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Interferon-γ priming is involved in the activation of arginase by oligodeoxinucleotides containing CpG motifs in murine macrophages

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Summary

Recognition of microbial products by macrophages (Mϕ) stimulates an inflammatory response and plays a critical role in directing the host immune response against infection. In the present work, we showed for the first time that synthetic oligodeoxynucleotides containing unmethylated cytosine guanine motifs (CpG) are able to stimulate, in the presence of interferon-γ (IFN-γ), both arginase and inducible nitric oxide synthase (iNOS) in murine Mϕ. Unexpectedly, IFN-γ, a cytokine believed to be an inhibitor of arginase activity, intervened in the activation of this enzyme. A significant increase in arginase activity was observed upon a short pre-incubation (1 hr) with IFN-γ and subsequent CpG stimulation. Therefore, a very interesting observation of this study was that the CpG-mediated arginase activity is dependent on IFN-γ priming. The increase in arginase activity as a result of stimulation with CpG plus IFN-γ was correlated with augmented expression of the arginase II isoform. The use of pharmacological specific inhibitors revealed that arginase activity was dependent on p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated protein kinase (ERK), but independent of c-Jun N-terminal kinase (JNK) activation. This report reveals a singular effect of the combination of CpG and IFN-γ, one of the mayor cytokines produced in response to CpG administration *in vivo*.

Keywords: arginase; CpG; interferon-γ; macrophages/monocytes; Toll-like receptors

Introduction

It is well known that unmethylated cytosine guanine motifs contained in bacterial DNA or synthetic oligonucleotides CpG-DNA are powerful immunostimulatory molecules. Initially, CpG-DNA were believed to be predominantly pro-inflammatory molecules, stimulating a T helper 1 (Th1)-like response dominated by the release of interleukin (IL)-12 and interferon-γ (IFN-γ).[1] However, in recent years there has been a spate of interest in some anti-inflammatory properties of CpG-DNA. Pulmonary inflammation decreased in response to lipopolysaccharide (LPS) after systemic exposure to CpG-DNA.[2] CpG-DNA were found to stimulate the production of IL-10,[3–5] increase the expression of indoleamine 2,3-dioxygenase (IDO), an enzyme linked to the suppression of T-cell-mediated immunity,[6,7] and enhance the shedding of the tumour necrosis factor-α (TNF-α) receptor in macro-

Abbreviations: BMMϕ, bone marrow-derived macrophages; CHX, cycloheximide; CpG, synthetic oligodeoxynucleotides containing unmethylated cytosine guanine motifs; CpG-DNA, unmethylated cytosine guanine motifs contained in bacterial DNA or synthetic oligonucleotides; ECL, enhanced chemiluminescence; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated protein kinase; EU, endotoxin unit; FBS, fetal bovine serum; GpC, non CpG; HRP, horseradish peroxidase; IDO, indoleamine 2,3-dioxygenase; IFN-γ, interferon-γ; IL, interleukin; iNOS, inducible nitric oxide synthase; ISPF, alpha-isonitrosopropiophenone; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; Mϕ, macrophage; MAPK, mitogen-activated protein kinase; M-CSF, macrophage colony-stimulating factor; NO, nitric oxide; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; SOCS, suppressors of cytokine signaling; Th1, T helper 1; TLR, Toll-like receptor; TNF-α, tumour necrosis factor-α.

phages (Mϕ) as a counterinflammatory effect.[8] In addition, CpG-DNA induced suppressors of cytokine signaling (SOCS-1 and SOCS-3) to modulate cytokine responses in antigen-presenting cells.[9] Moreover, although the recognition of CpG-DNA and LPS by Mϕ share many elements that mediate the inflammatory response [IL-1, IL-6, IL-12, TNF-α and nitric oxide (NO)], CpG-DNA seems to be less toxic *in vivo*, a characteristic that has been observed in several human clinical trials.[10,11] Therefore, low toxicity of CpG-DNA is an attractive feature for use of CpG-DNA as a vaccine adjuvant and in other therapeutic strategies. Exactly, how CpG-DNA regulate discrete anti-inflammatory elements remains uncertain. For instance, CpG-DNA stimulate NO secretion in macrophages, but it is unknown if CpG-DNA are able to modulate NO production in order to avoid the production of toxic levels. Arginase and inducible nitric oxide synthase (iNOS) share a common substrate, L-arginine, and an overwhelming body of evidence indicates that arginase works as a modulator of NO production by siphoning off substrate.[12–14] Therefore, we sought to determine the capability of synthetic oligodeoxynucleotides containing unmethylated cytosine guanine motifs (CpG) to modulate arginase activity in bone marrow-derived macrophages (BMMϕ). The data presented in this work established that, in addition to their capacity to stimulate NO production, CpG also effectively induce arginase activity. Surprisingly, we observed that both CpG-mediated arginase and NO induction occurred only in the presence of IFN-γ. As IFN-γ has been considered to be an inhibitor of the arginase activation by cytokines or LPS,[15–17] the data presented here shed new light on the NO–arginase regulation complexity. Furthermore, the effect of CpG plus IFN-γ on arginase activity revealed a singular effect of the combination of CpG and IFN-γ, one of the mayor cytokines produced in response to CpG administration *in vivo*.

Materials and methods

Mice

Experiments were performed using 8–10-week-old female BALB/c mice originally obtained from the Bioterio de la Facultad de Ciencias Veterinarias Universidad Nacional de la Plata (Argentina). The Institutional Experimentation Animal Committee (authorization no. 15-07-62010 and HCD resolution 450/07) approved the animal handling and experimental procedures.

