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Regulation of IFN Regulatory Factor-7 and IFN-α Production by Enveloped Virus and Lipopolysaccharide in Human Plasmacytoid Dendritic Cells[1]

Jihong Dai,* Nicholas J. Megjugorac,† Sheela B. Amrute,* and Patricia Fitzgerald-Bocarsly[2]*†

Human plasmacytoid dendritic cells (PDC) are a major source of IFN-α upon exposure to enveloped viruses and TLR-7 and TLR-9 ligands. Although IFN regulatory factor-7 (IRF-7) is known to play an essential role in virus-activated transcription of IFN-α genes, the molecular mechanisms of IFN-α production in human PDC remain poorly understood. We and others have recently reported high constitutive levels of IRF-7 expression in PDC as compared with other PBMC. In this study, we demonstrate that both LPS and HSV up-regulate the expression of IRF-7 in PDC, and that this enhancement of IRF-7 is dependent on NF-κB activation. The NF-κB inhibitors MG132 and pyrrolidinedithiocarbamate efficiently inhibited the induction of IRF-7 by HSV or LPS, and also down-regulated the constitutive expression of IRF-7 in PDC and blocked the HSV-induced production of IFN-α. In addition, we found that nuclear translocation of IRF-7 occurred rapidly in response to HSV stimulation, but not in response to LPS, which is consistent with the stimulation of IFN-α production by virus and not by LPS. Although LPS by itself was not able to induce IFN-α production, it led to rapid up-regulation of TLR-4 on PDC and increased the magnitude and accelerated the kinetics of HSV-induced IFN-α production in PDC, providing a mechanism that might be operative in a scenario of mixed infection. In contrast to the current concept of IFN-α regulation established in cell lines, this study strongly supports the immediate availability of high constitutive levels of IRF-7 expression in PDC, and suggests an activation required for IRF-7 that contributes to IFN-α production in virus-stimulated PDC. ***The Journal of Immunology*****, 2004, 173: 1535–1548.**

The type I IFN family represents one of the best characterized models of virus-inducible gene activation (1). Within several hours after host infection by viruses or some bacteria, production of type I IFN is markedly induced, and this IFN then exerts various physical and biochemical effects, including antiviral and immunomodulatory activities. These early responses play an important role in innate defense against infectious agents (2–4). Induction of type I IFN is regulated at the transcriptional level. The presence of IFN regulatory factor (IRF)[3]-like binding sites in the promoter region of IFN-β and IFN-α genes implicated the IRF factors as direct regulators of IFN gene induction (5–9).

Within the IRF family, the essential and distinct roles of IRF-3 and IRF-7 for IFN-$\alpha\beta$ gene induction have been made clearer by recent gene disruption/introduction studies (9, 10). IRF-3 is expressed constitutively in the cytoplasm in a variety of cells. Upon viral infection, IRF-3 is activated and translocates from the cytoplasm to the nucleus. In the nucleus, IRF-3 assembles with other transcription factors and contributes to the induction of specific defense genes, including IFN-β (11). IRF-7 was first described to bind and repress the EBV Qp promoter for EBV nuclear Ag 1, which contains an IFN-stimulated regulatory element-like element (12). Soon after, IRF-7 was shown to be an important component in IFN-α induction (13–15). IRF-7 is primarily expressed in cells of lymphoid origin at a low level. Its expression has been shown to be up-regulated by IFN, LPS, and viral infection (13, 16). Like IRF-3, IRF-7 also undergoes virus-induced phosphorylation and nuclear translocation (13, 14). It has been shown that IRF-7 alone was able to activate both IFN-α and IFN-β genes, whereas IRF-3 itself mainly activates the IFN-β gene (11, 15). In support of this hypothesis, ectopic expression of IRF-7 has been shown to reconstitute virus-mediated IFN-α production in fibroblasts, suggesting that IRF-7 is critical for the induction of virally induced IFN-α gene expression (10).

The major IFN-α-producing cells (IPC) in human blood were originally designated as natural IPC (NIPC). NIPC are rare cells, representing only ~0.1% of the PBMC (17, 18). Although rare, the NIPC are enormously potent producers of IFN-α: each functional NIPC can produce between 1 and 2 IU of IFN-α (3–10 pg) in response to enveloped viruses (18). In contrast, monocytes, which can produce IFN-α in response to a much more limited range of viruses, produce 5- to 10-fold less IFN-α on a per cell basis (18, 19). Although there was clear evidence developed over the course of several years that NIPC belong within the dendritic cell (DC) lineage (20–23), only recently have DC subsets in peripheral blood been clearly described, thus allowing definition of NIPC as plasmacytoid DC (PDC) (24–26). PDC were first described as a subset of DC in peripheral blood distinguished from myeloid DC by their lack of expression of CD11c (27, 28). They were subsequently further identified to be HLA-DR$^+$, lineage (CDs 3, 14, 16, 56, 20)$^-$, CD123 (IL-3-Ra) bright. Most recently, these cells have

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[3] Abbreviations used in this paper: IRF, IFN regulatory factor; BDCA, blood DC Ag; DC, dendritic cell; IKK, IκB kinase; IPC, IFN-α-producing cell; MFI, mean fluorescence intensity; MOI, multiplicity of infection; NIPC, natural IPC; PDC, plasmacytoid DC; PDTC, pyrrolidinedithiocarbamate; RT, room temperature; SV, Sendai virus; TBK, tank-binding kinase.

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been found to express a unique marker, the C-type lectin blood DC Ag-2 (BDCA-2) and another marker, BDCA-4 (29, 30). A second distinct population of circulating DC, myeloid DC, are HLA-DR$^+$, lineage$^-$, CD11c$^+$, and CD123^{dim} (24, 27). Myeloid DC produce relatively little IFN-α in response to viral stimulus, but were recently reported to produce IFN-α in response to dsRNA (25, 31). In contrast, PDC do not respond to dsRNA and lack the TLR-3 receptor to which it binds.

The molecular mechanisms of IFN-α induction have been investigated intensively in mouse model systems, human cell lines, and monocyte-derived DC. However, due to their paucity in peripheral blood, relatively little is known about the induction of IFN-α in PDC. Because of their unique ability to produce large quantities of IFN-α in response to a wide range of enveloped viruses and CpG, and their ability to respond well to both live and UV-inactivated enveloped viruses, unlike other IFN-α-producing cells (reviewed in Ref. 32), we hypothesized that it is likely that induction of IFN-α in these cells is distinct from induction of the pathway in other cells. Previous studies demonstrate that IRF-7 gene expression is under the control of IFN-$\alpha\beta$ signaling (14, 16). In mouse embryo fibroblasts, IFN-α4 and IFN-β prime for further type I IFN expression in response to viruses through IRF-7 induction, suggesting that IFN-$\alpha\beta$ is required to amplify its own expression in vitro through a positive feedback loop (13, 14). We and others have recently demonstrated high constitutive levels of IRF-7 expression in PDC as compared with other PBMC subpopulations (33–35).

In this study, we have used the combination of four-color intracellular flow cytometry and immunofluorescence microscopy to study the protein levels of IRF-7 to further investigate the molecular mechanisms of virus-induced IFN-α production in human PDC. We demonstrate that: 1) enveloped virus, but not LPS, is able to induce the production of IFN-α in PDC; 2) IRF-7 appears to be regulated at two distinct levels in PDC: while expression of the IRF-7 is induced by both virus and LPS, probably via the NF-κB pathway, the *trans* activation function of IRF-7 is only modulated by virus stimulation, not by LPS; and 3) although LPS by itself is not able to induce IFN-α production, it can potently modify both the magnitude and the kinetics of virus-induced IFN-α production in PDC.

Materials and Methods

Virus and cell lines

HSV-1 strain 2931 was originally obtained from C. Lopez, then at the Sloan-Kettering Institute (New York, NY). Sendai virus, strain Sendai/Cantell, was obtained from Charles River Laboratories (Spafas, MA). HSV-1 was grown in VERO cells (American Type Culture Collection, Manassas, VA) and titered by plaque-forming assay. Influenza virus A (Flu), PR8 strain (1×10^6 PFU/μl), was kindly provided by T. Moran (Mount Sinai Medical Center, New York, NY). All virus stocks were stored at −70°C until use.

Reagents

PerCP- or allophycocyanin-conjugated anti-HLA-DR, FITC-conjugated anti-lineage, allophycocyanin-conjugated anti-CD11c, allophycocyanin- or PE-conjugated anti-CD123, and FITC- or allophycocyanin-conjugated anti-CD14 were purchased from BD Biosciences (Sunnyvale, CA). PE-conjugated anti-BDCA-2 was purchased from Miltenyi Biotec (Auburn, CA). PE-conjugated anti-TLR-4 and isotype control IgG were purchased from eBioscience (San Diego, CA). Unconjugated polyclonal rabbit anti-IRF-7, anti-IRF-3, and normal rabbit IgG (as a control) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). FITC-conjugated goat anti-rabbit IgG was purchased from BD Pharmingen (Sunnyvale, CA). LPS isolated from *Pseudomonas aeruginosa* (<1% protein) was purchased from Sigma-Aldrich (St. Louis, MO). Highly purified LPS (containing only 0.08% protein) from *Escherichia coli* K235 was kindly provided by S. Vogel (University of Maryland, Baltimore, MD). The NF-κB inhibitors pyrrolidinedithiocarbamate (PDTC) and MG132 were purchased from Sigma-Aldrich.

Preparation of PBMC

PBMC were isolated by Ficoll-Hypaque density centrifugation (Lymphoprep; Accurate Chemical & Scientific, Westbury, NY) from fresh heparinized peripheral blood obtained with informed consent from healthy volunteers. The human studies were approved by the Institutional Review Board of the New Jersey Medical School. The PBMC were resuspended in RPMI 1640 medium containing 10% heat-inactivated FBS (Gemini Biosciences, Woodland Hills, CA), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 25 mM HEPES, and enumerated electronically with a Coulter Counter series Z1 (Coulter Electronics, Hialeah, FL). Cells were stimulated at a concentration of 2×10^6/ml by HSV (multiplicity of infection (MOI) of 1), Sendai virus (SV) (16 hemagglutinating U/ml), Flu (MOI of 1), LPS (10 ng/ml), or purified LPS (10 ng/ml).

