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Immunology

Interleukin 18 together with interleukin 12 inhibits IgE production by induction of interferon-γ production from activated B cells

(helminth/cytokine)

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ABSTRACT **Interleukin 18 (IL-18), originally called interferon (IFN)-γ-inducing factor, is a recently cloned cytokine of approximately 18 kDa synthesized by Kupffer cells and activated macrophages. The major activity associated with this molecule is the induction of IFN-γ production from anti-CD3-activated T helper 1 cells in the presence of IL-12. B cells produce IgG1 and IgE when stimulated with anti-CD40 and IL-4. Here we show that a combination of IL-12 and IL-18 induces anti-CD40-activated B cells to produce IFN-γ, which inhibits IL-4-dependent IgE and IgG1 production and enhances IgG2a production without inhibiting the B cell proliferative response. We also show that 24.3% of B cells became positive for cytoplasmic IFN-γ after being stimulated with IL-12 and IL-18. Furthermore, we show that, like splenic T cells stimulated with anti-CD3, IL-12, and IL-18, B cells produced high level of IFN-γ in response to anti-CD40, IL-12, and IL-18. Injection of a mixture of IL-12 and IL-18 into mice inoculated with *Nippostrongylus brasiliensis* or injected with anti-IgD induced IFN-γ-producing cells that inhibit IgE production in them. Furthermore, B cells obtained from normal mice could develop into IFN-γ-producing cells in IFN-$\gamma^{-/-}$ host mice in response to *in vivo* treatment with IL-12 and IL-18. These results indicate that IFN-γ from activated B cells differentially regulates IgG1/IgE and IgG2a responses *in vitro* and *in vivo*, indicating that B cells act as regulatory cells in the immune response. Present results suggested that injection of IL-12 and IL-18 could present a unique approach for the treatment of allergic disorders.**

The activation, proliferation, and differentiation of B cells are highly regulated events in which the action of T cells and their soluble products plays a major role (1–3). However, a few studies have suggested that activated B cells also may regulate immune response by production of interleukin (IL)-10 that inhibits T helper 1-mediated immune response (4, 5). IL-18, originally called interferon gamma (IFN-γ)-inducing factor, is a recently cloned cytokine of approximately 18 kDa synthesized by Kupffer cells and activated macrophages (6). IL-18 acts on T helper 1-type T cells and in combination with IL-12 strongly induces them to produce IFN-γ (6). Recently a cDNA for human IL-18 has been cloned (7). It has been shown to stimulate T cells and NK cells to produce IFN-γ and enhances Fas ligand expression (6–8). In this study we sought to determine whether or not IL-18 by itself or in combination with IL-12 could stimulate B cells to produce IFN-γ and thus potentially act as regulatory cells in the immune response.

MATERIALS AND METHODS

Animals and Reagents. Virus-free C57BL/6, BALB/c, or BALB/c *nu/nu* female mice, 8–12 weeks of age, were used. Homozygous IFN-γ knockout (IFN-$\gamma^{-/-}$) mice were established and maintained at the Laboratory Animal Research Center, Institute of Medical Science, University of Tokyo. Recombinant mouse IL-12 and IL-18 were generous gifts from Hayashibara Biochemical Laboratories (Okayama, Japan). Recombinant mouse IFN-γ was purchased from PharMingen. Rat anti-mouse IFN-γ (R4–6A2) (9) and rat anti-mouse CD40 (LB429) (10) antibodies were purified in our laboratory. Goat anti-IgD antisera were kindly provided by Fred Finkelman (University of Cincinnati, Cincinnati, OH). Fluorescein isothiocyanate (FITC)-rat anti-mouse B220 (RA3–6B2), FITC-rat anti-mouse IFN-γ (XMG 1.2), and phycoerythrin-rat anti-mouse IL-2 receptor β chain (IL-2Rβ) (TMβ-1), were purchased from PharMingen. Magnetic beads coated with rat anti-mouse B220 antibody were purchased from PerSeptive Diagnostics (Cambridge, MA).

