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Immunology

Generation of nitric oxide and induction of major histocompatibility complex class II antigen in macrophages from mice lacking the interferon γ receptor

(targeted gene deletion/tumor necrosis factor/granulocyte–macrophage colony-stimulating factor/interleukin 4/*Mycobacterium bovis*)

RYUTARO KAMIJO*, DEBORAH SHAPIRO*, JUNMING LE*, SUI HUANG†, MICHEL AGUET†, AND JAN VILČEK*

*Department of Microbiology and Kaplan Cancer Center, New York University Medical Center, New York, NY 10016; and †Institute of Molecular Biology I, University of Zurich, 8093 Zurich, Switzerland

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ABSTRACT **Availability of mice with a targeted disruption of the interferon γ (IFN-γ) receptor gene (IFN-γR$^{0/0}$ mice) made it possible to examine parameters of macrophage activation in the absence of a functional IFN-γ receptor. We asked to what extent other cytokines could replace IFN-γ in the induction of nitric oxide or major histocompatibility complex class II antigen (Ia) expression in peritoneal macrophages. In thioglycollate-elicited macrophages from wild-type mice, tumor necrosis factor (TNF) alone was virtually ineffective in inducing release of NO_2^- (the endproduct of nitric oxide generation), but TNF enhanced NO_2^- release in the presence of IFN-γ. In macrophages from IFN-γR$^{0/0}$ mice, which were unresponsive to IFN-γ, TNF completely failed to stimulate NO_2^- release. The stimulatory actions of IFN-α/β on NO_2^- release were indistinguishable in wild-type and IFN-γR$^{0/0}$ macrophages: IFN-α/β was ineffective on its own, showed marginal stimulation of NO_2^- release in combination with TNF, and was moderately effective in the presence of lipopolysaccharide. The level of constitutive Ia antigen expression was not significantly different in peritoneal macrophages from wild-type and IFN-γR$^{0/0}$ mice. An increased Ia expression was induced by IL-4 and granulocyte–macrophage colony-stimulating factor in both wild-type and IFN-γR$^{0/0}$ macrophages, but the magnitude of this induction was less than with optimal concentrations of IFN-γ in macrophages from wild-type mice. IFN-α/β showed only a minor stimulatory effect on Ia expression in both wild-type and IFN-γR$^{0/0}$ macrophages. Simultaneous treatment of wild-type macrophages with IFN-α/β and IFN-γ reduced the IFN-γ-induced Ia expression in wild-type macrophages, but IFN-α/β did not show an inhibitory effect on IL-4- or granulocyte–macrophage-colony-stimulating factor-induced Ia expression in either wild-type or IFN-γR$^{0/0}$ macrophages. The important role of IFN-γ in the regulation of the induced expression of major histocompatibility complex class II antigen was confirmed by showing that after systemic infection with the BCG strain of *Mycobacterium bovis* resident peritoneal macrophages from IFN-γR$^{0/0}$ mice had a lower level of Ia expression than macrophages from wild-type mice. The inability of other cytokines to substitute fully for IFN-γ in macrophage activation helps to explain the earlier observed decreased resistance of IFN-γR$^{0/0}$ mice to some infections.**

