Appl Biochem Biotechnol (2010) 160:1179–1187
DOI 10.1007/s12010-009-8643-7

Role of Toll-Like Receptor 3, RIG-I, and MDA5 in the Expression of Mesothelial IL-8 Induced by Viral RNA

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Received: 22 December 2008 / Accepted: 6 April 2009 /
Published online: 27 May 2009

Abstract Interleukin-8 (IL-8) is a chemokine that has been shown to be a potent chemoattractant for polymorphonuclear neutrophils from the vascular compartment into the pleural space during infectious pleural effusions. Mesothelial cells express the viral receptors Toll-like receptor 3 (TLR3), RIG-I, and MDA5. Activation of these receptors by viral RNA exemplified by poly (I:C) RNA leads to a time- and dose-dependent increase of mesothelial IL-8 synthesis. To show the specific effect of viral receptors, knockdown experiments with short interfering RNA specific for TLR3, RIG-I and MDA5 were performed. This novel finding of functional expression of these viral sensors on human mesothelial cells may indicate a novel link between viral infections and mesothelial inflammation and indicates a pathophysiologic role of viral receptors in these processes.

Keywords IL-8 · MDA5 · Poly (I:C) RNA · RIG-I · Toll-like receptor 3

Introduction

Neutrophil infiltration into the pleural space is a prominent and characteristic feature of an early and acute inflammatory response in several pleural diseases. Parapneumonic effusions and empyema pleural fluids contain high levels of interleukin-8 (IL-8) [1]. Bacterial pneumonia is a major cause of parapneumonic pleural effusions [2, 3]. The onset of parapneumonic pleural effusions is accompanied by an influx of leukocytes from the vascular compartment into the pleural space [4, 5]. VEGF is a key mediator in pleural

This work was supported by a grant from the Else-Kröner-Fresenius-Stiftung to Matthias Sauter and Thomas Sitter and from the Deutsche Nierenstiftung to Markus Wörnle.

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effusion formation. Source of pleural VEGF is the pleural mesothelium [6]. The pleural mesothelium is a monolayer of metabolically active cells, which play an important role in regulating influx of inflammatory cells as well as in processes of fibrosis and fibrinolysis [7–9]. Mesothelial cells (MC) secrete cytokines and chemokines, which promote extravasation of leukocytes into the pleural space during infection and inflammation [10, 11]. Several stimuli, including lipopolysaccharide, TNF-α, and IL-1β initiate a mesothelial synthesis of IL-8 [10–12]. IL-8 plays an important role in migration and direction of polymorphonuclear leukocytes across the mesothelium [13, 14]. MC express intracellular adhesion molecule-1 and vascular cellular adhesion molecule-1 [15, 16]. Migration of leukocytes from the blood across the mesothelium is mediated by these adhesion molecules [17]. The expression of these adhesion proteins has been shown to be important in neutrophil and macrophage adherence to MC, respectively [16]. The synthesis of IL-8 by MC is an important factor that controls the direction of the migration of neutrophils across the mesothelium [18]. Beside bacterial infections of the lung, viral infections can cause severe problems. Viral infections cause airway inflammatory disease and exacerbate asthma and chronic obstructive pulmonary disease. Especially in immunosuppressed patients, pleural effusion caused by viral infections is a common complication. In patients with human immunodeficiency virus infection pulmonal Kaposi's sarcoma, a human herpesvirus 8 (HHV8)-related neoplasm and HHV8-related primary effusion lymphoma impair the course of disease [19–21]. In non-immunosuppressed patients, viral mesothelial infections are responsible for the transformation to malignant mesothelioma in patients without asbestos exposition or are an important cofactor in asbestos exposition [22–24].

Toll-like receptors (TLRs) are an essential part of the innate immune system. TLRs recognize conserved pathogen-associated molecular patterns and are expressed not only on immune cells but also on a number of non-immune cells [25, 26]. An expression of several TLRs on human MC was shown before [27]. TLRs recognize molecular patterns associated with microbial pathogens and induce an immune response [25, 28]. In mice, 11 members of the TLR family (TLR1-11) have been identified and ten in humans so far. Each TLR recognizes a distinct component of an infectious agent [25, 26, 29].

