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Activation of an Immunoregulatory and Antiviral Gene Expression Program in Poly(I:C)-Transfected Human Neutrophils

Nicola Tamassia,* Vincent Le Moigne,* Marzia Rossato,* Marta Donini,* Stephen McCartney,† Federica Calzetti,* Marco Colonna,† Flavia Bazzoni,* and Marco A. Cassatella2*

Neutrophils, historically known for their involvement in acute inflammation, are also targets for infection by many different DNA and RNA viruses. However, the mechanisms by which they recognize and respond to viral components are poorly understood. Polynosinic:polycytidylic acid (poly(I:C)) is a synthetic mimic of viral dsRNA that is known to interact either with endosomal TLR3 (not expressed by human neutrophils) or with cytoplasmic RNA helicases such as melanoma differentiation-associated gene 5 (MDA5) and retinoic acid-inducible gene I (RIG-I). In this study, we report that intracellularly administered poly(I:C) stimulates human neutrophils to specifically express elevated mRNA levels encoding type I IFNs, immunoregulatory cytokines, and chemokines, such as TNF-α, IL-12p40, CXCL10, CXCL8, CCL4, and CCL20, as well as classical IFN-responsive genes (IRG), including IFIT1 (IFN-induced protein with tetratricopeptide repeats 1)/IFN-stimulated gene (ISG)56, G1P2/ISG15, PKR (dsRNA-dependent protein kinase), and IFN-regulatory factor (IRF)7. Investigations into the mechanisms whereby transfected poly(I:C) promotes gene expression in neutrophils uncovered a crucial involvement of the MAPK-, PKR-, NF-κB-, and TANK (TNF receptor-associated NF-κB kinase)-binding kinase (TBK1)/IRF3-signaling transduction pathways, as illustrated by the use of specific pharmacological inhibitors. Consistent with the requirement of the cytoplasmic dsRNA pathway for antiviral signaling, human neutrophils were found to constitutively express significant levels of both MDA5 and RIG-I, but not TLR3. Accordingly, neutrophils isolated from MDA5-deficient mice had a partial impairment in the production of IFN-β and TNF-α upon infection with encephalomyocarditis virus. Taken together, our data demonstrate that neutrophils are able to activate antiviral responses via helicase recognition, thus acting at the frontline of immunity against viruses.

P

olymorphonuclear neutrophils (PMNs)3 are cells of the innate immune system that are crucial for host defense because of their capacity to release a variety of proteases, massive amounts of reactive oxygen species, and bactericidal proteins (1). In addition to their classical proinflammatory functions, PMNs are able to synthesize and release, upon appropriate stimulation, numerous cytokines and chemokines, both in vitro and in vivo (2). Microbial products are known to strongly induce cytokine/chemokine gene expression and production in neutrophils, mainly via the stimulation of pattern recognition receptors such as Toll-like receptors (TLRs) (3). Among TLR agonists, LPS, the natural TLR4 ligand, represents one of the most effective and well-studied stimuli for neutrophil-derived cytokines/chemokines, as demonstrated by its ability to activate the production of IL-1, TNF-α, CXCL8, CCL3, CCL4, CCL19, CCL20, and IL-12p40 (2, 4). Recent studies (5), aimed at clarifying the molecular mechanisms whereby the LPS- TLR4 complex transduces its signals in human neutrophils, have uncovered that LPS does not mobilize the so-called “MyD88-independent pathway” (6). In fact, it was observed that LPS fails to induce type I IFN (IFN-β and/or IFN-α) production in PMNs (5), thereby preventing the autocrine/paracrine induction of STAT1 tyrosine phosphorylation that is ultimately responsible for the transcriptional activation of a host of antimicrobial and antiviral IFN-responsive genes (IRG) (7). It was further shown that in human PMNs the MyD88-independent pathway is not mobilized as a consequence of the inability of LPS to activate the TANK (TNF receptor-associated NF-κB kinase)-binding kinase (TBK1) and, in turn, the phosphorylation of IFN regulatory factor (IRF)3 (5), two phenomena essential for the transcriptional induction of IFN-β (8, 9). Curiously, lack of TBK1 activation occurs despite constitutive expression of the intracellular signaling and regulatory components involved along the MyD88-independent cascade, including TRAM (TRIF-related adapter molecule), TRIF (TRIF-domain-containing adapter-inducing IFN-β), NAP1 (NF-κB-activating kinase-associated protein 1), TNFR-associated factor (TRAF)3, TBK1, IκB kinase (IKK)α, and IRF3 in neutrophils (5). In view of the fact that the other known TLR-dependent transduction pathway, the “TLR3/TRIF-dependent pathway”, which ultimately signals through TBK1 and IRF3 (10, 11), is also nonfunctional in neutrophils for their lack of TLR3 expression (12, 13), it remains intriguing to explain why human PMNs constitutively express TBK1 and IRF3.

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Abbreviations used in this paper: PMN, polymorphonuclear neutrophil; CHX, cycloheximide; DC, dendritic cell; EMCV, encephalomyocarditis virus; IFIT1, IFN-induced protein with tetratricopeptide repeats 1; IKK, IκB kinase; IRF, IFN regulatory factor; IRG, IFN-responsive gene; ISG, IFN-stimulated gene; MDA5, melanoma differentiation-associated gene 5; MMP, matrix metalloproteinase; MINE, mean normalized expression; MOI, multiplicity of infection; mono-DC, monocye-derived DC; PKR, dsRNA-dependent protein kinase; poly(I:C), polynosinic/polyricidylic acid; PT, primary transcript; RIG-I, retinoic acid-inducible gene I; TBK, TANK (TNF receptor-associated NF-κB kinase)-binding kinase; TRAF, TNFR-associated factor; TRIF, TIR-domain-containing adapter-inducing IFN-β.