Generation of BMMϕ

Bone marrow cells were obtained by flushing the femurs and tibias of the female BALB/c mice with RPMI-1640 (GIBCO Cell Culture Systems, Rockville, MD). Cells were cultured in RPMI-1640 containing 10% heat-inactivated fetal bovine serum (FBS) (Natocor, Carlos Paz, Argentina), 100 μg/ml of penicillin, 100 U/ml of streptomycin and the supernatant of L929 fibroblasts, at a final concentration of 10% (v/v), as a source of macrophage colony-stimulating factor (M-CSF) that drives cell differentiation towards a 95% pure population of BMMϕ, identified by flow cytometric analysis as CD11b-positive cells.

Reagents

Mouse recombinant IFN-γ was obtained from R&D Systems (Minneapolis, MN). L-arginine hydrochloride, protease inhibitors, Triton X-100, alpha-isonitrosopropiophenone (ISPF), chloroquine, polymyxin B, cycloheximide (CHX), sulphanilamide and naphthylethylene diamine dihydrochloride were obtained from Sigma-Aldrich (St Louis, MO). Ultrapure LPS (from *Escherichia coli* K12) was obtained from InvivoGen (San Diego, CA). The mitogen-activated protein kinase (MAPK) inhibitors SB203580 and PD98059 were purchased from Calbiochem (San Diego, CA), and SP600125 was obtained from Sigma-Aldrich.

Synthetic oligodeoxynucleotides

The synthetic oligodeoxynucleotides used were: 1826 (CpG), TCCATGACGTTCCTGACGTT; and 1745 non-CpG (GpC), TCCAATGAGCTTCCTGAGTCT. The CpG motifs are underlined. All oligodeoxynucleotides were synthesized with a nuclease-resistant phosphorothioate backbone and contained no LPS contaminants (Operon Technologies-Alameda, CA). In addition, we performed a standard Limulus amebocyte lysate assay (BioWhittaker Inc., Walkersville, MD) which showed that the endotoxin content of the oligodeoxynucleotides after reconstitution was less than 1 endotoxin unit (EU)/ml.

BMMϕ culture

BMMϕ were cultured in RPMI-1640 in the absence of phenol red (Sigma-Aldrich) and supplemented with 10% (v/v) heat-inactivated FBS, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 100 μg/ml of penicillin and 100 U/ml of streptomycin at 37° in a moist atmosphere of 5% CO_2 in air. Unless otherwise mentioned, 0·5 ml of BMMϕ suspension, at 1×10^6 cells/ml, was seeded in 48-well tissue-culture plates (GREINER Bio One, Frickenhausen, Germany) for 48 hr and stimulus was added to the culture medium to give the following final concentrations: 0·3 μM CpG or GpC, 25 ng/ml of recombinant IL-4, 1 μg/ml of LPS and 50 IU/ml of recombinant IFN-γ. In some experiments BMMϕ were pre-incubated with inhibitors. In these cases parallel-control experiments were performed by adding the vehicle solution (dimethyl sulphoxide). Cell viability was assessed by Trypan Blue exclusion.

Arginase enzyme activity and protein expression assays

Arginase activity was measured in cell lysates, as described by Corraliza *et al.*,[18] with a few modifications. Briefly, cells were lysed with 0·1% Triton X-100 plus protease inhibitors for 30 min. Equal volumes of Tris-HCl (25 mM)–$MnCl_2$ (10 mM) buffer and lysate were mixed, and the enzyme was activated by heating for 10 min at 55°. Arginine hydrolysis was conducted by incubating the cell lysates with L-arginine (pH 9·7) at 37° for 60 min. The reaction was stopped upon the addition of 400 μl of $H_2SO_4/H_3PO_4/H_2O$ (1:3:7, v/v/v). The urea concentration was measured at 540 nm after the addition of 25 μl of ISPF (dissolved in 100% ethanol), followed by heating at 100° for 40 min. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of urea/min.

To detect arginase isoform protein, 6×10^6 BMMϕ were incubated for 48 hr. Then, cells were lysed in 100 μl of buffer containing 150 mM NaCl, 10 mM Tris–HCl (pH 7·5), 0·5% Triton X-100 and protease inhibitors. The protein contents were determined using the Bradford assay (Bio-Rad, Hercules, CA). One-hundred micrograms of each sample (except for liver, for which we used 50 μg) were resolved by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) in a 12% gel, and then transferred to a nitrocellulose membrane (Millipore, Billerica, MA). Membranes were incubated with rabbit anti-arginase I or II and followed by incubation with a HRP-conjugate anti-rabbit IgG. Protein was detected using the enhanced chemiluminescence (ECL) system (Amersham Biosciences, Little Chafont, UK). The specificity of the bands was demonstrated using liver lysate (arginase I-positive control) and kidney lysate (arginase II-positive control). Antibody against arginase I and II were a kind gift of Dr Tomomi Gotoh.[19] As a loading control, the blots were reprobed with antibody against α-tubulin (Sigma-Aldrich).

NO production assay

NO was measured using the Griess reagent. Briefly, 100 μl of culture supernatant was reacted with 200 μl of reagent (1% sulphanilamide/0·1% naphthylethylene diamine dihydrochloride/2·5% H_3PO_4) at room temperature (25°) for 10 min, after which the absorbance at 540 nm was determined.

Measurement of cytokine levels

The levels of IL-10 and IL-12 in culture supernatants were measured using capture enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's recommendations (BD PharMingen, San Diego, CA). The concentrations are expressed in relation to standard curves constructed by assaying serial dilutions of the respective standard cytokine.

Phosphorylated p38 and p42/44 MAPK protein expression assays

BMMϕ were incubated for 24 hr with medium containing 2% FBS but deprived of the M-CSF factor. Then, BMMϕ were stimulated, harvested and lysed at the indicated time-points. Equal amounts of samples were resolved by SDS–PAGE in a 10% gel and electrotransferred to nitrocellulose membranes. The membranes were incubated with antibody against phospho-p38, phospho-p42/44 and phospho-p46/54 (Cell Signaling Technology, Beverly, MA) followed by incubation with a HRP-conjugate anti-rabbit IgG. The reaction was visualized using the ECL system. As a loading control, the membranes were stripped and reprobed with antibody against p42/44 or p38 and α-tubulin.

