Cell type-specific activation of mitogen-activated protein kinases by CpG-DNA controls interleukin-12 release from antigen-presenting cells

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Activation of antigen-presenting cells (APCs) by invariant constituents of pathogens such as lipopolysaccharide (LPS) or bacterial DNA (CpG-DNA) initiates immune responses. We have analyzed the mitogen-activated protein kinase (MAPK) pathways triggered by CpG-DNA and their significance for cytokine production in two subsets of APCs, i.e. macrophages and dendritic cells (DCs). We found that CpG-DNA induced extracellular signal-regulated kinase (ERK) activity in macrophages in a classic MEK-dependent way. This pathway up-regulated tumor necrosis factor production but down-regulated interleukin (IL)-12 production. However, in DCs, which produce large amounts of IL-12, CpG-DNA and LPS failed to induce ERK activity. Consistent with a specific negative regulatory role for ERK in macrophages, chemical activation of this pathway in DCs suppressed CpG-DNA-induced IL-12 production. Overall, these results imply that differential activation of MAP kinase pathways is a basic mechanism by which distinct subsets of innate immune cells regulate their effector functions.

Keywords: CpG-DNA/IL-12/innate immunity/MAP kinase/TNF

Introduction

Antigen-presenting cells (APCs), such as macrophages and dendritic cells (DCs), carry out at least two important tasks during immune responses towards pathogens: first, they represent a primary barrier against pathogens through their antimicrobial activity, and secondly, they provide the basis for development of adaptive immune responses. In contrast to cells of the adaptive immune system, innate immune cells do not require clonal expansion and receptor rearrangement of antigen-specific cells but recognize pathogens through structurally conserved, invariant microbial constituents, so-called pattern recognition factors (PRFs). Recognition of these factors by APCs activates the cells to exert various effector functions. In macrophages, direct antimicrobial functions seem to dominate

their anti-infectious repertoire (reviewed by Fearon, 1997). These functions include the production of cytotoxic substances as well as the secretion of large amounts of inflammatory cytokines such as tumor necrosis factor (TNF), interleukin (IL)-6 and, to a lesser extent, IL-12.

The main task of DCs appears to be the control of the adaptive immune system through appropriate antigen presentation. Immature DCs process protein antigens from pathogens and present peptides that serve as a stimulus for specific T-cell receptors (for a review, see Cella *et al.*, 1997). Either direct recognition of PRFs or T-helper cell-mediated CD40 ligation activates APCs to transit to professional APCs via up-regulation of major histocompatibility complex (MHC) class II and co-stimulatory molecules and secretion of large amounts of IL-12 and TNF.

Bacterial cell wall components, e.g. lipopolysaccharides (LPS) and peptidoglycans (Hoffmann *et al.*, 1999) are well characterized PRFs. Over the last few years, bacterial DNA has also been recognized as a powerful stimulus of innate immune cells *in vivo* and *in vitro* (for a review, see Wagner, 1999). Its immune-stimulatory potential is based on the presence of unmethylated CpG-‘motifs’, which often contain the nucleotide sequence purine-purine-CG-pyrimidine-pyrimidine (Krieg *et al.*, 1995). These sequences are present in bacterial DNA at almost the statistically expected frequency but are evolutionarily suppressed (‘CG suppression’) and methylated in vertebrate DNA (Sved and Bird, 1990). Recognition of this invariant structural feature by the vertebrates’ innate immune cells qualifies bacterial CpG-DNA as a PRF. The stimulatory principle contained in double-stranded DNA can be transferred to single-stranded oligonucleotides (ODNs) that contain the described CpG motif (CpG ODN) (Yamamoto *et al.*, 1992). Commonly, DNA with immune-stimulatory capacity based on CpG-‘motifs’ is referred to as CpG-DNA.

In macrophages and bone marrow-derived dendritic cells (BMDDCs), CpG-DNA has been shown to induce release of the cytokines TNF, IL-6 and IL-12 (Stacey *et al.*, 1996; Sparwasser *et al.*, 1997a, 1998). While in macrophages the pro-inflammatory cytokines TNF and IL-6 quantitatively dominate IL-12, DCs secrete large amounts of IL-12 (Häcker *et al.*, 1998; Sparwasser *et al.*, 1998). Additionally, CpG-DNA induces maturation of DCs, characterized by up-regulation of MHC class II and co-stimulatory molecules such as CD80 and CD86, which results in highly efficient antigen presentation (Sparwasser *et al.*, 1998). Induction of this DC maturation accompanied by the production of large amounts of IL-12 might explain the exceptional potency of CpG-DNA as an adjuvant (Lipford *et al.*, 1997a).

We have investigated signal transduction pathways activated by CpG-DNA. Recently, we and others found

that CpG-DNA signaling involves activation of NF- κ B and induction of the stress kinases c-Jun N-terminal kinase (JNK) 1/2 and p38 in macrophages, DCs and B cells (Stacey *et al.*, 1996; Häcker *et al.*, 1998; Yi and Krieg, 1998; for a review, see Häcker, 1999).

In this study, we focused on MAPK pathways induced by CpG-DNA and LPS in APCs displaying a distinct differentiation status, i.e. macrophages and DCs, with the emphasis on the role of the ERK pathway for release of the cytokines IL-12 and TNF.

