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CpG-DNA-specific activation of antigen-presenting cells requires stress kinase activity and is preceded by non-specific endocytosis and endosomal maturation

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Unmethylated CpG motifs in bacterial DNA, plasmid DNA and synthetic oligodeoxynucleotides (CpG ODN) activate dendritic cells (DC) and macrophages in a CD40–CD40 ligand-independent fashion. To understand the molecular mechanisms involved we focused on the cellular uptake of CpG ODN, the need for endosomal maturation and the role of the stress kinase pathway. Here we demonstrate that CpG-DNA induces phosphorylation of Jun N-terminal kinase kinase 1 (JNKK1/SEK/MKK4) and subsequent activation of the stress kinases JNK1/2 and p38 in murine macrophages and dendritic cells. This leads to activation of the transcription factor activating protein-1 (AP-1) via phosphorylation of its constituents c-Jun and ATF2. Moreover, stress kinase activation is essential for CpG-DNA-induced cytokine release of tumor necrosis factor α (TNFα) and interleukin-12 (IL-12), as inhibition of p38 results in severe impairment of this biological response. We further demonstrate that cellular uptake via endocytosis and subsequent endosomal maturation is essential for signalling, since competition by non-CpG-DNA or compounds blocking endosomal maturation such as chloroquine or bafilomycin A prevent all aspects of cellular activation. The data suggest that endosomal maturation is required for translation of intraendosomal CpG ODN sequences into signalling via the stress kinase pathway, where p38 kinase activation represents an essential step in CpG-ODN-triggered activation of antigen-presenting cells.

Keywords: antigen-presenting cell/CpG-DNA/p38 MAP kinase/SAPK

Introduction

For decades, bacterial genomic DNA has been considered to be immunologically inert. Recent evidence, however, suggests that the immune system via pattern recognition detects prokaryotic DNA as a signal for 'infectious danger'.

The first reports on the immunostimulatory properties of bacterial DNA date back to Tokunaga and associates who succeeded in attributing the tumoricidal effects of Bacillus Calmette Guerin (BCG) to mycobacterial DNA. A DNA-rich fraction extracted from BCG exhibited anti-tumor activity *in vivo*, augmented natural killer (NK) cell activity and triggered *in vitro* type 1 and type 2 interferon release from murine spleen or human peripheral blood leukocytes (PBL). All these activities were DNase sensitive (Shimada *et al*., 1985; Yamamoto *et al*., 1992). While vertebrate DNA lacks immunostimulatory effects, certain synthetic oligonucleotide sequences were found to mimic the stimulatory effects of mycobacterial DNA (Yamamoto *et al*., 1992). Bacterial DNA was also reported to induce B-cell activation and immunoglobulin secretion, while vertebrate DNA did not (Messina *et al*., 1991). Unexpected sequence-specific immunostimulatory effects were also noted with anti-sense oligodeoxynucleotides (ODN) (Tanaka *et al*., 1992; Branda *et al*., 1993). Using sequence-specific synthetic ODN to trigger B-cell mitogenicity, Krieg and associates iteratively defined unmethylated CpG motifs displaying 5′Pu-Pu-CpG-Pyr-Pyr3′ nucleotide sequences as biologically active (Krieg *et al*., 1995). Unmethylated CpG motifs are common in bacterial DNA, but are suppressed, as well as being methylated, in vertebrate DNA (Bird, 1986; Sved and Bird, 1990). The concept thus emerged that unmethylated CpG dinucleotides in the context of selective flanking bases are recognized by cells of the immune system to discriminate pathogen-derived foreign DNA from self DNA (Krieg *et al*., 1995; Pisetsky, 1996).

Bacterial DNA and biologically active CpG ODN activate powerfully cells of the innate immune system such as macrophages and immature dendritic cells (DCs) to upregulate major histocompatibility complex (MHC) class II and co-stimulatory molecules, to transcribe cytokine mRNAs, and to secrete pro-inflammatory cytokines including tumor necrosis factor α (TNFα), interleukins IL-1, IL-6 and IL-12 (Stacey *et al*., 1996; Lipford *et al*., 1997a; Sparwasser *et al*., 1997a,b). Conversion of immature DCs to professional antigen-presenting cells (APC) (Sparwasser *et al*., 1998) might explain the strong adjuvant effect of CpG-DNA in promoting *in vivo* productive Th1 responses (Chu *et al*., 1997; Lipford *et al*., 1997b; Roman *et al*., 1997; Davis *et al*., 1998; Zimmermann *et al*., 1998). This adjuvant effect has also been noted in genetic vaccination protocols using plasmid expression vectors (vDNA) as a source of antigen. Insertion of non-coding singular CpG ODN motifs into the backbone of vDNA conferred immunogenicity to otherwise poorly immunogenic vectors, as if an adjuvant were 'built in' (Sato *et al*., 1996; Klinman *et al*., 1997; Roman *et al*., 1997).

While the phenomenon of CpG-ODN-mediated activation of APC such as macrophages and DCs becomes

increasingly documented, the molecular mechanisms causing APC activation are poorly understood. There is evidence that CpG-DNA binds to cell-surface receptors which subsequently transduce stimulatory signals (Liang *et al*., 1996), a view challenged by others (Krieg *et al*., 1995). Furthermore, CpG-DNA may generate reactive oxygen species (ROS) which precedes nuclear factor kappa B (NFκB) activation (Yi *et al*., 1998). In order to define components involved in CpG-ODN-mediated signal integration leading to APC activation, we examined its cellular uptake, endosomal localization and the activity of the stress kinase signalling pathway. Our results indicate that activation of APC by CpG-DNA is mediated, at least in part, by the stress kinase pathway. Furthermore, CpG-ODN-specific activation of the stress kinase pathway requires endosomal translocation and maturation.

Results

vDNA and CpG ODN induce cytokine release in DCs and the macrophage cell line ANA-1

The observations that vDNA and CpG ODN activate macrophages (Stacey *et al*., 1996; Sparwasser *et al*., 1997b) and that the immunogenicity of vDNA *in vivo* depends on CpG motifs (Sato *et al*., 1996), suggested that DCs would be activated by vDNA. To investigate this, we isolated bone marrow-derived dendritic cells (BMDDC) and incubated these cells with vDNA (pBluescript), which contains at least two characterized immunostimulatory CpG sequences in the ampicillinase gene (Sato *et al*., 1996). vDNA induces substantial release of the cytokines IL-12 and TNFα (Figure 1A and B). CpG-specific methylation of the plasmid totally abolishes this capacity, demonstrating the requirement of unmethylated CpG motifs in bacterial DNA for BMDDC activation. Figure 1 also shows that this principle can be mimicked by CpG ODN, as only ODN containing a CpG motif (1668; Krieg *et al*., 1995), but not an ODN with inverted CpG (GpC ODN) induces cytokine release. Stimulation of BMDDC with lipopolysaccharide (LPS), a bacterial cell-wall component and known stimulus for dendritic cells, is shown for comparison.

Because of the difficulty in transfecting primary cells, we examined whether the principal activities of CpG-DNA, i.e. induction of cytokines such as TNFα and IL-12 can be reproduced on the transfectable macrophage cell line ANA-1. Both vDNA and CpG ODN induce IL-12 and TNFα release in a strictly CpG-dependent manner (Figure 1C and D). Cytokine production was similar in magnitude to that triggered by LPS. The ability to respond to CpG-DNA is not restricted to the cell line ANA-1. Stimulation of the macrophage cell lines RAW 264.7 and J774 as well as primary peritoneal macrophages with CpG-DNA revealed essentially identical results (data not shown). We conclude from these data that ANA-1 cells can be used to investigate the principal molecular activities of CpG-DNA.

