Uncorrected Version. Published on April 27, 2009 as DOI:10.1189/jlb.0908560

JLB

Article

IFN-α amplifies human naïve B cell TLR-9-mediated activation and Ig production

L. Giordani,[,1] M. Sanchez,[†] I. Libri,* M. G. Quaranta,* B. Mattioli,* and M. Viora*[,1,2]*

Departments of *Therapeutic Research and Medicine Evaluation and [†]Cell Biology and Neurosciences, Istituto Superiore di Sanità, Rome, Italy

RECEIVED SEPTEMBER 19, 2008; REVISED MARCH 12, 2009; ACCEPTED MARCH 13, 2009. DOI: 10.1189/JLB.0908560

ABSTRACT

TLRs are a family of molecules that function as sensors for the detection of pathogens. TLR-9, expressed on B cells and pDCs, recognizes CpG motifs of unmethylated bacterial DNA and plays a role in the development of autoimmunity. The present study was designed to investigate the effects of IFN-α in combination with CpG ODN on the activation of CD27$^-$ naïve B cells and on Ig production. We provide evidence that CpG ODN not only induces a total and T-dependent, specific IgM response by naïve B cells but also their phenotypic differentiation in plasma cells, as demonstrated by the up-regulation of CD38 expression. We found that TLR-9 stimulation with CpG ODN induces IL-1β, TNF-α, IL-10, and IL-6 production. Interestingly, we also found that CpG ODN induces naïve B cell maturation into memory cells, as demonstrated by the induction of CD27, AID mRNA expression, and IgG production. More importantly, our results demonstrate that IFN-α amplifies the inductive effect of CpG ODN on naïve B activation and on Ig production through a mechanism involving TLR-9/MyD88-dependent signaling. Moreover, we found that IFN-α enhances the frequency of CpG ODN-induced memory B cells. Our results may contribute to clarify the events promoting IFN-α-induced amplification of naïve B cell activation via TLR-9 for a better understanding of the pathogenesis of autoimmune disorders and may guide treatments targeting this pathway within B cells. *J. Leukoc. Biol.* 86: 000–000; 2009.

Introduction

TLRs are key molecules involved in the innate recognition of conserved structures found in a broad range of pathogens, and their activation triggers innate and adaptive immune responses directed against invading pathogens [1, 2]. The members of the TLR family are expressed differently on human immune cells, and their stimulation triggers different immune responses depending on the specific expression pattern of these receptors. Human B cells express a limited set of TLRs including TLR-9, whose natural ligands are unmethylated CpG motifs characteristic of bacterial DNA.

In the last years, many progresses in the understanding of human CD27$^-$ naïve B cell activation have been performed. Ruprecht and Lanzavecchia [3] demonstrated that TLR stimulation represents a third signal required for activation of human naive B cells. Successively, Huggins et al. [4] showed that CpG ODN, agonist of TLR-9, in combination with IL-2, IL-10, and IL-15, induces proliferation and survival of naïve B cells and their differentiation in plasma cells. A more recent study has provided evidence that even in the absence of exogenous cytokines, CpG ODN provides sufficient signals for induction of naïve B cell activation [5]. Indeed, this study showed that in the absence of BCR triggering and T cell help, the CpG ODN stimulation induces proliferation and survival of naïve B cells, increases their antigen-presentation function, and induces their differentiation in IgM-SC. On the contrary, CpG ODN did not induce naïve B cells to undergo isotype-switching or to acquire a memory or plasma cell surface phenotype [5].

Although these last data contribute to revise the activation model of naïve B cells, the mechanisms that regulate naïve B cell proliferation and function are still incompletely understood and somewhat controversial.

In humans, the pDCs expressing TLR-9 recognize bacterial and viral nucleic acids. Activation of pDCs by TLR-9 leads to secretion of type I IFN (IFN-α and IFN-β), which influencing protein synthesis, cell growth, and survival, establishes an antiviral state. In addition, TLR-9 triggering induces an activated phenotype in pDCs, which together with the production of type I IFNs and other secreted cytokines and chemokines, promotes T cell immunity by directing Th1 polarization and by inducing a cytotoxic T cell response [6–8]. Type I IFNs also support humoral immunity. Indeed, IFN-α enables B cells to undergo isotype-switching and mature into ASC [9]. However, type I IFNs also play an important role in the development of autoimmune responses, and several evidences revealed that

Abbreviations: AID=activation-induced cytidine deaminase, AP=alkaline phosphatase, APC=allophycocyanin, ASC=antibody-secreting cell(s), HDL=high-density lipoprotein(s), KLH=keyhole limpet hemocyanin, MFI=mean fluorescence intensity, MP=mannoprotein, ODN=oligodeoxynucleotide, pDC=plasmacytoid dendritic cell, SC=secreting cell(s)

1. These authors were principal investigators.

2. Correspondence: Department of Therapeutic Research and Medicine Evaluation, Istituto Superiore di Sanità Viale Regina Elena, 299 00161 Rome, Italy. E-mail: viora@iss.it

IFN-α contributes to the pathogenesis of systemic lupus erythematosus [10, 11].

Thus, as antigen-independent activation of naïve B cells is one of the mechanisms responsible for some autoimmune diseases and in light of the recent findings revising the naïve B cell activation model, this study was designed to investigate the effects of CpG stimulation alone and in combination with IFN-α on the activation of $CD27^-$ naïve B cells and on Ig production in the absence of BCR stimulation.

MATERIALS AND METHODS

Reagents

CpG-B ODN 2006 (sequence: 5′ TCG TC G TTT TGT CGT TTT GTC GTT 3′) was purchased from Operon Biotechnologies (Cologne, Germany) and used at 2.5 μg/ml. Dose-response experiments were performed to define the optimal dose of human rIFN-α (Alexis Biochechemicals, San Diego, CA, USA). Different concentrations of rIFN-α (500–10000 U/ml) in combination with CpG ODN were added to naïve B cell cultures. The maximal number of IgM-SC was obtained at IFN-α doses between 7000 and 10,000 U/ml. Therefore, all experiments were performed using the concentration of 8000 U/ml.

Anti-human IL-6-, anti-TNF-α-, anti-IL-1β-, and anti-IL-10-neutralizing antibodies were purchased by R&D Systems (Minneapolis, MN, USA). *Candida albicans* (strain BP, serotype A) cells and MP purified from the *C. albicans* cell wall were prepared as described previously [12, 13]. *Candida parapsilosis* was a kind gift from Dr. E. Giacomini (Istituto Superiore di Sanità, Italy). KLH was purchased from Sigma-Aldrich (Milano, Italia). HDL were obtained from the plasma of healthy volunteers by preparative ultracentrifugation as described previously [14].

MyD88 homodimerization inhibitory peptide Set was purchased from Imgenex (San Diego, CA, USA). MyD88 homodimerization inhibitory peptide and the control peptide were used at a final concentration of 100 μM.

Cell isolation and culture

PBMC were isolated from buffy coats obtained from healthy volunteer blood donors by the Transfusion Center, Università Degli Studi "La Sapienza" (Rome, Italy). Total B cells were positively selected with anti-CD19 MicroBeads (Miltenyi Biotec, Bergisch-Gladbach, Germany) on an LS+ column. Highly purified naïve B cells were isolated by cell sorting using a FACS (FACSAria, Becton Dickinson, San Diego, CA, USA) by labeling $CD19^+$ B cells with FITC or APC-conjugated anti-CD27 and PE-conjugated anti-IgD and collecting $CD27^-$ B cells, and cells labeled with fluorochome-conjugated isotype antibodies were used to gate nonspecific fluorescence signals, and dead cells were excluded on the basis of propidium iodide (5 μg/ml, BD Biosciences, Franklin Lakes, NJ, USA) fluorescence intensity. The purity of naïve B cells was >98.5% as analyzed by flow cytometry. We evaluated the contamination of pDC in the naive B cell pool. We performed experiments to quantify the number of pDCs and to monitor their activation state. To this aim, in the naive B cell pool purified by cell sorting, we analyzed the expression of BDCA2 as a phenotypic marker of pDCs and of CD80 and CD86 as activation markers of pDCs. We found that pDC contamination was consistently below 0.03% within purified naïve B cells, and this subpopulation did not express CD80 and CD86 markers (data not shown).