Isolation of PDC

PDC were isolated by MACS using the BDCA-4 DC isolation kit from Miltenyi Biotec. PBMC were resuspended in MACS buffer (PBS with 0.5% BSA and 2 mM EDTA; Sigma-Aldrich). PDC were labeled with anti-BDCA-4 Ab coupled to colloidal paramagnetic microbeads and passed twice through a magnetic separation column (LS and MS column; Miltenyi Biotec). The purity of isolated PDC (lineage-negative, CD11c-negative, HLA-DR-positive, and CD123-positive cells) was >95%. Viability of enriched PDC was >95% as determined by trypan blue exclusion.

ELISPOT assays

ELISPOT assays were used to determine the frequency of IPC using a simplification of a previously described method (36). PBMC were suspended in RPMI 1640 containing 10% FCS and incubated with HSV-1 for 6 h at 37°C in a 5% CO_2 incubator. Ninety-six-well microtiter plates with mixed cellulose ester membrane bottoms (MultiScreen HA plates; Millipore, Bedford, MA) were coated with the IgG fraction of ammonium sulfate-precipitated bovine anti-human IFN-α antiserum (GlaxoSmithKline, Breckenridge, U.K.) in PBS for at least 5 h at room temperature (RT). The plates were then washed three times with PBS. The cells were washed and added at 1×10^5/well, and then incubated for 12–16 h at 37°C in a 5% CO_2 incubator to allow production and capture of IFN-α. The plates were then washed twice with PBS and six times with PBS-T (PBS with 0.05% Tween 20; Sigma-Aldrich). A murine anti-human IFN-α mAb (293 mAb (provided by G. Alm, Uppsala, Sweden), which recognizes multiple species of IFN-α) was added at 2 μg/ml. This was followed by 1-h incubation at RT with goat antiserum to murine IgG conjugated to HRP (The Jackson Laboratory, Bar Harbor, ME). The plates were again washed, as described, and then treated with the HRP substrate 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich). The spots were enumerated with the aid of a dissecting microscope, and the frequencies were calculated and expressed as IPC per 10^4 PBMC.

Intracellular flow cytometry for IRF-7 and IFN-α with surface phenotyping

Intracellular flow for IRF-7 and IFN-α was performed using indirect cytoplasmic staining combined with surface staining. Cells at a concentration of 2×10^6 cells/ml were stimulated in 1-ml vol in tubes (for PBMC) or in 100 μl in tubes (for purified PDC) with virus or LPS for 4–6 h at 37°C in a 5% CO_2 incubator. For detection of IFN-α, brefeldin A (Sigma-Aldrich) was added at a final concentration of 5 μg/ml at 4 h, and cells were incubated for an additional 2 h. The cells were washed with cold 0.1% BSA (Sigma-Aldrich) in PBS, blocked with 5% heat-inactivated human serum, and stained with the appropriate fluorochrome-conjugated cell surface marker Abs for 20 min at 4°C. The cells were then washed and fixed with 1% paraformaldehyde (Fisher Scientific, Hampton, NH) in PBS overnight at 4°C. The cells were washed twice with 2% FCS-PBS and permeabilized with 0.5% saponin (Sigma-Aldrich) in 2% FCS-PBS for 15 min at RT. Cells were then incubated with rabbit anti-human IRF-7 (400 ng), IRF-3 (2000 ng), or 50 ng of biotinylated 293 mAb in a final volume of 50 μl for 30 min at RT. Normal rabbit IgG was used for isotype controls for the IRF-3 and 7 at the same concentrations. The cells were washed twice with permeabilization buffer, and incubated 30 min with goat anti-rabbit IgG FITC or streptavidin-quantum red (Sigma-Aldrich) for IRF-7 and IFN-α, respectively. The cells were washed and fixed with 1% paraformaldehyde

in PBS, and analyzed using FACSCalibur flow cytometry with CellQuest Analysis software (BD Biosciences).

RT-PCR analysis

Total RNA from PBMC was prepared using the TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA), according to the manufacturer's instruction. Total RNA from purified PDC was extracted using an RNeasy Mini Kit (Qiagen, Santa Clarita, CA). The sequences of the primers were as follows: IFN-α1, sense, 5′-GTACTGCAGAATCTCTCCTTTCTCCTG-3′ and antisense, 5′-GTGTCTAGATCHGACAACCTCCCAGGCACA-3′; β-actin, sense, 5′-ACAATGAGCTGCTGGTGGCT-3′ and antisense, 5′-GATGGGCACAGTGTGGGTGA; TLR-4, sense, 5′-CTGCAATGGAT CAAGGACCA-3′ and antisense, 5′-TCCCACTCCAGGTAAGTGTT-3′. First-strand cDNA was synthesized with oligo(dT) primers. From this mixture of cDNAs, human IFN-α1 and TLR-4 and β-actin DNA were amplified by PCR using the primer sets. The resulting PCR products from the RT-PCR were resolved on 2% agarose gels and visualized with ethidium bromide.

Fluorescence microscopy

Purified PDC (2×10^5 cells/100 μl) were treated with HSV (MOI of 1) or LPS (10 ng/ml) for 2 h. After stimulation, PDC were stained with PE-labeled BDCA-2 and then fixed overnight at 4°C. The fixed cells were seeded on glass slides by cytospin, then permeabilized with 0.2% Triton X-100. Cells were blocked with wash buffer containing 3% BSA and 10% normal goat serum for 30 min and incubated with the primary Ab, anti-human IRF-7 at a 1/20 dilution, or normal rabbit IgG, as an isotype control. After washing, cells were incubated with FITC-conjugated anti-rabbit IgG, and 4′,6′-diamidino-2-phenylindole at 50 ng/ml. Following extensive washing of the slides, coverslips were placed with PBS. Slides were scanned under fluorescence with an Olympus (Melville, NY) fluorescence microscope with digital image capture.

Statistical analysis

Data were analyzed using the Statview statistics program. Significant differences between groups were determined by ANOVA, with values of $p < 0.05$ considered significant.

Results

Identification of PDC in human PBMC

The established protocol for the identification of PDC within PBMC is to gate on lineage$^-$, HLA-DR$^+$, CD11c$^-$, and CD123$^+$ cells. However, this four-color gating limits the availability of fluorescent channels to evaluate intracellular cytokine or transcription factor expression in the cells. PDC can also be identified in peripheral blood as the cells that are HLA-DR$^+$ and CD123^{bright} (35, 37, 38) (Fig. 1). Using multicolor gating, we demonstrate that very similar populations are defined by first gating on *lin*$^-$/DR$^+$ cells, followed by CD123^{bright}/CD11c$^-$ gating (Fig. 1*A*, gates R2 and R3) as obtained using CD123^{bright}/HLA-DR$^+$ cells (gate R5). In this analysis, the CD123^{bright} PDC from gate R3 are painted green, then located in gate R2 and R5. Similar results were found in PBMC after stimulation with HSV (Fig. 1*B*), confirming the identification of PDC in PBMC by using CD123 and HLA-DR.

IRF-7 expression in PDC is increased by virus or LPS stimulation

As reported recently, IRF-7 mRNA and protein are constitutively expressed even in freshly isolated PDC (33–35) and are induced by virus or LPS in human PBMC and mouse macrophages (13, 39).

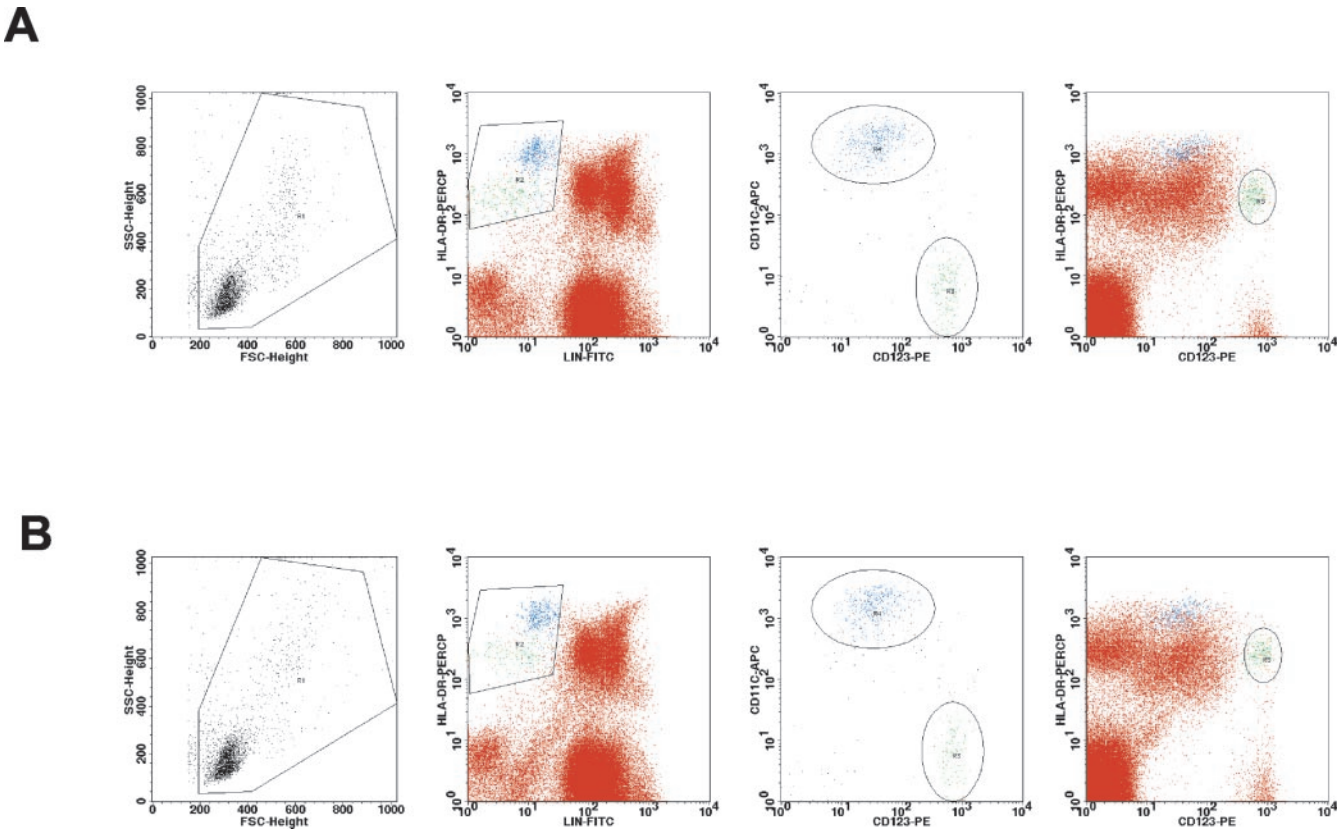

FIGURE 1. Identification of PDC in PBMC. PBMC were prepared from peripheral blood of normal healthy volunteers and analyzed by four-color flow cytometry for expression of lineage, HLA-DR, CD11c, and CD123. PBMC unstimulated (*A*) and stimulated with HSV for 6 h (*B*) were first gated for forward and side scatter (R1), then analyzed for lineage vs HLA-DR (R2). The lineage-negative, DR$^+$ cells were further analyzed for expression of CD123 and CD11c, and the PDC were defined as CD123$^+$, CD11c$^-$ (R3). PDC were directly gated as CD123$^+$/HLA-DR$^+$ cells in PBMC (R5, 474 events, 0.16% of unstimulated PBMC; 358 events, 0.12% of stimulated PBMC). The CD123$^+$/CD11c$^-$ cells from R3 (475 events, 0.16% of unstimulated PBMC; 346 events, 0.12% of stimulated PBMC) were painted green and traced in the gates R2 and R3. Results are from a representative routine experiment.

