The stromal component in rheumatoid arthritis: CD55 expression, cell death and beyond

Karpus, O.N.

Publication date
2014

Document Version
Final published version

Citation for published version (APA):
1Departments of Experimental Immunology and Internal Medicine, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands; *Current address: GlaxoSmithKline Pharmaceuticals Research and Development, Stevenage, UK
INTRACELLULAR DELIVERY OF poly(I:C) INDUCES APOPTOSIS OF FIBROBLAST-LIKE SYNOVIOCYTES VIA AN UNKNOWN dsRNA SENSOR

Olga N. Karpus, Cheng-Chih Hsiao, Hanneke de Kort, Paul P. Tak*, and Jörg Hamann

Submitted for publication
ABSTRACT

Objective
Fibroblast-like synoviocytes (FLS) express functional membranous and cytoplasmic sensors for double-stranded RNA (dsRNA). Notably, FLS undergo apoptosis upon transfection with the synthetic dsRNA analog poly(I:C). We here studied the mechanism of intracellular poly(I:C) recognition and subsequent cell death in FLS.

Methods
FLS from patients with rheumatoid arthritis were transfected with poly(I:C) or 3pRNA, complexed with Fugene. Knockdown of dsRNA sensors (MDA5, RIG-I), adaptor proteins (IPS, STING, TRIF), and interferon receptor (IFNAR) was performed using SMART pool siRNAs. TBK1 was blocked using the inhibitor BX795. Induction of the interferon response genes MDA5, RIG-I, and CXCL10 was measured by RT-PCR. Cell death was detected by flow-cytometry, Western blot, and TUNEL assay.

Results
FLS responded similarly to poly(I:C) or 3pRNA transfection, however only intracellular delivery of poly(I:C) induced significant cell death, accompanied by upregulation of pro-apoptotic proteins Puma and Noxa, caspase 3 cleavage, and nuclear segregation. Knockdown of the DExD/H-box helicase MDA5 did not affect the response to intracellular poly(I:C); in contrast, knockdown of RIG-I abrogated the response to 3pRNA. Knockdown of the downstream adaptor proteins IPS, STING, and TRIF or inhibition of TBK1 did not affect the response to intracellular poly(I:C), while knockdown of IFNAR blocked intracellular poly(I:C)-mediated signaling and cell death.

Conclusions
A so far unknown intracellular sensor recognizes dsRNA and induces apoptosis in FLS.
INTRODUCTION

Fibroblast-like synoviocytes (FLS) are a unique type of stromal cells, which line and organize the synovial tissue, and produce components of the synovial fluid. The synovium of healthy individuals comprises a few layers of FLS, which build the intimal lining. In chronic inflammatory conditions, such as rheumatoid arthritis (RA), the amount of these cells increases dramatically due to higher proliferation and less susceptibility to apoptosis\(^1\). Activated FLS are responsible for cartilage degradation and bone erosion\(^2\). Moreover, they attract and activate leukocytes from the circulation, causing a viscous cycle of synovial tissue inflammation.

Like other cell types, FLS are equipped with various molecules sensing molecular patterns, associated with evading pathogens or damage to the tissue. Others and we previously showed that FLS from RA synovial tissue express functional dsRNA sensors TLR3, MDA5, and RIG-I\(^3-5\). Membranous TLR3 recognizes extracellular dsRNA, including the synthetic analog poly(I:C)\(^6\). MDA5 and RIG-I are cytoplasmic DExD/H-box helicases with specificity for linear and 5'-triphosphated dsRNA (3pRNA), respectively. However, recent reports showed that different cell types may engage other DExD/H-box helicases than MDA5 to sense dsRNAs of different length\(^7\). DExD/H-box helicases belong to a family of proteins with RNA-binding and -remodeling functions\(^8\), yet, the exact role of many of these proteins is still unknown. By means of their two caspase-recruitment domains (CARD), MDA5 and RIG-I interact with the adaptor protein IPS-1, also known as MAVS, VISA, or CARDIF\(^9\). Engagement of IPS-1 by activated MDA5 or RIG-I leads to formation of a TBK1-IKK\(\varepsilon\) complex\(^10\) that phosphorylates the transcription factors IRF-3 and IRF-7\(^11\). Phosphorylated IRFs migrate into the nucleus where they induce production of IFN\(\alpha/\beta\), which upon secretion bind to the type I IFN receptor (IFNAR), thereby initiating expression of a plethora of IFN-response genes (IRGs). Notably, triggering of dsRNA sensors can also directly lead to apoptosis. Intracellular delivery of 3pRNA and poly(I:C) has been shown to induce cell death of melanoma cells in vitro\(^12, 13\) and of dendritic cells in an animal model of multiple sclerosis\(^14\).