***In Vivo* Treatment of Mice.** IFN-$\gamma^{+/+}$ C57BL/6 mice (5 per group) or IFN-$\gamma^{-/-}$ C57BL/6 mice (4 per group) were either not treated or treated with s.c. injection of 100 μl of goat anti-IgD antiserum or s.c. inoculation of 700 *Nippostrongylus brasiliensis* (Nb) third-stage larvae on the first day of experiment. Anti-IgD-injected or Nb-inoculated IFN-$\gamma^{+/+}$ mice were injected daily with IL-12 (50–100 ng/mouse) and/or IL-18 (500 ng/mouse) for 6 and 12 days, respectively. Serum IgE levels were measured at 7 and 13 days after anti-IgD-injection and Nb-inoculation, respectively. In some experiments, IFN-$\gamma^{+/+}$ mice, IFN-$\gamma^{+/-}$ mice or IFN-$\gamma^{-/-}$ mice administered with highly purified B cells (10^8/mouse) from IFN-$\gamma^{+/+}$ C57BL/6 mice, were injected i.p. daily for 4 days with IL-12 (100 ng/mouse) and IL-18 (1,000 ng/mouse). Spleen cells, B cells, and non-B cells were obtained from such treated mice and examined for their expression of IFN-γ mRNA by reverse transcription–PCR (RT–PCR).

B and T Cell Preparation. Highly purified splenic B cells were prepared from BALB/c mice pretreated with anti-asialo-GM1, which was used to eliminate NK cells, followed by passage of spleen cells over a Sephadex G10 column and two rounds of complement-mediated lysis of T cells with mono-

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Abbreviations: IL, interleukin; IFN, interferon; IFN-$\gamma^{-/-}$, IFN-γ knockout, FITC, fluorescein isothiocyanate; Nb, *Nippostrongylus brasiliensis*; RT–PCR, reverse transcription–PCR.

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clonal anti-Thy-1.2 and anti-Lyt-1.2 antibodies (11) This procedure routinely yields cells that are >99% surface IgM, B220, and Ia positive and <1% CD3 positive. Highly purified splenic T cells were prepared from anti-asialo-GM1-treated mice by passing their spleen cells through a nylon wool column (12), followed by treatment of resultant cells with a mixture of magnetic beads coated with monoclonal antibodies against B cells and macrophages to remove residual B cells and macrophages as detailed previously (13), yielding 99% CD3 positive cells.

Intracellular Cytokine Staining. For analysis of intracellular IFN-γ positive B cells, we followed the modified protocol of immunofluorescent staining of intracellular cytokines for the flow cytometric analysis described by Vikingson and Muller (14). Briefly, highly purified B cells (2×10^6/ml per well) were cultured with or without anti-CD40 antibody (0.5 μg/ml) in the presence or absence of 10 ng/ml each of IL-12 and IL-18 for 84 h with a pulse of 3 μg/ml monensin during the final 12 h to inhibit IFN-γ secretion (15). Such treated B cells first were stained with phycoerythrin-conjugated rat anti-mouse B220 and followed by fixation with 4% (wt/vol) paraformaldehyde in PBS and permeabilization of cell membrane with ice-cold PBS containing 1% fetal calf serum plus 0.1% saponin. Resultant cells were further stained with 0.5 μg of FITC-conjugated rat anti-mouse IFN-γ antibody in the presence or absence of excess IFN-γ (10 μg/ml) and analyzed for their proportion of cytoplasmic IFN-γ positive B cells by two-color flow cytometrical analysis by FACScan (Becton Dickinson). The percentages shown represent the proportion of IFN-γ positive cells among B220 positive cells. Quadrants were set on the basis of stained profiles in the presence of IFN-γ.

Cell Cultures. Purified B cells (10^5/0.2 ml per well), cultured with anti-CD40 (0.5 μg/ml) alone or with anti-CD40 and IL-12 and/or IL-18 (37 pg/ml to 27 ng/ml) in the presence or absence of anti-IFN-γ antibody (1.25 to 20 μg/ml) for 24 h, were followed by additional stimulation with 5,000 units/ml IL-4 for 7 days. Supernatants in triplicate cultures were collected at 4 or 8 days after the initiation of the culture, and quantitative immunoassays for secreted IFN-γ or IgE, IgG1, IgG2a and IgM, respectively, were performed by using specific two-site ELISA, with reference standard curve prepared using known amounts of rIFN-γ, or IgE, IgG1, IgG2a and IgM (13). In some experiments, highly purified B cells (2×10^6/ml per well) cultured with or without anti-CD40 antibody (0.5 μg/ml) or splenic T cells (2×10^6/ml per well) cultured in 24-well anti-CD3 (10 μg/ml) coated plates were stimulated with IL-12 (20 ng/ml) and/or IL-18 (20 ng/ml) for 72 h. Supernatants were measured for IFN-γ contents by ELISA.

Analysis of Expression of IFN-γ mRNA. Total RNA was prepared from cells using the guanidinium method. As positive control, mRNA extracted from total spleen cells from mice treated with anti-CD3 antibody 90 min earlier were used (16). For analysis of expression of IFN-γ mRNA, mRNAs were amplified by a modified standard RT–PCR amplification procedure as described in our previous paper (16).