Interferon γ (IFN-γ) is an immunoregulatory cytokine whose major actions include the activation of mononuclear phagocytes, induction of expression of major histocompatibility complex (MHC) class I and II molecules on a variety of cells, activation of natural killer cells, promotion of cytotoxic lymphocyte maturation, and stimulation of immunoglobulin secretion by B cells (for review, see refs. 1–3). Other cytokines can substitute for IFN-γ in many of these actions. For example, IFN-α/β is a potent inducer of MHC class I antigens (2) and can, at least partly, replace IFN-γ in its ability to activate mononuclear phagocytes for antimicrobial and antitumor activities (4, 5). Some features of macrophage activation characteristic for IFN-γ also can be mediated by tumor necrosis factor (TNF), granulocyte–macrophage colony-stimulating factor (GM-CSF), or interleukin 4 (IL-4) (6–8). In view of this redundancy it has been difficult to determine how essential IFN-γ is for the mediation of its recognized actions, especially in the intact organism. The technique of targeted gene deletion (9, 10) is proving useful for the elucidation of functions of major cytokines (11–13), and the generation of mice with a homozygous disruption of the IFN-γ receptor gene (IFN-γR$^{0/0}$ mice) has made it possible to start dissecting the precise roles of IFN-γ in immune responses and host defenses (14). Although cells from tissues of IFN-γR$^{0/0}$ mice are unresponsive to IFN-γ, the mice are viable and show no overt phenotypic abnormalities up to the age of 14 mo. The immune system in IFN-γR$^{0/0}$ mice appears to develop normally, and mutant mice were able to mount a normal cytotoxic lymphocyte response upon infection with vaccinia virus and a potent T-cell response on infection with lymphocytic choriomeningitis virus. IFN-γR$^{0/0}$ mice also were able to produce virtually undiminished antibody titers upon immunization with some antigens, although the level of IgG2a and, to a lesser extent, of IgG3 antibodies was decreased in comparison with wild-type mice. However, IFN-γR$^{0/0}$ mice showed severe defects in the early defense against *Listeria monocytogenes* and vaccinia virus (14). Doses of *L. monocytogenes* or vaccinia virus that were not lethal for wild-type mice killed a majority of IFN-γR$^{0/0}$ mice by 5 days after inoculation, and greatly increased numbers of *L. monocytogenes* colony-forming units or of vaccinia virus plaque-forming units were recovered from the organs of mutant mice, compared with wild-type mice.

Mononuclear phagocytes are known to be essential for the defense against protozoa, fungi, helminths, as well as intracellular bacteria, including *L. monocytogenes* (15). Macrophages were also shown to play a central role in resistance to many viruses (for review, see ref. 16). Recent evidence indicates that much of the antimicrobial activity of mouse macrophages is mediated by the synthesis of nitric oxide and possibly other reactive nitrogen intermediates, resulting from the induction of nitric oxide synthase by macrophage-activating agents (17–22). Whether nitric oxide plays a role in antiviral defenses is not known. Macrophages also play an important role as antigen-presenting cells during the initiation

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Abbreviations: IFN, interferon; TNF, tumor necrosis factor; GM-CSF, granulocyte–macrophage colony-stimulating factor; IL-2 and IL-4, interleukin 2 and 4, respectively; BCG, bacillus Calmette–Guérin; LPS, lipopolysaccharide.

of specific immune responses, and much of the latter function involves the interaction of MHC class II molecules with $CD4^+$ T cells. IFN-γ is the archetypic macrophage activation factor, identified as the cytokine most important in their antimicrobial activity and most active as an inducer of nitric oxide generation (4, 7, 23). Furthermore, IFN-γ is known to be the most potent inducer of MHC class II molecules on macrophages and other cells (2, 24, 25). Availability of IFN-$\gamma R^{0/0}$ mice offered the opportunity to evaluate the ability of other cytokines to replace IFN-γ as inducers of nitric oxide and MHC class II antigen. In contrast to earlier studies, the complete absence of a functional IFN-γ receptor on cells from IFN-$\gamma R^{0/0}$ mice has made it possible to rule out any contribution of endogenous IFN-γ to the actions examined in our experiments.

MATERIALS AND METHODS

Animals. Mice with a deletion in the gene coding for IFN-γ receptor have been generated as reported (14). The gene was disrupted by inserting the neomycin resistance gene into exon V, which encodes an extracellular, membrane-proximal portion of the receptor. Homozygous IFN-$\gamma R^{0/0}$ or wild-type $(129/Sv/Ev \times C57BL/6)F_2$ mice, aged between 2 and 10 mo, were used in the experiments. C57BL/6 mice (The Jackson Laboratory) were used in some experiments.