To evaluate the role of IRF-7 in human PDC, we examined the protein level of IRF-7 expression in PDC induced by different virus and LPS stimulations. Due to the paucity of PDC in blood, we used intracellular staining for IRF-7 protein combined with cell surface staining, as recently described (35). After stimulation with HSV, SV, Flu, or LPS for 2, 4, and 6 h, the cells were surface stained with anti-CD123 and HLA-DR for identification of PDC, followed by fixation and intracellular staining for IRF-7. In confirmation of our earlier findings (35), IRF-7 was expressed constitutively at very high levels in PDC as compared with monocytes, which expressed only low levels of IRF-7 (Fig. 2, *A* and *B*). Compared with unstimulated PDC, there was a modest enhancement of IRF-7 expression that was only observed after 4-h incubation with virus or LPS. The IRF-7 levels remained high in the virus-stimulated samples at 6 h, but the levels of IRF-7 in the unstimulated PDC decreased. Compared with the relative levels of IRF-7 at 4 h, there was no significant increase of IRF-7 levels at 6 h after virus or LPS stimulation. The IRF-7 expression in PDC was enhanced by treatment of PBMC with 10 ng/ml LPS, and this enhancement followed a similar time course as for the virus-induced up-regulation of IRF-7 (Fig. 2, *C* and *D*).

Virus, but not LPS, induces IFN-α production in PDC

Because IRF-7 plays an essential role in virus-activated transcription of IFN-α genes (13), and LPS up-regulates the expression of IRF-7 in PDC, we next examined whether LPS is able to initiate IFN-α production in PDC. Previous experiments demonstrated that 4-h incubation with enveloped virus, followed by washing, plating in ELISPOT plates, and overnight incubation led to detectable IPC, with the peak response occurring after 6-h preincubation, followed by overnight in the plates (data not shown). Therefore, for these studies, PBMC were incubated with virus or LPS for 4 or 6 h, then washed and plated overnight for synthesis and capture of IFN-α. As shown in Fig. 3*A*, there were some spots (2.1–14.4

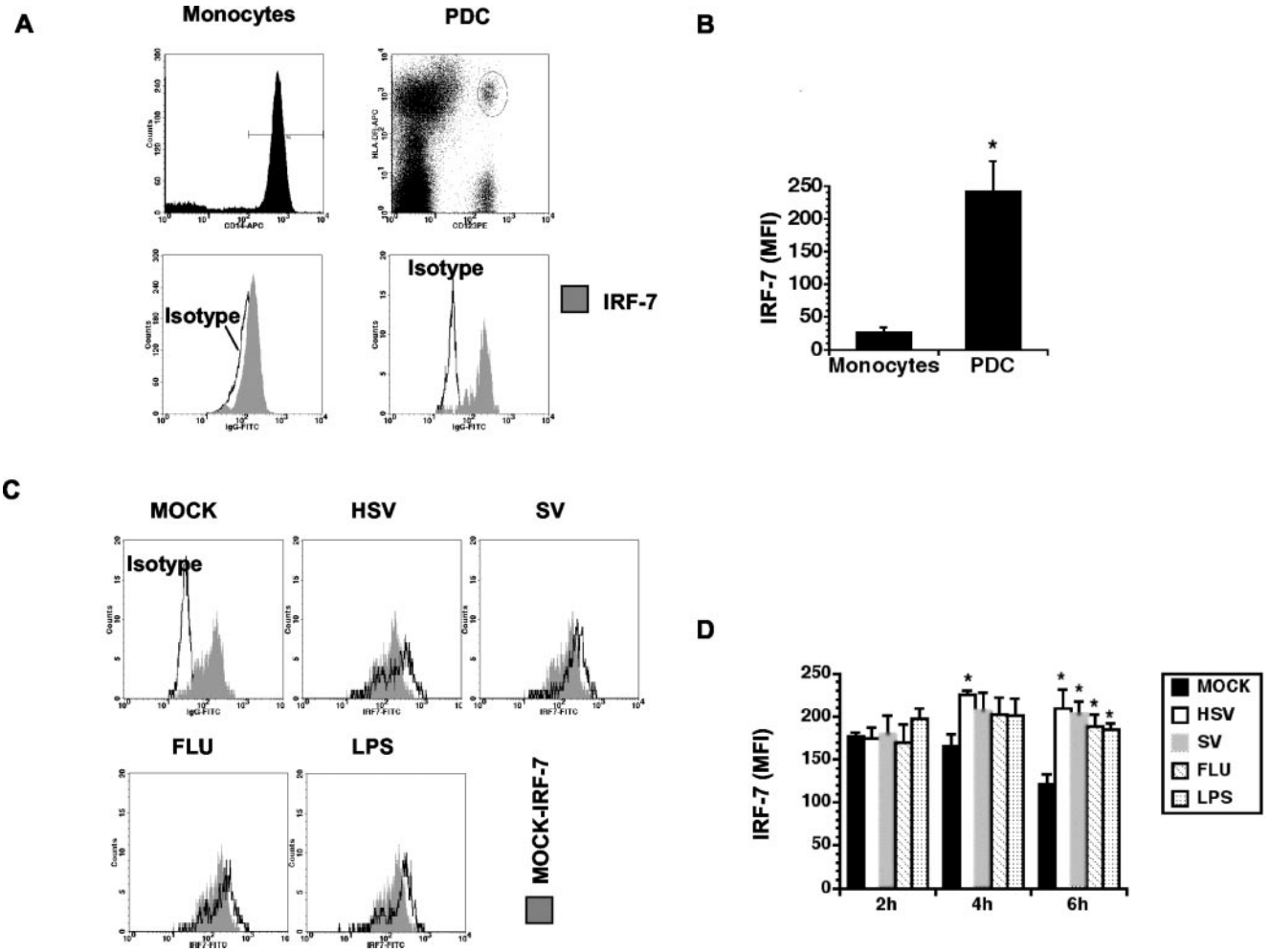

FIGURE 2. PDC express high levels of IRF-7 that are up-regulated by virus or LPS. *A* and *B*, High levels of constitutive IRF-7 expression in PDC compared with the expression of IRF-7 in monocytes. PBMC were surface stained CD123 PE and HLA-DR allophycocyanin for identification of PDC populations, and CD14 allophycocyanin for monocytes, followed by fixation and intracellular staining for IRF-7. The cells were gated on PDC and monocyte populations, respectively (*A*, *upper panels*). Histogram overlays compare expression of IRF-7 (gray filled histogram) with isotype IgG control (black line) in *A*, *lower panels*. Data are representative of three independent experiments in *A* and means of the MFI of IRF-7 minus means of isotype controls ± SEM of three independent experiments in *B*. Significant differences ($p < 0.05$) are indicated by an asterisk. *C*, Expression of IRF-7 is increased by virus or LPS. A total of 2×10^6/ml PBMC was stimulated with HSV (MOI = 1), SV (16 hemagglutinating U/ml), Flu (MOI = 1), or LPS (10 ng/ml) for 6 h, followed by staining for PDC and IRF-7 in *A*. Histogram overlays compare expression of IRF-7 in PDC between mock (gray filled histograms) and PBMC stimulated with different viruses or LPS (black lines). Data are representative of five independent experiments. *D*, Time course of expression of IRF-7 intracellular in PDC after stimulation with viruses or LPS. The means of MFI of IRF-7 minus means of isotype controls ± SEM of $n = 5$ independent experiments are shown. Significant differences ($p < 0.05$) between mock and PBMC stimulated with different virus or LPS are indicated by an asterisk.

