

## **Dendritic Cell Survival and Maturation Are Regulated by Different Signaling Pathways**

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### **Summary**

Although dendritic cell (DC) activation is a critical event for the induction of immune responses, the signaling pathways involved in this process have not been characterized. In this report, we show that DC activation induced by lipopolysaccharide (LPS) can be separated into two distinct processes: first, maturation, leading to upregulation of MHC and costimulatory molecules, and second, rescue from immediate apoptosis after withdrawal of growth factors (survival). Using a DC culture system that allowed us to propagate immature growth factor-dependent DCs, we have investigated the signaling pathways activated by LPS. We found that LPS induced nuclear translocation of the nuclear factor (NF)- $\kappa$ B transcription factor. Inhibition of NF- $\kappa$ B activation blocked maturation of DCs in terms of upregulation of major histocompatibility complex and costimulatory molecules. In addition, we found that LPS activated the extracellular signal-regulated kinase (ERK), and that specific inhibition of MEK1, the kinase which activates ERK, abrogated the ability of LPS to prevent apoptosis but did not inhibit DC maturation or NF- $\kappa$ B nuclear translocation. These results indicate that ERK and NF- $\kappa$ B regulate different aspects of LPS-induced DC activation: ERK regulates DC survival whereas NF- $\kappa$ B is responsible for DC maturation.

Key words: dendritic cells • maturation • survival • mitogen activated protein kinases • nuclear factor  $\kappa$ B

Dendritic cells (DCs) are now recognized as major players in the regulation of immune responses to a variety of antigens, including bacterial and viral agents (1). DCs direct both the quality and the extent of the immune response, but the ability of DCs to activate naive T cells depends on their maturation. Fully mature DCs have a high surface expression of MHC and costimulatory molecules and are located in lymphoid organs. In contrast, immature DCs are mainly distributed in tissues interfacing with the external environment where they capture and process antigens with high efficiency (1). After microbe internalization and inflammation, DC leave the tissue to reach the lymphoid organs. During this migration the DC undergo maturation and acquire the ability to prime T cells. This second functional stage is irreversible and is followed by apoptosis (2). Among the inflammatory signals that induce DC maturation, LPS, a Gram-negative bacterial cell wall component, has been shown to fully activate DC both in vitro and in vivo (2–4).

To oppose bacterial infections and inflammation, DCs have to respond rapidly to changes in their microenvironment. Most types of signals induce cellular responses by

binding to specific cell-surface receptors that respond to occupancy by triggering one or more signal transduction pathways (5). One of the most common responses to receptor engagement is the activation of transcription factors and the synthesis of new proteins. Among the transcription factors, the active heterodimer p50/p65 form of nuclear factor (NF)- $\kappa$ B plays a central role in immunological processes by inducing expression of a variety of genes involved in inflammatory responses (6). In macrophages, NF- $\kappa$ B can be activated by exposure to LPS as well as by inflammatory cytokines (TNF- $\alpha$  and IL-1) and viral infections (7–9). Mature DCs express high levels of the NF- $\kappa$ B family of transcription factors (10) and signaling by members of the TNF- $\alpha$  receptor family, such as CD40 and RANK, results in activation of NF- $\kappa$ B (1). Other transcription factors are regulated by signal transduction pathways that involve enzymatic cascades of mitogen-activated protein (MAP) kinases. The latter are activated by many receptors, as well as by environmental stresses, and have been shown to mediate both mitogenic and apoptotic responses (11–14). LPS has been shown to activate the extracellular signal-regulated kinase, c-Jun NH<sub>2</sub>-terminal kinase (JNK), and p38 MAP

kinases in murine macrophages (15–17) but, to date, the signal transduction pathways activated during DC maturation have not been characterized.