Reagents. Recombinant murine IFN-γ (1×10^7 units/mg) and interleukin 2 (IL-2) (2.5×10^6 BRMP units/mg) were obtained from Genzyme. Natural murine IFN-α/β (9.8×10^6 units/mg) was purchased from Lee Biomolecular Laboratories (San Diego). Recombinant murine TNF-α (6×10^6 units/mg) was from Masafumi Tsujimoto (Suntory, Osaka). Murine recombinant IL-4 (5×10^7 units/mg) and GM-CSF (1×10^7 units/mg) were purchased from Biosource International (Camarillo, CA). Thioglycollate broth, lipopolysaccharide (LPS) from *Escherichia coli* 0127:B8, *N*-(1-naphthyl)ethylenediamine, sulfanilamide, and sodium nitrite were obtained from Sigma. Rat anti-mouse Ia monoclonal antibody M5/114 was donated by Jeannette Thorbecke (New York University Medical Center). Goat anti-rat IgG conjugated with fluorescein isothiocyanate was purchased from Boehringer Mannheim. Fetal bovine serum was purchased from GIBCO. The endotoxin level of the fetal bovine serum lot used was 0.3 endotoxin unit/ml, as determined by the *Limulus* amebocyte lysate assay. Bacillus Calmette–Guérin (BCG) was from Connaught Laboratories. ELISA kits for the determination of murine IFN-γ levels were purchased from Genzyme.

Macrophage Culture. Mice were killed 4 days after i.p. injection with 2 ml of 4% thioglycollate broth. Peritoneal exudate cells were harvested in 10 ml of phosphate-buffered saline (PBS). Cells were washed and resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin at 100 units/ml, and streptomycin at 100 μg/ml (complete medium). Macrophage cultures were prepared as follows. For the determination of NO_2^- release, adherent monolayers were obtained by plating 1×10^5 cells per well in 96-well microplates (Falcon 3072, Becton Dickinson). Peritoneal cells were allowed to adhere for 30 min at 37°C and washed three times with complete medium to remove nonadherent cells. Adherent monolayers were treated by cytokines with or without LPS for 48 hr at 37°C in 5% CO_2. For quantitation of Ia antigen expression, 1×10^7 cells were allowed to adhere to 150-mm tissue culture dishes for 30 min at 37°C and then washed four times with complete medium. Adherent cells were detached by incubation with 0.2% EDTA/PBS for 15 min at 4°C. After washing cells with complete medium, 1×10^6 cells were cultured in loosely capped polypropylene centrifuge tubes (Corning 25319-15) with cytokines for 48 hr at 37°C in 5% CO_2.

Nitrite Assay for Estimation of Nitric Oxide Production by Macrophages. NO_2^- concentration in the medium was measured by a microplate assay method, as described by Ding *et al.* (4). Briefly, 100 μl of culture supernatant was collected from each well of 96-well microplates and incubated for 10 min at room temperature with an equal volume of Griess reagent (1% sulfanilamide/0.1% naphthylethylene diamine dihydrochloride/2.5% H_3PO_4). Absorbance at 570 nm was measured on microplate reader MR5000 (Dynatech). To calculate the NO_2^- concentration, sodium nitrite was used as a standard.

Flow Cytometric Analysis. Cells were treated with anti-Ia monoclonal antibody for 30 min at 4°C in 1.5-ml Eppendorf tubes. After two washes with PBS/0.01% sodium azide/3% fetal bovine serum, the cells were treated with a 1:50 dilution of fluorescein isothiocyanate-conjugated goat anti-rat antibody for 30 min at 4°C. After three washes, flow cytometric analysis was done on a FACScan apparatus (Becton Dickinson). Cells stained with the second antibody alone were used as controls.

