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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 signalling

Running title: *IFN β mRNA regulation in transfected neutrophils*

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Abstract

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 have delineated that human neutrophils are unable to induce the transcription of IFN β in response to TLR4 stimulation. Since neutrophils do not express PKC ϵ , a molecule recently reported as essential for initiating the MyD88-independent/TRIF-dependent pathway, we optimized an electroporation method to transfect PKC ϵ 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 PKC ϵ -overexpressing neutrophils but also in cells transfected with a series of empty DNA plasmids: LPS, however, further upregulated the IFN β transcript levels in plasmid-transfected neutrophils, regardless of PKC ϵ 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- κ B, activated by LPS. Additional immunoblotting and coimmunoprecipitation studies not only revealed that neutrophils constitutively express various cytosolic DNA sensors, including IFI16, LRRFIP1 and DDX41, but also identified IFI16 as 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, data raise questions on the role of PKC ϵ in driving the MyD88-independent/TRIF-dependent response, while they indicate that human neutrophils are able to recognize and respond to microbial cytosolic DNA.

Introduction

The innate immune system, to which polymorphonuclear neutrophils (PMN) belong, is highly specialized in its capacity to recognize foreign pathogens, due to the expression of evolutionary conserved families of receptor proteins, the “pattern recognition receptors” (PRR) (1). The latter include, among others, the Toll like receptors (TLRs)(2), which all 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 lipopolysaccharide (LPS)(7). However, unlike human monocytes, endotoxin-activated neutrophils neither express type I IFN (IFN β and/or IFN α), nor IFN-dependent genes (IRG), such as CXCL10 or CXCL9 (8). This has been recently attributed to the inability of neutrophils to mobilize the so-called “myeloid differentiation factor-88 (MyD88)-independent/TIR domain-containing adapter inducing IFN β (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 TIRAP/MAL (TIR domain containing adapter protein/myeloid differentiation factor-88 adapter-like protein) mediate a rapid and early activation of the transcription factor NF- κ B, 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- κ B and IRF (IFN-regulatory factor)-3 transcription factors in MyD88 $^{-/-}$ mice, that ultimately lead to the expression of IFN β and, subsequently, to an IFN β -dependent STAT1 activation (1). They include, for instance, a number of antiviral genes, Th1-activating chemokines such as MIG (monokine induced by IFN γ)/ CXCL9, IP-10 (IFN-inducible protein-10)/CXCL10

and I-TAC (IFN-inducible T-cell alpha chemoattractant)/ CXCL11 and anticancer molecules such as TRAIL (TNF-related apoptosis-inducing ligand) (9). Along the “MyD88-independent” pathway, TRAM (TIR domain-containing adapter-inducing IFN β -related adapter molecule) and TRIF (TIR domain-containing adapter inducing IFN β) adaptor proteins both transduce the activation of redundant protein kinases, namely TBK1 (TRAF family associated NF- κ B binding kinase [TANK]-binding kinase-1) and IKK (I κ B kinase, IKK)- ϵ , which phosphorylate IRF-3 on Ser/Thr residues (10). As a result, IRF-3 dimerizes, translocates to the nucleus, associates with other coactivators and ultimately contributes to activate IFN β gene transcription (11). Interestingly enough, the “MyD88-independent/TRIF-dependent” cascade seems to be initiated by a rapid activation of PKC ϵ 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 then cause TRAM disappearance from the membrane, in concomitance with the activation of a downstream signaling cascade that would finally lead to the activation of the TBK1-IRF3 axis and ultimately the transcriptional induction of IFN β (12).

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

Materials and Methods

Antibodies – PKC ϵ (sc-214), phospho-PKC ϵ (Ser 729) (sc-12355), PKC β II (sc-210), IRF-3 (sc-9082), NF- κ B p65 (sc-372), NF- κ B p50 (sc-7178), I κ B- α (sc-371), IFI16(sc-8023), DDX41 (sc-166225) and LRRFIP1 (sc-135917) antibodies (Abs) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). TBK1 (no. 3013), phospho-IRF3 (Ser 396) (no. 4947), phospho-NF- κ B p65 (Ser536) (no. 3031) and phospho-p44/42 MAPK (no. 9106) Abs were from Cell Signaling (Beverly, MA, USA), while anti- β -tubulin (T5293) and anti-actin (A5060) Abs were from Sigma (Saint Louis, MO, USA). TBK1 monoclonal mAbs (IMG-139A) and rabbit polyclonal STING Abs (IMG-6422A) were from Imgenex (San Diego, CA, USA), while rabbit polyclonal ISG15 Abs were kindly provided by Dr. Arthur L. Haas (Louisiana State University Health Sciences Center, New Orleans, LA, USA).

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 experiments presented in this paper (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 with or without stimuli [including 100 ng/ml ultrapure *Escherichia coli* LPS (0111:B4, from Alexis), 10 μ M R848 (InvivoGen), 50 μ g/ml poly(I:C) (InvivoGen), 10 ng/ml phorbol-myristate acetate (PMA) (Sigma) or 100 U/ml IFN γ (R&D Systems, Minneapolis, MN, USA)], 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% CO₂ atmosphere. After the desired incubation

period, cells were collected, spun at $300 \times g$ for 5 min and cell pellets 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 μ l of lipofectamine 2000 (Invitrogen) complexed with 0.8 μ g poly(dA:dT) (Sigma) or 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-PKC ϵ [kindly provided by Professor P. Parker (Cancer Research UK, London Research Institute)] 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 nucleofactor kit (Lonza) and the Amaxa nucleofactor II device (Lonza) according to the reagent instructions, yet with minor modifications. After isolation, 5×10^6 neutrophils were resuspended in 100 μ l of complete nucleofactor solution containing 2.5-15 μ g DNA (or equal volumes of PBS, for mock-transfection) and then transferred to a nucleoporation cuvette. Electroporation was performed using the Y001 program present in the nucleofactor II device. Cells were then recovered and let stand for 5 min in 2 ml of human monocyte nucleofactor medium supplemented with 2 mM glutamine and 10 % FBS. Thereafter, neutrophils were washed once with PBS to remove broken cells and then 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 preincubated for 30 min with different inhibitors, including BAY 117082, pyrrolidine

dithiocarbamate (PDTC), sc-514 (Calbiochem, San Diego, CA) or 10 μ M MG132 (Sigma), prior to the subsequent stimulation with LPS.

Reverse transcription quantitative real-time PCR (RT-qPCR) – RNA isolation and reverse transcription were accomplished as previously described (19). RT-qPCR was performed using SYBR[®] Premix Ex Taq (Takara) and gene-specific primers (purchased from Invitrogen, Carlsbad, CA, USA) available in the public database RTPriemerDB (<http://medgen.ugent.be/rtprimerdb/index.php>) under the following entry codes: IFN β (3542), TNF α (3551), CXCL8/IL-8 (3553), CXCL10/IP-10 (3537), G1P2/ISG15 (3547), IFIT1 (3540), and GAPDH (3539). Data were calculated with Q-Gene software (www.BioTechniques.com) 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 *et al.* (20). Data were analysed by FLOWJO software (Tree Star Inc., Ashland, OR).