RESULTS

Combination of IL-12 and IL-18 Inhibits IgE and IgG1 Production from Anti-CD40 plus IL-4-Stimulated B Cells. Highly purified B cells (≈99% surface IgM^+ and $B220^+$) were prepared from BALB/c mice and cultured with anti-CD40 antibody and IL-4, which induce B cells to produce IgE/IgG1 and IgM (17). NK cells were depleted by previous injection of the mice with anti-asialo-GM 1 (11). In Fig. 1*A*, we show the effect of IL-12 and/or IL-18 addition on this IL-4-regulated isotype switching. We find that IL-12 stimulation partially inhibited IL-4-induced Ig synthesis, whereas IL-18 stimulation did not affect this Ig response. However, the combination of 500 pg to 1 ng per ml each of IL-12 and IL-18 inhibited IgE and IgG1 production almost completely without inhibiting anti-CD40 plus IL-4-induced B cell growth responses (data not shown). This combination also inhibited IgM production markedly. Moreover, IL-18 with IL-12 induced a 3-fold enhancement in IgG2a synthesis, especially when IL-12 and IL-18 were used at high concentrations.

Because IgG1/IgE and IgG2a responses depend upon IL-4 and IFN-γ (18, 19), respectively, IL-18 with IL-12 might have

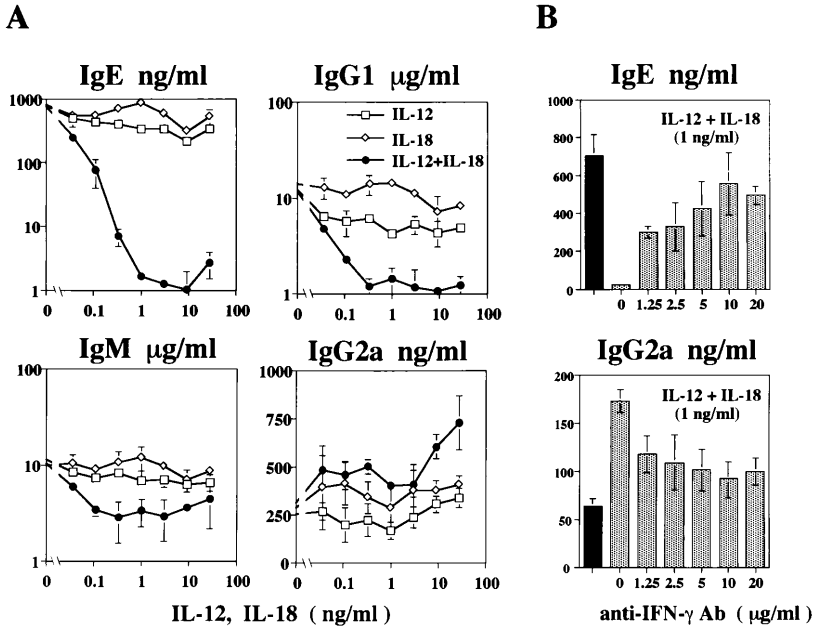

FIG. 1. Effects of IL-12 and/or IL-18 on Ig production by anti-CD40 and IL-4 stimulated B cells in the absence (*A*) or presence (*B*) of anti-IFN-γ antibody. (*A*) Purified B cells (10^5/0.2 ml per well) were cultured with anti-CD40 antibody (LB429, IgG2a; 0.5 μg/ml) in the presence of recombinant murine IL-12 and/or IL-18 (37 pg/ml to 27 ng/ml) for 24 h, followed by additional stimulation with 5,000 units/ml IL-4 for 7 days. (*B*) B cells (10^5/0.2 ml per well) cultured with anti-CD40 antibody (0.5 μg/ml) or anti-CD40 plus IL-12/IL-18 (1 ng/ml) at various concentrations of anti-IFNγ antibodies (R4–6A2; 1.25 to 20 μg/ml) for 24 h were further incubated with IL-4 (5,000 units/ml) for 7 days. Quantitative immunoassays for secreted IgE, IgG1, IgG2a, and IgM were performed by using an avidin-biotin microtiter ELISA. Results are mean ± 1 SD.