Results

CpG-DNA induces ERK activity in primary macrophages and the macrophage cell line RAW264.7

Previously, we showed that CpG-DNA activates the stress kinases JNK and p38 in macrophage cell lines and primary BMDDCs (Häcker *et al.*, 1998). To decipher further the signaling events involved in APC activation, we investigated the role of the classic MAPKs, the ERKs, in cells stimulated with CpG-DNA. Although this is a pathway that has been characterized mainly during growth factor-dependent signaling, it also has been shown to be used by other PRFs such as LPS and peptidoglycans (Dziarski *et al.*, 1996; Sanghera *et al.*, 1996). Initially, we stimulated the macrophage cell line RAW264.7 with CpG ODN or a control oligonucleotide where the CpG has been inverted to GpC (GpC ODN). This control ODN allows differentiation between specific, CpG-dependent events and non-specific effects of the ODN backbone (Sparwasser *et al.*, 1997b). As shown in Figure 1A, stimulation of the cells with CpG ODN led to a rapid induction of ERK activity that peaked after 30 min to a >5-fold induction over background and declined slowly afterwards. In contrast, the control GpC ODN did not induce significant ERK activity, confirming that ERK activation depended on the bacterial 'CpG-motif'.

To ensure that the observation made in a cell line was representative of primary macrophages, we next examined ERK activation in peritoneal washout cells (PWCs). PWCs were collected and cultured overnight. The cells were then stimulated with CpG ODN, GpC ODN or, as a positive control, with LPS, a known inducer of ERK activity in macrophages (Sanghera *et al.*, 1996). Again, strong activation of ERK was observed upon CpG ODN or LPS stimulation, while GpC ODN failed to induce ERK activity (Figure 1B). Activation of ERK as measured by this *in vitro* kinase assay (Figure 1A and B) correlated directly with ERK phosphorylation at their activation site, Thr202/Tyr204, as determined by Western blotting (data not shown).

The upstream activating kinases of ERK are the MAPK/ERK kinases (MEK) 1/2, which are activated by phosphorylation at Ser217/221 (Alessi *et al.*, 1994). Using antibodies that recognize specifically the Ser217/221-phosphorylated form of MEK1/2, we examined activation of MEK1/2 by CpG ODN. As shown in Figure 1C, clear induction of phosphorylation and thus activation of MEK1/2 could be demonstrated in CpG ODN-activated RAW264.7 cells. LPS was again used as a positive control. To show that the ERK activity induced by CpG ODN in macrophages is dependent on MEK1/2, we transfected

Fig. 1. CpG ODN induce ERK activity in RAW264.7 cells and primary macrophages in a MEK-dependent way. (A) A total of 2×10^6 RAW264.7 cells were stimulated in complete culture medium for the indicated times with CpG ODN or GpC ODN (1 μ M). Then, cells were lysed and kinase activity was determined by immune complex kinase assay with antibodies to ERK-2 and myelin basic protein (MBP) as substrate. The radioactivity incorporated into MBP was quantitated after SDS-PAGE by PhosphorImager analysis. The values obtained were normalized against the activity in non-stimulated cells and are presented as fold induction. (B) PWCs were prepared as described in Materials and methods. Stimulation was performed in complete culture medium. Cells were stimulated with CpG ODN or GpC ODN (1 μ M) or LPS (10 ng/ml) for the time indicated. After stimulation, cells were lysed and kinase activity was determined by immune complex kinase assay with antibodies to ERK-2 and MBP as substrate. Quantification was performed as described in (A). (C) RAW264.7 cells were stimulated with 1 μ M CpG ODN or 10 ng/ml LPS for the times indicated. Cell lysates were prepared and analyzed by Western blotting with antibodies against total MEK1/2 and against the Ser217/221 phosphorylated forms of MEK1/2. (D) RAW264.7 cells were transiently transfected with an expression vector for HA-tagged ERK-2 or HA-tagged JNK-1, together with either a control vector or an expression vector for MEKdn as indicated. At 24 h post-transfection, cells were left untreated or were stimulated with 1 μ M CpG ODN for 20 min. After stimulation, cells were lysed and kinases were immunoprecipitated with antibodies to the HA tag. *In vitro* kinase assays were performed with MBP or GST-Jun(79) as substrate.

RAW264.7 cells with a dominant-negative form of MEK (MEKdn) and activated these cells with CpG ODN. As shown in Figure 1D, MEKdn inhibited activation of ERK. This inhibition was specific as activation of JNK was not reduced in the presence of MEKdn (Figure 1D). Taken

together, these results demonstrate that CpG-DNA induces ERK activity via a classic MEK-dependent pathway.

CpG-DNA-induced MEK/ERK activity regulates expression of TNF and IL-12

As a next step, we addressed the question of the biological significance of this ERK activation. Recently, we and others have noted that CpG-DNA induces the production of large amounts of TNF but small amounts of IL-12 in macrophage cell lines and primary peritoneal macrophages (Sparwasser *et al.*, 1997b; Sweet *et al.*, 1998). Secretion of both cytokines upon CpG ODN stimulation requires p38 activity (Häcker *et al.*, 1998). While no selective inhibitor of ERK is available at present, the MEK inhibitor PD98059 can be used to inhibit MEK and subsequent MEK-dependent ERK activation (Dudley *et al.*, 1995). Hence we examined secretion of TNF and IL-12 upon CpG ODN stimulation in RAW264.7 cells in the presence of PD98059. As shown in Figure 2A, inhibition of ERK activation resulted in a significant suppression of TNF release. However, unexpectedly, inhibition of the ERK pathway resulted in a dose-dependent increase of IL-12 secretion, up to >10-fold (Figure 2A). Comparable results, i.e. strong up-regulation of IL-12 in the presence of PD98059, were obtained with PWCs (data not shown). Recently, another potent MEK inhibitor, UO126, has become available. In contrast to PD98059, which primarily seems to inhibit MEK1/2 activation by blockade of the access of activating enzymes, UO126 is able to inhibit the activated, phosphorylated form of MEK1/2 (Favata *et al.*, 1998). Qualitatively, the same results were obtained with UO126 as with PD98059, although its effects on cytokine production were more pronounced with respect to both TNF suppression and up-regulation of IL-12 (Figure 2B).