CpG-DNA induces transcriptional activity of AP-1 via phosphorylation of c-Jun

The activating protein-1 (AP-1), a transcription factor complex comprised of members of the Fos-, Jun- and ATF- (activating factor) families, is not only involved in

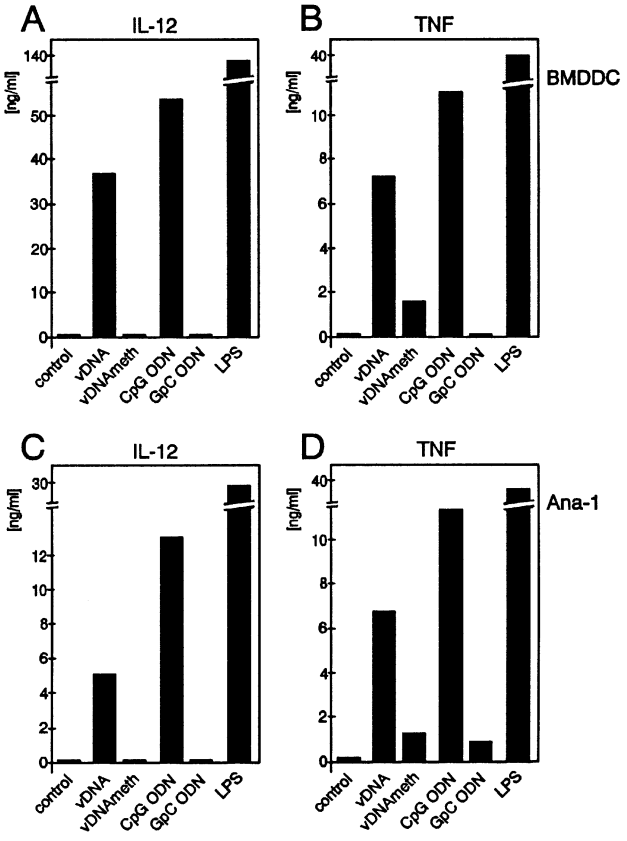

Fig. 1. vDNA and CpG ODN activate BMDDC and ANA-1 cells in a strictly CpG-dependent manner. (**A** and **B**) BMDDC (2.5×10^5) were washed and seeded in 0.5 ml complete culture medium containing 10% fetal calf serum (FCS) without granulocyte/macrophage colony-stimulating factor (GM-CSF) and stimulated with vDNA or methylated vDNA (vDNAmeth) (20 μg/ml), CpG ODN or GpC ODN (20 nM) or LPS (10 ng/ml) for 8 h and cytokine release of IL-12 (A) and TNFα (B) was measured by ELISA. (**C** and **D**) ANA-1 cells (2.5×10^5) were washed and seeded in 0.5 ml complete culture medium containing 10% FCS and stimulated with vDNA or methylated vDNA (vDNAmeth) (20 μg/ml), CpG ODN or GpC ODN (2 μM) or LPS (10 ng/ml) for 20 h and the supernatants were analysed for cytokine production of IL-12 (C) and TNFα (D).

the regulation of various immediate early genes and the expression of certain cytokines (Ray *et al*., 1989; Dendorfer *et al*., 1994; Cockerill *et al*., 1995; Cella *et al*., 1997; Karin *et al*., 1997) but also integrates signals from different signal transduction pathways (for review see Su and Karin, 1996). We therefore investigated whether upregulation of AP-1 activity represents a primary event triggered in APC by unmethylated CpG-DNA motifs.

To test for AP-1 transcriptional activity, ANA-1 macrophages were transiently transfected with a reporter construct containing the luciferase gene with a minimal promoter under the control of three TPA responsive element (TRE) (AP-1) consensus sites. Both vDNA and CpG ODN induce transcriptional activity of AP-1, provided vDNA is not methylated and the CpG motif is not inverted to a GpC motif (Figure 2A). Induction of AP-1 activity was similar in magnitude to LPS, a known inducer of AP-1 (Hambleton *et al*., 1996).

Since the transfection procedure itself transiently activates macrophages, we also established RAW264.7 cells, containing the AP-1-luciferase reporter cassette stably integrated. Figure 2B and C shows, for one representative