Statistical analysis

Data were analyzed using GRAPHPAD PRISM software (GraphPad Software, San Diego, CA). The data were analyzed using a one-way analysis of variance (ANOVA) followed by Tukey's post-test for multiple comparisons and the Student's *t*-test for two groups. All data were considered statistically significant if P values were $< 0{\cdot}05$.

Results

CpG induced arginase activity in BMMϕ in the presence of IFN-γ

Some anti-inflammatory properties have been recently described for CpG-DNA.[2–9] Thus, we wondered if CpG would induce arginase activity. First, we cultured BMMϕ with medium alone, or with medium containing CpG, IFN-γ or CpG plus IFN-γ, for 48 hr. Although CpG alone did not induce a statistically significant increase in arginase activity, CpG plus IFN-γ consistently increased arginase activity (Fig. 1a). In Fig. 1b, arginase activity fold induction under stimulation with CpG plus IFN-γ was compared graphically with the arginase activity fold induction in BMMϕ stimulated with IL-4 (the most powerful known stimulus for arginase). It is important to note that IFN-γ alone did not activate this enzyme (Fig. 1a,b). Conversely to CpG plus IFN-γ neither GpC alone nor GpC plus IFN-γ increased arginase activity (Fig. 1c).

Internalization of CpG-DNA into an acidified endosomal compartment is a prerequisite for ligand binding to Toll-like receptor 9 (TLR9) and signaling transduction.[20] Chloroquine, which interferes with endosomal acidification, markedly inhibited CpG-mediated arginase activity in a dose-dependent manner, but the increase in arginase activity mediated by LPS was unaffected, thus demonstrating the specificity of this inhibition (Fig. 1d).

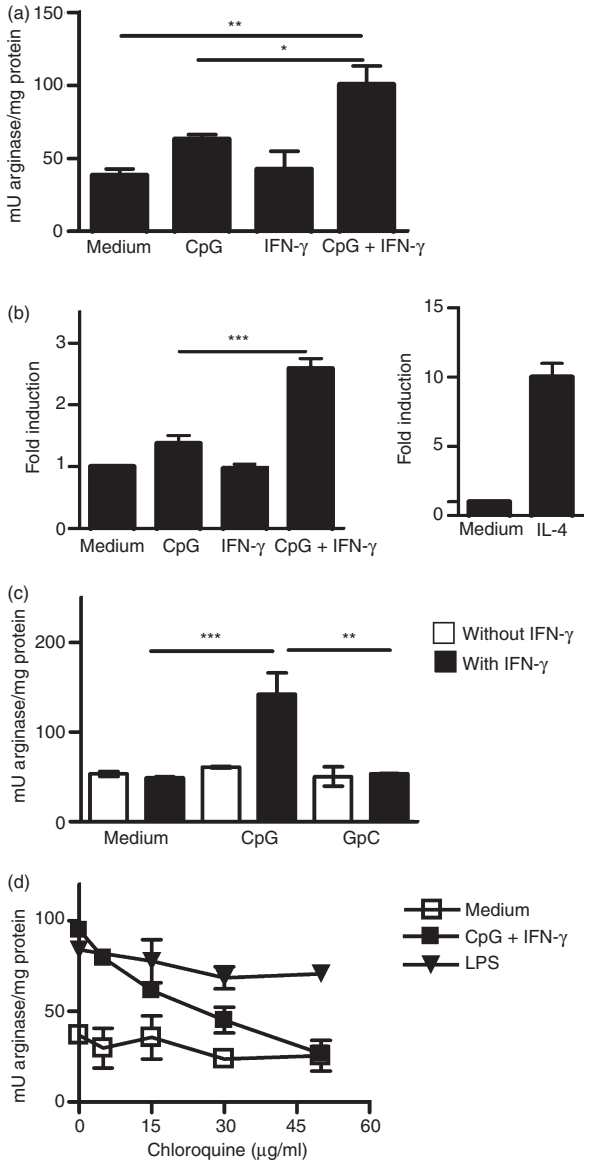

Figure 1. Synthetic oligodeoxynucleotides containing unmethylated cytosine guanine motifs (CpG) induced arginase activity in bone marrow-derived macrophages (BMMϕ) in the presence of interferon-γ (IFN-γ). (a) BMMϕ were cultured with medium, 0·3 μM CpG, 50 IU/ml of IFN-γ or 0·3 μM CpG plus 50 IU/ml of IFN-γ for 48 hr, after which cell lysates were prepared for arginase activity analysis. Arginase activity was assessed by determining the concentration of urea produced after incubation of lysates with L-arginine for 1 hr, as described in the *Materials and methods*. Results are expressed as mU of enzyme activity per mg of protein lysate, or (b) as fold induction from BMMϕ cultured with medium alone. (c) BMMϕ were cultured with medium, 50 IU/ml of IFN-γ, 0·3 μM CpG, CpG plus 50 IU/ml of IFN-γ, 0·3 μM non CpG (GpC), or GpC plus 50 IU/ml of IFN-γ. (d) BMMϕ were pre-incubated for 1 hr in the presence or absence of the indicated concentrations of chloroquine and were then stimulated as in (c). The results shown in (a) and (b) are mean ± standard error of 35 independent experiments. The results shown in (c) and (d) represent the mean ± standard deviation and are representative of three independent experiments. $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$. IL-4, interleukin-4.

Taken together, these observations suggest that CpG plus IFN-γ increased arginase activity by a mechanism specific to CG sequences and dependent on endosome maturation/acidification, consistent with the classical activation of TLR9.