TLR3 recognizes dsRNA of viral origin as exemplified by polyriboinosinic/polyribocytidylic acid (poly (I:C) RNA), a synthetic analogue of viral dsRNA [30, 31]. TLR3 expression is not restricted to leukocytes but also occurs in non-immune organs, including kidney, lung, and bowel [32, 33]. Beside TLR3, the helicases retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) may also act as sensors of viral infections through recognition of viral dsRNA and may up-regulate type I interferons [34, 35]. RIG-I and MDA5 play a role in the expression of inflammatory chemokines induced by poly (I:C) RNA in airway epithelial cells [36].

We could previously show that human MC express TLR3, RIG-I, and MDA5 [27]. Activation of these viral receptors leads to synthesis of certain cytokines and chemokines as well as VEGF and type I interferons [27, 37]. Now, we demonstrate that poly (I:C) RNA stimulation of MC results in up-regulation of mesothelial IL-8 expression.

Materials and Methods

Medium M199 and newborn calf serum were obtained from Gibco BRL (Germany), and tissue culture plates were from Costar (USA). Human serum was prepared from freshly collected blood of healthy donors and stored at −20 °C. Fibronectin from human serum and trypsin were purchased from Boehringer (Germany), and collagenase type II was from

Worthington (USA). Monoclonal antibodies against cytokeratin 8 and 18 and against vimentin were a gift from Dr. G. van Muijen (University of Nijmegen, The Netherlands).

Cell Culture Experiments

Human MC were isolated from the omental tissue of consenting patients undergoing elective surgery as described previously [38]. The studies have been reviewed and approved by the national ethics committee. Cells were grown in fibronectin-coated dishes in M199 medium supplemented with 25 mmol/L Hepes (pH 7.3), 2 mmol/L glutamine, 10% (vol/vol) human serum, 10% (vol/vol) newborn calf serum (heat-inactivated), penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37 °C under 5% CO_2/95% air atmosphere. The medium was replaced every 2 to 3 days. Subcultures were obtained by trypsin/ethylenediaminetetraacetic acid treatment at a split ratio of 1:3. Cells from omental tissue were pure MC as assessed by their uniform cobblestone appearance at confluence, by the absence of von Willebrand factor and the uniform positive staining for cytokeratins 8 and 18 and for vimentin. For the experiments, confluent cultures were used at the second or third passage, and cells were always refed 48 h before the experiment with M199 medium, supplemented with 2% (vol/vol) human serum and antibiotics. Confluent cells have been demonstrated to be in a non-proliferative state under these conditions [39]. Incubation of cells with doses of tested compounds for up to 48 h did not have any significant effect on cell viability as tested by vital cell staining with acridine orange and ethidium bromide and by LDH release (cell viability exceeded 95%).

Real-Time RT-PCR Analysis

Real-time RT-PCR analysis was done as described [40]. For real-time quantitative RT-PCR, 2 μg of isolated total RNA underwent random primed reverse transcription using a modified Moloney murine leukemia virus reverse transcriptase (Superscript; Life Technologies, Germany). In parallel, 2 μg aliquots was processed without reverse transcription to control for contaminating genomic DNA. Real-time RT-PCR was performed on a TaqMan ABI 7700 Sequence Detection System (PE Applied Biosystems, Germany). rRNA was used as reference gene. All water controls were negative for target and housekeeper. Sequences with following gene bank accession numbers served for the design of the pre-developed Taq Man assay reagents (PDAR) or primers and probe, purchased from Applied Biosystems: NM003265/U88879 (human TLR3), NM014314 (human RIG-I), NM0022168 (human MDA5), Z11686 (human IL-8/CXCL8), and M33197 (human GAPDH).

