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IFN-α/β enhances BCR-dependent B cell responses

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Abstract

Type I interferon (IFN-I) is constitutively produced in the bone marrow (BM), and induced at sites of inflammation and following infection by viruses or microorganisms. We have previously shown that IFN-I regulates the generation and selection of normal B cell populations in the BM. In the present work, we assess the effects of IFN-I on mature B cell function by monitoring the responses of IFN-α/β-treated murine splenic B cells to apoptotic, mitogenic and activating stimuli. A similar analysis is performed on BM mature B cells obtained from wild-type or IFN-I receptor-deficient mice. IFN-α/β is shown to induce B cells to a state of partial activation characterized by the up-regulation of CD69, CD86 and CD25 molecules in the absence of either proliferation or terminal differentiation. B cells treated with IFN-α/β show an increased survival and resistance to Fas-mediated apoptosis. IFN-α/β also enhances B cell responses to BCR ligation such as calcium fluxes, IgM internalization, induction of activation markers and proliferation. These results indicate that in addition to its inhibitory effect on viral replication and T cell apoptosis, IFN-α/β plays an essential role during an inflammatory response by lowering the threshold for B cell induction, thereby promoting fast and polyclonal antibody responses.

Introduction

IFN are potent antiviral cytokines produced in response to viral infection. There are two broad categories, called type I (IFN-I) and type II (IFN-II), which were originally distinguished on the basis of the nature of the producing cells. IFN-II is secreted exclusively by NK and T lymphocytes, while IFN-I is produced by a large spectrum of different cell types, mostly components of the innate immune system (1–3). Unlike IFN-II, which is encoded by a single gene, the IFN-γ, IFN-I is a family of gene products which differ in structure and expression patterns, but all bind to a single specific IFN-I receptor (IFN-IR) (4). In mammals, several IFN-α, and single IFN-β and IFN-ω genes encode the various members of IFN-I. Expression of IFN-I follows infection with viruses, mycoplasma and bacteria, and can be experimentally induced by non-infectious agents such as nucleic acids and glucocorticoid hormones (5–8). In turn, IFN-IR ligation induces the expression of numerous genes such as MHC class I, IFN regulatory factor-1, double-stranded RNA-dependent protein kinase, intracellular adhesion molecule-1, oligoadenylate synthetase and MxA, in a large spectrum of different cell types. Most of these proteins promote the elimination of virally infected cells or act as inhibitors of viral replication (1).

Although best known for activation of macrophages and NK cells (9,10), IFN-α/β has clear pleiotropic effects on the immune system. In addition to its induction during infection, IFN-I is constitutively produced in primary lymphoid organs (bone marrow and thymus) where it interferes with lymphocyte differentiation (11,12). At early stages of lymphocyte development, it impairs IL-7-dependent proliferation of pre-B and pre-T cells (13). Later during B cell differentiation, it is responsible for the low threshold of sensitivity to IgM ligation characteristic of both mature and immature B cells in the bone marrow (14). As a direct consequence of this regulation, it determines the stringency of B cell repertoire selection and the number of IgM-secreting B cells in the bone marrow (BM) (15). During an immune response, IFN-α/β also appears to prolong the survival of activated T cells and may promote the generation of memory T cells (16–18). However, studies on resistance to viral infection of mice deficient for either the IFN-IR (IFN-IR$^{-/-}$) or the IFN-γ receptor provided a rough general-

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ization that B cell responses are predominantly controlled by IFN-I, while IFN-γ regulates T cell activities (19). The authors demonstrated that IFN-I is necessary for the resolution of viral infections controlled by antibodies, but has little or no impact on viral infections that are largely controlled by non-antibody-dependent mechanisms. Little is known, however, on the direct effect of IFN-I on B cell function in the course of an immune response and B cell responses to IFN-I have been sparsely studied.

In order to evaluate the role of IFN-α/β in B lymphocyte responses, we have investigated the direct effect of IFN-I on mature B cells. The responses of freshly isolated B lymphocytes, treated or not with IFN-α/β, to apoptotic, proliferative and activating stimuli have been compared. The results indicate that IFN-α/β induces B cells to a state of partial activation associated with a higher sensitivity to further stimulation through the BCR. The finding that IFN-IR acts as an amplifier of the BCR signal suggests that IFN-I enhances B cell function during an inflammatory response, and thus serves as a bridge between the innate and acquired immune responses.

Methods

Mice

C57BL/6 originally purchased from Iffa-Credo (l'Abresle, France), IFN-IR-deficient mice (19) (a gift from Michel Aguet) and control 129Sv wild-type animals were bred in our animal facilities. All mice were analyzed between 6 and 12 weeks of age.