For preparation of highly purified, unmanipulated, naïve B cells, the naïve B cell isolation kit II (Miltenyi Biotec) was used. The negatively selected naïve B cells consisted of 98.8–99.8% IgD^+ $CD27^-$ cells, and the viability was >98%. pDC contamination was consistently below 0.07% within the purified naïve B cells.

For induction of a IgM response, purified naïve B cells ($IgD^+/CD27^-$) were cultured at a density of 5×10^5 cells/ml in 12-well round-bottom plates in IMDM (Sigma Chemical Co., St. Louis, MO, USA), supplemented with 5% heat-inactivated FCS and 5% heat-inactivated *C. albicans*-absorbed human AB serum and 50 U/ml penicillin and streptomycin. Purified naïve B cells were stimulated with CpG ODN, with IFN-α or with a combination of both. Where indicated, *C albicans* antigen was added in the form of 0.5×10^6/ml. The cultures were incubated at 37°C in a humidified 5% CO_2 incubator. After 4 days, cells were assayed for IgM-SC and IgG-SC and anti-*C. albicans*-ASC by ELISPOT assay. Ig-SC and anti-*C. albicans*-ASC were expressed as the number of recovered cells at the end of the culture period. Viable cell count was performed on the harvested samples by trypan blue staining. To evaluate Ig, class-switching cells were cultured for 9 days, and IgG were detected by ELISA.

ELISPOT assay

Ig-SC and anti-*C. albicans*-ASC were enumerated by a modification of the ELISPOT test [15]. To enumerate ISC, flat-bottomed microtiter plates (96-well; M129A, Dynatech, Alexandria, VA, USA) were coated overnight at 4°C with 0.1 ml/well optimal dilution of goat anti-human IgG and/or IgM (Southern Biotechnology, Birminghan, AL, USA) in carbonate-bicarbonate buffer, pH 9.7. To enumerate anti-*C. albicans*-ASC, flat-bottomed microtiter plates were coated overnight at 4°C with 10 μg/ml MP purified from *C. albicans* in carbonate-bicarbonate buffer, pH 9.7. Plates were washed twice with PBS-0.05% Tween 20 (Sigma Chemical Co.) and incubated for 1 h at 37°C with PBS containing 3% gelatin (Sigma Chemical Co.) as a blocking agent. The plates were rinsed, and the cells were then added and incubated at 37°C in 5% CO_2 incubator for 3 h. The wells were washed subsequently and then incubated overnight at 4°C with 100 μl/well of an optimal dilution of AP-conjugated goat anti-human IgG or IgM (Southern Biotechnology). After extensive washings with PBS-Tween, 100 μl 1 mg/ml AP substrate 5-bromo-4-chloro-3-indolylphosphate (Sigma Chemical Co.) in 1 M 2-amino-2 methyl-1-propanol buffer (Sigma Chemical Co.) containing 5 mM $MgCl_2$, 0.01% Triton X-405 (Sigma Chemical Co.), and 0.01% NaN_3 was added to each well. The plates were incubated for 1 h at room temperature. The supernatants were then discarded and the wells rinsed with deionized water. The plates were allowed to dry, and the spots were enumerated under a stereomicroscope with a 40-fold magnification.

Flow cytometry

Analysis of cell surface markers was performed in a direct immunofluorescence assay using the following mouse mAb: FITC-conjugated anti-CD20, anti-CD27, anti-CD38, and anti-CD86 (BD PharMingen, San Jose, CA, USA) and PerCp-conjugated anti-HLA-DR (BD PharMingen). Negative controls included directly labeled isotype-matched irrelevant mAb. Flow cytometric data were acquired on a Becton Dickinson FACSAria and were analyzed using BD FACSDiva software (BD Biosciences).

ELISA for IgG and cytokine production

To evaluate Ig class-switching, we measured after 9 days of culture the IgG production in cell supernatants by ELISA. Briefly, 96-well plates (Costar, Corning, NY, USA) were coated overnight with optimal dilution of goat anti-human IgG. After washing with PBS/0.05% Tween and blocking with PBS/gelatin 3% (Sigma Chemical Co.), plates were incubated for 2 h with the supernatants of the cultured cells. The wells were washed subsequently and then incubated for 1 h with 100 μl/well of an optimal dilution of AP-conjugated goat anti-human IgG (Southern Biotechnology). The assay was developed with *p*-nitrophenyl phosphate tablets (Sigma-Aldrich) as a chromogenic substrate. Total amounts of Ig were derived from a standard curve.

Cell culture supernatants were recovered 24, 48, and 72 h after stimulation. IL-6, IL-10, IL-1β, and TNF-α levels were measured by ELISA (Euroclone, Pero, Italy) according to the manufacturer's instructions.

Real-time PCR

Detection of TLR-9, MyD88, AID, and GAPDH mRNA expression was performed by RT-PCR. Total RNA was extracted using the RNeasy Mini kit

(Qiagen, Valencia, CA, USA). cDNA was synthesized with a cDNA synthesis kit (Bioline, London, UK). PCR assays were performed in duplicate using the Sensi Mix dT (Quantace, London, UK) and SYBR Green (Quantace), according to the manufacturer's instructions, on an ABI PRISM 7700 sequence detector (Applied Biosystems, Foster City, CA, USA). The amount of mRNA was normalized relative to the amount of GAPDH mRNA. The sequences of the primers used were: TLR-9 forward (5′-ACAACAACATCCACAGCCAAGTGTC-3′) and reverse (5′-AAGGCCAGGTAATTGTCACGGAG-3′); MyD88 forward (5′-AGAGGCGTGACAGTGCTACA-3′) and reverse (5′-TGTAGCGGAGGAAGAGCAAT-3′); AICDA (AID) forward (5′-AGAGGCGTGACAGTGCTACA-3′) and reverse (5′-TGTAGCGGAGGAAGAGCAAT-3′); GAPDH forward (5′-ACCCACTCCACCTTTGA-3′) and reverse (5′-TGACAAAGTGGTCGTTGAGGG-3′). All primers were purchased from Operon Biotecnologies (Cologne, Germany).

Statistical analysis

Statistical analysis was calculated using a two-tailed paired Student's *t*-test. A *P* value of <0.05 was considered statistically significant. Statistical analyses were performed using GraphPad software (GraphPad Software, San Diego, CA, USA).