In vitro studies of immature mouse DCs have been hampered because it has not been possible to arrest spontaneous DC maturation and cell death (18). Recently, we have described a DC culture system (D1 cells) that allows us to maintain the immature DC phenotype in vitro. DC proliferation is growth factor dependent, and maturation can be induced by inflammatory signals and by bacteria (2, 19), thus mimicking the in vivo response (3). Survival of the immature D1 cells is maintained by a mixture of cytokines contained in the DC conditioned medium (CM). Deprivation of CM causes D1 cells to undergo apoptotic cell death within 48 h. Using this unique system, we were able to investigate the kinetics of DC maturation and survival and to identify some of the molecular events involved in these processes. Here it is shown that, in addition to inducing DC maturation, LPS arrests DC proliferation and promotes DC survival after CM deprivation. We also found that LPS activates both the ERK MAP kinase and the NF- $\kappa$ B transcription factor. The two signal transduction pathways are independent and regulate different aspects of LPS-induced DC activation.

## Materials and Methods

**Cells and Reagents.** The D1 cells were derived from murine splenic DCs and maintained in vitro as growth factor-dependent immature DCs (2, 19). D1 cells were grown in complete IMDM supplemented with 30% R1 CM containing 30 ng/ml GM-CSF as previously described (2).

LPS (*Escherichia coli* serotype 026:B6) and *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) were purchased from Sigma Chemical Co. (St. Louis, MO). MEK inhibitor PD98059 was from BIOMOL Research Labs., Inc. (Plymouth Meeting, PA).

**Cell Stimulation and Preparation of Cell Lysates.**  $5 \times 10^6$  D1 cells were resuspended in 1 ml of modified Hepes-buffered saline (20), warmed to 37°C, and stimulated with LPS (10  $\mu$ g/ml) for the indicated times. Where indicated, the cells were pretreated at 37°C with 50 or 100  $\mu$ M MEK inhibitor PD98059 for 30 min or with 15  $\mu$ M TPCK for 1.5 h before being stimulated with LPS. Reactions were stopped by adding ice-cold PBS containing 1 mM  $\text{Na}_3\text{VO}_4$  and then centrifuging the cells for 3 min in the cold. The cells were pelleted and then solubilized in buffers containing 1% Triton X-100 or NP-40 as well as protease and phosphatase inhibitors. Detergent-insoluble material was removed by centrifugation.

**In Vitro Kinase Assays.** In vitro kinase assays for ERK, JNK, and MAP kinase-activated protein (MAPKAP) kinase-2 have been described previously (21). For ERK assays, lysates from  $5 \times 10^6$  D1 cells were immunoprecipitated with an agarose-conjugated antibody that recognizes ERK2, and to a lesser extent ERK1 (antibody C-14; Santa Cruz Biotechnology Inc., Santa Cruz, CA). For JNK and MAPKAP kinase-2 assays, lysates from  $5 \times 10^6$  D1 cells were immunoprecipitated with an antibody to either JNK1 (antibody C-17; Santa Cruz Biotechnology) or MAPKAP kinase-2 (Upstate Biotechnology Inc., Lake Placid, NY) and immune complexes were collected on protein A-Sepharose (Sigma Chemical Co.). After washing, immune complexes were incubated with  $^{32}\text{P}$ - $\gamma$ -ATP and either myelin basic protein

(MBP; Sigma Chemical Co.) for ERK assays, a glutathione S-transferase (GST)-c-Jun fusion protein (21) for JNK assays, or recombinant hsp 25 (StressGen, Victoria, British Columbia, Canada) for MAPKAP kinase-2 assays. The samples were separated by SDS-PAGE and the transfer of  $^{32}\text{P}$  to the substrates was quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

**Assessment of Apoptosis by Flow Cytometry.** Cells were preincubated or not with 50  $\mu$ M PD98059 for 30 min and then with 10  $\mu$ g/ml LPS for 8, 24, or 48 h. Cells were detached, double stained with FITC-conjugated Annexin V (PharMingen, San Diego, CA) and 1.25  $\mu$ g/ml propidium iodide (Sigma Chemical Co.), and analyzed by flow cytometry.

