

## **Murine Macrophages Secrete Interferon $\gamma$ upon Combined Stimulation with Interleukin (IL)-12 and IL-18: A Novel Pathway of Autocrine Macrophage Activation**

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### **Summary**

Interferon (IFN)- $\gamma$ , a key immunoregulatory cytokine, has been thought to be produced solely by activated T cells and natural killer cells. In this study, we show that murine bone marrow-derived macrophages (BMM $\Phi$ ) secrete large amounts of IFN- $\gamma$  upon appropriate stimulation. Although interleukin (IL)-12 and IL-18 alone induce low levels of IFN- $\gamma$  mRNA transcripts, the combined stimulation of BMM $\Phi$  with both cytokines leads to the efficient production of IFN- $\gamma$  protein. The macrophage-derived IFN- $\gamma$  is biologically active as shown by induction of inducible nitric oxide synthase as well as upregulation of CD40 in macrophages. Our findings uncover a novel pathway of autocrine macrophage activation by demonstrating that the macrophage is not only a key cell type responding to IFN- $\gamma$  but also a potent IFN- $\gamma$ -producing cell.

**Key words:** macrophage • interferon  $\gamma$  • interleukin 12 • interleukin 18 • innate immunity

Interferon (IFN)- $\gamma$  regulates a variety of important immunological programs. It is the predominant cytokine during Th1-dominated immune reactions, participates importantly during antigen presentation, and is the prototypical macrophage-activating cytokine. Consequently, a pivotal role of IFN- $\gamma$  in the clearance of various intracellular pathogens has been amply demonstrated (1). One of the key events during innate immune reactions is the production of IL-12 mainly by macrophages (2). IL-12 induces NK cells to rapidly secrete IFN- $\gamma$ , which then acts back to activate macrophages early in an immune response. Furthermore, IL-12 induces IFN- $\gamma$  production by T cells and is the key cytokine driving Th1 cell differentiation.

A recently discovered novel cytokine, IL-18, shares many functional properties with IL-12. IL-18 was described originally as a Kupffer cell-derived costimulating factor essential for the production of IFN- $\gamma$  in a murine LPS-induced shock model (3). IL-18 has been cloned recently (4), and was shown subsequently to induce IFN- $\gamma$  in human (5) and murine (6) T cells. Moreover, strong synergistic effects between IL-12 and IL-18 in the induction of IFN- $\gamma$  secretion of T cells (5, 6) or NK cells (7) were described.

A few reports described the secretion of low levels of IFN- $\gamma$  by murine (8, 9) or human (10) macrophages stimulated with IFN- $\gamma$  itself (8), IL-12 (9), or *Mycobacterium tuberculosis* (10). Because these findings are at variance with the widely accepted view that T cells and NK cells are the sole producers of IFN- $\gamma$ , the general significance of these

observations remained uncertain. A recent report demonstrated the secretion of IFN- $\gamma$  even by B cells upon combined stimulation with IL-12 and IL-18 (11). In this study, we demonstrate that these two cytokines synergistically induce macrophages to secrete large amounts of IFN- $\gamma$  which is biologically active in an autocrine fashion. The data suggest that macrophage activation may operate by an autocrine positive feedback loop involving multiple cytokines, including IFN- $\gamma$ .

### **Materials and Methods**

**Medium and Reagents.** All cell culture was performed in DMEM supplemented with 10% heat-inactivated FCS, 2 mM l-glutamine, 60  $\mu$ M 2-mercaptoethanol, 1 mM sodium pyruvate, 1 $\times$  nonessential amino acids, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin (GIBCO BRL, Paisley, UK).

Recombinant murine IFN- $\gamma$  was obtained from Genentech Inc. (South San Francisco, CA), IL-12 was purchased from R&D Systems (Abingdon, UK), and IL-18 and TNF- $\alpha$  from Pepro-Tech, Inc. (London, UK). Biotinylated anti-CD40 (clone 3/23) and rIgG2a isotype control antibodies were obtained from PharMingen (San Diego, CA). Streptavidin-PE was purchased from GIBCO BRL.