RESULTS

Type I IFN amplifies Ig production by TLR-9-stimulated naïve B cells

We first evaluated the ability of CpG ODN to induce Ig production by naïve B cells in the absence of BCR triggering and T cell help. To this aim, naïve B cells (IgD^+, $CD27^-$) were sorted from $CD19^+$ B lymphocytes, and cell purity was >98.5% (**Fig. 1A**). Naïve B cells were stimulated with the polyclonal B cell activator CpG ODN, and Ig response was evaluated by enumerating IgA-, IgG-, and IgM-SC using an isotype-specific ELISPOT assay. As shown in Figure 1B and as demonstrated previously by Jiang et al. [5], CpG ODN alone drove naïve B

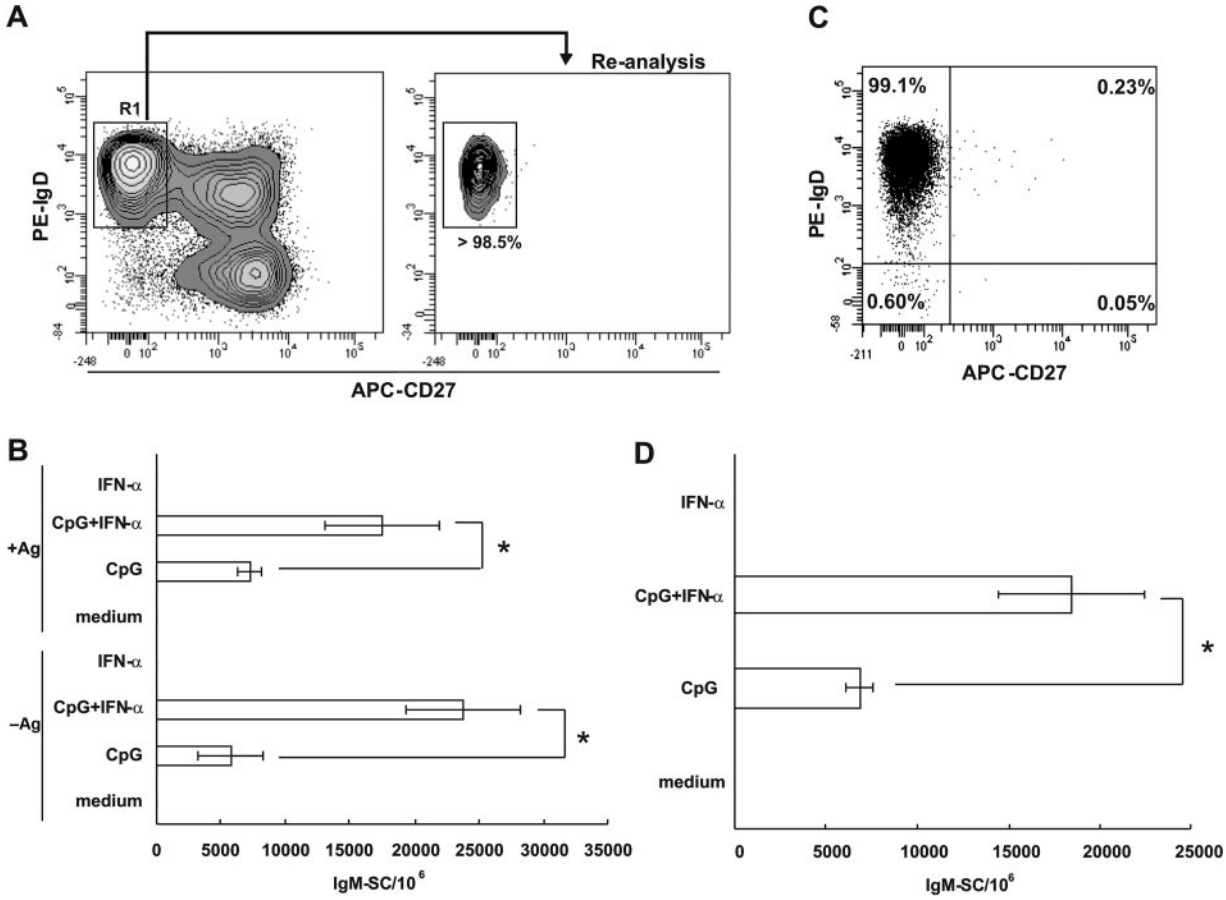


Figure 1. $CD27^-IgD^+$ naïve B cells are induced to produce IgM by TLR-9 stimulation, and IFN-α amplifies this response. (A) Purification of IgD^+CD27^- naïve B cells. Enriched $CD19^+$ cells were stained with PE-conjugated anti-IgD and APC-conjugated anti-CD27 antibodies. Naïve B cells were identified by positive expression of IgD and negative expression of CD27 (R1-gated cells). This population was purified by cell sorting, and reanalysis is shown in the right dot plot. Cells labeled with fluorochrome-conjugated isotype antibody were used to gate nonspecific fluorescence signals, and dead cells were excluded on the basis of propidium iodide fluorescence intensity. (B) Naïve B cells were incubated with or without antigen (Ag) and were left untreated or treated with CpG ODN or IFN-α or their combination. After 4 days, total IgM-SC were enumerated by ELISPOT assay. Data are shown as mean ± SEM of four independent experiments. (C) Untouched, selected, naïve B cells were generated from PBMC by negative selection and were identified by positive expression of IgD and negative expression of CD27. (D) Untouched, selected, naïve B cells were incubated without antigen and were left untreated or treated with CpG ODN or IFN-α or their combination. After 4 days, total IgM-SC were enumerated by ELISPOT assay. Data are shown as mean ± SEM of three independent experiments. (B and D) *, $P < 0.05$.

cells to secrete IgM. As the peak level of IgM production was reached after 4 days of culture (data not shown), the subsequent experiments were performed at this time frame. As expected, naïve B cells did not secrete IgA and IgG (data not shown).

It has been demonstrated that type I IFNs potently enhance the primary antibody response to a soluble protein and promote isotype-switching [9]. More recently, Poeck et al. [16] reported that pDC, antigen, and CpG ODN license human B cells for plasma cell differentiation and Ig production in the absence of T cell help. Therefore, we checked whether IFN-α was able to enhance the induction of the CpG ODN-induced IgM response. As shown in Figure 1B, IFN-α significantly enhanced the CpG ODN-induced IgM-SC number. On the other hand, IFN-α alone did not exert any effect. Similar results were obtained when naïve B cells were stimulated by a CpG ODN/IFN-β combination (5000 U/ml; data not shown).

As anti-CD19 and anti-IgD antibodies, used to positively select naïve B cells, could induce cross-linking of their respective ligands on the B cell surface, lowering the threshold of activation, we performed experiments using a negative selection kit to isolate naive B cells. Untouched, selected IgD^+CD27^- naïve B cells (Fig. 1C) were capable of producing IgM in response to CpG-ODN stimulation, and the addition of IFN-α increased this response significantly (Fig. 1D). Of note, the extent of IgM production was comparable with that obtained using positively selected naïve B cells in the same culture conditions (Fig. 1D).

To better verify the antigen independence of the CpG ODN-induced Ig response, naïve B cells were incubated with the T-dependent antigen *C. albicans* alone or in combination with CpG ODN or IFN-α or the CpG ODN/IFN-α combination, and Ig response was evaluated at Day 4 by an ELISPOT assay. We found that naïve B cells stimulated with antigen and CpG ODN maturated in a number of IgM-SC, similar to that matured with CpG ODN alone, thus demonstrating that the IgM response is antigen-independent. Similarly, naïve B cells cultured with antigen, CpG ODN, and IFN-α produced a similar level of IgM of the corresponding cultures in the absence of antigen. As expected, naïve B cells incubated in the presence of antigen alone or in combination with IFN-α did not produce IgM (Fig. 1B).

These results suggest that CpG, in the absence of BCR triggering and T cell help, induces IgM production by naïve B cells, and IFN-α increases the IgM response significantly.