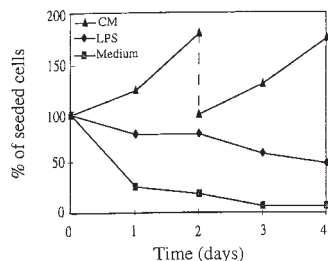
**Antibodies, Cytokine Assays, and Flow Cytometry Analysis.** At different time points after LPS activation, D1 cells were detached with PBS/2 mM EDTA and incubated with one of the following mAbs: anti-(I-A<sup>d</sup>/I-E<sup>d</sup>) or anti-CD86 (B7.2; PharMingen). Staining was carried out in the presence of 2-4G2 (anti-CD32) antibody supernatant to block Fc receptor binding. The cells were washed and analyzed using the FACScan<sup>®</sup> (Becton Dickinson, San Jose, CA). Culture supernatants were collected 24 h after treatment with LPS in the presence or absence of indicated concentrations of PD98059, and TNF- $\alpha$  was measured by TNF- $\alpha$  DuoSet ELISA following manufacturer's instructions (Genzyme Corp., Cambridge, MA).

**NF- $\kappa$ B Nuclear Translocation.** D1 cells were pretreated or not with 50  $\mu$ M MEK inhibitor or 15  $\mu$ M TPCK and activated with LPS (10  $\mu$ g/ml) for 30 min, 1 h, or 4 h. Cells were then washed and plated on poly-L-Lysine-treated coverslips and processed for immunofluorescence. Coverslips were stained with rabbit anti-p65 (Rel A), purchased from Santa Cruz Biotechnology. Anti-rabbit CY3 conjugate was from Nycomed Amersham plc (Little Chalfont, Buckinghamshire, UK).

## Results and Discussion

**LPS Induces both Survival and Maturation of DC.** Murine Langerhans cells purified from the skin as well as bone marrow-derived DCs have a limited life-span in culture even in the presence of granulocyte-macrophage colony stimulating factor (1). Cultured DCs undergo spontaneous maturation and cell death (18). We have described a DC culture system (D1 cells) that allows us to maintain the immature DC phenotype in vitro. Growth of D1 cells is supported by a pool of cytokines present in CM. We have previously shown that incubation of D1 cells with LPS induced functional maturation of the cells (2), here we have shown that it also arrests the cell cycle, and promotes survival after CM deprivation (Fig. 1). More than 50% of the cells were still viable 5 d after growth-factor withdrawal, whereas in the absence of LPS, cells died within 48 h. This indicates that LPS promotes survival of DCs.

**LPS Activates ERK Kinases.** To understand the molecular mechanisms involved in DC maturation, we investigated the signaling pathways activated by LPS in D1 cells. MAP kinases are activated by many receptors, as well as by environmental stresses, and have been shown to mediate both mitogenic and apoptotic responses (11–14). There are three main families of MAP kinases that are involved in signal transduction, the ERKs, the JNKs, and the p38 kinases.



**Figure 1.** LPS induces growth arrest and survival of D1 cells after CM deprivation. Cells were incubated either in medium or CM with or without 10  $\mu$ g/ml of LPS for the indicated times. The number of viable cells remaining at the different time points is shown as a percentage of the cells seeded at time 0.

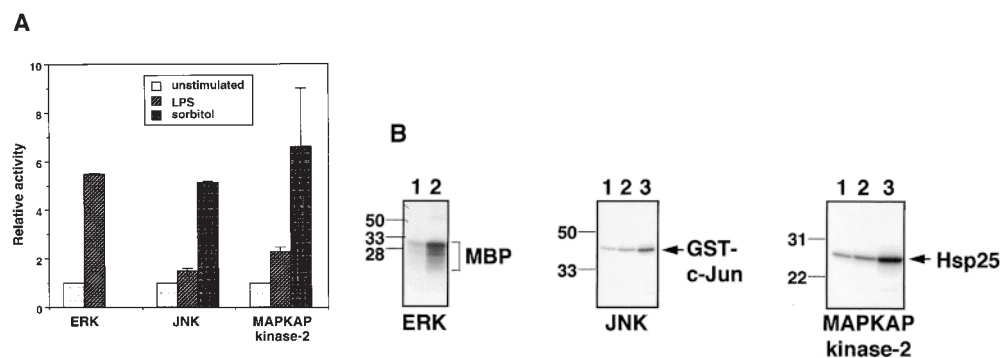
These kinases are activated by MAP kinase kinases (MKKs), which phosphorylate threonine and tyrosine residues in the Thr-X-Tyr activation motif (22, 23). Upon activation, MAP kinases can migrate to the nucleus where they phosphorylate and activate transcription factors. Each MAP kinase family targets a different set of transcription factors.

