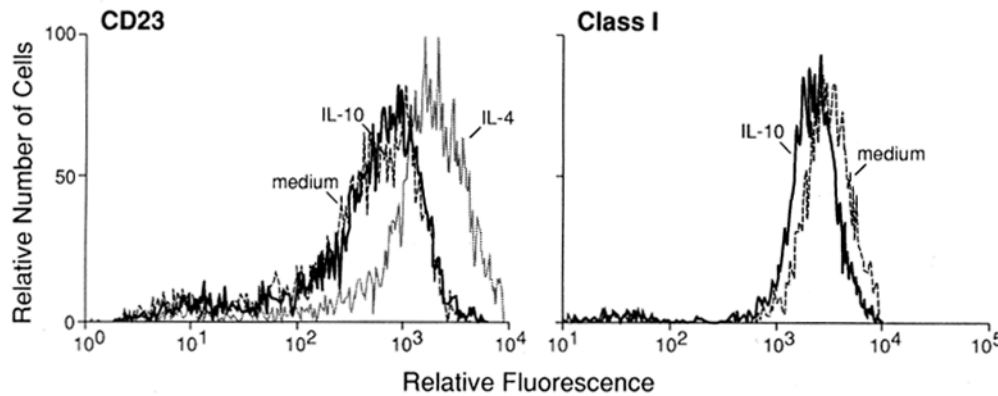
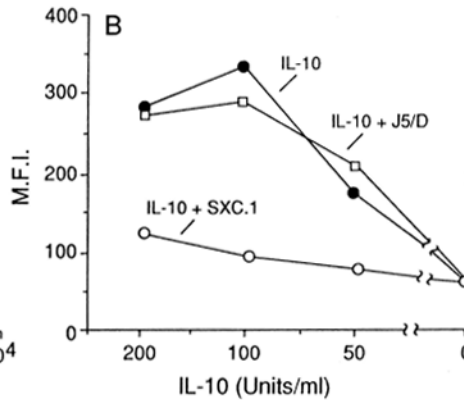
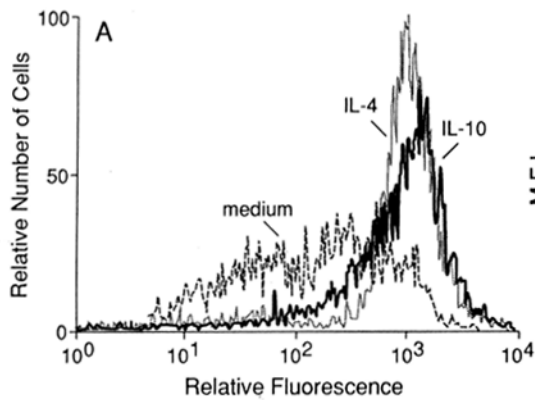


Figure 1. Effect of IL-10 on class II MHC antigen expression on small dense B lymphocytes from unstimulated BALB/c mice. (A) Splenic B cells were cultured for 22 h either in medium alone, in medium supplemented with 200 U/ml affinity-purified *E. coli*-expressed mouse rIL-10, or in medium supplemented with 100 U/ml purified rIL-4. Class II MHC antigen expression was evaluated using biotinylated anti-I-A^d antibody plus SA-PE. (B) Splenic B cells were cultured for 22 h with various concentrations of affinity-purified *E. coli*-expressed rIL-10 alone (●), in the presence of 50 μg/ml anti-IL-10 (SXC1) antibody (○) or an isotype control (J5/D) (□). Mean fluorescence intensities were calculated from 3,000 cells.

Figure 2. IL-10 does not induce CD23 or class I MHC antigen expression on B cells. Small splenic B cells were cultured for 22 h alone, with rIL-10 (200 U/ml), or with rIL-4 (100 U/ml). (Left) Small B cells from BALB/c mice were stained with biotinylated anti-CD23 antibody plus SA-PE. (Right) Small B cells from CBA/J mice were stained with biotinylated anti-H-2K^b antibody plus SA-PE.

dense B cells (Fig. 2). In fact, a small but detectable decrease in class I MHC antigen expression was observed after culture of B cells with IL-10 (Fig. 2).

IL-10 Does Not Upregulate Class II Antigen Expression on XID B Cells. While B cells from several normal strains of mice (e.g., BALB/cByJ, and DBA/2J) responded to IL-10 by upregulating class II MHC antigen expression, small dense splenic B cells from the immunodeficient XID mouse seemed incapable of this response. As shown in Fig. 3, class II antigen expression on XID splenic B cells from CBA/N × DBA/2 male mice was upregulated after IL-4 stimulation, but was unaltered after culture with IL-10. In contrast, the normal control population of CBA/N × DBA/2 female mouse B cells showed significant upregulation of class II MHC antigen expression after either IL-4 or IL-10 stimulation (Fig. 3).