**Animals and Generation of Bone Marrow-derived Macrophages.** Mice of strains AKR/N, C57BL/6, and 129Sv and mice homozygous for a targeted mutation of the IFN- $\gamma$  receptor (IFN- $\gamma$ R<sup>-/-</sup>; 129Sv) (12) were obtained from the specific pathogen-free animal facilities of the Max-Planck-Institut and were used between 6 and 8 wk of age.

Macrophages were derived from bone marrow cells (BMM $\Phi$ ) as described previously (12).

**IFN- $\gamma$  Assay, Nitric Oxide Measurement, and Analysis of CD40 Expression by FACS<sup>®</sup>.** IFN- $\gamma$  was determined by a commercially available (PharMingen) sandwich ELISA test according to the manufacturer's protocol. The measuring range of the ELISA test was 0.5–100 ng/ml. Nitric oxide (NO) was measured as nitrite using the Griess reagent as described previously (13).

To assess CD40 expression by FACS<sup>®</sup>,  $2 \times 10^5$  BMM $\Phi$  were preincubated with FcBlock<sup>™</sup> (PharMingen) and stained with biotinylated anti-CD40 mAb or isotype control (biotin rIgG2a) for 30 min on ice. After another wash step, cells were stained with Streptavidin-PE for 30 min on ice and subsequently analyzed on a FACScan<sup>®</sup> (Becton Dickinson, Mountain View, CA), gating on viable cells by propidium iodide counterstaining. All washing and staining steps were performed in PBS/2% FCS.

**Reverse Transcription PCR.**  $5 \times 10^6$  BMM $\Phi$  were seeded and stimulated in 55-mm Petriperm<sup>®</sup> hydrophob Petri dishes (Heraeus GmbH, Hanau, Germany) in a final volume of 5 ml. At the indicated time points, cells were harvested with a rubber policeman, and total cellular RNA was prepared by the method of Chomczynski and Sacchi (14). Reverse transcription was performed by standard procedures using Moloney murine leukemia virus reverse transcriptase (Pharmacia GmbH, Freiburg, Germany).

0.01–1  $\mu$ l of the resulting cDNA (adjusted to a concentration of 50 ng/ $\mu$ l input RNA) was then amplified by PCR (annealing temperature 58°C, 1.5 mM MgCl<sub>2</sub>) for 35 cycles. The sequences for the primers used are as follows:  $\beta$ -actin sense primer, 5' TG-GAATCCTGTGGCATCCAT GAAAC 3', and  $\beta$ -actin antisense primer, 5' TAAAACGCAGCTCAGTAACAGTCCG 3', generating a 348-bp PCR product; and IFN- $\gamma$  sense primer, 5' GCTCTGAGACAATGAACGCT 3', and IFN- $\gamma$  antisense primer, 5' AAAGAGATAATCTGGCTCTGC 3', generating a 227-bp PCR product. The PCR products were run on a 1.5% agarose gel and visualized by ethidium bromide staining.

