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IFN-β Expression Is Directly Activated in Human Neutrophils Transfected with Plasmid DNA and Is Further Increased via TLR-4–Mediated Signaling

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Upon LPS binding, TLR4 activates a MyD88-dependent pathway leading to the transcriptional activation of proinflammatory genes, as well as a MyD88-independent/TRIF-dependent pathway, responsible for the transcriptional induction of IFN-β. Previous findings delineated that human neutrophils are unable to induce the transcription of IFN-β in response to TLR4 stimulation. Because neutrophils do not express protein kinase C ε, a molecule recently reported as essential for initiating the MyD88-independent/TRIF-dependent pathway, we optimized an electroporation method to transfect PKCε into neutrophils with very high efficiency. By doing so, a significant IFN-β mRNA expression was induced, in the absence of LPS stimulation, not only in PKCε-overexpressing neutrophils but also in cells transfected with a series of empty DNA plasmids; however, LPS further upregulated the IFN-β transcript levels in plasmid-transfected neutrophils, regardless of PKCε overexpression. Phosphoimmunoblotting studies, as well as chromatin immunoprecipitation assays targeting the IFN-β promoter, revealed that IFN-β mRNA induction occurred through the cooperative action of IRF3, activated by transfected DNA, and NF-κB, activated by LPS. Additional immunoblotting and coimmunoprecipitation studies revealed that neutrophils constitutively express various cytosolic DNA sensors, including IFN-inducible protein 16, leucine-rich repeat (in Flightless I) interacting protein-1, and DDX41, as well as that IFN-inducible protein 16 is the intracellular receptor recognizing transfected DNA. Consistently, infection of neutrophils with intracellular pathogens, such as *Bartonella henselae*, *Listeria monocytogenes*, *Legionella pneumophila*, or adenovirus type 5, promoted a marked induction of IFN-β mRNA expression. Taken together, these data raise questions about the role of PKCε in driving the MyD88-independent/TRIF-dependent response and indicate that human neutrophils are able to recognize and respond to microbial cytosolic DNA. *The Journal of Immunology*, **2012, 189: 1500–1509.**

The innate immune system, to which polymorphonuclear neutrophils belong, is highly specialized in its capacity to recognize foreign pathogens as a result of the expression of evolutionarily conserved families of receptor proteins: the pattern recognition receptors (1). The latter include, among others, the TLRs (2), which are constitutively expressed and functional in human neutrophils, with the exception of TLR3 (3–5). For instance, TLR4, which is the specific receptor for endotoxin (6), potently triggers TNF-α, CXCL8, CCL3, CCL4, CCL19, CCL20, IL-1ra, and IL-12p40 mRNA expression and production in neutrophils stimulated with LPS (7). However, unlike human monocytes, endotoxin-activated neutrophils do not express type I IFN (IFN-β and/or IFN-α) or IFN-dependent genes, such as CXCL10 or CXCL9 (8). This was recently attributed to the inability of neutrophils to mobilize the so-called "MyD88-independent/TRIF-dependent" pathway (8). In fact, it has emerged from studies mainly performed in macrophages or dendritic cells of gene-targeted mice that LPS triggers two classes of genes via TLR4 (1). One class is defined as MyD88 dependent, because it is not induced in MyD88$^{-/-}$ mice and mostly includes proinflammatory mediators, such as TNF-α, IL-1, IL-12p40, and CXCL8 (1). In this pathway, MyD88 and TIR domain-containing adapter protein/MyD88 adapter-like protein mediate a rapid and early activation of the transcription factor NF-κB, which is essential for transcriptional induction of the above-mentioned proinflammatory genes (1). The other class of genes is defined as MyD88-independent, because it relies on a more delayed activation of both NF-κB and IRF3 transcription factors in MyD88$^{-/-}$ mice that ultimately leads to the expression of IFN-β and, subsequently, to IFN-β–dependent STAT1 activation (1). It includes, for instance, a number of antiviral genes; Th1-activating chemokines, such as monokine induced by IFN-γ/CXCL9, IFN-inducible protein-10/CXCL10, and IFN-inducible T cell α chemoattractant/CXCL11; and anticancer molecules, such as TRAIL (9). Along the MyD88-independent pathway, TRAM (TIR domain-containing adapter-

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Abbreviations used in this article: ChIP, chromatin immunoprecipitation; DAI, DNA-dependent activator of IRF; IFI16, IFN-inducible protein 16; LRRFIP1, leucine-rich repeat (in Flightless I) interacting protein-1; MNE, mean normalized expression; MOI, multiplicity of infection; PDTC, pyrrolidine dithiocarbamate; pEGFP, enhanced GFP plasmid; PI, propidium iodide; PKC, protein kinase C; poly(dA:dT), poly(deoxyadenylic-deoxythymidylic) acid; poly(I:C), polyinosinic-polycytidylic acid; PRL, prolactin; qPCR, quantitative PCR; RIG-I, retinoic acid-inducible gene; STING, stimulator of IFN genes; TBK1, TRAF family-associated NF-κB–binding kinase-binding kinase-1; TRAM, TIR domain-containing adapter-inducing IFN-β–related adapter molecule; T4SS, type IV secretion system.

inducing IFN-β–related adapter molecule) and TRIF adapter proteins both transduce the activation of redundant protein kinases (i.e., TRAF family-associated NF-κB–binding kinase-binding kinase-1 [TBK1] and IκB kinase-ε), which phosphorylate IRF3 on Ser/Thr residues (10). As a result, IRF3 dimerizes, translocates to the nucleus, associates with other coactivators, and ultimately contributes to activate IFN-β gene transcription (11). Interestingly, the MyD88-independent/TRIF-dependent cascade seems to be initiated by a rapid activation of protein kinase C (PKCε in response to LPS, which, according to recent findings, would lead to phosphorylation of TRAM on serine residues (12). Such a post-translational modification would cause TRAM disappearance from the membrane concomitantly with activation of a downstream signaling cascade that would finally lead to activation of the TBK1–IRF3 axis and, ultimately, the transcriptional induction of IFN-β (12).

In this study, given the absence of PKCε in human neutrophils (13–16), we verified whether the lack of PKCε expression is the defect that prevents activation of the LPS-triggered MyD88-independent/TRIF-dependent pathway. For this purpose, we developed a transfection procedure to overexpress PKCε in human neutrophils and ultimately test whether we could rescue the MyD88-independent/TRIF-dependent pathway. Although our experiments disproved a role for PKCε in driving the MyD88-independent/TRIF-dependent response, they helped us to subsequently demonstrate that, similarly to all cell types tested to date, human neutrophils constitutively express various cytosolic DNA sensors. Consequently, we found that neutrophils can promptly express IFN-β mRNA upon recognition of transfected DNA or upon infection with intracellular pathogens, such as *Bartonella henselae*, *Listeria monocytogenes*, *Legionella pneumophila*, or adenovirus type 5.

Materials and Methods

Abs

PKCε (sc-214), p-PKCε (Ser^{729}; sc-12355), PKCβII (sc-210), IRF3 (sc-9082), NF-κB p65 (sc-372), NF-κB p50 (sc-7178), IκB-α (sc-371), IFN-inducible protein 16 (IFI16; sc-8023), DDX41 (sc-166225), and leucine-rich repeat (in Flightless I) interacting protein-1 (LRRFIP1; sc-135917) Abs were from Santa Cruz Biotechnology (Santa Cruz, CA). TBK1 (no. 3013), p-IRF3 (Ser^{396}; no. 4947), p–NF-κB p65 (Ser^{536}; no. 3031), and p-p44/42 MAPK (no. 9106) Abs were from Cell Signaling (Beverly, MA); anti–β-tubulin (T5293) and anti-actin (A5060) Abs were from Sigma (St. Louis, MO). TBK1 mAbs (IMG-139A) and rabbit polyclonal stimulator of IFN genes (STING) Abs (IMG-6422A) were from IMGENEX (San Diego, CA); rabbit polyclonal ISG15 Abs were kindly provided by Dr. Arthur L. Haas (Louisiana State University Health Sciences Center, New Orleans, LA).