Inoculation with BCG and Determination of Ia Antigen Expression on Resident Macrophages. Freeze-dried live bacteria were reconstituted with PBS/0.025% Tween 80. Mice were inoculated in the tail vein with 0.2 ml of bacterial suspension containing 2×10^7 viable cells. Mice were killed 2 weeks after BCG inoculation, and cells were harvested from the peritoneal cavity by washing with 10 ml of PBS. After centrifugation the whole-cell population was resuspended in PBS/0.01% sodium azide/3% fetal bovine serum and treated with anti-Ia monoclonal antibody and fluorescein isothiocyanate-conjugated second antibody, as described in the preceding paragraph. Ia antigen expression was determined by FACScan analysis. Mean fluorescence intensity was determined after gating out the nonmacrophage cell populations.

RESULTS AND DISCUSSION

NO_2^- Generation in Macrophages from Wild-Type and IFN-$\gamma R^{0/0}$ Mice. Thioglycollate-elicited macrophages were incubated with various doses of murine IFN-γ in the presence or absence of LPS (100 ng/ml), and aliquots of the culture supernatants were collected after 48 hr for the assay of NO_2^- concentration (4). NO_2^- is the stable endproduct of nitric oxide generation. No stimulation of NO_2^- release was seen with any dose of IFN-γ (up to 1000 units/ml was used) in cells from IFN-$\gamma R^{0/0}$ mice, thus confirming the absence of functional IFN-γ receptors (data not shown). The cytokines GM-CSF, IL-2, and IL-4 were completely ineffective in inducing NO_2^- release in wild-type macrophages both in the presence and absence of LPS (data not shown); their action on IFN-$\gamma R^{0/0}$ macrophages was not examined. In the experiment summarized in Fig. 1 we compared the stimulation of NO_2^- release from thioglycollate-elicited peritoneal macrophages by IFN-γ or IFN-α/β, used either singly or in the presence of TNF, LPS, or a combination of TNF and LPS. Our data obtained in macrophages from wild-type mice are in full agreement with the results of Ding *et al.* (4) generated in macrophages from CD_1 mice. TNF, although not effective in the absence of added IFN (except for a very weak stimulatory effect seen in the presence of LPS that may be due to the presence of traces of endogenous IFN-γ), acted cooperatively with IFN-γ. A synergy between TNF and IFN-γ was apparent with the lower dose of IFN-γ (50 units/ml) used. Addition of TNF to cultures treated with IFN-γ and LPS produced virtually no further stimulation of NO_2^- release. IFN-α/β did not stimulate a significant NO_2^- release in the absence of LPS, and little or no cooperativity between IFN-α/β and TNF was seen (except for a weak stimulation by TNF in the presence of IFN-α/β at 1000 units/ml). The