Immunoprecipitations and immunoblots – Whole cell extracts were prepared either using the RNeasy mini kit (Qiagen) as described (21), for immunoblots, or according to the chromatin immunoprecipitation (ChIP) assay procedure (22), for IFI16, LRRFIP1 and DDX41 immunoprecipitations. Cytoplasmic extracts for PKC ϵ and TBK1 immunoprecipitations were prepared by the nitrogen cavitation procedure and processed exactly as previously described (23). Blotted proteins were detected and quantified using the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA) (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 μ g of anti-IRF3 or 5 μ g of anti-NF- κ B p65/p50 Abs, while for cytoplasmic DNA sensor/plasmid DNA co-immunoprecipitations, whole cell extracts, prepared from 5×10^6 pEGFP-transfected neutrophils, were immunoprecipitated using 5 μ g of α DDX41, α IFI16 and α LRRFIP1 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 co-immunoprecipitated material was then subjected to qPCR analysis using the following specific primers (purchased from Invitrogen): IFN β promoter forward: TCCCAGGAACTCAATGAAGG; IFN β promoter reverse: GTGTCGCAATGGAGTGTTGT; CXCL8 promoter forward: CTTAGTGGGGTTGAAAGTGAC; CXCL8 promoter reverse: AAGAAATAGTCACTCACCCAAG; pEGFP-backbone forward: ACGGCATCAAGGTGAACTTC; pEGFP-backbone reverse: GCTTCTCGTTGGGGTCTTTG; pEGFP-GFP forward: TGCCATAGCCTCAGGTTACTC; pEGFP-GFP reverse: GACGCTCAGTGGAACGAAAAC. PRL promoter forward: AGGGAAACGAATGCCTGATT; PRL promoter reverse: GCAGGAAACACACTTCACCA. pEGFP-backbone and pEGFP-GFP primers of pEGFP (Accession Number: U55763) amplify, respectively, the 3854-3999 bp and the 1091-1259 bp regions.

Infection of neutrophils – 2×10^6 high purity neutrophils were infected with 10 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 RT-qPCR analysis. At 1 h post-infection, 50 μ g/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 (AdV-GFP, a kind gift by Prof. B.M. Foxwell (Imperial College, London, UK)]. Bacterial strains were all grown to mid-logarithmic phase as it follows: wild type *L. monocytogenes* (WT, 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, Italy)] 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); *B. henselae* Houston-1 (from ATCC 49882) were grown in Schneider's medium supplemented with 10 % FCS and 2 mM glutamine, at 37° C with 5 % CO₂ (25). Prior to infection, bacteria were pelleted and washed twice with PBS.

Statistical analysis – Data are expressed as means \pm SE. Statistical evaluation was performed using student *t* test or 1-way ANOVA followed by Tukey's *post hoc* test. Values of $P < 0.05$ were considered statistically significant.

Results

Overexpression of PKC ϵ in human neutrophils by electroporation – Immunoblot and immunoprecipitation analyses of either cytoplasmic cavitates (26) (Supplemental Fig. 1A, *top panel*), or whole cell lysates (not shown), confirmed (13-16) that neutrophils do not express PKC ϵ , unlike autologous monocytes. Moreover, PKC ϵ protein expression in both neutrophils and monocytes was found to be, respectively, neither inducible nor upregulated, by a 24 h-treatment with either LPS or IFN γ (Supplemental Fig. 1B). Therefore, to clarify whether the absence of PKC ϵ might be responsible for the inability of human neutrophils to mobilize the “MyD88-independent/TRIF-dependent” pathway, we attempted to overexpress PKC ϵ in these cells. A series of preliminary experiments were, however, necessary to develop a reliable transfection protocol, since neutrophils are notoriously extremely difficult to manipulate for exogenous gene expression, due to their inability to proliferate and to survive in culture for long periods. We were ultimately successful by modifying a recently reported nucleofection protocol (27), with which the authors were able to transfect both GFP and p47^{phox}-GFP proteins into neutrophils. By our procedure, we could transfect a pmaxGFP plasmid into neutrophils with a greater efficiency than the reported 5 % (27) (see the fluorescence microscopy image displayed in the *left* panel of Supplemental Fig 2A). Accordingly, flow cytometry analysis revealed that the percentage of GFP-positive neutrophils augmented in correlation with increasing amounts of transfected pmaxGFP plasmid (Supplemental Fig. 2B). For example, more than 50 % of GFP-positive neutrophils were measured by nucleofecting 15 μ g pmaxGFP plasmid (Supplemental Fig. 2B). Importantly, such remarkable levels of GFP-positive neutrophils were obtained as early as 4 h post-nucleofection and remained stable for up to 20 h of culture (Supplemental Fig. 2C). However, since neutrophils became mostly apoptotic after 20 h (nearly 80 % were PI-positive) (Supplemental Fig. 2D), whereas they were substantially alive up to 7-8 h post-

transfection (75-80 % of PI-negative 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 PKC ϵ (pEGFP-PKC ϵ), which produced a *de novo* expression of remarkable quantities of exogenous PKC ϵ -GFP (Fig. 1A-B). Indeed, pEGFP-PKC ϵ -transfected neutrophils displayed specific PKC ϵ -immunoreactive signals, unlike the cells transfected with the corresponding empty plasmid (pEGFP) (Fig. 1A). Again, expression levels of exogenous PKC ϵ correlated with the amounts of transfected plasmid (Fig. 1B): for instance, the levels of exogenous PKC ϵ -GFP expressed in 2×10^6 neutrophils transfected with 15 μ g PKC ϵ -GFP plasmid were comparable to the levels of endogenous PKC ϵ present in 1×10^6 monocytes (Fig. 1A-B). Importantly, PKC ϵ -GFP overexpressed in neutrophils underwent serine phosphorylation in response to PMA stimulation, as revealed by the use of specific anti-phospho-PKC ϵ Abs (Fig. 1C). Similar data could be extrapolated by using anti-PKC ϵ Abs which detect a doublet (28), the slower migrating band likely corresponding to phosphorylated PKC ϵ – 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 substantially similar to those observed in non-transfected cells (Fig. 1C). Taken together, our data demonstrate that it is possible to successfully transfect PKC ϵ -GFP in neutrophils and that exogenous PKC ϵ -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 PKC ϵ could restore the “MyD88-

independent/TRIF-dependent” pathway, neutrophils were incubated 4 h post PKC ϵ -GFP- or pEGFP-plasmid transfection and then stimulated with 100 ng/ml ultrapure LPS for additional 90 min (see the *upper panel* Figure 2A), to investigate the state of IFN β and CXCL10 mRNA expression by RT-qPCR. In line with our previous study (8), no IFN β or CXCL10 mRNA induction occurred in non-transfected neutrophils stimulated with LPS, as opposed to the induction of two classical MyD88-dependent genes, namely TNF α and CXCL8 (Fig. 2A, *lower panels*). By contrast, both IFN β and CXCL10 (but not TNF α or CXCL8) gene expression was significantly increased by LPS in PKC ϵ -GFP-overexpressing neutrophils (Fig. 2A, *lower panels*), as if the “MyD88-independent/TRIF-dependent” pathway was rescued by the introduction of an exogenous PKC ϵ . 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 PKC ϵ -GFP-overexpressing neutrophils (Fig. 2A, *center panels*). Unexpectedly, neutrophils transfected with either PKC ϵ -GFP or pEGFP plasmids were found to express elevated amounts of IFN β , CXCL10, TNF α and CXCL8 transcripts even in the absence of any LPS stimulation (Fig. 2A), thus 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 PKC ϵ -GFP plasmid, or *E.coli* DNA, data not shown), a time-dependent increase of IFN β and CXCL10 mRNA is already detectable in neutrophils after 45 min, that augments 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-DNA (29), was found to directly trigger 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 also prove that the stimulating agent is genuine DNA and not a contaminating product derived