Cell purification and culture

Highly purified granulocytes (neutrophils > 96.5%, eosinophils < 3%, n = 30) and Percoll-purified monocytes (n = 15) were isolated and prepared under endotoxin-free conditions from buffy coats of healthy donors, as previously described (17). Ficoll-Paque–isolated neutrophils were further enriched by positively removing any eventual contaminating cells, to reach > 99.7% purity (high purity neutrophils), at least once for each type of experiment presented in this article (18). Immediately after purification, neutrophils were either subjected to the transfection procedure (see below) or suspended in RPMI 1640 medium supplemented with 10% low-endotoxin FBS (<0.5 EU/ml; BioWhittaker, Verviers, Belgium), treated or not with stimuli (including 100 ng/ml ultrapure *Escherichia coli* LPS [0111:B4; Alexis], 10 μM R848 [InvivoGen], 50 μg/ml polyinosinic-polycytidylic acid [poly(I:C); InvivoGen], 10 ng/ml PMA [Sigma], or 100 U/ml IFN-γ [R&D Systems, Minneapolis, MN]), and then plated either in 6/24-well tissue culture plates (Nunc, Roskilde, Denmark) or in polystyrene flasks (Orange, Trasadingen, Switzerland) to be cultured at 37°C, 5% CO_2 atmosphere. After the desired incubation period, cells were collected and spun at 300 × g for 5 min, and cell pellets were either extracted for total RNA or lysed for protein analysis, as described below.

HEK293T cells (German Collection of Microorganism and Cell Cultures, Braunschweig, Germany) were cultured in 24-well plates using DMEM medium (Lonza) supplemented with 10% FBS and transfected with 2 μl Lipofectamine 2000 (Invitrogen) complexed with 0.8 μg poly(deoxyadenylic-deoxythymidylic) acid [poly(dA:dT); Sigma] or enhanced GFP plasmid (pEGFP; Clontech). All reagents used were of the highest available grade and were dissolved in pyrogen-free water for clinical use.

Transfection of neutrophils

Neutrophils were transfected with different types of plasmids, including pmaxGFP (Lonza), pEGFP, and pEGFP-PKCε (kindly provided by Professor P. Parker, Cancer Research UK, London Research Institute, London, U.K.) previously purified using the EndoFree Plasmid Maxi Kit (QIAGEN), as well as with *E. coli* DNA (#D4889; RNA and contaminant free, according to the Sigma datasheet, as well as to our own checking), poly(dA:dT), and poly(I:C). Transfection was performed using the human monocyte nucleofector kit and the Amaxa nucleofector II device (both from Lonza), according to the reagent instructions, yet with minor modifications. After isolation, 5×10^6 neutrophils were resuspended in 100 μl complete nucleofector solution containing 2.5–15 μg DNA (or equal volumes of PBS for mock transfection) and then transferred to a nucleoporation cuvette. Electroporation was performed using the Y001 program in the nucleofector II device. Cells were then recovered and left for 5 min in 2 ml human monocyte nucleofector medium supplemented with 2 mM glutamine and 10% FBS. Thereafter, neutrophils were washed once with PBS to remove broken cells and subjected to flow cytometry analysis using a FACScan flow cytometer (Becton-Dickinson, Franklin Lakes, NJ) to determine GFP expression. In selected experiments, transfected neutrophils were incubated for 30 min with different inhibitors, including BAY 117082, pyrrolidine dithiocarbamate (PDTC), sc-514 (Calbiochem, San Diego, CA), or 10 μM MG132 (Sigma) prior to stimulation with LPS.

Quantitative PCR

RNA isolation and reverse transcription were accomplished as previously described (19). Quantitative PCR (qPCR) was performed using SYBR Premix Ex Taq (Takara) and gene-specific primers (Invitrogen, Carlsbad, CA) available in the public database RTPrimerDB (http://medgen.ugent.be/rtprimerdb/index.php) under the following entry codes: IFN-β (3542), TNF-α (3551), CXCL8/IL-8 (3553), CXCL10/IFN-inducible protein-10 (3537), G1P2/ISG15 (3547), IFIT1 (3540), and GAPDH (3539). Data were calculated with Q-Gene software (http://www.gene-quantification.de/download.html) and are expressed as mean normalized expression (MNE) units after GAPDH normalization.

Apoptosis assessment

Apoptosis of transfected neutrophils was determined, as previously described (5), by the propidium iodide (PI) staining procedure, according to the "quick method" described by Riccardi and Nicoletti (20). Data were analyzed by FlowJo software (TreeStar, Ashland, OR).

Immunoprecipitations and immunoblots

Whole-cell extracts were prepared for immunoblots using the RNeasy mini kit (QIAGEN), as described (21), or according to the chromatin immunoprecipitation (ChIP) assay procedure (22) for IFI16, LRRFIP1, and DDX41 immunoprecipitations. Cytoplasmic extracts for PKCε and TBK1 immunoprecipitations were prepared using the nitrogen cavitation procedure and processed exactly as described previously (23). Blotted proteins were detected and quantified using the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE) (24).

ChIP assays

ChIP experiments were performed as described elsewhere (22), with minor modifications. For immunoprecipitation of transcription factors, nuclear extracts from 10^7 sonicated neutrophils were incubated with 7 μg anti-IRF3 or 5 μg anti–NF-κB p65/p50 Abs, whereas for cytoplasmic DNA sensor/plasmid DNA coimmunoprecipitations, whole-cell extracts prepared from 5×10^6 pEGFP-transfected neutrophils were immunoprecipitated using 5 μg αDDX41, αIFI16, and αLRRFIP1 Abs. In all ChIP experiments, protein recruitment to the prolactin (PRL) promoter (a gene that is completely silent in our cell types) was used as negative control. The coimmunoprecipitated material was then subjected to qPCR analysis using the following specific primers (purchased from Invitrogen): IFN-β promoter forward: 5′-TCCCAGGAACTCAATGAAGG-3′, IFN-β promoter reverse: 5′-GTGTCGCAATGGAGTGTTGT-3′; CXCL8 promoter forward: 5′-CTTAGTGGGGTTGAAAGTGAC-3′, CXCL8 promoter reverse: 5′-AAGAAATAGTCACTCACCCAAG-3′; pEGFP-backbone

forward: 5′-ACGGCATCAAGGTGAACTTC-3′, pEGFP-backbone reverse: 5′-GCTTCTCGTTGGGGTCTTTG-3′; pEGFP-GFP forward: 5′-TGCCATAGCCTCAGGTTACTC-3′, pEGFP-GFP reverse: 5′-GACGCTCAGTGGAACGAAAAC-3′; and PRL promoter forward: 5′-AGGGAAACGAATGCCTGATT-3′, PRL promoter reverse: 5′-GCAGGAAACACACTTCACCA-3′. pEGFP-backbone and pEGFP-GFP primers of pEGFP (accession number: U55763, http://www.ncbi.nlm.nih.gov/genbank/) amplify the 3854–3999-bp and 1091–1259-bp regions, respectively.

Infection of neutrophils

A total of 2×10^6 high-purity neutrophils was infected with 10 multiplicity of infection (MOI) *L. monocytogenes*, *L. pneumophila*, or *B. henselae* and then cultured in 24-well tissue culture plates for up to 6 h prior to RNA extraction and qPCR analysis. At 1 h postinfection, 50 μg/ml gentamicin was added to limit the growth of extracellular bacteria. In other experiments, neutrophils were infected with 1000 MOI adenovirus type 5 (E1 deleted, E3 defective, encoding for GFP; a kind gift of Prof. B. M. Foxwell, Imperial College, London, U.K.). Bacterial strains were all grown to mid-logarithmic phase as follows: wild-type *L. monocytogenes* (ATCC 19115) were grown in brain–heart infusion medium (Difco, Detroit, MI); *L. pneumophila* (serogroup 1 NCTC 12821, kindly provided by Prof. M.C. Zotti, University of Torino) were grown in N-(2-acetamido)-2-aminoethanesulfonic acid buffered yeast extract broth supplemented with 0.4 mg/ml L-cysteine and 0.135 mg/ml ferric nitrate (Sigma); and *B. henselae* Houston-1 (ATCC 49882) were grown in Schneider's medium supplemented with 10% FCS and 2 mM glutamine at 37°C with 5% CO_2 (25). Prior to infection, bacteria were pelleted and washed twice with PBS.