Significance of the CpG-DNA-induced ERK activity for regulation of TNF and IL-12 p40 mRNA levels and promoter activity

IL-12 in its active form, IL-12 p70, consists of a heterodimer of the proteins p40 and p35 (Gubler *et al.*, 1991; Wolf *et al.*, 1991). While IL-12 p35 is expressed more ubiquitously, IL-12 p40 expression appears to be restricted to cell types that express biologically active IL-12 and is strongly inducible by LPS and CD40 ligation in certain cell types (D'Andrea *et al.*, 1992; Shu *et al.*, 1995). The data available suggest that IL-12-inducing stimuli act via enhancement of IL-12 p40 promoter activity (Murphy *et al.*, 1995; Ma *et al.*, 1996; Plevy *et al.*, 1997). TNF production is regulated at both the transcriptional and post-transcriptional level (for a review, see Beutler and Kruys, 1995). To define further the level of cytokine regulation by CpG-DNA-induced MEK/ERK activity, we measured mRNA levels by RNase protection assays and promoter activity using RAW264.7 cells, stably transfected with a luciferase gene under the control of either the TNF or the IL-12 p40 promoter. RAW264.7 cells were stimulated with CpG ODN in either the absence or presence of the MEK inhibitors PD98059 or UO126, and RNA was prepared and subjected to RNase protection assays using TNF or IL-12 p40 sequences as probes. As shown in Figure 2C, CpG ODN induces rapid up-regulation of TNF mRNA. Both PD98059 and UO126 led

to a slight decrease in constitutive mRNA levels, but no substantial effect on CpG ODN-induced up-regulation of TNF mRNA was observed (Figure 2C). These results correlate well with experiments using the RAW264.7 TNF promoter transfectants: even at the highest concentration of PD98059 (50 μ M, as used in the RNase protection assay), no significant effect on CpG ODN-induced TNF promoter activity was observed (Figure 2D). We conclude from these experiments that CpG-DNA-induced ERK activity up-regulates TNF primarily at a post-transcriptional level. This result is in accordance with published observations on LPS-induced TNF secretion, where ERK activation acted primarily at a post-transcriptional level (Bhat *et al.*, 1998).

In contrast to the regulation of TNF, CpG ODN-induced up-regulation of both IL-12 p40 mRNA and IL-12 p40 promoter activity is controlled by ERK signaling: inhibition of MEK by PD98059 or UO126 led to a strong increase in CpG ODN-induced IL-12 p40 mRNA levels (Figure 2C). In direct correlation with this up-regulation, PD98059 strongly enhanced the CpG ODN-induced activity of the IL-12 p40 promoter in stably transfected RAW264.7 cells (Figure 2D).

To control further for the possibility that the observed effects of the MEK inhibitors were due to the inhibition of other kinases, we also examined the effect of dominant-negative MEK-1 and of a constitutively active Raf mutant on the IL-12 p40 promoter. RAW264.7 cells were transiently transfected with the IL-12-p40 promoter-luciferase construct and either dominant-negative MEK-1 or Raf-BXB, the constitutively active kinase domain of RAF. RAF BxB on its own can activate MEK1/2 and subsequently ERK1/2 (Kyriakis *et al.*, 1992). The results, shown in Figure 2E and F, confirm the findings obtained with the MEK inhibitor: inhibition of MEK activity using the dominant-negative construct also increased the CpG ODN-induced IL-12 p40 promoter activity in a dose-dependent way (Figure 2E). Furthermore, activation of MEK by the upstream kinase Raf-1 decreased the capacity of CpG ODN to induce the IL-12 p40 promoter (Figure 2F).

Taken together, these data show that CpG ODN-driven activation of the ERK pathway triggers TNF secretion at a post-transcriptional level but negatively regulates IL-12 p40 production at the promoter level.

Priming of macrophages with interferon- γ does not affect CpG ODN-induced ERK activity

The data presented here clearly indicate that IL-12 production is negatively regulated by CpG-DNA-activated ERK. Earlier data, on the other hand, had demonstrated that CpG-DNA also activates NF- κ B and stress kinase pathways, i.e. signaling pathways that stimulate IL-12 production (Stacey *et al.*, 1996; Häcker *et al.*, 1998). This constellation suggests that IL-12 secretion depends on the balance of positive and negative stimuli. While in macrophages CpG ODN appear to activate both stimulatory and inhibitory pathways, there may exist situations, or cell types, where other external stimuli shift the balance and prime the cells towards selective IL-12 production. For example, in primary macrophages and macrophage cell lines, efficient induction of IL-12 p40 promoter activity and protein secretion by LPS is dependent on interferon- γ (IFN- γ) priming (Ma *et al.*, 1996). We therefore speculated that

Fig. 2. IL-12 and TNF expression is regulated by the MAPK/ERK pathway. (A) A total of 2×10^5 RAW264.7 cells were seeded in 0.5 ml of complete culture medium and stimulated with CpG ODN (1 μ M) for 8 h. Release of TNF (left) and IL-12 (right) was measured by ELISA. Where PD98059 was used, cells were pre-incubated for 30 min before stimulation with the concentrations of PD98059 indicated. (B) RAW264.7 cells were treated as described in (A). Instead of PD98059, the MEK inhibitor UO126 was used at the concentrations indicated. Release of TNF (left) and IL-12 (right) was measured by ELISA. (C) A total of 2×10^6 RAW264.7 cells were seeded in 5 ml of complete culture medium and incubated for 16 h. Then cells were stimulated in the presence or absence of the MEK inhibitors PD98059 (50 μ M) or UO126 (5 μ M) with CpG ODN (1 μ M) for 3, 6 or 9 h. After stimulation, total RNA was prepared and mRNA levels were analyzed by RNase protection analysis using TNF, IL-12 p40 and GAPDH as probes. Where PD98059 or UO126 was used, cells were pre-treated for 30 min before stimulation. Where PD98059 or UO126 was used without subsequent stimulation by CpG ODN, cells were incubated for 9.5 h in the presence of the inhibitor before RNA preparation. (D) RAW264.7 cells with a stably integrated TNF promoter-luciferase reporter (left) or an IL-12 p40 promoter-luciferase reporter (right) were stimulated for 8 h with CpG ODN (1 μ M). Where PD98059 was used, cells were pre-incubated for 30 min before stimulation with the concentrations of PD98059 indicated. After stimulation, luciferase activity was measured. Each column represents the mean of two independent stimulations. (E and F) RAW264.7 cells were transiently transfected with the IL-12 p40 promoter-luciferase reporter and increasing amounts of either an expression vector for MEKdn (E) or an expression vector for RAF BxB (F). The overall amount of DNA per transfection was held constant by adding the appropriate control vectors. At 18 h post-transfection, cells were left untreated or were stimulated with 1 μ M CpG ODN for 8 h and luciferase activity was determined. Each column represents the mean of three independent stimulations; error bars are standard deviation.