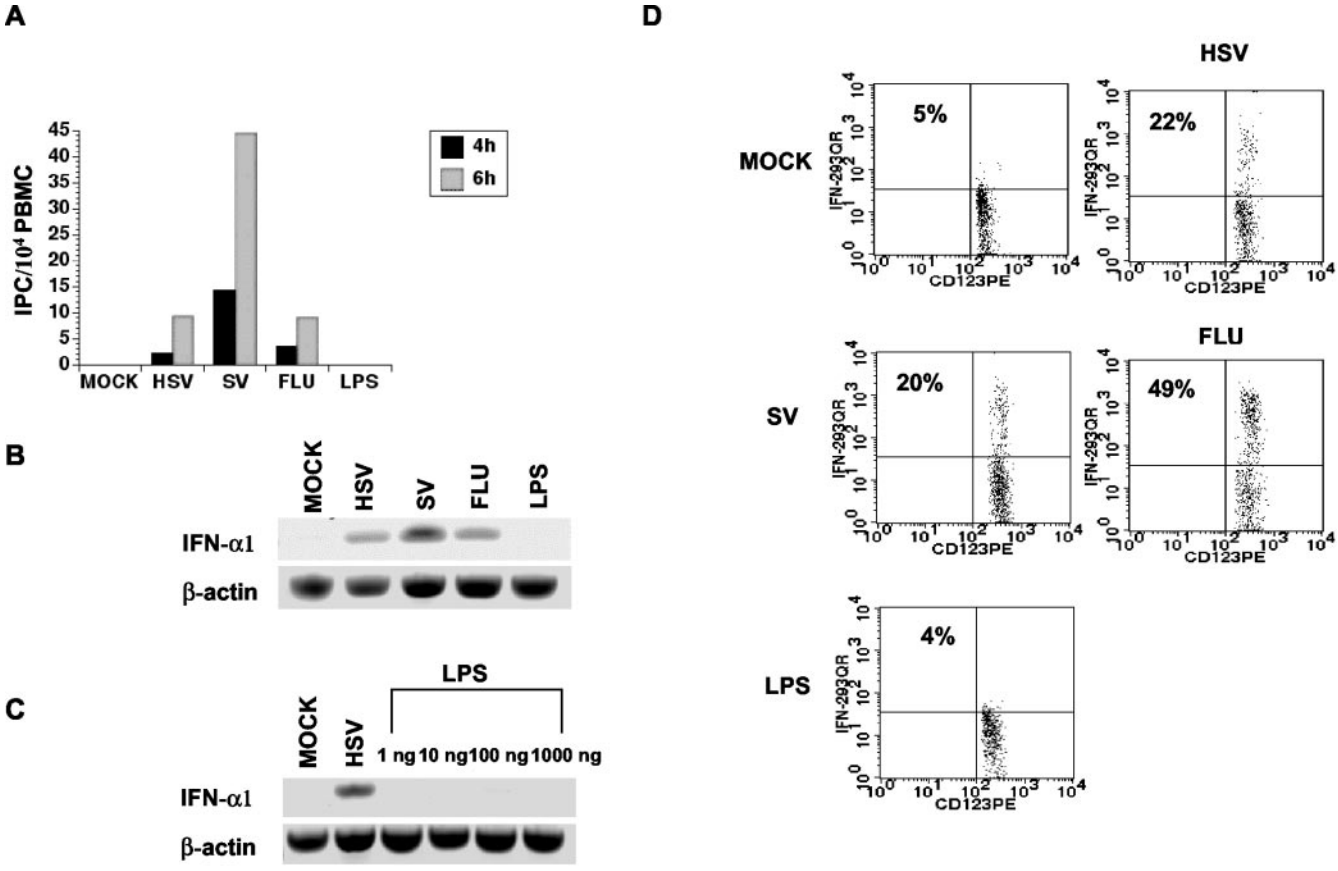

FIGURE 3. Virus, but not LPS, induces IFN-α production in PDC. *A*, 2×10^6/ml PBMC were stimulated with virus (in amounts as described in Fig. 1) or 10 ng/ml LPS for 4 and 6 h. Frequency of IFN-α-producing cells was determined by ELISPOT. Data represent the average values of triplicate samples. The experiment was repeated twice with similar results. *B* and *C*, PBMC (2×10^6/ml) were stimulated with virus, as described in Fig. 1, or LPS for 4 h, then RNA was extracted and run for IFN-α1 or β-actin by RT-PCR. Expression of IFN-α1 mRNA in PBMC after stimulation with virus or 10 ng/ml LPS is shown in *B* and with HSV or different doses of LPS in *C*. *D*, Intracellular flow cytometry of IFN-α-producing cells after stimulation of PBMC with different virus or 10 ng/ml LPS for 6 h. Cells were gated on the PDC population, as in Fig. 2*A*. Numbers indicate percentage of IFN-α-positive cells (*upper right quadrant*). Data are representative of five independent experiments.

IPC/10^4 PBMC) detected after 4-h initial stimulation with enveloped virus, with many more IFN-α spots (9.3–45.6 IPC/10^4) observed at 6 h. In agreement with our previous studies, SV induced many more spots than HSV or Flu, because both PDC and monocytes respond to SV with IFN-α production (19, 23, 40, 41), while primarily PDC produce IFN-α in response to HSV or Flu. Notably, neither unstimulated cells nor LPS-treated cells produced IFN-α (Fig. 3*A*). We also examined the levels of IFN-α1 mRNA expression in PBMC because IFN-α1 was found to be the major transcript expressed in both monocytes and PDC (35). As shown in Fig. 3*B*, the expression of IFN-α1 mRNA was induced after 4-h stimulation with enveloped virus; however, IFN-α1 was not expressed at detectable levels in LPS-treated or unstimulated cells (Fig. 3*C*). To confirm these results in PDC, intracellular IFN-α protein expression was measured. We found IFN-α production was induced in PDC stimulated with HSV, SV, or Flu. In contrast, LPS did not induce IFN-α production (Fig. 3*D*). These results confirm the findings of others (34, 42, 43) and indicate that LPS is not able to induce IFN-α production in PDC even though it enhances IRF-7 expression.

Purified LPS up-regulates IRF-7 expression in PDC, and is inhibited by polymyxin B

To confirm that the enhancement of IRF-7 expression in PDC was not due to impurities of LPS, we evaluated the ability of highly purified LPS to induce IRF-7 in PDC. Compared with the expression of IRF-7 in HSV-stimulated-PDC, IRF-7 expression was enhanced to even higher levels in response to 10 ng/ml purified LPS (Fig. 4, *A* and *B*). However, as found with the less pure LPS, the purified LPS did not induce IFN-α production in PDC or PBMC (data not shown).

To examine whether LPS or HSV enhances IRF-7 expression in purified PDC, PDC were purified from PBMC by positive selection with anti-BDCA-4 microbeads and stimulated with LPS or HSV. IRF-7 was modestly increased in both HSV- and LPS-stimulated PDC after 6-h incubation (Fig. 4, *D* and *E*), which is consistent with our observations in Fig. 2. When LPS was added in combination with polymyxin B, a molecule that is known to bind LPS and neutralize its effects (44), there was no up-regulation of IRF-7 in the PDC (Fig. 4*F*). Therefore, the effects of LPS on IRF-7 expression in PDC were most likely due to stimulation by LPS and not to secondary effects.

Up-regulation of TLR-4 gene expression in PDC by LPS

Recent studies have demonstrated the importance of signaling through TLR in innate immune response to microbes (reviewed in Ref. 45). Signaling through TLR is essential for DC maturation induced by bacteria and other pathogens. Genetic analysis has identified TLR-4 as a primary receptor for LPS (46, 47). Previous

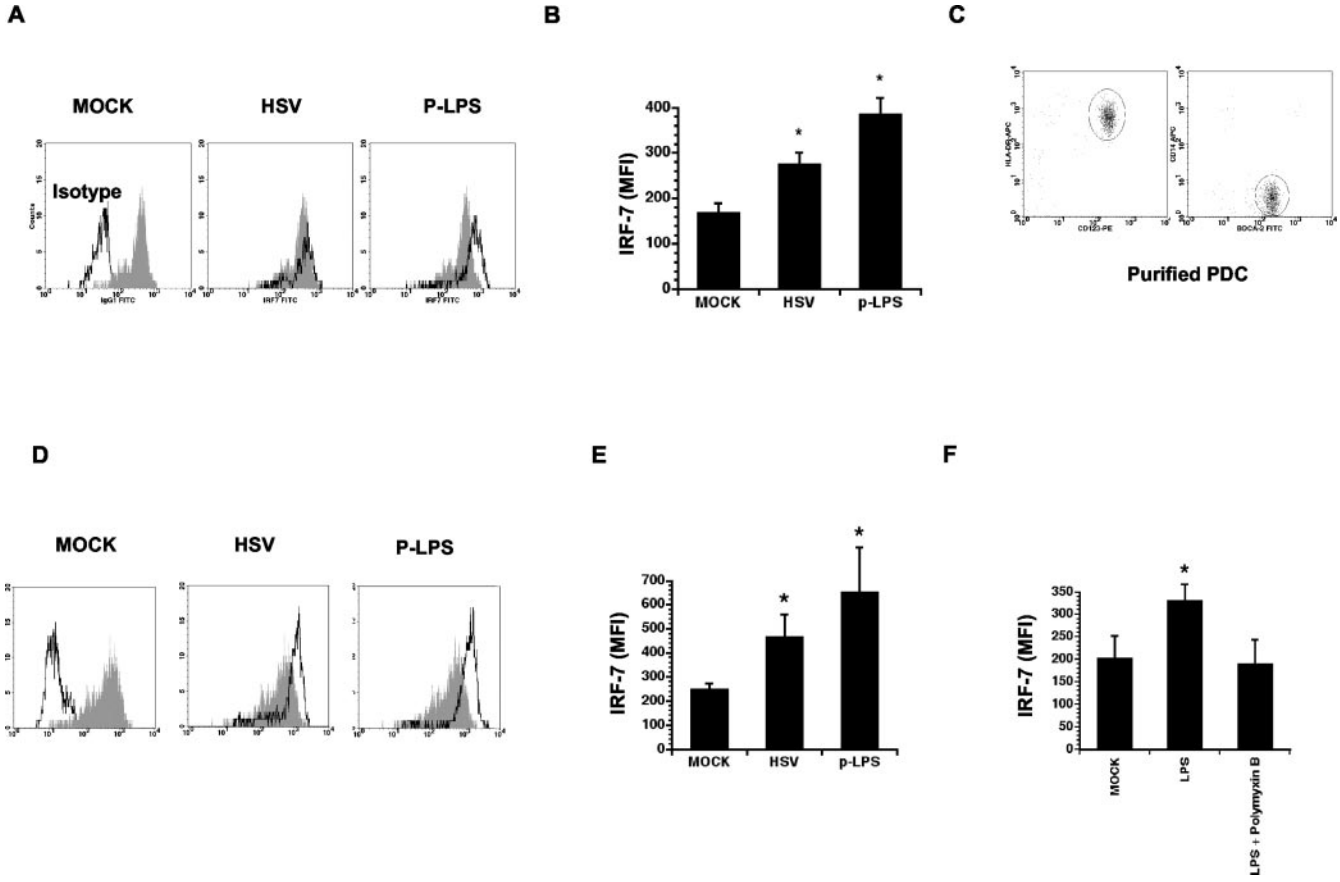

FIGURE 4. Purified LPS up-regulates IRF-7 expression in PDC. *A* and *B*, 2×10^6/ml PBMC were stimulated with high purity LPS (10 ng/ml) and HSV for 6 h. Histogram overlays compare IRF-7 expression between mock (gray filled histograms)- and HSV or purified LPS (P-LPS)-stimulated PDC (black lines) after gating on PDC populations. Rabbit IgG was used as a control (data not shown). Data are representative of three independent experiments in *A*. Means of MFI of IRF-7 minus means of isotype controls ± SEM of the three independent experiments are shown in *B*. Significant differences ($p < 0.05$) are indicated by an asterisk. *C–E*, LPS up-regulates IRF-7 expression in purified PDC. PDC were isolated from PBMC by using BDCA-4-positive magnetic beads isolation; the purity of the PDC populations exceeded 95%, and the contamination with $CD14^+$ cells was <1% (*C*); *D* and *E*, the purified PDC from *C* were stimulated with HSV or LPS, and levels of IRF-7 expression were determined by flow cytometry. *F*, Polymyxin B neutralizes the up-regulation of IRF-7 by LPS in purified PDC. *D–F*, 2×10^5 purified PDC in 100 μl were stimulated with LPS (10 ng/ml) and HSV (MOI = 1) for 6 h or with LPS in the presence of 1 μg/ml polymyxin B. Cells were surface stained with CD123 PE and HLA-DR allophycocyanin for identification of PDC populations, followed by fixation and intracellular staining for IRF-7. Data are representative of three independent experiments in *D*. Means of MFI of IRF-7 minus means of isotype controls ± SEM of the three independent experiments are shown in *E* and *F*. Significant differences ($p < 0.05$) are indicated by an asterisk.