Statistical analysis

Data are expressed as mean ± SE. Statistical evaluation was performed using the Student *t* test or one-way ANOVA followed by the Tukey post hoc test. The *p* values < 0.05 were considered statistically significant.

Results

Overexpression of PKCε in human neutrophils by electroporation

Immunoblot and immunoprecipitation analyses of either cytoplasmic cavitates (26) (Supplemental Fig. 1A, *top panel*) or whole-cell lysates (data not shown) confirmed (13–16) that neutrophils do not express PKCε, unlike autologous monocytes. Moreover, PKCε protein expression in neutrophils and monocytes was not inducible or upregulated, respectively, by 24 h-treatment with either LPS or IFN-γ (Supplemental Fig. 1B). Therefore, to clarify whether the absence of PKCε might be responsible for the inability of human neutrophils to mobilize the MyD88-independent/TRIF-dependent pathway, we attempted to overexpress PKCε in these cells. However, a series of preliminary experiments was necessary to develop a reliable transfection protocol, because neutrophils are extremely difficult to manipulate for exogenous gene expression as a result of their inability to proliferate and to survive in culture for long periods. Ultimately, we achieved success by modifying a recently reported nucleofection protocol (27), with which the investigators were able to transfect both GFP and $p47^{phox}$-GFP proteins into neutrophils. By our procedure, we transfected a pmaxGFP plasmid into neutrophils with a greater efficiency than the reported 5% (27) (see the fluorescence microscopy image in Supplemental Fig. 2A, *left panel*). Accordingly, flow cytometry analysis revealed that the percentage of GFP^+ neutrophils correlated with the amounts of transfected pmaxGFP plasmid (Supplemental Fig. 2B). For example, >50% of GFP^+ neutrophils were measured by nucleofecting 15 μg pmaxGFP plasmid (Supplemental Fig. 2B). Importantly, such remarkable levels of GFP^+ neutrophils were obtained as early as 4 h postnucleofection, and they remained stable for up to 20 h of culture (Supplemental Fig. 2C). However, because neutrophils became mostly apoptotic after 20 h (nearly 80% were PI^+; Supplemental Fig. 2D), whereas they remained alive for up to 7–8 h posttransfection (75–80% of PI^- cells) (Supplemental Fig. 2D), all subsequent experiments were performed using neutrophils that were first transfected for 4 h and then stimulated with LPS for no more than 3 h.

The validity of our experimental procedure was confirmed by transfecting neutrophils with a plasmid encoding a GFP-tagged full-length PKCε (pEGFP-PKCε), which produced de novo expression of remarkable quantities of exogenous PKCε-GFP (Fig. 1A, 1B). Indeed, pEGFP-PKCε–transfected neutrophils displayed specific PKCε-immunoreactive signals, unlike the cells transfected with the corresponding empty plasmid (pEGFP) (Fig. 1A). Again, expression levels of exogenous PKCε correlated with the amounts of transfected plasmid (Fig. 1B): for instance, the levels of exogenous PKCε-GFP expressed in 2×10^6 neutrophils transfected with 15 μg PKCε-GFP plasmid were comparable to the levels of endogenous PKCε present in 1×10^6 monocytes

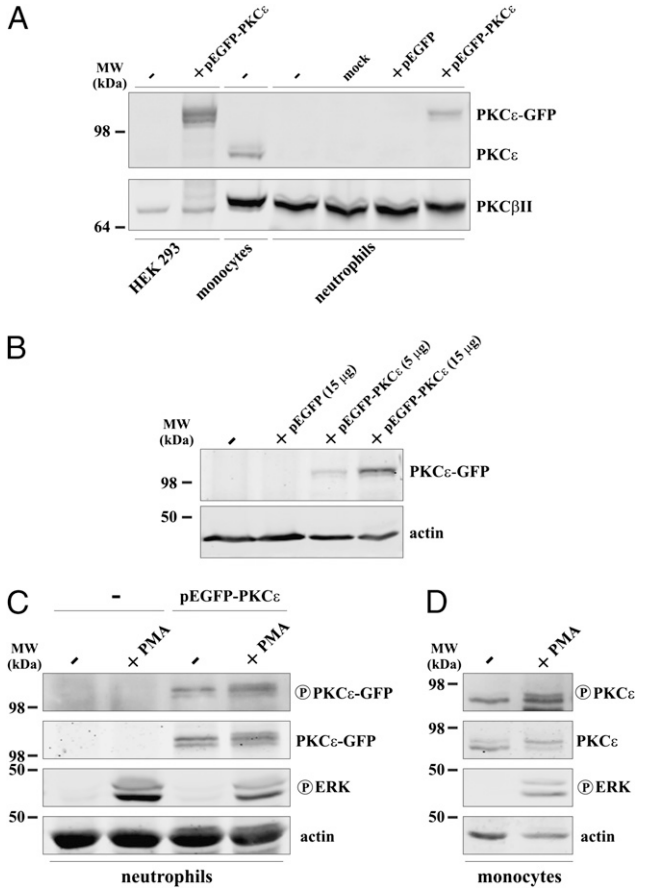

FIGURE 1. Transgenic expression of PKCε-GFP in human neutrophils. (**A**) Neutrophils were electroporated with 15 μg pEGFP-PKCε, 15 μg pmaxGFP, or PBS (i.e., mock electroporated), as described in *Materials and Methods*. After 4 h, neutrophil and monocyte whole-cell extracts were prepared and, together with extracts from HEK293T cells, were transfected with pEGFP-PKCε or not and electrophoresed (50 μg neutrophils, 30 μg monocytes, 10 μg for HEK293T) for immunoblotting studies using Abs specific for PKCε and PKCβII. Panels depict a representative experiment of three independent ones. (**B**) Neutrophils were electroporated with 5 or 15 μg of pEGFP-PKCε or with 15 μg of pEGFP. After 4 h, whole-cell extracts were prepared and then electrophoresed for PKCε and actin immunoblotting. Panels depict a representative experiment (n = 2). (**C**) Neutrophils were electroporated with 15 μg of pEGFP-PKCε for 4 h, treated with 5 ng/ml PMA, and then lysed after an additional 20 min. Whole-cell extracts were prepared, electrophoresed, and immunoblotted using Abs specific for p-PKCε, total PKCε, p-ERK(p42/p44), and actin. (**D**) Whole-cell extracts prepared from monocytes stimulated for 20 min with 5 ng/ml PMA were processed as in (C) and used as positive control. For (C) and (D), data show one representative experiment of two.

(Fig. 1A, 1B). Importantly, PKCε-GFP overexpressed in neutrophils underwent serine phosphorylation in response to PMA stimulation, as revealed by the use of specific anti–p-PKCε Abs (Fig. 1C). Similar data could be extrapolated by using anti-PKCε Abs that detect a doublet (28), with the more slowly migrating band likely corresponding to phosphorylated PKCε, as clearly evidenced in monocytes (Fig. 1D). Importantly, transfected neutrophils displayed an unaltered capacity to respond to external agonists, as proven by the findings that PMA triggered ERK phosphorylation in electroporated neutrophils at levels very similar to those observed in nontransfected cells (Fig. 1C). Taken together, our data demonstrate that it is possible to successfully transfect PKCε-GFP in neutrophils and that exogenous PKCε-GFP can be rapidly phosphorylated by PMA.