**RNA In Situ Hybridization.** In situ hybridization was performed using a digoxigenin-labeled riboprobe corresponding to the region between nucleotides 371 and 1075 of the mouse IFN- $\gamma$  cDNA, using a protocol modified from Vignaud et al. (15). In brief, BMM $\Phi$  were cultured on glass chamber slides (Nunc, Inc., Naperville, IL), washed in PBS, fixed in 4% paraformaldehyde for 3 min, permeabilized in 100 mM glycine for 20 min, followed by a short wash in water, and postfixed in 4% paraformaldehyde for 5 min. The cells were then hybridized overnight at 55°C with 1  $\mu$ g/ml of the riboprobe in 40% formamide, 5 $\times$  SSC, 1 $\times$  Denhardt's, 100  $\mu$ g/ml denatured herring sperm DNA, and 100  $\mu$ g/ml tRNA. After extensive washing in 2 $\times$  SSC, 50% formamide at 50°C, unspecific antibody binding was blocked by incubation at room temperature for 1 h in TBS (25 mM Tris-HCl, pH 7.5, 140 mM NaCl, 2.7 mM KCl) containing 10% sheep serum. Alkaline phosphatase-conjugated antidigoxigenin antibodies (Boehringer Mannheim, Mannheim, Germany) were then applied at 4°C for 5 h in TBS containing 1% sheep serum. The cells were then washed extensively in TBS, equilibrated with 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM MgCl<sub>2</sub>, and the alkaline phosphatase activity was developed with BCIP/NBT (Boehringer Mannheim) for 6 h. The preparations were mounted, and the cells were photographed under Nomarski optics.

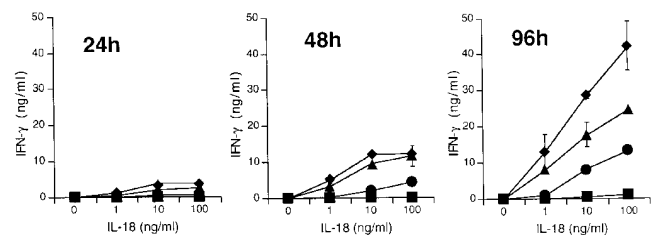
## Results

**IL-12 and IL-18 Stimulate BMM $\Phi$  to Secrete Large Amounts of IFN- $\gamma$ .** AKR/N-BMM $\Phi$  were stimulated with various

concentrations of IL-12 or IL-18 alone or in combination (Fig. 1). At different time points, the IFN- $\gamma$  concentrations in the supernatants were determined by ELISA. The stimulation of the macrophages with IL-12 or IL-18 alone did not result in detectable IFN- $\gamma$  secretion. In sharp contrast, both cytokines synergized strongly to induce readily detectable levels of IFN- $\gamma$  in the supernatants at all time points tested. The cytokine accumulated from 3.7 ng/ml per  $10^5$  cells at 24 h to 42 ng/ml per  $10^5$  cells IFN- $\gamma$  at 96 h. Stimulation of the macrophages with IL-12 and IL-18 at 96 h was still suboptimal, as a plateau of IFN- $\gamma$  secretion has not been reached. The induced levels of IFN- $\gamma$  are comparable to those reached upon anti-CD3 stimulation of the prototypical Th1 T cell clone AE7 (81 ng/ml per  $10^5$  cells, data not shown). The ability of the macrophages to secrete IFN- $\gamma$  is not restricted to the AKR/N strain of mice. We also tested BMM $\Phi$  of C57BL/6 (see also Fig. 3 A) and BALB/c (data not shown) mice with similar results. To demonstrate the purity of our BMM $\Phi$  population and to exclude the possibility of contaminating NK, T, or B cells in our assay, we analyzed the BMM $\Phi$  by FACS<sup>®</sup>. The population was consistently found to be homogeneously positive for F4/80, MAC-1, I-A<sup>k</sup>, B7.1, and CD16/32. T cell antigens (CD3, CD4, and CD8), NK cell marker (DX5), and also NK1.1 for C57BL/6 mice) or B cell antigens (B220, CD19, and CD5) were uniformly negative (data not shown).

We also assayed the commonly used mouse macrophage cell lines RAW 264.7, J774, RAW 309Cr, P388D1, and IC 21. In contrast to BMM $\Phi$ , none of the five cell lines produced IFN- $\gamma$  upon stimulation with IL-12 and IL-18 (data not shown).

