Activation of interferon regulatory factor-3 via toll-like receptor 3 and immunomodulatory functions detected in A549 lung epithelial cells exposed to misplaced U1-snRNA

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ABSTRACT

U1-snRNA is an integral part of the U1 ribonucleoprotein pivotal for pre-mRNA splicing. Toll-like receptor (TLR) signaling has recently been associated with immunoregulatory capacities of U1-snRNA. Using lung A549 epithelial/carcinoma cells, we report for the first time on interferon regulatory factor (IRF)-3 activation initiated by endosomally delivered U1-snRNA. This was associated with expression of the IRF3-inducible genes interferon-β (IFN-β), CXCL10/IP-10 and indoleamine 2,3-dioxygenase. Mutational analysis of the U1-snRNA-activated IFN-β promoter confirmed the crucial role of the PRDIII element, previously proven pivotal for promoter activation by IRF3. Notably, expression of these parameters was suppressed by bafilomycin A1, an inhibitor of endosomal acidification, implicating endosomal TLR activation. Since resiquimod, an agonist of TLR7/8, failed to stimulate A549 cells, data suggest TLR3 to be of prime relevance for cellular activation. To assess the overall regulatory potential of U1-snRNA-activated epithelial cells on cytokine production, co-cultivation with peripheral blood mononuclear cells (PBMC) was performed. Interestingly, A549 cells activated by U1-snRNA reinforced phytohemagglutinin-induced interleukin-10 release by PBMC but suppressed that of tumor necrosis factor-α, indicating an anti-inflammatory potential of U1-snRNA. Since U1-snRNA is enriched in apoptotic bodies and epithelial cells are capable of performing efferocytosis, the present data in particular connect to immunobiological aspects of apoptosis at host/environment interfaces.

INTRODUCTION

With one million copies per nucleus U1-snRNA is the most abundant small nuclear RNA (snRNA) in eukaryotic cells. The highly conserved molecule is the defining component of the U1-ribonucleoprotein (RNP) that, by recognizing the 5′ splice site of precursor mRNA, plays a vital role in the fundamental task of RNA processing (1,2). The concept that U1 RNP, in particular U1-snRNA, may in addition exhibit immunomodulatory properties originates from observations that relate autoantibodies targeting U1-RNP to secretion of type I interferons (IFN) and the pathogenesis of systemic lupus erythematosus (SLE) (3–6). Specifically, U1-snRNA has recently been shown to induce release of type I IFN from plasmacytoid dendritic cells (4–6) as well as interleukin (IL)-6 and IL-8 from endometrial cells (7).

In context of SLE, it has been suggested that extranuclear U1-snRNA, in other words misplaced U1-snRNA, delivers its activating/regulatory signals to competent cells through endosomal uptake during phagocytosis of U1-RNP-containing immune complexes (4,6). A different scenario that may apply to diverse pathophysiological conditions is based on the observation that U1 RNP enriches in apoptotic bodies (8–13). Accordingly, it is reasonable to assume that uptake of apoptotic bodies during efferocytosis (14,15) likewise delivers U1-snRNA to the signaling machinery located at the endosomal compartment.

Composed of 164 nucleotides and characterized by stretches of double-stranded RNA (1), U1-snRNA is potentially detected by several sensors of innate immunity.
Endosomal Toll-like receptor (TLR)-3 and TLR7/8 recognize double- and single-stranded RNA, respectively (16–20). Those receptors have been previously associated with cellular activation by misplaced U1-snRNA (4,5,7). Endosomal delivery of U1-snRNP in fact mediates production of IFN-α by human peripheral blood mononuclear cells. Notably, pretreatment of U1-snRNP complexes with RNase A, but not with proteinase, nullified IFN-α release identifying U1-snRNA as the signaling principle (4). In addition to endosomal TLRs, protein kinase R (PKR) (17) as well as retinoic acid inducible gene-I (RIG-I) and melanoma differentiation-associated gene-5 (MDA5) are activated by RNA. However, it is unlikely that the latter two, both members of the family of RNA helicases (17,21), are involved in sensing misplaced U1-snRNA. Specifically, activation of RIG-I appears to be mediated primarily by RNA molecules containing a 5′-triphosphate moiety (22), which is uncommon to eukaryotic RNA, including U1-snRNA. Recent data moreover indicate that activation of MDA5 demands extended stretches of double-stranded RNA (23). Notably, these are substantially longer than those found in U1-snRNA.

Epithelial cells are a vital component of the innate immune system and determine course of diseases at host/environment interfaces (24,25). Notably, epithelial cells are also capable of executing the fundamental program of efferocytosis (14,15,26,27). Since current knowledge on activation of epithelial cells by misplaced U1-snRNA is merely fragmentary, we set out to systematically investigate effects of endosomally-delivered U1-snRNA on human A549 lung epithelial cells.