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Recent advances have provided a clearer picture of the pathways by which the innate immune system senses bacterial and viral pathogens, and, additionally, how this leads to production of type I IFNs (14). These phenomena are primarily mediated not only by the engagement of specific endosomal TLRs, such as TLR3, TLR7, TLR8 and TLR9, but also by TLR-independent cascades, especially in the case of viral RNA recognition in the cytosol. The latter pathways involve two cytosolic RNA helicases, RIG-I (retinoic acid-inducible gene I) and MDA5 (melanoma differentiation-associated gene 5, also called Helicard) (15, 16). Recognition of different viruses by MDA5 and RIG-I is mediated by differences in ligand specificity between the two sensors. RIG-I has been shown to bind preferentially to 5′-phosphorylated RNA as well as poly-uridine motifs (17), while MDA5 recognizes dsRNA. However, according to a very recent study, it appears that viral dsRNAs and polyinosinic:polycytidylic acid (poly(IC)) may differentially activate both RIG-I and MDA5, depending on their length (18). RIG-I recognizes specific sets of RNA viruses belonging to Flaviviridae, Paramyxoviridae, Orthomyxoviridae, and Rhabdoviridae (15, 19, 20), whereas MDA5 is specifically required for the recognition of picornaviruses such as encephalomyocarditis virus (EMCV), Theiler’s virus, and Mengo virus or intracellular poly(IC) dsRNA (16, 19). Additionally, MDA5 has been shown to play a role in the measles virus-induced activation of IFN-β mRNA synthesis (21). Other viruses such as orthoreovirus, dengue virus, and West Nile virus have been shown to be recognized by both MDA5 and RIG-I (22). A third helicase, LGP2, lacks caspase activation and recruitment domains (CARDs) and, while it likely induces RIG-I responses (23, 24), its real function remains to be definitively established. Once engaged by their ligands, both RIG-I and MDA5 bind through CARDs to mitochondrial IPS-1 (also known as MAVS, VISA, or Cardif) (25–28) and initiate signaling cascades that lead to TBK1, IKKe, IRF3, IRF7, NF-κB, and AP-1 activation and, in turn, IFN-α/β expression. Interestingly, both RIG-I and MDA5 expression appear essential for IFN-α/β production by fibroblasts, conventional dendritic cells (DCs), and macrophages, but not plasmacytoid DCs that instead operate through the TLR pathways only (19).

In this study, we show that a number of RNA helicase-mediated signal transduction cascades are potentially operative in human neutrophils, as revealed by the capacity of intracellularly administered poly(I:C) to activate the TBK1, p38 MAPK, ERK, JNK, and PKR (dsRNA-dependent protein kinase) kinases, the IRF3 and NF-κB transcription factors, and, in turn, the expression of many antiviral and immunoregulatory molecules.

Materials and Methods

Antibodies

Anti-IRF3 Abs were from Active Motif (catalog no. 39033) or Santa Cruz Biotechnology (sc-9082); TBK1 mAbs were from Imgenex (IMG-139A), whereas rabbit polyclonal TBK1 Abs were a kind gift from Dr. Sarah M. McWhirter (Harvard University, Cambridge, MA). Rabbit polyclonal IFIT1 (IFN-induced protein with tetratricopeptide repeats 1) and anti-IRF3 Abs were from Cell Signaling Technology, Antibody-Stat1 (catalo (sc-346), -ERK1 (sc-93), and anti-IRF3 (154), -IκB-α (sc-371), -PKR (sc-6282), and -IRF-7 (sc-9083) Abs were from Santa Cruz Biotechnology. Rabbit polyclonal anti-phospho-PKR Abs were from BioSource International (44-668), mouse monoclonal anti-IFN-β Abs were from Sigma-Aldrich (clone no. A5060), and anti-actin Abs were from Sigma-Aldrich (H9260).

Cell purification and culture

Granulocytes (neutrophils >96.5%, eosinophils <3%) and Percoll-purified monocytes were isolated under endotoxin-free conditions from buffy coats of healthy donors (4, 5). Monocyte-derived dendritic cells (mono-DC) were prepared as described (4). In some experiments, Ficol-Paque-isolated neutrophils were further enriched to reach a 99.9% purity by positively removing eventual contaminating cells (T cells, NK cells, B cells, monocytes, DCs, platelets, eosinophils, or erythrocytes) with Abs against CD3, CD56, CD19, CD36, CD49d, and Gly-A using a custom-made EasySep kit (StemCell Technologies). 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), reconstituted with or without 0.02–250 μg/ml poly(I:C) (either from InvivoGen or from Amersham Pharmacia Biotech), 2 μg/ml of Escherichia coli RNA (Sigma-Aldrich), 100 ng/ml Ultra-Pure E. coli LPS (0111:B4 strain, from InvivoGen), 0.1–1000 U/ml IFN-β (Betaliferon from Schering Berlin), and then plated either in 6/24-well tissue culture plates (Nunc) or in polystyrene flasks (Greiner Bio-One) for their culture at 37°C, 5% CO₂ atmosphere. In selected experiments, neutrophils were preincubated for 30 min with 20 μg/ml cycloheximide (CHX), 2 mM 2-aminopurine, 50 μM resveratrol, 50 μM trans-stilbene, 5 μg/ml brefeldin A (all from Sigma-Aldrich), 20 μM PD98059, 20 μM SP600125, and 10 μM SB203580 (from Alexis Biochemicals) before transfection or stimulation. After the desired incubation period, neutrophils were collected and spun at 800 × g for 5 min. The resulting supernatants were immediately frozen in liquid nitrogen and stored at −80°C, while the corresponding pellets were either extracted for total RNA or lysed for protein analysis or other assays. All reagents used were of the highest available grade and were dissolved in pyrogen-free water for clinical use (4, 5).