TLR-9 stimulation induces antigen-specific antibody production by naïve B cells in the absence of BCR triggering, and IFN-α amplifies this response

As CpG ODN is a polyclonal B cell activator, we measured the induction of a specific antibody response to *C. albicans* in cultures of CpG ODN-treated naïve B cells, with or without antigen. We found that naïve B cells treated with CpG without antigen stimulation were capable of producing IgM specific to *C. albicans* and that IFN-α enhanced this response significantly (**Fig. 2A**). Interestingly, the number of IgM-ASC specific to *C. albicans* was similar to that obtained stimulating naïve B cells with CpG in the presence of antigen. Once again, the addition of IFN-α to naïve B cells stimulated with CpG and *C. albicans* increased the number of specific IgM-ASC significantly. As expected, naïve B cells incubated with antigen, IFN-α, or their combination did not produce anti-*C. albicans*-specific IgM. To better demonstrate that CpG plus IFN-α induce naïve B cells to produce IgM antibody, which were truly *C. albicans*-specific, we used the following experimental approach: Naïve B cells stimulated with CpG and IFN-α were tested in plastic-coated wells with MP from *C. albicans* in the presence of increasing concentrations of soluble MPs from *C. albicans* or *C. parapsilosis*

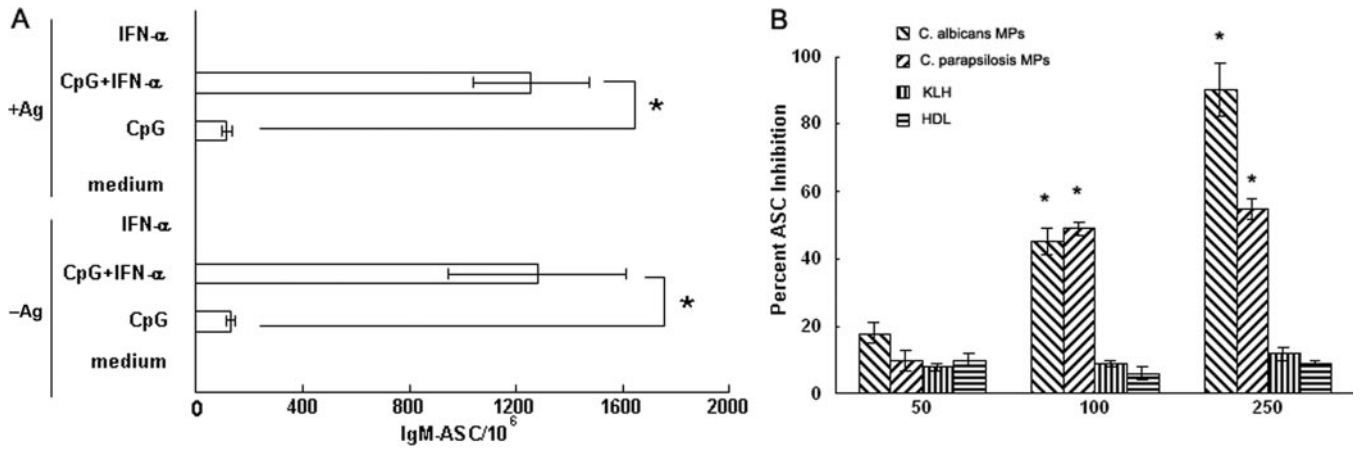


Figure 2. Naïve B cells in response to TLR-9 stimulation and in the absence of BCR triggering are induced to mount a specific antibody response, and IFN-α amplifies this effect. (A) Naïve B cells were incubated with or without antigen and were left untreated or treated with CpG ODN, IFN-α, or their combination. After 4 days, anti-*C. albicans* IgM-ASC were enumerated by ELISPOT assay. (B) Naïve B cells were stimulated with CpG plus IFN-α, and after 4 days, anti-*C. albicans* IgM-ASC were enumerated by ELISPOT. Assays were performed on plates coated with *C. albicans* MP in the presence or absence of increasing amounts of soluble MP from C. *albicans*, *C. parapsilosis*, KLH, or HDL. Results are expressed as percentage of inhibition of the number of anti-*C. albicans* IgM-ASC with respect to cultures performed in the absence of soluble antigens. Mean ± SEM of three independent experiments is shown. (A and B) *, $P < 0.05$.

or in the presence of increasing concentrations of KLH or HDL unrelated control antigens. The rationale of this experiment derives from the known distribution of cross-reacting mannan epitopes among different species of *Candida* [17]. *C. albicans* MPs inhibited spot formation in a dose-dependent manner up to 98%, and *C. parapsilosis* MPs inhibited the number of the specific IgM-SC significantly, up to 50%. In agreement with this result, we have demonstrated previously that *C. albicans* and *C. parapsilosis* share MP epitopes [18]. On the other hand, no inhibition was found when increasing concentrations of KLH or HDL, unrelated control antigens, were added (Fig. 2B).

These results indicate that to mount a specific Ig response to a T-dependent antigen, naïve B cells require TLR-9 stimulation independently of BCR triggering and T cell help.

TLR-9 stimulation triggers phenotypic differentiation of naïve B cells in plasma cells, and IFN-α amplifies this effect

It is well known that during plasma cell development, naïve B cells up-regulate activation markers, down-regulate CD20, and up-regulate CD38 expression. Thus, to verify whether Ig production induced by CpG ODN alone or in combination with IFN-α was accompanied by an early naïve B cell maturation in plasma cells, we examined B cell surface marker expression. To this aim, naïve B cells were left untreated or were treated with IFN-α, CpG ODN, or their combination. As shown in **Figure 3A**, we found that after 18 h of culture, CpG ODN induced an up-regulation of the activation marker CD86 (from 4.8% to 27% of positive cells) and an up-regulation of the plasma cell maturation marker CD38 (MFI from 650 to 900) and CD20 (MFI from 5000 to 9100). IFN-α alone induced a slight increase of the percentage of CD86-positive cells, and it did not increase the percentage of CD38- and CD20-positive cells. IFN-α, in combination with CpG ODN, increased surface expression of CD86 further, up to 35% of positive cells, and of CD38 expression (MFI=1600) and abrogated the effect of CpG ODN on CD20 surface expression partially (MFI=6800).

These results indicate that TLR-9 stimulation induces an early phenotypic differentiation of naïve B cells in plasma cells, and IFN-α potentiates this effect. Consistent with the increased expression of the CD38 surface molecule on naïve B cells treated with the double combination, the highest number of IgM-SC was found in the naïve B cell cultures treated with the double combination.

To better define plasma cell differentiation, we examined CD38 and HLA-DR cell surface expression after 4 days of culture, coincident with the peak level of IgM production. To this aim, naïve B cells were left untreated or were treated with IFN-α, CpG ODN, or their combination. As shown in Figure 3, B and C, we found that CpG increased HLA-DR and CD38 surface expression on total B cells. Furthermore, we found that CpG ODN induced a B cell subpopulation, up-regulating CD38 and down-regulating levels of HLA-DR, thus acquiring a phenotypic differentiation in plasma cells (0.52±0.09% of HLA-DR^{low}/CD38^{high} cells). IFN-α, in combination with CpG ODN, increased the frequency of HLA-DR^{low}/CD38^{high} plasma cells further (2.55±0.18% of HLA-DR^{low}/CD38^{high} cells; Fig. 3, B and D). IFN-α alone induced a slight increase of the percentage of HLA-DR^{low}/CD38^{high} plasma cells. Interestingly, the percentage of HLA-DR^{low}/CD38^{high} plasma cells induced in response to CpG ODN or CpG ODN plus IFN-α stimulation closely fit with the frequency of IgM-SC, evaluated by ELISPOT assay, in the corresponding cultures shown in Figure 1B.

IFN-α amplifies cytokine production by TLR-9-stimulated naïve B cells

As CpG stimulates the secretion of cytokines by naïve B cells [16], we analyzed the pattern of the production of these mediators in our cultures. To this aim, naïve B cells were cultured with CpG ODN, IFN-α, or their combination, and after 24, 48, and 72 h, IL-6, IL-10, TNF-α, and IL-1β accumulation in culture supernatants was measured.

As shown in **Figure 4**, CpG ODN induced IL-6, IL-10, TNF-α, and IL-1β production from naive B cells after 24, 48, and 72 h of culture. IFN-α alone did not exert any effect, and enhanced CpG ODN induced cytokine production significantly at all considered time-points. Moreover, we found a significant time-dependent increase of IL-10 production in the culture of naïve B cells stimulated with CpG ODN plus IFN-α.