IFN- γ priming might inhibit ERK activation and thus lead to secretion of high levels of IL-12. To this end, we examined the effect of IFN- γ priming on IL-12 production by CpG ODN-stimulated macrophages. Figure 3A shows that IFN- γ priming also strongly enhances the CpG ODN-induced IL-12 production. This effect is accompanied by a strong increase in promoter activity (not shown). However, as in unprimed cells, inhibition of MEK by PD98059 in IFN- γ -primed cells led to a substantial increase in IL-12 secretion (Figure 3A). These results suggested that IFN- γ treatment would not change ERK activity in response to CpG ODN. Indeed, as shown in Figure 3B, no difference in ERK activity could be detected in IFN- γ -treated versus untreated cells. Hence, the IFN- γ priming

for IL-12 secretion by macrophages does not seem to involve ERK.

In dendritic cells, CpG ODN activates stress kinases JNK and p38 but fails to induce ERK activity

There is evidence that *in vivo* the main source of IL-12 is not macrophages but DCs (De Smedt *et al.*, 1996; Sousa *et al.*, 1997). *In vitro*, DCs secrete TNF in amounts comparable with macrophages, but up to two orders of magnitude more IL-12 (compare Figures 3A and 5A).

In order to see whether the balance of positive and negative regulatory signals is different in DCs, we prepared primary DCs from bone marrow cells (Inaba

Fig. 3. IFN- γ priming effect on CpG ODN-stimulated RAW264.7 cells. (A) A total of 2×10^5 RAW264.7 cells were seeded in 0.5 ml of complete culture medium with or without 10 ng/ml IFN- γ as indicated. After 12 h, cells were stimulated with CpG ODN (1 μ M) for 8 h in the presence or absence of PD98059. Where PD98059 was used, cells were pre-incubated for 30 min before stimulation. The concentration of IL-12 was measured by ELISA. (B) A total of 1.5×10^6 RAW264.7 cells were seeded in 2 ml of complete culture medium with or without 10 ng/ml IFN- γ as indicated. After 12 h, cells were stimulated with CpG ODN (1 μ M) for 15 or 60 min and ERK-2 kinase activity determined in an immune complex kinase assay with antibodies to ERK-2 and MBP as substrate. The radioactivity incorporated into MBP was quantitated after SDS-PAGE by PhosphorImager analysis. The values obtained were normalized against the radioactivity obtained in non-stimulated cells and are presented as fold induction.

et al., 1992; Lutz *et al.*, 1999). After 8 days of *in vitro* culture in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF), these cells displayed a phenotype characteristic for this type of DC culture: the majority of cells were positive for CD11c (Figure 4A) and responded to CpG ODN or LPS by up-regulating MHC class II expression (Figure 4A) and expression of CD80, CD40 and CD86 (not shown; Sparwasser *et al.*, 1998).

Stimulation of DCs with CpG ODN and LPS induced robust activation of JNK and p38 activity (Figure 4B) as suggested by earlier results (Häcker *et al.*, 1998). This activity peaked after 15–30 min (Figure 4C), a similar time frame as observed in macrophages (Häcker *et al.*, 1998). In contrast to the macrophages, however, ERK activation could not be detected in DCs. As a control, DCs were stimulated with phorbol ester [phorbol 12-myristate 13-acetate (PMA)]. Figure 4B and C shows that PMA did induce rapid and strong activation of ERK but no significant JNK or p38 activity. Because DCs were generated in GM-CSF-conditioned medium, we also performed experiments to analyze whether GM-CSF would

Fig. 4. FACS analysis and PRF-induced kinase activity of BMDDCs. (A) BMDDCs were prepared as described in Materials and methods. After 9 days of *in vitro* culture, cells were washed and either left untreated (upper panel) or stimulated with CpG ODN (1 μ M, middle panel) or LPS (10 ng/ml, lower panel) for 20 h. Cells were stained with antibodies to CD11c (left, bold) or MHC class II (right, bold) and the appropriate isotype controls (fine), and analyzed by flow cytometry. (B) BMDDCs were used after 9 days of *in vitro* culture. A total of 5×10^6 cells each were stimulated in complete culture medium with CpG ODN (1 μ M), LPS (10 ng/ml) or PMA (10 ng/ml) for the times indicated. Then, lysates were prepared and split, and immune complex assays were performed on different aliquots. As substrates, MBP was used for ERK-2, GST-Jun(79) for JNK1/2 and GST-MAPKAP kinase II for p38. (C) The radioactivity incorporated into MBP (ERK-2), GST-Jun (JNK1/2) and GST-MAPKAP kinase II (p38) obtained in (B) was quantitated by PhosphorImager analysis. The values obtained were normalized to the radioactivity in non-stimulated cells and are presented as fold activation.