Lining FLS are known for their resistance to apoptosis in situ\(^15\). Remarkably, we recently found that transfection with poly(I:C), but not with 3pRNA, induces cell death in FLS\(^5\). In the current study, we further explored this finding. We show that FLS upregulate IRGs upon transfection with both, poly(I:C) and 3pRNA. However, in contrast to other cell types, FLS neither required MDA5 for the recognition of intracellular poly(I:C) nor did they need the known adaptor proteins IPS, STING, and TRIF or the TBK1-IKK\(\varepsilon\) complex. The type I IFN receptor was required for both intracellular poly(I:C)-mediated signaling and cell death. We conclude that another, to be identified dsRNA sensor facilitates the recognition of intracellular poly(I:C) that causes cell death in FLS.

MATERIALS AND METHODS

Isolation and culture of FLS

Synovial tissue was obtained by needle arthroscopy from patients with RA\(^16\). The Medical Ethics Committee of the Academic Medical Center approved this study, and all patients gave written informed consent. Single cell suspensions were generated by finely mincing freshly isolated tissue, followed by treatment with 0.5 mg/ml collagenase type VIII (Sigma-Aldrich, Zwijndrecht, Netherlands) for 2 h at 37°C. The obtained cell suspension was cultured in Dulbecco’s Eagle’s
medium (DMEM; 1 g/l D-glucose, Invitrogen, Bleiswijk, Netherlands), supplemented with 10% heat inactivated fetal calf serum (FCS; GE Healthcare, Colbe, Germany), L-glutamine, HEPES, and antibiotics (penicillin, gentamicin, and streptomycin; all from Gibco, Breda, Netherlands). Non-adherent cells were removed after 24 h, and adhering cells were grown to sub-confluence and splitted subsequently (1:3) by trypsinization. Cells were expanded in 6-well plates to 70-90% confluence and used for experiments from passage 3 until passage 9; at that time, cultures were free of macrophages and other non-fibroblasts.

**Delivery of dsRNA**
For intracellular delivery, 0.1–1 µg/ml of high molecular weight (HMW) poly(I:C) (Invivogen, Toulouse, France) or 0.1–1 µg/ml 3pRNA (kindly provided by Prof. G. Hartmann and Dr. M. Schlee, University Hospital Bonn, Germany) were mixed with the transfection reagent Fugene HD (Roche, Mannheim, Germany), according to the manufacturer’s protocol, and added to the culture medium. For extracellular delivery, 10 µg/ml poly(I:C) was added to the medium without Fugene.

**siRNA knockdown**
For transient siRNA transfection, SMART Pool siRNAs targeting the dsRNA sensors MDA5 and RIG-I, the adaptor molecules IPS, STING, and TRIF, the IFN-α/β receptor (IFNAR), and a non-target control (NT) were purchased from Thermo Scientific (Darmstadt, Germany). Transfection was performed according to the manufacturer’s protocol. The optimal concentrations of siRNA and transfection reagent were determined by testing a range of 5–100 nM siRNA and 0.5–2.5 µl/ml transfection reagent Dharmafect 2 (DF2). With 5 nM of specific siRNA and 0.5 µl/ml DF2, we obtained efficient knockdown without affecting cell survival. 48 h after siRNA delivery, FLS were transfected with poly(I:C) or 3pRNA; 18 h later, knockdown efficiency and expression of response genes were evaluated.

**Pharmacological inhibition**
To block TLR3 signaling and TBK1 complex formation, 20 µM chloroquine (inhibitor of endosomal acidification; Invivogen) or 200 nM BX795 (TBK1 inhibitor; Invivogen) were added to the medium 1 h prior to dsRNA delivery.