IFN-γ requirement for arginase induction differs between CpG and LPS stimulation

Considering that LPS can induce arginase,[17,21] similar experiments were performed in the presence of polymyxin B, a known LPS inhibitor, in order to exclude any putative LPS contamination. The results presented in Fig. 2(a) show that polymyxin B did not interfere with the arginase activity of BMMϕ stimulated with CpG plus IFN-γ, but completely abolished, in a dose-dependent manner, the arginase activity stimulated with LPS.

On the other hand, it has been reported that whereas IFN-γ plus LPS produce additive effects in the induction of iNOS, IFN-γ abolishes the capacity of LPS to induce arginase activity. To verify this difference in the IFN-γ requirement for LPS or CpG arginase induction, BMMϕ were cultured with medium, IFN-γ, CpG or CpG plus IFN-γ, and LPS or LPS plus IFN-γ for 48 hr. In accordance with a previous report, IFN-γ abolished LPS-mediated arginase activity, whereas arginase activity was induced by CpG only in the presence of IFN-γ (Fig. 2b,c).

CpG plus IFN-γ co-induced arginase and iNOS activities

Whereas the activity of iNOS makes it well suited as a cellular defense effector against invading microorganisms, persistent activation can lead to toxic levels of NO production.[22,23] Several reports have mentioned that arginase acts as an iNOS regulator.[12–14] To examine the relationship between these two enzymes under CpG plus IFN-γ stimulation we conducted a time course assay. At each time-point, BMMϕ were harvested to determine arginase activity in lysates and NO production in the supernatants as an iNOS activity indicator. As illustrated in Fig. 3, and in agreement with a previous report,[24] CpG alone was unable to stimulate NO production, whereas a substantial increase of NO secretion occurred in the presence of IFN-γ. However, there were some evident differences regarding the activation of arginase and iNOS. NO was detected at 18 hr, increased with time and reached a maximum at 48 hr, after which NO accumulation remained stable for 48 hr longer. On the other hand, arginase activity began to increase more slowly (between 18 and 24 hr) and increased almost linearly over 96 hr, reaching a level about fourfold higher than the basal level. Taking into account these results, the decrease in NO formation occurring between 48 and 96 hr of incubation might be attributed to the continually increasing activity of arginase.

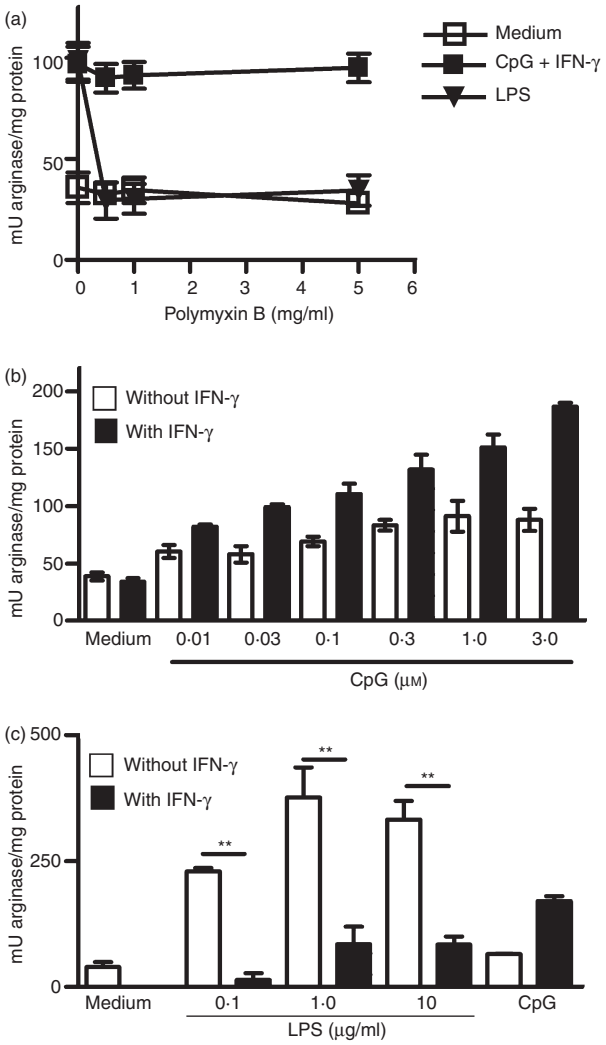

Figure 2. Interferon-γ (IFN-γ) requirement for arginase induction differs between synthetic oligodeoxynucleotides containing unmethylated cytosine guanine motifs (CpG) and lipopolysaccharide (LPS) stimulation. (a) Bone marrow-derived macrophages (BMMϕ) were pre-incubated for 1 hr in the presence or absence of the indicated concentrations of polymyxin B, and then stimulated for 48 hr with medium, 0·3 μM CpG plus 50 IU/ml of IFN-γ, or 1 μg/ml of LPS. (b) BMMϕ were stimulated with the indicated concentrations of CpG in the presence or absence of 50 IU/ml of IFN-γ for 48 hr, after which cell lysates were prepared for arginase activity analysis. (c) BMMϕ were stimulated with the indicated concentrations of LPS in the presence or absence of 50 IU/ml of IFN-γ or with 0·3 μM CpG plus 50 IU/ml of IFN-γ for 48 hr. Data in (a)–(c) represent the mean ± standard deviation and are representative of three independent experiments. $^{**}P < 0{\cdot}01$.