To verify whether the increased cytokine production in naïve B cell cultures stimulated with CpG ODN in combination with IFN-α was responsible for the increased Ig production in the same cultures, we added different amounts of the following neutralizing antibodies: anti-human IL-6 (1 μg/ml and 10 μg/ml), anti-human IL-1β (0.1 μg/ml and 0.4 μg/ml), and anti-human TNF-α (from 0.02 μg/ml to 0.4μg/ml) and anti-human IL-10 (from 0.1 μg/ml to 10 μg/ml) antibodies. The combination of all antibodies was also tested. The results obtained did not give any evidence of an inhibitory effect induced by neutralizing antibodies on a CpG ODN/IFN-α-induced increase of Ig responses (data not shown).

This suggests that the up-regulation of cytokine production by naïve B cells in response to the double stimulation of CpG plus IFN-α is not in charge of the increased Ig response observed in the same cultures.

IFN-α alone and in combination with CpG ODN enhances the expression of MyD88 in naïve B cells amplifying their functions

It is well known that TLR-9 signaling requires the adaptor molecule MyD88 [19]. Therefore, we examined the impact of CpG ODN, IFN-α, and their combination on the expression levels of TLR-9 and MyD88 mRNA in naïve B cells. As we demonstrated that naïve B cells stimulated with a CpG ODN or CpG ODN/IFN-α combination showed an early up-regulation of CD38 expression (Fig. 3), we planned to study gene expression in the first hours of the culture during the inductive phase of the Ig response. To this aim, naïve B cell cultures were stimulated with CpG ODN, IFN-α, or their combination, and after 30 min, 1 h, 2 h, and 4 h, the expression levels of TLR-9 and MyD88 mRNA were analyzed by quantitative real-time PCR.

As shown in **Figure 5A**, we found that IFN-α alone or in combination with CpG ODN significantly up-regulated, within

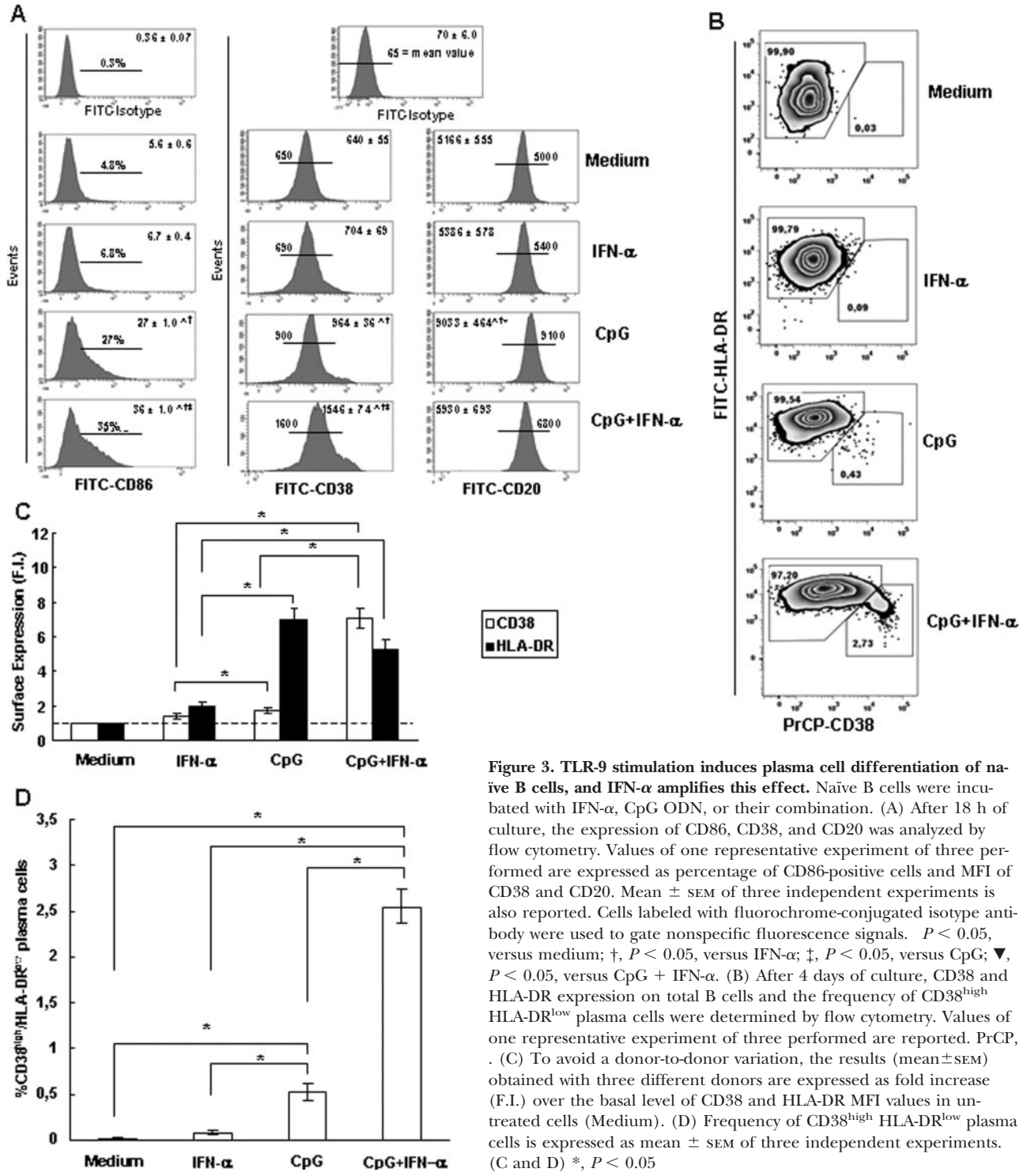


Figure 3. TLR-9 stimulation induces plasma cell differentiation of naïve B cells, and IFN-α amplifies this effect. Naïve B cells were incubated with IFN-α, CpG ODN, or their combination. (A) After 18 h of culture, the expression of CD86, CD38, and CD20 was analyzed by flow cytometry. Values of one representative experiment of three performed are expressed as percentage of CD86-positive cells and MFI of CD38 and CD20. Mean ± SEM of three independent experiments is also reported. Cells labeled with fluorochrome-conjugated isotype antibody were used to gate nonspecific fluorescence signals. $P < 0.05$, versus medium; †, $P < 0.05$, versus IFN-α; ‡, $P < 0.05$, versus CpG; ▼, $P < 0.05$, versus CpG + IFN-α. (B) After 4 days of culture, CD38 and HLA-DR expression on total B cells and the frequency of $CD38^{high}$ $HLA\text{-}DR^{low}$ plasma cells were determined by flow cytometry. Values of one representative experiment of three performed are reported. PrCP, . (C) To avoid a donor-to-donor variation, the results (mean±SEM) obtained with three different donors are expressed as fold increase (F.I.) over the basal level of CD38 and HLA-DR MFI values in untreated cells (Medium). (D) Frequency of $CD38^{high}$ $HLA\text{-}DR^{low}$ plasma cells is expressed as mean ± SEM of three independent experiments. (C and D) *, $P < 0.05$

30 min, the expression of MyD88, reaching a peak level after 2 h. On the other hand, CpG ODN alone did not affect the expression of MyD88. Differently, the expression of TRL-9 was not modified significantly by CpG or by IFN-α, alone or in combination (Fig. 5B).

These results show clearly that the enhancement of Ig production, induced by IFN-α in combination with CpG ODN, is associated with an early increase of MyD88 mRNA expression in the same cultures.