**MATERIALS AND METHODS**

**Materials**

Bafilomycin A₁ (Baf), phytohemagglutinin (PHA) and cycloheximide (CHX) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Recombinant flagellin (Flg, *Salmonella* munchen), resiquimod/R-848 (Rq) and lipopolysaccharide (LPS) (E. coli, serotype R515) were from Alexis Biochemicals (Lausen, Switzerland). Polyinosinic:polycytidylic acid [poly(I:C) or PIC] was purchased from Amersham Biosciences/GE Healthcare (Munich, Germany). Tumor necrosis factor-α (TNF-α) was kindly provided by the Knoll AG (Ludwigshafen, Germany). IL-1β was from Invitrogen/Biosource (Karlsruhe, Germany).

**Cultivation of A549 and DLD-1 cells**

Human A549 lung epithelial/carcinoma cells were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Cells were maintained using RPMI 1640, supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 10% heat-inactivated FCS and 10 mM HEPES buffer (GIBCO-BRL, Eggenstein, Germany). This same culture medium was used for experiments (except for cocultivation with PBMC, see below). Human DLD-1 colon epithelial/carcinoma cells were obtained from the Centre for Applied Microbiology & Research (Salisbury, United Kingdom).

For maintenance and experiments, cells were cultured in DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated FCS (GIBCO-BRL). For experiments, A549 or DLD-1 cells grown on 6-well polystyrene plates (Greiner, Frickenhausen, Germany) were incubated using the aforementioned culture media at ≈80% confluence. Cultivation of cells was performed at 37°C and 5% CO₂.

**Isolation of peripheral blood mononuclear cells (PBMC), standard cultivation and co-cultivation with A549 cells**

The study protocol and consent documents were approved by the ‘Ethik Kommission’ of the University Hospital Goethe-University Frankfurt. Informed consent was obtained from volunteers. Healthy donors had abstained from taking drugs for 2 weeks prior to the study. PBMC were freshly isolated from peripheral blood using Histopaque®-1077 (Sigma-Aldrich) according to the manufacturer’s instructions. For standard cultivation of PBMC, cells were resuspended in RPMI 1640 supplemented with 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin and 1% human serum (Invitrogen, Karlsruhe, Germany) and seeded at 3 × 10⁶ cells/ml in round-bottom polypropylene tubes (Greiner).

For subsequent cocultivation with PBMC, A549 cells at ≈80% confluence cultured in 6-well polystyrene plates (Costar, Bodenheim, Germany) were transfected with U1-snRNA as described below. After the 4 h transfection procedure and medium change to PBMC coculture medium (see below), 9 × 10⁶ PBMC seeded into Transwell-Clear inserts (0.4 μm pore size, Costar) were placed on top of the A549 cultures. Immediately before cocultivation, PBMC had been resuspended in PBMC coculture medium [RPMI 1640 supplemented with 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin and 3% heat-inactivated FCS (GIBCO-BRL)]. Cocultivation was performed for 72 h in a total volume of 3 ml of the aforementioned coculture medium.

**Cloning and preparation of human U1- and U2-snRNA by in vitro transcription**

The human U1-snRNA gene (V00591, nt 394–557) was cloned into the pCDNA3-vector (BglII, HindIII). A T7 promoter sequence was introduced directly in front of the human U1-snRNA gene. The following primers were used for cloning purposes: forward, 5'-TCAGATCTTTAT ACGACTCACTATAAGGGATACCTTACCTGGCAGGG G-3'; reverse, 5'-CTAAGCTTTCAGGGAAAGCGCGAG ACG-3'. The identity of the cloned fragment was confirmed by sequencing. U1-snRNA was transcribed using the MEGAscript T7 High Yield Transcription Kit (Ambion; Austin, TX, USA) according to manufacturer’s instructions. The DNA template was digested by DNase I provided with the kit. In order to eliminate 5′-triphosphate moieties from *in vitro* transcribed U1-snRNA that may artificially activate RIG-I (22), the molecule was treated with shrimp alkaline phosphatase (SAP; Promega, Mannheim, Germany). For that purpose, a protocol proven to effectively remove radiolabeled 5′-phosphate ends from *in vitro* transcribed U1-snRNA by at least
97% was used (data not shown). In an optimized protocol, 1 μg transcribed U1-snRNA was treated with 0.3 U SAP in presence of 4 U RNase inhibitor (Applied Biosystems, Darmstadt, Germany) at 37°C for 1 h. To inactivate SAP, samples were incubated at 65°C for 15 min. Subsequently, U1-snRNA was precipitated by phenol/chloroform extraction and isopropanol and washed with 70% ethanol. U1-snRNA was dissolved in nuclease- and pyrogen-free water. Size and integrity of U1-snRNA was visualized by gel electrophoresis and its concentration was determined by ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA). For cloning, in vitro transcription, and preparation of U2-snRNA (NR_002716, nt 1–187) the same methodology was used as for U1-snRNA (forward primer, 5'-TCAGATCTTTATCGACTGATTTAGG GATCGCTTCTCAGGCTTTGG-3'; reverse primer, 5'-C TAAAGCTTTGCTACCCGTGGAGG-3'). The identity of the cloned U2-snRNA fragment was confirmed by sequencing. U1- and U2-snRNA preparations were aliquoted and stored at −80°C.