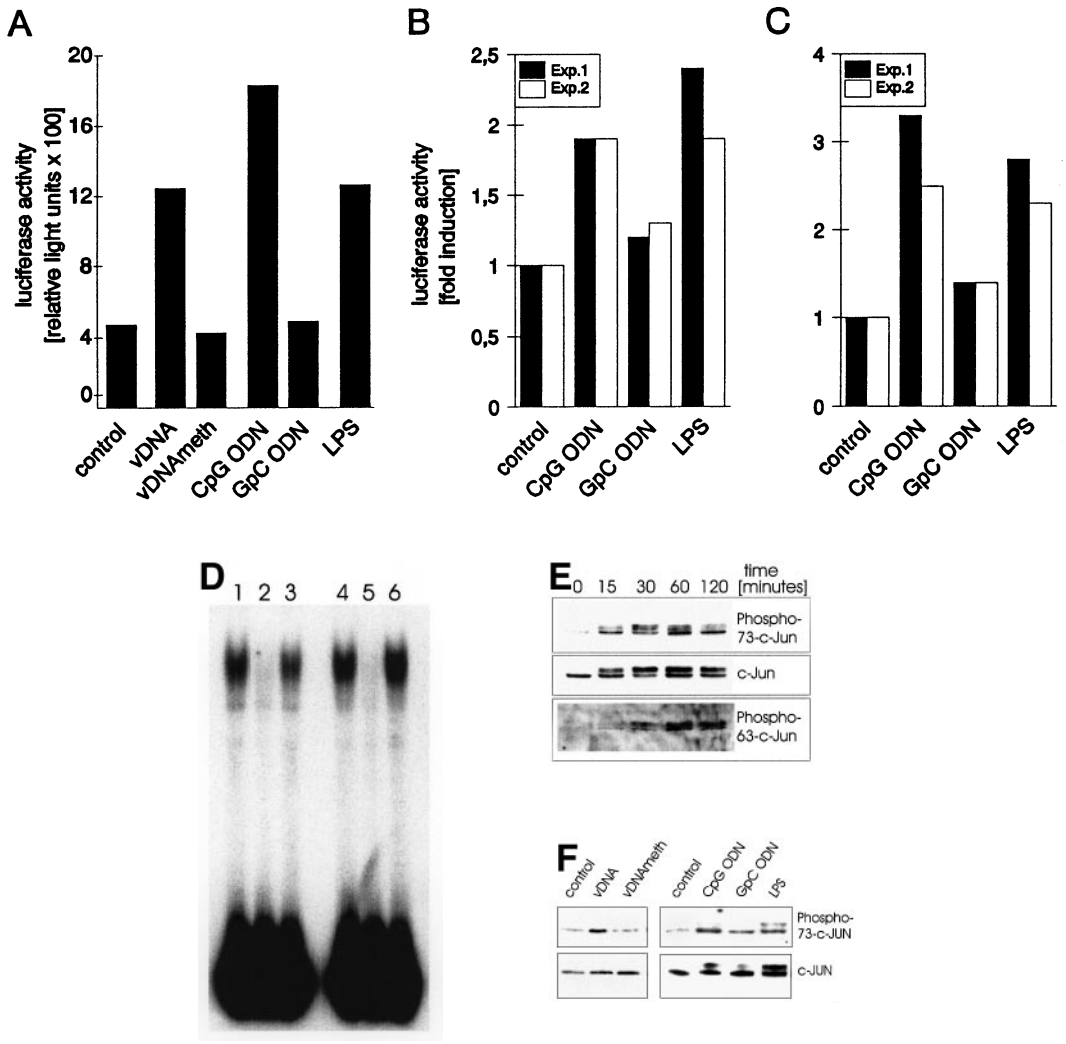

Fig. 2. CpG-DNA stimulates transcriptional activity of AP-1 and phosphorylation of c-Jun. ANA-1 and RAW264.7 cells were grown in complete culture medium. (**A**) Cells were transfected with a TRE (AP-1)-luciferase reporter plasmid and stimulated with vDNA or methylated vDNA (vDNAmeth) (15 μg/ml), CpG ODN or GpC ODN (1 μM) or LPS (10 ng/ml) as indicated and luciferase activity was measured. (**B** and **C**) RAW264.7 cells with a stably integrated AP-1-luciferase reporter gene were stimulated for 4 h (B) or 24 h (C) with CpG ODN or GpC ODN (1 μM) or LPS (10 ng/ml) as indicated and luciferase activity was measured. Two independent experiments are shown. (**D**) Electromobility shift assays of protein extracts from unstimulated cells (lanes 1–3) or cells stimulated for 4 h with 2 μM CpG ODN (lanes 4–6) using the TRE (AP-1) motif-containing oligonucleotide. Cells were lysed and nuclear protein extracts were prepared as described (Schmid *et al*., 1991). The specificity of AP-1 DNA binding was confirmed in competition experiments using a 20-fold excess of unlabeled oligonucleotide, TRE (AP-1) (lanes 2 and 5) or an unrelated oligonucleotide spanning an SP-1 binding site (lanes 3 and 6). (**E**) Western blot analysis of c-Jun phosphorylation. Cells were stimulated with 2 μM CpG ODN for the times indicated. Cell lysates were analysed by Western blotting with antibodies against total c-Jun and antibodies against Ser73- or Ser63-phosphorylated forms of c-Jun. (**F**) Cells were stimulated for 1 h with 20 μg/ml vDNA or methylated vDNA, 2 μM CpG ODN or GpC ODN or 10 ng/ml LPS. Phosphorylation of c-Jun was assessed by Western blotting using antibodies against the Ser73-phosphorylated form of c-Jun or an antibody against total c-Jun.

clone, that induction of transcriptional activity of AP-1 by CpG-DNA or LPS is not a result of the preceding transient transfection. Four hours after stimulation, clear induction of AP-1-controlled luciferase activity can be detected (Figure 2B). The activity increases up to 24 h (Figure 2C) and declines slowly thereafter (not shown). Both, kinetics and height of AP-1 induction were comparable for CpG-DNA and LPS on several independent clones (data not shown).

AP-1 activity can be regulated at different levels. It is controlled at the transcriptional level for c-Fos and c-Jun as well as by phosphorylation which enhances the transactivating potency of the complex (for review see Su and Karin, 1996). For c-Jun and ATF2, critical phosphorylation sites are well defined (Pulverer *et al*., 1991; Smeal *et al*., 1991, 1992; Gupta *et al*., 1995; van Dam *et al*., 1995). To examine whether AP-1 transcriptional activity correlates with enhanced TRE (AP-1) binding activity, we performed electromobility shift assays with nuclear extracts from unstimulated or CpG ODN-stimulated cells. Figure 2D shows that basal AP-1 binding activity only slightly increased over a 4 h period of stimulation with CpG ODN. Confirmation that the AP-1 complex contains c-Jun was demonstrated in a supershift assay (data not shown). We concluded from these data that increase of AP-1-binding activity is not a prerequisite for AP-1-dependent immediate early responses in macrophages. As such, the data suggested that induction of AP-1 activity

may be due to activating phosphorylation. We therefore focused on the phosphorylation sites Ser73 and Ser63 of c-Jun, known to be critical for the transactivating potency of AP-1 (Pulverer *et al*., 1991; Smeal *et al*., 1991, 1992).

As shown by Western blotting, c-Jun becomes phosphorylated within minutes at positions Ser73 and Ser63 upon stimulation of the cells with CpG ODN, while the overall amount of c-Jun increases only slightly (Figure 2E). Phosphorylation of c-Jun at Ser73 is CpG-sequence-dependent (Figure 2E). As a positive control, LPS, a known stimulus of c-Jun N-terminal kinases in macrophages (Hambleton *et al*., 1996) is included (Figure 2F).

CpG-DNA triggered c-Jun phosphorylation within minutes. In contrast, both the overall amount of c-Jun protein and TRE (AP-1) binding activity only slightly increased during the overall time period of the experiments (Figure 2D and E). Therefore, we concluded that AP-1-dependent immediate early responses of macrophages to CpG ODN are primarily controlled by c-Jun phosphorylation.

CpG-DNA activates stress kinases

JNK1/2 (Karin, 1995), also termed stress-activated protein kinases (SAPK) (Kyriakis *et al*., 1994) are activated by distinct extracellular stimuli including IL-1, TNFα, LPS and UV light (Hibi *et al*., 1993; Bird *et al*., 1994; Sluss *et al*., 1994; Westwick *et al*., 1994; Hambleton *et al*., 1996). JNK kinase 1 (JNKK1/SEK1/MKK4; Sanchez *et al*., 1994; Derijard *et al*., 1995; Lin *et al*., 1995) has been identified as the upstream kinase phosphorylating and activating JNK. CpG-DNA triggers the kinase activity of JNK in macrophages several-fold, as is the case with LPS (Figure 3A). Phosphospecific antibodies directed against the phosphorylation site of JNKK1 (Thr223) identified this kinase as one upstream target of CpG ODN signalling (Figure 3B). In addition, p38, another SAPK originally identified as a kinase activated by LPS (Han *et al*., 1993, 1994) and also a target of JNKK1 *in vivo* (Sanchez *et al*., 1994; Derijard *et al*., 1995; Lin *et al*., 1995), shows similar kinetics of activation via phosphorylation at Thr180/Tyr182 (Figure 3B). These data identify JNKK1, JNK and p38 as immediate early targets of CpG ODN.

ATF2, a substrate of both p38 and JNK1/2 (Gupta *et al*., 1995; Raingeaud *et al*., 1996), is also rapidly phosphorylated in response to CpG-DNA and LPS at Thr69/Thr71, the regulatory sites that confer transcriptional activity to this protein (Gupta *et al*., 1995; Livingstone *et al*., 1995) (Figure 4). Withdrawal of serum, which contains the LPS-binding protein, abolishes the ability of LPS to signal through its receptor CD14. Therefore the ability of LPS to induce ATF2 phosphorylation is dependent on serum. In contrast, the activity of CpG-DNA is independent of added serum components (Figure 4).