IL-10 Enhances In Vitro Viability of Small Dense B Cells. Highly purified small dense B cells from unstimulated BALB/c

mouse spleens were cultured for various times with 30 U/ml highly purified natural IL-10 from activated D10 cells, rIL-4 at 100 U/ml as a positive control, or medium alone. Viability was evaluated by trypan blue exclusion throughout a 4-d culture period (Fig. 4 A). Alternatively, small B cells were cultured with various dilutions of COS7 cell-expressed mouse rIL-10 (ranging from 150 to 0.15 U/ml), affinity-purified rIL-4, or mock transfection supernatant from COS7 cells. Viability was measured by MTT assay at 3 d of culture (Fig. 4 B). Both assays showed that coculture with IL-10 produced a marked enhancement of B cell viability. Identical results of enhanced mouse B cell viability were obtained using affinity-purified rIL-10 expressed in *E. coli* (data not shown). The level of IL-10-enhanced B cell viability was comparable with that obtained when B cells were cultured with IL-4 (Fig. 4). Antibodies to IL-10 or IL-4 blocked the enhanced B cell viability induced by each specific cytokine, but not by the other cytokine

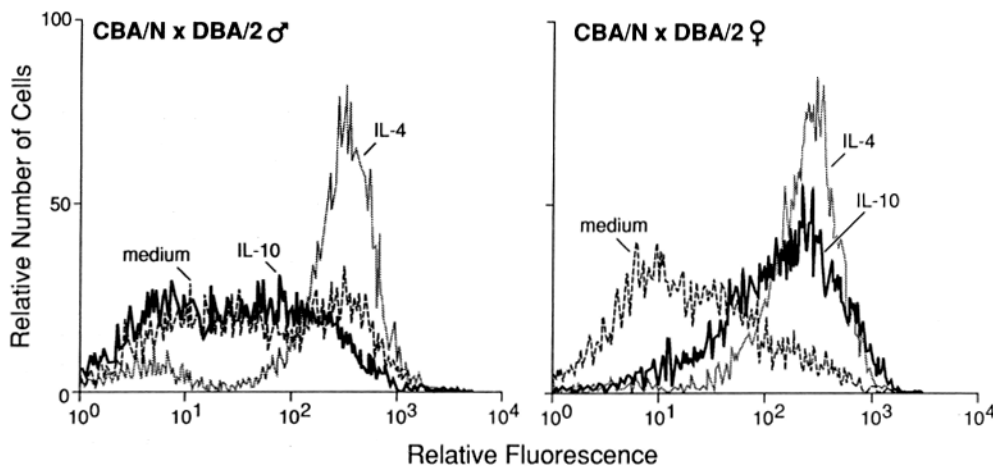


Figure 3. Effects of IL-4 and IL-10 on class II MHC antigen expression on B cells from XID mice. Small splenic B cells from defective male or normal female (CBA/N × DBA/2) F1 mice were cultured for 22 h either alone, with rIL-10 (200 U/ml), or with rIL-4 (100 U/ml).

(Fig. 5), suggesting that both cytokines acted independently in this assay. No additive or synergistic effect was observed when IL-10 and IL-4 were both added to the B cell cultures (data not shown). While IL-10 enhanced the viability of small dense B cells, it did not induce their proliferation as evaluated by [³H]thymidine incorporation (Fig. 6). Similarly, IL-10 did not affect B cell proliferation induced by the polyclonal B cell activator, LPS (Fig. 6).

Effect of BCRFI Protein on Small Dense B Cells. As mentioned above, the sequence of IL-10 is highly homologous to that of an EBV open reading frame designated BCRFI (8). Furthermore, IL-10 and BCRFI protein share the property of suppressing cytokine synthesis by activated Th1 cells (4, 9). We therefore tested whether BCRFI protein also resembled IL-10 in terms of its effects on small dense B cells. BCRFI protein expressed in COS7 cells caused a marked enhancement of the *in vitro* viability of small dense murine B cells (Fig. 7). The level of viability enhancement was comparable with that induced by human rIL-10, although both of these stimuli were less effective than mouse IL-10 in enhancing viability of mouse B cells (Fig. 7). It is not surprising that a

functional level BCRFI is more closely related to human IL-10 than to mouse IL-10, since BCRFI and human IL-10 are both evolved to act on human cells and BCRFI shows a higher degree of homology to human IL-10 than to mouse IL-10 (12). The results shown in Fig. 7 indicate that comparable amounts of each stimulus were effective at enhancing B cell viability. The maximal responses were obtained with 50 U/ml mouse IL-10, human IL-10, or BCRFI, with units in each case referring to their respective activities in the murine cytokine synthesis inhibition assay (4).