LPS acquires the capacity to upregulate IFN-β mRNA expression in plasmid DNA-transfected neutrophils

To assess whether transfected PKCε could restore the MyD88-independent/TRIF-dependent pathway, neutrophils were incubated 4 h post–PKCε-GFP plasmid or pEGFP plasmid transfection and then stimulated with 100 ng/ml ultrapure LPS for an additional 90 min (Fig. 2A, *upper panel*) to investigate the state of IFN-β and CXL10 mRNA expression by qPCR. In line with our previous study (8), no IFN-β or CXL10 mRNA induction occurred in nontransfected neutrophils stimulated with LPS, as opposed to the induction of two classical MyD88-dependent genes: *TNF-α* and *CXCL8* (Fig. 2A, *lower panels*). In contrast, both IFN-β and CXCL10 (but not TNF-α or CXCL8) gene expression was significantly increased by LPS in PKCε-GFP–overexpressing neutrophils (Fig. 2A, *lower panels*), as if the MyD88-independent/TRIF-dependent pathway was rescued by the introduction of an exogenous PKCε. However, this presumed rescue also occurred in neutrophils transfected with a pEGFP plasmid, with increased amounts of IFN-β and CXCL10 transcripts in response to LPS at levels comparable to those measured in PKCε-GFP–overexpressing neutrophils (Fig. 2A, *center panels*). Unexpectedly, neutrophils transfected with either PKCε-GFP or pEGFP plasmids expressed elevated amounts of IFN-β, CXCL10, TNF-α, and CXCL8 transcripts, even in the absence of LPS stimulation (Fig. 2A), indicating that the simple introduction of DNA into neutrophils strongly promotes their gene activation. Accordingly, data depicted in Fig. 2B clearly demonstrate that, upon transfection with pEGFP plasmid (or PKCε-GFP plasmid or *E. coli* DNA, data not shown), a time-dependent increase in IFN-β and CXCL10 mRNA is already detectable in neutrophils after 45 min, which increases thereafter up to 7 h. Also, neutrophil electroporation with poly(dA:dT), a chemically synthesized DNA commonly used to study the immunological response to B-form DNA (29), directly triggered IFN-β and CXCL10 mRNA expression (Fig. 2C, *lower panels*). The latter experiments not only demonstrate that the ability of neutrophils to recognize foreign DNA is not restricted to plasmid DNA, but they also prove that the stimulating agent is genuine DNA and not a contaminating product derived from the plasmid-isolation procedure (e.g., bacterial RNA). Moreover, additional stimulation with LPS of poly(dA:dT)-electroporated neutrophils again resulted in an upregulation in the levels of IFN-β and CXCL10 transcripts relative to nontransfected neutrophils (Fig. 2C, *lower panels*). Such an LPS-mediated effect was also observed in neutrophils transfected with *E. coli* DNA (Fig. 2C, *lower panels*) but, interestingly, not with poly(I:C) (Fig. 2C, *lower panels*), a synthetic analog of viral dsRNA that we previously showed to very strongly upregulate IFN-β mRNA expression when electroporated into human neutrophils (5). Altogether, our data demonstrate that the mere DNA/plasmid DNA transfec-

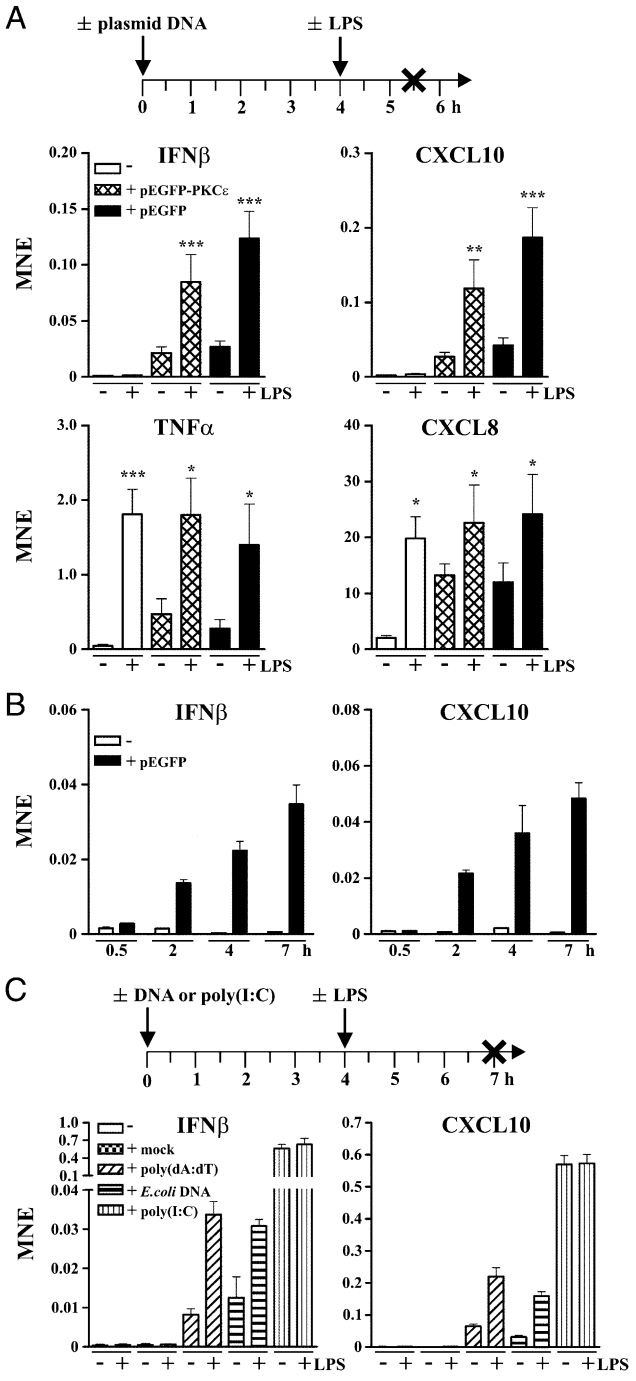

FIGURE 2. Modulation of IFN-β mRNA expression in plasmid-transfected neutrophils incubated or not with LPS. Neutrophils were electroporated with 15 μg pEGFP-PKCε, 15 μg pEGFP (empty vector) (**A**, **B**), 15 μg of poly(dA:dT), 15 μg *E. coli* DNA, 2 μg of poly(I:C), or PBS (mock electroporated) (**C**) and then incubated for 4 h before the addition of 100 ng/ml LPS (A, C) or incubated for the times indicated (B). Total RNA was extracted after an additional 90 min (A) or 180 min (C) post-LPS stimulation or as indicated (B) and then analyzed for IFN-β, CXCL10, TNF-α, CXCL8, and GAPDH mRNA expression by qPCR. The schemes illustrating the experimental protocols used are shown in the *uppermost panels* of (A) and (C). Gene expression is depicted as MNE ± SE ($n = 6$) after GAPDH normalization of triplicate reactions for each sample. For (B) and (C), results shown are from one representative experiment of at least three performed. Asterisks indicate a significant increase exerted by LPS. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

tion of neutrophils markedly activates the expression of IFN-β and CXCL10 mRNA. Our data also demonstrate that LPS further upregulates the expression of IFN-β and CXCL10 mRNA in DNA/plasmid DNA-transfected neutrophils, yet in a manner that appears independent from the overexpression of exogenous PKCε-GFP.