To verify whether IFN-α enhances the IgM response to CpG ODN stimulation by an up-regulation of MyD88 expression, we performed experiments using a peptide that interferes with the homodimerization of MyD88 Toll/IL-1R domains. To this aim, naïve B cells were incubated for 24 h, alone or with a

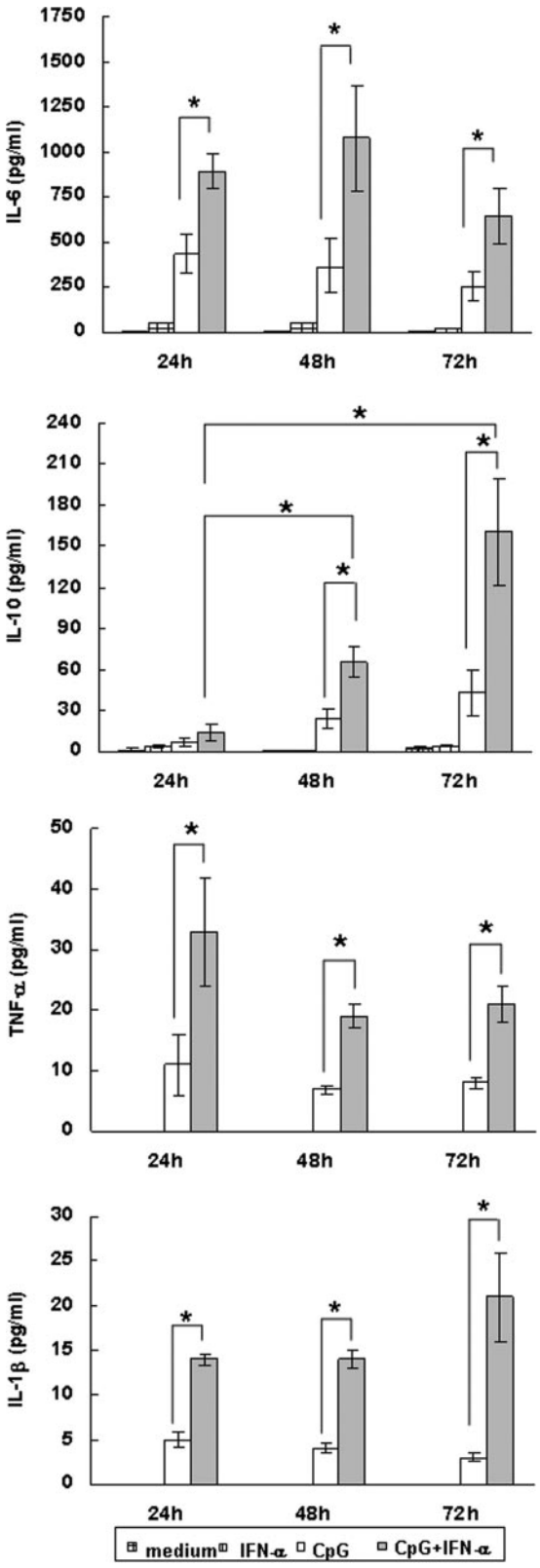


Figure 4. TLR-9 stimulation induces cytokine production by naïve B cells, and IFN-α enhances this effect. Naïve B cells were incubated with IFN-α, CpG ODN, or their combination. After 24, 48, and 72 h, IL-6, IL-10, TNF-α, and IL-1β were measured by ELISA. Data are shown as mean ± SEM of three independent experiments. *, $P < 0.05$

control peptide, or with MyD88 homodimerization inhibitory peptide prior to the stimulation with CpG ODN and IFN-α. As shown in Figure 5C, we found that the addition of the MyD88 homodimerization inhibitory peptide induced an inhibition (90%) of the number of IgM-SC with respect to the control cultures. Of note, the control peptide did not exert any effect.

These results show clearly that the enhancement of Ig production, induced by IFN-α in combination with CpG ODN, is MyD88-dependent.

To better verify if the early IFN-α-induced up-regulation of MyD88 expression is responsible for the ability of IFN-α to enhance CpG ODN-induced signals, we preincubated naïve B cells with IFN-α for 2 h, and then, cells were washed and CpG ODN added to the culture. We then evaluated IgM and cytokine production. As shown in Figure 5D, the preincubation with IFN-α induced a significant increase of IgM production by naive B cells with respect to CpG ODN alone. Similarly, preincubation of naïve B cells with IFN-α increased IL-6, IL-1β, IL-10 (Fig. 5E), and TNF-α (data not shown) production significantly in the culture supernatant with respect to CpG ODN alone. Notably, a similar level of IgM and cytokine production was obtained when IFN-α was added to the cultures together with CpG ODN.

These results suggest that IFN-α promptly enhances MyD88 expression, making naïve B cells more susceptible to CpG stimulation, thus amplifying their functions.

TLR-9 stimulation induces CD27$^+$ memory B cells, and IFN-α increases their percentage

To ascertain whether the CpG-induced Ig production was accompanied by evidence of naïve B cell maturation into memory B cells, we examined B cell surface expression of CD27 after stimulation with CpG ODN, alone or in combination with IFN-α. We found that 4.7% of naïve B cells matured into CD27$^+$ memory cells after 4 days of CpG stimulation, and 8.3% of naïve B cells matured into CD27$^+$ memory cells after CpG ODN/IFN-α stimulation (**Fig. 6A**). Similarly to Jiang et al. [5], we found that CpG ODN markedly enhanced HLA-DR expression on naïve B cells, enhancing their antigen-presenting function, and we found that CD27$^+$ memory B cells showed a lower HLA-DR surface expression. Of note, we observed that IFN-α, in combination with CpG ODN, partially reduced the effect of CpG ODN on HLA-DR expression by naïve and memory B cells.

It is well established that AID is required for the induction of class-switch recombination at the Ig loci and for somatic hypermutation [20, 21]. These two processes are T cell-dependent and occur in the germinal centers of secondary lymphoid organs [22]. As a specific antibody response to the T-dependent *C. albicans* antigen (Fig. 2) as well as the maturation in memory B cells (Fig. 6A) was induced in cultures of CpG ODN-stimulated naïve B cells, we investigated whether the stimulation with CpG ODN can induce AID expression. To this aim, RNA was extracted from naïve B cells incubated with CpG ODN for 4 days, and AID expression was analyzed by real-time PCR. Results were normalized using GAPDH as an endogenous control and were plotted as fold increase to AID expression in untreated naïve B cells. We found that the basal