**IL-12 and IL-18 Induce IFN- $\gamma$  mRNA in BMM $\Phi$ .** To address the level of regulation of IFN- $\gamma$  synthesis, we stimulated BMM $\Phi$  with IL-12 (10 ng/ml) or IL-18 (50 ng/ml), alone or in combination, and prepared mRNA after 2, 8, and 24 h of stimulation. IFN- $\gamma$  mRNA expression was then evaluated by reverse transcription (RT)-PCR (Fig. 2 A). In the case of BMM $\Phi$  stimulated with IL-12 plus IL-18, a prominent band of IFN- $\gamma$  mRNA appeared as early as 2 h after addition of the cytokines, and increased further at later time points after stimulation. The PCR product

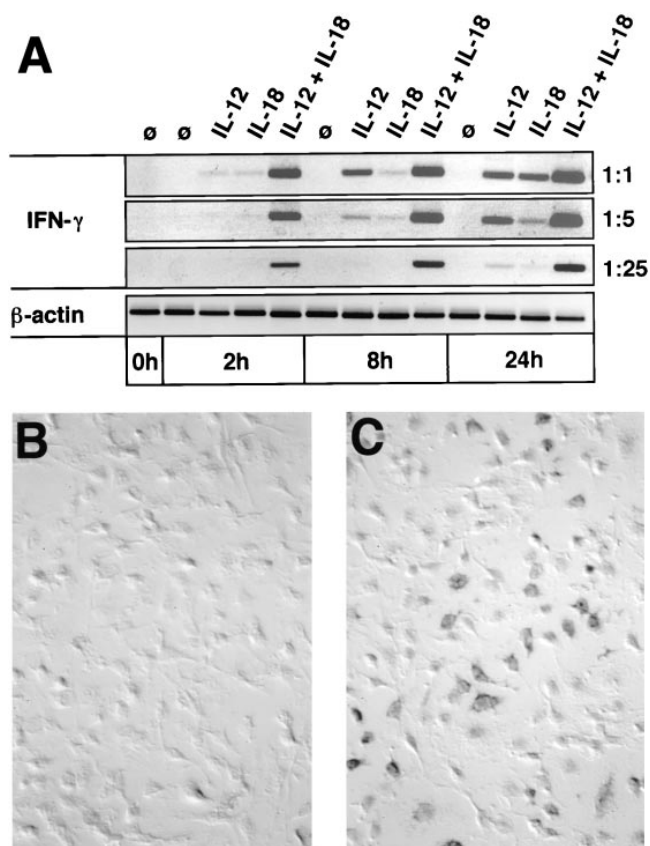


**Figure 1.** IL-12/IL-18-induced IFN- $\gamma$  synthesis of BMM $\Phi$ .  $10^5$  AKR/N-BMM $\Phi$  were stimulated in a final volume of 200  $\mu$ l in 96-well flat-bottomed plates with increasing concentrations of IL-12 alone (squares, no IL-12; circles, 0.001 ng/ml; triangles, 0.1 ng/ml; diamonds, 10 ng/ml), IL-18 alone, or both cytokines together in a final volume of 200  $\mu$ l in 96-well flat-bottomed plates. At the indicated time points, culture supernatants were harvested and analyzed for IFN- $\gamma$  concentration by ELISA. Data represent means of triplicate with SD indicated. Similar results were obtained in a total of three independent experiments.

was proven to result from IFN- $\gamma$  mRNA by sequencing (data not shown). Surprisingly, although no secreted IFN- $\gamma$  was detectable in the supernatant of BMM $\Phi$  stimulated with IL-12 alone or IL-18 alone, IFN- $\gamma$  mRNA was clearly induced. This induction appears to be more prominent in the case of stimulation with IL-12 than with IL-18 and increases over time during the culture period. Nevertheless, as shown semiquantitatively by titrating the input cDNA of the PCR reaction, the induction of IFN- $\gamma$  mRNA by combined stimulation with IL-12 and IL-18 is clearly far more efficient, consistent with the synergism observed at the protein level. To estimate the frequency of IFN- $\gamma$ -producing macrophages within the whole population, we performed RNA in situ hybridization experiments (Fig. 2, B and C). After 18 h of stimulation with IL-12 plus IL-18, ~25–30% of the BMM $\Phi$  stained strongly positive for IFN- $\gamma$  mRNA (Fig. 2 C), whereas unstimulated control BMM $\Phi$  were homogeneously negative (Fig. 2 B). Control stainings with the riboprobe in sense orientation also yielded completely negative results (data not shown).