Identification of IRF3 as the critical factor for the transcription of IFN-β mRNA in plasmid DNA-transfected neutrophils

Subsequent experiments were aimed at elucidating the molecular bases responsible for the induction of IFN-β mRNA expression in neutrophils transfected with plasmid DNA, as well as for its further upregulation by LPS. For this purpose, we analyzed the activation status of IRF3, which represents a crucial intermediate for the transcriptional induction of IFN-β (11). Using the protocol depicted in the scheme of Fig. 3A (*upper panel*), we found that the mere pEGFP transfection of neutrophils triggered a direct ser396 phosphorylation of IRF3 (Fig. 3A, *center panel*) that was relatively weak after 2 h but became very strong after 5 h (for a quantitative densitometric analysis see Fig. 3A, *lower panel*, n = 3). Notably, similar results were obtained with the PKCε-GFP plasmid (data not shown), whereas a 1-h stimulation of DNA-transfected neutrophils with LPS did not augment the levels of ser396-phosphorylated IRF3 (Fig. 3A). Consistent with these findings were the results from IRF3 ChIP assays, which revealed a strong recruitment of IRF3 to the IFN-β promoter in plasmid-transfected neutrophils (Fig. 3B, *lower panel*) and that such recruitment is not increased further by subsequent LPS stimulation (Fig. 3B, *lower panel*). The specificity of the IRF3 ChIP assay was proved by the fact that the PRL promoter was not amplified under the same experimental conditions (Fig. 3B, *lower panel*), as well as by the fact that matched control Abs did not coprecipitate any IFN-β or PRL promoter (data not shown). Altogether, data demonstrate that, although the transfection of neutrophils with plasmid DNA directly activates the phosphorylation of IRF3 and its binding to the IFN-β promoter, LPS remains unable to modify the activation status of IRF3, even if it upregulates the accumulation of IFN-β transcripts. It follows that the upregulatory effect of LPS at the level of IFN-β gene expression in plasmid-transfected neutrophils does not occur via the MyD88-independent/TRIF-dependent cascade.

Identification of the transcription factors promoting the expression of IFN-β mRNA in plasmid DNA-transfected neutrophils upon LPS stimulation: role of NF-κB

Because NF-κB is a transcription factor cooperating with IRF3 in promoting IFN-β transcription (30), we subsequently analyzed its activation status in plasmid-transfected neutrophils treated or not with LPS for 45 min (Fig. 4A, 4B). By doing so, we observed considerable levels of phosphorylated NF-κB p65 (Fig. 4A) and diminished amounts of IκBα (Fig. 4B) in neutrophils transfected with DNA plasmids for 4 h and 45 min. Moreover, the addition of LPS to 4-h plasmid-transfected neutrophils resulted in a further increase in the levels of NF-κB p65 phosphorylation (Fig. 4A) and IκBα degradation (Fig. 4B) after 45 min (for a quantitative densitometric analysis see Fig. 4A, 4B, *lower panels*, n = 3). Importantly, ChIP assays performed according to the scheme depicted in Fig. 4C, using Abs toward NF-κB p65 (Fig. 4D) and NF-κB p50 (Fig. 4E), highlighted a crucial role for NF-κB in driving the effect of LPS on IFN-β mRNA expression in plasmid-transfected neutrophils. In fact, both p50 and p65 NF-κB subunits were strongly recruited to the IFN-β promoter upon LPS stimulation of plasmid-transfected neutrophils (Fig. 4D, 4E, *left panels*). In contrast, no recruitment of either NF-κB p50 or NF-κB p65 to the IFN-β promoter was observed in nontransfected neutrophils treated with LPS (Fig. 4D, 4E, *left panels*), consistent with the inability of LPS to induce IFN-β gene expression under the latter conditions (8). However, under the latter conditions, both NF-κB p65 and NF-κB p50 bound strongly to the CXCL8 promoter (Fig. 4D, 4E, *right panels*). Notably, in plasmid-transfected neutrophils, the recruitment of NF-κB p65 and NF-κB p50 to the CXCL8 promoter, although negligible after 60 min of LPS stimulation (Fig. 4D, 4E, *right panels*), was ≥3-fold higher after 30 min (data not shown), in line with the enhanced CXCL8 mRNA expression observed in parallel (Fig. 2A).

The role of activated NF-κB as a crucial mediator of the additional upregulation of IFN-β mRNA expression by LPS in

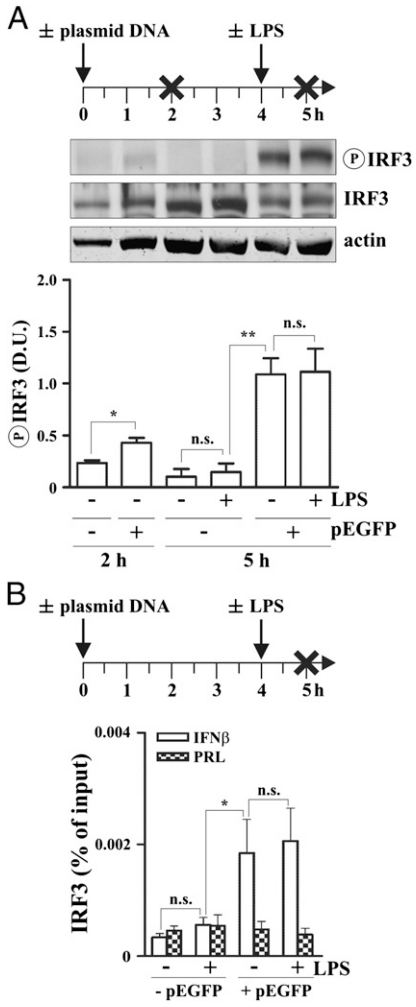

FIGURE 3. Activation of IRF3 in plasmid-transfected neutrophils. (**A**) Neutrophils were electroporated with 15 μg pEGFP and cultured for up to 5 h, the last hour in the presence or the absence of 100 ng/ml LPS. Neutrophils were lysed at the 2- and 5-h time points (*uppermost panel*) and whole-cell extracts were electrophoresed and immunoblotted using anti–p-IRF3 (Ser^{396}), anti-IRF3, and anti-actin Abs. The *center panel* shows a representative immunoblot (n = 3), and the *lower panel* displays the densitometric quantification of p-IRF3 levels (normalized by total IRF3; n = 3) by LI-COR Odyssey software, expressed as densitometric units (D.U.). (**B**) Neutrophils were electroporated with 15 μg pEGFP and cultured for 5 h, the last hour in the presence or absence of 100 ng/ml LPS. Cells were then processed for ChIP analysis, as described in *Materials and Methods*. Enrichment of IFN-β and PRL promoters in coprecipitated DNA was analyzed by qPCR using promoter-specific primers. Data from qPCR are expressed as percentages over input DNA (mean ± SE; n = 3). $*p < 0.05$, $**p < 0.01$ versus nontransfected cells.