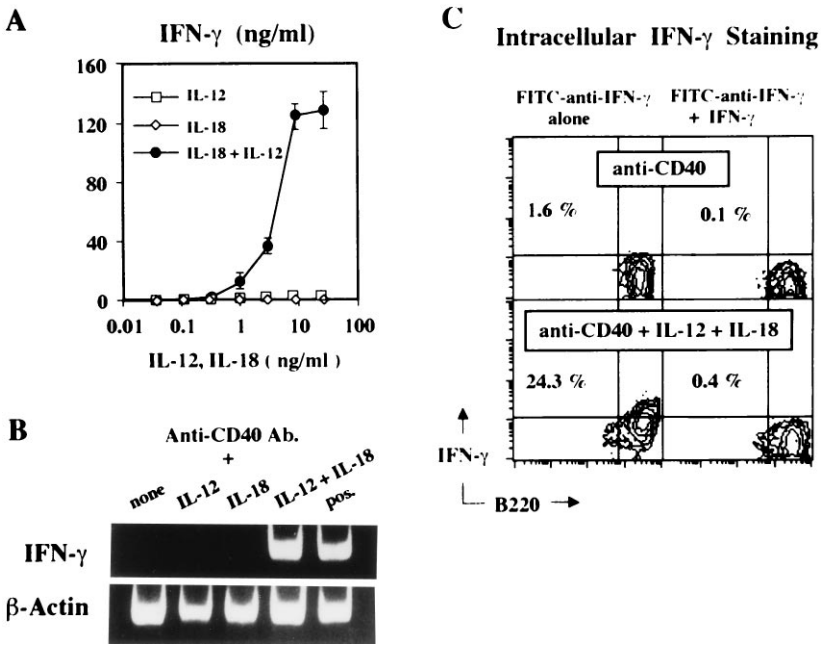

FIG. 2. IFN-γ production from purified B cells in response to anti-CD40, IL-12, and IL-18. (*A*) Highly purified B cells were cultured at 10^5 in flat-bottomed 96-well plates in 200 μl of culture medium with anti-CD40 antibody (0.5 μg/ml) in the presence of various concentrations of recombinant murine IL-12 (□), IL-18 (◇), or a combination of IL-12/IL-18 (●) (37 pg/ml to 27 ng/ml) for 96 h. Supernatants in triplicate culture were harvested and tested for their concentrations of IFN-γ by ELISA. Results are mean ± 1 SD. (*B*) One microgram of total RNAs from B cells stimulated with anti-CD40 antibody (0.5 μg/ml) alone, or anti-CD40 plus 10 ng/ml each of IL-18 or IL-12, or its combination for 72 h were amplified by modified standard RT–PCR amplification procedure. (*C*) Intracellular IFN-γ staining of B cells stimulated with anti-CD40 antibody alone or with anti-CD40, IL-12, and IL-18 for 96 h were performed and analyzed as described in *Materials and Methods*.

differentially regulated these Ig responses by inducing IFN-γ from anti-CD40-activated B cells. As shown in Fig. 1*B*, we tested this possibility by adding an anti-IFN-γ antibody and found that it enhances IgE production but inhibits IgG2a production, indicating that endogenous IFN-γ reciprocally regulates these Ig responses in a dose-dependent manner.

Combination of IL-12 and IL-18 Induces IFN-γ Production from B Cells *in Vitro*. Even though the B cell preparations were very highly purified by cell surface marker analysis (see above), we sought to assess any possible T cell and NK cell contamination by hybridizing Northern blots with T-cell receptor Cβ probe (86T5) and CD16 probe, respectively. We found no hybridization (data not shown), consistent with the cell surface marker analysis. Furthermore, the resultant cells had no detectable $B220^-IL\text{-}2R\beta^+$ cells (NK cells) or $Mac\text{-}1^+$ cells (macrophages) on FACS analysis. Therefore, it appears that anti-CD40-stimulated B cells produce IFN-γ in response to IL-12 plus IL-18.

To show that these activated B cells produce IFN-γ and to determine whether or not this IFN-γ production requires stimulation with anti-CD40, we incubated B cells with or without anti-CD40 in the presence of various doses of IL-12, IL-18, or IL-12/IL-18 for 3 or 4 days. While stimulation of B cells with anti-CD40 alone or with anti-CD40 and IL-12 or IL-18 produced little or no IFN-γ mRNA and protein, stimulation with anti-CD40 plus IL-12/IL-18 strikingly induced B cells to synthesize IFN-γ mRNA as well as biologically active IFN-γ (Fig. 2*A* and *B*). Measurement of IFN-γ mRNA expression by RT–PCR in $B220^+$ cells purified from anti-CD40 plus IL-12/IL-18-stimulated B cells by using magnetic beads coated with rat anti-mouse B220 antibody clearly revealed that such activated B cells also strongly expressed IFN-γ mRNA (data not shown). Levels of IFN-γ in the culture supernatants of B cells stimulated with IL-12/IL-18 in the presence and absence of anti-CD40 revealed that anti-CD40 stimulation induced ≈3-fold increase in the level of secreted IFN-γ (Table 1). Similar results were obtained when B cells prepared from anti-asialo-GM1-treated BALB/c *nu/nu* mice were stimulated with IL-12 and IL-18 in the presence or absence of anti-CD40 (Table 1). We also compared the capacity of T cells and B cells to produce IFN-γ. Like T cells incubated with anti-CD3, IL-12, and IL-18, B cells produced high levels of IFN-γ when they were similarly stimulated (Table 1).