**Autocrine Macrophage Stimulation by Macrophage-derived IFN- $\gamma$ .** To test the biological activity of IFN- $\gamma$  produced endogenously by BMM $\Phi$  stimulated with IL-12 plus IL-18, we tested two different indicators of IFN- $\gamma$ -mediated macrophage activation. First, IFN- $\gamma$  is known to synergize with TNF- $\alpha$  in the induction of inducible NO synthase (iNOS) with concomitant production of nitric oxide (NO) in macrophages (16). Therefore, we stimulated BMM $\Phi$  of AKR/N and C57BL/6 mice with optimal concentrations of IL-12, IL-18, or IL-12 plus IL-18 and titrated increasing concentrations of TNF- $\alpha$  into these cultures. Control cultures received IFN- $\gamma$ . As a readout for iNOS induction, we determined nitrite, the stable end product of NO, in the supernatants after 96 h of stimulation (Fig. 3 A). IFN- $\gamma$  was measured simultaneously in the supernatants. In macrophages exposed to IL-12 plus IL-18, increasing concentrations of TNF- $\alpha$  induced iNOS very efficiently, although somewhat less than upon optimal stimulation with exogenously added IFN- $\gamma$ . Experiments with mice lacking the IFN- $\gamma$  receptor (IFN- $\gamma$ R $^{-/-}$ ) proved that endogenously produced IFN- $\gamma$  is responsible for the induction of iNOS: although IFN- $\gamma$ R $^{-/-}$ -BMM $\Phi$  stimulated with IL-12 plus IL-18 plus TNF- $\alpha$  secreted IFN- $\gamma$  comparable to control IFN- $\gamma$ R $^{+/+}$ -BMM $\Phi$  (129Sv), no nitrites were detectable in the supernatant of the IFN- $\gamma$ R $^{-/-}$ -BMM $\Phi$ , reflecting a failure of iNOS induction when IFN- $\gamma$  is unable to signal via its receptor (Fig. 3 B). Indeed, nitrite accumulation reflected iNOS induction, as demonstrated by the addition of the iNOS inhibitor 1-monomethyl-L-arginine, which completely abolished detectable nitrites without influencing IFN- $\gamma$  induction (data not shown).