**Stimulation with U1-, U2-snRNA, or poly(I:C) by transfection**

For stimulation by endosomal delivery (28,29) of U1-, U2-snRNA (see Figure 9), or poly(I:C), A549 cells (or DLD-1 cells, see Figure 7) were transfected as described below by using the cationic liposome lipofectamine-2000 (Invitrogen). To control for hypothetical RIG-I activation by remnant uncleaved 5'-triphosphate ends (22) within SAP-treated U1/U2-snRNA preparations, an additional ‘U1/U2-snRNA control’ (U1/U2ctr) was performed. This U1/U2ctr consists of in vitro transcribed U1/U2-snRNA containing 5'-triphosphate ends (no SAP treatment) at a final concentration of 3% (see above) of the SAP-treated U1/U2-snRNA amount used for stimulation of cells. U1/U2ctr thus controls for residual 5'-triphosphate ends in the SAP-treated U1/U2-snRNA preparation. For transfection, U1-snRNA, U1ctr, U2-snRNA, U2ctr or poly(I:C) were pre-incubated with 4 μl lipofectamine-2000 per sample for 20 min in order to allow formation of nucleic acid/lipofectamine-2000 complexes. For stimulation purposes, complexes were then added to cells under serum-free conditions. Control cells were exposed to lipofectamine-2000 in the absence of nucleic acids (indicated as mock transfection). Unless otherwise indicated, supernatants containing lipofectamine-2000 were removed after 4 h. Thereafter, cells were washed twice with PBS and 2 ml of fresh culture medium (containing FCS) was added. Incubation periods indicated throughout the study are understood as being started at the time of lipofectamine-2000 addition to cells. By using 32P-labeled U1-snRNA (see below), the fraction of U1-snRNA actually taken up by A549 cells during this 4 h transfection procedure was determined to be 65.7% ± 7.2%. We also like to point out that the amount of U1-snRNA on average delivered into a single cell by the current transfection protocol can be regarded as roughly in range of the endogenous molecule. Based on the molar mass of U1-snRNA and the presence of ≥10^6 molecules per cell (1) the amount of endogenous U1-snRNA per cell can be estimated as ≈93 fg. Based on the transfection efficiency of the protocol described above and the transfected cell numbers, we deduce the transfected amount of U1-snRNA to be 86 fg per cell (for transfection of 0.1 μg/ml U1-snRNA).

In addition to immunoregulatory properties, TLR3/IRF3-dependent signaling appears to be capable of promoting apoptosis under certain conditions (30–32). Twenty four hours after U1-snRNA transfection, negligible cytotoxicity was evident compared to mock-transfection in A549 cell cultures as detected by light microscopy (Figure 1C, inset). Quantification of cell layer total protein content (determined as a measure of cell detachment resulting from apoptotic cell death) showed a slight tendency towards decrease in A549 cell cultures exposed to U1-snRNA for 24 h [89.2% ± 11.1% total protein for exposure to U1-snRNA (0.1 μg/ml) compared to mock-transfected control set as 100%, n = 9]. A549 cell viability as detected by the WST-1 assay (Roche Diagnostics, Mannheim, Germany) showed a similar tendency after a 24 h exposure to U1-snRNA at 0.1 μg/ml (84.4% ± 15.6% viability for exposure to U1-snRNA compared to mock-transfected control set as 100%, n = 9). This tendency to slightly reduced viability resulting from extended activation by U1-snRNA did not reach statistical significance in both assays performed (analyzed by unpaired Student’s t-test on raw data).

**Analysis of in vitro transcribed and transfected U1-snRNA integrity**

U1-snRNA was synthesized in vitro using T7 RNA polymerase (as described above) in the presence of [γ-32P]UTP (800 Ci/mmol; Perkin Elmer, Rodgau, Germany). After SAP-treatment, A549 cells were transfected (as described above) with 32P-labeled U1-snRNA. In order to proof integrity of U1-snRNA, 1000 cpm of in vitro transcribed U1-snRNA (not transfected into cells) and 1000 cpm of total cellular RNA isolated from U1-snRNA transfected cells (after 4 h) were separated on a 5% polyacrylamide/8 M urea gel and analyzed by PhosphoImager (Fuji, Straubenhardt, Germany). One thousand cpm of a 32P-labeled glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) probe (184 nt) served as size-control (U1-snRNA: 164 nt).