Western Blot Analysis

For western blot analysis, MC were lysed with RadioImmunoPrecipitation assay buffer (50 mM Tris–HCL, pH 8; 150 mM NaCl; 1% NP-40; 0.5% sodium deoxycholate; 0.1% SDS) containing protease inhibitors. The lysates were cleared by centrifugation at 13,000×g for 15 min at 4 °C, and protein concentration was determined with DC protein assay kit (BioRad Laboratories, USA). Proteins (40 μg/mL) were separated by sodium dodecyl sulfate-polyacrylamide on an 8% polyacrylamide gel and transferred to a PVDF membrane for western blotting (Immobilon-P Transfer Membrane, Millipore). The membranes were blocked in 3% milk solution in Tris-buffered saline-Tween 20 (TLR3) or 5% bovine serum albumin (RIG-I) for 1 h and probed overnight with a monoclonal antibody to human TLR3

(Acris Antibodies, Germany) or RIG-I antibody (monoclonal antibody against RIG-I was a gift from Dr. Simon Rothenfusser, Ludwig-Maximilians University Munich, Germany). After washing four times in TBST, the membranes were incubated with secondary purified rabbit anti-mouse IgG [H&L]-HRP (TLR3) or secondary peroxidase-conjugated affinipure goat anti-rat IgG+IgM [H&L] (RIG-I) for 1 h and washed again four times with TBST. Signals were visualized with a Western Lightning Chemiluminescence reagent plus kit (PerkinElmer, USA).

ELISA

ELISA for IL-8 was performed on cell culture supernatants using commercial assay kits (Quantikine®, R&D Systems, USA) and following the providers' instructions.

Knockdown of Gene Expression with Short Interfering RNA

Predesigned short interfering RNAs (siRNAs) specific for TLR3, RIG-I, and MDA5 were purchased from Ambion (Japan). Transfection of siRNA into the cells was performed using siPORT-NeoFX transfection agent (Ambion) as described before [36]. Scrambled siRNA was used as the nonspecific negative control of siRNA (Ambion). Cells were pretreated with siRNA for 24 h and washed once using cell culture medium to remove remaining transfection agent. Poly (I:C) RNA was mixed to cell culture medium as indicated.

Statistical Analysis

Values are provided as mean±SEM. Statistical analysis was performed by unpaired *t* test if applicable or by the ANOVA analysis. Significant differences are indicated for p values <0.05 (*) or 0.01 (**), respectively.

Results

Effect of Poly (I:C) RNA on Synthesis of Mesothelial IL-8

MC express the viral receptors TLR3, RIG-I, and MDA5 under basal conditions [27, 37]. In order to test the effect of activation of viral receptors on mesothelial IL-8 synthesis, MC were grown under standard conditions and stimulated with poly (I:C) RNA (5 μg/ml) for different time intervals (3–48 h). IL-8 synthesis was analyzed by RT-PCR PCR after 3, 6, 9, 12, and 24 h (Fig. 1a) and ELISA after 24 and 48 h (Fig. 1b). Poly (I:C) RNA stimulation leads to a time-dependent increase of IL-8 synthesis analyzed by RT-PCR with the highest increase at 12 h. By ELISA, an increase of IL-8 synthesis was shown up to 48 h.

When MC were stimulated with poly (I:C) RNA in different concentrations (0.5 to 10 μg/ml) for 24 h, a concentration-dependent effect on IL-8 synthesis was shown by RT-PCR (Fig. 2a) and by ELISA (Fig. 2b).

Effect of Transfection with siRNA Specific for TLR3, RIG-I, and MDA5 on Synthesis of Poly (I:C) RNA-Induced IL-8 Production

MC constitutively expressed mRNA for TLR3, RIG-I, and MDA5 [27, 37]. Transfection of MC with siRNA specific for TLR3, RIG-I, and MDA5 caused remarkable down-regulation