**Lentiviral knockdown**
Lentiviral plasmids containing shRNA against human MDA5 and control shRNA (Mission shRNA; Sigma) were transfected into 293T cells using FuGene6 (Roche). Transfected cells were cultured in DMEM containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. Culture media containing lentiviral particles were collected after 2 days and added 2 ml supplemented with 8 µg/ml polybrene to 30 x 10^5 FLS cultured in 6-well plates. Cells were selected over 14 days with 1 µg/ml puromycin, transfected with poly(I:C), and tested for knockdown efficiency and expression of response genes as described above.

**Real time PCR**
Total RNA was isolated using the Qiagen RNA extraction kit (Qiagen, Venlo, Netherlands) and quantified on a Nanodrop (Thermo Scientific). 100 ng total RNA was reverse transcribed using oligo(dT), random hexamers, and M-MuLV reverse transcriptase from First Strand cDNA Synthesis.
kit (Thermo Scientific) according to manufacturer’s protocol. Transcript levels of the dsRNA sensors MDA5 and RIG-I, the adaptor proteins IPS, STING, and TRIF, and the response genes TNF, IFNβ, and CXCL10 (IP10) were analyzed by quantitative PCR with the StepOnePlus Real-Time PCR system using Fast SYBR Green Master Mix (Applied Biosystems, Carlsbad, CA, USA). Primer sequences are depicted in Supplementary Table 1. Gene transcription was normalized to 18S rRNA (ΔCt). Fold change expression was calculated using the 2-ΔΔCt method.

**Analysis of apoptosis by flow cytometry**

Cell death of FLS transfected with poly(I:C) or 3pRNA was quantified by collecting floating and attached cells 48 h after transfection. To block apoptosis or necroptosis, the pan-caspase inhibitor QVD or necrostatin-1 were added 30 min before transfection with poly(I:C). Attached cells were washed with PBS, detached using trypsin-EDTA (Gibco), washed with PBA (PBS/0.5% bovine serum albumin (BSA)), pooled with washed cells from supernatant, and incubated with annexin-V-FITC (1:100; IQ Products, Groningen, Netherlands) for 30 min at 4°C in calcium buffer. Before measurement, 5 ng/ml propidium iodide (PI; Sigma) was added. Cells were analyzed on a FACSCalibur (BD), and results were analyzed using FlowJo software (Tree Stars, Ashland, OR, USA). Annexin V+, annexin V+PI+, and annexin V PI+ cells were considered as dead/apoptotic cells.

**Analysis of apoptosis and knockdown efficiency by Western blot**

FLS, plated in 6-well plates and transfected with siRNAs for 48 h and/or poly(I:C) or 3pRNA for 24 h, were washed with PBS and collected directly in a 1 x NuPAGE sample buffer (Invitrogen), supplemented with 5% β-mercaptoethanol (Sigma). Lysates were separated by SDS-PAGE, using approximately 3 x 10⁴ cells per lane, and blotted on a Immobilon-FL polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA) by wet transfer. For analysis of apoptosis, membranes were stained overnight using antibodies specific for caspase 3 (#9662; Cell Signaling/Bioke, Leiden, Netherlands), caspase 8 (clone 5F7; MBL/SanBio BV Biologicals, Uden, Netherlands), Bid (#2002; Cell Signaling), Puma (#4976; Cell Signaling), or Noxa (IMG-349A; Imgenex, San Diego, CA, USA). For analysis of siRNA knockdown, membranes were stained overnight with antibodies specific for RIG-I (clone Alme-1; Enzo Life Sciences, Lausen, Switzerland) or MDA5 (#5321; Cell Signaling). For detection, HRP-conjugated secondary antibodies (Dako, Heverlee, Belgium) and chemiluminescent substrate (Roche) were used. Analysis was performed by digital imaging with a charge-coupled device (CCD) camera-based imager (GE Healthcare).

**CellTracker and TUNEL assay**

To visualize the cytoplasm, FLS were mixed with CellTracker Green (Invitrogen), according to manufacturer’s protocol, cultured on chamber slides to 70-90% confluence, and transfected with 1 μg/ml poly(I:C) or 3pRNA. 48 h later, adherent cells were fixed in acetone, washed and mounted with Vectashield containing 4’,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Amsterdam, Netherlands). For TUNEL assay, FLS cultured on chamber slides and transfected with poly(I:C) or 3pRNA as above were fixed after 48 h in 4% paraformaldehyde. Sections were permeabilized with 0.2% TritonX in PBS, pre-incubated in 1 x Reaction Buffer, and incubated with 4 pmol/μl Biotin-11-dUTP and 3.2 U/ml of TdT (all Fermentas) for 90 min at 37°C. Biotin-11-dUTP incorporation was visualized by streptavidin-Alexa 594 and nuclei were co-stained with DAPI.
Images were obtained using a fluorescence microscope (DMRA; Leica, Rijswijk, Netherlands) and Image Pro Plus software (Media Cybernetics, Rockville, MD, USA).