Since LPS has been shown to activate the ERK, JNK, and p38 MAP kinases in murine macrophages (15–17), we investigated whether LPS activated MAP kinases in the D1 cells. The D1 cells were incubated with LPS and *in vitro* kinase assays were performed to measure the activity of ERK1/2, JNK1, and MAPKAP kinase-2, a downstream target of the p38 MAP kinase. We chose to assay MAPKAP kinase-2 activity instead of directly assaying p38 MAP kinase activity since the MAPKAP kinase-2 assay is more sensitive. Activation of MAPKAP kinase-2 has been shown to be entirely dependent on p38 activation (reference 24; Sutherland, C.L., and M.R. Gold, unpublished results). We found that exposing D1 cells to LPS for 15 min resulted in a fivefold activation of the ERK MAP kinases (Fig. 2). In contrast, LPS caused very little activation ( $\sim$ 1.5-fold stimulation) of the JNK MAP kinase and only modest activation (2–2.5-fold stimulation) of MAPKAP kinase-2. Thus, the ERK MAP kinases appear to be the major MAP kinase target of LPS signaling in D1 cells.

**ERK Is Involved in DC Survival but not Maturation during LPS Treatment.** To evaluate the role of ERK in both DC survival and DC maturation, we investigated the effect of a highly selective inhibitor of the ERK pathway on D1 cells.

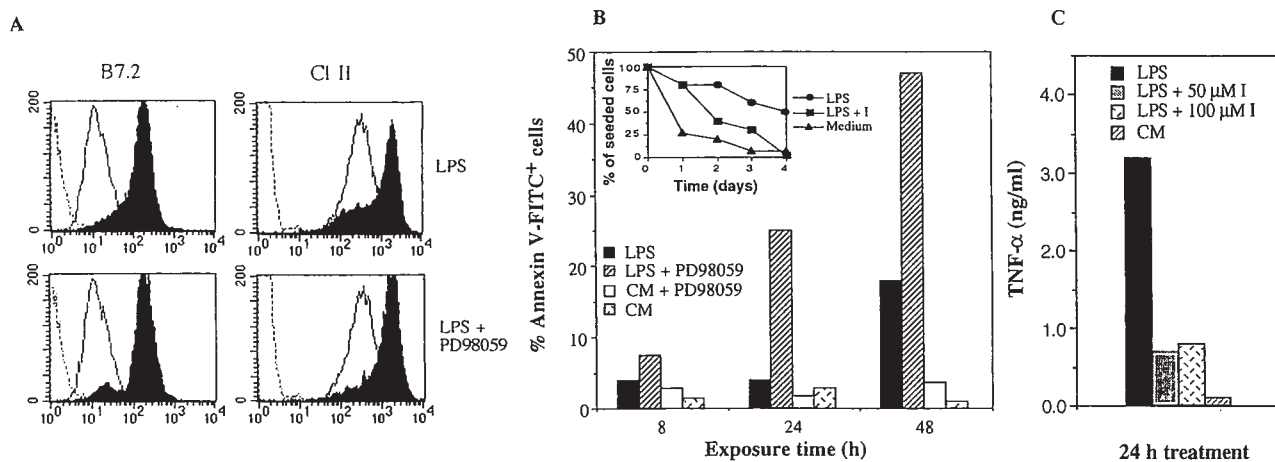
PD98059 (25) is a specific inhibitor of MEK1, the kinase that phosphorylates and activates the ERK kinases. DC maturation correlates with the upregulation of a panel of surface markers including MHC and costimulatory molecules. We found that pretreating D1 cells with the MEK inhibitor PD98059 had no effect on the ability of LPS to increase the surface expression of MHC and costimulatory molecules (Fig. 3 A). Thus ERK activation is not involved in DC maturation.