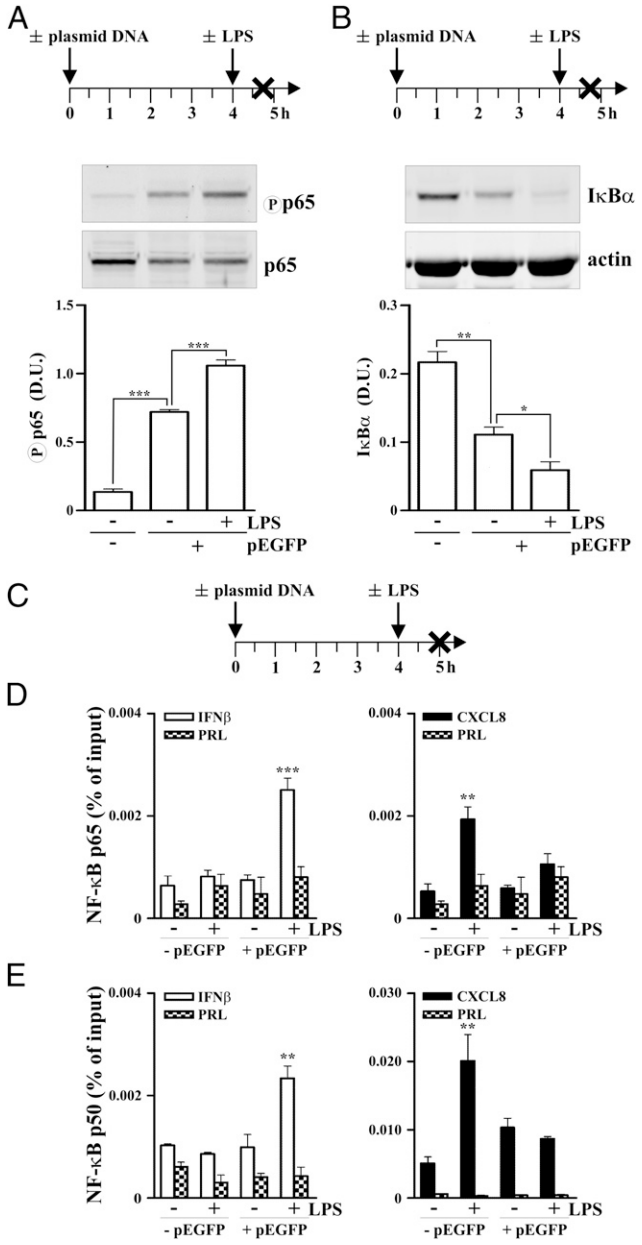

FIGURE 4. LPS-mediated NF-κB activation and binding to the IFN-β promoter in plasmid-transfected neutrophils. (**A**, **B**) Neutrophils were electroporated with 15 μg pEGFP and cultured for 4 h and 45 min, with the last 45 min in the presence or absence of 100 ng/ml LPS (as depicted in the *top panels*). Whole-cell extracts were prepared, electrophoresed, and immunoblotted using anti-p–NF-κB p65 (Ser^{536}), anti-total NF-κB p65 (A), anti-IκBα, and anti-actin Abs (B). Shown is a representative immunoblot split into two parts ($n = 3$) (*center panels*). The graphs (*lower panels*) illustrate the densitometric quantification of p–NF-κB p65 levels (normalized by the total NF-κB p65) (A) and IκBα levels (normalized by the total actin) (B), expressed as mean ± SE ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Neutrophils were treated as in (**C**), and processed for ChIP analysis using anti–NF-κB p65 (**D**) or anti–NF-κB p50 (**E**) Abs. Enrichment in IFN-β, CXCL8, and PRL promoter in coprecipitated DNA was analyzed by qPCR using promoter-specific primers. Data from qPCR are expressed as percentages over input DNA (mean ± SE; $n = 3$). $^{**}p < 0.01$, $^{***}p < 0.001$ versus control or nontransfected cells.

plasmid-transfected neutrophils was additionally supported by the effect of four chemical NF-κB blockers: BAY 117082, an inhibitor of IκBα phosphorylation and degradation (31); PDTC, an inhibitor of NF-κB activation (32); sc-514, a selective IκB kinase-2 inhibitor (33) (Fig. 5); and MG132, a proteasomal inhibitor (34) (data not shown). In fact, although they had no effect on the induction of IFN-β mRNA directly triggered by the simple plasmid transfection, all of them significantly reduced the upregulatory effect of LPS on IFN-β mRNA expression (Fig. 5). Altogether, our data suggest that the increased expression of IFN-β mRNA observed in response to LPS in plasmid-transfected neutrophils is likely the consequence of the LPS-triggered recruitment of p65 and p50 NF-κB subunits to the IFN-β promoter, which, for unknown reasons, does not take place in nontransfected neutrophils.

Intracellular recognition of plasmid DNA by human neutrophils is dependent on IFI16

Finally, we aimed at defining how human neutrophils recognize transfected plasmid DNA. Initially, we investigated the role of TLR9, because this molecule is constitutively expressed and responsive in human neutrophils (4, 35). TLR9 recognizes unmethylated CG dinucleotides (CpG) present in the extracellular space within the endosomal compartment (36); consequently, drugs able to block endosome acidification (37), such as chloroquine and bafylomicin A1, inhibit its ability to activate NF-κB and to induce cytokines (37, 38). Experiments in which neutrophils were treated with the two inhibitors for 30 min prior to plasmid DNA electroporation (for 4 h) revealed that both chloroquine and bafylomicin A1 do not prevent the induction of IFN-β or TNF-α gene expression (Supplemental Fig. 3A, *left* and *center panels*). In contrast, both inhibitors markedly suppressed the induction of TNF-α mRNA expression in R848-stimulated neutrophils (Supplemental Fig. 3A, *right panel*), thereby proving that they were functioning appropriately. Then we tested the role of RNA polymerase III, which functions as an indirect intracellular DNA sensor (39, 40). In fact, RNA polymerase III recognizes AT-rich DNA and consequently synthesizes uncapped 5′ triphosphate-bearing RNA, which, in turn, serves as an agonist for retinoic acid-inducible gene (RIG-I) (39, 40), which is constitutively expressed by human neutrophils (Fig. 6A) (5). Once again, a specific RNA polymerase III inhibitor-based approach, using ML-60218 (39, 40), did not suppress the induction of IFN-β mRNA exerted by plasmid DNA or poly(dA:dT) electroporation (Supplemental Fig. 3B). In contrast, ML-60218 dose dependently reduced the

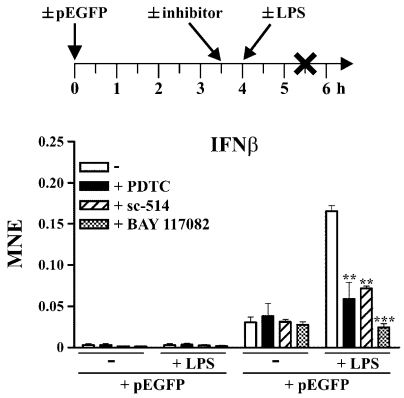

FIGURE 5. Effect of NF-κB inhibitors on the upregulation of IFN-β mRNA expression triggered by LPS in plasmid-transfected neutrophils. Neutrophils were electroporated with 15 μg of pEGFP; after 3.5 h of culture, they were incubated with 300 μM PDTC, 100 μM sc-514, or 5 μM BAY 117082. After 30 min, neutrophils were stimulated with 100 ng/ml LPS for an additional 90 min (*upper panel*). Total RNA was then extracted and analyzed for IFN-β and GAPDH mRNA expression by qPCR. IFN-β mRNA expression is depicted as MNE ± SE ($n = 3$) after GAPDH normalization of triplicate reactions for each sample. $^{**}p < 0.01$, $^{***}p < 0.001$.

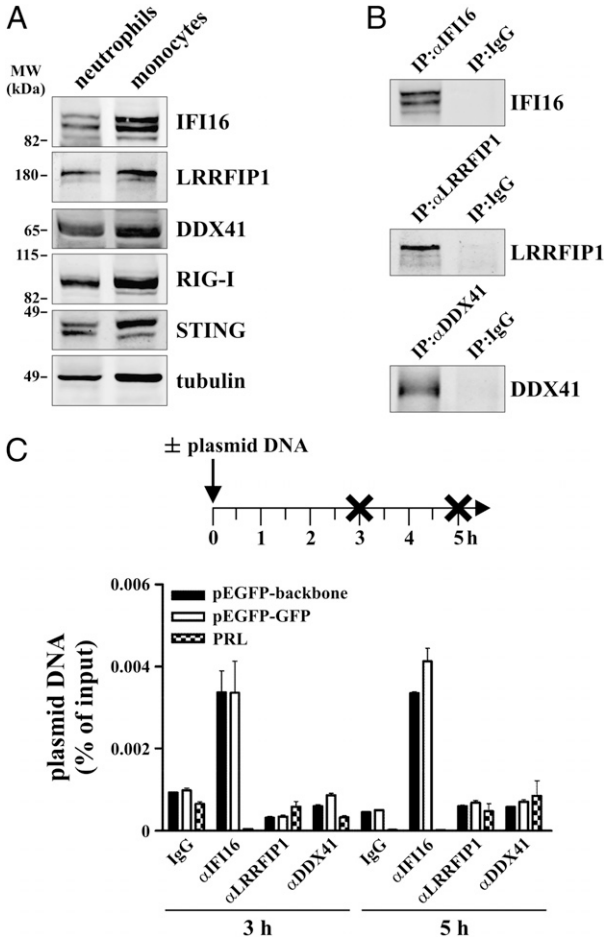

FIGURE 6. Neutrophil recognition of intracellular plasmid DNA is dependent on IFI16. (**A**) Whole neutrophil and monocyte extracts (60 μg) were electrophoresed and immunoblotted using Abs specific for IFI16, LRRFIP1, DDX41 RIG-I, STING, and tubulin. (**B**) Whole neutrophil lysates (500 μg), prepared using the ChIP assay buffer, were subjected to immunoprecipitation with IFI16, LRRFIP1, DDX41, or anti-mouse IgG1 (control) Abs. Representative immunoblot experiments are shown ($n = 3$) for (A) and (B). (**C**) Neutrophils were transfected with pEGFP, cultured for 3 and 5 h, and then disrupted by ChIP assay buffer. Whole-cell lysates were then subjected to immunoprecipitation with IFI16, LRRFIP1, DDX41, or anti-mouse IgG1 control Abs. Finally, coimmunoprecipitated DNA was purified and analyzed by qPCR using two sets of primers specific for pEGFP (pEGFP-backbone and pEGFP-GFP). Data from qPCR are expressed as percentages over input DNA. Results are from one experiment representative of four.