Statistical analysis
Differences between groups were calculated by paired t-test. Values are expressed as mean ± SEM. A two-tailed p value of less than 0.05 was considered to represent a significant difference.

RESULTS
Transfection of FLS with the dsRNA analogs poly(I:C) or 3pRNA induces a similar immune response, but only intracellular poly(I:C) triggers apoptosis

Previous studies showed that the DExD/H-box helicases MDA5 and RIG-I, which sense poly(I:C) and 3pRNA, respectively, are present and functional in FLS. To test whether FLS respond differently to these synthetic dsRNA analogs, we transfected primary human RA FLS with 0.1 μg/ml poly(I:C) or 3pRNA for 4 h and 18 h, and measured the induction of known IRGs and DNA sensors by qPCR. Both, poly(I:C) and 3pRNA, had a similar effect (Figure 1 and Supplemental Figure S1B).

Figure 1. Transfection with poly(I:C) or 3pRNA evokes similar induction of IRGs in FLS. RA-derived FLS were stimulated for 4 h or 16 h with 0.1 μg/ml poly(I:C) or 3pRNA, complexed with fugene (FG). Transcript levels of response genes were quantified by qPCR, using 18S rRNA as reference gene (mean ± SEM, n=3-4).
In line with our previous observations, FLS detached 48 h after transfection with poly(I:C), but not 3pRNA, indicating a loss in viability. To quantify the differences in cell death induced by poly(I:C) or 3pRNA, we stained the cells with annexin V and PI. Poly(I:C) at 1 μg/ml induced significantly more cell death (up to 75%) in comparison to 3pRNA (up to 20%) (Figure 2A), which was inhibited by the pan-caspase inhibitor QVD, but not by the necroptosis inhibitor necrostatin-1 (Figure 2B). In line herewith, cleavage of caspase 3 was detectable after transfection of FLS with 0.1 or 1 μg/ml poly(I:C), but not 3pRNA (Figure 2C/D). Pretreatment of the cells with QVD reduced the amount of cleaved caspase 3 (Figure 2D). To test whether cell death occurred due to extrinsic apoptosis, we analyzed caspase 8 and BID cleavage, which was not affected by transfection with poly(I:C) (Figure 2C). Caspase activation via the intrinsic, mitochondrial pathway of apoptosis is preceded by upregulation of proapoptotic BH3-only proteins, such as Noxa and Puma. We found upregulation of Puma and Noxa after transfection with poly(I:C), but not 3pRNA (Figure 2C).

Alternatively, apoptosis of FLS after poly(I:C) transfection was demonstrated by TUNEL staining, showing segregated nuclei with incorporated label (Figure 2E, upper panel). When we

Figure 2. Transfection with poly(I:C), but not with 3pRNA, induces apoptosis in FLS. RA-derived FLS were stimulated for 48 h (A, B, E) or 24 h (C, D) with the indicated concentrations (μg/ml) of poly(I:C) or 3pRNA, complexed with fugene (FG). (A) Quantification of annexin V+ and/or PI+ dead cells by flow cytometry (mean ± SEM, n³5). (B) Quantification of annexin V+ and/or PI+ dead cells after pre-incubation with the pan-caspase inhibitor QVD (20 μM) or the necroptosis inhibitor necrostatin-1 (20 μM) (mean ± SEM, n=3-4). (C) Western blot analysis of caspase 3 and 8, and the pro-apoptotic proteins BID, Noxa, and Puma. Cleaved caspases are indicated with arrows. MDA5 was used as an IFN response control. One representative out of three experiments is shown. (D) Analysis of caspase 3 cleavage after pre-incubation of cells with QVD. One representative experiment is shown. (E) TUNEL (upper panel) and CellTracker (lower panel) staining demonstrates nuclear segregation in apoptotic cells after poly(I:C), but not 3pRNA, treatment. *, p<0.05; ***, p<0.001
stained cells with CellTracker and co-stained nuclei with DAPI, we observed the same segregated nuclei that appeared only after transfection with poly(I:C), but not 3pRNA (Figure 2E, lower panel).