studies have suggested that expression of TLR by human PDC is restricted to TLR-1, TLR-6, TLR-7, TLR-9, and low levels of TLR-10 (48, 49). However, it has been described that microbial stimuli affect the expression of their cognate TLR (50–52), and TLR-4 gene expression can be up-regulated by LPS in immature mouse DC (53). We therefore examined the expression of TLR-4 on human PDC. TLR-4 message expression was examined in purified PDC. As shown in Fig. 5*B*, TLR-4 mRNA expression was expressed at low levels or was undetectable in unstimulated PDC, but clearly discernible as after 2-h stimulation with purified LPS. Surface expression of TLR-4 was not detected in unstimulated DC, but was modestly up-regulated following stimulation with purified LPS (Fig. 5*A*). In contrast, monocytes constitutively expressed surface TLR-4, and this expression was unaffected by LPS treatment (Fig. 5*A*, *right panel*). These results confirmed that the expression of TLR-4 on PDC was up-regulated by purified LPS within a short period of time, which is consistent with the increased expression of IRF-7 following LPS stimulation (Fig. 4*A*), and suggests that the canonical TLR-4-mediated pathway is most likely involved in the enhancement of IRF-7 expression in response to LPS. Interestingly, HSV stimulation of the PDC also resulted in up-regulation of TLR-4 mRNA (Fig. 5*B*).

Effects of NF-κB pathway inhibitors on expression of IRF-7

The LPS/TLR-4 interaction has been described to result in NF-κB activation (54). An NF-κB site located next to the TATA box on the IRF-7 promoter was reported to be responsible for the induction of the IRF-7 gene by 12-*O*-tetradecanoylphorbol-13-acetate (55). In addition, the proinflammatory cytokine TNF-α also induces IRF-7 expression that is dependent upon the same NF-κB site (56). We therefore conducted experiments to test whether the NF-κB pathway is involved in the expression of IRF-7 by PDC in response to LPS or enveloped virus using the NF-κB inhibitors PDTC and MG132. As demonstrated in Fig. 6, *A* and *B*, the induction of IRF-7 by either LPS or HSV was inhibited to levels even lower than the constitutive expression of IRF-7 by 6-h treatment with 20 μM PDTC or MG132. Furthermore, IFN-α production by HSV-stimulated PDC was abrogated in the presence of either PDTC or MG132 (Fig. 6, *A* and *B*). Similar results were also found using purified PDC (Fig. 6, *B* and *C*). However, when PDTC

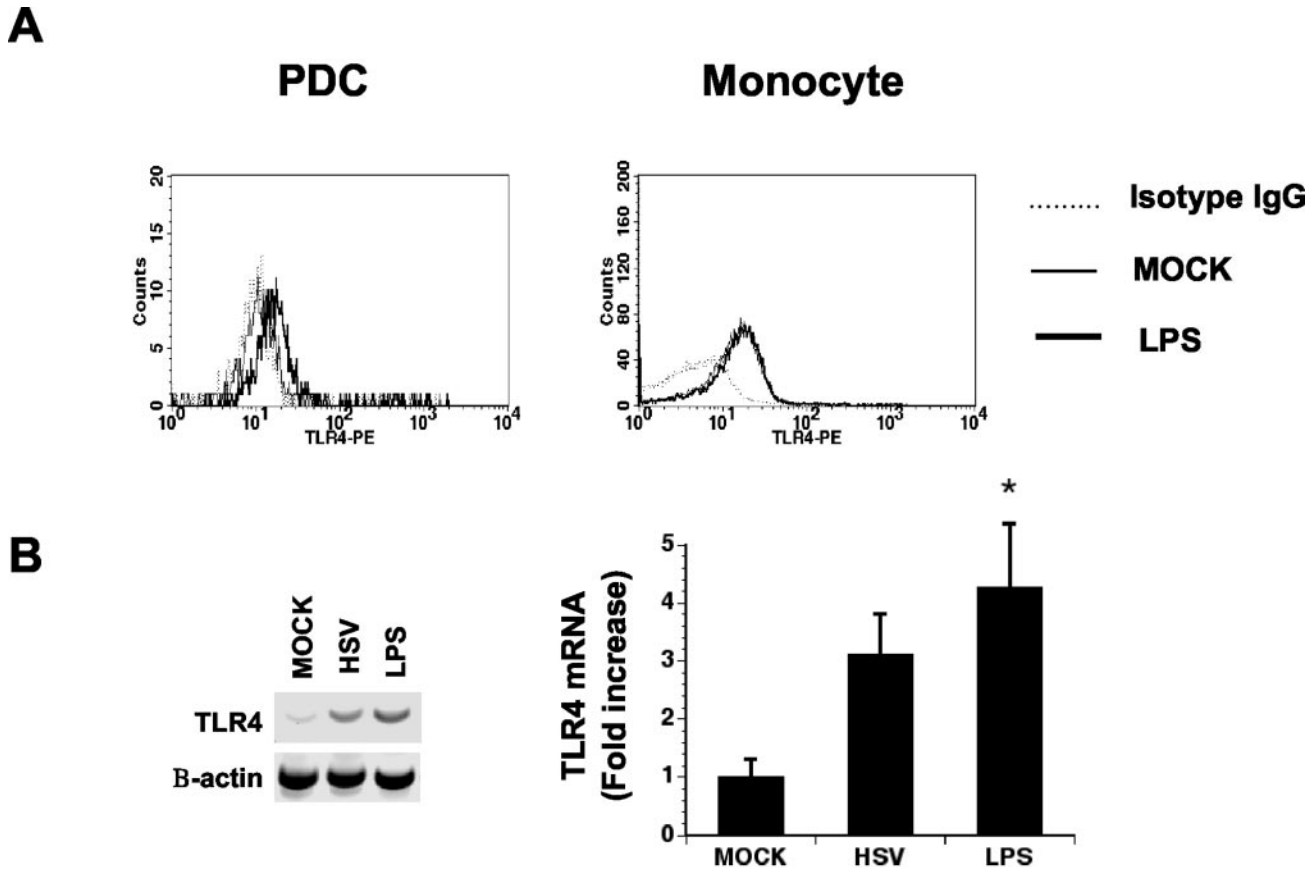

FIGURE 5. LPS induces TLR-4 expression in PDC. *A*, 2×10^6/ml PBMC were stimulated with high purity LPS (10 ng/ml) for 3 h. Cells were surface stained with TLR-4 or isotype IgG. CD123 allophycocyanin and HLA-DR PerCP were used for identification of PDC populations, and CD14 FITC for monocytes. Data are representative of experiments performed with three donors. *B*, PDC were isolated from PBMC by using BDCA-4-positive magnetic beads isolation; the purity of the PDC populations exceeded 95%. A total of 2×10^5 purified PDC in 100 μl was stimulated with HSV (MOI = 1) or LPS (10 ng/ml) for 2 h; message was extracted; and mRNA expression of TLR-4 and β-actin was analyzed by RT-PCR. Data are representative of experiments performed on three donors. mRNA levels are expressed as fold increase (experimental value/control value).

or MG132 was added 4 h after the initiation of stimulation with HSV, a point when the gene expression of IFN-α was already induced by HSV (Fig. 3*B*), there was no inhibition of HSV-induced IFN-α production by PDTC or MG132 (Fig. 7).

We further asked whether the NF-κB inhibitors affected the constitutive levels of IRF-7 expression and whether they had any effect on IRF-3 expression. As shown in Fig. 8, after 1- or 4-h incubation with PDTC, the IRF-7 expression was down-regulated in either unstimulated or HSV-stimulated PDC. The same was seen with 4-h incubation with MG132, which inhibits NF-κB by inhibiting the proteasome. In contrast, neither drug inhibited the expression of IRF-3. These inhibitors did not induce significant cytotoxicity, as demonstrated by using annexin/7-aminoactinomycin D staining (data not shown). Taken together, these results suggest that the NF-κB pathway is involved in the IRF-7 expression stimulated by virus or LPS and also in the constitutive IRF-7 expression in PDC, but is not involved in the expression of IRF-3.

HSV, but not LPS, induces nuclear translocation of IRF-7 in PDC

To determine whether HSV-induced production of IFN-α is associated with activation of IRF-7, nuclear translocation experiments were undertaken. PDC were isolated from PBMC, stimulated with HSV or LPS, and examined for the expression of nuclear vs cytoplasmic IRF-7 by immunofluorescence. As shown in Fig. 9, a diffuse staining of IRF-7 was seen in both the cytosol and nucleus of unstimulated PDC, consistent with our previous findings (35). Following 2-h stimulation with HSV, a very bright nuclear staining of IRF-7 was observed. In contrast, there was no increase in nuclear IRF-7 staining after LPS stimulation; however, a high fluorescence of IRF-7 was observed in the cytosol of the LPS-stimulated PDC, in agreement with the LPS-induced up-regulation of IRF-7, as shown by flow cytometry (Fig. 4). These results clearly demonstrate that although HSV and LPS both up-regulate the expression of IRF-7, activation of IRF-7 occurred in HSV-, but not in LPS-treated human PDC.