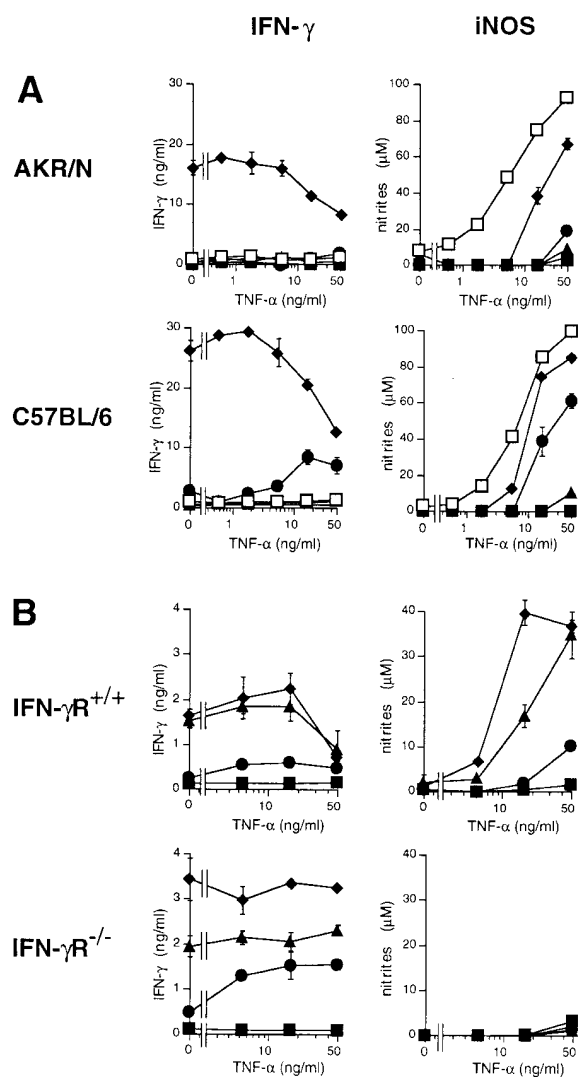
Two additional interesting observations were consistently made. First, with increasing concentrations of TNF- $\alpha$ , IFN- $\gamma$  levels stimulated with IL-12 plus IL-18 decrease (Fig. 3, A and B). IFN- $\gamma$ R $^{-/-}$ -BMM $\Phi$  are an exception to this rule (Fig. 3 B), pointing to a possible role of enhanced consumption of IFN- $\gamma$  upon increasing concentrations of TNF- $\alpha$ . The phenomenon is unrelated to the simulta-



**Figure 2.** IL-12/IL-18-induced IFN- $\gamma$  mRNA in BMM $\Phi$ . (A)  $5 \times 10^6$  AKR/N-BMM $\Phi$  were seeded in 55-mm Petriperm<sup>®</sup> hydrophob plates and either cultivated in medium alone (control,  $\emptyset$ ) or stimulated with 10 ng/ml IL-12 alone, 50 ng/ml IL-18 alone, or with both cytokines together. At the indicated time points, cells were harvested, mRNA was extracted, and cDNA was prepared as described in Materials and Methods. 1, 0.2, or 0.04  $\mu$ l of input cDNA was amplified by PCR with IFN- $\gamma$ -specific primers. As a control,  $\beta$ -actin mRNA was also amplified with 0.01  $\mu$ l input cDNA. One of three independent experiments, yielding identical results, is shown. (B and C)  $10^6$  AKR/N-BMM $\Phi$  were cultivated on glass chamber slides with medium alone (B) or with 10 ng/ml IL-12 plus 50 ng/ml IL-18 (C). After 18 h, RNA in situ hybridization for IFN- $\gamma$  mRNA was performed as described in Materials and Methods;  $\times 200$ .

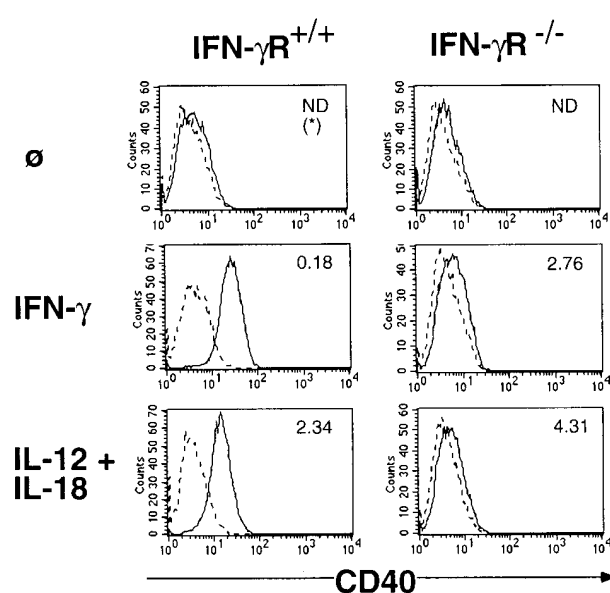
neously produced NO, known to be potentially autotoxic to the secreting macrophage itself (16), because L-monomethyl-L-arginine had no influence on the observed decrease of IFN- $\gamma$  (data not shown). Second, in the case of the C57BL/6-BMM $\Phi$  (Fig. 3 A), IL-12 alone synergizes with TNF- $\alpha$  to induce IFN- $\gamma$  as well as iNOS. In AKR/N mice, a different and/or less efficient synergism seems to operate: in five independent experiments, a slight induction of nitrites was noted consistently upon costimulation with 50 ng/ml TNF- $\alpha$ , whereas IFN- $\gamma$  in the supernatant was either undetectable or only marginally elevated (e.g., 1.5 ng/ml in Fig. 3 A).