**FLS do not recognize intracellular poly(I:C) through MDA5**

To determine whether FLS recognize intracellular poly(I:C) through MDA5, we applied siRNA knockdown. At 25 nM, siRNA against MDA5 (siMDA5) and a non-target sequence (siNT) reduced viability of FLS and increased expression of CXCL10 (Supplemental Figure S2A/C). At a reduced

![Figure 3. Knockdown of dsRNA sensor MDA5 or TLR3 in FLS does not abrogate the response of FLS to intracellular poly(I:C).](image)

(A, B) Knockdown of MDA5 with 5 nM siRNA in RA-derived FLS was performed for 48 h, prior to stimulating cells for 18 h (A) or 24 h (B) with 0.1 μg/ml poly(I:C), complexed with fugene (FG). Knockdown of MDA5 and expression of RIG-I and CXCL10 mRNA (A; mean ± SEM, n=4) and MDA5 and RIG-I protein (B shows one representative out of four experiments) was analyzed by qPCR and Western blot analysis, respectively. (C, D) RA-FLS were pretreated with 10 μM chloroquine; 1 h later, cells were stimulated for 18 h (C) or 24 h (D) with poly(I:C), complexed with FG. 10 μg/ml extracellular poly(I:C), a TLR3 ligand, was used as positive control. Expression of RIG-I, MDA5, and CXCL10 mRNA (C; mean ± SEM, n=3) and MDA5 protein (D shows one representative out of two experiments) was analyzed. (E, F) Knockdown of RIG-I with 5 nM siRNA in RA-derived FLS was performed for 48 h, prior to stimulating cells for 18 h (E) or 24 h (F) with 0.1 μg/ml 3pRNA, complexed with FG. Knockdown of RIG-I and expression of MDA5 and CXCL10 mRNA (E; mean ± SEM, n=6) and MDA5 and RIG-I protein (F shows one representative out of four experiments) was analyzed. *, p<0.05; ***, p<0.005
concentration of 5 nM, these side effects disappeared (Supplemental Figure S2B and Figure 3A); therefore, in all further experiments, we used 5 nM siRNA.

Knockdown of MDA5 was confirmed at mRNA level (Figure 3A). However, after silencing MDA5 and transfecting cells with poly(I:C), we still observed upregulation of the IRGs RIG-I and CXCL10 (Figure 3A). Knockdown of MDA5 and upregulation of RIG-I upon transfection with poly(I:C) were confirmed at protein level (Figure 3B). In line with the preserved responsiveness, knockdown of MDA5 did not rescue cells from poly(I:C)-induced apoptosis (Supplemental Figure S2B). To exclude involvement of TLR3 in sensing intracellular poly(I:C), we blocked TLR3 function with chloroquine, an inhibitor of endosomal acidification. Chloroquine treatment did not affect signaling after transfection with poly(I:C) either (Figure 3C/D), suggesting a TLR3-independent route of intracellular poly(I:C) recognition. As a positive control, we knocked down RIG-I, confirmed at

Figure 4. Knockdown of adaptor proteins IPS, STING, and TRIF or inhibition of TBK1 does not abrogate the response of FLS to intracellular poly(I:C). (A–D) Knockdown of IPS, STING, and TRIF with 5 nM siRNA in RA-derived FLS was performed for 48 h, prior to stimulating cells for 18 h (A–C) or 24 h (D) with 0.1 μg/ml poly(I:C), complexed with fugene (FG). Knockdown of adaptor proteins and expression of MDA5 and CXCL10 mRNA (A–C; mean ± SEM, n=4-5), and MDA5 protein (D shows one representative out of two experiments) was analyzed by qPCR and Western blot analysis, respectively. siRNA against IFNAR was used as a positive control. (E) RA-FLS were pretreated with the TBK1 inhibitor BX795 (200 nM), 1 h later, cells were stimulated for 24 h with poly(I:C), complexed with FG. 10 μg/ml extracellular poly(I:C) was used as a positive control. Shown is one representative out of two experiments. (F) Knockdown of TRIF and IFNAR with 5 nM siRNA for 48 h or inhibition of TBK1 with 200 nM BX795 for 1 h was performed prior stimulation of cells with 0.1 μg/ml poly(I:C), complexed with FG. 48 h later, annexin V+ and/or PI+ dead cells were quantified by flow cytometry (mean ± SEM, n=4). *, p<0.05
mRNA (Figure 3E) and protein (Figure 3F) level. After silencing RIG-I and transfecting cells with 3pRNA, upregulation of MDA5 (Figure 3E/F) and CXCL10 (Figure 3E) was diminished.