Cell purification and culture

Comparisons between BM cells originating from wild-type or IFN-IR$^{-/-}$ mice were performed using the 129Sv animals, while analyses of splenocytes treated with IFN-α/β was achieved with cells prepared from C57BL/6 mice, unless otherwise mentioned. Cell suspensions were prepared by grinding spleens with nylon mesh tissue or by flushing whole BM from femurs and tibias with a 23-gauge needle in HBSS (Gibco/BRL, Gaithersburg, MD) containing 2% FCS (Gibco/BRL). When required, following T cell depletion by mAb anti-Thy1 (from the J1J hybridoma) and complement (Low-Tox rabbit complement; Hornby, Cedarlane, Ontario, Canada), splenocytes were separated on five layers of Percoll gradient (Pharmacia, Uppsala, Sweden). The 60–70% interface contained resting (small, high-density) cells while activated (large, low-density) cells were recovered at the 50–60% interface. FACS analyses for FSC and staining with anti-B220 indicated a purity of at least 95 and 80% for resting and activated B220$^+$ cells respectively. Cell cultures were performed in RPMI 1640 (Gibco/BRL) supplemented with 10% FCS (Gibco/BRL), streptomycin (50 μg/ml), penicillin (50 U/ml), 2-mercaptoethanol (50 μM), HEPES buffer (10 mM, pH 7.3) and sodium pyruvate (1 mM) (all from Gibco/BRL). Pre-incubation with IFN-α/β (Sigma-Aldrich, Madrid, Spain) lasted for 16 or 24 h prior to initiation of each assay unless otherwise indicated and the concentration of IFN-α/β was maintained at 400 U/ml during the course of the experiment. Control cultures were maintained for the same time in media alone. Actinomycin D (Sigma) was used at 4 μg/ml. Bulk cultures were set at 2×10^6 cells/ml, high-density cultures at 10^7 cells/ml and proliferation assay at 2.5 or 5×10^5 cells/ml.

Antibodies, cytokines and FACS analyses

In vitro assays of BCR ligation used antibodies directed against the μ chain of mouse Ig, either monoclonal rat IgG (purified in the laboratory from the hybridoma 331.12 (20)), goat polyclonal F(ab′)$_2$ (Pierce, Rockford, IL) or affinity-isolated goat polyclonal Ig (Sigma). The rat anti-mouse CD40 antibody was produced in the laboratory using the FGK45 hybridoma (a gift from J. Andersson, Basel Institute of Immunology, Switzerland). Lipopolysaccharide (LPS) from *Salmonella typhimurium* was purchased from Sigma. Culture supernatant from the transfected 3T3-IL-4 cell line (a gift from A. Cumano, Pasteur Institute, France) diluted 1/100 was used as a source of IL-4. All labeled antibodies used for flow cytometry analyses were purchased from Becton Dickinson (Mountain View, CA). Single-cell suspensions of BM or splenic B cells were stained with various antibodies coupled to FITC, phycoerythrin, biotin or allophycocyanin at previously determined concentrations. Biotinylated antibodies were revealed by streptavidin–phycoerythrin (Becton Dickinson). Dead cells were excluded from analysis by gating out propidium iodide (Sigma) stained cells. Data were collected from 5 to 10×10^3 cells on a FACScan or a FACSCalibur flow cytometer (Becton Dickinson) and analyzed using the CellQuest program.

Measure of apoptosis by TUNEL

The measures of apoptosis were performed using a TUNEL kit (Roche Diagnostics, Mannheim, Germany) following the instructions of the manufacturer. The cells were stained with phycoerythrin–-B220 antibody prior to fixation. Acquisition was completed on a FACSCalibur flow cytometer (Becton Dickinson). All results were confirmed by propidium iodide staining of ethanol fixed cells.

Measurements of proliferation

[^{3}H]Thymidine (ICN, Aurora, OH; sp. act. 5 Ci/mol) uptake was performed at day 3 or 4 of culture by adding 1 μCi/culture, 6 h before harvest. Pulsed cultures were harvested on glass-fiber filter paper using a 96-well microtiter plate harvester (TOMTEC; Pharmacia/LKB, Bromma, Sweden). After drying, the filters were immersed in scintillation fluid and counted in a Rack-Beta Plate liquid scintillation counter (Pharmacia/LKB). The data represent the mean and SD of three cultures expressed as c.p.m. per culture.

Measurements of Ca^{2+} influxes

A protocol adapted from Sei and Akora (21) was followed. Briefly, 10^7 cells/ml were incubated for 20 min at 37°C in HBSS (Gibco/BRL) containing 4 μg/ml Fluo-3 AM (Molecular Probes, Eugene, OR). Cells were then diluted 5-fold in HBSS containing 1% FCS and incubated for 40 min at 37°C. Following surface staining with phycoerythrin–anti-B220 antibody, the cells were washed and resuspended at 2×10^6/ml in HEPES buffer (137 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 5 mM glucose, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1 g/ml BSA and 10 mM HEPES). Before each test, cells were incubated during 10 min

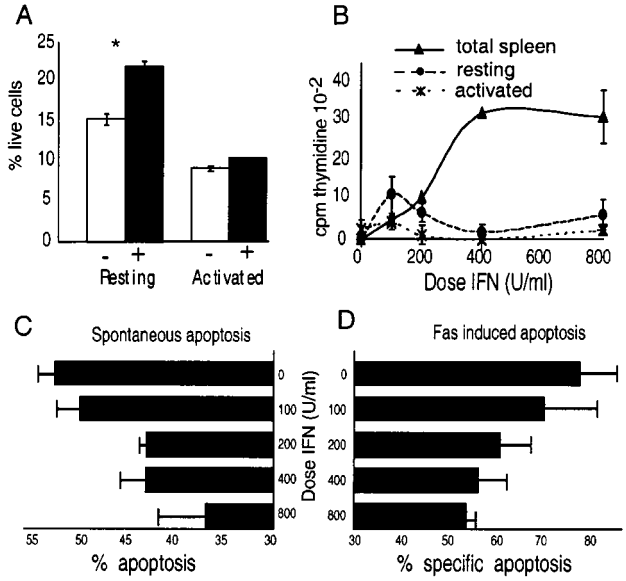

Fig. 1. IFN-α/β enhances B lymphocyte survival and resistance to apoptosis. (A) Percentages of surviving cells in purified resting or activated B lymphocytes cultured for 48 h in the presence (+) or absence (–) of 400 U/ml IFN-α/β. Histograms represent the mean of three independent experiments ± SD (*$P < 0.01$). (B) High-density cultures (10^7 cells/ml) were set for 24 h in the presence of increasing concentrations of IFN-α/β and then labeled with [^{3}H]thymidine for 6 h. The data represent the mean of the c.p.m. incorporated by the cells in three different culture wells ± SD. (C and D) Evaluation by the TUNEL method of B220$^+$ cell apoptosis among splenocytes cultured for 48 h in medium (C) or of purified B cells after 48 h stimulation with anti-CD40 mAb followed by 7 h anti-Fas mAb treatment (D) in the presence of increasing doses of IFN-α/β. The data in (D) are expressed as percentages of specific apoptosis = (% measured apoptosis – % apoptosis without anti-Fas mAb)/(100 – % apoptosis without anti-Fas mAb) × 100.