CpG plus IFN-γ induced up-regulation of arginase type II protein

In mammals, two distinct arginase isoforms are expressed in Mϕ: the cytosolic arginase I and the mitochondrial arginase II, which are identical to the liver-type and kidney-type arginase, respectively. We examined arginase

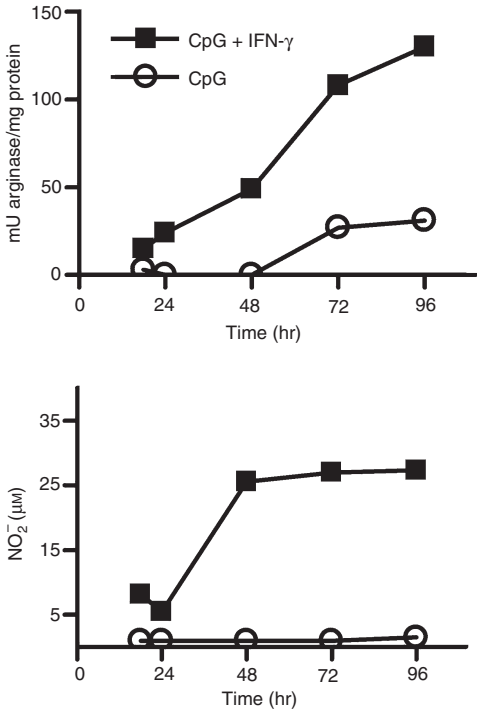

Figure 3. Synthetic oligodeoxynucleotides containing unmethylated cytosine guanine motifs (CpG) plus interferon-γ (IFN-γ) co-induced arginase and inducible nitric oxide synthase (iNOS) activities. Bone marrow-derived macrophages (BMMϕ) were incubated with medium, 0·3 μM CpG, or 0·3 μM CpG plus 50 IU/ml of IFN-γ for the indicated period of time. For each well, arginase activity was measured in lysates and nitric oxide (NO) concentration was measured in supernatants. Data represent the mean (stimulated minus unstimulated BMMϕ control) and are representative of three independent experiments.

I and II protein levels in BMMϕ by western blot. CpG plus IFN-γ increased the expression of arginase II but not of arginase I. In addition, the expression of arginase II protein was revealed in unstimulated BMMϕ, consistent with the basal arginase activity observed in these cells (Fig. 4a). A densitometry assay demonstrated that the increased expression of arginase II was consistent with the observed magnitude of arginase activity induction (Fig. 4b). Therefore, the CpG plus IFN-γ-mediated arginase activity might be primarily caused by an increase in arginase II expression.

CpG plus IFN-γ-mediated arginase activity was dependent on IFN-γ priming but independent of the autocrine IL-10 effect

IFN-γ coördinates diverse cellular programmes through transcriptional regulation and integration of other signalling pathways.[25] We found that CpG increased arginase activity in the presence of IFN-γ, whereas other reports have described the inhibitory effects of IFN-γ on arginase activity.[15–17] Therefore, to rule out the possibility that

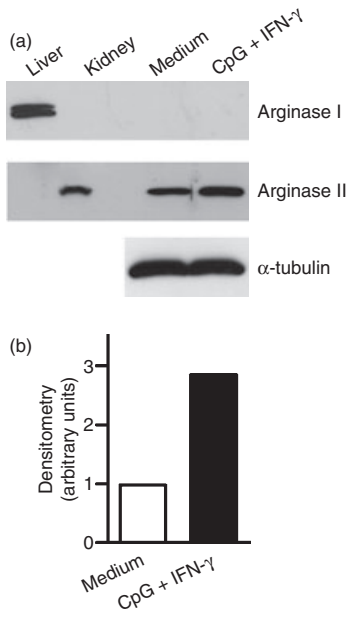

Figure 4. Synthetic oligodeoxynucleotides containing unmethylated cytosine guanine motifs (CpG) plus interferon-γ (IFN-γ) induced the up-regulation of arginase type II protein. (a) Bone marrow-derived macrophages (BMMϕ) were stimulated or not with 0·3 μM CpG plus 50 IU/ml of IFN-γ for 48 hr. Equal amounts of whole-cell lysates (100 μg/lane) were subjected to electrophoresis on a 12% sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE). Western blots were performed using specific antibodies against arginase type I and arginase type II. To normalize the loaded samples, antibody against α-tubulin was used as a control that is not affected by the treatment. Mouse liver (50 μg/lane) and kidney (100 μg/lane) lysates were used as positive controls for arginase I and arginase II, respectively. (b) Densitometric evaluation is expressed as arbitrary units. Data in (a) and (b) are representative experiments of two performed.

IFN-γ inhibition had actually occurred at higher concentrations, we performed an IFN-γ dose–response curve. As depicted in Fig. 5(a), no inhibition of arginase activity was observed in spite of increasing concentrations of IFN-γ, confirming that arginase activity was induced by CpG only in the presence of IFN-γ.

Previous findings reported that IFN-γ primes Mϕ to CpG-DNA for some functions.[24] Thus, we carried out a priming assay to evaluate the role of IFN-γ in arginase activation. BMMϕ were prestimulated with medium, CpG or IFN-γ alone for 1 hr. Then, BMMϕ were carefully washed and stimulated with the indicated stimulus for 48 hr (Fig. 5b). A statistically significant increase in arginase activity was only observed when BMMϕ were pre-incubated with IFN-γ and then stimulated with CpG. In contrast, BMMϕ pre-incubated with CpG alone and stimulated with IFN-γ did not have any positive effect on arginase activity. These observations demonstrated that CpG plus IFN-γ-mediated arginase activity is dependent on IFN-γ priming. This priming phenomenon might not be a general requisite to mediate the change from resting BMMϕ to pre-activated BMMϕ, which are highly receptive to subsequent CpG stimulation, because the data presented in Fig. 5(c) show that BMMϕ were effectively activated by CpG alone and secreted a significant amount of IL-12 and IL-10. Bearing this in mind, we attempted to define the role of IFN-γ priming in this arginase activation. For that, BMMϕ were cultured in the presence of the protein synthesis inhibitor CHX. As illustrated in Fig. 5(d), CHX blocked the induction of arginase activity that occurred as a result of IFN-γ priming. These results suggest that a rapidly IFN-γ-stimulated and *de novo*-synthesized protein may trigger arginase induction in CpG-stimulated BMMϕ that could be consistent with an indirect stimulatory mechanism. Considering that earlier studies showed that IL-10 increases the arginase activity in Mϕ,[12,26] and that IL-10 secretion is stimulated in CpG-activated Mϕ (Fig. 5c and ref.[3]), we wondered whether IL-10 has a role in CpG plus IFN-γ-mediated arginase activity. Therefore, we determined the IL-10 concentration in the supernatant using ELISA. The results in Fig. 5(e) clearly show an inverse pattern regarding IL-10 production and arginase activity: CpG alone induced practically no arginase activity but stimulated IL-10 secretion, whereas CpG plus IFN-γ increased arginase activity but dramatically reduced IL-10 secretion. On the basis of these results, it seems unlikely that IL-10 participates, at least directly, in the CpG plus IFN-γ-mediated arginase activity. Therefore, the ability of CpG plus IFN-γ to induce arginase activity might occur by a mechanism independent of an autocrine IL-10 effect.