To further validate the observation that MDA5 is not the principal sensor for intracellular poly(I:C) in FLS, we silenced the gene using lentiviral shRNAs. Efficient knockdown of MDA5 did not abrogate the induction of the IRGs RIG-I and CXCL10 upon intracellular poly(I:C) (Supplemental Figure S2D/E).

The response of FLS to intracellular poly(I:C) is independent of the adaptor proteins IPS, STING, and TRIF or TBK1 complex
dsRNA sensors signal through the adaptor proteins IPS, STING, and TRIF. To test the contribution of these molecules to the intracellular recognition of poly(I:C), we knocked them down individually and in combination using siRNAs. Knockdown efficacy was confirmed at mRNA level (Figure 4A-C). Silencing of IPS, STING, and TRIF did not change the response to intracellular poly(I:C), as indicated by induction of the IRGs MDA5 and CXCL10 (Figure 4A-C). Only silencing TRIF slightly reduced the induction of CXCL10 (Figure 4C). We also did not observe changes in the response to intracellular poly(I:C) after combined knockdown of STING and IPS, or TRIF and IPS (Supplemental Figure S3A/B). Western blot analysis confirmed these findings (Figure 4D). In contrast, knockdown of IFNAR (IFNα/β receptor) blocked the induction of MDA5, indicating that the recognition of intracellular poly(I:C) initiates a type I IFN loop, upregulating IRGs (Figure 4D).

Adapter proteins downstream of dsRNA sensors commonly trigger the formation of a TBK1-IKKε complex that phosphorylates transcription factors IRF3 and IRF7. Blockade of this complex using the TBK1 inhibitor BX795 blocked the TLR3-dependent response to extracellular poly(I:C), but did not inhibit the response to intracellular poly(I:C) (Figure 4E). We concluded that the cellular response of FLS to intracellular poly(I:C) does not require MDA5, the adaptor proteins IPS, STING, or TRIF, and the formation of a TBK1-IKKε complex.

In line with the observation that intracellular poly(I:C) does not signal through known dsRNA sensors in FLS, cell death was still induced in the knock-down settings tested in this study (Figure 4F and Supplementary Figure 2B). Notably, silencing IFNAR and, to a lower extend, TRIF reduced apoptosis, indicating a role of type IFN signaling in the induction of cell death.

DISCUSSION

RA FLS are remarkably resistant to apoptosis, which has been linked to deficiencies in extrinsic and intrinsic pathways. These blockages can be bypassed in vitro by forced expression of pro-apoptotic Puma. On the other hand, transfection of primary FLS with recombinant DNA or RNA induces cell death and, therefore, is difficult to achieve; the mechanism underlying this restriction is fairly unknown. We here show that upon transfection with the dsRNA analog poly(I:C), FLS undergo apoptosis, associated with upregulation of the BH3-only proteins Puma and Noxa, and caspase-3 cleavage. We also show that the initiation of cell death by intracellular poly(I:C) is independent of the well-established sensor MDA5.

There are several possibilities how poly(I:C) signaling may induce apoptosis. Firstly, transcription factors downstream of the receptor for poly(I:C) may cause cell death directly. IRF-3 and IRF-7 are known transcription factors downstream of several dsRNA and dsDNA sensors; however, other
IRFs also may play a role. In gastric cancer cells, IRF-1 induces transcription of pro-apoptotic Puma, which results in apoptosis. Moreover, in genetically modified mice and human cell lines, IRF-3 was shown to interact with the pro-apoptotic protein Bax, translocate it to the mitochondria, and induce the intrinsic apoptotic pathway. Therefore, IRF-3 has two activities upon activation: it translocates to the nucleus to induce transcription of IFNα/β, and it migrates to the mitochondria to induce apoptosis. Secondly, IRGs, induced upon type I IFN receptor engagement, may trigger FLS apoptosis. There are more than a hundred genes upregulated in response to type I IFNs. In dendritic cells, type I IFNs induce apoptosis through BH3-only proteins, involving Bim and either Puma, Noxa, or Bid. Two other IRGs that might be of interest are RNA-dependent protein kinase R (PKR) and 2’, 5’-oligoadenylatesynthetase (OAS). Both require binding to dsRNA for their function, upon which they evoke cell effects directly without further signaling. PKR (also known as eIF2 kinase) autophosphorylates and forms homodimers, which are able to phosphorylate various cellular substrates and block initiation of translation or induce apoptosis. OAS activates the endoribonuclease RNAse L, which is able to degrade cellular RNAs and induce apoptosis.