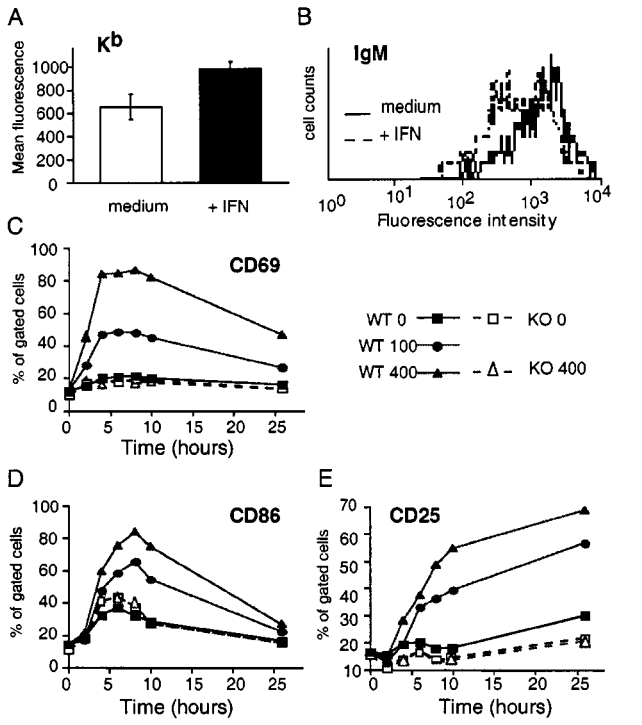

Fig. 2. IFN-α/β induces the expression of several surface markers in a dose-dependent manner. (A and B) Induction of class I MHC K^b expression (A) and decreased expression of mIgM (B) on wild-type splenocytes after 48 (A) and 16 (B) h cultures in the presence of 400 U/ml IFN-α/β. (C and D) Kinetics of expression of the surface markers CD69, CD86 and CD25. Splenocytes from wild-type (WT) or IFN-IR$^{-/-}$ (KO) mice were treated for various times with 0, 100 or 400 U/ml of IFN-α/β as indicated. Marker expression was assessed by flow cytometry on gated live B220$^+$ cells. These results are representative of at least five experiments.

at 37°C. The stimulation was performed with mAb anti-μ 331.12 at doses ranging from 0.1 to 10 μg/ml.

Results

IFN-α/β promotes survival of B cells

Culture of splenocytes or purified lymphocytes in the presence of IFN-α/β consistently resulted in the recovery of a higher number of B lymphocytes. Thus, after 48 h culture in the absence of stimuli other than IFN-α/β, the number of cells recovered increased from 15 to 23% ($P < 0.01$) of the seeded resting B cells (Fig. 1A). In contrast, IFN-α/β treatment did not modify the recovery of large B cells, a population of lymphocytes naturally activated *in vivo*. This increased yield was attributed to increased survival rather than proliferation since addition of IFN-α/β to high-density cultures (10^7 cell/ml) of purified splenic B lymphocytes (resting or activated) did not induce any significant proliferation (Fig. 1B). The IFN-α/β stimulated incorporation of [^{3}H]thymidine observed in total splenocytes cultures (Fig. 1B) was due to non-B cells, as assessed by CFSE staining and FACS analyses (data not shown).

The addition of IFN-α/β also resulted in a dose-dependent protection of B lymphocytes from spontaneous apoptosis. In control cultures, ~55% of B220$^+$ cells undergo apoptosis after 48 h, whereas in the presence of IFN-α/β spontaneous apoptosis was reduced to 37% (Fig. 1C). Similarly, addition of IFN-α/β protected activated B cells from apoptosis induced by engagement of Fas receptor (CD95), once again in a dose-dependent manner (Fig. 1D). Thus, after culture for 48 h in the presence of anti-CD40 mAb followed by 7 h with anti-Fas-R mAb, at the highest concentration (800 U/ml) of IFN-α/β, apoptotic cells in the B220$^+$ population were reduced from some 80% in the control to 55%. In contrast, irradiation induced apoptosis was not affected by IFN-α/β (data not shown). These findings indicate that IFN-α/β selectively affects different pathways of programmed cell death. Taken together, our results indicate that IFN-α/β does not induce proliferation of either resting or activated B lymphocytes and, instead, increases survival of resting B cells, presumably by protecting them from undergoing apoptosis.