CpG plus IFN-γ-mediated arginase activity was dependent on p38 and ERK MAPK activation, but independent of JNK

A universal outcome of engaging all TLRs is activation of the MAPK signaling pathways.[27] To investigate whether arginase activity after stimulation with CpG plus IFN-γ occurred through the classical MAPK activation pathways, we used pharmacological specific inhibitors in culture. Arginase activity was markedly suppressed by SB203580 and PD98059, p38 MAPK and extracellular signal-related protein kinase (ERK)-specific inhibitors, respectively, but not by SP600125, a c-Jun N-terminal kinase (JNK) inhibitor (Fig. 6a), suggesting that p38 and ERK, but not JNK, signaling pathways are involved in the induction of arginase activity by CpG plus IFN-γ.

To corroborate the result obtained from pharmacological inhibition, we analyzed the phosphorylation status of p38 and p44/p42, as an indication of activation, by western blot. Consistent with the pharmacological sensitivity observed, p38 and p42/p44 were substantially phosphorylated when stimulated with CpG plus IFN-γ (Fig. 6b). The kinetics of induction of p44/p42 phosphorylation was

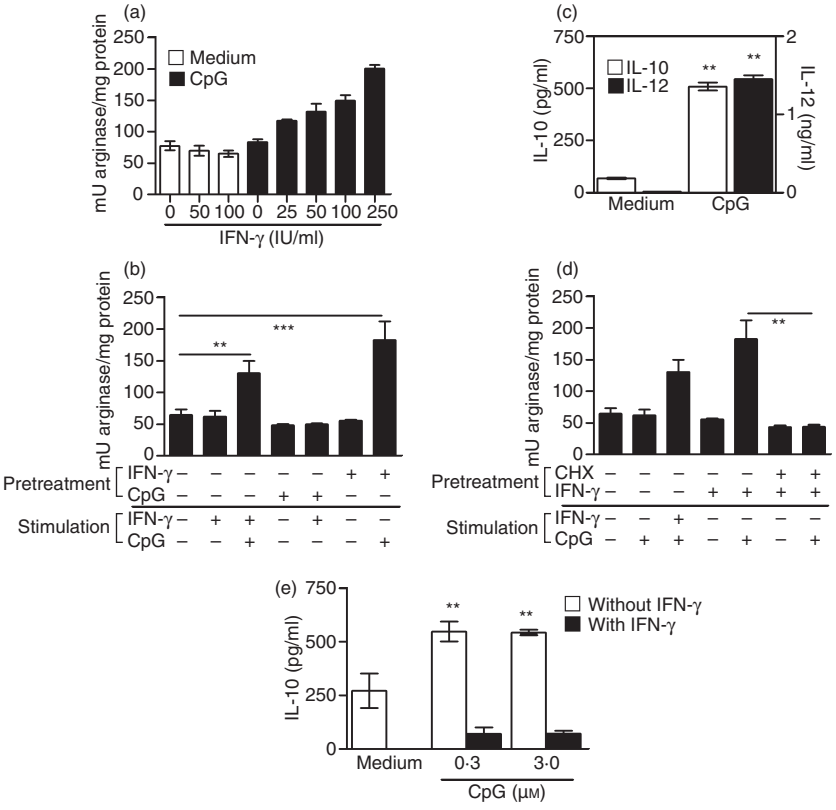

Figure 5. Synthetic oligodeoxynucleotides containing unmethylated cytosine guanine motifs (CpG) plus interferon-γ (IFN-γ)-mediated arginase activity was dependent on IFN-γ priming but independent of the autocrine interleukin (IL)-10 effect. (a) Bone marrow-derived macrophages (BMMϕ) were incubated for 48 hr with medium or with 0·3 μM CpG plus the indicated concentrations of IFN-γ, after which cell lysates were prepared for arginase activity analysis. (b) BMMϕ were pre-incubated with medium, 0·3 μM CpG, or 50 IU/ml of IFN-γ for 1 hr, washed three times and cultured for a further 48 hr with the indicated stimulus. (c) BMMϕ were cultured with medium alone or with 0·3 μM CpG for 48 hr. IL-10 and IL-12 production were measured in supernatants by enzyme-linked immunosorbent assay (ELISA). (d) Where indicated, BMMϕ were pre-exposed to 10 μg/ml of cycloheximide (CHX) for 30 min. Then, BMMϕ were prestimulated in the presence or absence of IFN-γ for 1 hr, washed and stimulated with IFN-γ or CpG for a further 48 hr. (e) IL-10 was measured in supernatants of BMMϕ incubated with medium, 3·0 μM CpG, or CpG plus 50 IU/ml of IFN-γ for 48 hr. Results shown are the mean ± standard deviation and are representative of three independent experiments. $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$.

rapid, but transient, while p38 phosphorylation was more sustained.

The capacity of the mentioned drugs to inhibit p38, ERK (p42/44) and JNK (p46/54) phosphorylation was verified by western blot analysis, as shown in Fig. 6(c).