Essentially the same results, i.e. JNK activation, p38 activation and phosphorylation of c-Jun and ATF2 were obtained after CpG-DNA-mediated stimulation of the cell line RAW 264.7 (data not shown). To investigate whether the signalling pathways activated by CpG-DNA on the macrophage cell lines also operate on primary APC, we analysed the effect of CpG ODN on BMDDC. Figure 5

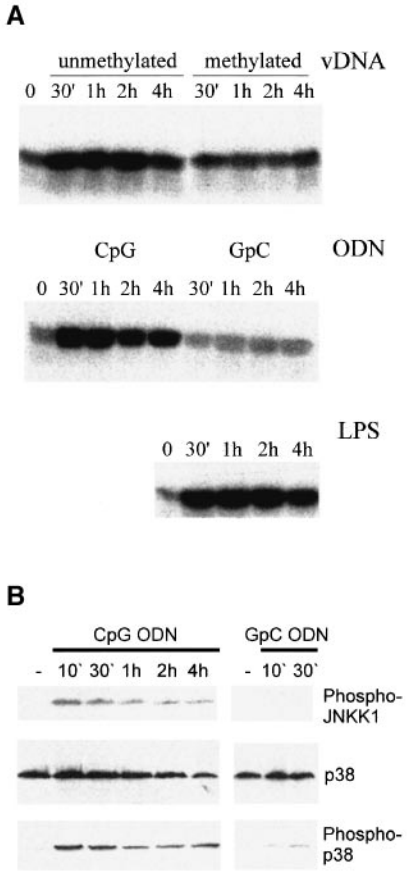

Fig. 3. CpG-DNA activates JNK1/2, p38 and JNKK1. (**A**) ANA-1 cells were stimulated for the indicated times with vDNA or methylated vDNA (30 μg/ml each), CpG ODN or GpC ODN (2 μM each) or LPS (1 μg/ml). Then cells were lysed and kinase activity was determined by immune complex kinase assay with GST-jun(79) as substrate. Glutathione *S*-transferase (GST)-jun(79) (top panel) or antibodies against JNK1/2 (middle and bottom panels) were used to precipitate JNK1/2. (**B**) ANA-1 cells were grown for 36 h in serum-reduced medium (0.5% FCS) and stimulated with CpG ODN or GpC ODN (2 μM each) for the indicated time, lysed and subjected to Western blot analysis using antibodies against p38, the Thr223-phosphorylated form of JNKK1/2 or the Thr180/Tyr182-phosphorylated form of p38.

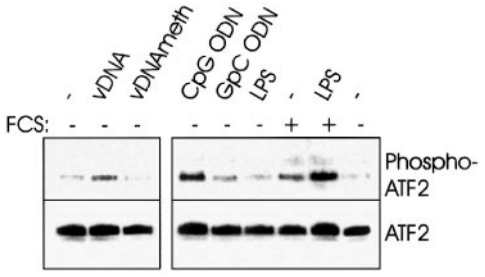

Fig. 4. CpG-DNA-induced ATF2 phosphorylation does not depend on the presence of serum-derived factors. ANA-1 cells were grown in serum-reduced medium (0.5% FCS) for 36 h, washed twice and plated for 2 h in medium without FCS. Cells were then stimulated for 15 min with 20 μg/ml vDNA or methylated vDNA, 2 μM CpG ODN or GpC ODN or 10 ng/ml LPS in the presence or absence of 0.5% FCS as indicated. After stimulation, cells were lysed and subjected to Western blot analysis using antibodies against ATF2 or the Thr69/71-phosphorylated form of ATF2.

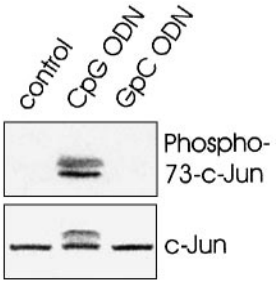

Fig. 5. CpG ODN induces phosphorylation of c-Jun in primary DCs. BMDDC were stimulated in complete culture medium for 20 min with 2 μM CpG ODN or GpC ODN and subjected to Western blot analysis using Ser73-phospho-c-Jun-specific antibodies or antibodies against total c-Jun.

shows that stimulation of these cells resulted in phosphorylation of Ser73 of c-Jun in a strictly CpG-dependent manner. However, stimulation of stress kinases by CpG-DNA is not at all a general phenomenon. Stimulation of NIH 3T3 fibroblasts with CpG ODN failed to induce any stress kinase activity, while TNFα as control induced robust activation of JNK (data not shown).

CpG ODN-induced cytokine release is dependent on p38 activity

As a next step, we examined whether the CpG-DNA-induced stress kinase pathways are relevant for APC effector functions. It is known that p38 plays a crucial role in cytokine release. This kinase has originally been defined by its specific inhibitor SB203580 (Lee *et al*., 1994; Cuenda *et al*., 1995), which belongs to a class of cytokine biosynthesis inhibitors called cytokine-suppressive anti-inflammatory drugs (CSAID). Concentrations of SB203580, previously shown to selectively block p38 (Cuenda *et al*., 1995), severely affected CpG ODN-induced production of TNFα in BMDDC (Figure 6). Moreover, IL-12 secretion is also heavily suppressed by p38 inhibition. Similar results were obtained with the cell lines ANA-1 and RAW264.7 (data not shown). These data implicate p38 activation as essential for CpG ODN-triggered cytokine release by APC.

Non-specific endocytosis of CpG-DNA

The fast kinetics of kinase activation, the cell-type selectivity and the high degree of sequence specificity imply the existence of a specific CpG receptor upstream of the stress kinase pathway. We therefore focused on the upstream mechanism of CpG-DNA-induced signalling. Previous studies using antisense ODN suggested that ODN are endocytosed into acidic vesicles and then further transported to the cytosol and nucleus of cells (Tonkinson and Stein, 1994). Fluorescein isothiocyanate (FITC)-labelled, biologically active CpG ODN are taken up by macrophages and localize in the endosomal–lysosomal compartment in a time-dependent fashion (Figure 7A and E). Very little or no staining is apparent on the plasma membrane or in the cytosol or nucleus even hours after first signalling events such as stress kinase activity can be measured. This uptake is not CpG-specific as non-CpG-containing, biologically inactive ODN as the ODN pZ2

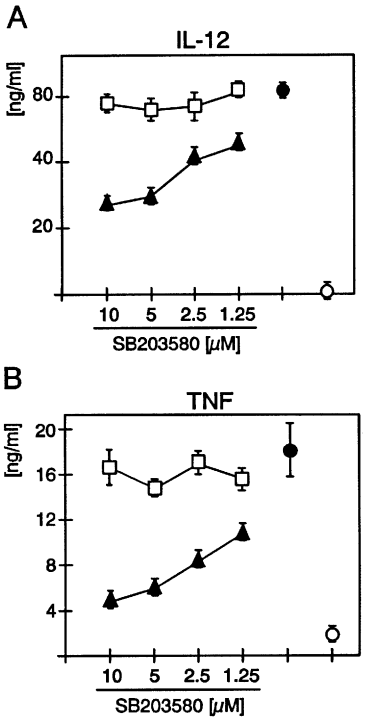

Fig. 6. CpG ODN induced cytokine release depends on p38 activity. BMDDC were pre-incubated for 1 h with the indicated concentration of the p38 inhibitor SB203580 (triangles) or the equivalent amount of its solvent DMSO (squares). After stimulation with 1 μM CpG ODN for 4 h, IL-12 (**A**) and TNFα (**B**) release was determined by ELISA. As controls, cells were not stimulated (open circles) or stimulated with CpG ODN (closed circles) in the absence of SB203580 and DMSO. Error bars indicate duplicate ELISA values.

are endocytosed in a comparable manner (data not shown). Furthermore, non-CpG ODN such as ODN pZ2 effectively block the uptake of labelled CpG ODN (Figure 7B and E). This implied that the uptake, i.e. endosomal translocation of CpG ODN is CpG motif-independent and hence can be competed for by non-CpG ODN. On the other hand it shows, that a receptor-like structure is required for endosomal translocation. As shown below, competitive blockade of cellular uptake (endosomal translocation) directly correlates with inhibition of cell activation by CpG ODN.