IFN-α/β induces the expression of various B cell activation markers

To further characterize the physiological status of IFN-α/β-treated B splenocytes, we measured the expression level of several surface markers associated with B cell activation. In

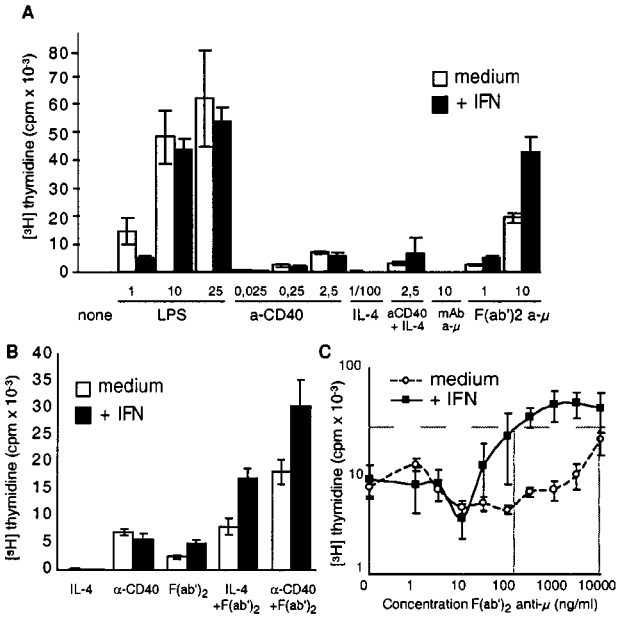

Fig. 3. IFN-α/β enhances the proliferation induced by BCR engagement. (A and B) Purified resting B lymphocytes (5×10^5/ml) were cultured for 72 h with the indicated stimuli. [^{3}H]Thymidine was added 6 h before the end of the culture. Numbers under the *x*-axis indicate concentration (μg/ml); $F(ab')_2$ = $F(ab')_2$ fragments of polyclonal anti-μ antibody; mAb anti-μ = monoclonal anti-μ antibody 331. In (B) the concentration is 1 μg/ml for $F(ab')_2$ anti-μ and 2.5 μg/ml for anti-CD40. (C) Total splenocytes (2.5×10^5/ml) were cultured with 4 μg/ml anti-CD40 mAb and various doses of $F(ab')_2$ anti-μ fragments. [^{3}H]Thymidine was added 6 h before the end of the culture. The data represent the mean c.p.m. incorporated by the cells in three different culture wells ± SD.

agreement with what has been reported for various cell types (22,23), wild-type splenic B cells treated with IFN-α/β for 48 h up-regulate class I molecule expression (K^b, shown in Fig. 2A). The level of IgM expressed at the cell membrane was decreased (Fig. 2B), a feature often associated with BCR-mediated activation. Expression of CD69 was rapidly induced and peaked at ~6 h after initiation of the treatment (Fig. 2C). Induction of CD86 (B7-2) was maximal after 8 h (Fig. 2D), whereas expression of CD25 (the α chain of the IL-2 receptor) was detectable on up to 60% of treated B cells after 10–12 h of incubation (Fig. 2E). Expression of MHC class II, CD38 or CD62 ligand remained unaffected by IFN-α/β treatment (data not shown). While the percentages of B cells induced for expression of K^b, CD69, CD86 or CD25 was dependent on the concentration of IFN-α/β added to the media, the levels of expression per cell remained constant. In addition, the induction was transient and resolved after 24–48 h. The same pattern of expression was obtained when purified resting B cells were analyzed (not shown), indicating that IFN-α/β directly stimulates B cells. In each assay, lymphocytes prepared from mice deficient for the type I IFN receptor (IFN-IR$^{-/-}$) served as controls for the specificity of the responses. Wild-type lymphocytes cultured with medium alone and IFN-IR$^{-/-}$ splenocytes treated or not with IFN-α/β showed equivalent levels of expression for each of the markers tested. We conclude that IFN-I directly and specifically induces a transient activation of B cells.

Similarly to IFN-I treatment, a 6–8 h stimulation by LPS, anti-CD40 mAb, IL-4 or $F(ab')_2$ anti-μ fragments results in readily detectable induction of CD69, CD25 and CD86, while incubation with IL-15 does not induce CD25 expression (data not shown). LPS-mediated activation is associated with cellular proliferation and differentiation of resting B cells to plasma cells, while the anti-CD40 and $F(ab')_2$ anti-μ-mediated signals, although mitogenic, do not result in significant Ig secretion. In contrast, purified B cells treated with IFN-α/β or IL-4 do not proliferate or undergo terminal differentiation (Fig. 3 and data not shown). Taken together, these results indicate that, in 5–10 h, IFN-α/β induces B cells to a state of partial activation.

IFN-α/β enhances proliferation mediated by BCR ligation

We next tested whether IFN-α/β affects B cell responses to further activating stimuli. In a first step, we monitored the proliferative responses of purified resting B cells treated overnight with IFN-α/β and further stimulated by known mitogenic and differentiating factors. IFN-α/β was used at 400 U/ml, a low dose shown above to induce maximal apoptosis inhibition (Fig. 1 C and D) and surface marker expression (Fig. 2). As shown in Fig. 3(A), IFN-α/β had no detectable effect on the proliferation induced by LPS, anti-CD40 mAb or anti-CD40 + IL-4. In contrast, IFN-α/β enhanced the responses of B cells to a mitogenic goat polyclonal $F(ab')_2$ anti-mouse μ chain but did not modify the [^{3}H]thymidine uptake in cultures stimulated by various non-mitogenic anti-μ reagents such as the mAb 331 (Fig. 3A) and polyclonal Ig used or not in conjunction with anti-Fc blocking mAb (not shown). In addition, co-stimulation by anti-CD40 (or IL-4) and $F(ab')_2$ anti-μ induced a strong synergetic proliferative response, further enhanced by IFN-α/β (Fig. 3B).