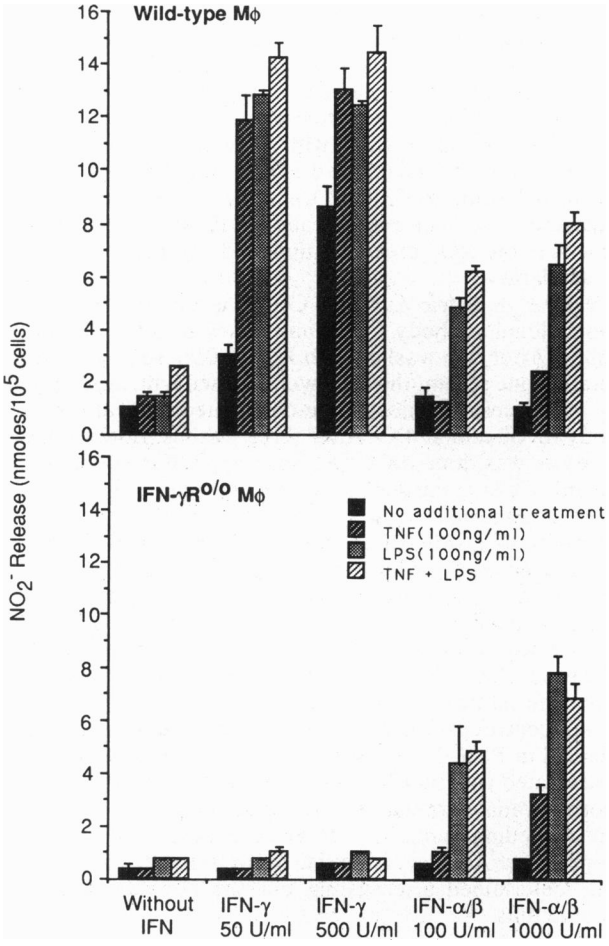

FIG. 1. NO_2^- release from macrophages of wild-type and IFN-$\gamma R^{0/0}$ mice after treatments with IFN-γ, IFN-α/β, and/or TNF. Thioglycollate-elicited peritoneal macrophages were incubated with the agents indicated. The amount of NO_2^- released into the culture medium after 48 hr was quantitated.

maximal level of NO_2^- release induced by IFN-α/β in the presence of LPS was only about half of the maximal level produced after treatments with IFN-γ. Macrophages from IFN-$\gamma R^{0/0}$ mice were not affected by IFN-γ and were virtually unresponsive to TNF, underscoring the lack of effectiveness of TNF in the absence of IFN. However, the responses of wild-type and IFN-$\gamma R^{0/0}$ macrophages to IFN-α/β were indistinguishable, indicating that the induction of NO_2^- release by IFN-α/β is completely independent of functional IFN-γ.

One conclusion that can be drawn from the experiment shown in Fig. 1 is that even at high doses IFN-α/β cannot fully replace IFN-γ in stimulating the generation of NO_2^- by macrophages. In addition, the effectiveness of TNF as an inducer of NO_2^- release largely depends on the simultaneous presence of IFN-γ. In IFN-γ-stimulated wild-type macrophages TNF can substitute for LPS as a second signal for the stimulation of NO_2^- release, but TNF is much less effective than LPS as a second signal in combination with IFN-α/β. Other experiments have shown that in cultures of macrophages from C57BL/6 mice neutralizing antibodies to TNF significantly reduced the stimulatory activity of LPS on the IFN-γ-induced NO_2^- release, suggesting that TNF is an important mediator of LPS action in this system (D.S., R.K., and J.V., unpublished observations).

The inability of other cytokines to substitute for IFN-γ in stimulating the generation of nitric oxide is likely to be relevant to the observed defect in the early resistance of IFN-$\gamma R^{0/0}$ mice to *L. monocytogenes* (14). IFN-γ as well as TNF was shown to be important in the defense against *Listeria* in the mouse (15, 26–28). In the intact organism both IFN-γ (29) and TNF (27) are produced during the early stages of *Listeria* infection, and they are thought to exert their protective effects within the first 3 days after inoculation (15, 28). The interdependence of IFN-γ and TNF is likely to be reflected also at the level of TNF production because IFN-γ is known to promote TNF-α generation by monocytes/macrophages in culture (30, 31) and to prime TNF-α production in *Listeria*-infected mice (32). Hence the absence of a functional IFN-γ receptor not only diminishes responsiveness to TNF (Fig. 1), it would also be expected to reduce the generation of TNF-α in the intact organism, thereby further diminishing host resistance to intracellular pathogens. Recent experiments have shown that IFN-$\gamma R^{0/0}$ mice also have a significantly reduced resistance to infection with *Leishmania major* (J. Louis and M.A., unpublished data); the role of nitric oxide in the leishmanicidal action of macrophages has been extensively documented in tissue culture and in the intact organism (19, 22).