In contrast, we found that MEK activity was essential for DC survival. We found that the ability of LPS to prevent apoptosis of D1 cells was dramatically reduced when the cells were pre-treated with the MEK inhibitor PD98059 (Fig. 3 B). Apoptosis of D1 cells due to growth factor withdrawal was analyzed using annexin V-FITC, which detects the appearance of the early apoptotic marker phosphatidylserine on the cell surface. D1 cells cultured in medium alone, in the absence of CM, underwent rapid apoptosis with 20% of the cells binding annexin V-FITC after 8 h (data not shown). In the presence of LPS, the number of apoptotic D1 cells was reduced to  $<$ 5% after 24 h and  $\sim$ 18% after 48 h (Fig. 3 B). However, when the D1 cells were pretreated with the MEK inhibitor, the ability of LPS to prevent apoptosis was greatly reduced with 25% of the cell becoming apoptotic after 24 h and nearly 50% being apoptotic after 48 h (Fig. 3 B). The same feature was observed when viability of the cells was analyzed at different time points (Fig. 3 B, *inset*). Although PD98059 blocked the ability of LPS to prevent apoptosis of D1 cells, it did not block the ability of CM to prevent apoptosis. This shows that PD98059 is not toxic to the D1 cells, that its ability to block LPS-induced survival of D1 cells is a specific effect, and that the ability of CM to prevent D1 cell apoptosis does not depend on the MEK/ERK pathway. Thus, activation of the MEK/ERK pathway is required for LPS, but not CM, to prevent apoptosis of D1 cells due to growth factor withdrawal. The role of ERK in promoting the survival of D1 cells is consistent with the finding that ERK activation is also essential for preventing apoptosis of PC-12 cells after growth factor withdrawal (14).



**Figure 2.** Activation of MAP kinases by LPS. D1 cells were incubated with or without 10  $\mu$ g/ml LPS for 15 min. As a positive control for activation of the stress-activated MAP kinase pathways (the JNK and p38/MAPKAP kinase-2 pathways), D1 cells were exposed to high osmolarity conditions by treating them with 0.6 M sorbitol for 15 min. *In vitro* kinase assays were performed using MBP as a substrate for ERK, GST-c-Jun as a substrate for JNK, and hsp25 as a substrate for MAPKAP kinase-2. (A) MAP

kinase activities relative to those in unstimulated cells (defined as 1.0). The transfer of  $^{32}$ P to the various substrates was quantitated with a PhosphorImager. The data represent the average and range of two independent experiments. (B) Representative autoradiographs of the *in vitro* kinase assays for ERK, JNK, and MAPKAP kinase-2. Lane 1, unstimulated cells; lane 2, LPS-stimulated cells; lane 3, sorbitol-treated cells.



**Figure 3.** MEK inhibitor does not influence maturation but induces apoptosis of DCs. (A) Flow cytometric analysis of B7.2 and MHC class II in D1 cells. Cells were preincubated or not with 50  $\mu$ M PD98059 for 30 min and then treated with 10  $\mu$ g/ml of LPS for 24 h in medium alone (IMDM + 10% FCS). Histograms: *black*, treated cells; *white*, untreated cells; *broken outline*, isotype control. (B) DCs were incubated with 10  $\mu$ g/ml LPS in the presence or absence of 50  $\mu$ M PD98059 (*I*) in medium alone or with or without 50  $\mu$ M PD98059 in CM. Cells were double stained with annexin V-FITC and propidium iodide and analysed by flow cytometry. Percentage of single positive cells for annexin V-FITC has been plotted. In the inset the number of viable cells remaining at the different time points is shown as a percentage of the cells seeded at time 0. (C) TNF- $\alpha$  production induced by LPS is inhibited by PD98059. Cells were preincubated with 50 or 100  $\mu$ M inhibitor (*I*) and then treated for 24 h with 10  $\mu$ g/ml LPS. Culture supernatants were tested by ELISA for the presence of TNF- $\alpha$ .