In contrast, quantities of BCRFI protein varying from 25 to 250 U/ml did not significantly alter B cell class II antigen expression when compared with B cells cultured in COS7 mock supernatants or medium alone (Fig. 8 C). Both mouse and human IL-10 within that concentration range were capable of maximal upregulation of B cell class II antigen expression (Fig. 8, A and B). Thus, BCRFI appeared capable of mediating only one of the two B cell stimulatory properties of IL-10 described in this report. This conclusion is supported by results obtained in multiple experiments, using several preparations of rBCRFI.

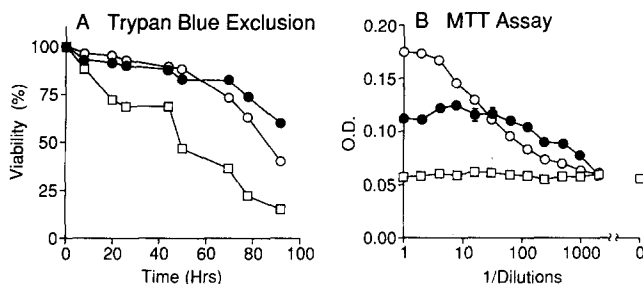


Figure 4. IL-10 enhances *in vitro* B cell viability. (A) Small dense B cells were cultured in medium alone (□), in partially purified D10 cell-derived IL-10 (30 U/ml) (○), or in rIL-4 (100 U/ml) (●) for various times. Cell viability was determined by trypan blue exclusion. (B) Small dense B cells were cultured for 3 d in various dilutions of COS7 cell-expressed mouse rIL-10 starting at 150 U/ml (●), in mock supernatant added at identical dilutions (□), or in various dilutions of rIL-4 starting at 100 U/ml (○). Cell viability was measured by MTT assay. The OD readings are proportional to the number of viable cells.

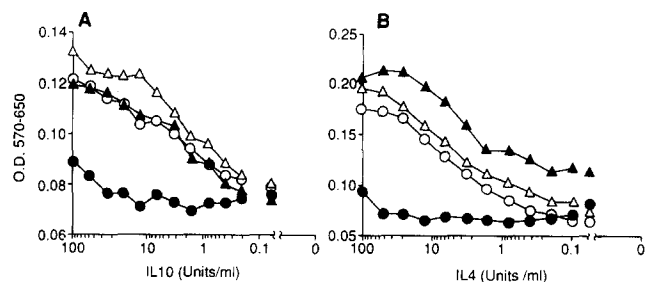


Figure 5. IL-10 and IL-4 enhance *in vitro* B cell viability independently. Small splenic B cells from BALB/c mice were cultured for 3 d with various concentrations of IL-10 or IL-4. (A) Cells were cultured in affinity-purified *E. coli*-expressed rIL-10 (○), IL-10 plus 10 μg/ml anti-IL-10 antibody SXC.1 (●), IL-10 plus 10 μg/ml anti-IL-4 antibody 11B11 (▲), or IL-10 plus 10 μg/ml SXC.1 isotype control antibody J5/D (Δ). (B) Cells were cultured in rIL-4 (○), IL-4 plus 10 μg/ml 11B11 (●), IL-4 plus 10 μg/ml SXC.1 (▲), or IL-4 plus 10 μg/ml 11B11 isotype control antibody GL113 (Δ).

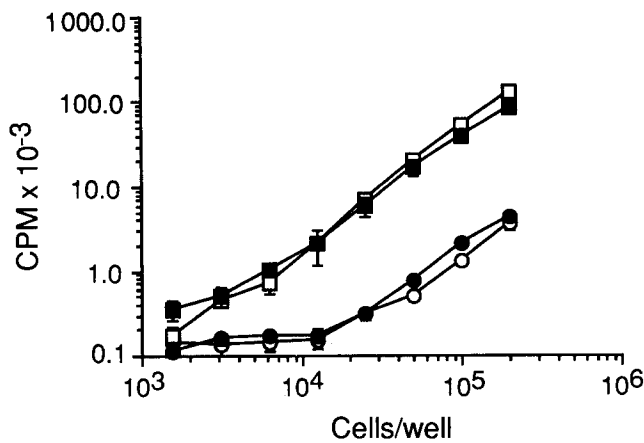


Figure 6. IL-10 does not induce proliferation of small dense B cells. Small dense B cells were cultured for 3 d at various cell concentrations in medium alone (○), in affinity-purified *E. coli*-expressed mouse rIL-10 (50 U/ml) (●), in LPS (50 µg/ml) (□), or in LPS (50 µg/ml) plus IL-10 (50 U/ml) (■). Cell proliferation was measured by [³H]thymidine incorporation (cpm).