To distinguish further between uptake, endosomal maturation and CpG-dependent signalling, we used the compounds bafilomycin A and chloroquine. Chloroquine and bafilomycin A block endosomal maturation primarily through inhibition of vesicular acidification. While chloroquine, a strong base, directly leads to a pH shift in the endosomal vesicles (Ohkuma and Poole, 1981), bafilomycin A specifically blocks vesicular hydrogen ion pumps (Yoshimori *et al*., 1991). Recently, it has been shown that CpG-ODN-mediated mitogenicity to B cells is chloroquine sensitive (Macfarlane and Manzel, 1998). As shown in Figure 7C, bafilomycin A did not block uptake of FITC–CpG ODN, however, the magnitude of endosomal accumulation was reduced (Figure 7C and E). Chloroquine also did not inhibit cellular uptake but enhanced the overall fluorescence signal within the endosomes (Figure 7D and

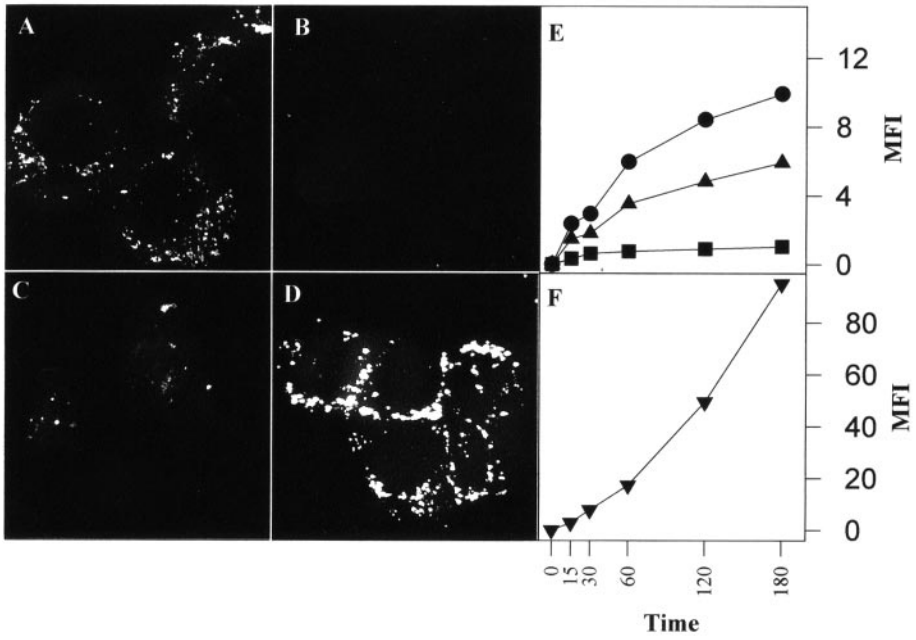

Fig. 7. Uptake of labelled CpG ODN by J774 macrophage cells. J774 cells were incubated with 1 μM FITC–CpG ODN, plus or minus inhibitors, at 37°C for 2 h and visualized by fluorescence microscopy. Cells were not pre-incubated (**A**) or pre-incubated with either 3 μM non-CpG ODN (**B**), 30 nM bafilomycin A (**C**) or 3 μg/ml chloroquine (**D**) before incubation with labeled FITC–CpG ODN. (**E** and **F**) The uptake kinetics of biotinylated CpG ODN into macrophages, plus or minus inhibitors are shown. (E) Biotinylated CpG ODN uptake without inhibitor (●), after pre-incubation with 30 nM bafilomycin A (▲) or when competed by 3 μM non-CpG ODN (■). (F) Biotinylated CpG ODN uptake after pre-incubation with 3 μg/ml chloroquine (▲).

F). Thus, these compounds neither block the putative cell-surface receptor engagement nor cellular uptake.

Inhibition of CpG ODN uptake or endosomal maturation blocks APC activation

Next we analysed the effects of blocking CpG ODN uptake (by non-CpG ODN) or endosomal maturation (by chloroquine or bafilomycin A) on APC activation. As 'read-out', both cytokine production and the respective cytokine promoter activities were measured. Promoter activities were analysed using stable RAW264.7 transfectants, containing the TNFα-, or the IL-12-p40-promoter in front of the luciferase gene. Blockade of CpG ODN uptake into macrophages by non-CpG ODN competition dose-dependently inhibited TNFα, IL-6 and IL-12 secretion (Figure 8A–C), correlating with the promoter activities of the TNFα and IL-12 promoter (Figure 9A and B), but did not interfere with LPS-induced cytokine release (Figure 8D–F) or promoter activity of TNFα (Figure 9A). Bafilomycin A or chloroquine also blocked CpG ODN-mediated cytokine release in a dose-dependent manner (Figure 8), as well as the respective promoter activities (Figure 9). It is noteworthy that neither compound altered cytokine output or promoter activity triggered by LPS (Figures 8 and 9).

To ensure that the cytokine output of the cells examined under the various conditions was not a peculiar feature to these cell lines, TNFα and IL-12 release from BMDDC was investigated. Table I shows that these primary cells display essentially the same pattern of response as the cell lines in the presence of non-CpG ODN, chloroquine or bafilomycin A.

Inhibition of endosomal maturation blocks immediate early p38 kinase activation

Since stress kinase p38 activity represents an important event in CpG-ODN-triggered signalling where it controls TNFα and IL-12 production (see Figure 6), we investigated whether inhibition of endosomal maturation prevents p38 activation. Figure 10 shows that p38 phosphorylation occurs in response to CpG ODN or LPS in BMDDC. Non-CpG ODN, although endocytosed (not shown), do not induce activation of p38 (Figure 10D). Blockade of CpG ODN uptake by competition with non-CpG ODN (Figure 10A) as well as inhibition of endosomal maturation by chloroquine (Figure 10B) inhibits p38 phosphorylation in a dose-dependent manner. In contrast, LPS signalling is not affected by non-CpG ODN (Figure 10C). Importantly, the capacity of non-CpG ODN to inhibit uptake and APC activation by CpG ODN was not a peculiar feature to the sequence of the non-CpG ODN used (pZ2). Although the degree of competition varied between different non-CpG ODN, all of the ODN tested so far ($n = 10$) exhibited this blocking activity (data not shown).

Taken together, these data define endosomal translocation and endosomal maturation as essential steps preceding 'translation' of CpG ODN motifs into 'signalling'.