Isolation and infection of murine neutrophils

Neutrophils were isolated from bone marrow of 6–8-wk-old wild-type and MDA5−/− mice, using a discontinuous Percoll gradient according to standard procedures (29). At the end of the preparation, neutrophils (>95% purity as determined by Hema-3 staining) were suspended in RPMI 1640 at 5 × 10⁶ cells/ml and infected with 10 MOI (multiplicities of infection) EMCV, or left unstimulated in RPMI 1640 for 4 or 20 h, after which time culture supernatants and cell pellets were isolated and analyzed.

Intracellular delivery of poly(I:C) to neutrophils

Transfection of neutrophils with poly(I:C) or RNA was performed using the Amaxa Biosystems nucleoporator, according to the method described by Johnson et al. (30), with minor modifications. After isolation, 10⁶ neutrophils were resuspended in 100 μl complete nucleofector solution, containing 0.4–10 μg poly(I:C), and then transferred to a nucleoporation cuvette. For mock-transfection, PBS was used to replace poly(I:C). After electroporation, cells were immediately transferred into 6/24-well tissue culture plates at 5 × 10⁶ cells/ml and cultured as described in the Results. Morphological analyses and photomicrographs of transfected neutrophils were made 1 h after poly(I:C) transfection or LPS stimulation using phase-contrast optics.

Analysis of mediator concentration

Cytokine concentrations in cell-free supernatants were measured by specific human ELISA kits for IFN-β (from either BioSource International or PBL Biomedical Laboratories) and CXCL8 (ImmuTools), IFN-α (PBL Biomedical Laboratories), TNF-α, IL-12p70, CCL10, CCL20, and CCL4 (R&D Systems), or murine ELISA kits for IFN-β (PBL Biomedical Laboratories) and cytokometric bead array for TNF-α (BD Biosciences). Detection limits of these ELISA were: 2 U/ml for human IFN-β, 30 pg/ml for human IL-12p70, 12 pg/ml for human IFN-γ, 15 pg/ml for human CXCL8, TNF-α, CCL10, CCL11, and CCL4, 5 pg/ml for murine IFN-β, and 10 pg/ml for murine TNF-α. Measurement of the content of gelatinase/matrix metalloproteinase (MMP)-9, lactoferrin, and β-glucuronidase in neutrophil-derived supernatants was performed by ELISA (R&D Systems) or functional assays (31).

Real-time RT-PCR and primary transcript (PT) real-time RT-PCR

Real-time RT-PCR and PT real-time RT-PCR have been performed as described (32, 33) using gene-specific primer pairs (purchased from Invitrogen) available under the following entry code in the public database RPMinusDB (mdgen.ugent.be/rtprimerdb/): human 9570 (3253), GAPDH (3537), IFIT1 (3540), PT-IFIT1 (3912), and IFIT2 (3910), by guest on April 14, 2017 http://www.jimmunol.org/ Downloaded from
IL-1ra (3544), IFN-α (all genes) (3541), IFN-β (3542), IRF7 (3546), PTTIRF7 (3914), G1P2/ISG15 (3547), PT-IRF7 (3913), TNF-α (3551), TRAIL/TFNSF5 (3552), CCL2 (3533), CCL4 (3535), CCL20 (3911), PKR (3915), RIG-I (3916), MDA5 (3917), LGP2 (3918), and TLR3 (3919); murine IFN-β (3957), CXCL10 (3956), TNF-α (3954), IFIT1 (3955), and GAPDH (3958). Data were calculated with Q-Gene software (www.BioTechniques.com) and are expressed as mean normalized expression (MNE) units after GAPDH normalization.

Flow cytometry
Freshly isolated neutrophils, mono-DC, or mock/poly(I:C)-electroporated neutrophils were centrifuged, suspended in 100 μl of PBS containing 0.2% albumin, and blocked with 10% complement-inactivated human serum. Cells were then incubated with 10 μg/ml of mouse anti-human TLR3 (MCA2267, Abd Serotec), together with control mouse IgG1 (10 μg/ml) (BD Pharmingen), for 30 min at 4°C. For intracellular staining, cells were pretreated with a permeabilizing solution (Fix and Perm, Caltag Laboratories) and then incubated with TLR3 mAbs or control mouse IgG1 for 30 min at room temperature. After washing twice with PBS, biotinylated secondary sheep anti-mouse IgG (Sigma-Aldrich) and subsequently PE-conjugated streptavidin (BD Biosciences) were added to the cells and further incubated for 30 min at 4°C. In another group of experiments, neutrophils were directly double stained with CD11b-PE (catalog no. 301306) and CD62L-FITC (catalog no. 304804) in combination with their relative isotype controls (BioLegend) (34). Cytofluorimetric analysis was performed on a FACScan flow cytometer (BD Biosciences) using CellQuest software (35).

Estimation of apoptotic cells
Apoptosis of mock/poly(I:C)-electroporated neutrophils was determined by propidium iodide staining, as previously described (36). Briefly, neutrophils (106) were washed once with PBS and then suspended in 1.5 ml hypotonic fluorochrome solution (50 μg/ml propidium iodide in 0.1% sodium citrate plus 0.1% Triton X-100) for DNA detection. Samples were then placed in the dark at 4°C for at least 1 h before analysis by flow cytometry. Propidium iodide fluorescence of 10,000 individual nuclei per sample was acquired and analyzed with a FACSscan flow cytometer using CellQuest software (31).