Our previous work demonstrated that IFN-α/β amplifies the inhibition of LPS-induced terminal differentiation by BCR ligation (14). We tested whether it would also increase the sensitivity of B cells to anti-μ stimulation in the anti-CD40 proliferation assay. Control or IFN-α/β-treated splenocytes were stimulated with a fixed amount of anti-CD40 mAb together with various doses of $F(ab')_2$ anti-μ fragments and incorporation of [^{3}H]thymidine was monitored on day 3 (Fig. 3C). For each cell population, proliferation followed a dose–response relationship that reached the same plateau. However, evaluation of the $F(ab')_2$ concentration resulting in 50% of the maximum proliferation (dotted line in Fig. 3C), revealed that IFN-α/β-treated splenocytes are 100 times more sensitive to anti-μ-mediated proliferation than untreated splenocytes. Since IFN-β is constitutively produced in the BM (11), the same co-stimulation was performed on freshly isolated BM cells prepared from wild-type and IFN-IR$^{-/-}$ mice. For a low concentration of $F(ab')_2$ anti-μ, cells deficient for the IFN-IR proliferated less than wild-type lymphocytes (data not shown), thus confirming that IFN-I enhances B cell proliferative response to $F(ab')_2$ anti-μ. These analyses therefore indicate that IFN-α/β endows B lymphocytes with a higher sensitivity to proliferative responses induced by their antigen receptor ligation.

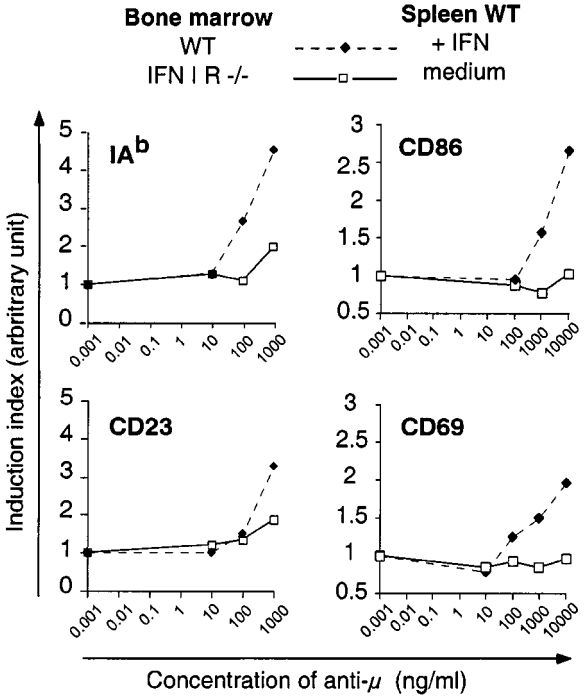

Fig. 4. IFN-α/β enhances the expression of various surface markers following BCR engagement. BM cells from wild-type or IFN-IR$^{-/-}$ mice as well as wild-type splenocytes previously treated for 24 h with 400 U/ml IFN or not were cultured overnight with various doses of anti-μ mAb. For both tissues, the levels of expression of nine different surface markers were assessed by flow cytometry after gating on live B220^{bright} mature B cells. Typical results obtained for I-A^b, CD23, CD86 and CD69 expression in BM (left) or spleen (right) are shown. Expression of the other studied markers was not affected by IFN-I. The induction index takes into account both the intensity of fluorescence and the percentage of positive cells, all normalized for the background expression in absence of stimulation: induction index = (% positive cells × median of intensity)$_{experiment}$/(% positive cells × median of expression)$_{control}$.

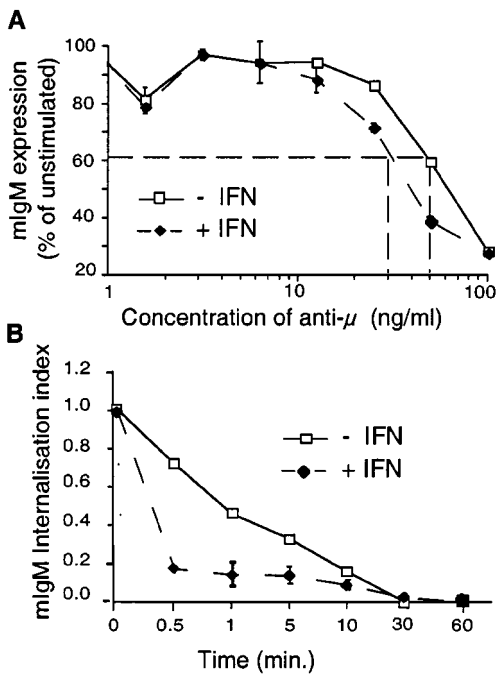

Fig. 5. IFN-α/β increases IgM receptor internalization induced by BCR binding. Splenocytes were cultured overnight in media alone or containing 400 U/ml of IFN-α/β before the assay. Surface IgM expression was assessed by flow cytometry analyses gated on live B220$^+$ cells. (A) Internalization after 16 h of culture in the presence of various doses of the non-mitogenic anti-μ mAb. The data is expressed as a percentage of the level of mIgM expression (fluorescence median) in absence of ligand. (B) Kinetics of mIgM internalization after stimulation with 50 ng/ml anti-μ mAb. The internalization index derives from the arbitrary value of 1 for the level of surface IgM expression (fluorescence median) in non-stimulated cells and 0 for cells stimulated for 60 min. In (A) and (B) each point represent the mean ± SD of three independent cultures.