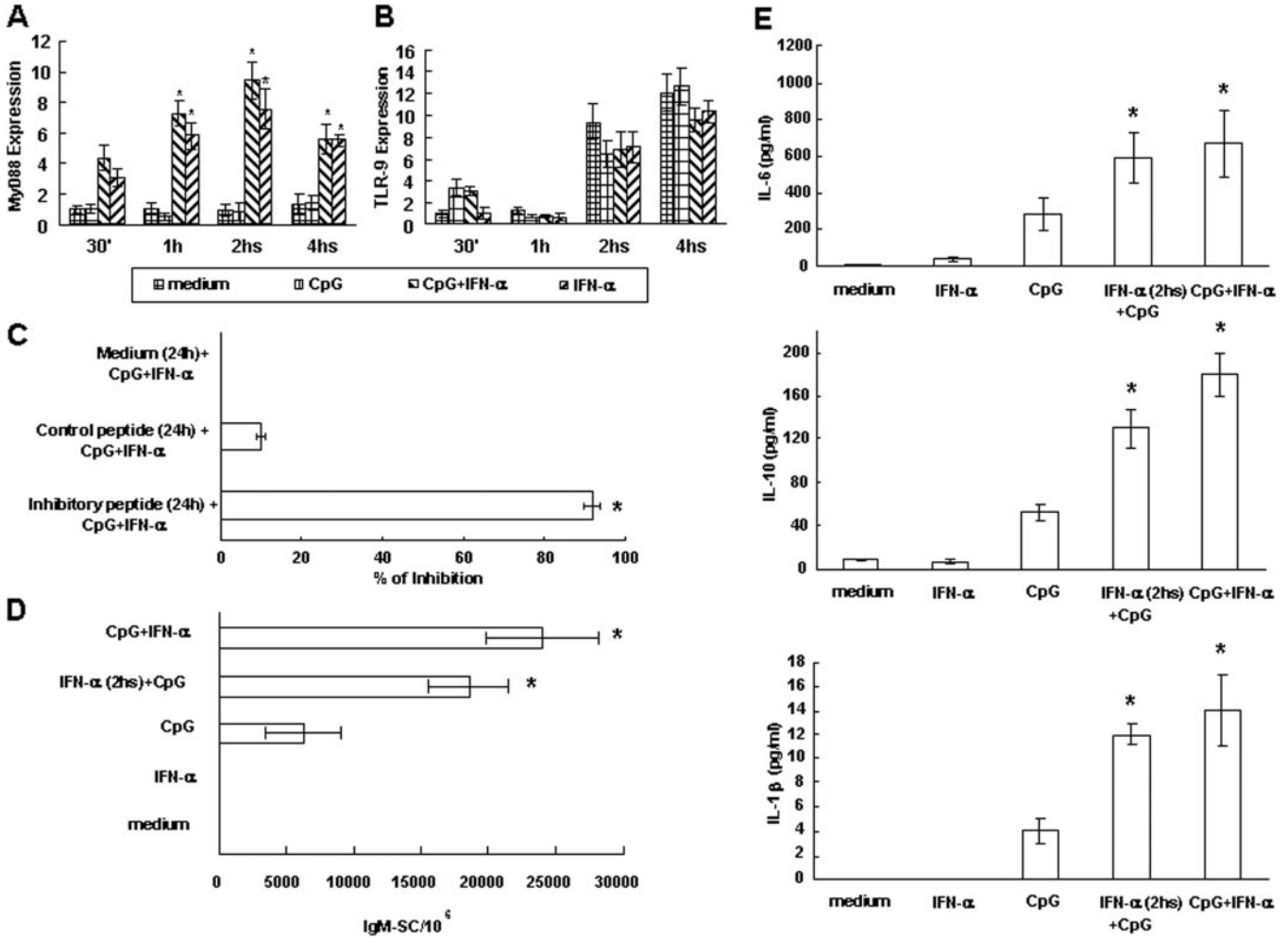


Figure 5. IFN-α alone and in combination with CpG ODN enhances the expression of MyD88 in naïve B cells up-regulating their functions. Naïve B cells were left untreated or were treated with CpG ODN, IFN-α, or with their combination, and after 30 min, 1 h, 2 h, and 4 h, the expression levels of mMyD88 (A) and mTLR-9 (B) were analyzed by quantitative real-time PCR. The amount of mRNA was normalized relative to the amount of GAPDH mRNA. Results are representative of three independent experiments. Data shown mean ± SEM of triplicate values. (A) *, $P < 0.05$. (C) Naïve B cells were left untreated or were preincubated for 24 h with MyD88 homodimerization inhibitory peptide or control peptide before CpG ODN + IFN-α treatment. After 4 days, IgM-SC were enumerated by ELISPOT assay. Data are shown as mean ± SEM of three independent experiments. Naïve B cells were left untreated or were treated with CpG ODN, IFN-α, or their combination or were preincubated for 2 h with IFN-α before CpG ODN treatment. (D) After 4 days, IgM-SC were enumerated by ELISPOT assay. Data are shown as mean ± SEM of three independent experiments. (E) After 72 h of culture, IL-6, IL-10, and IL-1β were measured by ELISA. Data are shown as mean ± SEM of three independent experiments. (C–E) *, $P < 0.05$, versus CpG alone.

level of AID mRNA was strongly increased by CpG ODN stimulation (Fig. 6B).

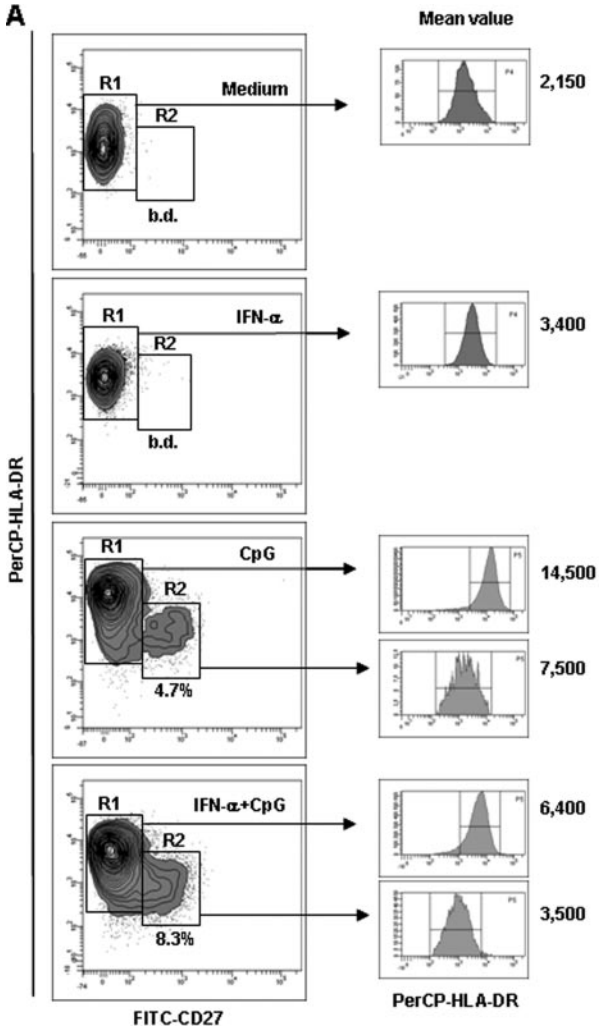
To provide the functional evidence of class-switching, we measured the levels of IgG in culture supernatants of naïve B cells stimulated by CpG ODN at Day 9. We found that CpG ODN induced a significant IgG production by naïve B cells (Fig. 6C).

These results indicate that CpG ODN alone induces class-switching, and IFN-α enhances the frequency of memory B cells.

DISCUSSION

In this study, we demonstrate for the first time that CpG ODN not only drives human highly purified CD27$^-$ naïve B cells to differentiate in plasma cells, as demonstrated by the up-regulation of CD38 expression levels and the down-regulation of HLA-DR expression levels, but also induces naïve B cells to maturate into memory cells, as assessed by the induction of CD27, AID mRNA expression, and IgG production. More importantly, we found that IFN-α selectively up-regulates MyD88 mRNA expression, amplifying the inductive effect of CpG ODN on cytokine production and IgM response by CD27$^-$ naïve B cells. Moreover, we found that IFN-α enhances the frequency of CpG ODN-induced memory B cells.

Our experimental setting measures the intrinsic capacity of naïve B cells to respond to TLR-9 signals, excluding the modulation induced by exogenous cytokines, helping to better understand naïve B cell biology.