To examine the proportion of IFN-γ-producing cells, we stained B cells for cytoplasmic IFN-γ and analyzed them by FACS analysis (14). B cells were cultured with or without anti-CD40 antibody in the presence or absence of 10 ng/ml each of IL-12 and IL-18 for 84 h with a pulse of 3 μg/ml monensin during the final 12 h to inhibit IFN-γ secretion (15). As shown in Fig. 2*C*, 24.3% of B cells cultured with anti-CD40 plus IL-12/IL-18 are cytoplasmic IFN-γ positive, whereas only 1.6% of B cells cultured with anti-CD40 alone are positive for IFN-γ. That the intracellular IFN-γ staining is specific is indicated by the fact that it is completely blocked by the

Table 1. IFN-γ production by B cells stimulated with IL-12 and/or IL-18

	IFN-γ production, ng/ml				
	B cells				T cells
	Without anti-CD40		With anti-CD40		With anti-CD3
Stimulus	BALB/c	*nu/nu*	BALB/c	*nu/nu*	BALB/c
Medium	<0.1	<0.1	<0.1	<0.1	2.50
IL-12	0.42	<0.1	0.55	<0.1	16.7
IL-18	<0.1	<0.1	<0.1	<0.1	3.64
IL-12 + IL-18	45.7	33.7	112	86.7	122

Highly purified B cells (2×10^6/ml per well) from BALB/c or BALB/c *nu/nu* mice were stimulated with IL-12 (20 ng/ml) and/or IL-18 (20 ng/ml) in the absence or presence of anti-CD40 (0.5 μg/ml) for 72 h. Splenic T cells (2×10^6/ml per well) from BALB/c mice were stimulated with IL-12 and/or IL-18 in 24-well plates coated with anti-CD3 (10 μg/ml) for 72 h. IFN-γ contents in culture supernatants were examined by ELISA.

preincubation of the conjugated antibody with excess recombinant mouse IFN-γ (Fig. 2*C*, *Right*). Failure to stain cytoplasmic IFN-γ in B cells with FITC-conjugated rat Ig class-matched control antibody further substantiated this specificity of the intracellular IFN-γ staining (data not shown). Obtained results (data not shown) concerning the contribution of anti-CD40 stimulation in induction of IFN-γ-producing cells revealed that stimulation with anti-CD40 enhanced IFN-γ production by causing an increase in the number of IFN-γ-producing cells without affecting the proportion of IFN-γ-producing cells. Thus, a substantial fraction of B cells expresses cytoplasmic IFN-γ after stimulation with IL-12 and IL-18.

Combination of IL-12 and IL-18 Inhibits IgE Production by Induction of IFN-γ-Producing Cells *in Vivo*. Next we examined whether daily i.p. injections of IL-12 and/or IL-18 could inhibit IgE production in mice inoculated with Nb or injected with anti-IgD antibody (20) by the induction of IFN-γ-producing B cells. We used IFN-$\gamma^{+/+}$ and IFN-$\gamma^{-/-}$ C57BL/6 mice. As shown in Fig. 3*A* and *B* and consistent with the previous report (20, 21), injection of IL-12 (50 ng/mouse) into Nb-inoculated- or anti-IgD-injected-IFN-$\gamma^{+/+}$ mice markedly inhibited IgE production but significantly enhanced IgG2a production (data not shown), whereas injection of IL-18 (500 ng/mouse) only modestly inhibited IgE production in these mice. However, daily i.p. injection of both IL-12 and IL-18 into Nb-inoculated- or anti-IgD-injected-IFN-$\gamma^{+/+}$ mice almost completely inhibited IgE production (Fig. 3 *A* and *B*, *Left*) but markedly enhanced IgG2a production (data not shown). Like anti-IgD-injected-IFN-$\gamma^{+/+}$ mice, IFN-$\gamma^{-/-}$ mice produced IgE in response to anti-IgD. However, injection of a mixture of IL-12 and IL-18 failed to inhibit IgE production in such anti-IgD-treated IFN-$\gamma^{-/-}$ mice (Fig. 3*B*, *Right*). To our surprise, such injection rather enhanced IgE production in anti-IgD-treated IFN-$\gamma^{-/-}$ mice (Fig. 3*B*, *Right*). Furthermore injection of anti-IFN-γ antibody reversed the IL-12 and IL-18-induced IgE inhibition in the normal mice (data not shown). These results strongly indicate that IFN-γ from IL-12/IL-18-stimulated cells can suppress IgE production. Indeed as shown in Fig. 3*C*, spleen cells from anti-IgD and IL-12-treated mice produced low levels of IFN-γ, whereas anti-IgD, IL-12, and IL-18-treated mice produced high levels.