from the plasmid isolation procedure, such as for instance bacterial RNA. Moreover, a further 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 non-transfected 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 which 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 transfection 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 PKC ϵ -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 up-regulation by LPS. For such a purpose, we analyzed the activation status of IRF3, which represents a crucial intermediate for the transcriptional induction of IFN β (11). By using the protocol depicted in the scheme of Figure 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 *lower panel* of Fig. 3A, n=3). Notably, similar results were obtained with the PKC ϵ -GFP plasmid (data not shown), while 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 further increased by the 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*), and by the fact that matched control antibodies did not coprecipitate any IFN β or PRL promoter (not shown). Altogether, data demonstrate that while 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- κ B – Since NF- κ B is a transcription factor cooperating with IRF3 in promoting IFN β transcription (30), we subsequently analyzed its activation status in plasmid-transfected neutrophils treated with or without LPS for 45 min (Fig. 4, *panels A and B*). By doing so, we observed considerable levels of phosphorylated NF- κ Bp65 (Fig. 4A) and diminished amounts of I κ B α (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 of the levels of NF- κ B p65 phosphorylation (Fig. 4A) and I κ B α degradation (Fig. 4B) after 45 min (for a quantitative densitometric analysis see *lower panels* of Fig. 4A and 4B, n=3). Importantly, ChIP assays performed according to the scheme depicted in Fig. 4C, using antibodies towards NF- κ Bp65 (Fig. 4D) and NF- κ Bp50 (Fig. 4E), highlighted a crucial role of NF- κ B in driving

the effect of LPS on IFN β mRNA expression in plasmid-transfected neutrophils. In fact, both p50 and p65 NF- κ B subunits were strongly recruited to the IFN β promoter upon LPS-stimulation of plasmid-transfected neutrophils (Fig. 4D and 4E, *left panels*). On the other hand, no recruitment of either NF- κ Bp50 or NF- κ Bp65 to the IFN β promoter was observed in non-transfected neutrophils treated with LPS (Fig. 4D and 4E, *left panels*), consistent with the inability of LPS to induce IFN β gene expression under the latter conditions (8). Under the latter conditions, however, both NF- κ Bp65 and NF- κ Bp50 were found to strongly bind to the CXCL8 promoter (Fig. 4D and 4E, *right panels*). In plasmid-transfected neutrophils, notably, the recruitment of NF- κ Bp65 and NF- κ Bp50 to the CXCL8 promoter, although negligible after 60 min of LPS-stimulation (Fig. 4D and 4E, *right panels*), was at least three-fold higher after 30 min (data not shown), in line with the enhanced CXCL8 mRNA expression observed in parallel (Figure 2A).

The role of activated NF- κ B as crucial mediator of the additional upregulation of IFN β mRNA expression by LPS in plasmid-transfected neutrophils was additionally supported by the effect of four chemical NF- κ B blockers, namely BAY 117082, an inhibitor of I κ B α phosphorylation and degradation (31), PDTC, an inhibitor of NF- κ B activation (32), sc-514, a selective IKK-2 inhibitor (33) (Fig. 5), and MG132, a proteasomal inhibitor (34) (not shown). In fact, while 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). All in all, 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- κ B subunits to the IFN β promoter, which, for unknown reasons, does not take place in not-transfected neutrophils.

Intracellular recognition of plasmid DNA by human neutrophils is dependent on IFI16 (interferon-inducible protein 16) – Finally, we aimed at defining how human neutrophils recognize transfected plasmid DNA. Initially, we investigated the role of TLR9, since 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) and consequently drugs able to block endosome acidification (37), such as chloroquine and bafylomicin A1, inhibit its ability to activate NF- κ B and to induce cytokines (37-38). Experiments in which neutrophils were pretreated 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*). By contrast, both inhibitors markedly suppressed the induction of TNF α mRNA expression in R848-stimulated neutrophils (Supplemental Fig. 3A, *right panel*), therefore proving that they were appropriately functioning. 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), that is constitutively expressed by human neutrophils (Fig. 6A) (5). Once again, a specific RNA polymerase III inhibitor-based approach, by 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,). By contrast, ML-60218 dose-dependently reduced the induction of IFN β mRNA in 293T cells transfected with poly(dA:dT) (Supplemental Fig. 3C), as expected (39-40). We could thus conclude that neither TLR9, nor RNA polymerase III, function as the intracellular receptor(s) recognizing plasmid DNA in electroporated neutrophils. On the other hand, we could confirm that neutrophils express IFI16 (41), one of the newly identified intracellular receptor 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), similarly to what originally described in the nuclear extracts of IFN γ -treated HL-60 cells (41). Under the same conditions, we could also detect, in both neutrophils and monocytes, two more recently discovered DNA sensors, namely leucine-rich repeat (in Flightless I) interacting protein-1 (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 stimulator of IFN genes (STING) (Fig. 6A), a transmembrane protein located in the endoplasmic reticulum that is crucial for DNA-mediated signalling (45-48).

To attempt identifying 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. Precisely, we immunoprecipitated IFI16, LRRFIP1 and DDX41 after DNA transfection (Fig. 6B), and then analyzed, by real-time qPCR, which of these receptors plasmid DNA was stably bound to (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, that, according to the literature (49) are known to activate IFN β gene expression *via* cytosolic sensing of their DNA. As shown in Figure 7A, all intracellular bacteria under examination triggered a remarkable IFN β and CXCL10 mRNA expression in neutrophils infected for 6, but not 3, h. Similarly, all bacteria upregulated the expression of TNF α (Fig. 7A) and CXCL8 mRNA (data not shown), indicating they are able to activate a wide spectrum of neutrophil genes upon their recognition. A time-dependent induction 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 peculiar 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 PKC ϵ as a crucial molecule for initiating the MyD88-independent/TRIF-dependent pathway (12), and having confirmed that PKC ϵ results undetectable in human neutrophils (13-16), we attempted to overexpress PKC ϵ with the aim to restore the “MyD88-independent/TRIF-dependent” pathway in human neutrophils. By optimizing a previously described electroporation method (27) to obtain more than 50 % GFP-positive cells, we were able to successfully transfect PKC ϵ in neutrophils. However, despite of the very high amounts of exogenous PKC ϵ protein expressed by transfected neutrophils, the results of our subsequent experiments did not permit us to conclude that overexpressed PKC ϵ is instrumental in rescuing the “MyD88-independent/TRIF-dependent” pathway. In fact, while LPS did acquire the capacity to upregulate the expression of IFN β and CXCL10 mRNA (as well as ISG15 and IFIT-1 mRNA, N.T. and M.A.C., unpublished observations) in PKC ϵ -transfected neutrophils, a similar phenomenon was also 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 PKC ϵ -transfected neutrophils, further demonstrates that the MyD88-independent/TRIF-dependent cascade was not activated in DNA-nucleofected neutrophils. Assuming that overexpressed PKC ϵ was fully functional in neutrophils (in this regard, we show that PKC ϵ underwent serine phosphorylation upon cell treatment with PMA), a further implication of our results is that PKC ϵ 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 PKC ϵ in driving the MyD88-independent/TRIF-dependent response has

been recently questioned also by Parker *et al.* (50), who demonstrated that PKC ϵ associates with MyD88 and that PKC ϵ phosphorylation is important for the NF- κ B activation *via* TLR2, which, unlike TLR4, does not utilize the TRIF or TRAM adaptors (51).