Fig. 5. Influence of PD98059 and PMA on CpG ODN-induced IL-12 and TNF release by BMDDCs. **(A)** A total of 2×10^5 BMDDCs (day 9 of *in vitro* culture) were washed and seeded in 0.5 ml of complete culture medium and stimulated with CpG ODN (1 μ M) in the presence or absence of different concentrations of PD98059 as indicated, either for 3 h (left side) or 7 h (right side). Release of IL-12 was measured by ELISA. Where PD98059 was used, cells were pre-incubated for 30 min before stimulation. **(B)** BMDDCs were stimulated with CpG ODN (1 μ M) for 7 h in the presence or absence of different concentrations of PMA as indicated, and cytokine release of IL-12 (left side) or TNF (right side) was determined by ELISA. PMA and CpG ODN were added at the same time.

affect ERK activation. In a series of different culture conditions such as GM-CSF starvation or serum reduction for time periods up to 24 h, we consistently observed stress kinase activation by CpG ODN and LPS but never found significant ERK activation (not shown). In contrast, we reproducibly obtained a slight reduction in ERK activity at earlier time points after stimulation with CpG ODN (Figure 4B and C). Because we suspected that this might be due to activation of phosphatases, we pre-treated cells with CpG ODN, followed by stimulation with PMA. However, under these conditions, no reduction in PMA-induced ERK activity was observed, arguing against involvement of phosphatases acting directly on ERK (data not shown). It does not rule out the possibility that upstream phosphatases are activated by CpG-DNA, which impact on the basal level of ERK activity.

As expected from the results of the kinase assays, IL-12 secretion was essentially unchanged in the presence of the MEK inhibitor (Figure 5A). Because of the rapid production of IL-12 by DCs, two time points were investigated, 3 and 7 h of stimulation, with the same result (Figure 5A). Notably, the absolute levels of IL-12 secreted by DCs were up to 100-fold higher than that produced by the same number of macrophages (compare Figures 3A and 5A).

Since PMA in DCs induced ERK but not JNK or p38 activity (Figure 4A and B), we tested its influence on IL-12 secretion. As shown in Figure 5B, activation of

ERK by PMA led to a dramatic decrease of IL-12 secretion to levels similar to those observed in macrophages. Corresponding to the findings in macrophages, activation of the ERK pathway somewhat enhanced TNF secretion (Figure 5B).

These results show that two PRFs, CpG-DNA and LPS, activate the stress kinases JNK and p38 in DCs but, in contrast to macrophages, do not induce ERK activity in these cells. In accordance with this, inhibition of the ERK activation did not affect IL-12 release in DCs. Activation of ERK by PMA, however, strongly diminished IL-12 production. We conclude that the specific pattern of kinase activation in DCs upon stimulation by PRFs leads to secretion of high levels of IL-12.

Discussion

The first report on the immunostimulatory potential of bacterial DNA dates back to the work by Tokunaga and colleagues who found that the main immunogenic fraction of a mycobacterial lysate consists of genomic DNA (Tokunaga *et al.*, 1984). Since that time, substantial progress has been made in the attempt to understand the immunostimulatory potency of CpG-DNA *in vitro* and *in vivo*. The molecular mechanisms of how CpG-DNA stimulates immune cells, however, are less clear. Its unusual biochemical structure and its potential to interact with the host's DNA by hybridization has been the basis for much speculation (for a review, see Pisetsky, 1996). However, the recently demonstrated activation of classic, receptor-driven signaling cascades such as the NF- κ B and stress kinase pathways by CpG-DNA seems to favor a model in which CpG-DNA binds to a sequence-specific cellular receptor and activates signal transduction pathways in a 'traditional' manner like other receptor ligands (for a review, see Häcker, 1999).

CpG-DNA activates the MAP kinase ERK in a MEK-dependent way

The data presented here reveal that another classic signaling cascade, the MAP/ERK signal transduction pathway, is activated by CpG-DNA in primary macrophages and the macrophage cell line RAW264.7. This observation further supports the view that CpG-DNA acts as a classic receptor-dependent ligand. To our knowledge, the only other attempt to investigate MAPK/ERK signaling by CpG ODN was the work by Yi and colleagues who reported that no ERK phosphorylation was detectable by Western blotting in CpG ODN-stimulated J774 macrophage cells (Yi and Krieg, 1998). Their finding is in accordance with the fact that J774 cells produce large amounts of IL-12 upon stimulation with CpG ODN and LPS (Lipford *et al.*, 1997b, and our own unpublished observations). J774 is referred to commonly as a macrophage cell line but its constitutive capacity for IL-12 secretion, even without IFN- γ priming, in response to CpG ODN or LPS, separates these cells from primary macrophages and RAW264.7 macrophage cells.

Other stimuli known to induce ERK activity are mainly growth factors such as epidermal growth factor (EGF), colony-stimulating factor-1 (CSF-1) and GM-CSF (for reviews, see Boonstra *et al.*, 1995; Hamilton, 1997; de Groot *et al.*, 1998). However, it is important to note that

in macrophages not only CpG-DNA but also other PRFs such as LPS and peptidoglycans induce NF- κ B, ERK and stress kinase activity (Dziarski *et al.*, 1996; Sanghera *et al.*, 1996). We conclude that this broad pattern of kinase activation characterizes PRF-dependent signaling in macrophages.

CpG-DNA-induced ERK activity controls production of TNF and IL-12

An important role for both TNF and IL-12 during immune responses to pathogens is well documented. As well as its role during organogenesis (Neumann *et al.*, 1996), TNF is important for the immune defense against intracellular bacteria such as *Listeria* (Pfeffer *et al.*, 1993) and mycobacteria (Smith *et al.*, 1997). After stimulation with LPS, TNF is secreted by macrophages and DCs in comparable amounts and is regulated at the transcriptional as well as the post-transcriptional level (Raabe *et al.*, 1998). Both ERK and p38 have been shown to up-regulate TNF production at a post-transcriptional level following stimulation by LPS (Beutler and Kruys, 1995; Prichett *et al.*, 1995). In accordance, CpG-DNA-triggered ERK activity also appears to up-regulate TNF release at a post-transcriptional level (Figure 2C and D).