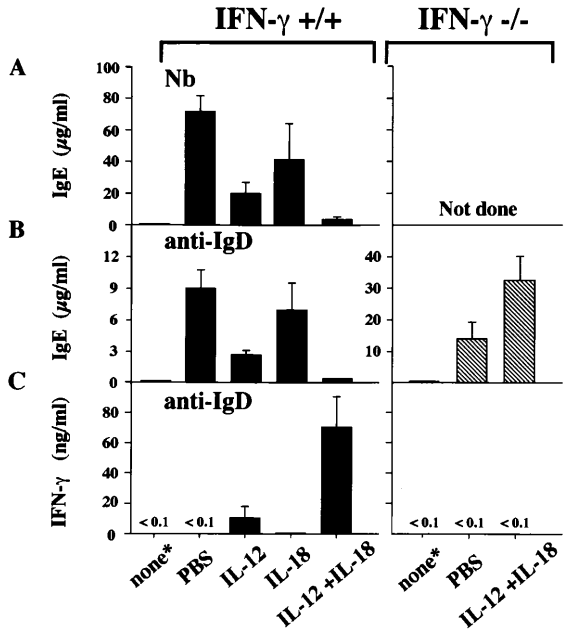

FIG. 3. Inhibition of IgE production by IL-12/IL-18 in mice. (*A* and *B*) IFN-$\gamma^{+/+}$ C57BL/6 mice (5 per group) were either not treated (*) or treated with s.c. inoculation of 700 Nb third-stage larvae or s.c. injection of 100 μl of goat anti-IgD antiserum on the first day of experiment. Nb-inoculated or anti-IgD injected IFN-$\gamma^{+/+}$ mice were treated with daily injections of IL-12 (50 ng/mouse) and/or IL-18 (500 ng/mouse) for 12 and 6 days, respectively. IFN-$\gamma^{-/-}$ C57BL/6 mice (4 per group) also were either not treated or treated with s.c. injection of 100 μl of goat anti-IgD antiserum followed by daily injection of PBS or IL-12 (100 ng/ml) and IL-18 (500 ng/ml). Serum IgE levels were measured at 13 and 7 days after Nb-inoculation and anti-IgD injection, respectively. (*C*) Spleen cells (2×10^6) prepared from the IFN-$\gamma^{+/+}$ mice (5 per group) or IFN-$\gamma^{-/-}$ mice (4 per group) injected with ant-IgD 7 days previously followed by daily injection of PBS, IL-12, IL-18, or IL-12 and IL-18, were cultured for 48 h. Supernatants were examined for their IFN-γ activity. Results are mean + 1 SD.

Combination of IL-12 and IL-18 Induces IFN-γ-mRNA-Expressing B Cells *in Vivo*. To show whether IL-12 and IL-18 stimulate B cells to produce IFN-γ *in vivo,* we injected IL-12 (100 ng/mouse) and IL-18 (1,000 ng/mouse) into IFN-$\gamma^{+/+}$ or IFN-$\gamma^{+/-}$ mice or IFN-$\gamma^{-/-}$ mice transferred with IFN-$\gamma^{+/+}$ B cells (10^8/mouse). As shown in Fig. 4, daily i.p. injection of IL-12 and IL-18 induced IFN-γ mRNA almost equally in whole