Whatever the case may be, herein we also show that the mere transfection of neutrophils with plasmid DNA (regardless of the presence of a PKC ϵ 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 not only confirmed this hypothesis, but also 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: i) plasmid DNA transfection promotes a time-dependent recruitment of IRF3 to the IFN β promoter; ii) the addition of LPS to plasmid transfected neutrophils does not increase such IRF3 recruitment; iii) while cytosolic DNA markedly activates NF- κ B in the cytoplasm, it is unable to promote NF- κ B recruitment at the IFN β promoter; iv) LPS promotes the binding of NF- κ B to the IFN β promoter in plasmid-transfected neutrophils but not in non-transfected cells; v) NF- κ B 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- κ B 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 Figure 4). In fact, given the closed proximity of the IRF3 binding site to the NF- κ B locus within the IFN β promoter, it is possible that the binding of NF- κ B occurs only

when IRF3 is already bound. In this regard, it has been already demonstrated that the binding of IRF3 to the IFN β promoter produces a nucleosome shift which functions as a prerequisite for the binding of NF- κ B and AP1 to the IFN β promoter (52). In other words, the nucleosome remodeling exerted by IRF3 might unmask the NF- κ B binding sites present in the IFN β promoter, in this way allowing NF- κ B binding and consequent increased IFN β transcription. All in all, our data are in accordance with the view that, even if not essential unlike IRF3 (11), NF- κ B 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 necessitate to be stimulated by two concurrent but different stimulatory pathways to optimally express a given gene: for instance 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 which are, at molecular levels, the reasons for such a LPS-inability, some speculations can be made. First, because we have shown that poly(I:C)-transfection itself represents one of the most potent stimulatory condition for gene expression in neutrophils (5), it is plausible that IFN β and CXCL10 mRNA levels are already induced at the potential maximum levels, and are therefore not further increasable. Second, and in agreement with the former effects, poly(I:C)-transfection activates NF- κ B so powerfully (5) that LPS might result unable to further activate it. However, the fact that LPS maintains the capacity to promote lactoferrin release (58) in poly(I:C)-transfected neutrophils (N.T. and M.A.C., 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 (ODN) in terms of CXCL8 production and inhibition of apoptosis since they express their cognate receptor, TLR9 (4). Other findings have 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). Among the intracellular sensors that recognize exogenous DNA, that to date include TLR9 (36), RIG-I through RNA polymerase III (39-40), absent in melanoma 2 (AIM2) (61-64), LRRFIP1 (43), DNA-dependent activator of IRFs (DAI) (65), IFI16 (42) and DDX41 (44). Herein, we provide evidence that human neutrophils constitutively express, other than RIG-I (5), also DDX41, LRRFIP1 and IFI16, in the latter case confirming previous findings (41). We also show that neutrophils constitutively express STING, a transmembrane protein that is 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), since 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 AIM2, a member of the PYHIN (pyrin and HIN200 domain-containing) protein family, since, based on the literature, this receptor is not essential for type I IFN induction by transfected DNA, while it is crucial for inflammasome activation and interleukin 1 β (IL-1 β) secretion (61-64). Moreover, we excluded the involvement of both TLR9 and RNA polymerase III by taking the advantage of using specific inhibitors. However, by performing co-immunoprecipitation

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 perfectly consistent with the biological features of IFI16 that, differently from TLR9, DAI and RNA pol III, does not recognize specific DNA structure (42), and that, through the activation of NF- κ B and IRF3, is known to induce a strong production of proinflammatory cytokines and type I IFN in response to transfection with DNA motifs (42). The presence of RIG-I, DDX41 and LRRFIP1 in neutrophils provides, in any case, solid molecular bases explaining our additional findings on the capacity of *L. monocytogenes*, *L. pneumophila* and adenovirus type 5 to induce the expression of IFN β and other ISG mRNA in infected neutrophils, since those pathogens require, for such a function, DDX41 plus LRRFIP1, RIG-I, and DDX41 plus RIG, respectively. On the other hand, we show that neutrophils express IFN β mRNA also following infection with *B. henselae*, a gram-negative facultative intracellular microorganism that can invade many cells (69-70). Among others, *Bartonella* utilizes several virulence factors for its interaction with host cells, including the VirB/D4 type IV secretion systems (T4SSs), which has been recently shown to be able of mediating plasmid DNA and protein transfer into eukaryotic host cells (71). Since 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 polIII/RIG cascade (40), it is therefore tempting to speculate that *B. henselae* might induce IFN β mRNA through mechanisms similar to those utilized by *L. pneumophila*.

Whatever the case is, 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 paper 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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Footnotes

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Figure legends

FIGURE 1. Transgenic expression of PKC ϵ -GFP in human neutrophils. (A) Neutrophils were electroporated with 15 μ g pEGFP-PKC ϵ , 15 μ g pmaxGFP or PBS (i.e., mock electroporated) as described in M&M. After 4 h, neutrophil and monocyte whole cell extracts were prepared and, together with extracts from HEK 293T cells, transfected with pEGFP-PKC ϵ or not, were electrophoresed (50 μ g neutrophils, 30 μ g monocytes, 10 μ g for HEK 293T) for immunoblotting studies using antibodies specific for PKC ϵ and PKC β II. Panels depict a representative experiment out of three independent ones. (B) Neutrophils were electroporated with 5 or 15 μ g of pEGFP-PKC ϵ , or with 15 μ g of pEGFP. After 4 h, whole cell extracts were prepared and then electrophoresed for PKC ϵ and actin immunoblotting. Panels depict a representative experiment (n=2). (C) Neutrophils were electroporated with 15 μ g of pEGFP-PKC ϵ for 4 h, treated with 5 ng/ml PMA and then lysed after additional 20 min. Whole cell extracts were prepared, electrophoresed and immunoblotted using antibodies specific for phospho-PKC ϵ , total PKC ϵ , phospho p42/p44 and actin. (D) Whole cell extracts prepared from monocytes stimulated for 20 min with 5 ng/ml PMA were processed as for (C) and used as positive control. For (C, D), data show one experiment representative of two ones.