TREATMENT	% of CD27⁺ B cells	MFI of CD27⁺/HLA-DR^{low} B cells
Medium	b.d.	2,170 ± 116
IFN-α	b.d.	3,221 ± 297
CpG	4.5 ± 0.4	7,617 ± 394*†
CpG+IFN-α	7.5 ± 0.5‡	3,200 ± 434

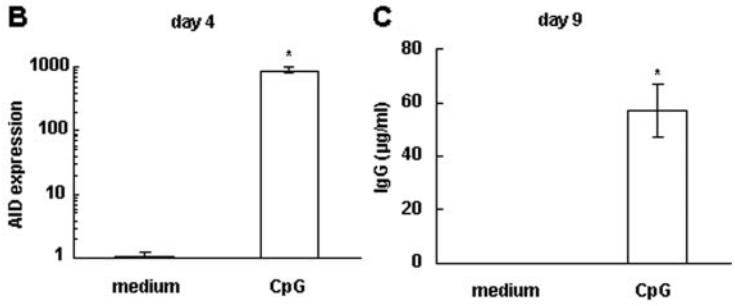


Figure 6. TLR-9 stimulation induces CD27$^+$ memory B cells, and IFN-α increases their percentage. Naïve B cells were incubated alone or in the presence of IFN-α, CpG ODN, or their combination. (A) After 4 days of culture, the frequency of memory B cells and the MFI of HLA-DR expression on memory B cells were analyzed by flow cytometry. One representative experiment of three performed is shown. Mean ± SEM of three independent experiments is also reported. b.d., Below detection; , $P < 0.05$, versus medium; †, $P < 0.05$, versus IFN-α; •, $P < 0.05$, versus CpG + IFN-α. (B) After 4 days of culture, AID expression level was analyzed by quantitative real-time PCR. The amount of mRNA was normalized relative to the amount of GAPDH mRNA. Results are representative of three independent experiments. Data shown mean ± SEM of triplicate values. (C) After 9 days, IgG levels were measured by ELISA. Data are shown as mean ± SEM of three independent experiments. (B and C) *, $P < 0.05$.

Two sets of experiments were performed to verify whether the induction of the Ig response was independent of BCR stimulation. Indeed, naïve B cells stimulated with CpG ODN alone maturated in a number of IgM-SC, similar to that matured in the presence of CpG ODN plus antigen. Again, in cultures of naive B cells triggered by CpG ODN alone, we measured a specific antibody response to the *C. albicans* T-dependent antigen whose magnitude was similar to the specific antibody response induced in cultures of naïve B cells triggered by CpG ODN plus antigen. Therefore, in our experimental system, neither BCR activation nor CD4 T cell-derived costimulation via CD40 ligand nor T cell-derived cytokines was required for Ig production by naïve B cells.

In contrast with our findings, Capolunghi et al. [23] reported that IgM production was almost undetectable in the supernatant of naïve B cells stimulated with CpG ODN.

It is now well accepted that IFN-α plays a crucial role in host immune responses against infections [8, 9, 24] and in the pathogenesis of autoimmune diseases [25]. pDCs constitutively express TLR-9, and upon stimulation by different viruses [26–28] as well as bacterial components such as CpG ODN oligonucleotides [29–31], they produce a large amount of type I IFNs. Thus, TLR-9 signaling plays a direct role on naïve B cells, inducing Ig production with a possible risk for autoimmunity, and an indirect role on pDCs, inducing IFN-α production, which increasing IgM production, further amplifies the susceptibility to autoimmune disorders. In addition, several case reports have emerged describing autoimmune conditions developed during IFN-α therapy [32]. Our results also demonstrate that CpG-ODN is able to support differentiation of naïve B cells into isotype-switched IgG producing plasma cells, increasing deleterious consequences of TLR-9-induced autoreactivity.

Here, we demonstrate that TLR-9 stimulation not only induces naïve B cells to produce IgM and to mount a specific IgM response to a T-dependent antigen but also drives their maturation into CD27$^+$ memory B cells. CpG ODN-mediated induction of the Ig response is accompanied by plasma cell phenotypic differentiation of CD27$^-$ naïve B cells. CpG-ODN stimulation induced an early up-regulation of CD86, a marker of B cell activation, and an up-regulation of CD38, a marker of plasma cell development. Surprisingly, we found a CpG ODN-induced up-regulation of CD20 expression, a cellular marker whose decrease represents a hallmark to define the in vitro

plasma cell differentiation. This finding is consistent with the report that CpG ODN up-regulated the expression of CD20 on the human malignant B cell surface [33]. Moreover, Jiang et al. [5] also reported an up-regulation of CD40, CD80, and HLA-DR expression by CpG ODN stimulation on naïve B cells. In our experimental system, CpG ODN drives naïve B cells to mature into IgM-SC with the $CD38^{high}$ $HLA\text{-}DR^{low}$ plasma cell phenotype. Contrasting with our own observation, Jiang et al. [5] reported that CpG ODN-expanded naïve B cells did not mature into $CD27^+$ memory B cells nor undergo isotype-switching or plasma cell surface phenotype acquisition.

We found that the IFN-α-induced increase of the Ig response via TLR-9 is accompanied by an amplification of naïve B cell activation evaluated as cytokine production and as an expression of surface markers. Of note, in the double combination, IFN-α up-regulated the CpG ODN-induced effect on CD86 and CD38 expression and down-regulated the CpG ODN-induced effect on CD20 expression. Moreover, in the double combination, IFN-α increased the frequency of $CD27^+$ memory B cells.

Germinal centers are known to be associated with the T-dependent antibody response, and experimental evidence indicates that it is the main site in which high-affinity, antibody-secreting long-lived or short-lived plasma cells and memory B cells are generated [34]. Thus, we hypothesize that IgG- and IgM-producing plasma cells and $CD27^+$ memory B cells derived from in vitro CpG ODN-stimulated naïve B cells may develop from a possible activation model operative in germinal centers. It will be interesting to study whether naïve B cells, in response to TLR-9 stimulation, develop into long-lived or short-lived plasma cells and memory cells and whether BCR stimulation is limiting for generation of long-lived or short-lived cells. Moreover, as IFN-α amplifies the inductive effect of CpG ODN on total and specific Ig responses, we hypothesize that pDCs can substitute T cell-derived costimulation by producing IFN-α. Therefore, pDCs can be responsible for supporting a humoral immune response during infection in vivo. Interestingly, pDCs are found in multiple compartments relevant for immune response, including blood, lymphoid tissues [35], and inflamed skin [36, 37], and in solid tumor tissue [38–40].

Our results demonstrate that IFN-α enhances the inductive effect of CpG on Ig response through a mechanism involving an early up-regulation of MyD88 mRNA expression without TLR-9 mRNA modulation.

Moreover, we found that resting human naïve B cells have baseline levels of TLR-9 and MyD88 mRNA expression, which at all time-points considered, was not modified by CpG ODN stimulation. Thus, in the inductive phase of the Ig response to CpG ODN stimulation, the preformed gene expression of the adaptor molecule MyD88 and of TLR-9 does not limit naïve B cell activation, ensuring their differentiation in Ig-producing plasma cells. IFN-α, in combination with CpG ODN, by the up-regulation of MyD88 gene expression, increased the responsiveness to the TLR-9 ligand.

TLR-9 regulation in naïve B cells stimulated with CpG ODN is controversial. Bernasconi et al. [41] showed that naïve B cells, in response to CpG ODN, do not modify transcript copy numbers of TLR-9 mRNA, and other groups reported an increase and a decrease of TLR-9 mRNA levels [16, 42, 43]. Recently, Huggins et al. [4] demonstrated an up-regulation of TLR-9 protein expression after CpG ODN stimulation without a molecular analysis of gene expression. Similarly to our results, Bekeredjian-Ding [44] demonstrated that MyD88 is up-regulated in B cells by IFN-α.

As for the in vivo relevance, our results indicate that the prolonged exposure of naïve B cells to high concentrations of TLR-9 agonists, as well as the ability of the TLR-9 ligand CpG ODN to activate naïve B cells directly without critical checkpoints, i.e., BCR stimulation, cytokines, and T cell costimulation, could predispose individuals to autoimmune reactivity. This is also emphasized by our finding that IFN-α amplifies the inductive effect of CpG ODN on activation and Ig response by naïve B cells, further increasing the risk for development of autoimmune conditions.

Taken together, our findings, besides contributing to elucidate the mechanisms that regulate naïve B cell biology, contribute to better understand the role of IFN-α in the pathogenesis and course of autoimmune disorders. Moreover, this knowledge may be important for the design of new therapeutic strategies targeting the TLR-9 pathway within B cells.

ACKNOWLEDGMENT

This research has been funded by a grant to M. V. from the Italian Ministry of Health.

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KEY WORDS:
cytokines · CpG ODN