Laser confocal microscopy
Cytospins of mock- or poly(I:C)-transfected neutrophils were processed essentially as previously described (5).

EMSA
Transcription factor binding analyses were performed by incubating cell extracts in binding buffer in the presence of labeled oligonucleotide probes, exactly as described (37).

Immunoblotts, native gels, and in vitro TBK1 kinase assays
After stimulation, neutrophils (5 × 106/condition) were diluted in ice-cold PBS and centrifuged twice at 300 × g for 5 min at 4°C. Whole cell extracts were prepared by lysis with RIPA (radioimmunoprecipitation assay) buffer, frozen, and stored at −80°C (5). For phospho-tyrosine STAT1 detection, neutrophils were lysed in a different buffer (38). Small aliquots of the various samples were routinely processed for protein content determination, by using a protein assay kit (Bio-Rad). For Western blot analysis, whole-cell extracts were subjected to immunoblotting by standard procedures. Nitrocellulose membranes were firstly blocked for 1 h at room temperature in TBS/T (20 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.1% Tween 20) containing 5% BSA, and then incubated overnight at 4°C in the presence of specific primary Abs in the same buffer. Ab binding was detected by using HRP-conjugated anti-mouse or anti-rabbit IgG (Amersham Pharmacia Biotech), and revealed using the ECL system (Amersham Pharmacia Biotech), according to the manufacturer’s instructions. Native PAGE was performed as previously described (5) using whole-cell extracts, according to the procedure of Iwamura et al. (39). Immunoprecipitation of TBK1 for the in vitro kinase assay and anti-TBK1 immunoblotting were done as described (5), with analysis and quantification of gel radioactivity performed by Instant Imager (Packard Instruments).

Statistical analysis
Data are expressed as means ± SE. Statistical evaluation was performed by Student’s t test for paired data and was considered to be significant if p < 0.05.

Results
Human neutrophils transfected with poly(I:C) express mRNAs encoding for IFN-β and other antiviral and immunoregulatory products. Preliminary experiments confirmed and extended previous information (13) on the inability of human neutrophils to respond to extracellular poly(I:C) (Fig. 1). Real-time RT-PCR experiments documented, in fact, that addition of exogenous poly(I:C) to neutrophil cultures, at concentrations corresponding to 50 μg/ml (and up to 250 μg/ml, data not shown), fail to elicit the mRNA induction of cytokines and chemokines, including type I IFN, TNF-α, IL-12p40, CXCL10 (Fig. 1A), TRAIL, IL-1ra, CCL2, and CCL20, in addition to CXCL8 and CCL4 (13) (not shown), or of classical IRG, such as IFIT1/ISG56, G1P2/ISG15, PKR, and IRF7 (Fig. 1A). In contrast, monocytes incubated with exogenous poly(I:C) displayed elevated levels of IFN-β, CXCL10, IFIT1 (Fig. 1B), IFN-α, G1P2/ISG15, PKR, and IRF7 (data not shown), in agreement with previous information (40). Similarly, neutrophils incubated with LPS or IFN-β were found to accumulate elevated transcript levels of TNF-α and IL-12p40, or IFIT1, ISG15, PKR, and IRF7, respectively (Fig. 1A), indicating that they were responsive to appropriate stimuli. Consistent with their unresponsiveness to extracellular poly(I:C), circulating neutrophils constitutively express neither surface nor intracellular TLR3, unlike mono-DC (Fig. 1C).

Because it has been recently shown that the transfection of poly(I:C) into target cells strongly activates the production of type I IFNs and IRG via cytoplasmic MDA5 and/or RIG-I (15, 16, 18), in subsequent experiments we examined whether also neutrophils could respond to transfected poly(I:C). For such a purpose, intracellular delivery of poly(I:C) was successfully achieved by electroporation, as already reported to function in human neutrophils by others (30). In our hands, mock- and poly(I:C)-electroporated cells did not substantially decrease their viability within the time-frame of our studies (up to 7 h) (Fig. 2A), and they displayed typical morphological changes (Fig. 2B), as well as alterations of CD11b and CD62L expression (Fig. 2C), that are manifested by activated neutrophils (34). As a result, transfection of poly(I:C) into the cytoplasm of neutrophils was found to dramatically induce the mRNA expression for a variety of genes, including IFN-β and all IFN-α, antiviral proteins/IRG such as IFIT1, ISG15, PKR, IRF7 (Fig. 3), and IFIT2/ISG54 (not shown), cytokines, and chemokines, for instance TNF-α, IL-12p40, CXCL10, and CCL20 (Fig. 3), but not TRAIL or IL-1ra (not shown). Using optimal concentrations of poly(I:C) (corresponding to 2 μg/ml) (Fig. 3A), expression of most (but not all) genes peaked after 4 h posttransfection (Fig. 3B), reaching in most cases levels of induction much higher than those observed in LPS-, IFN-β- (Fig. 1A), or IFN-γ-stimulated neutrophils (5). Such strong effects were specific for the transfected poly(I:C), as they were not observed in mock- (Fig. 3, A and B) or tRNA-electroporated cells (not shown), and are genuinely attributable to neutrophils, as identical results were obtained by using highly pure (>99.9%) neutrophil populations isolated through negative selection of magnetically labeled leukocytes (not shown). Intracellular poly(I:C) is known to activate MDA5 and RIG-I, depending on its length (16, 18, 19). Consistent with these findings, human neutrophils were found to constitutively express MDA5 and RIG-I transcripts (Fig. 3C), as well as MDA5 and RIG-I Ags (Fig. 3D), at levels comparable to those present in autologous monocytes. Higher levels of MDA5 expression were, however, detectable in mono-DC, in line with previous information (41). Neutrophils were also found to express substantial levels of LPG2 mRNA, which, similarly to the MDA5 and RIG-I transcripts, were further inducible by poly(I:C) transfection (not
shown). In contrast, a constitutive TLR3 mRNA was not observed in neutrophils, consistent with the data shown in Fig. 1B and in other reports (12, 13). Collectively, these data demonstrate that intracellular, but not extracellular, administration of poly(I:C) potently stimulates human neutrophils to express high levels of immunostimulatory and antiviral genes, presumably via interaction with cytoplasmic MDA5 and/or RIG-I.