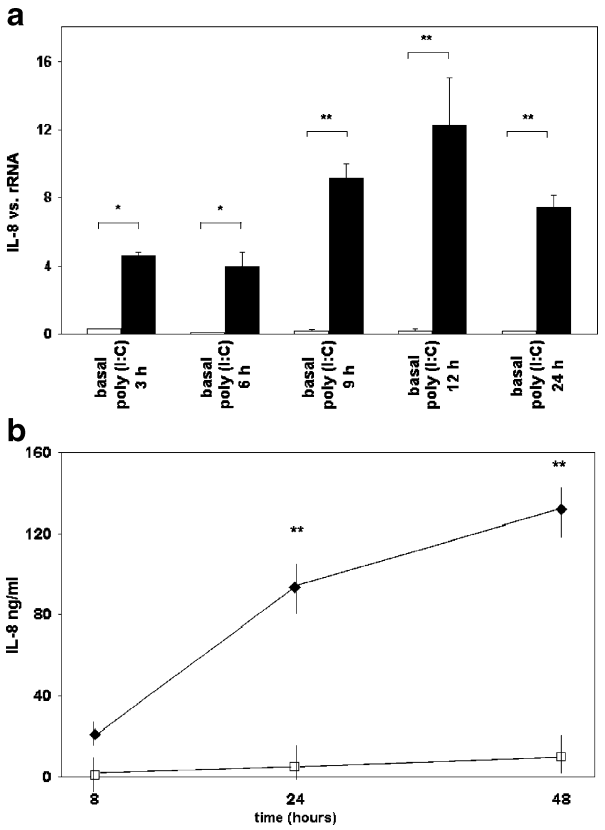

Fig. 1 Poly (I:C) RNA up-regulates mRNA expression and release of mesothelial IL-8. MC were stimulated with and without (basal) poly (I:C) RNA (5 μg/ml) for different time intervals (3–48 h). mRNA expression for IL-8 was analyzed by real-time RT-PCR (**a**). Release of IL-8 was detected by ELISA (**b**). Results are mean±SEM of three independently performed experiments, and ribosomal RNA served as reference gene

of basal gene expression of TLR3 (86%), RIG-I (89%), and MDA5 (90%; Fig. 3a). For protein analysis, cells were transfected with siRNAs specific for TLR3 and RIG-I, incubated in culture medium for 24 h, lysed, and examined for the TLR3 and RIG-I protein levels by western blotting (Fig. 3b). Basal protein expression of TLR3 was inhibited by siRNA specific for TLR3. When cells were additionally stimulated after transfection with poly (I:C) RNA (5 μg/ml) for 24 h, expression of TLR3 was increased by poly (I:C) RNA stimulation. This effect on TLR3 expression could be decreased by siRNA for TLR3. When protein expression of RIG-I was analyzed, no significant difference on protein level was observed between basal expression of RIG-I and cells cultivated under standard conditions and transfected with siRNA specific for RIG-I. Poly (I:C) RNA (5 μg/ml) increased protein expression of RIG-I in a small amount, an effect which could be clearly reduced by siRNA specific for RIG-I. Hek 293 cells served as a positive control. Because there is no antibody against MDA5 available, which is working in western blot analysis of MDA5, protein expression for MDA5 could not be examined.

MC were stimulated with poly (I:C) RNA (5 μg/ml) for 24 h. siRNA specific for TLR3 reduced poly (I:C) RNA-induced mRNA expression of IL-8 shown by RT-PCR significantly (Fig. 3c). siRNA specific for MDA5 and RIG-I had no effect on poly (I:C) RNA-induced expression of IL-8. When IL-8 release was analyzed by ELISA (Fig. 3d), the poly (I:C) RNA-induced increase of IL-8 synthesis was reduced by siRNA specific for TLR3. siRNA specific for MDA5 and RIG-I had no effect on poly (I:C) RNA-induced