MHC Class II Antigen Expression on Thioglycollate-Elicited Peritoneal Macrophages from Wild-type and IFN-$\gamma R^{0/0}$ Mice. The ability of IFN-γ to induce the expression of MHC class II antigens on macrophages and other cells has been amply documented (24, 25). In contrast, IFN-α/β is generally much less potent than IFN-γ in inducing MHC class II antigen expression (for review, see ref. 2). Simultaneous treatment of murine macrophages with IFN-γ and IFN-α/β was shown to reduce IFN-γ-induced MHC class II antigen expression (33, 34). In agreement with these earlier observations, we found that IFN-γ was a potent inducer of Ia expression in peritoneal macrophages from wild-type mice, whereas IFN-α/β produced a barely detectable stimulation (Fig. 2). Higher doses of IFN-γ or IFN-α/β did not produce a significantly greater stimulation (data not shown). In addition, simultaneous treatment with IFN-α/β significantly reduced IFN-γ-induced Ia expression in wild-type macrophages (Fig. 2). Macrophages

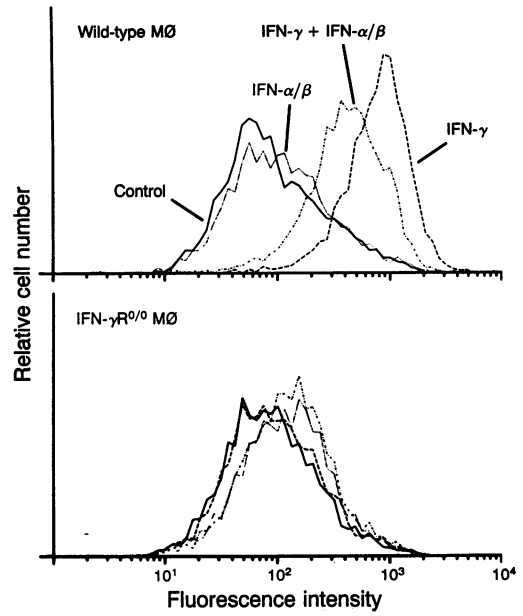

FIG. 2. MHC class II antigen expression on peritoneal macrophages from wild-type and IFN-$\gamma R^{0/0}$ mice after treatment with IFN-γ and/or IFN-α/β. Thioglycollate-elicited peritoneal macrophages were treated with IFN-γ (100 units/ml) and/or IFN-α/β (100 units/ml) for 48 hr. MHC class II antigen on macrophages was detected by treating the cells with rat anti-Ia monoclonal antibody and goat anti-rat IgG conjugated with fluorescein isothiocyanate. Flow cytometric analysis was done on a FACScan.

from IFN-$\gamma R^{0/0}$ mice failed to respond to IFN-γ, whereas IFN-α/β did produce a slight stimulation. It is interesting that no significant difference was seen in this and other experiments in the constitutive level of Ia antigen expression on untreated (control) thioglycollate-elicited macrophages from wild-type and IFN-$\gamma R^{0/0}$ mice. In addition, no clear differences between wild-type and IFN-$\gamma R^{0/0}$ mice were found when the level of Ia expression was examined on peritoneal macrophages immediately after their isolation by adherence to plastic dishes (data not shown). These results suggest that endogenous IFN-γ is not an important factor in the regulation of constitutive levels of MHC class II antigen expression.

In addition to IFN-γ and IFN-α/β, other cytokines were earlier shown to stimulate MHC class II antigen expression on macrophages or some other types of cells (8, 35–38). In preliminary experiments, we have examined the ability of TNF, IL-4, and GM-CSF to induce Ia expression on thioglycollate-elicited peritoneal macrophages from conventional C57BL/6 mice. Although IL-4 and GM-CSF showed Ia-inducing activity in these cells, TNF did not. Subsequently, the stimulation of Ia antigen expression by IL-4 and GM-CSF, at concentrations found to produce a maximal increase in preliminary experiments, was compared with thioglycollate-elicited macrophages from wild-type and IFN-$\gamma R^{0/0}$ mice (Fig. 3). Both IL-4 and GM-CSF caused a moderate stimulation of Ia antigen expression, with a similar stimulatory effect seen in wild-type and IFN-$\gamma R^{0/0}$ macrophages. These findings indicate that the stimulatory actions of IL-4 and GM-CSF must be independent of IFN-γ. Unlike the inhibitory effect exerted by IFN-α/β on IFN-γ-induced Ia antigen expression (see Fig. 2), simultaneous treatment with IL-4 or GM-CSF and IFN-α/β did not reduce the level of Ia antigen expression below that seen with IL-4 or GM-CSF alone. The latter finding suggests that the pathways of the stimulatory actions of IL-4 or GM-CSF are different from those used by IFN-γ. The major conclusion drawn from the results shown in Fig. 3 (and from other experiments not shown) is that IL-4 and GM-CSF can act in the absence of functional IFN-γ, but the maximal level of Ia antigen expression induced on peritoneal macrophages by these cytokines is lower than that produced by IFN-γ.