As a second system to demonstrate the functional potential of the BMM $\Phi$ -derived IFN- $\gamma$ , we assayed the IFN- $\gamma$ -mediated upregulation of CD40. Resting BMM $\Phi$  are negative or only slightly positive for CD40, whereas stimulation with IFN- $\gamma$  for 48 h leads to an upregulation of this costimulatory molecule (M. Munder, unpublished observa-



**Figure 3.** Endogenously produced IFN- $\gamma$  induces autocrine macrophage stimulation: iNOS induction. (A)  $10^5$  AKR/N-BMM $\Phi$  were cultivated in a final volume of 200  $\mu$ l in 96-well flat-bottomed plates with medium alone (filled squares) or stimulated with 10 ng/ml IL-12 alone (circles), 50 ng/ml IL-18 alone (triangles), with both cytokines together (diamonds), or with 10 ng/ml IFN- $\gamma$  (open squares) in a final volume of 200  $\mu$ l in 96-well flat-bottomed plates. To allow for synergistic iNOS induction, increasing concentrations of TNF- $\alpha$  were titrated into each culture. After 96 h, supernatants were harvested, and IFN- $\gamma$  as well as nitrites were determined as described in Materials and Methods. Data represent means of triplicate with SD indicated. Similar results were obtained in a total of four independent experiments. (B)  $10^5$  BMM $\Phi$  of IFN- $\gamma$ R<sup>+/+</sup> (129Sv) or IFN- $\gamma$ R<sup>-/-</sup> strains of mice were stimulated with increasing concentrations of IL-12 plus IL-18 (squares, no cytokines; circles, 0.1/0.5 ng/ml; triangles, 1/5 ng/ml; diamonds, 10/50 ng/ml, respectively) in a final volume of 200  $\mu$ l in 96-well flat-bottomed plates. Increasing concentrations of TNF- $\alpha$  were titrated into each culture. After 96 h, supernatants were harvested, and IFN- $\gamma$  as well as nitrites were determined as described in Materials and Methods. Data represent means of triplicate with SD indicated. One of two independent experiments with similar results is shown.

tion). The same induction was noted on IFN- $\gamma$ R<sup>+/+</sup>-BMM $\Phi$  (129Sv) stimulated with IL-12 plus IL-18. Again, endogenously produced IFN- $\gamma$  was responsible for this effect, as demonstrated by the lack of CD40 induction on IFN- $\gamma$ R<sup>-/-</sup>-BMM $\Phi$  stimulated with IL-12 plus IL-18



**Figure 4.** Macrophage-derived IFN- $\gamma$  is biologically active: CD40 up-regulation.  $1.5 \times 10^6$  BMM $\Phi$  of IFN- $\gamma$ R<sup>+/+</sup> (129Sv) or IFN- $\gamma$ R<sup>-/-</sup> strains of mice were seeded in 35-mm Petriperm<sup>®</sup> hydrophob plates in a final volume of 1.5 ml. They were either not stimulated (Ø, medium only) or stimulated with 10 ng/ml IFN- $\gamma$  (positive control) or with the combination of 10 ng/ml IL-12 plus 50 ng/ml IL-18. After 48 h, BMM $\Phi$  were harvested with a rubber policeman and analyzed by FACS<sup>®</sup> for CD40 up-regulation as described in Materials and Methods. (\*) Simultaneous determination of IFN- $\gamma$  concentration (ng/ml) in the 48-h supernatant. ND, Not determined. One of two independent experiments with similar results is shown.