Thirdly, recently reported MDA5-independent inflammasome activation by intracellular poly(I:C) might influence cell viability of FLS. However, IL-1β, the product of inflammasome activation, increases metabolic activity of FLS.

The specific induction of FLS apoptosis by intracellular poly(I:C), but not 3pRNA, likely is due to interaction with an as yet unknown dsRNA sensor. Our data indicate that neither the adaptor proteins IPS and STING nor the downstream TBK1 complex are involved in the recognition of poly(I:C) in FLS. The concept of MDA5 as the only signaling receptor for intracellular poly(I:C) has been challenged recently by the discovery of other protein complexes, recognizing long-form poly(I:C). Liu and colleagues showed that the helicases DDX1, DDX21, DHX9, and DHX36 form complexes with IPS and TRIF to sense dsRNA in myeloid cells. While FLS express several of these helicases, our data indicate that the central adapter IPS is not required for cytosolic poly(I:C) sensing in FLS. Moreover, we found, for the first time to our knowledge, that MDA5 is not involved in the recognition of intracellular poly(I:C). Our data are not fully conclusive regarding the adapter TRIF. TRIF knockdown moderately affected intracellular poly(I:C)-induced upregulation of CXCL10, but not MDA5, and it reduced cell death. TRIF was reported to be involved in the formation of a caspase 8-RIP1 signaling complex, a ripoptosome, which, depending on its composition, can activate apoptosis and/or necroptosis. A TRIF-dependent mechanism of sensing cytosolic poly(I:C) in FLS therefore cannot be excluded and warrants further investigation.

Cell apoptosis after poly(I:C) transfection is commonly seen in various cancers, but not in non-malignant cells, which often require a second, apoptosis-inducing stimulus, such as ligation of FAS. FLS possess certain features of transformed cells, such as invasive behavior and overexpression of oncogenes. Susceptibility of FLS to poly(I:C)-induced apoptosis through a cell type-specific mechanism may open up novel therapeutic possibilities, aiming at the reduction of hyperplasia of the intimal lining in RA.

ACKNOWLEDGEMENTS

We thank the Host Defense and Rheumatology groups at our departments for sharing reagents and cells.
CHAPTER 4