IFN-α/β amplifies BCR-mediated induction of surface activation markers

Cross-linking of the BCR at the surface of a resting mature B cell has been shown to induce expression of various molecules such as B7-2 and MHC II (24,25). To further characterize the synergy of BCR and IFN-IR signals, wild-type splenocytes treated or not for 24 h with IFN-α/β as well as BM cells prepared either from IFN-IR$^{-/-}$ or wild-type animals were incubated for 16 h with various doses of the non-proliferative anti-μ mAb. In both spleen and BM, measurement of the induction of several surface molecules was restricted to mature B cells (B220^{high}). Since IFN-α/β alone induces the transient up-regulation of several activation markers on wild-type cells, all measurements of surface marker expression following BCR engagement were normalized to the level of expression before BCR stimulation. The dose-dependent induction of the four surface markers I-A^b (MHC-II), CD23, CD69 and CD86 following treatment of both spleen and BM mature B cells with the anti-μ mAb was dramatically enhanced by IFN-α/β, either added in the cultures (splenocytes) or locally produced (wild-type BM). Typical profiles of induction for each molecule are shown in Fig. 4. Noteworthy, the same expression profiles were obtained after stimulation of BM mature B cells or IFN-I-treated B splenocytes. This result indicates that a concentration of 400 U/ml *in vitro* reflects closely the IFN-α/β amount actually present in the BM. The expression levels of other activation markers (CD25, CD38 and CD44) or differentiation markers associated with membrane (m) IgM triggering (CD19, CD22) remained constant after BCR ligation, independently of the presence or absence of IFN-α/β (data not shown). These results indicate that IFN-α/β and BCR ligation act in synergy to stimulate B cells.

IFN-α/β and BCR internalization

Down-modulation of mIgM is an early event following BCR ligation that is dependent on the concentration of ligand and readily detectable at very low doses of non-proliferating anti-μ mAb (Fig. 5A). In order to monitor IgM internalization by FACS analyses, we first confirmed that binding of the BCR by the non-mitogenic 331 anti-μ mAb used at saturating concentrations at 4°C does not interfere with the surface IgM–FITC staining. The concentrations of anti-μ mAb required to induce 50% of the maximum BCR internalization after 16 h of culture were only reduced from 50 to 30 ng/ml when cultures were pretreated with IFN-α/β (dashed lines in Fig. 5A). This minimal

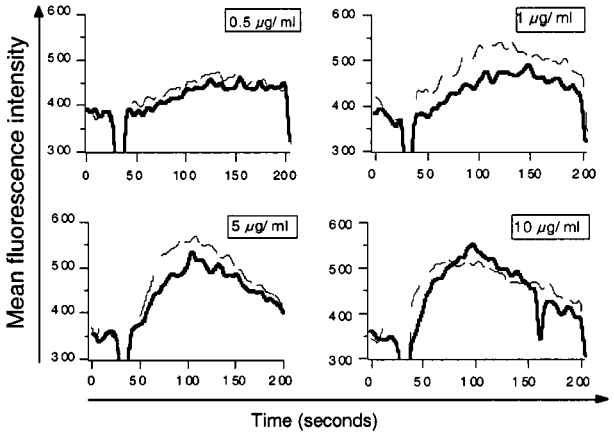

Fig. 6. IFN-α/β enhances the calcium flux that follows BCR engagement. Splenocytes previously incubated overnight with 400 U/ml of IFN-α/β (broken line) or medium alone (bold line) were loaded with Fluo-3 AM and intracellular calcium fluxes were monitored on a FACScan after stimulation with various doses of anti-μ mAb (indicated in the frames). Analyses are gated on live B220$^+$ lymphocytes. The results shown are representative of three experiments.

difference may well reflect the low sensitivity of the assay. We monitored the rate of BCR internalization in both treated and untreated splenic B cell populations using various concentrations of anti-μ established above as discriminatory. Figure 5(B) shows an example of such an assay, where the concentration of anti-μ antibody was set at 50 ng/ml. Pre-treatment of purified resting B cells with IFN-α/β decreased the time lapse between addition of anti-μ and maximum IgM internalization from 10 min to 30 s. These results reinforce the notion that IFN-α/β acts as an amplifier of BCR triggering and suggest that this cytokine interferes with early events of the B cell response.

IFN-α/β enhances BCR signaling events

One of the earliest signaling events following BCR ligation is the induction of an intracellular Ca^{2+} influx. The amplitude of this response has been shown to be dependent on the number of ligated receptors (26). To further elucidate at which step of the BCR signaling pathway IFN-α/β signal interferes, we measured the Ca^{2+} influxes induced by various concentrations of anti-μ mAb on splenic B cells preliminarily treated or not with IFN-α/β (Fig. 6). In both cases, 0.5 μg/ml was the minimal concentration of anti-μ necessary to record a calcium flux, while at 10 μg/ml the response was at its maximum, defining the plateau of the dose-dependent response. In that range of concentrations, splenic B cells sensitized by IFN-α/β consistently displayed a higher or faster response when compared to untreated cells. Comparison of the response obtained after 1 or 5 μg/ml anti-μ treatment reveal that the non-treated cells needed to receive a 5 times higher concentration of anti-μ mAb to display the same signal as IFN-α/β-treated cells.