Discussion

In this report we show that stimulation of APC by CpG-DNA is initiated by the uptake of the CpG-DNA into endosomes. Endosomal maturation is required for subsequent activation of the stress kinase pathway. Stress kinase activation represents a restriction point for CpG-DNA-induced APC stimulation. We conclude that transla-

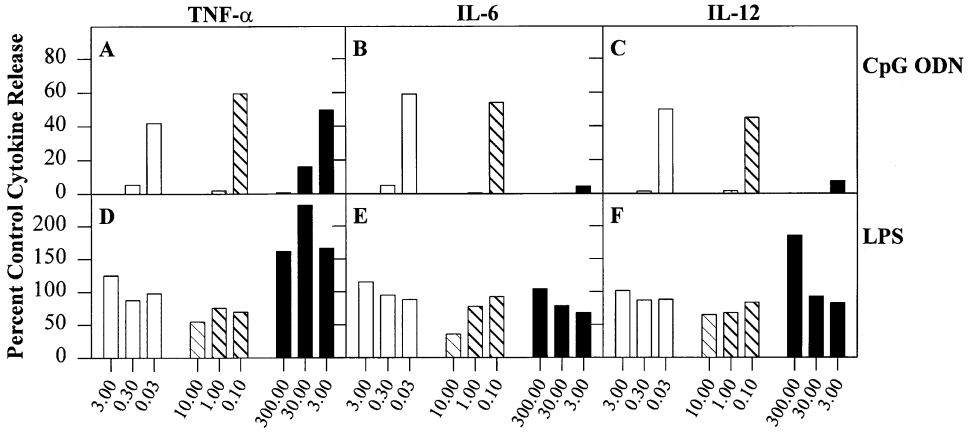

Fig. 8. Inhibition of macrophage cytokine release by blockade of CpG ODN uptake or endosomal maturation. J774 cells were stimulated for 6 h with CpG ODN (1 μM) (A–C) or LPS (10 ng/ml) (D–F) in the presence or absence of different inhibitors and the level of secretion of TNFα (**A** and **D**), IL-6 (**B** and **E**) and IL-12 (**C** and **F**) was determined. The inhibitors, non-CpG ODN (open bars, concentration in μM), chloroquine (hatched bars, concentration in μg/ml) or bafilomycin A (closed bars, concentration in nM) were added to the cells 15 min prior to stimulation. Values are given as percentage of the cytokine output in the absence of inhibitors.

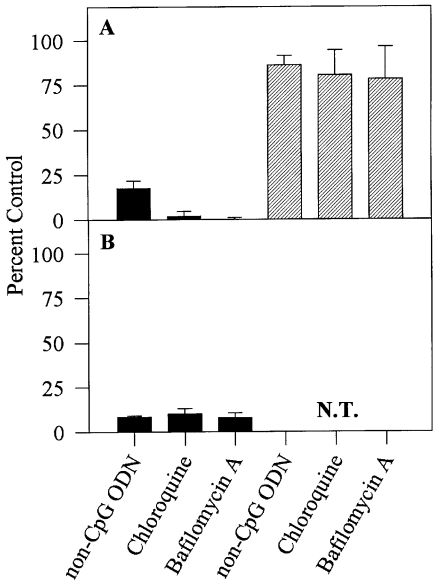

Fig. 9. Inhibition of macrophage signal transduction by blockade of CpG ODN uptake or endosomal maturation. RAW264.7 cells, stably transfected with vectors which utilize the TNFα promoter (**A**) or the IL-12 p40 promoter (**B**) to drive the expression of a luciferase gene, were stimulated for 3 h with CpG ODN (1 μM, closed bars) or LPS (10 ng/ml, hatched bars) to induce luciferase activity measured as arbitrary light units in the presence or absence of the indicated inhibitors (non-CpG ODN, 3 μM; chloroquine, 3 μg/ml; bafilomycin, 30 nM). The results are given as percent control which represents the mean arbitrary light units (n = 3 determinations) in the presence of inhibitor divided by the uninhibited reading. The values represent mean and standard deviations of three determinations. N.T. = not tested.

tion of CpG motifs into stress kinase signalling is conveyed by as yet undefined intracellular structures operating downstream of endosomal maturation.

AP-1 and stress kinase activation

AP-1 appeared to be a potential target for CpG stimulation for various reasons: firstly, this transcription factor is influenced by and integrates different MAP kinase pathways, and secondly, CpG-DNA and LPS induce in APC a similar pattern of effector functions and LPS is known to activate AP-1 (Hambleton *et al.*, 1996). Because we were interested in primary events triggered by CpG-DNA, we concentrated on early time points after activation. Our data clearly show that CpG-DNA induces transcriptional activity of AP-1, yet AP-1-binding activity, i.e. the amount of AP-1 complex, is not induced significantly during the critical time period. However, since phosphorylation of c-Jun at its transactivating sites takes place within minutes we conclude that c-Jun phosphorylation explains the immediate early activation of AP-1. In accordance with these findings, the kinases upstream of c-Jun, JNK1/2 and JNKK1 were found to be activated. Furthermore, p38, a stress kinase originally cloned as a LPS responsive kinase (Han *et al.*, 1993, 1994), as well as its downstream substrate ATF2 (Raingeaud *et al.*, 1996) were also found to be activated by CpG-DNA.

Experiments with specific inhibitors of p38 such as SB203580 or related pyridinyl imidazole drugs have suggested a role for this kinase in the induction of cytokines including TNFα, IL-6 and interferon-γ, as well as the adhesion molecule VCAM-1 (Prichett *et al.*, 1995; Beyaert *et al.*, 1996; Pietersma *et al.*, 1997; Torres *et al.*, 1997). Interestingly, the drugs seem to affect these responses by different mechanisms. For VCAM-1 and TNFα, interference with translation of their mRNAs has been reported (Prichett *et al.*, 1995; Pietersma *et al.*, 1997). For IL-6, reduced mRNA levels have been found (Beyaert *et al.*, 1996) and for interferon-γ, a reduced promoter activity has been observed (Rincon *et al.*, 1998). However, to our knowledge, the p38 targets responsible for these divergent effector functions have not been identified. Here we show that not only TNFα release, but also secretion of IL-12, a macrophage and DC-derived cytokine involved in the control of T helper 1 (Th1) responses, requires p38 activity. Recently, Rincon and colleagues have demonstrated a crucial role for p38 activity in Th1 T-cell-dependent interferon-γ production, an intrinsic function of the adaptive immune system (Rincon *et al.*, 1998). The dependency of IL-12 secretion on p38 activity complements this view from the perspective of the innate immune system. Clearly, more information is required to assess whether the outcome of a complex

Table I. CpG-ODN- or LPS-induced cytokine release from BMDDC

	Stimulus	Blocking agent			
		None	Non-CpG ODN	Chloroquine	Bafilomycin
TNFα	CpG ODN	29.0	<1.0	<1.0	2.5
	LPS	28.0	36.4	22.4	25.0
IL-12	CpG ODN	73.5	9.1	<1.0	6.0
	LPS	44.0	46.0	49.3	25.0

BMDDC were stimulated with CpG ODN (1 μM) or LPS (10 ng/ml) for 3 h in the presence or absence of non-CpG ODN (3 μM), chloroquine (10 μg/ml) or bafilomycin A (30 nM) and cytokine release of TNFα or IL-12 were determined. Values are given as ng/ml of cytokine output and represent the mean of duplicate determinations.