LPS accelerates virus-induced IFN-α production in PDC

Concomitant infection with both bacteria and virus is a common clinical situation. Because LPS is able to increase the expression of IRF-7 in PDC, we asked whether LPS can modulate virus-induced IFN-α production in PDC. As shown in Fig. 10*A*, pretreatment of PBMC with LPS 2 h before HSV, SV, or Flu stimulation efficiently increased the population of PDC that produce IFN-α. As shown in Fig. 10*C*, the mean fluorescence intensity (MFI) of IFN-α in HSV-stimulated PDC increased in a time-dependent manner (1–6 h), indicating an accumulation of IFN-α within the PDC. Pretreatment of PBMC with LPS did not significantly increase the amount of IFN-α produced at peak (as defined by MFI) (Fig. 10*C*), but did significantly accelerate the production of IFN-α such that the percentage of IPC had already plateaued by 2 h in the LPS-pretreated

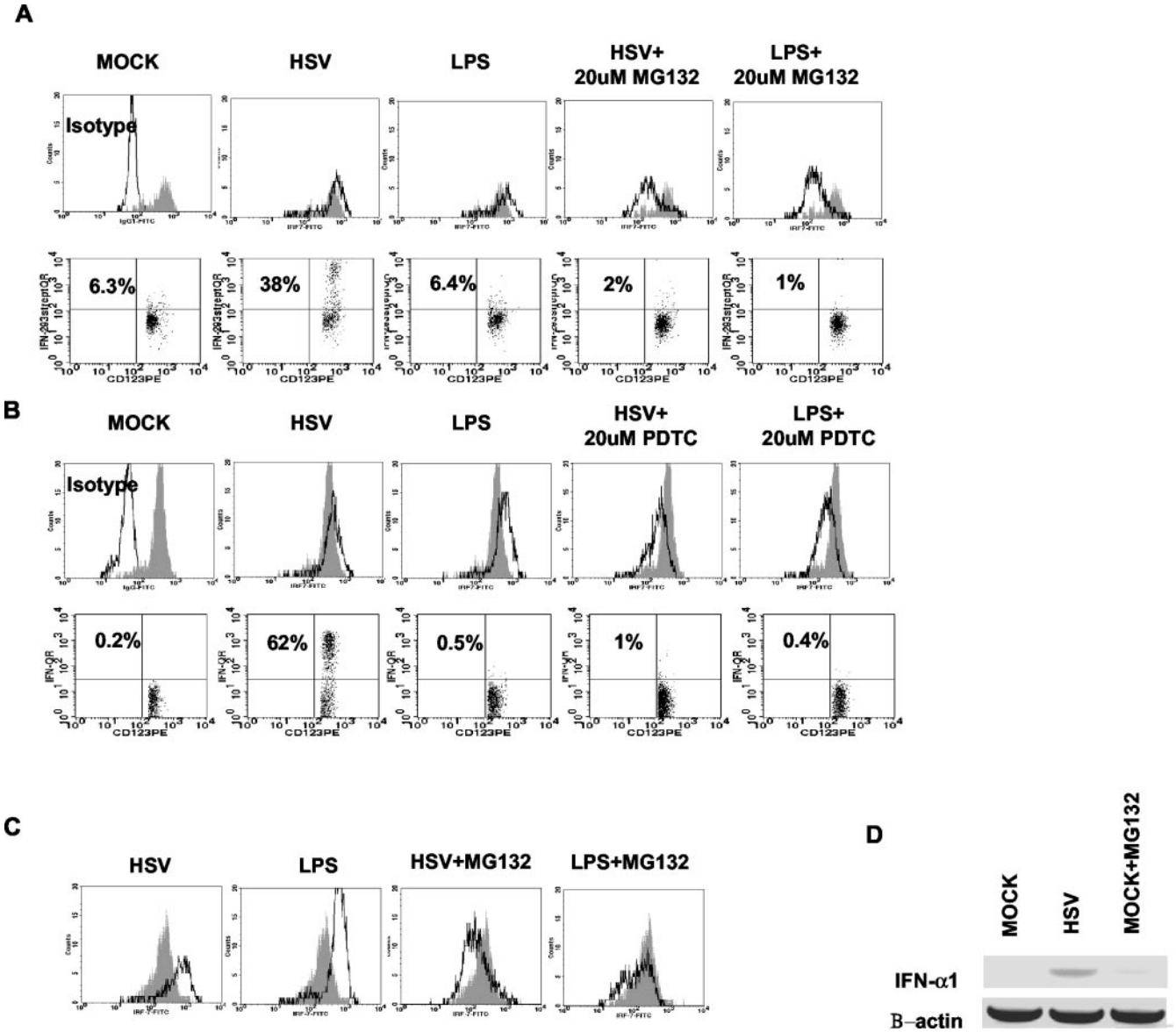

FIGURE 6. The NF-κB inhibitors PDTC and MG132 inhibit both IRF-7 and IFN-α expression in PDC. PBMC (*A* and *B*) or purified PDC (*C*) were stimulated with HSV or LPS for 6 h in the presence or absence of 20 μM MG132 (*A* and *C*) or 20 μM PDTC (*B*). The histogram overlays compare IRF-7 expression between mock (gray filled histograms) and differently treated PDC (black lines) after gating on PDC populations. Rabbit IgG was used as an isotype control. Numbers shown on the flow cytometric dot plot represent percentage of PDC that produced IFN-α (cells in *upper right quadrant*). Data in *A–C* are representative of three independent experiments. *D*, Purified PDC (≥96% pure PDC and ≤0.3% monocytes) were stimulated with HSV 6 h in the presence or absence of 20 μM MG132, then RNA was extracted and run for IFN-α1 or β-actin by RT-PCR. Data are representative of two independent experiments.

cells (Fig. 10*B*). Together, these results suggest that LPS treatment of PDC accelerates or primes virus-induced IFN-α production.

Discussion

In the present study, we have confirmed that PDC constitutively express high protein levels of IRF-7, consistent with recent studies by us and others (33–35). In contrast, monocytes expressed much less IRF-7 and produce 5- to 10-fold less IFN-α on a per cell basis and in response to a much more limited range of viruses (18, 19, 35). In addition, we found that enveloped viruses (including HSV, SV, and Flu) and LPS can increase the expression of IRF-7 in PDC; however, only virus, but not LPS, induced IFN-α production in these cells. This latter observation is in agreement with the findings of others: that LPS is a poor inducer of IFN-α production in human PDC (34, 42, 43). Moreover, the data demonstrate that the induction of IRF-7 by stimulation with LPS is insufficient to induce IFN-α production; rather, there is a distinct pathway for virus to induce IFN-α production in PDC.

Several studies have shown that in response to viral infection, IRF-7 is activated through phosphorylation and is transported to the nucleus, where it participates in the activation of type I IFN gene transcription (13, 14, 16, 57). By blocking phosphorylation and nuclear accumulation of IRF-7, a Kaposi's sarcoma-associated herpesviral immediate-early protein, open reading frame 45, efficiently inhibits the activation of type I IFN genes during viral infection (58). Phosphorylation is a common thread in response to infection; however, the underlying mechanisms leading to phosphorylation-dependent transcriptional activity are distinct and depend, at least in part, on distinct cellular kinases. IRF-3, for example, was found to be activated by phosphorylation on a specific

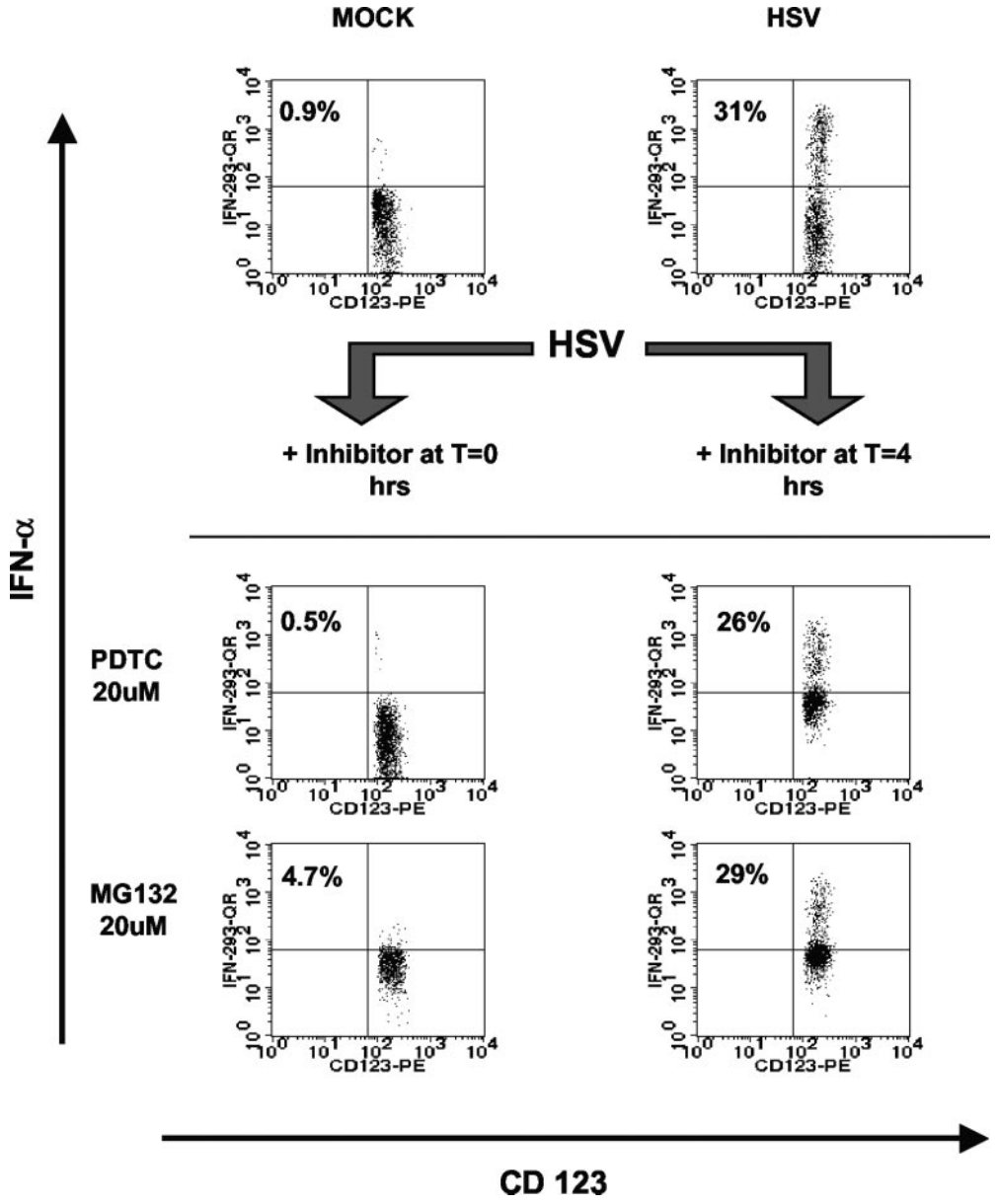

FIGURE 7. Neither PDTC nor MG132 inhibits IFN-α expression in PDC when added late after HSV stimulation. PBMC at 2×10^6/ml were stimulated with HSV at an MOI of 1. MG132 or PDTC at a concentration of 20 μM was added at the beginning or 4 h after the initiation of HSV stimulation. Cells were surface stained with CD123 PE and HLA-DR allophycocyanin for identification of PDC populations, followed by fixation and intracellular staining for IFN-α. Numbers shown on the flow cytometric dot plot represent percentage of PDC that produced IFN-α (cells in *upper right quadrant*). Data are representative of three independent experiments.

serine residue, Ser^{396}, following Sendai virus infection and dsRNA treatment, but not LPS (59). The kinase for IRF-7 phosphorylation is still unknown.