De novo synthesis is necessary to mediate the type I IFN effect on BCR triggering

Our results presented above indicate that IFN-α/β mediates rapid activation of B cells and affect BCR triggering. To determine whether the latter is a consequence of the former or results from a direct interaction between the IFN-IR and the BCR signaling pathway, we first established the time lapse necessary for IFN-I to increase the up-regulation of activation markers that follow BCR engagement. Analyses of resting B splenocyte responses to anti-μ stimulation at various time after IFN-α/β treatment revealed that incubation for at least 8 h is necessary for IFN-I to exert its effect on BCR-mediated induction of surface markers expression (as shown for CD69 and CD85 in Fig. 7A). In another set of experiments, resting B splenocytes were maintained for 10 h in the presence of actinomycin D, a strong inhibitor of RNA synthesis, together or not with IFN-α/β. As shown in Fig. 7(B), in the absence of *de novo* synthesis, the level of mIgM expression is lower and not affected by IFN-I. Moreover, cells stimulated for 1 min with various concentration of anti-μ down-modulated mIgM level equally whether they were treated or not with IFN-I (Fig. 7B).

Taken together, these results demonstrate that the effect of IFN-α/β on BCR triggering requires *de novo* synthesis, and hence is not the mere result of signaling events intersection and/or overlap, but rather a consequence of the pre-activation of the B cell.

Discussion

IFN-I is produced during the immune responses to a large spectrum of microorganisms. It has wide pleiotropic effects that include the prevention of viral replication by inhibition of the DNA replication machinery in infected cells, and the activation of macrophages and NK cells (9,10). Its participation in the adaptive immune response is still unclear, although IFN-α/β has been shown to regulate both CD4 and CD8 T cell activation and survival (7,16–18). The present work shows that IFN-α/β induces B cell partial activation and resistance to Fas-mediated apoptosis, and increases their sensitivity to BCR ligation, therefore demonstrating an additional role for this cytokine.

Our results showing an anti-apoptotic effect of IFN-α/β on resting B cells are in accordance with previous studies conducted on B cell lines. Inhibition of BCR-mediated apoptosis by IFN-α/β in Ramos cells (which are phenotypically representative of germinal center B cells) has been correlated with increased expression of the anti-apoptotic Bcl-2 and Bcl-X_L genes (27). In addition, IFN-α activates the NF-κB transcription factor, protecting human Daudi cells against pro-apoptotic stimuli such as virus infection and BCR cross-linking (28). Several studies indicate that IFN-α/β is a T cell survival and/or anti-apoptotic factor (7,16–18), an effect that appears to be restricted to activated and memory lymphocytes (17). In contrast, our results indicate that IFN-α/β increases the survival of resting B cells and does not significantly affect naturally activated B cell recovery after culture. However, B cells activated through the CD40 receptor, a mimic of T cell help in germinal centers, show increased resistance to Fas-mediated apoptosis when treated with IFN-α/β. This apparent discrepancy may reflect the particular nature of naturally activated B cells, which do not seem to result from *bona fide* inductive T–B cell interaction and cycle poorly [e.g. (29,30)]. In turn, these results suggest that IFN-α/β may affect the outcome of Fas-mediated B cell

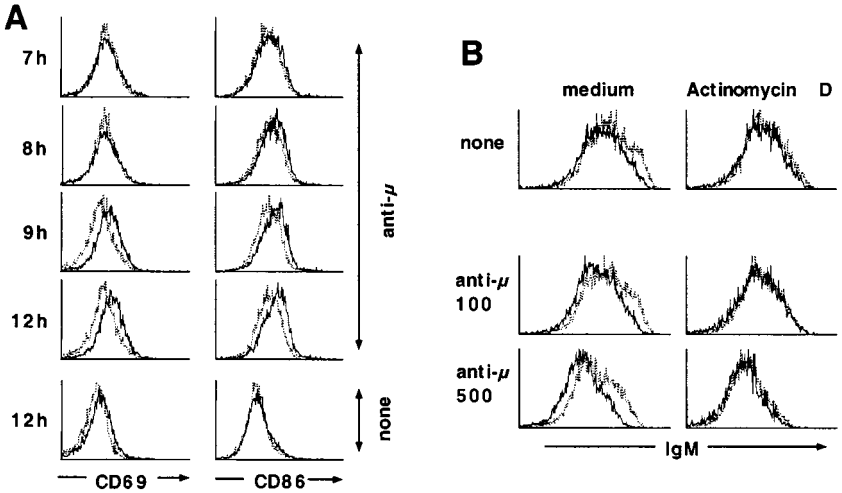

Fig. 7. *De novo* synthesis is necessary to mediate IFN-α/β effects on BCR triggering. (A) Resting B splenocytes were treated for the indicated times with (plain line) or without (dotted line) 400 U/ml of IFN-α/β. At the end of the incubation, stimulation with 1 μg/ml polyclonal goat Ig anti-μ lasted for 12 h. (B) Cells were treated with IFN-α/β or not for 10 h as in (A), except that, where indicated, actinomycin D was added at the beginning of the experiment. Stimulation with the same polyclonal goat Ig anti-μ preparation used at the specified concentrations lasted for 1 min. Histograms representative of three different experiments show CD69 and CD86 (A) or mIgM (B) expression.

selection during germinal center reactions. Analyses of the dynamic of Ig affinity maturation and evaluation of *in situ* cell death in the germinal center during the course of a T-dependent immune response in IFN-IR$^{-/-}$ mice will help clarify this point.

We show that IFN-α/β treatment leads to increased B cell survival and up-regulation of several activation markers, although it does not induce B cell proliferation. The increased survival and clonal expansion of CD8^{+} T cells mediated by IFN-α/β has been attributed to the effect of IL-15 produced by activated macrophages. Purified B cells respond to IFN-α/β no differently than whole spleen cell populations. It is therefore unlikely that a much-reduced population of activated macrophages could secrete significant amounts of IL-15, which would account for the effects we monitored following IFN-α/β treatment. Moreover, splenocytes cultured in medium supplemented with IL-15 instead of IFN-α/β do not show any increase in CD25 expression. Thus, in our assay, IL-15 does not appear to mediate the effects of IFN-α/β.