FIGURE 2. Modulation of IFN β mRNA expression in plasmid transfected-neutrophils incubated with or without LPS. Neutrophils were electroporated with 15 μ g pEGFP-PKC ϵ , 15 μ g pEGFP (empty vector) (A, B), 15 μ g of poly(dA:dT), 15 μ g *E.coli* DNA, 2 μ g of poly(I:C) or PBS (mock electroporated) (C), and then incubated either for 4 h (A, C) before the addition of 100 ng/ml LPS, or for the times indicated only (B). Total RNA was extracted after further 90 (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 RT-qPCR. In the *upper parts* of panels (A) and (C) are reported the schemes illustrating the experimental protocols utilized. Gene expression is depicted as MNE \pm SE (n=6) after GAPDH normalization of triplicate reactions for each sample. Asterisks indicate a significant increase exerted by LPS. * p<0.05, *** p< 0.001. For B and C, depicted results are from one representative experiment of at least three independent ones.

FIGURE 3. Activation of IRF3 in plasmid-transfected neutrophils. (A) Neutrophils were electroporated with 15 μ g 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 point (as depicted in the *upper* scheme) and whole cell extracts then electrophoresed and immunoblotted using anti-phosphoIRF3 (Ser 396), anti-IRF3 and anti-actin Abs. The *center panel* shows a representative immunoblot (n=3), while the *lower panel*, displays the densitometric quantification of phospho-IRF3 levels (normalized by the total IRF3, n=3) by LI-COR Odyssey software, expressed as densitometric units (D.U.). Asterisks indicate a significant increase of phospho-IRF3 levels over not-transfected cells. * p<0.05, ** p< 0.01. (B) Neutrophils were electroporated with 15 μ g pEGFP and cultured for 5 h, the last hour in the presence or the absence of 100 ng/ml LPS. Cells were then processed for ChIP analysis as described in M&M. 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 \pm SE, n=3). Asterisks indicate a significant increase of IRF3 recruitment over not-transfected cells. * p<0.05.

FIGURE 4. LPS-mediated NF- κ B activation and binding to the IFN β -promoter in plasmid-transfected neutrophils. (A, B) Neutrophils were electroporated with 15 μ g

pEGFP and cultured for 4 h and 45 min, the last 45 min in the presence or the absence of 100 ng/ml LPS (as depicted in the *upper* schemes). Whole cell extracts were prepared, electrophoresed and immunoblotted using anti-phospho-NF- κ Bp65 (Ser536), anti-total NF- κ Bp65 (**A**), anti-I κ B α and anti-actin antibodies (**B**). Shown is a representative immunoblot split into two parts (n=3). The *lower* graphs in (**A**) and (**B**) illustrate the densitometric quantification of the phospho-NF- κ Bp65 levels (normalized by the total NF- κ Bp65) (**A**), and of the I κ B α levels (normalized by the total actin) (**B**), expressed as mean \pm SE (n=3). Asterisks indicate significant differences among the various experimental conditions (* p<0.05, ** p< 0.01, *** p< 0.001), as indicated. For panels (**D**) and (**E**), neutrophils were treated as depicted in the scheme of panel (**C**), and processed for ChIP analysis using anti-NF- κ Bp65 (**D**), or anti-NF- κ Bp50 (**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 \pm SE, n=3). Asterisks indicate a significant increase of NF- κ B recruitment over control or not-transfected cells. ** p<0.01, *** p< 0.001.

FIGURE 5. Effect of NF- κ B inhibitors on the upregulation of IFN β mRNA expression triggered by LPS in plasmid-transfected neutrophils. Neutrophils were electroporated with 15 μ g of pEGFP and, after 3.5 h of culture, incubated with 300 μ M PDTC, 100 μ M sc-514 or 5 μ M BAY 117082. After 30 min, neutrophils were stimulated with 100 ng/ml LPS for further 90 min (as depicted in the *upper* scheme). Total RNA was then extracted and analyzed for IFN β and GAPDH mRNA expression by RT-qPCR. IFN β mRNA expression is depicted as MNE \pm SE (n=3) after GAPDH normalization of triplicate reactions for each sample. Asterisks indicate a significant inhibition by the inhibitor. ** 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 μ g) were electrophoresed and immunoblotted using antibodies specific for IFI16, LRRFIP1, DDX41 RIG-I, STING and tubulin. (B) Whole neutrophil lysates (500 μ g), prepared using the ChIP assay buffer, were subjected to immunoprecipitation with IFI16, LRRFIP1, DDX41 or anti-mouse IgG1 (control) antibodies. For (A) and (B), representative immunoblot experiments are shown (n=3). (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 antibodies. Finally, co-immunoprecipitated DNA was purified and analyzed by qPCR using two different 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.

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*, *L. pneumophila* at MOI 10. Total RNA was then extracted and analyzed for IFN β , CXCL10, TNF α and GAPDH mRNA expression by RT-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 RT-qPCR. Gene expression in A and B is depicted as MNE after GAPDH normalization of triplicate reactions for each sample (MNE \pm SE). Depicted results are from one experiment representative of at least three independent ones.