Although phosphorylated IRF-7 activity can be mimicked by substitution of individual serine residues with phosphomimetic aspartate residues (60), the exact sites of virus-induced phosphorylation within the regulatory domains have not been definitively established. Direct study of IRF-7 phosphorylation in PDC is difficult due to the low frequency of these cells in peripheral blood and the prohibitively high numbers of PDC from a single donor that are required for detection of phosphorylated IRF-7 by Western blot. However, because nuclear translocation occurs following IRF-7 phosphorylation, fluorescence microscopy studies of IRF-7 translocation can provide information regarding IRF-7 activation. In this study, we found that the translocation of IRF-7 occurred in PDC within 2 h of stimulation with HSV, which is earlier than when IRF-7 enhancement was observed in virus-stimulated PDC (at 4 h). This is consistent with other results reported in CpG DNA-stimulated human PDC: that the nuclear translocation of constitutive IRF-7 and the IFN-α gene expression were induced earlier than IRF-7 expression (34). It is likely that virus directly activates IRF-7 that is constitutively expressed at high levels in PDC to trigger the transcription of IFN-α genes. Interestingly, although LPS significantly up-regulated the expression of IRF-7 in PDC, no nuclear translocation of IRF-7 was induced by LPS. This suggests that the effects of virus on PDC leading to IFN-α production are mediated through mechanisms that are distinct from those of LPS activation. For example, virus may trigger a distinct kinase cascade for the induction of IFN-α. Recently, it has been reported that three kinases (IκB kinase (IKK)β, IKKϵ, and tank-binding kinase

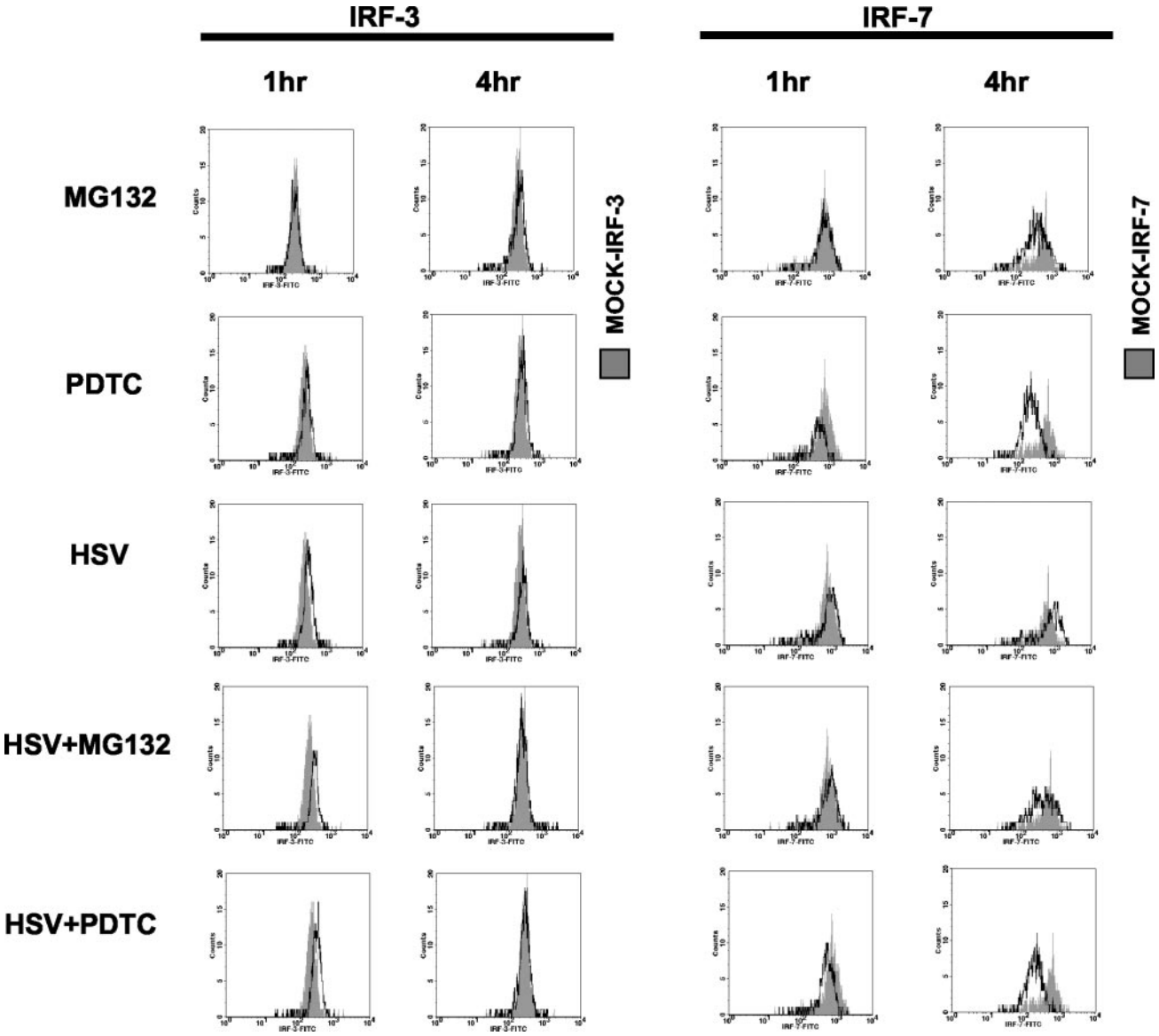

FIGURE 8. PDTC and MG132 down-regulate constitutive IRF-7, but not IRF-3 expression in PDC. PBMC (2×10^6/ml) were incubated in the presence or absence of 20 μM PDTC or MG132 with or without HSV stimulation for 1 and 4 h. The histogram overlays compare IRF-3 (*left panel*) and IRF-7 expression (*right panel*) between mock (gray filled histograms) and differently treated PDC (black lines) after gating on PDC populations. Rabbit IgG was used as an isotype control (data not shown). Data are representative of three independent experiments.

(TBK)1) activate NF-κB, whereas only IKKϵ and TBK1 are components of the virus-activated kinase that activates IRF-3 and IRF-7 by phosphorylation (61, 62). It is possible that HSV may activate IRF-7 via IKKϵ or TBK1 in PDC. This will be an interesting issue to be addressed in future studies.

PDC have been reported to express TLR-9, but not TLR-4 (48, 49). However, in our study, PDC clearly responded to LPS with the up-regulation of IRF-7. The response also occurred in purified PDC. The up-regulation of IRF-7 was even higher in response to highly purified LPS than to the commercial preparation (data not shown) and was sensitive to polymyxin treatment. By its priming for the accumulation of IRF-7, LPS was able to modify both the magnitude and the kinetics of virus-induced IFN-α production. Moreover, the surface and mRNA expression of TLR-4 were rapidly up-regulated in PDC in response to LPS, which may help to explain the effect of LPS on the induction of IRF-7 in PDC. Although we could not totally rule out the possibility that the low levels of TLR-4 message in the purified PDC were contributed to by the small numbers of contaminating monocytes (typically 0.3–0.5%), its up-regulation on the surface of the LPS-stimulated PDC, but not monocytes, suggests the TLR-4 expression by the PDC. Moreover, although the surface expression of TLR-4 was low in the LPS-primed PDC, these levels are similar to the levels induced on airway epithelial cells by respiratory syncytial virus, and which were shown to be physiologically active (63). However, as the purity of PDC was 95%, we cannot rule out the possibility of an indirect effect of cytokines contributed by the small numbers of contaminating cells.

The observation that the NF-κB site in the human IRF-7 promoter is sufficient to induce the expression of IRF-7 provided new information on the regulation of the IRF-7 gene (56). The activation of NF-κB is quite well understood: NF-κB is present as an inactive complex with its regulatory subunit IκB. IκB-α is phosphorylated at specific serine residues by the signal-activated IκB kinase complex. Then the phosphorylated IκB-α is specifically recognized and polyubiquitinated to become a substrate for

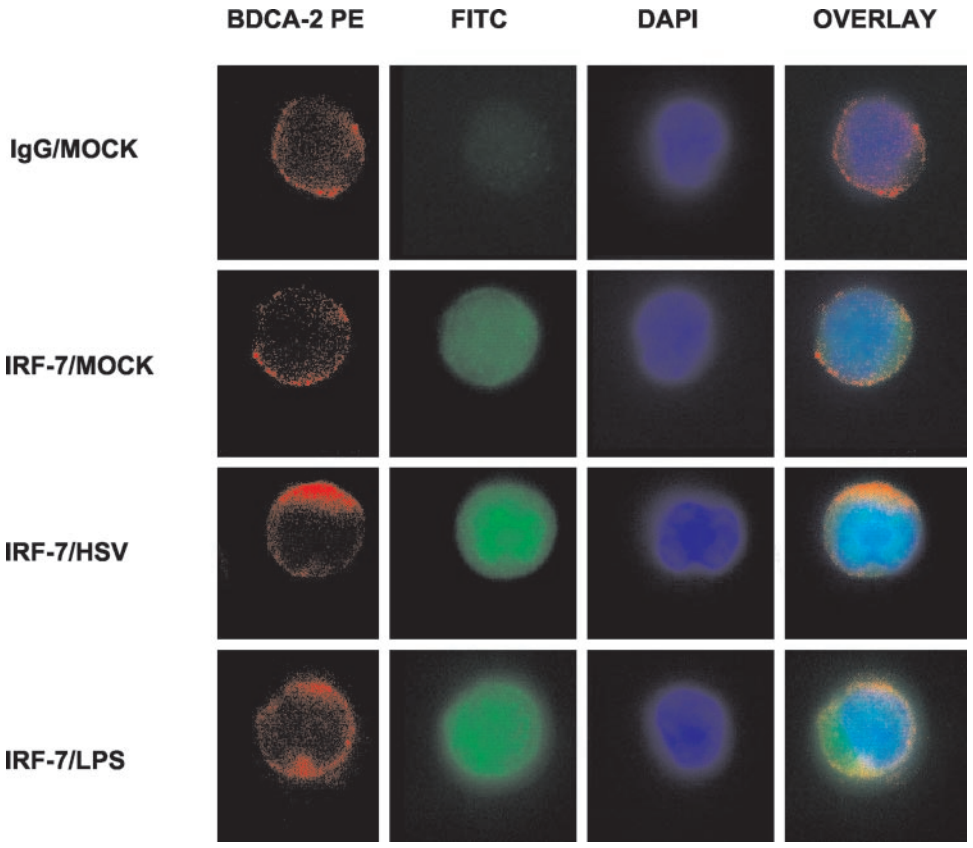

FIGURE 9. HSV, but not LPS, induces nuclear translocation of IRF-7 in purified PDC. PDC were purified by positive selection of BDCA-4-positive cells, as described above. A total of 2×10^5 purified PDC in 100 μl was stimulated with HSV or purified LPS for 2 h. PDC were identified by surface staining with BDCA-2 PE, and IRF-7 expression was measured using rabbit anti-human IRF-7 Ab, and detected via a FITC-conjugated anti-rabbit IgG Ab. Normal rabbit IgG was used as an isotype control (*first row*). The 4′,6′-diamidino-2-phenylindole staining was used to demonstrate the location of the nucleus.