MHC Class II Antigen Expression on Resident Macrophages from BCG-Infected Wild-Type and IFN-$\gamma R^{0/0}$ Mice. To determine whether IFN-γ is important for the induced expression of Ia antigen in the intact organism, we compared MHC class II antigen expression on resident macrophages from mice infected with the BCG strain of *Mycobacterium bovis*. In preliminary experiments we determined that BCG infection induced detectable levels of IFN-γ in the sera of wild-type mice. At 2 weeks after BCG infection the serum level of IFN-γ was 820 ± 295 pg/ml (which corresponds to ≈4 reference units/ml), whereas uninfected wild-type mice had undetectable levels of IFN-γ (<64 pg/ml). Ia expression was quantitated on resident macrophages obtained from wild-type or IFN-$\gamma R^{0/0}$ mice 2 weeks after inoculation with BCG (Fig. 4). Mean fluorescence intensity corresponding to Ia antigen expression was much higher on macrophages from wild-type mice (678.2 ± 120.4) than on IFN-$\gamma R^{0/0}$ macrophages (266.1 ± 84.5); comparison by a two-tailed unpaired *t* test showed that this difference was statistically highly significant ($P = 0.0001$). Other experiments showed that the difference in the level of MHC class II antigen expression between macrophages from wild-type and IFN-$\gamma R^{0/0}$ mice was seen only after infection with BCG because resident macrophages obtained from uninfected wild-type or IFN-$\gamma R^{0/0}$ mice expressed similar levels of Ia antigen (data not shown). These results indicate that, at least after infection with BCG, IFN-γ is the major factor responsible for the increased Ia antigen expression on macrophages, and that in this system other cytokines cannot fully substitute for the absence of functional IFN-γ.