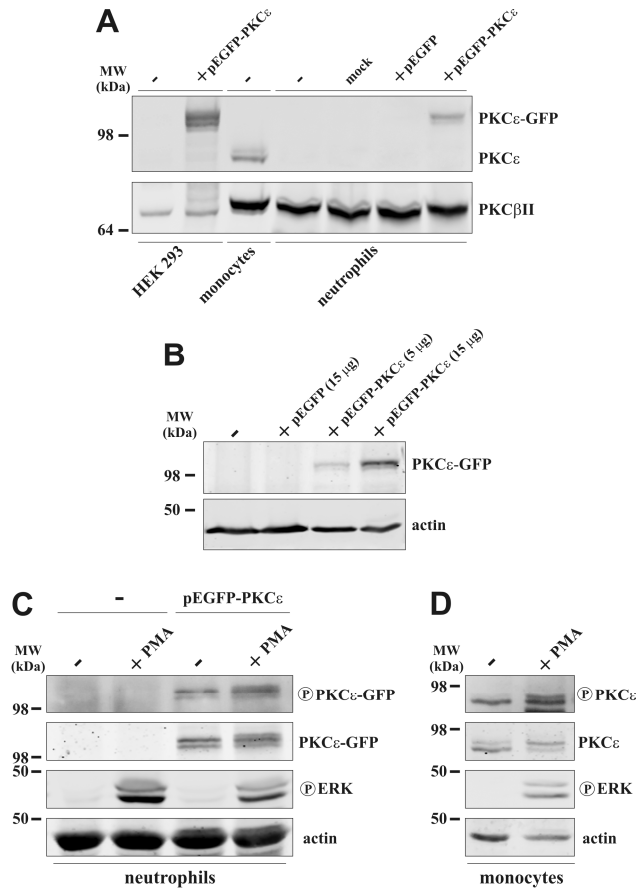


Figure 1

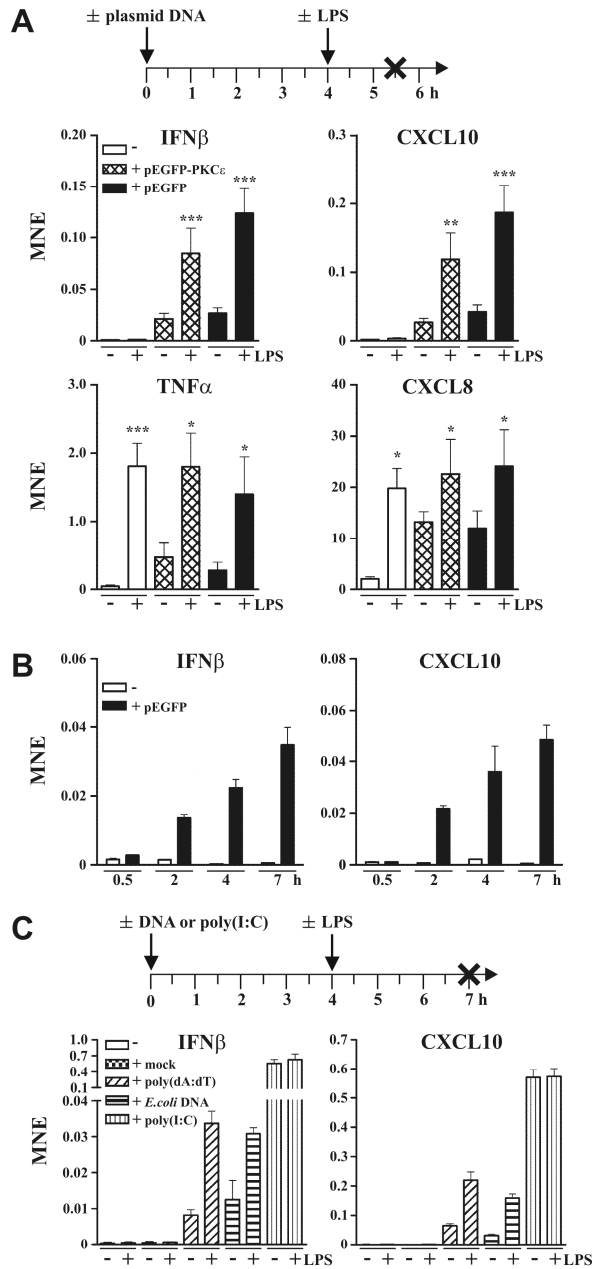


Figure 2

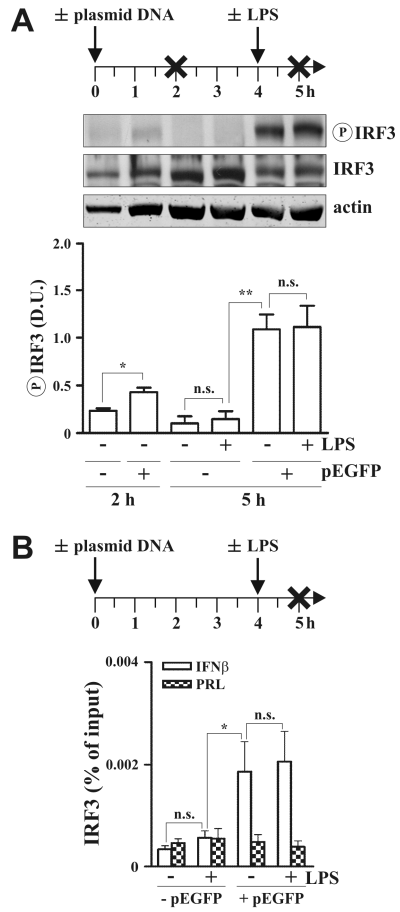


Figure 3

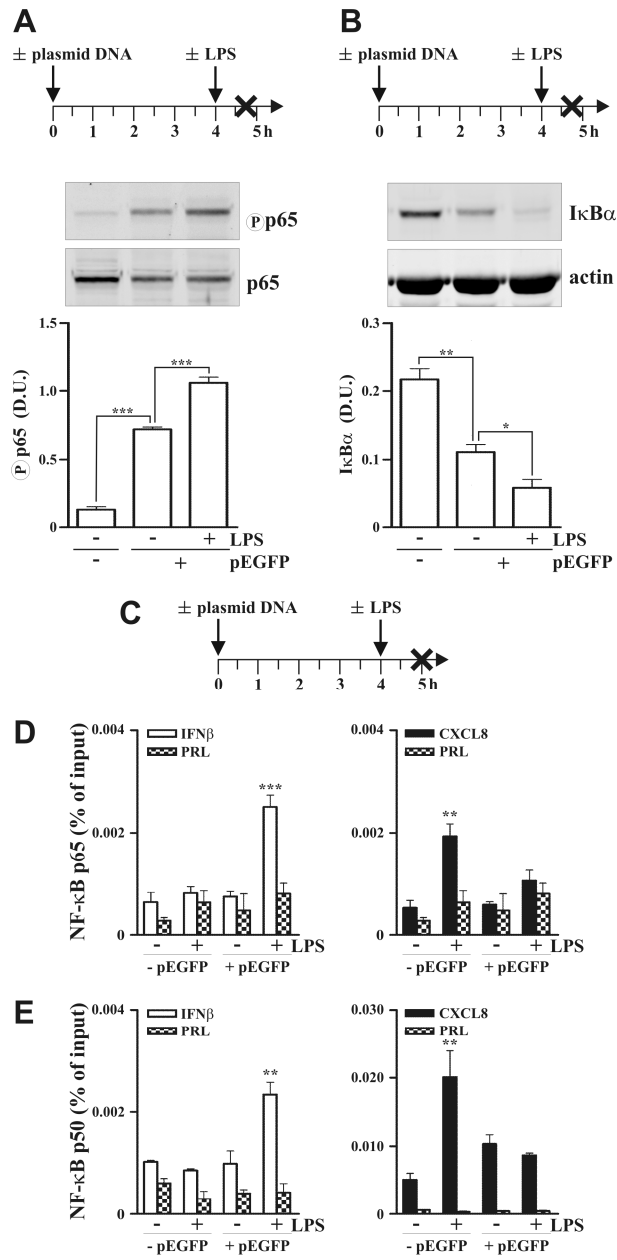


Figure 4

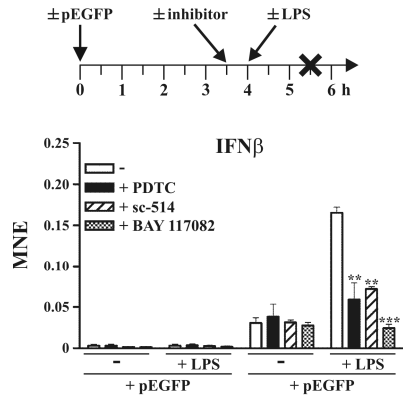


Figure 5

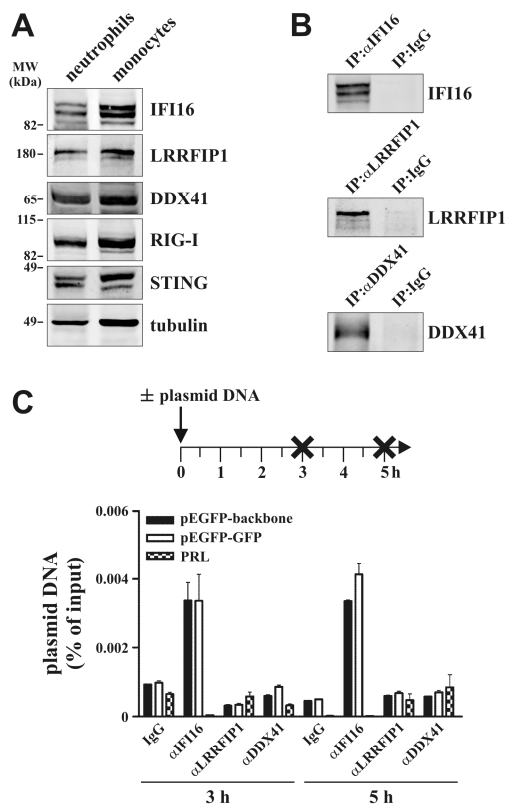


Figure 6

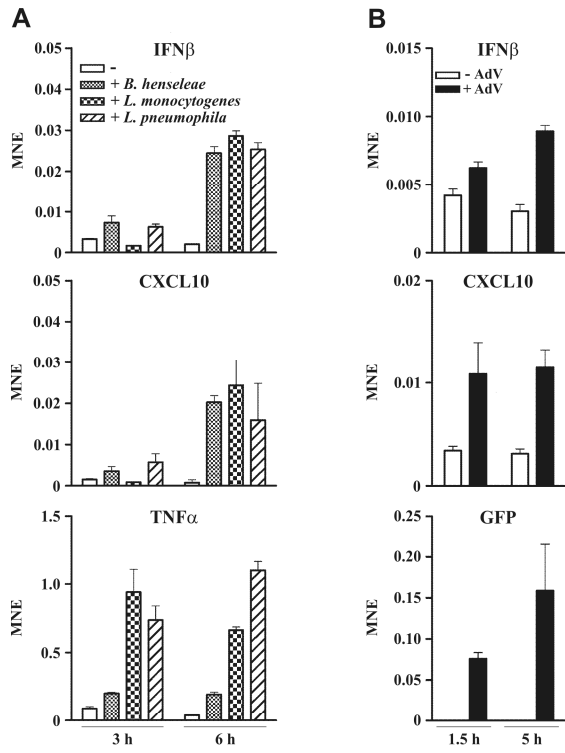
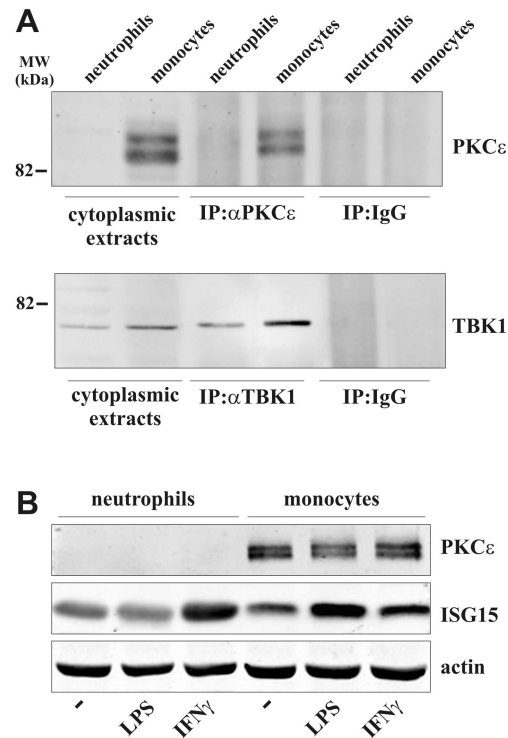


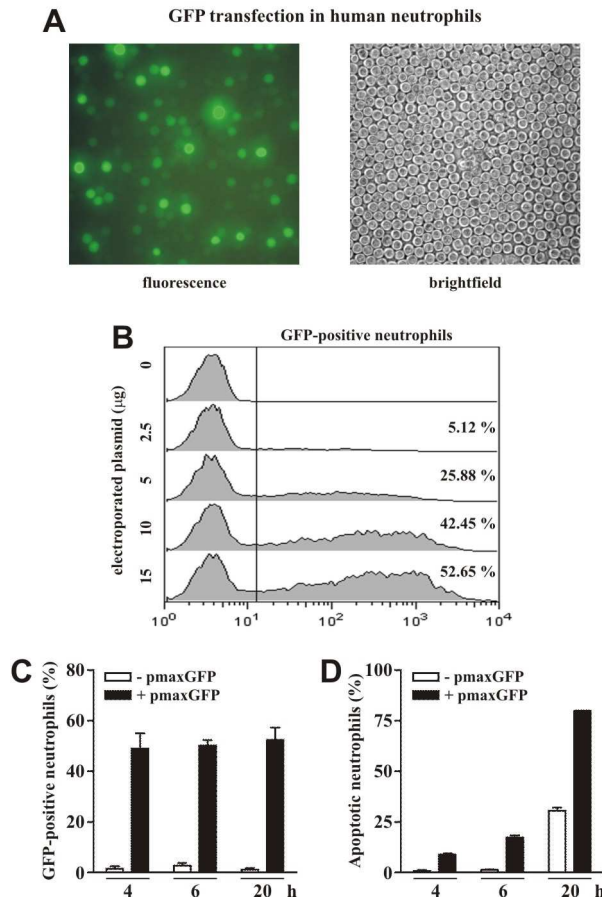
Figure 7

Supplemental Figure 1



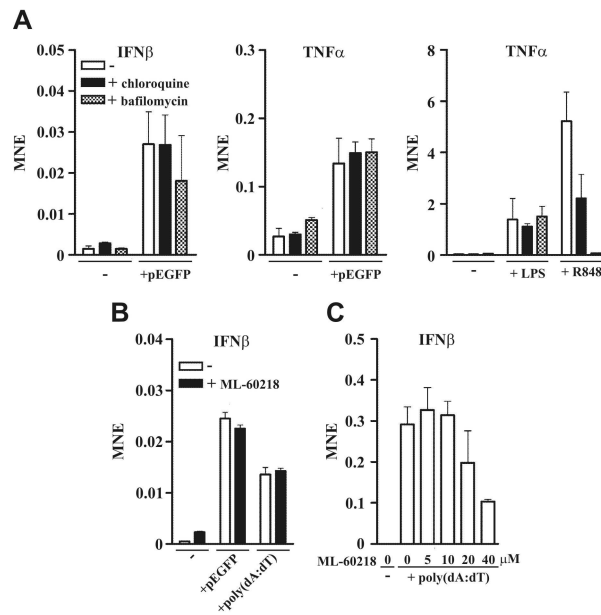
Supplemental Figure 1. PKC ϵ protein is not detectable in human neutrophils. (A) Cytoplasmic extracts prepared by nitrogen cavitation (see M&M) from neutrophils and autologous monocytes were either electrophoresed (50 μ g neutrophils, 30 μ g monocytes) or immunoprecipitated (500 μ g from neutrophils, 300 μ g from monocytes) with specific anti-PKC ϵ and TBK1. Samples were also immunoprecipitated with anti-rabbit IgG control antibodies. A representative immunoblot experiment out of three with similar results is shown. Panel (A) shows that PKC ϵ is not detectable in either cytoplasmic neutrophil cavitates (*upper* panel). On the other hand, TBK1, which has a MW comparable to that of PKC ϵ , is readily detected in neutrophil samples (*lower* panel), proving that our samples were correctly prepared. (B) Neutrophils and autologous monocytes were incubated with 100 ng/ml LPS or 100 U/ml IFN γ for 24 h. Whole cell extracts were then prepared, electrophoresed (50 μ g neutrophils, 30 μ g monocytes) and immunoblotted using antibodies specific for PKC ϵ , ISG15 and actin. Panels display one experiment representative of three with similar results. Data demonstrate that the levels of ISG15, but not PKC ϵ , protein are upregulated by IFN γ in both neutrophils and monocytes, as well as by LPS in monocytes only, in line with the fact that ISG15 is a “MyD88-independent/TRIF-dependent” gene.