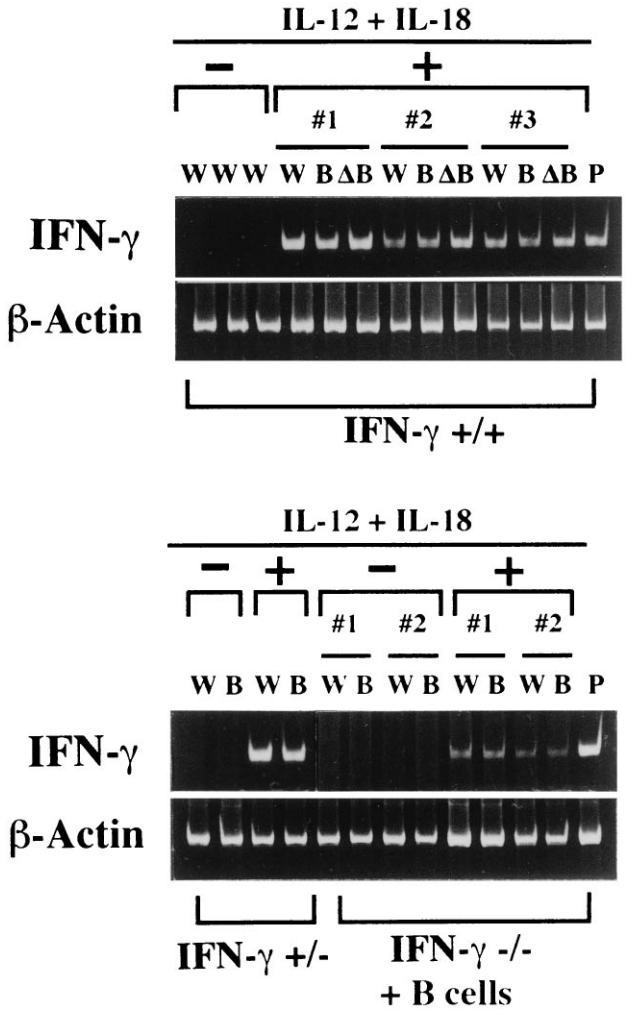

FIG. 4. Induction of spleen cells, B cells, and non-B cells to develop into cells expressing IFN-γ mRNA by injection of IL-12 and IL-18. Highly purified B cells (10^8) from IFN-$\gamma^{+/+}$ C57BL/6 mice were transferred into IFN-$\gamma^{-/-}$ mice. IL-12 (100 ng/mouse) and IL-18 (1,000 ng/mouse) were injected i.p. every day for 4 days into IFN-$\gamma^{+/+}$ mice (Nos. 1–3), IFN-$\gamma^{+/-}$ mice or IFN-$\gamma^{-/-}$ mice (Nos. 1, 2) administered with IFN-$\gamma^{+/+}$ B cells. mRNAs extracted from whole spleen cells (W), $B220^+$ cells (B), and $B220^-$ cells (ΔB) fractionated from whole spleen cells by using magnetic beads coated with rat anti-mouse B220 antibody were amplified by RT–PCR for analysis of expression of IFN-γ mRNA. Flow cytometric analysis revealed that more than 95% of beads bound cells (B) and less than 0.5% of beads-unbound cells (ΔB) were surface IgM positive.

spleen cells, positively selected B cells by using anti-B220 antibody coated magnetic beads and B cell-depleted (ΔB) spleen cells in IFN-$\gamma^{+/+}$ (Nos. 1–3) or IFN-$\gamma^{+/-}$ mice. Furthermore, such treatment also induced IFN-γ mRNA expression in whole spleen cells as well as positively selected B cells from IFN-$\gamma^{-/-}$ mice transferred with B cells from normal mice (Nos. 1, 2), although the level of IFN-γ mRNA expression in these cells was low. We also examined the capacity of IL-12 (100 ng/mouse) and IL-18 (1,000 ng/mouse) injected into IFN-$\gamma^{-/-}$ mice either transferred with IFN-$\gamma^{+/+}$ B cells or not to inhibit anti-IgD-induced IgE production. We found that injection of IL-12 and IL-18 inhibited anti-IgD-induced IgE production (60% inhibition) only in IFN-$\gamma^{-/-}$ mice transferred with IFN-$\gamma^{+/+}$ B cells. These results taken together indicate that IL-12 and IL-18 activate B cells to produce IFN-γ that may differentially regulate IgG1/IgE and IgG2a responses *in vivo* in an autocrine or paracrine manner.

DISCUSSION

B cells have been recognized as cells that produce Ig after being stimulated with antigen and T helper cells. However, several reports have demonstrated that activated B cells also produce cytokines such as IL-1 (22), IL-6 (23, 24), and IL-10 (4, 5, 24). IL-18 has been originally described as a factor that enhances IFN-γ production from T helper 1 cells, particularly in the presence of IL-12 (6). IL-18 also markedly stimulates IFN-γ production in nylon wool-purified splenic T cells in the presence of immobilized anti-CD3 antibody and IL-12 (Table 1) (6). As noted in Figs. 1 and 2, anti-CD40, IL-12, and IL-18-stimulated-B cells produce significant quantities of IFN-γ, and this inhibits IL-4-dependent IgG1 and IgE production *in vitro*. We also show that B cell-derived-IFN-γ can enhance IgG2a production *in vitro* (Fig. 1). Thus IL-12 and IL-18-stimulated B cells act as regulatory cells that differentially regulate IgG1/IgE and IgG2a responses by production of IFN-γ *in vitro*.