Discussion

This report identifies IL-10 as a novel B cell stimulatory factor. Highly purified natural or rIL-10 can upregulate class II MHC antigen expression and enhance in vitro viability of small dense splenic B cells from unstimulated mice. Previous studies have shown that both of these activities are also medi-

ated by IL-4 (16, 19–22). We believe, however, that IL-10 and IL-4 induce these B cell stimulatory activities by independent mechanisms. Anti-IL-10 antibodies blocked IL-10- but not IL-4-enhanced B cell viability and vice versa. Similarly, while both IL-10 and IL-4 mediate upregulation of class II MHC antigen expression on resting B cells, only the latter cytokine upregulates CD23 expression on small dense B cells (Fig. 2; references 22–24). These data argue against the possibility that IL-10-mediated upregulation of B cell class II MHC antigen expression is due to endogenously generated IL-4. The effects of IL-4 and IL-10 on upregulating class II MHC antigen expression are further distinguished by our experiments using the immunodeficient XID gene-bearing mice. The results showed that XID B cells upregulate class II antigen expression after stimulation with IL-4, but not IL-10. One possible explanation for the unresponsiveness of XID B cells to IL-10 is that they may lack functional IL-10 receptor(s) or associated signal-transducing molecules. Alternatively, it is possible that the observed induction of class II antigen expression on the majority of splenic B cells (Figs. 1 and 3) is critically dependent on the presence of Ly-1⁺ B cells, a numerically small subpopulation of normal splenic B cells, which are absent from XID mice (25). Experiments to evaluate these possibilities are in progress. The lack of class II MHC antigen induction on XID B cells suggests a possible role for IL-10 in thymus-independent type II antigen responsiveness and/or expansion of Ly-1⁺ B cells, processes which are known to be deficient in XID mice (25, 26).

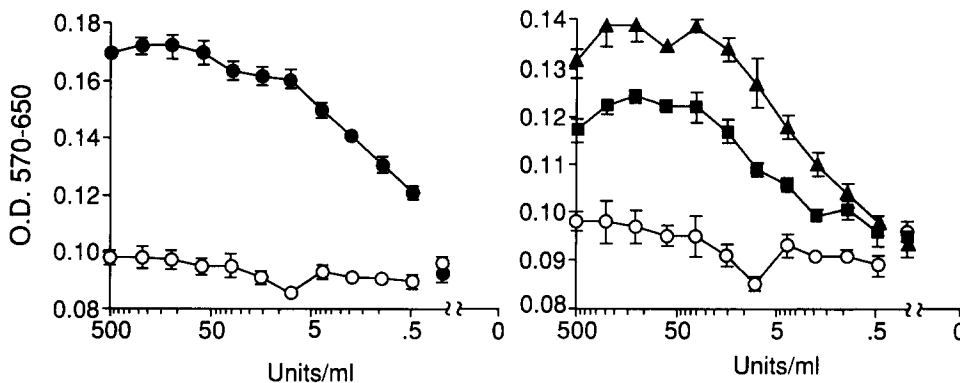


Figure 7. Effect of BCRFI protein on in vitro B cell viability. Small dense B cells were cultured for 3 d in COS7 cell-expressed mouse rIL-10 (●), human rIL-10 (▲), BCRFI (■), or mock supernatant from COS7 cells (○). Cell viability was evaluated by MTT assay.