In immune responses, IL-12 plays a central role as a link between the innate and adaptive immune systems (for a review, see Trinchieri, 1998). As well as its direct effects on natural killer (NK) and T cells for IFN- γ release and enhancement of their lytic activity, IL-12 is key to polarization of the immune system towards a primary Th-1 response. Moreover, it has profound effects on hemopoiesis and, synergistically with other hemopoietic growth factors such as Flt-3 ligand, promotes maturation of primitive bone marrow precursors and proliferation of DCs (Shurin *et al.*, 1998). These effects might account for the outstanding role of IL-12 as an adjuvant for T- and B-cell-dependent immune responses (for a review, see Lu *et al.*, 1999).

Biologically active IL-12 (p70) consists of a heterodimer of two proteins of 40 kDa (p40) and 35 kDa (p35) (Gubler *et al.*, 1991; Wolf *et al.*, 1991). A basal level of IL-12 p35 seems to be expressed constitutively by many cell types in different tissues. However, IL-12 p40 expression appears to be restricted to cell types that produce biologically active IL-12 such as macrophages, granulocytes and DCs (D'Andrea *et al.*, 1992). Moreover, p40 expression is induced by external stimuli such as intracellular bacteria and bacterial constituents like LPS and CpG-DNA (D'Andrea *et al.*, 1992; Häcker *et al.*, 1998). In this study, we focused on the regulation of p40. Among the cell types and stimuli investigated so far, IL-12 p40 expression seems to be controlled mainly by its promoter activity. We have reported that p38 activity induced by CpG-DNA controls IL-12 production (Häcker *et al.*, 1998), a finding that has been confirmed by Lu and colleagues who demonstrated that MKK3-dependent p38 activation induces IL-12 p40 promoter activity and cytokine release (Lu *et al.*, 1999). However, the target of p38 that mediates IL-12 p40 expression has not been identified yet.

Our analysis of the role of the MAPK/ERK pathway for IL-12 yielded the unexpected finding that ERK activity suppressed IL-12 production. IL-12 release correlated directly with IL-12 p40 mRNA levels and promoter

activity, implying that the regulation of IL-12 production by the ERK pathway occurs primarily at the transcriptional level. Because of technical constraints, the studies of cytokine release and promoter activity were performed using the MEK inhibitor PD98059 and dominant-negative MEK1, respectively. Since both agents specifically block MEK1/2, we cannot, at present, completely rule out the possibility that the inhibitory influence on IL-12 production is achieved via an unknown target of MEK1/2, independent of ERK. Further work is required to clarify this issue.

One of the next steps will be to identify the promoter element in the IL-12 p40 promoter that confers its MAPK dependency. At present, it would be rather speculative to name any other binding site or transcription factor as a candidate. While three major positive regulatory sites in the IL-12 p40 promoter have been described, an Ets site (Ma *et al.*, 1996), a NF- κ B half-site (Murphy *et al.*, 1995) and a C/EBP element (Plevy *et al.*, 1997), the only suggestion put forward for a negative regulatory transcription factor is the C/EBP β isoform LIP (Plevy *et al.*, 1997). This variant arises by alternative translation initiation of the C/EBP β mRNA and lacks the transactivation domain of C/EBP β . Heterodimerization of LIP with full-length C/EBP β attenuates transcriptional activity, suggesting a negative regulatory function of LIP (Descombes and Schibler, 1991). Notably, LIP has been demonstrated to block LPS-induced IL-12 p40 promoter activity when expressed in RAW264.7 macrophages (Plevy *et al.*, 1997). However, the signaling pathways impinging on this alternative translation initiation are unknown.

Cell type-dependent activation of the MAPK pathways by pattern recognition factors regulates IL-12 release

The ability of the MAPK/ERK pathway to negatively regulate IL-12 production pointed towards the possibility that situations or cell types exist where this negative signal is eased off in favor of IL-12 production. We first investigated the effect of IFN- γ pre-treatment of macrophages. IL-12 p40 mRNA transcription and protein secretion by macrophages are strongly enhanced by IFN- γ priming (Ma *et al.*, 1996). How IFN- γ acts at the molecular level is less clear. In macrophages, we also observed a strong up-regulation of IL-12 p40 promoter activity and cytokine release upon CpG ODN stimulation after priming with IFN- γ . However, this activity could still be enhanced substantially by inhibition of MEK1/2. Accordingly, IFN- γ pre-treated RAW264.7 cells did not exhibit a significantly altered magnitude or kinetics of ERK activation in response to CpG ODNs. Thus, the priming effect of IFN- γ appears not to be due to altered MEK/ERK activation. Several previous reports have also addressed the question of how IFN- γ priming might induce IL-12 up-regulation; however, with different results. While Ma *et al.* (1996) described an Ets-binding site as a critical element conferring IFN- γ responsiveness, Murphy *et al.* (1995) found increased NF- κ B-binding activity upon IFN- γ pre-treatment. Moreover, Plevy *et al.* (1997) failed to detect any increased promoter activity upon IFN- γ priming and therefore speculated that mRNA stabilization might be involved. Importantly, the experimental settings used in these reports were different with respect to the cell lines (RAW264.7 versus J774), the species of the IL-12 p40 promoter

(mouse versus human) and the stimuli used (LPS versus heat-killed *Listeria monocytogenes*). Overall, these reports suggest that more than one mechanism might be involved, although their relative contributions to the priming effect of IFN- γ cannot be estimated yet. However, as activating pathways of IL-12 p40, such as the NF- κ B- and p38-activating pathways, are becoming elucidated, this knowledge will contribute to our understanding of IFN- γ priming.