Discussion

During the last decade, there has been increasing recognition of the immunostimulatory properties of CpG-DNA and its safety profile is an attractive feature for its use in therapeutic strategies. Initially, CpG-DNA were believed to be predominantly pro-inflammatory molecules; however, more recently, some anti-inflammatory properties have been described.[2–9] The overwhelming body of evidence which indicates that arginase plays a key role as an iNOS regulator prompted us to investigate the relationship between these two enzymes in CpG-activated BMMϕ. Our data revealed, for the first time, that CpG is able to induce arginase activation and that this occurred in the presence of IFN-γ, considered to be an inhibitor of the arginase activation by cytokines or LPS.[15–17] Therefore, IFN-γ differentially affects the responses to LPS and CpG. In the presence of IFN-γ, Mϕ have been reported to respond to LPS by secreting great quantities of NO but arginase production was practically abolished (ref.[17] and Fig. 2c), whereas we demonstrated here that BMMϕ in the presence of IFN-γ responded to CpG by activating simultaneously iNOS and arginase. Although the effects of LPS and CpG-DNA on Mϕ are similar (both induce the production of NO and the pro-inflammatory cytokines IL-1, IL-6, IL-12 and TNF-α),[10,11] their toxicities differ greatly.[1] In consequence, our observation of the effect of CpG plus IFN-γ on arginase activity demonstrates another difference between LPS and CpG pathways that could help to explain the lower toxicity found for CpG.

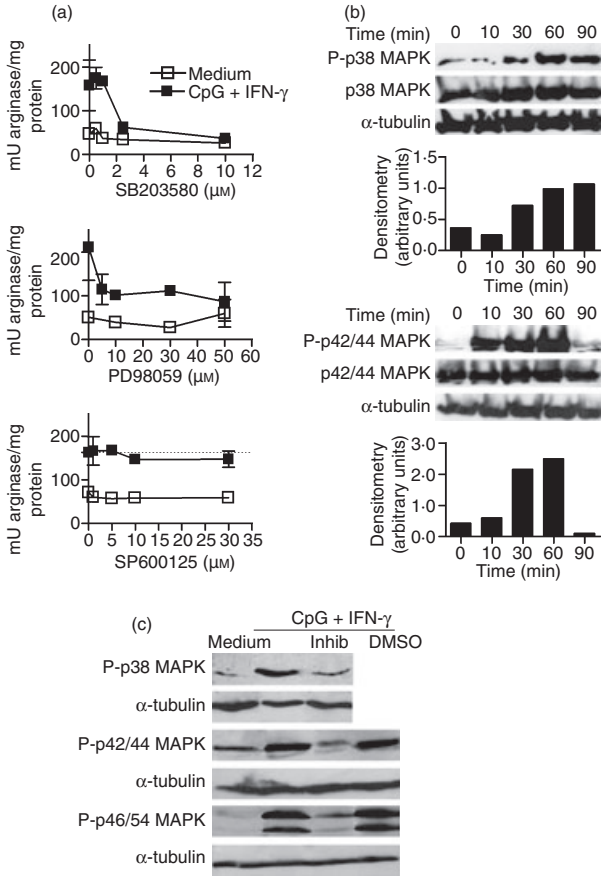

Figure 6. Synthetic oligodeoxynucleotides containing unmethylated cytosine guanine motifs (CpG) plus interferon-γ (IFN-γ)-mediated arginase activity was dependent on p38 and extracellular signal-regulated protein kinase (ERK) mitogen-activated protein kinase (MAPK) activation but independent of c-Jun N-terminal kinase (JNK). (a) Bone marrow-derived macrophages (BMMϕ) were pre-incubated with the indicated concentration of the MAPK specific inhibitors SB203580, PD98059 and SP600125 for 1 hr. Then, the indicated stimulus was added to the medium and BMMϕ were cultured for a further 48 hr before cell lysates were prepared for arginase activity analysis. Results represent the mean ± standard deviation of a representative experiment of three, with similar results obtained on each occasion. (b) BMMϕ (2×10^6 cells) were deprived of the macrophage colony-stimulating factor (M-CSF) and cultured with medium containing 2% fetal bovine serum (FBS) for 24 hr. Then, BMMϕ were stimulated with 0·3 μM CpG plus 50 IU/ml of IFN-γ for the indicated period of time. Cells were harvested, lysed and subjected to electrophoresis in a 10% sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE). Western blots were performed using antibodies against phospho-p38 (P-p38) or phospho-p42/44 (P- p42/44). Blots were stripped and reprobed for p38 or p42/44 and α-tubulin as loading control. The figure shows a representative experiment of three, with similar results obtained on each occasion. (c) BMMϕ were treated as in (b) but first were pre-incubated for 1 hr with the inhibitor for the indicated MAPK: SB203580 (10 μM), PD98059 (25 μM), SP600125 (20 μM) or dimethylsulphoxide (DMSO). Then, cells were stimulated with CpG plus IFN-γ for a further 1 hr. Western blots were performed using antibodies against phospho-p38 (P-p38), phospho-p42/44 (P-p42/44) or phospho-p46/54 (P-p46/54). Blots were reprobed for α-tubulin as a loading control. The figure shows a representative experiment of two, with similar results obtained on each occasion. Inhib, inhibitor.

In the present study we demonstrated that while CpG plus IFN-γ-mediated arginase activity increased almost linearly over 96 hr, the accumulation of NO in supernatants increased for only 48 hr. It has previously been demonstrated that iNOS activity is mainly dependent on the extracellular L-arginine supply[28,29] and that arginase regulates the iNOS activity by siphoning off the substrate. Our results suggest that the decrease in NO formation as a result of stimulation with CpG plus IFN-γ between 48 and 96 hr could be related to the continually increasing arginase activity. This possibility is also supported by the fact that this regulatory mechanism has been described as particularly important when the exogenous L-arginine concentration is low. For example, L-arginine is dramatically reduced in plasma during inflammatory events, in wound fluids and at other inflammatory sites.[14,30,31] Furthermore, the chronology of these events with a delayed onset of arginase induction may be of great importance. The production of NO may first have positive effects on infectious diseases by favoring microbiostasis-microbial killing and vasodilatation. Following this, arginase may avoid exacerbated NO secretion, and promote wound healing and tissue remodeling.