The responses of B cells to antigen begin with a Ca^{2+} flux, receptor internalization and antigen uptake, then proceed to antigen processing, induction of several 'activation' surface molecules, and finally result in proliferation and differentiation to antibody-secreting cells. The earlier events lead B cells to a state of competence for cognate interaction with primed T_h cells, which in turn induces proliferation and differentiation into effector cells. We show that pre-treatment of B lymphocytes with IFN-α/β increases Ca^{2+} flux, receptor internalization as well as MHC II expression following BCR ligation. Similarly, expression of several activation markers, including B7-2, a molecule necessary for interactions with T cells, is dramatically increased. Finally B cell proliferative response to co-ligation of the BCR and CD40 molecules, an experimental mimicry of T–B cell interaction, was also enhanced by IFN-α/β. It is worth recalling that the level of IgM at the B cell surface is substantially decreased after overnight culture with IFN-α/β. Therefore the higher sensitivity to BCR ligation after IFN-α/β treatment does not depend on the number of available receptors at the cell surface and, thus, should result from modifications of the cell responsiveness, most probably related to its partial-activation status. The increase of sensitivity to BCR ligation characterized here is of the same order of magnitude as the one we previously reported measuring the inhibition of IgM secretion after LPS stimulation (14,31). The doses of IFN-α/β required to monitor such effects (100–400 U/ml) are relatively low and therefore close to physiological levels. Moreover, the effect is similar when considering peripheral B cells treated *ex vivo* with IFN-α/β and mature BM B cells from wild-type animal, a tissue where IFN-α/β is constitutively produced (11). Finally, because the various IFN-I share a single specific receptor, the comparison between BM cells prepared from either wild-type mice, where IFN-α/β is constitutively expressed (11), or IFN-IR$^{-/-}$ animals, where cells cannot respond to this cytokine, indicates that the role of IFN-I on B cells responses is of physiological relevance.

Our finding that IFN-α/β enhances B lymphocyte responses to antigen receptor ligation and acts as a regulator of B cell function is reminiscent of the role that has been attributed to C3 when binding to the CD19/CD21/CD81 co-receptor complex (32). Co-receptors are molecules physically associated with the BCR and their co-ligation modulates the strength of the antigen receptor signal. This regulatory function has never been attributed to a cytokine receptor. The parameters we varied in our analyses were both the dose of IFN-α/β and the expression of the IFN-IR; therefore we conclude that IFN-IR upon binding IFN-α/β, acts as an amplifier of BCR-mediated responses.

There are two possible ways that binding of IFN-α/β to the IFN-IR could influence BCR signaling. It may exert a direct effect on intermediates of the BCR signaling pathway or induce changes in the expression of a number of components—both intracellular and at the cell surface—

increasing the likelihood that a BCR–ligand binding event will produce a functional signal. The former hypothesis suggests that the BCR and IFN-IR signaling pathways share common elements. However, to date, none of the proteins identified in either of the signaling cascades seem to be identical (33,34). Our kinetic analyses indicate that pretreatment with IFN-α/β has to last for several hours before the cell sensitivity to BCR ligation is increased. Moreover, we show that inhibition of RNA synthesis prevents amplification of the BCR signal by IFN-α/β, demonstrating that it requires *de novo* synthesis.

It is striking that IFN-α/β treatment alone leads B cells to a physiological state closely resembling that induced by BCR triggering. Continuous BCR engagement has been suggested as a *sine qua non* requirement for B cell survival (35–37). It is thus possible that the small number (15%) of B cells recovered after 2 days of culture in medium alone survived through their possession of receptors recognizing serum proteins in the medium. Under this hypothesis, and as a direct consequence of a lower threshold of sensitivity to BCR ligation, the higher fraction of surviving cells in cultures containing IFN-α/β may correspond to a larger number of cells for which BCR engagement was strong enough to provide a survival signal. Experiments conducted in serum-free media supplemented with suboptimal doses of BCR ligand and/or involving monoclonal population of B cells sharing the same BCR specificity (purified from BCR transgenic mice) may help to clarify this issue.

Our present analyses, taken together with the previous works conducted on T cells (7,16–18), lead us to propose a new scheme for IFN-α/β function during an immune response. In the early step after infection, induction of IFN-α/β enhances B cell responses to antigen and to their subsequent interaction with helper T cells. Once the primary phase of the response is performed, IFN-α/β potentates survival of activated and memory T cells which are later responsible for the maintenance of an efficient immune response. These data thus provide a further functional link between the innate immune system largely associated with IFN-I and the acquired immune system.

The anti-proliferative properties of IFN-I have been exploited through the therapeutic usage of this cytokine in the treatment of viral infections or malignant disease (1). The analyses presented here suggest that these approaches may in addition augment B cell responses to viral or tumor antigens. However, an undesirable complication of the IFN-I therapy is the development of autoreactive antibodies, which frequently necessitates the interruption of the treatment [e.g. (38–41)]. The impaired Fas-mediated apoptosis, increased sensitivity to BCR activation as well as the apparent enhanced ability to receive T cell help evidenced here suggest that IFN-α/β direct effects on B cells are responsible for the production of autoreactive antibodies by these patients. Analyses of the role of IFN-I on the onset and development of a lupus-like syndrome in mice will test this hypothesis.

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Abbreviations

BM	bone marrow
IFN-IR	IFN type I receptor
LPS	lipopolysaccharide
mIgM	membrane IgM

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