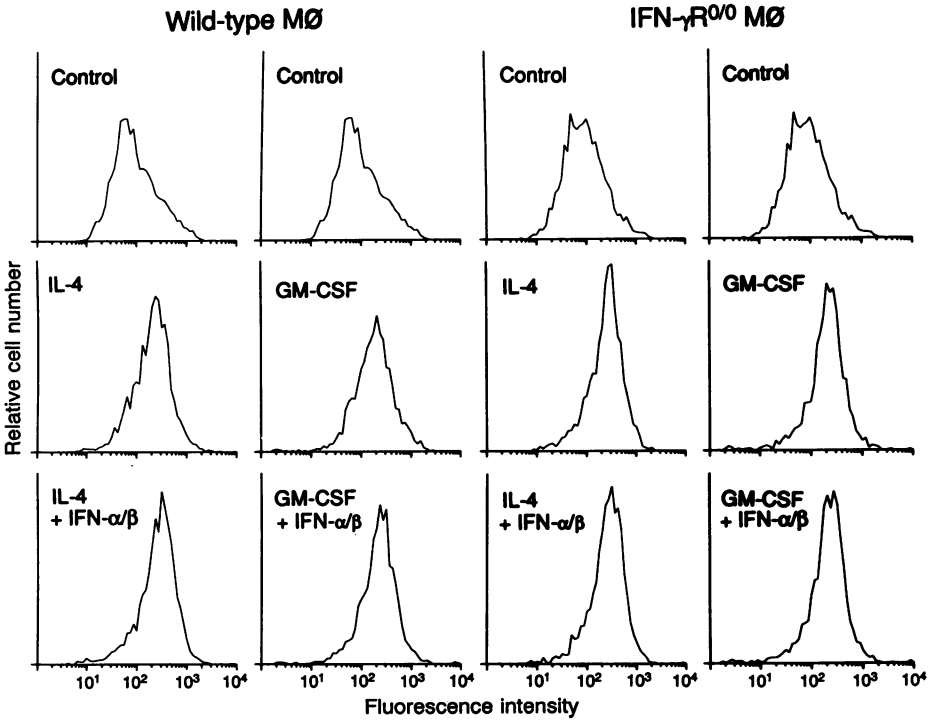

FIG. 3. Expression of MHC class II antigen on peritoneal macrophages from wild-type and IFN-$\gamma R^{0/0}$ mice after treatment with IL-4, GM-CSF, and IFN-α/β. Thioglycollate-elicited macrophages were treated with IL-4 (1000 units/ml) or GM-CSF (10 ng/ml), in the presence or absence of IFN-α/β (100 units/ml), for 48 hr. MHC class II antigen on macrophages was detected with the aid of rat anti-Ia monoclonal antibody and goat anti-rat IgG antibody conjugated with fluorescein isothiocyanate. Flow cytometric analysis was performed on a FACScan. Treatment with IFN-α/β alone produced a slight increase in fluorescence intensity in both wild-type and IFN-$\gamma R^{0/0}$ macrophages, as shown in Fig. 2.

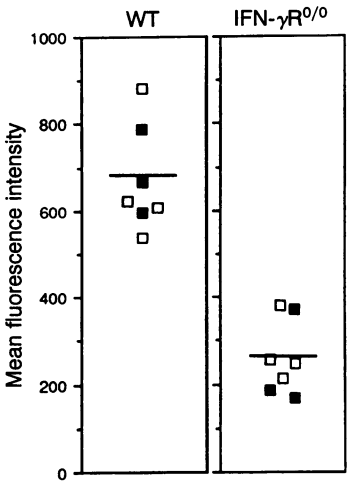

FIG. 4. MHC class II antigen expression on resident peritoneal macrophages from BCG-infected mice. Wild-type (WT, $n = 7$) and IFN-$\gamma R^{0/0}$ ($n = 7$) mice were inoculated in the tail vein with 0.2 ml of a bacterial suspension containing 2×10^7 BCG cells. At 2 weeks after infection, four mice from each group were injected i.v. with 25 μg of LPS (□); the remaining three mice (■) were not injected. All mice were killed 2 hr after the time of injection with LPS. (Injection with LPS had no apparent effect on the level of Ia antigen expression in either wild-type or IFN-$\gamma R^{0/0}$ mice.) Peritoneal exudate cells were harvested with 10 ml of PBS. The expression of Ia antigen on macrophages was analyzed by FACScan. Symbols indicate mean fluorescence intensity of each individual mouse. Horizontal lines indicate the average of mean fluorescence intensity per group.

Results shown in Figs. 2 and 3 indicate that although IFN-γ was the most potent stimulator of induced Ia expression on cultured peritoneal macrophages, the absence of a functional endogenous IFN-γ system did not reduce the constitutive level of Ia expression, and some other cytokines could, at least partly, replace IFN-γ as inducers of Ia antigen. These data suggest that MHC class II antigen presentation should not be completely impaired in IFN-$\gamma R^{0/0}$ mice, and this prediction is borne out by the demonstration that these mice were able to mount virtually undiminished immune responses to several types of antigenic stimuli (14). In contrast, the difference in the level of Ia antigen expression on resident peritoneal macrophages from wild-type and IFN-$\gamma R^{0/0}$ mice after BCG inoculation (Fig. 4) suggests a possible impairment in the generation of specific immune responses in the absence of a functional IFN-γ system in this mycobacterial infection.

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