Supplemental Figure 2



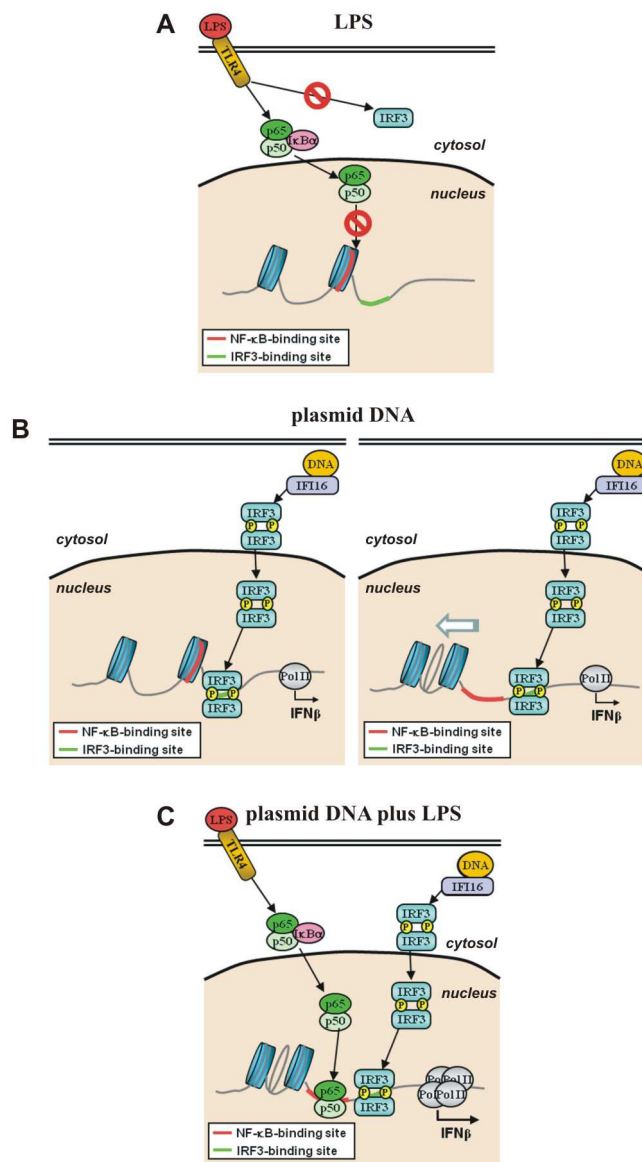
Supplemental Figure 2. Expression of transfected green fluorescent protein (GFP) in human neutrophils. (A) Human neutrophils were electroporated with a 15 μg GFP-encoding plasmid (pmaxGFP) as described in M&M and, after 6 h, cell images were captured by a fluorescence microscope, either in fluorescence (left panel), or in bright field (right panel), mode. (B) Neutrophils were electroporated with different amounts of pmaxGFP plasmid prior to measuring GFP expression levels after a 4 h-culture, by flow cytometry. Values within each plot indicate the percentage of GFP-positive neutrophils. A representative experiment out of three with similar results is shown. (C) Neutrophils were electroporated with 15 μg pmaxGFP plasmid and expression of GFP protein was detected after 4, 6 and 20 h by flow cytometry. Values (mean \pm SE, n=3) indicate the percentage of GFP-positive neutrophils. (D) Neutrophils were treated as described for panel C to evaluate their apoptotic state after different incubation times, as measured by the propidium iodide staining method. Values are expressed as mean \pm SE (n= 3).

Supplemental Figure 3



Supplemental Figure 3. Recognition of intracellular DNA by human neutrophils is independent from TLR9 or RNA polymerase III. (A) Neutrophils were pre-treated for 30 min with 5 μ g/ml chloroquine or 25 nM bafilomycin A1 and then either electroporated with 15 μ g of pEGFP (*left* and *center* panels), or incubated with 100 ng/ml LPS or 10 μ M R848 for 90 min (*right* panel). After 5 h (*left* and *center* panels), total RNA was extracted and analyzed for IFN β (*left* panels), TNF α (*right* and *center* panels) and GAPDH mRNA expression by RT-qPCR. (B) Neutrophils were pre-treated for 30 min with 30 μ M ML-60218 and then electroporated with either 15 μ g pEGFP or 15 μ g poly(dA:dT). After 5 h, total RNA was extracted and analyzed for IFN β and GAPDH mRNA expression by RT-qPCR. (C) HEK 293T cells were pre-treated for 2 h with different doses of ML-60218 and then transfected with 0.8 μ g/ml poly(dA:dT) complexed with lipofectamine. After 24 h, total RNA was extracted and analyzed for IFN β and β 2-microglobulin (β 2m) mRNA expression by RT-qPCR. For all panels, gene expression is depicted as MNE after GAPDH (A-B) or β 2m (C) normalization of triplicate reactions for each sample. Results are from one experiment representative of at least three independent ones.

Supplemental Figure 4



Supplemental Figure 4. Intracellular plasmid DNA activates IRF3 and favours the recruitment of LPS-activated NF- κ B to the IFN β promoter in human neutrophils. (A) Upon stimulation of human neutrophils with LPS, p65/p50 NF- κ B heterodimers, even if activated *via* the MyD88-dependent pathway, do not bind to the IFN β promoter since their binding sites are likely masked. No IRF3 activation occurs in LPS-stimulated neutrophils since the TRIF-dependent pathway is not functional. **(B)** Upon transfection, plasmid DNA binds to IFI16 and activates a signal transduction cascade that leads to IRF3 phosphorylation, dimerization and nuclear translocation. IRF3, in turn, is recruited to its binding sites within the IFN β promoter to induce IFN β transcription. Concomitantly, IRF3 presumably unmask NF- κ B recognition sequences within the same IFN β promoter *via* activation of unknown, local chromatin remodelling events. **(C)** As a result, p65/p50 NF- κ B heterodimers activated by LPS in DNA-transfected neutrophils become now able to bind the unmasked NF- κ B

recognition sequences and, cooperating with IRF3, promote the recruitment of more RNA polymerase II and consequently increase IFN β mRNA transcription.