Induction of antiviral genes in poly(I:C)-transfected neutrophils is IFN-β-independent

Having observed that poly(I:C)-transfected neutrophils express high levels of IFN-β mRNA (Fig. 3, A and B), it was important to establish whether poly(I:C)-transfected neutrophils could produce and release IFN-β itself into the extracellular medium. Such experiments were particularly relevant to perform not only in view of the key role of endogenous IFN-β in driving the induction of IRG genes in an autocrine manner as part of a classical antiviral gene expression program, but also in light of our recent findings showing that IFN-γ gene expression was not inducible in neutrophils stimulated with LPS and/or IFN-γ (5). Surprisingly, we could not detect any antigenic IFN-β in supernatants harvested from poly(I:C)-transfected neutrophils after 0.5, 1, 2, 6, and up to 21 h of culture (not shown). Additionally, no intracellular IFN-β was detectable in whole lysates of poly(I:C)-transfected neutrophils by immunoblotting (not shown). Similarly to antigenic IFN-β, neither the release of IFN-α, TNF-α, IL-12, CXCL10, CCL4, and CCL20 (not shown) nor an enhanced expression of immunodetectable ISG15 protein could be observed in poly(I:C)-transfected neutrophils (Fig. 4A), despite the concomitant high levels of the ISG15-related transcripts (Fig. 3, A and B). Expression of ISG15 protein, however, was augmented in neutrophils treated with exogenous IFN-β (Fig. 4B), as expected from findings in other cell types (42, 43). Unlike the case of the cytokines/chemokines mentioned above and of ISG15 as well, an up-regulated expression of antigenic IFIT1, PKR, and IRF7 was revealed in poly(I:C)-transfected neutrophils (Fig. 4A),
suggesting the possibility that their up-regulated mRNAs (Fig. 3, A and B) were efficiently translated in response to poly(I:C).

Hence, while it was unexpected to discover that IFN-β/H9252 mRNA induced in response to intracellular poly(I:C) in neutrophils is not translated into protein, it was evident that IFN-β/H9252 is dispensable for the induction of potential IRG genes such as CXCL10, IFIT1, IRF7, and ISG15 mRNAs. The latter notion was further supported by the following observations (Fig. 5): 1) kinetics of CXCL10, IFIT1, ISG15, and IRF7 transcriptional induction in poly(I:C)-transfected neutrophils are relatively rapid (Fig. 5A) and substantially precede the kinetics of IFN-β/H9252 mRNA induction (Fig. 3A); 2) kinetics of IFN-β (or all IFN-α) mRNA expression in poly(I:C)-transfected neutrophils do not precede but substantially overlap those of IRG genes (Fig. 3A); 3) preincubation of neutrophils with CHX (Fig. 5B) or brefeldin A (not shown) before poly(I:C) transfection did not suppress the up-regulation either of CXCL10, IFIT1, IRF7, and ISG15 mRNAs. The latter notion was further supported by the following observations (Fig. 5): 1) kinetics of CXCL10, IFIT1, ISG15, and IRF7 transcriptional induction in poly(I:C)-transfected neutrophils are relatively rapid (Fig. 5A) and substantially precede the kinetics of IFN-β mRNA induction (Fig. 3A); 2) kinetics of IFN-β (or all IFN-α) mRNA expression in poly(I:C)-transfected neutrophils do not precede but substantially overlap those of IRG genes (Fig. 3A); 3) preincubation of neutrophils with CHX (Fig. 5B) or brefeldin A (not shown) before poly(I:C) transfection did not suppress the up-regulation either of CXCL10, IFIT1, ISG15, and IRF7, or of IFN-β and TNF-α mRNA expression (Fig. 5B); 4) neither STAT1 tyrosine phosphorylation (Fig. 5C) nor STAT1-containing DNA-binding activities (Fig. 5D) were observed in neutrophils transfected with poly(I:C) and kept in culture for up to 4 h, thus ruling out any STAT1 activation by endogenous IFN-β or

FIGURE 3. Up-regulation of various cytokine, chemokine, and IRG expression in human neutrophils transfected with poly(I:C). Dose-dependent (A) and time-dependent (B) mRNA expression of various genes in poly(I:C)-transfected neutrophils are shown. Neutrophils were transfected either with (A) different concentrations of poly(I:C) (electroporated (EP) poly(I:C)) and cultured for 2 h, or with (B) 2 μg/ml poly(I:C) and incubated for the indicated times. Total RNA was extracted and analyzed for IFN-β, IFN-α (all genes), TNF-α, IL-12p40, CXCL10, CCL20, IFIT1, ISG15, PKR, and IRF7 and GAPDH mRNA expression by real-time RT-PCR. Gene expression is depicted as MNE units after GAPDH normalization of triplicate reactions for each sample. EP mock indicates electroporated neutrophils. Experiments depicted are representative of at least three independent ones. C, MDA5, RIG-I, LPG2, and TLR3 mRNA expression. Total RNA was extracted from neutrophils, autologous monocytes, and mono-DC and then analyzed for MDA5, RIG-I, LPG2, TLR3, and GAPDH mRNA expression by real-time RT-PCR. Gene expression is depicted as MNE units after GAPDH normalization of triplicate reactions for each sample. Data depicted are representative of two independent experiments. D, Antigenic MDA5 and RIG-I expression. Whole-cell extracts from neutrophils, autologous monocytes, and mono-DC were prepared, electrophoresed, and immunoblotted using Abs specific for MDA5, RIG-I, and actin. Depicted data are representative of three independent donors.