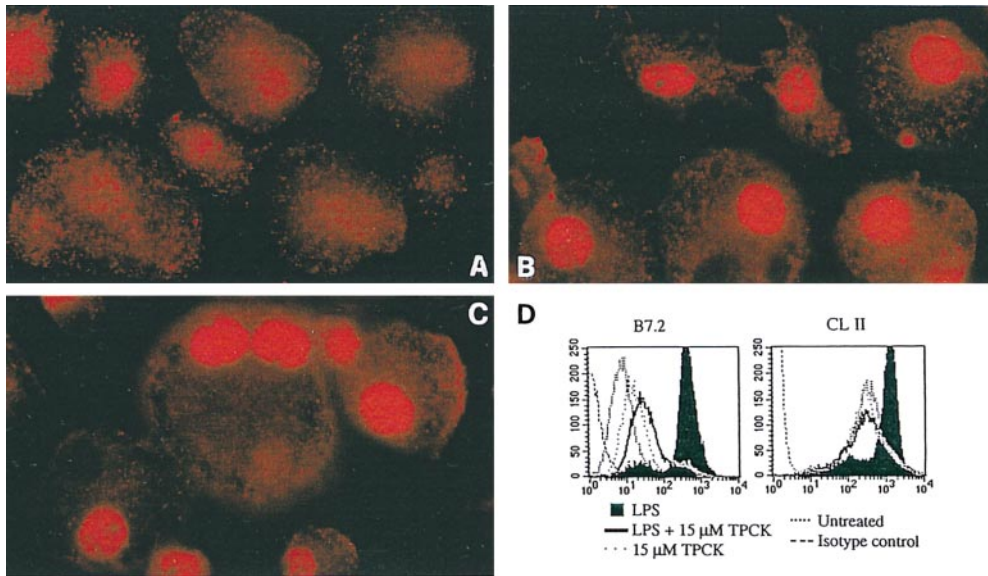
**ERK Activation Is Necessary to Promote TNF- $\alpha$  Production.** Since TNF- $\alpha$  has been shown to maintain the viability of Langerhans cells in culture (26), we investigated whether the MEK inhibitor blocked the ability of LPS to stimulate TNF- $\alpha$  production by D1 cells. Fig. 3 C shows that LPS causes a large increase in TNF- $\alpha$  release by D1 cells but that this response is substantially reduced in the presence of the MEK inhibitor PD98059. Thus, TNF- $\alpha$  production correlates with DC viability and the ability of LPS to prevent apoptosis of D1 cells.

**LPS Induces Nuclear Translocation of NF- $\kappa$ B.** Experiments using the MEK inhibitor PD98059 showed that the MEK/ERK pathway is essential for LPS to promote DC survival but not for LPS-induced DC maturation. To elucidate the signaling requirements for LPS-induced DC maturation, we investigated whether NF- $\kappa$ B is involved in this maturation program. The NF- $\kappa$ B transcription factor (p50-p65) is the prototype of a family of homodimeric and heterodimeric protein complexes comprised of subunits related to the c-rel protooncogene. Five mammalian proteins of the Rel/NF- $\kappa$ B family, NF- $\kappa$ B1 (p50, p105), NF- $\kappa$ B2 (p52, p100), Rel A (p65), Rel B, and Rel have been described so far (for review see references 27, 28). These proteins are widely expressed and regulate transcription by binding to decameric sequences ( $\kappa$ B motifs) that control transcription, particularly of proteins involved in immune and inflammatory responses (6, 29). Before stimulation, Rel/NF- $\kappa$ B is retained in the cytoplasm in an inactive form due to its binding to the inhibitor (I $\kappa$ B) proteins (27). In response to a number of different stimuli, I $\kappa$ B is first phosphorylated and then ubiquitinated and targeted to the proteasome for degradation. This allows Rel/NF- $\kappa$ B to translocate to the nucleus and activate transcription of target genes.