REFERENCES


Figure S1. Constitutive and induced expression of RNA and dsDNA sensors in FLS. (A) Expression of RNA and dsDNA sensors in non-stimulated RA-derived FLS analyzed by PCR. (B) RA-derived FLS were stimulated for 16 h with 0.1 μg/ml poly(I:C) or 3pRNA, complexed with fugene (FG). Transcript levels of dsDNA sensors were quantified by qPCR, using 18S rRNA as reference gene (mean ± SEM, n=3-4).
Figure S2. siRNA- and shRNA-mediated knockdown of MDA5 in FLS does not abrogate the response to intracellular poly(I:C). (A–C) Knockdown of MDA5 with 25 nM (A, C) or 5 nM of siRNA (B) in RA-derived FLS was performed for 48 h, prior to stimulation of cells for 48 h (A, B) or 18 h (C) with the indicated concentrations of poly(I:C), complexed with fugene (FG). Annexin V+ and/or PI+ dead cells were quantified by flow cytometry (A, B; mean ± SEM, n=3). In cells treated with 25 nM siRNA, knockdown of MDA5 and expression of RIG-I and CXCL10 mRNA was analyzed by qPCR (C; mean ± SEM, n=3). (D, E) Knockdown of MDA5 with lentiviral shRNAs in RA-derived FLS was performed. Cells were stimulated for 18 h (D) or 24 h (E) with poly(I:C) complexed FG. Knockdown of MDA5 and expression of RIG-I and CXCL10 mRNA (D) and MDA5 and RIG-1 protein (E), the latter by Western blot, was analyzed. *, p<0.05
Figure S3. Double knockdown of adaptor proteins IPS and STING or IPS and TRIF, and inhibition of TBK1 does not abrogate the response of FLS to intracellular poly(I:C). (A, B) Knockdown of IPS and STING or IPS and TRIF with each 5 nM siRNA in RA-derived FLS was performed for 48 h, prior to stimulation of cells for 18 h with 0.1 μg/ml poly(I:C), complexed with fugene (FG). Knockdown of adaptor proteins and expression of MDA5 and CXCL10 mRNA was analyzed by qPCR (mean ± SEM, n=3). (C) RA-FLS were pretreated with the TBK1 inhibitor BX795 (200 nM). 1 h later, cells were stimulated for 4 h with poly(I:C), complexed FG. 10 μg/ml extracellular poly(I:C) was used as a positive control. Expression of IFNβ and CXCL10 mRNA was analyzed (mean ± SEM, n=3). *, p<0.05; **, p<0.01.
**Supplemental Table 1. Sequences of primers used for real-time qPCR.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA5</td>
<td>TGTTCTCGTCACCAATGAAA</td>
<td>CTCCTGAACCACGTGTGAGCA</td>
</tr>
<tr>
<td>RIG-I</td>
<td>ATCCCA GTGTA TGAACAGCGAG</td>
<td>GCCCTGTACTTATACCCAATGTC</td>
</tr>
<tr>
<td>IPS</td>
<td>TCAGGAGCAGCAGACAGACAC</td>
<td>TGGAAGGAGACAGAGATGAGAGA</td>
</tr>
<tr>
<td>STING</td>
<td>CCGGACACTTGAAGGACATC</td>
<td>TACATCGAGGTTCTGTGTA</td>
</tr>
<tr>
<td>TRIF</td>
<td>TATTCGGAGCAGGTCACAA</td>
<td>AGCGGTGTGTATTTGAGAG</td>
</tr>
<tr>
<td>TNF</td>
<td>CCCATGTGTGAACACACCTT</td>
<td>TGAGGTACAGGCCCCTGTGAT</td>
</tr>
<tr>
<td>IFNβ</td>
<td>TCCAAATTGGCTCCTCTGTG</td>
<td>GCAGTATTTGACGCTCCCAT</td>
</tr>
<tr>
<td>CXCL10</td>
<td>CAGACATCTCTTCTCCACCTCTT</td>
<td>TGAATATTTGCTGCAAGCCCA</td>
</tr>
<tr>
<td>TREX1</td>
<td>GACCCCTTCAGACAGTCCAA</td>
<td>GTGTGTGAGTGGCCCAAGTGT</td>
</tr>
<tr>
<td>LGP2</td>
<td>CAGGGGAACACGCAGTCAA</td>
<td>ATACCCCGAGGGCTGTGTA</td>
</tr>
<tr>
<td>LRRFIP1</td>
<td>CAGCTGGCAGATTGACGCG</td>
<td>TGTGGGCGTGTTTTTCCTTT</td>
</tr>
<tr>
<td>IFI16</td>
<td>GTCCGAGAAACAGAGCTCAAGC</td>
<td>CCACTTCTTTGGGTCTGCA</td>
</tr>
<tr>
<td>DDX41</td>
<td>GACTCATCCTGCTGCCCTCG</td>
<td>TGTTACACCTGCTGGATG</td>
</tr>
<tr>
<td>DHX36</td>
<td>TGTTCTGCTGCTTACCCACCAAGCAG</td>
<td>CTTTCCAGCACCCTCAAAGG</td>
</tr>
<tr>
<td>ZBP1</td>
<td>CACAAACGGGGAGAAAAGACAT</td>
<td>TTGAAGTGCTGTCTTTGCTCTCT</td>
</tr>
<tr>
<td>Ku70</td>
<td>TCGTGCAACTCCAGACAGC</td>
<td>AACTTTGGGCAATGTCAAGT</td>
</tr>
<tr>
<td>AIM2</td>
<td>ACCTCTTCAGAGGAGGAAGGA</td>
<td>GTTCAGGTTACATGGAGGAGAG</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>GGACAACAAGCTCCGTGAAAGA</td>
<td>CAGAAAGTGAAGGCCCTCTCA</td>
</tr>
</tbody>
</table>