In the present work, we found that CpG plus IFN-γ-mediated arginase activity correlated with an increase in arginase II protein expression. Arginase II has been found to be involved in all the same functions as arginase I. These include the biosynthesis of polyamines and proline for the production of extracellular matrix, the participation as an anti-apoptotic effector and the modulation of NO synthesis in various organs.[32] Thus, it remains to be determined why CpG plus IFN-γ induced exclusively the arginase II isoform instead of arginase I or both together.

Unexpectedly, we observed that CpG-mediated arginase and iNOS co-induction only occurred in the presence of IFN-γ. This result might seem contradictory to other authors who have described an inhibitory effect of IFN-γ on arginase activity.[15–17] Despite this discrepancy, our finding is supported by a previous report which demonstrated that IFN-γ also participates in arginase induction in primary astrocytes.[33] In addition, in RAW 264·7 Mϕ, IFN-γ completely abolished cAMP-dependent induction of arginase I mRNA, but only a slight reduction was observed in cAMP induction of arginase II mRNA.[34] Interestingly, given the established inhibitory role played by IFN-γ in arginase activity, there are some inexplicable observations in *in vivo* models. For instance, although IFN-γ mRNA was markedly increased in the IL-10-deficient mice injected with schistosome egg, arginase I was induced to the same extent as that in wild-type animals.[16]

Meanwhile, in the *Citrobacter rodentium* model of colitis, associated with a strong mucosal Th1 response, both iNOS and arginase I were up-regulated in the colon of infected mice.[35] Although the precise reasons for differences in the role of IFN-γ in arginase regulation are not still clear, they may reflect several variants such as cell type, cell maturation-associated differentiation and the exogenous stimuli used.

It is very probable that IFN-γ elicited its effect very early in the pathway, because a short pretreatment of 1 hr was sufficient to produce an increase in arginase activity similar to that obtained when IFN-γ was added together with CpG. The efficacy of this brief stimulation with IFN-γ is consistent with the demonstration that the first wave of IFN-γ-induced transcription occurs within 15–30 min of treatment.[36] In addition, we observed that CHX blocked the induction of arginase activity observed under IFN-γ priming, which would be consistent with an indirect stimulatory mechanism. Thus, we investigated the role of IL-10 in CpG plus IFN-γ-mediated arginase activity. Considering the strong inhibition observed of IL-10 secretion in the presence of CpG plus IFN-γ, it seems unlikely that an autocrine IL-10 effect promotes the CpG plus IFN-γ-mediated arginase activity.

Furthermore, our results are in agreement with previous studies demonstrating that certain responses to CpG-DNA, such as arginine uptake and increased iNOS gene expression, occurred only after IFN-γ priming.[24] Traditionally, priming with IFN-γ was thought to mediate a change from the resting Mϕ to a pre-activated Mϕ that is highly receptive to a second activating signal. However, it is now known that Mϕ integrate multifarious signals to affect an appropriate cellular response in a more complex way.[37] In agreement with this, we observed that BMMϕ were effectively activated by CpG in the absence of IFN-γ and secreted significant amounts of IL-12 and IL-10. Thus, IFN-γ might not only be a prerequisite for BMMϕ activation but also might activate molecules or transcription factors shared with TLR9, thereby generating the cross-talk between these pathways to favour arginase activation.

It is well known that recognition of CpG-DNA by TLR9 initiates a signaling cascade that begins with the recruitment of the adaptor protein, MyD88, with the activation of MAPKs being one of the early biochemical signaling events.[27] The results presented here reveal that p38 MAPK and ERK, but not JNK, signaling pathways are involved in the induction of arginase activity by CpG plus IFN-γ. Interestingly, p38 and ERK have been involved in other CpG anti-inflammatory functions in Mϕ. A central negative-feedback role was described for ERK in the CpG-DNA-mediated Th1-type response by promoting IL-10 production, whereas p38 has been reported to be essential for the induction of both IL-10 and IL-12.[38] In addition, the induced expression of SOCS1 and SOCS3 by CpG-DNA were blocked partially when ERK and p38 were inhibited.[9] Also, p38 and ERK were involved in the activation of signaling pathways different from the canonical Jak-STAT1 for IFN-γ. This implies an association between the IFN-γ receptor and MyD88 that results in p38 activation[39] and the activation of ERK.[40,41] Our observations lend additional support to the hypothesis of IFN-γ signaling integration with pathogen-associated molecular patterns.

Collectively, the effect of CpG plus IFN-γ on arginase activity reveals an unsuspected complexity in the response of BMMϕ to the combination of CpG and IFN-γ, one of the principal and most relevant cytokines secreted *in vivo* in response to CpG-DNA. Furthermore, these data add support to the growing body of evidence that favour a dual role for IFN-γ activity.[42]

Finally, comparisons of different adjuvants in mouse models have demonstrated that CpG-DNA is unsurpassed at inducing Th1-type responses. What is more, CpG-DNA can overcome the Th2 bias associated with some disease states or in both very young and elderly mice.[43–46] However, administration of CpG-DNA also generates a response in order to avoid an exacerbated inflammation.[46,47] Therefore, the possibility that CpG-DNA could simultaneously or sequentially elicit both a pro-inflammatory and a counter anti-inflammatory response, similar to our *in vitro* data, is plausible and very interesting in terms of immune regulation during infection and in the improvement of vaccines.

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