Mature DCs express high levels of the NF- $\kappa$ B family of transcription factors (10) and signaling by members of the TNF- $\alpha$  receptor family, such as CD40 and RANK, results in activation of NF- $\kappa$ B (1). We found that a small proportion of activated Rel A protein was present in the nucleus of immature D1 cells, but that a 30-min treatment with LPS induced massive translocation of the p65 molecule to the nucleus (Fig. 4, A and B). LPS-induced nuclear translocation of p65 was not blocked by the MEK inhibitor, indicating that NF- $\kappa$ B activation does not depend on the MEK/ERK pathway (Fig. 4 C). This is consistent with recent findings that activation of NF- $\kappa$ B by TNF- $\alpha$  or IL-1 involves the NF- $\kappa$ B inducing kinase (NIK)/IKK kinase complex (27, 30), which is independent of the ERK pathway. This pathway is of particular interest because the number of catalytic steps is minimized in order to reduce the chances that low molecular weight metabolites such as those produced by microorganisms can suppress the immune response (27). Nevertheless, a recent report has shown that bacteria of the *Yersinia enterocolitica* strain can modulate the immune response of the host by interfering with activation of NF- $\kappa$ B transcription factor in J774 macrophage cell line (31). This strategy is used by the bacteria to suppress TNF- $\alpha$  production and this contributes to trigger macrophage cell death by apoptosis.

**NF- $\kappa$ B Activation Is Responsible for DC Maturation.** To test whether activation of NF- $\kappa$ B is involved in LPS-induced DC maturation, we used the serine protease inhibitor TPCK, which blocks nuclear translocation of Rel/NF- $\kappa$ B by preventing I $\kappa$ B- $\alpha$  degradation. Chloromethyl ketones such as TPCK have been shown to block NF- $\kappa$ B-dependent nitric oxide synthase in murine macrophages (32) and to induce apoptosis in murine B cells (33) by preventing Rel/





**Figure 4.** (A–C) Nuclear translocation of NF- $\kappa$ B following LPS activation. Cells were stained with a rabbit polyclonal antibody to p65 (Rel A) protein and with anti-rabbit Cy3 conjugated antibody. A, untreated cells; B, cells treated for 30 min with 10  $\mu$ g/ml LPS; C, cells preincubated for 30 min with 50  $\mu$ M PD98059 and then treated with 10  $\mu$ g/ml LPS for an additional 30 min. (D) Flow cytometry of LPS activated D1 cells. D1 cells were preincubated for 90 min with 15  $\mu$ M TPCK and then treated for 18 h with 10  $\mu$ g/ml LPS.

NF- $\kappa$ B translocation to the nucleus. We found that TPCK effectively blocked LPS-induced nuclear translocation of p65 (data not shown). Functionally, this correlated with inhibition of LPS-induced D1 cell maturation in that the ability of LPS to increase the cell surface expression of MHC and costimulatory molecules was blocked by TPCK treatment (Fig. 4 D). Inhibition of LPS-induced DC maturation by TPCK was dose dependent, with maximal inhibition at 20  $\mu$ M TPCK. However, this dose of TPCK decreased the viability of immature D1 cells, suggesting a role for other members of the Rel/NF- $\kappa$ B family in the growth factor-dependent survival of these cells. Consistent with this idea, a role for Rel B in the constitutive expression of  $\kappa$ B-containing housekeeping genes in DCs has been proposed based on studies done on rel B knockout mice (34, 35). Furthermore, a recent report has shown that in B lymphocytes NF- $\kappa$ B1 and c-Rel are used differently to regu-

late apoptosis and cell cycle progression in resting and activated cells (36).

DCs have the unique ability to sense the external world by capturing antigens. In the presence of inflammatory signals, maturing DCs abandon the inflamed site and reach the draining lymph node. During this process, DCs have to initiate two differentiation responses, one being maturation (upregulation of surface MHC and costimulatory molecules) and the other being survival in a growth-arrested state in the absence of growth factors. We have shown that LPS can promote both of these differentiation responses but that the two processes are mediated by different, independent signaling pathways. The ability of LPS to promote DC survival in the absence of growth factors is dependent on the MEK/ERK pathway, whereas the ability of LPS to induce DC maturation, in terms of upregulation of MHC II and B7.2, is dependent on nuclear translocation of the NF- $\kappa$ B transcription factor.

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