induction of IFN-β mRNA in HEK293T cells transfected with poly(dA:dT) (Supplemental Fig. 3C), as expected (39, 40). Thus, we concluded that neither TLR9 nor RNA polymerase III functions as the intracellular receptor(s) recognizing plasmid DNA in electroporated neutrophils. In contrast, we confirmed that neutrophils express IFI16 (41), one of the newly identified intracellular receptors for foreign DNA (42). Accordingly, immunoblotting experiments revealed that whole neutrophil and monocyte lysates contain IFI16, as a cluster of proteins of 85–95 kDa (Fig. 6A), similar to what was originally described in the nuclear extracts of IFN-γ–treated HL-60 cells (41). Under the same conditions, we also detected, in both neutrophils and monocytes, two more recently discovered DNA sensors: LRRFIP1, which promotes IFN-β gene expression through β-catenin engagement and not via IRF3 activation (43), and DDX41, a member of the DEXDc family of helicases (44) (Fig. 6A). Furthermore, we found that neutrophils also express STING (Fig. 6A), a transmembrane protein located in the endoplasmic reticulum that is crucial for DNA-mediated signaling (45–48).

In an attempt to identify the specific DNA sensor(s) binding transfected DNA in neutrophils, we used the strategy that allowed Zhang et al. (44) to identify the intracellular binding protein recognizing *L. monocytogenes* DNA. Specifically, we immunoprecipitated IFI16, LRRFIP1, and DDX41 after DNA transfection (Fig. 6B) and then analyzed, by qPCR, to which of these receptors plasmid DNA was stably bound (Fig. 6C). By doing so, we found that IFI16 functions as the intracellular DNA sensor that, in transfected neutrophils, is predominantly involved in recognizing plasmid DNA (Fig. 6C).

Induction of IFN-β mRNA expression by infection of neutrophils with intracellular pathogens

In a final series of experiments, we tested a number of intracellular pathogens, including *B. henselae, L. monocytogenes*, *L. pneumophila*, and adenovirus type 5, which, according to the literature (49) are known to activate IFN-β gene expression via cytosolic sensing of their DNA. As shown in Fig. 7A, all intracellular bacteria under examination triggered a remarkable IFN-β and CXCL10 mRNA expression in neutrophils infected for 6 h but not 3 h. In addition, all bacteria upregulated the expression of TNFα (Fig. 7A) and CXCL8 mRNA (data not shown), indicating that

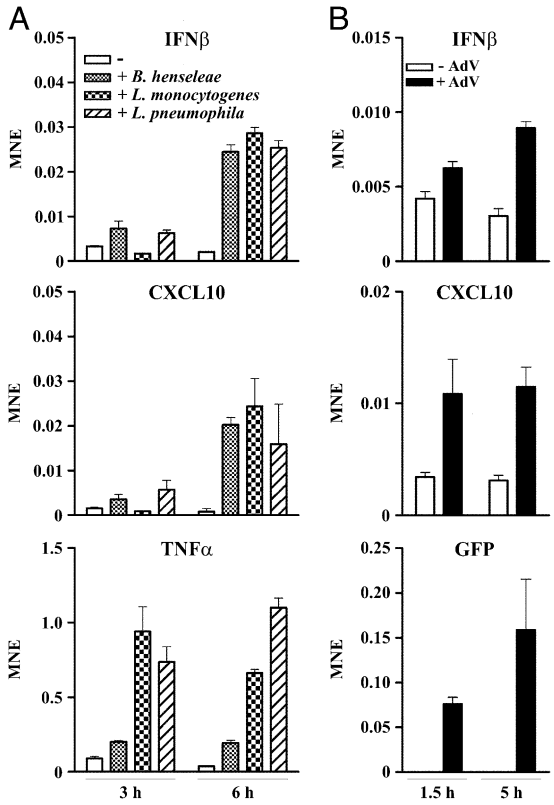

FIGURE 7. Induction of IFN-β mRNA expression in neutrophils infected by various intracellular pathogens. (**A**) Neutrophils were infected for 3 and 6 h with *B. henselae*, *L. monocytogenes*, or *L. pneumophila* at MOI 10. Total RNA was then extracted and analyzed for IFN-β, CXCL10, TNF-α, and GAPDH mRNA expression by qPCR. (**B**) Neutrophils were infected with adenovirus type 5 at MOI 1000. Total RNA was extracted after 1.5 and 5 h of incubation and analyzed for IFN-β, CXCL10, GFP, and GAPDH mRNA expression by qPCR. Gene expression in (A) and (B) is depicted as MNE after GAPDH normalization of triplicate reactions for each sample (MNE ± SE). Depicted results are from one experiment representative of at least three independent ones.

they are able to activate a wide spectrum of neutrophil genes upon their recognition. A time-dependent induction of IFN-β and CXCL10 mRNA expression was also observed in neutrophils infected with adenovirus type 5 (Fig. 7B). The latter phenomenon was genuine, as testified by the amplification of transcripts encoding GFP, which are virus specific (Fig. 7B).

Discussion

A characteristic feature that differentiates human neutrophils from monocytes in terms of LPS responsiveness is that neutrophils are unable to mobilize the MyD88-independent/TRIF-dependent cascade upon TLR4 engagement (8). In this study, in light of the identification of PKCε as a crucial molecule for initiating the MyD88-independent/TRIF-dependent pathway (12), and having confirmed that PKCε is undetectable in human neutrophils (13–16), we attempted to overexpress PKCε with the aim to restore the MyD88-independent/TRIF-dependent pathway. By optimizing a previously described electroporation method (27) to obtain >50% GFP^+ cells, we were able to successfully transfect PKCε in neutrophils. However, despite the very high amounts of exogenous PKCε protein expressed by transfected neutrophils, the results of our subsequent experiments did not permit us to conclude that overexpressed PKCε is instrumental in rescuing the MyD88-independent/TRIF-dependent pathway. In fact, although LPS acquired the capacity to upregulate the expression of IFN-β and CXCL10 mRNA (as well as ISG15 and IFIT-1 mRNA [N. Tamassia and M.A. Cassatella, unpublished observations]) in PKCε-transfected neutrophils, a similar phenomenon was observed in neutrophils transfected with *E. coli* DNA, poly(dA:dT), or a variety of empty plasmids but not in mock-transfected cells. Moreover, the fact that LPS did not modify the state of IRF3 activation/phosphorylation in either plasmid- or PKCε-transfected neutrophils further demonstrates that the MyD88-independent/TRIF-dependent cascade was not activated in DNA-nucleofected neutrophils. Assuming that overexpressed PKCε was fully functional in neutrophils (in this regard, we show that PKCε underwent serine phosphorylation upon cell treatment with PMA), an additional implication of our results is that PKCε may not be as critical for the activation of the MyD88-independent/TRIF-dependent cascade as previously proposed (12). Consistent with such a notion, the role of PKCε in driving the MyD88-independent/TRIF-dependent response was also questioned recently by Parker and colleagues (50), who demonstrated that PKCε associates with MyD88 and that PKCε phosphorylation is important for NF-κB activation via TLR2, which, unlike TLR4, does not use the TRIF or TRAM adapters (51).