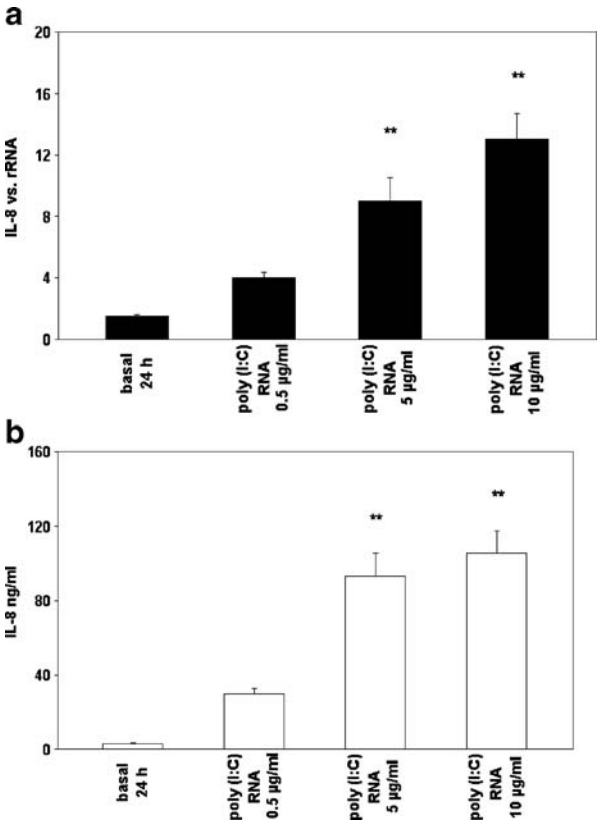

Fig. 2 Poly (I:C) RNA up-regulates mRNA expression and release of mesothelial IL-8 in a dose-dependent manner. MC were stimulated with different concentrations (0.5, 5, 10 µg/ml) of poly (I:C) RNA for 24 h. mRNA expression for IL-8 was analyzed by real-time RT-PCR (**a**). Release of IL-8 was detected by ELISA (**b**). Results are mean± SEM of three independently performed experiments, and ribosomal RNA served as reference gene

release of IL-8. Negative control with scrambled RNA had no effect on poly (I:C) RNA-induced changes of IL-8 production.

Discussion

TLR3, RIG-I, and MDA5 are known as receptors for dsRNA. Viral dsRNA has been recognized as a major ligand for TLR3 in many cell types, including DCs and non-immune cells such as intestinal epithelial cells, fibroblasts, and mesangial cells [32, 41]. This is considered to serve as an anti-viral system by generating type I interferons. In addition to TLR3, a role for RIG-I and in MDA5 in viral infections was shown for several cell types [34–36]. It has been recently shown that also single-stranded RNA can act as a ligand for TLR3. That viral RNA generated during the reproduction process of DNA viruses may bind to TLR3 seems to be supposable [42]. We could previously demonstrate an expression of TLR3, RIG-I, and MDA5 on human MC. Stimulation with poly (I:C) RNA leads to an increased synthesis of cytokines such as IL-6 and chemokines such as MCP-1/CCL2 and RANTES/CCL5 as well as an increased synthesis of type I interferons [27]. In addition, poly (I:C) RNA stimulation resulted in an up-regulation of mesothelial VEGF synthesis [37]. The cytokines and chemokines mentioned above may all play a role during viral infection and inflammation of the pleura. Mesothelial VEGF synthesis is a key mediator for