FIGURE 4. Expression of IFIT1, IRF7, PKR, and ISG15 proteins in poly(I:C)-transfected and IFN-β-treated neutrophils. Neutrophils were transfected with 2 μg/ml poly(I:C) (electroporated (EP) poly(I:C)) and then cultured for up to 6 h (A) or incubated with 1000 U/ml IFN-β for up to 20 h (B). Whole-cell extracts were then prepared, electrophoresed, and immunoblotted using Abs specific for IFIT1, IRF7, PKR, ISG15, and actin. EP mock indicates electroporated neutrophils. Depicted data are representative of four independent experiments.
IFN-α; and 5) exogenous IFN-β, even when used at doses as low as 2 U/ml (corresponding to the lower concentrations detected by two different IFN-β ELISAs used), effectively induces either STAT1 tyrosine phosphorylation (Fig. 5E) or IL-1ra mRNA expression (Fig. 5F) (44) in both mock- and poly(I:C)-transfected neutrophils. The latter responses are important because they indicate that the procedures of transfection and/or poly(I:C) administration do not alter the responsiveness of neutrophils to exogenous IFN-β. Collectively, these data demonstrate that, in neutrophils transfected with poly(I:C), the up-regulation of most (if not all) target genes occurs directly, that is, without the need for the production of endogenous mediators.

**Activation of the TBK1-IRF3 axis in neutrophils transfected with poly(I:C)**

To elucidate the molecular basis underlying the capacity of transfected poly(I:C) to activate the expression of target genes in neutrophils, we initially analyzed the activation status of IRF3, the transcription factor that, under these conditions, is considered crucial for IFN-β induction (45). As shown in Fig. 6A, IRF3 was detected in native PAGE as a single, fast migrating band (corresponding to the functionally inactive form) in mock-transfected or, as previously described (5), in LPS-stimulated neutrophils. In contrast, dimerization of IRF3 was readily detected in whole extracts prepared from neutrophils.
dose-dependently transfected with poly(I:C) (Fig. 6A), in which it appeared as early as after 15 min (not shown), reached maximal intensity after 120 min, and then slowly decreased (Fig. 6B). IRF3 activation was also evidenced by laser confocal microscopy experiments, which allowed us to directly visualize nuclear-translocated IRF3 in poly(I:C)-transfected neutrophils (Fig. 6C).

Since TBK1 is the kinase downstream of either MDA5 or RIG-I engagement responsible for IRF3 serine phosphorylation (25), a process essential for IRF3 dimerization (46), we subsequently investigated whether TBK1 is activated in poly(I:C)-transfected neutrophils. By performing immunocomplex kinase assays (5), we observed that TBK1 displays easily detectable autophosphotransferase and phosphotransferase activities in cellular fractions of neutrophils transfected with poly(I:C) (Fig. 6D), which reached strongest catalytic activities 60 min post-transfection (Fig. 6E). The crucial role of IRF3 for the induction of not only IFN-β but also CXCL10, IFIT1, and ISG15 mRNAs in poly(I:C)-transfected neutrophils was additionally demonstrated by experiments performed with resveratrol and stilbene (Fig. 6F), two inhibitors of the TBK1-IRF3 axis (47). Specificity of the latter drugs was demonstrated by the fact that they did not influence the direct effect of IFN-β on CXCL10, IFIT1, and ISG15 mRNA expression (Fig. 6G), which is mediated by STAT1 activation (48–50). Altogether, these data unequivocally demonstrate that in neutrophils transfected with poly(I:C), TBK1 and its substrate, IRF3, are rapidly activated and play a critical role for the transcriptional induction of target genes.

**Additional signaling pathways activated in neutrophils transfected with poly(I:C)**

We subsequently analyzed whether other signaling cascades (25) are triggered in poly(I:C)-transfected neutrophils. We could observe, for instance, that poly(I:C) transfection provokes a dramatic activation of NF-κB (as revealed by induction of IkB-α degradation) (37) that was readily detectable in neutrophils within 15–30 min and persisted up to 120 min (Fig. 7A). Such kinetics are noticeably more sustained than those observed in neutrophils incubated with LPS, in which NF-κB activation/IkB-α degradation are more transient (5, 37). We could also detect highly phosphorylated p38 MAPK, ERK, and JNK in poly(I:C)-transfected neutrophils, which were maintained in their phosphorylated/activated form for up to 3 h (Fig. 7B). Furthermore, a remarkable phosphorylation of PKR was also detectable in poly(I:C)-transfected neutrophils (Fig. 7B); the latter was clearly independent from the augmented expression of PKR protein levels observed under the same conditions (Fig. 5). The importance of these pathways were illustrated by the remarkable suppressive effects exerted by inhibitors of p38 MAPK (SB203580) and ERK (PD98059) on the induction of TNF-α and CCL20, but not IFN-β, CXCL10 (Fig. 7C), CCL4, or other IRG (not shown) mRNAs in poly(I:C)-transfected neutrophils. Similarly, inhibitors of JNK (SP600125) and PKR (2-aminopurine, G)
2-AP) significantly inhibited poly(I:C)-mediated induction of TNF-α mRNA, without affecting CCL20 mRNA (Fig. 7C). Furthermore, the inability of 2-AP to affect the induction of IFN-β mRNA by transfected poly(I:C) rules out the involvement of PKR (another known receptor of poly(I:C)) in the transcription of IFN-β, in accord with previous studies (51).