DCs represent a subclass of cells of the hemopoietic lineage whose main task for adaptive immune responses appears to be uptake and presentation of antigen to T cells. Upon maturation to professional APCs, they synthesize membrane-bound co-stimulatory molecules and secrete cytokines. As such, DCs harbor the potential to induce a Th-1 polarization of emerging T-cell responses. *In vitro*, a commonly used source of DCs is mouse bone marrow. After culture of bone marrow with GM-CSF for 8–10 days, a phenotypically homogeneous, CD11c-expressing DC population can be recovered (Inaba *et al.*, 1992; Lutz *et al.*, 1999). CpG ODN- or LPS-driven stimulation of this population results in up-regulation of MHC class II molecules, CD40, CD80 and CD86 (Sparwasser *et al.*, 1998). We used this source of DCs to study the MAPK pathways during PRF stimulation. One key element in the effector repertoire of DCs is the production of IL-12. Notably, DCs appear to secrete *in vitro* more IL-12 than any other cell type investigated, including macrophages, keratinocytes and polymorphonuclear leukocytes (D'Andrea *et al.*, 1992; Aragane *et al.*, 1994; Cassatella *et al.*, 1995; Koch *et al.*, 1996). In fact, under identical experimental conditions, PRF-stimulated DCs produce ~20–100 times more IL-12 than peritoneal macrophages, yet secrete comparable amounts of TNF. This pattern of cytokine production is the same for both CpG-DNA and LPS (not shown). These results suggested that the MAPK/ERK pathway may be differentially active in DCs versus macrophages.

Indeed, upon stimulation with PRF, DCs produced large amounts of IL-12 and activated stress kinases, but not ERK. The MEK inhibitor PD98059 had no influence on IL-12 secretion by DCs, yet stimulation of ERK using PMA reduced IL-12 secretion dramatically but selectively in that TNF production was not reduced. Overall, these data suggest that the absence of ERK activation allows DCs to produce large amounts of IL-12.

A recent report describes that under certain conditions even DCs are able to activate ERK in response to LPS (Rescigno *et al.*, 1998). The DC population used in that study had been derived from mouse spleen by long-term culture with GM-CSF and a 3T3 fibroblast-conditioned medium. Under these culture conditions, a DC population survived and proliferated for >100 days (Winzler *et al.*, 1997). The DCs we used were cultured with GM-CSF only, reached a final differentiation stage and stopped proliferating after ~10 days. The regulation of the MAPK/ERK pathway therefore appears to be different even in DCs dependent upon their stage of differentiation. The kinase activation pattern in response to PRF may be another parameter to define maturation stages within the lineage of DCs.

As outlined above, IL-12 has a physiological role during initiation of the host's immune defense against pathogens.

It has been suggested that an inappropriate production of IL-12 may condition for immune deviation, such as autoimmune diseases (Adorini *et al.*, 1996). Therefore, understanding of the signaling pathways controlling IL-12 may aid the development of new therapeutic strategies.

Materials and methods

Cell culture and generation of BMDDCs

The murine macrophage cell line RAW264.7 was cultured in LowTox Clicks/RPMI (Biochrom, Berlin, Germany) supplemented with 10% (v/v) fetal calf serum (FCS; Seromed^R, Biochrom), 50 μ M 2-mercaptoethanol and antibiotics [penicillin G (100 IU/ml of medium) and streptomycin sulfate (100 IU/ml of medium)]. This medium is referred to as complete culture medium.

BMDDCs from C57BL/6 mice were prepared as described (Häcker *et al.*, 1998; Lutz *et al.*, 1999). Briefly, femurs of mice were rinsed with cell culture medium using a syringe with a 27 gauge needle. Bone marrow cells (3×10^6) were seeded into a 100 mm tissue culture Petri dish in complete medium supplemented with GM-CSF (200 U/ml). Cells were used at day 9 of *in vitro* culture when mature DCs (CD11c⁺, GR-1⁻) represented >90% of the resulting cell population. For stimulation, non-adherent cells were used.

Peritoneal washout cells were prepared by peritoneal lavage of untreated C57BL/6 mice. Cells were plated in complete culture medium and, 3 and 8 h after plating, cells were washed extensively and non-adherent cells were removed. After 24 h of culture, 2×10^6 adherent cells were used for each stimulation and the following *in vitro* kinase assay.

Flow cytometry analysis

Cells were washed in phosphate-buffered saline (PBS)/2% FCS and incubated with anti-Fc γ RII/III monoclonal antibodies (clone 2.4G2, Pharmingen) for 20 min to block unspecific, Fc receptor-mediated binding of monoclonal antibodies. Then cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-MHC II monoclonal antibodies (clone 2G9, Pharmingen) or phycoerythrin (PE)-conjugated anti-CD11c monoclonal antibodies (clone HL3, Pharmingen). After staining, cells were washed and live cells were analyzed on a FACSCaliburTM flow cytometer (Becton Dickinson).

Determination of cytokine secretion

Cytokine levels in culture supernatants were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits (TNF- α and total mIL-12 DuoSet) according to the manufacturer's instructions (Genzyme, Germany). Each value shown represents the mean of duplicate values.

Preparation of plasmids and reagents

All plasmids used were grown in the *Escherichia coli* strain DH5 α and prepared using the 'EndoFree Plasmid kit' from Qiagen according to the manufacturer's instructions. Phosphothioate-stabilized CpG ODN (TCC-ATG-ACG-TTC-CTG-ATG-CT) and GpC ODN (TCC-ATG-AGC-TTC-CTG-ATG-CT) (Krieg *et al.*, 1995) were purchased from TIB MOLBIOL (Berlin, Germany), LPS (*E.coli*) and PMA were from Sigma, mouse IFN- γ was from Pharmingen and PD98059 was from Alexis Biochemicals, UO126 was from Promega.