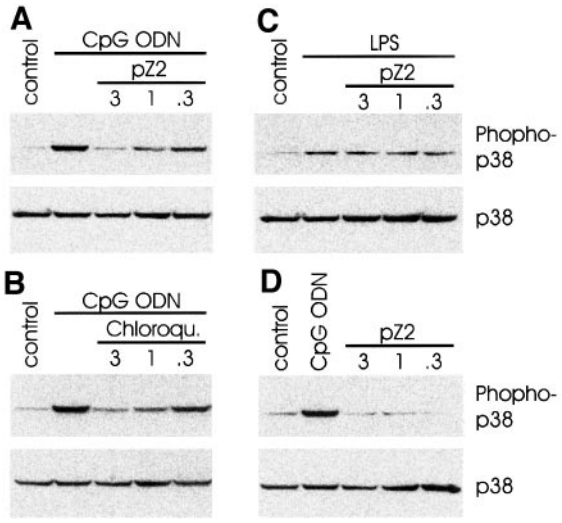

Fig. 10. CpG-ODN-induced phosphorylation of p38 is blocked by non-CpG ODN (pZ2) and chloroquine. BMDDC were stimulated with CpG ODN (1 μM) (**A** and **B**) or LPS (10 ng/ml) (**C**) for 30 min in the presence or absence of the non-CpG ODN pZ2 (A and C) or chloroquine (B) as indicated and phosphorylation of p38 was investigated by phosphospecific antibodies. (**D**) control of pZ2 alone in comparison to CpG ODN alone. The concentration of pZ2 is given in micromoles, and the concentration of chloroquine in μg/ml. Inhibitors were added 15 min before stimulation. Loading of comparable amounts of protein was confirmed by antibodies against total p38 as indicated.

immune reaction can be reduced to the activity of a single kinase.

Cellular uptake and endosomal maturation

Endosomal translocation and downstream signalling of CpG ODN is efficiently blocked by non-CpG ODN (Figures 7–10). Thus, cellular uptake of CpG ODN is not sequence-specific. The ability of unrelated ODN to block cell-surface binding of FITC-conjugated non-CpG ODN has been observed previously (Tonkinson and Stein, 1994). Based on competition analysis it was argued that ODN uptake was receptor mediated. Several cell-surface receptors lacking sequence specificity have been shown to engage ssDNA, including CD11b/CD18 integrins and scavenger receptors (Kimura *et al*., 1994; Benimetskaya *et al*., 1997). Our data show that CpG-DNA enters cells via cell-surface proteins which bind DNA non-specifically and internalize it into an endosomal compartment. The competition of non-CpG-DNA with CpG-DNA sequences may explain why genomic bacterial DNA and plasmid DNA, containing long stretches of non-CpG-DNA are less efficient in activating APCs compared with synthetic CpG ODN (Sparwasser *et al*., 1997b; unpublished observations).

Endosomal maturation may be defined as pH-dependent evolution of early endosomes to lysosomal compartments (reviewed in Mellman *et al*., 1986). The compounds bafilomycin A and chloroquine block this evolution, the former by antagonizing intravesicular hydrogen pumps (Yoshimori *et al*., 1991), and the latter by partitioning into acidified vesicles and acting as neutralizing base buffer (Ohkuma and Poole, 1981). Neither compound significantly altered cellular uptake but modulated endosomal accumulation of ODN, possibly by altering trafficking of lysosomal enzymes and receptors (Chapman and Munro, 1994) or by affecting efflux pathways. Endosomal vesicle compartments with varying ODN efflux rates have been described previously (Tonkinson and Stein, 1994). Using a variety of read-out systems such as cytokine production, cytokine-promoter activity and immediate early activation of the stress kinase p38, we consistently observed that all these CpG ODN-driven responses were sensitive to bafilomycin A and chloroquine (see Figures 8–10). The blockade of CpG ODN uptake by non-CpG ODN and the observation that uptake alone into the cell is not sufficient for signalling strongly suggests that uptake is a prerequisite for CpG-dependent signalling. The exquisite sensitivity of CpG-driven responses to the compounds chloroquine and bafilomycin A identifies endosomal maturation as a critical step for translation of CpG ODN sequences into cellular signalling. This pH-dependent intracellular step precedes not only activation of the stress kinase pathway but also generation of ROS associated with NFκB translocation (Yi *et al*., 1998). The process from uptake of material to the delivery into its final destination is generally referred to as 'endosomal maturation' and such maturation is necessary for activation of APC by CpG-DNA. More specifically, inhibitors of endosomal acidification were found to prevent initiation of downstream events which implies that a shift to low pH is necessary. Such pH changes could either trigger a dissociation of CpG-DNA from a non-specific (cell surface) receptor or enable binding of CpG-DNA to a specific (endosomal) receptor. Alternatively, a less well-defined molecular mechanism downstream of acidification might be responsible for DNA-signal transduction.

As for bacterial DNA, the mechanism by which sensitive cells respond to the bacterial product LPS has not been defined. Nevertheless, several features distinguish CpG-DNA from LPS: (i) CpG-DNA-induced signalling still

functions in cells and animals which are LPS resistant (Sparwasser *et al*., 1997b); (ii) LPS requires the presence of the LPS-binding protein in serum and hence LPS signalling is sensitive towards serum withdrawal while CpG-DNA is not (Figure 4); and (iii) CpG-DNA, but not LPS, is sensitive towards inhibitors of endosomal maturation such as bafilomycin A or chloroquine. According to this, the initiation point of CpG- and LPS-dependent signalling must be different. However, as we show here, these two structurally unrelated compounds seem to utilize similar signal transduction pathways such as the SAPK pathway further downstream, leading to a similar pattern of effector function.

In conclusion, we have demonstrated that cellular uptake via non-specific endocytosis and subsequent endosomal maturation precede activation of members of the stress kinase pathway, triggered by CpG-DNA. After endosomal maturation, CpG-DNA appears to engage specific receptors which are able to discriminate CpG-motif sequences. Identification of CpG ODN-specific 'binding structures' will be the next step towards understanding the immunobiology of CpG-DNA.

Materials and methods

Cell culture and generation of BMDDC

Murine macrophage cell lines ANA-1, RAW264.7 and J774 were cultured in LowTox Clicks/RPMI 1640 (Biochrom, Berlin, Germany) supplemented with 10% (v/v) FCS (Hyclone Lab. Inc., Logan, UT), 50 μM 2-mercaptoethanol and antibiotics [penicillin G (100 IU/ml of medium) and streptomycin sulfate (100 IU/ml of medium)]. In this manuscript, this medium is referred to as complete culture medium.

BMDDC from C57BL/6 mice were prepared as described (Inaba *et al*., 1992). 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 onto a 100 mm tissue culture Petri dish in complete medium supplemented with GM-CSF (2 U/ml) (Zal *et al*., 1994). Cells were used between day 7 and day 10 when mature DC (GR-1$^-$, MHC class II^{high}, CD86^{high}) represented 30–40% of the resulting cell population. For stimulation, non-adherent cells were used. Stimulation of this cell population with LPS or CpG-DNA leads to expression of the DC marker CD11c on ~80% of the cells. Approximately 20% of the cells exhibit an immature, CD14 negative phenotype.

Determination of cytokines

Cytokine levels were determined using commercially available ELISA kits (TNFα and total nIL-12-Duoset) according to the instructions of the manufacturer (Genzyme, Germany). Each value shown represents the mean of duplicate values.