As T cells require costimulation with IL-12 to respond to IL-18 by striking production of IFN-γ (Table 1) (6), B cells also require stimulation with IL-12 to become responsive to IL-18. Furthermore, as splenic T cells stimulated with immobilized anti-CD3, IL-12 and IL-18 produce high levels of IFN-γ (122 ng/ml), B cells stimulated with IL-12/IL-18 produce a comparable level of IFN-γ (112 ng/ml) (Table 1). Thus, both B cells and T cells seem to require IL-12 to be responsive to IL-18 that strikingly enhances IFN-γ production from IL-12-stimulated B cells and T cells. We also demonstrated that anti-CD40 is not prerequisite for inducing IFN-γ-producing B cells, although stimulation with anti-CD40 enhances IFN-γ production from B cells (Table 1).

Finkelman *et al.* (21) reported that IL-12 inhibits IgE production *in vivo* via endogenous IFN-γ, although the property of IFN-γ producing cells is not elucidated. Li *et al.* (25) recently have reported IL-12 stimulates human B cells to produce IFN-γ *in vitro*. Here we show that IL-12 and IL-18 strongly activates B cells to produce IFN-γ *in vitro* (Fig. 2) and *in vivo* (Fig. 4). We have described that anti-CD40-activated B cells have the capacity to produce IFN-γ in response to IL-12 and IL-18, and this IFN-γ inhibits IL-4-dependent IgG1 and IgE responses *in vitro* (Figs. 1, 2). Furthermore, to reveal the proportion of IFN-γ producing B cells, we performed intracellular IFN-γ staining. 24.3% of B cells became positive for cytoplasmic IFN-γ after being stimulated with anti-CD40, IL-12, and IL-18 (Fig. 2*C*). We used anti-B220 to detect B cells. Similar results also were obtained when B cells incubated with IL-12 and IL-18 were stained with a combination of phycoerythrin-anti-IgM and FITC-anti-IFN-γ (data not shown). We also demonstrated injection of a mixture of IL-12 and IL-18 induces IFN-γ that inhibits IgE production in Nb-inoculated or anti-IgD-injected mice (Fig. 3).

To reveal the relative contribution of B cell-derived IFN-γ in this IL-12 and IL-18-induced IgE suppression *in vivo*, we tested whether B cells prepared from nontreated IFN-$\gamma^{+/+}$ mice could develop into IFN-γ-producing cells after being stimulated with IL-12 and IL-18 in IFN-$\gamma^{-/-}$ mice and inhibited anti-IgD-induced IgE production. As expected, enriched B cells from IFN-$\gamma^{-/-}$ mice treated with IL-12/IL-18 did not express IFN-γ-mRNA (data not shown), whereas enriched B cells from IFN-$\gamma^{-/-}$ mice administered with IFN-$\gamma^{+/+}$ B cells and treated with IL-12/IL-18 expressed IFN-γ mRNA (Fig. 4). Furthermore, injection of IL-12/IL-18 into IFN-$\gamma^{-/-}$ mice injected with anti-IgD did not inhibit IgE (Fig. 3), whereas the same treatment of IFN-$\gamma^{-/-}$ mice administered with IFN-$\gamma^{+/+}$ B cells and injected with anti-IgD showed partial but significant inhibition of IgE production (60% suppression). These results indicate that B cells can develop into IFN-γ-producing cells after being stimulated with IL-12 and IL-18 *in vivo* and regulate isotype switching.

Injection of IL-12/IL-18 into anti-IgD-injected IFN-$\gamma^{+/+}$ mice inhibited IgE production almost completely (Fig. 3). However, same treatment of IFN-$\gamma^{-/-}$ mice administered with IFN-$\gamma^{+/+}$ B cells and injected with anti-IgD inhibited partially. Two reasons may account for this partial inhibition. First, the transferred B cells failed to develop fully into IFN-γ-producing cells. We suspect that injected B cells were widely distributed and activated poorly compared with far more abundant host B cells that are activated with CD40 ligand in peripheral lymphoid organs. Second, paracrine IFN-γ production possibly from IL-12 and IL-18-stimulated T and/or NK cells also regulate patterns of Ig isotype produced from B cells. Indeed, as shown in Fig. 4, injection of IL-12/IL-18 into normal mice induces strong IFN-γ-mRNA in both B cells and non-B cells. Thus, we conclude that, like T helper 1 cells, B cells also can act as regulator cells that play a physiologic role in Ig isotype regulation.

As IgE responses are an important factor in allergic responses, we also would suggest that IL-12/IL-18 treatment could present a unique approach for the treatment of allergic disease. The potentiality that B cells produce IFN-γ also may have enormous implications concerning understanding the regulation of immune response against infection as well as the traditional division of cell-mediated and humoral immunity.

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