**Discussion**

In this study, we report that the intracellular administration of poly(I:C) into human neutrophils elicits a cascade of events that ultimately activate the expression of immunoregulatory and antiviral genes. Accordingly, we show that poly(I:C) transfection of neutrophils induces a dramatic accumulation of transcripts encoding various cytokines and chemokines, including IFN-β, all IFN-α, CXCL10, CCL2, CCL4, CCL20, IL-12p40, and TNF-α, as well as IFIT1, and TNF-α mRNA expression by real-time RT-PCR. Gene expression is depicted as MNE units after GAPDH normalization of triplicate reactions for each sample. B. Extracellular IFN-β and TNF-α, as detected by ELISA in neutrophil-derived supernatants. Values indicate the means ± SE calculated from six independent experiments. *, p < 0.05.

**Induction of IFN-β and TNF-α production in murine neutrophils infected by EMCV**

In a subsequent series of experiments, we initially analyzed whether human neutrophils could be infected and activated by a murine strain of EMCV, an RNA virus that produces dsRNA that is recognized by MDA5. However, no remarkable responses by neutrophils incubated with murine EMCV (used at 1–10 MOI, for up to 20 h) were found, as measured in terms of 1) induction of IFN-β and TNF-α production, 2) IRG gene expression (CXCL10 and IFIT1), and 3) viability changes. Because the lack of EMCV responsiveness by human neutrophils might be explained by several potential reasons, including, for example, the species specificity of the virus and/or the lack of a specific receptor for virus entry, we did not further proceed with these experiments. We examined, instead, whether neutrophils isolated from wild-type and MDA5-deficient mice could effectively respond to EMCV (Fig. 8). These latter experiments revealed that bone marrow-derived neutrophils display a strong induction of IFN-β, CXCL10, IFIT1, and TNF-α mRNA expression (Fig. 8A), as well as IFN-β and TNF-α production (Fig. 8B), upon infection with EMCV. Surprisingly, such EMCV-mediated upregulatory effects were only partially dependent on MDA5 in neutrophils (Fig. 8), unlike what has been previously observed using bone marrow-derived macrophages and DCs (16). Taken together, these data show that murine neutrophils release IFN-β and TNF-α upon EMCV infection, indicating that poly(I:C) transfection of neutrophils mimics a phenomenon that may occur in actuality.
classical antiviral molecules/IRG such as IFIT1, IFIT2, ISG15, PKR, and IRF7. Other genes potentially inducible by type I IFN, such as, for example, TRAIL (31), or IL-1rα mRNA (44) were not expressed in transfected neutrophils, consistent with the activation of a specific poly(I:C)-dependent gene expression program, independent on endogenous type I IFN (see below). Cell transfection with poly(I:C) is a well-established procedure that recapitulates the effects of direct infection by dsRNA viruses (52). Previous observations on targeted deleted mice (16, 19) have proven that MDA5 is the specific receptor for intracellular poly(I:C) in murine macrophages and DCs. More recently, however, it has been further demonstrated that viral dsRNAs and poly(I:C) may differentially activate RIG-I and MDA5, depending on their length (18). Consistent with a role of both MDA5 and RIG-I as potential cytosolic sensors for intracellular poly(I:C), we found that circulating neutrophils constitutively express MDA5 and RIG-I, both at mRNA and protein levels, in quantities comparable to those found in autologous monocytes. In contrast, we show that human neutrophils do not express TLR3 protein, in keeping with the lack of induction of type I IFN, CXCL10, CCL4, TNF-α, ISG15, IRF7, IFIT1, and PKR mRNA expression by exogenous poly(I:C), and extending previous observations (12, 13).

To strengthen the potential biological relevance of our observations, we tried to infect human and murine neutrophils with EMCV. While our attempts were unsuccessful with human cells, murine neutrophils were found to produce large amounts of either IFN-β or TNF-α upon infection with EMCV. Interestingly, the latter were detected at levels substantially comparable to those measured in supernatants harvested from an equivalent number of EMCV-infected macrophages (S. McCartney and M. Colonna, unpublished observations), but were only partially reduced in supernatants of neutrophils isolated by MDA5-deficient mice. This might suggest that the sensors involved in the recognition of dsRNA viruses (and likely of poly(I:C) as well) are different depending on the cell type, and that specifically in murine neutrophils MDA5 does not act singly. In this regard, it has recently been shown in human keratinocytes that multiple dsRNA detection pathways work together to mediate an antiviral state (53). Whatever the case, finding that neutrophils might potentially contribute to local or systemic IFN-β production extends our knowledge and provides important and useful information in the field of antiviral mechanisms.