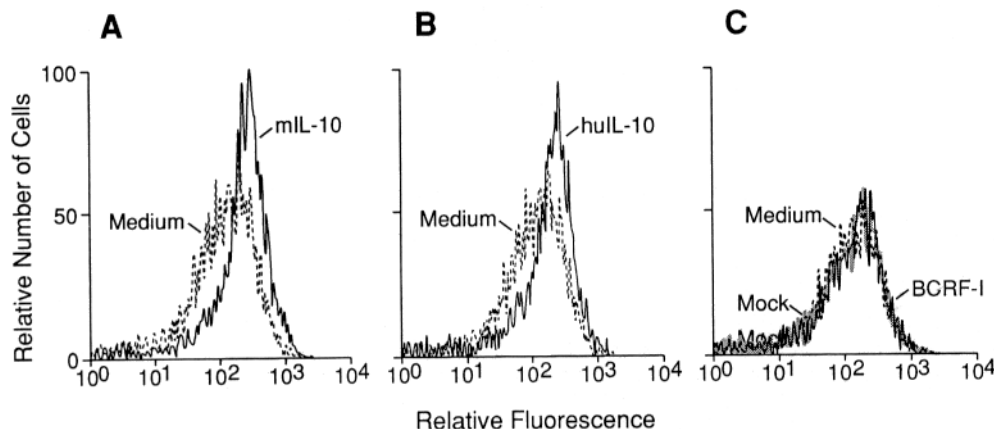


Figure 8. Effect of BCRFI on class II MHC antigen expression on small dense B cells. Splenic B cells from BALB/c mice were cultured for 22 h alone, or with 100 U/ml of COS7 cell-expressed mouse rIL-10, human rIL-10, BCRFI, or mock supernatant of same dilutions.

This study focuses on the ability of IL-10 to directly stimulate small dense B cells. Separate studies have previously identified three factors capable of directly stimulating small dense B cells: IL-4 (19, 20), BCAAF (27), and TRF2 (28). The latter two factors have not been purified to date and so their possible identity with IL-10 cannot be excluded. Indeed, BCAAF resembles IL-10, in terms of inducing class II MHC antigens, but not CD23 on small dense B cells (27), and TRF2 has been shown to be inactive on XID B cells (28). While other properties would appear to distinguish these factors from IL-10, e.g., their ability to induce cell division and/or Ig secretion by resting B cells, it must be recognized that in the absence of purification or cloning, such responses may reflect synergisms between multiple biologically active molecules.

This study also extends our understanding of the mechanisms that regulate class II antigen expression on APC, with IL-10 joining a group of cytokines that were previously shown to regulate class II expression on different cell types (19, 20, 29–31). An interesting picture concerning the interaction of Th cells and APC is beginning to emerge from these collective studies. IFN- γ , a specific product of Th1 cells (5), upregulates class II antigens on macrophages (30–32), but not on B cells (33) and suppresses IL-4-induced class II upregulation on B cells (33, 34). Conversely, IL-10 and/or IL-4, specific products of Th2 cells (4, 5), upregulate class II antigens on B cells (19, 20; this study) with little or no such effect on IFN- γ -activated macrophages (reference 20; Fiorentino, D. F., A. Zlotnik, P. Vieira, T. R. Mosmann, M. Howard, K. W. Moore, and A. O'Garra, manuscript submitted for publication), and suppress production of IFN- γ (4), a major activator of macrophage antigen-presenting function (30, 31). Thus, it would appear that a product of Th1 cells favors antigen presentation by macrophages, and products of Th2 cells

favor antigen presentation by B cells. Whether these preferences correspond to a role for macrophages versus B cells in determining the nature of the helper T cell response generated in an immune response remains to be established.

Our data also provide further information concerning the relationship between the mammalian cytokine IL-10 and the EBV-encoded BCRFI protein. Both IL-10 and BCRFI suppress cytokine production by Th1 cells (9). Our experiments suggest that both proteins enhance in vitro viability of small dense B cells, but only IL-10 upregulates class II MHC antigens on B cells. One explanation for these data is that BCRFI protein induces signals at a lower efficiency than IL-10, with this efficiency being sufficient to trigger the thresholds of the CSIF and B cell viability enhancement assays, but insufficient to trigger the class II antigen-inducing effects. An alternative theory is that IL-10 contains at least two functional epitopes, one of which mediates CSIF activity and enhancement of B cell viability, and the other which induces class II MHC antigens on B cells. The data obtained with BCRFI protein would be explained if only the first of these functional epitopes has been captured by EBV. This latter proposal of two functional epitopes is supported by separate studies that indicate that wide concentration ranges of BCRFI-encoded protein fail to mediate two other activities of IL-10, specifically growth costimulation of mature and immature murine thymocytes (35), and of mast cells (12). If the theory of two functional epitopes is correct, it is interesting that the two IL-10 properties captured by the EBV protein, i.e., suppression of Th1-specific cytokines such as IFN- γ and enhancement of B cell viability, are the properties most likely to enhance viral survival in the host, since EBV grows in B cells, and IFN- γ is a major host defense against EBV transformation of B cells (36, 37).

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