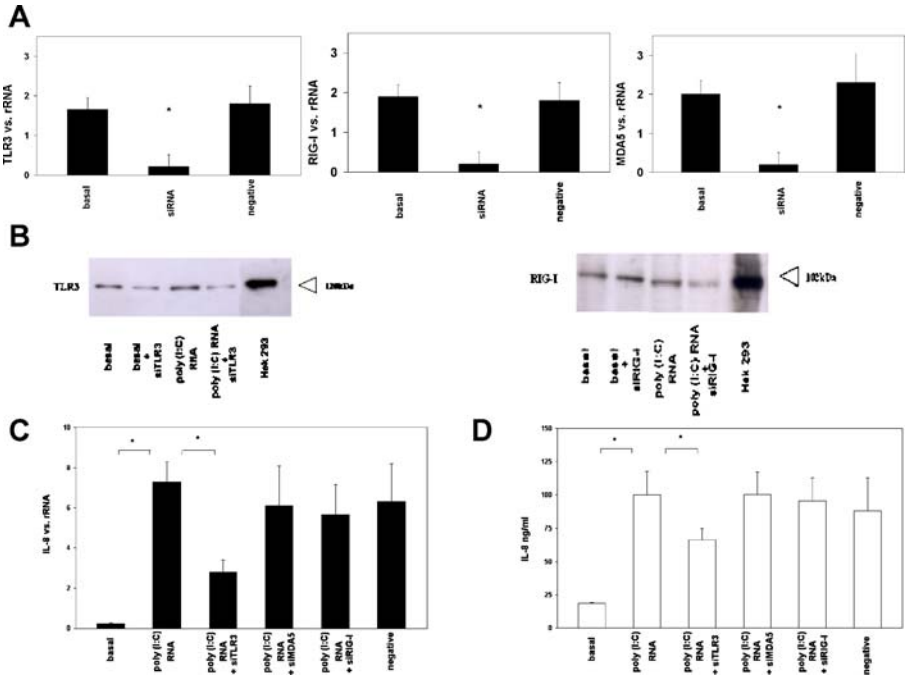

Fig. 3 Effect of transfection with siRNA specific for TLR3, MDA5, and RIG-I on poly (I:C) RNA-induced synthesis of IL-8. Transfection of MC with siRNA specific for TLR3, RIG-I, and MDA5 caused remarkable down-regulation of basal gene expression of TLR3 (86%), RIG-I (89%), and MDA5 (90%) shown by RT-PCR (**a**). For protein analysis, cells were cultured under standard conditions and transfected with siRNAs specific for TLR3 and RIG-I for 24 h, lysed, and examined for the TLR3 and RIG-I protein levels by western blotting (**b**). Cells were additionally stimulated after transfection with poly (I:C) RNA (5 μg/ml) for 24 h, and expression of TLR3 and RIG-I was analyzed with and without transfection with the respective siRNAs for TLR3 and RIG-I. Results shown are from one of two independently performed experiments, which showed reproducible results. siRNA specific for TLR3 reduced poly (I:C) RNA (5 μg/ml) induced mRNA expression of IL-8 significantly shown by RT-PCR (**c**). siRNA specific for MDA5 and RIG-I had no effect on poly (I:C) RNA-induced expression of IL-8. siRNA specific for TLR3 decreased poly (I:C) RNA-induced release of IL-8, shown by ELISA (**d**), but not significantly. siRNA specific for MDA5 and RIG-I had no effect on poly (I:C) RNA-induced release of IL-8. Negative control with scrambled RNA (negative) had no effect on poly (I:C) RNA-induced changes of IL-8 production. Values are means±SEM of four independently performed series of experiments

pleural effusion formation and plays a critical role in tumorgenesis and progression of malignant mesothelioma [6, 43].

The present study shows that viral infections of MC exemplified by poly (I:C) RNA lead to an increased synthesis of mesothelial IL-8. This effect was dose and time dependent. IL-8 plays a key role in direction and migration of leukocytes across the mesothelium [13, 14]. Migration of inflammatory cells into the peritoneal cavity contributes to the initiation, amplification, and resolution of peritoneal inflammation during peritonitis [44]. During viral infection of the lung, IL-8 expressed by pleural mesothelial cells may be responsible for direction of leukocytes into the pleura and thereby contributes to formation of pleural effusions and pleural inflammation. mRNA expression and protein production of IL-8 in response to viral infection is a differentially regulated and complex process. To show the specific role of the viral receptors, TLR3, RIG-I, and MDA5 knockdown experiments with

siRNA specific for TLR3, RIG-I, and MDA5 were performed, and IL-8 expression was analyzed after poly (I:C) RNA stimulation.

Poly (I:C) RNA-mediated expression of IL-8 shown by RT-PCR and ELISA was significantly inhibited by siRNA specific for TLR3 but not by siRNA specific for RIG-I or MDA5 arguing in favor of a predominant role of TLR3 in these processes.

Our observation of a mesothelial IL-8 synthesis after activation of viral receptors by viral RNA could describe a link between viral infections and mesothelial inflammation and further contribute to formation of pleural effusions.

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