(Fig. 4). BMM $\Phi$  of mice with 129 background are definitively less efficient in IFN- $\gamma$  production upon IL-12/IL-18 stimulation (Fig. 3 B, and Fig. 4) than BMM $\Phi$  of strains AKR/N or C57BL/6 (Fig. 1, and Fig. 3 A). Nevertheless, even these lower amounts of induced IFN- $\gamma$  clearly auto-activate the macrophages to upregulate iNOS and CD40.

## Discussion

These experiments were stimulated by the results of several authors showing synergism between IL-12 and IL-18 in the induction of IFN- $\gamma$  by human (5) and murine (6) T cells and murine NK cells (7). Remarkably, both cytokines were shown recently to cooperatively induce IFN- $\gamma$  secretion of murine B cells (11). We now extend these observations by demonstrating that macrophages become efficient IFN- $\gamma$ -producing cells upon combined stimulation with IL-12 and IL-18. The levels of IFN- $\gamma$  were of a similar magnitude as that produced by T cells, and we observe efficient autocrine macrophage activation by endogenously generated IFN- $\gamma$  in vitro.

We found that a considerable proportion but not all BMM $\Phi$  express IFN- $\gamma$  mRNA under our conditions of in vitro stimulation. Moreover, five established macrophage cell lines failed to produce IFN- $\gamma$  under these conditions. A detailed comparison (17) of various IFN- $\gamma$ -induced activation parameters between immortalized macrophage cell lines and ex vivo-derived peritoneal macrophages demon-

strated that each immortalized cell line exhibited only a part of the spectrum of the determined activation markers, whereas all were detected in normal macrophages. It is not clear whether these and our findings reflect a genuine quantitative or qualitative macrophage heterogeneity in the response to cytokines, and/or a loss of functional potency associated with prolonged tissue culture maintenance.

Although the combined stimulation of the BMM $\Phi$  with IL-12 and IL-18 is required to stimulate IFN- $\gamma$  secretion, both cytokines alone suffice in inducing detectable IFN- $\gamma$  mRNA. Whether this difference reflects merely a limitation of the ELISA sensitivity or hints towards unknown posttranscriptional regulatory mechanisms remains to be elucidated. Furthermore, Fultz et al. described the detection of IFN- $\gamma$  mRNA without secreted protein upon LPS stimulation of various types of murine macrophages, including BMM $\Phi$  (18), a finding we were unable to reproduce in the present study (not shown).

An interesting side-aspect of our work is the finding that IL-12 and TNF- $\alpha$ , similar to their synergistic effects in the

induction of IFN- $\gamma$  by NK cells (19), also cooperate in the induction of IFN- $\gamma$  secretion by BMM $\Phi$ . Among the three strains tested, this effect is seen only in C57BL/6-BMM $\Phi$ , possibly reflecting a genetic polymorphism related to the known genetic differences between inbred mice in the ability to generate Th1- or Th2-dominated immune responses. Thus, a detailed comparison of this phenomenon between different mouse strains might prove fruitful.

Our work demonstrates for the first time that macrophages secrete high levels of IFN- $\gamma$  upon combined stimulation with IL-12 and IL-18. Upon appropriate activation by pathogens or LPS, macrophages are known to be important producers of IL-12 (2) or IL-18 (3). Thus, our findings unravel a novel potential pathway of autocrine macrophage activation involving endogenously produced IFN- $\gamma$ . This pathway might play a pivotal role during early innate immune reactions, i.e., before the development of adaptive immunity, in infectious diseases (7, 10), in septic shock (3), as well as during autoimmune reactions (20).

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Received for publication 12 March 1998 and in revised form 6 April 1998.

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