Preparation of plasmids and reagents

Plasmid (pBluescript, Stratagene), grown in *Escherichia coli* strain DH5α and prepared using a plasmid purification kit according to the manufacturer's instructions (Qiagen) were incubated with or without Sss I (CpG) Methylase (New England Biolabs) until CpG methylation was complete (controlled by *Hpa*II digestion) and then purified again using Qiagen's EndoFree Plasmid kit. Phosphothioate stabilized CpG ODN (TCCATGACGTTCCTGATGCT) and GpC ODN (TCCATGAGCTTCCTGATGCT) (Krieg *et al*., 1995) and ODN pZ2 (CTCCTAGTGGGGGTGTCCTAT), as well as additionally modified ODN (biotinylated/FITC-labelled ODN) were purchased from TIB MOLBIOL (Berlin, Germany), LPS (*E.coli*) and phorbol 12-myristate 13-acetate (PMA) were from Sigma.

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

To investigate AP-1 transcriptional activity in transient assays, we used a plasmid containing a cassette of three TRE (AP-1) sites from the human collagenase gene in front of an interferon β-minimal promoter (–55 to +9) and the luciferase gene terminated by a SV40 poly(A) signal. This cassette was cloned into a plasmid containing a kanamycin resistance gene (pGFP-1, Clontech) thereby replacing the coding sequence of green fluorescent protein (GFP). ANA-1 cells (5×10^6) were transfected by electroporation with 20 μg reporter plasmid in 400 μl final volume (RPMI/25% FCS) at 220 V/960 μF in a Bio-Rad gene pulser. After electroporation, cells were washed and plated for 3 h at 37°C. Then cells were split and 5×10^5 cells each were stimulated in 0.5 ml growth medium with 15 μg/ml vDNA (pBluescript KS, Stratagene) or 1 μM phosphothioate-stabilized oligonucleotides or 10 ng/ml LPS for 36 h. Preparation of cell extracts and luciferase assays were performed according to the manufacturer's instructions (Promega).

To investigate activity of the TNFα- and IL-12 p40-promoter and AP-1 activity in stable RAW264.7 transfectants, the following plasmids were used. The TNFα promoter region was kindly provided by Victor Jongeneel. To obtain the TNFα luciferase reporter vector, a 1.2 kb *Bam*HI–*Xba*I fragment of the TNFα promoter was subcloned from pBLCAT3 (Luckow and Schutz, 1987) into the GL3 basic luciferase vector (Promega). The IL-12 p40 luciferase reporter vector was a generous gift of Kenneth Murphy and contains the –703 bp region of the IL-12 p40 gene (Murphy *et al*., 1995). To obtain the AP-1 luciferase reporter used here, the AP-1 luciferase cassette mentioned above was subcloned into the GL3 vector (Promega). Three kilobases upstream and in inverse direction, a PGK promoter driven neomycin gene was inserted for G418 selection.

Stable RAW264.7 transfectants were established by cotransfection (electroporation) of the TNFα and IL-12 reporter vectors and the pGFP-1 vector described (Clontech), containing a neomycin-resistance cassette in a ratio of 10:1 or the AP-1 reporter plasmid alone. One day after transfection, cells were overlaid by soft agar containing 0.4 μg/ml G418 (Gibco-BRL). G418 resistant clones were picked, expanded and tested for inducible luciferase activity. For this purpose the TNFα reporter transfectants were stimulated with LPS (10 ng/ml) for 8 h, IL-12 reporter transfectants were pre-incubated with interferon-γ (100 U/ml) for 12 h and stimulated for additional 8 h with LPS (10 ng/ml), AP-1 reporter transfectants were stimulated for 8 h with PMA (5 ng/ml). After stimulation, luciferase activity was determined.

Electromobility shift assay

To study AP-1-binding activity in ANA-1 cells, 10^7 cells were stimulated in complete culture growth medium with 2 μM CpG ODN for 4 h and nuclear protein extracts were prepared essentially as described (Schmid *et al*., 1991). To detect AP-1-binding activity, a double-stranded, ^{32}P-end-labeled oligonucleotide spanning a TRE motif was used (sense 5′-AGCTTACTCAGTACTAGTACG-3′ and antisense 5′-AATTCGTACTAGTACTGAGTA-3′). Labeled double-stranded probe (80 000 c.p.m.) was added to 10 μg of nuclear protein in the presence of 1 μg poly(dI–dC) as a non-specific competitor (Pharmacia). Binding reactions were carried out in 10 mM Tris–HCl pH 7.5, 100 mM NaCl, 4% glycerol for 15 min at room temperature. DNA protein complexes were resolved by electrophoresis on a 4% non-denaturing polyacrylamide gel in 1× Tris glycine EDTA buffer. Gels were dried and exposed to Kodak XAR-5 film. The specificity of AP-1 DNA binding was confirmed in competition experiments. Competition was performed using a 20-fold excess of unlabeled oligonucleotide containing the AP-1/TRE motif or an unrelated oligonucleotide spanning an SP-1 binding site.

JNK1/2 kinase assay and Western blotting

To determine kinase activity induced by CpG-DNA, cells were serum starved (0.5% FCS) for 16 h and stimulated as indicated in the figure legends. Lysates were prepared and kinase activity was determined essentially as described (Kieser *et al*., 1997). In brief, lysates were cleared by centrifugation at 10 000 *g* for 10 min and incubated with GST-jun(79) or antibodies to JNK1/2 to precipitate Jun N-terminal kinases. Subsequently, precipitates were incubated in kinase buffer in the presence of [γ-^{32}P]ATP. GST-jun(79) was used as substrate. Reactions were stopped by boiling in SDS sample buffer, resolved on a 10% SDS gel and visualized by autoradiography.

For Western blotting, cells were grown and stimulated as indicated in the figure legends. After stimulation cells were lysed in SDS sample buffer. Crude lysates were resolved on a 10% SDS gel and blotted onto nitrocellulose membranes. Membranes were probed with the indicated antibodies and visualized using enhanced chemiluminescence (ECL kit, Amersham) for detection. All antibodies were purchased from New England Biolabs.

ODN uptake

For these studies, the macrophage cell line J774 was used, as this cell line has the most outspread shape, improving the microscopic resolution

of different cellular compartments. J774 cells were plated at a density of 2×10^5 cells/well in 24-well flat bottom culture plates the day before analysis. For photographic documentation, FITC-labelled CpG ODN was incubated with J774 cells for 2 h at 37°C after which the cells were harvested, washed in cold phosphate-buffered saline (PBS) and cytospun onto glass slides. The slides were fixed with cold 95% ethanol and 5% acetic acid for 10 min, washed and mounted. The slides were imaged with Leica confocal microscope. For blocking treatments, cells were incubated with the blocking compound 15 min prior to FITC–CpG ODN addition. For fluorescence-activated cell sorter (FACS) analysis, biotinylated CpG ODN or pZ2 were incubated with cells for different time periods. The cells were then washed in PBS, fixed with 1.0% paraformaldahyde, washed again and incubated for 30 min with 0.1% saponin in PBS containing FITC-labelled strepavidin, washed again (all steps at 4°C) and analyzed on a Coulter Epics XL. Non-specific staining was determined by incubating in the absence of biotinylated ODN. Surface staining was determined by not including saponin.

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