It was surprising to find that human neutrophils transfected with poly(I:C) do not produce and, consequently, do not secrete any IFN-β, IFN-α, TNF-α, CXCL10, CCL4, CCL20, or IL-12, despite the elevated amounts of their mRNAs. The molecular reason(s) explaining why neutrophils do not produce IFN-β and the other cytokines and chemokines upon poly(I:C) transfection are intriguing. Interestingly, recent findings reported that human neutrophils do not produce type I IFN also upon exposure to Newcastle disease or Sendai viruses (54), which target cells via the RIG-I pathway (15, 19). It could be eventually hypothesized that cytokine/chemokine mRNAs are effectively translated, produced, and released by poly(I:C)-transfected neutrophils and yet are rapidly degraded by proteolytic enzymes released by neutrophils themselves into the extracellular medium. One of them might be gelatinase B/MMP-9, which is abundantly contained in tertiary granules of neutrophils, and which has been shown to cleave IFN-β and CXCL10 (55, 56).

Additional neutrophil-derived proteases shown to cleave TNF-α are elastase or cathepsin G (57, 58). Accordingly, we noticed that both mock transfection and (more efficiently) poly(I:C) transfection triggered rapid degranulation of neutrophils, as revealed by the detection of elevated amounts not only of MMP-9/gelatinase, but also of β-glucuronidase (marker of primary granules) and lactoferrin (marker of secondary granules), in neutrophil-derived cell-free supernatants (N. Tamassia and M. A. Cassatella, unpublished observations). Note, however, that we could also measure high levels of the CXCL8 chemokine in the same supernatants, which further increased when mock- or poly(I:C)-transfected neutrophils were stimulated by exogenous LPS (N. Tamassia and M. A. Cassatella, unpublished observations). Thus, even though the presence of other neutrophil-derived proteases selectively targeting IFN-β or other cytokines/chemokines cannot be excluded, the recovery of CXCL8, itself susceptible to MMP-9/gelatinase- or elastase-mediated degradation (59, 60), would rule out, in principle, the hypothesis that IFN-β or other cytokines/chemokines are not detectable because of degradation by gelatinase B/MMP-9. If so, our results would suggest that intracellular poly(I:C) delivers transcriptional signals to a wide variety of genes, only a fraction of which are subsequently translated into their products, for example, IFIT1, IRF7, or PKR as shown herein. Others, namely type I IFN, TNF-α, CXCL10, CCL4, CCL20, IL-12p40, and ISG15, are likely subject to specific translational control that might require additional signals (not provided by poly(I:C) itself) to be translated into proteins. In support of the latter hypothesis are previous studies describing that, in human neutrophils, several mRNAs are subjected to translational control; for example, VEGF mRNA in the case of LPS or LPS plus IFN-γ treatment (61), IL-6 receptor α subunit and retinoic acid receptor (RAR)-α mRNAs in response to platelet-activating factor (62), BAK mRNA in the case of IFN-γ- or TNF-α-treatment (63), and Bcl-xL (64) and TNF-α (our unpublished observations) mRNAs in the case of GM-CSF-treatment. Therefore, the identification of the factor(s) or elements shared by the translationally repressed IFN-β, IFN-α, TNF-α, CXCL10, CCL4, CCL20, IL-12p40, and ISG15 mRNAs may give some hints into the mechanisms controlling the rate of protein synthesis in poly(I:C)-transfected neutrophils. Recent findings provided, for instance, strong evidence for a repressive translational mechanism that impedes type I IFN production in plasmacytoid DCs, acting at the level of IFR7 mRNA translation (65). In this context, it should be also kept in mind that the steady-state levels of a given protein in a cell derive from the balance between the synthesis by translation vs the rate of degradation. On the basis of this assumption, the increased levels of antigenic IFIT, IRF7, or PKR in poly(I:C)-transfected neutrophils might more simply derive from a stabilization effect exerted by poly(I:C) on these proteins. While further studies are necessary to clarify all of these issues, our findings unequivocally imply that the mRNA induction of IFIT1, IRF7, PKR, ISG15, IFIT2, or CXCL10 (all classical IRG) in neutrophils transfected with poly(I:C) occurs independently from type I IFN, as also widely supported by the evidence provided in Fig. 5.

A final series of experiments were aimed at elucidating the molecular mechanism(s) whereby transfected poly(I:C) induces the expression of target genes. We document, for instance, that transfection of neutrophils with poly(I:C) rapidly triggers intracellular events leading to the phosphorylation/activation of p38 MAPK, ERK, JNK, and PKR kinases. Furthermore, we have also uncovered that poly(I:C) transfection of neutrophils elicits the activation of NF-κB and IRF3, two transcription factors that are crucial in driving the direct transcriptional induction of IFN-β (66). Interestingly, studies with specific inhibitors allowed us to define the involvement of phospho-p38 MAPK, phospho-JNK, and phospho-PKR in positively regulating the expression of TNF-α and CCL20 mRNAs, without affecting that of IFN-β, CXCL10, or CCL4. Additionally, the use of two different TBK1/IRF3 inhibitors (47) confirmed that IFN-β, CXCL10, IFIT1, and ISG15 are strongly dependent on IRF3 activation, as previously reported (67). With respect to IRF3, we also show that poly(I:C) transfection promotes...
the activation of TBK1, the kinase responsible for IRF3 phosphorylation, a process essential for triggering IRF3 dimerization and, in turn, nuclear translocation (8, 9). Worthy of note, the latter observations finally identify the biological relevance of the TBK1-IRF3 axis in human neutrophils, which, based on our recent findings (summarized in the Introduction) (5) remained elusive. Accordingly, the capacity of transfected poly(I:C) to activate TBK1 is also consistent with the fact that neutrophils constitutively express both the Traf3 and Napi1 proteins (5), which, together with Ips-1 and dependent mechanisms in lipopolysaccharide-treated neutrophils. IL-10 modulates cytokine gene transcription by protein synthesis-independent mechanism. Science 300: 1148-1151.