Luciferase reporter plasmid transfection, luciferase assay and generation of stable RAW264.7 transfectants

To investigate transcriptional activity of the IL-12 p40 promoter in transient assays, we used a plasmid containing the -703 bp region of the murine IL-12 p40 gene (Murphy *et al.*, 1995). This IL-12 p40 luciferase reporter vector was a generous gift from Kenneth Murphy. A total of $5-10 \times 10^6$ RAW264.7 cells were transfected by electroporation in a 400 μ l final volume (RPMI/25% FCS) at 280 V/960 μ F in a BioRad gene pulser. A 5 μ g aliquot of reporter plasmid was used together with different amounts of specific expression vectors as indicated in the figure legends. The overall amount of plasmid DNA was held constant at 20 μ g per electroporation by addition of the appropriate empty expression vector. After electroporation, cells were washed and split onto 24-well plates, 2×10^5 cells per well. At 18 h post-transfection, cells were stimulated in 0.5 ml of complete growth medium with 1 μ M phosphothioate-stabilized ODN for 8 h. Preparation of cell extracts and

luciferase assays were according to the manufacturer's instructions (Promega). The cDNA for dominant-negative MEK-1 (Ser221→Ala221; Cowley *et al.*, 1994), a kind gift from Roland Schmid, was expressed in pcDNA3 (Invitrogen). The cDNA for RAF BxB was contained in pcDNA3 (Invitrogen; Mischak *et al.*, 1996).

Generation of stable RAW264.7 transfectants and the plasmids used have been described (Hacker *et al.*, 1998). The following plasmids were used here; the TNF- α promoter region was kindly provided by Victor Jongeneel. To obtain the TNF- α luciferase reporter vector, a 1.2 kb *Bam*HI–*Xba*I fragment of the TNF- α promoter was subcloned from pBLCAT3 (Luckow and Schutz, 1987) into the GL3 basic luciferase vector (Promega). The IL-12 p40 luciferase reporter vector was the same as used for the transient assays (see above).

Stable RAW264.7 transfectants were established by co-transfection by electroporation of the described TNF- α and IL-12 reporter vectors and the pGFP-1 vector (Clontech), containing a neomycin resistance cassette in a ratio of 10 to 1. One day after transfection, cells were overlaid with soft agar containing 0.4 μ g/ml G418 (Gibco-BRL). G418-resistant clones were picked, expanded and tested for inducible luciferase activity.

In vitro kinase assay and Western blotting

After stimulation for different time periods as indicated in the figure legends, lysates (lysis buffer, see below) were prepared and kinase activity was determined essentially as described (Kieser *et al.*, 1997). Briefly, lysates were cleared by centrifugation at 10 000 g for 10 min and incubated with GST–Jun(79) to precipitate Jun N-terminal kinases, GST–MAPKAP kinase II to precipitate p38 or antibodies to ERK-2 (Santa Cruz Biotechnology, C-14) to precipitate ERK-2. For precipitation, GST fusion proteins were immobilized to glutathione–Sephadex and anti-ERK-2 antibodies to protein A–Sephadex. Subsequently, precipitates were washed carefully and incubated in kinase buffer (25 mM HEPES, 10 mM MgCl₂, 10 mM β -glycerophosphate, 0.5 mM EGTA, 0.5 mM sodium fluoride, 0.5 mM orthovanadate, 20 μ M ATP) in the presence of [γ -³²P]ATP. Myelin basic protein (MBP; Gibco-BRL) was used as substrate for ERK-2. Reactions were stopped by boiling in SDS sample buffer, resolved on a 10% SDS gel and analyzed by PhosphorImager analysis.

For kinase assays with hemagglutinin (HA)-tagged kinases, RAW264.7 cells were transfected with 5 μ g of expression vectors for HA-tagged ERK-2 and HA-tagged JNK-1, kind gifts from Michael Karin (Lin *et al.*, 1995). A 5 μ g aliquot of these vectors was used together with different amounts of specific expression vectors as indicated in the figure legends. The overall amount of plasmid DNA was held constant at 20 μ g per electroporation by adding the respective empty expression vector. After electroporation, cells were washed and split onto 6-well plates, 1.5×10^6 cells per well. At 18 h post-transfection, cells were stimulated in 2 ml of complete growth medium with 1 μ M phosphothioate-stabilized CpG ODN for 20 min. Then immune complex kinase assays were performed using antibodies to the HA tag (clone 12CA5, Boehringer Mannheim/Roche). Kinase assays were performed as described above. As substrate, MBP was used for HA-ERK-2, and GST–Jun(79) for HA-JNK-1.

For Western blotting, cells were grown and stimulated as indicated in the figure legends. After stimulation, cells were lysed in lysis buffer containing 25 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 10 mM pyrophosphate, 20 mM β -glycerophosphate, 10 μ g/ml leupeptin, 2 mM orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM sodium fluoride and 10 μ g/ml aprotinin. Lysates were cleared by centrifugation at 10 000 g for 10 min, boiled in SDS sample buffer, resolved on a 10% SDS gel and blotted onto nitrocellulose membranes. Membranes were probed with the indicated antibodies and visualized using enhanced chemiluminescence (ECL, Amersham) for detection. Antibodies to MEK1/2 and its Ser217/221-phosphorylated form were purchased from New England Biolabs.

RNAse protection assay (RPA)

RAW264.7 macrophages were stimulated as described in the figure legends and total RNA was isolated using peqGOLD TriFast reagent (Peqlab). A 20 μ g aliquot of total RNA was used for each reaction. The RPA was performed according to the manufacturer's instructions (RiboQuant RPA kit, Pharmingen). Probe sets mCK-2b and mCK-3 were used to detect mRNA expression of IL-12 p40 or TNF, respectively. The protected probes obtained were resolved on a polyacrylamide gel and analyzed by PhosphorImager analysis.

Acknowledgements

We thank Martin R. Vabulas and Ingrid Wess for assistance with the cell culture of BMDDCs and Eva Koch for excellent technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft SFB 391, Teilprojekt B-4 and the BMBF grant 0311675/CpG Immunopharmaceuticals GmbH.

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Received July 26, 1999; revised October 19, 1999;
accepted October 20, 1999