degradation by the proteasome, resulting in the liberation of NF-κB. Recently, three novel stimulators of IRF-7 gene expression, namely EBV latent membrane protein 1, 12-*O*-tetradecanoylphorbol-13-acetate, and TNF-α, have been found to regulate IRF-7 expression through the NF-κB pathway (55, 56, 64). It also has been shown that a supersuppressor of IκB is capable of blocking the induction of IRF-7 by latent membrane protein 1, and that overexpression of NF-κB (p65 plus p50) can induce the expression of IRF-7 (64). Both virus and LPS are known activators of the NF-κB pathway (65, 66) and capable of inducing IRF-7 expression (13, 39). The kinetics of IRF-7 enhancement in PDC by both virus and LPS were quite similar, and the NF-κB pathway inhibitor PDTC and proteasome inhibitor MG132 (which functionally serves as an NF-κB inhibitor) efficiently suppressed both virus- and LPS-mediated induction of IRF-7. Neither inhibitor induced significant cytotoxicity at the effective dose (data not shown). Moreover, the production of TNF-α was not observed in purified PDC after LPS stimulation (48, 49), suggesting that the induction of IRF-7 by virus or LPS could be a direct transcription event. Moreover, these chemicals neither inhibited IRF-3 expression in PDC when present from the initiation of culture, nor inhibited IFN-α induction in PDC when added 4 h after virus stimulation, again arguing against a toxic effect. It is well known that IRF-7 can be induced through the action of autocrine IFN produced in response to virus infection in fibroblast cells. Because IRF-7 is expressed at very low levels in these cells, the IFN induction in the early phase is required for an abundant expression of IRF-7 through IFN-αR feedback signaling. Then activated IRF-7 *trans* activates multiple IFN genes, in a delayed manner (14–16). Our data suggest that PDC already possess the transcriptional machinery to allow them to respond promptly to the viral stimulation. Similarly, unlike fibroblasts, mouse IPC can produce early IFN-α independent of IFN-αR feedback signaling (67). Most recently, Taniguchi and colleagues (68) indicated that this positive feedback mechanism may not be operational upon LPS stimulation of mouse bone marrow DC.

IRF-7 is reported to be short-lived ($t_{1/2}$ = 1 h) and must be continuously produced for its function on IFN-$\alpha\beta$ genes (13, 16). We observed that the levels of IRF-7 decreased in unstimulated PDC after incubation in vitro for 6 h, which suggests that the PDC are not able to efficiently maintain their IRF-7 expression in vitro. The NF-κB inhibitors PDTC and MG132 significantly down-regulated IRF-7 expression in unstimulated PDC, suggesting that the NF-κB pathway is also involved in maintaining the constitutive expression of IRF-7 in PDC.

To date, there is no evidence for a requirement for NF-κB to induce IFN-α genes, and the IFN-α genes lack NF-κB binding sites (69); however, binding sites for IRF proteins and a requirement for IRF protein activation are a common feature of IFN-α genes. The inhibitors PDTC and MG132 suppressed the induction of IRF-7 in PDC by HSV, and also down-regulated the constitutive expression of IRF-7 in PDC, and blocked the production of IFN-α induced by HSV. These effects are unlikely to be a result of inhibition of viral replication because HSV-induced IFN-α production is independent of HSV replication (70). Moreover, the inhibitors did not inhibit the production of IFN-α when they were added after

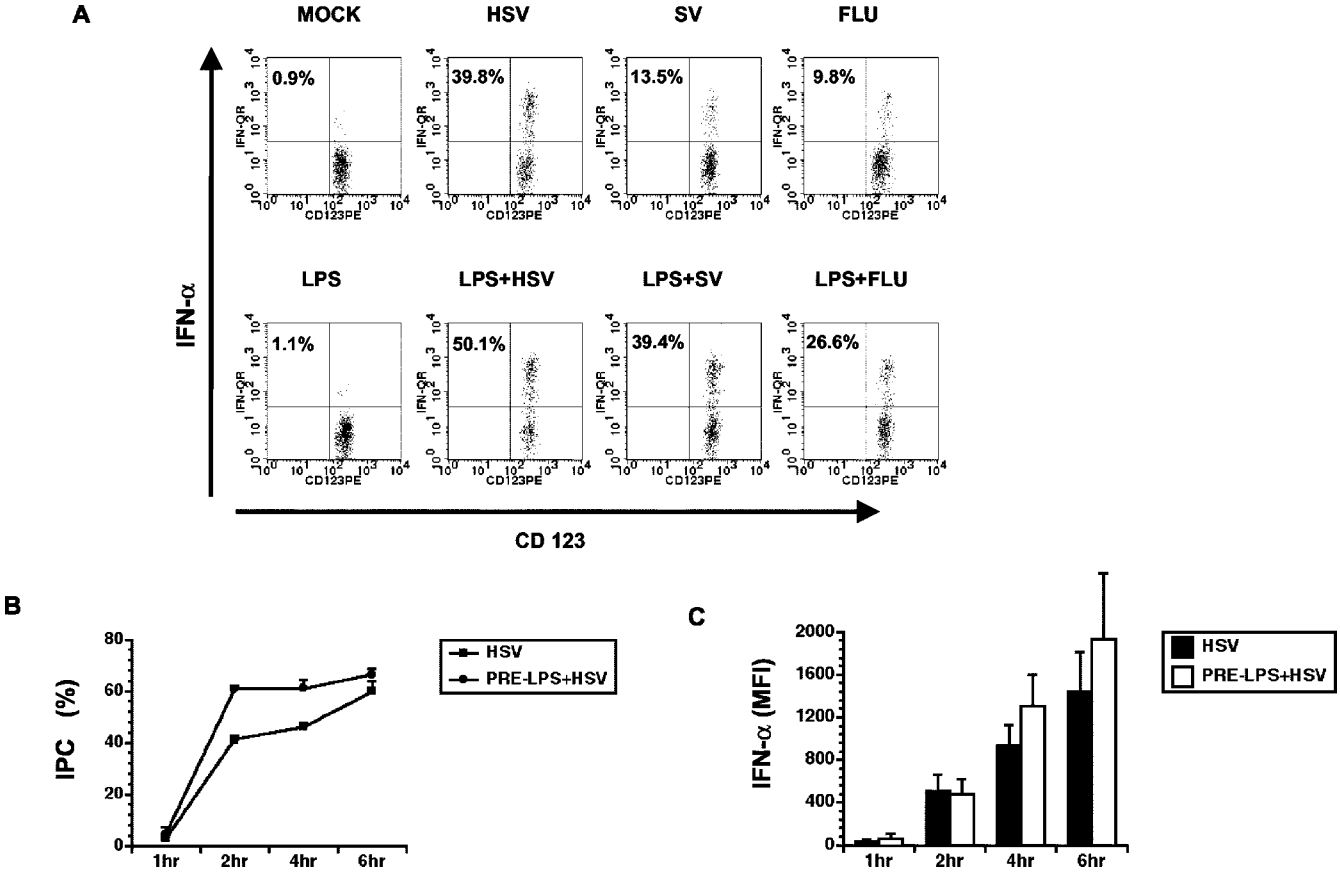

FIGURE 10. LPS augments and accelerates virus-induced IFN-α production in PDC. *A*, PBMC were incubated with or without LPS (10 ng/ml) for 2 h, followed by stimulation with HSV, SV, or Flu for an additional 4 h. Cells were surface stained with CD123 PE and HLA-DR allophycocyanin for identification of PDC populations, followed by fixation and intracellular staining for IFN-α. Numbers shown on the flow cytometric dot plots represent percentage of PDC that produced IFN-α (cells in *upper right quadrant*). Data are representative of three independent experiments. *B* and *C*, Time course of IFN-α production by LPS priming. A total of 2×10^6/ml PBMC was preincubated with or without LPS for 2 h, then stimulated with HSV for different time points. Cells were surface stained with CD123 PE and HLA-DR allophycocyanin for identification of PDC populations, followed by fixation and intracellular staining for IFN-α. The percentage of IPC was determined as in *B*. The increased MFI by LPS priming is shown in *C*. The means $\pm$ SEM of three independent experiments are shown.

the activation of IFN-α gene transcription had already been induced, further indicating that the high levels of constitutive IRF-7 expression in PDC are sufficient for triggering virus-induced IFN-α production.

Mixed infection is a clinical condition that occurs with high frequency in individuals who are immunocompromised as a consequence of HIV-1 infection, organ transplantation, or cancer. Notably, in PDC, although LPS by itself was unable to induce IFN-α production, it potently augmented and accelerated virus-induced IFN-α production. Because IRF-7 activation is required to induce IFN-$\alpha\beta$ gene induction by virus, the accumulation of IRF-7 by LPS priming could play a major role in LPS-mediated enhancement of IFN-α production in virus-stimulated PDC. Given the multiple roles of IFN-α in shaping both innate and adaptive immunity, and the central importance of PDC in bridging innate and adaptive immunity (71), enhanced IFN-α production in virus-stimulated PDC by LPS priming may provide an important protective mechanism during mixed infection.

In conclusion, we have presented data defining the critical role of IRF-7 in virus-induced IFN-α production in human PDC, and suggest two distinct regulatory pathways of IRF-7: the expression levels of IRF-7 in PDC can be increased by either virus or LPS via an NF-κB-dependent pathway, whereas the activation and translocation of IRF-7 in PDC are induced by a distinct pathway that is triggered by virus, but not LPS. The modification of virus-mediated IFN-α production by LPS priming further provides a potential protective mechanism in mixed infection.

Acknowledgments

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