Whatever the case may be, we also show in this study that the mere transfection of neutrophils with plasmid DNA (regardless of the presence of a PKCε-coding region within the vector) directly activates IRF3, promotes its recruitment to the IFN-β promoter, and, in turn, strongly induces the expression of *IFN-β* and other type I IFN-dependent genes (such as *CXCL10*, *ISG15*, and *IFIT-1*). Together with the findings described above, our data imply that, in plasmid-transfected neutrophils, LPS becomes able to further upregulate the expression of IFN-β mRNA without directly targeting IRF3. ChIP assays confirmed this hypothesis, as well as established the presumed molecular mechanisms underlying the induction of IFN-β mRNA, either by plasmid transfection or by LPS in plasmid-transfected neutrophils. Accordingly, we found that plasmid DNA transfection promotes a time-dependent recruitment of IRF3 to the IFN-β promoter; the addition of LPS to plasmid-transfected neutrophils does not increase such IRF3 recruitment; although cytosolic DNA markedly activates NF-κB in the cytoplasm, it is unable to promote NF-κB recruitment at the IFN-β promoter; LPS promotes the binding of NF-κB to the IFN-β promoter in plasmid-transfected neutrophils but not in nontransfected cells; and NF-κB inhibitors significantly suppress the upregulatory effect of LPS on IFN-β mRNA expression in transfected neutrophils. Based on these findings, we propose that the capacity of LPS to increase the expression of IFN-β mRNA in plasmid-transfected neutrophils relies on its ability to activate NF-κB, which, under conditions in which IRF3 is already present on the IFN-β promoter (i.e., after plasmid transfection), becomes readily recruited (see the scheme depicted in Supplemental Fig. 4). In fact, given the close proximity of the IRF3 binding site to the NF-κB locus within the IFN-β promoter, it is possible that binding of NF-κB occurs only when IRF3 is already bound. In this regard, it was demonstrated that binding of IRF3 to the IFN-β promoter produces a nucleosome shift that functions as a prerequisite for the binding of NF-κB and AP1 to the IFN-β promoter (52) (i.e., the nucleosome remodeling exerted by IRF3 might unmask the NF-κB binding sites present in the IFN-β promoter, in this way allowing NF-κB binding and consequent increased IFN-β transcription). Altogether, our data are in accordance with the view that, even if not essential unlike IRF3 (11), NF-κB plays an important role in modulating the degree of IFN-β promoter activation (11, 53). This is also in line with other experimental systems in which the induction of IFN-β results from a cooperative action of two different signals (54, 55). Similarly, the present data are consistent with previous findings demonstrating that human neutrophils often require stimulation by two concurrent, but different, stimulatory pathways to optimally express a given gene [e.g., IFN-γ plus LPS for CXCL10 (56) and IL-12 (57) expression or IL-10 plus LPS for IL-1ra (22) expression]. Curiously, LPS was unable to further upregulate the levels of IFN-β and CXCL10 transcripts in poly(I:C)-transfected neutrophils. Although we did not specifically explore, at the molecular levels, the reasons for this, some speculations can be made. First, because we showed that poly(I:C) transfection itself represents one of the most potent stimulatory conditions for gene expression in neutrophils (5), it is plausible that IFN-β and CXCL10 mRNA levels are already induced to the maximum and, therefore, cannot be increased further. Second, and in agreement with the former effects, poly(I:C) transfection activates NF-κB so powerfully (5) that LPS cannot further activate it. However, the fact that LPS maintains the ability to promote lactoferrin release (58) in poly(I:C)-transfected neutrophils (N. Tamassia and M.A. Cassatella, unpublished observations) indicates that, under the latter experimental conditions, TLR4 is expressed and functional.

This study also demonstrates that human neutrophils can promptly respond to transfected DNA, whether of bacterial or synthetic origin. In this context, our observations complement a series of previous studies describing the capacity of neutrophils to respond to exogenous DNA. Neutrophils, in fact, can readily respond to CpG oligodeoxynucleotides in terms of CXCL8 production and inhibition of apoptosis because they express their cognate receptor, TLR9 (4). Other findings demonstrated that extracellular bacterial DNA can trigger, through an unidentified receptor, another pathway functioning in a TLR9- and CpG-independent, but MyD88-dependent, manner (59). Our new observations additionally suggest that human neutrophils possess intracellular sensor system(s) that allow(s) the recognition of foreign and potentially dangerous DNA and, consequently, the induction of a distinct and potent immune response (60). The intracellular sensors that recognize exogenous DNA include TLR9 (36), RIG-I through RNA polymerase III (39, 40), absent in melanoma 2 (61–64), LRRFIP1 (43), DNA-dependent activator of IRFs (DAI) (65), IFI16 (42), and DDX41 (44). In this study, we provide evidence

that human neutrophils constitutively express, in addition to RIG-I (5), DDX41, LRRFIP1, and, confirming previous findings (41), IFI16. We also show that neutrophils constitutively express STING, a transmembrane protein essential for the signaling necessary for the production of IFN-β, via IRF3 activation, mediated by various cytosolic DNA sensors (45, 66). In this context, we did not investigate the role of DAI, the first identified cytosolic DNA sensor (65), because wild-type and DAI-deficient cells produce comparable amounts of type I IFN in response to cytosolic DNA (67) or poly(dA:dT) (68). We also did not focus on absent in melanoma 2, a member of the pyrin and HIN200 domain-containing protein family, because, based on the literature, this receptor is not essential for type I IFN induction by transfected DNA, whereas it is crucial for inflammasome activation and IL-1β secretion (61–64). Moreover, we excluded the involvement of both TLR9 and RNA polymerase III by using specific inhibitors. However, by performing coimmunoprecipitation studies of DDX41, LRRFIP1, and IFI16, and, presumably bound plasmid DNA, we were able to identify IFI16 as the critical intracellular sensor for plasmid DNA in human neutrophils. The latter findings are consistent with the biological features of IFI16, which, differently from TLR9, DAI, and RNA polymerase III, does not recognize specific DNA structure (42), and, which, through the activation of NF-κB and IRF3, is known to induce a strong production of proinflammatory cytokines and type I IFN in response to transfection with DNA motifs (42). In any case, the presence of RIG-I, DDX41, and LRRFIP1 in neutrophils provides a solid molecular basis to explain our additional findings on the ability of *L. monocytogenes*, *L. pneumophila*, and adenovirus type 5 to induce the expression of IFN-β and other ISG mRNA in infected neutrophils, because those pathogens require, for such a function, DDX41 plus LRRFIP1, RIG-I, and DDX41 plus RIG, respectively. In contrast, we show that neutrophils also express IFN-β mRNA following infection with *B. henselae,* a Gram-negative facultative intracellular microorganism that can invade many cells (69, 70). *Bartonella* uses several virulence factors for its interaction with host cells, including the VirB/D4 type IV secretion system (T4SS), which was recently shown to mediate plasmid DNA and protein transfer into eukaryotic host cells (71). Because VirB/VirD4 T4SS is related to the dot/Icm T4SS used by *L. pneumophila*, which is essential to trigger IFN-β expression (72), likely via the RNA polymerase III/RIG cascade (40), it is tempting to speculate that *B. henselae* induces IFN-β mRNA through mechanisms similar to those used by *L. pneumophila*.

Regardless, further studies are necessary to meticulously decipher which neutrophil sensor(s) is/are responsible for the recognition of foreign DNA from different sources and in which cell compartment they locate to do so. Nonetheless, the findings of this study shed new light on our understanding of the mechanisms by which human neutrophils recognize and respond to intracellular pathogens and, in turn, activate innate immune responses.

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Disclosures

The authors have no financial conflicts of interest.

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