**Digestion of U1-snRNA by benzonase and RNases A and T1**

U1-snRNA was exposed to benzonase (Novagen/Merck Chemicals, Nottingham, UK) or DNase-free RNases A/T1 (Roche Diagnostics) before transfection into cells. The used benzolase is a genetically engineered Serratia marcescens endonuclease digesting both, single- and double-stranded RNA. According to the manufacturer’s instructions, 0.1 μg U1-snRNA was incubated with 25 U benzonase at 37°C for 1 h. For digestion of U1-snRNA by RNases, the molecule was heated to 95°C for 5 min followed by addition of RNase A/T1 and digestion at 30°C for 1 h. The quality of U1-snRNA digestion was verified by gel electrophoresis.
Detection of mRNA by standard polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated using TRI-Reagent (Sigma-Aldrich) according to the manufacturer’s instructions. As IFN-β mRNA only consists of one single exon, it was impossible to design exon-spanning primers that exclude amplification of potentially contaminating genomic DNA. Therefore, all RNA isolates were first digested with RNase-free DNase I (Roche Diagnostics) according to the manufacturer’s instructions. After precipitation with 70% ethanol, 1 µg of total RNA was transcribed using random hexamer primers and Moloney virus reverse transcriptase (RT; Applied Biosystems) according to the manufacturer’s instructions. The following sequences were performed for PCR: 94°C for 10 min (1 cycle); 94°C for 1 min, 60°C (GAPDH, interferon-IFN)-β, PKR, toll-like receptor (TLR)-7, TLR8, RIG-I, 59°C (TLR5), 58°C [indoleamine 2,3-dioxygenase (IDO), CXCL10/IP-10], 56°C (TLR4), 55°C (MDA-5) or 47°C (TLR3) for 1 min and 72°C for 1 min (with the indicated numbers of cycles); final extension phase at 72°C for 7 min. Primer sequences and length of resulting amplicons: GAPDH (F): 5'-ACCACAGTC CATGCCCATC-3', GAPDH (R): 5'-TCCACACCCT GTTGCTGT-3', 452 bp, 24 cycles; IDO (F): 5'-GAT CCAAATAGGCCCTGACT-3', IDO (R): 5'-CAGCAT GTCTCCACCAAG-3', 454 bp, 31–33 cycles; IFN-β (F): 5'-AGCTGAGCGTTCCTCAAGAAG-3', IFN-β (R): 5'-A GTCTCATCTCCAGCAGTG-3', 110 bp, 30–32 cycles; TLR8 (F): 5'-CCTTGCTAACTGCTTTCAG-3', TLR8 (R): 5'-AGCTGCAGCAGTTCCAGAAG-3', 222 bp, 30–33 cycles; PKR (F): 5'-GGCCAGAGCAGATTTTGTCG-3', PKR (R): 5'-TATCTGAGATGATCGCTCCC-3', 150 bp, 39 cycles; TLR3 (F): 5'-GTCATCCAACAGAATCAT-3', TLR3 (R): 5'-GATTAATTCCTTCGCTT-3', 466 bp, 39 cycles; TLR4 (F): 5'-ATGGAGCTGAATTTC TACAAAATCC-3', TLR4 (R): 5'-GCTTCTGTAACACT TGAATGTC-3', 271 bp, 35 cycles; TLR5 (F): 5'-CTA GAAGTCTCCCTGCTTACTAGA-3', TLR5 (R): 5'-AA GGGAAGAGTAAAGCAGT-3', 226 bp, 35 cycles; TLR7 (F): 5'-GGATGGAAACACAGCTACTAGA-3', TLR7 (R): 5'-TAGGGACGCGCTGACATTG-3', 260 bp, 39 cycles; TLR8 (F): 5'-CAGAATAGCAGGCAA CATACA-3', TLR8 (R): 5'-AGTGCAAGTCGTC ATTCAAAAAGG-3', 625 bp, 39 cycles; RIG-I (F): 5'-GAGCA GGAGATTCGGAAGA-3', RIG-I (R): 5'-AGAGGGACGAGCTCTACCTGTCGTCG-3', 477 bp, 39 cycles; MDA5 (F): 5'-CAGAAAGAGCTGGTCTGCT-3', MDA5 (R): 5'-GTGCTGATCTCCAAACTACA-3', 335 bp, 39 cycles. Identity of amplicons was confirmed by sequencing (310 Genetic Analyzer, Applied Biosystems). Note the white banding pattern seen on agarose gels (Figures 1AB, 2AB, 3C, 4AE and 7B) is due to the loading dye.

Analysis of IL-18 binding protein (IL-18BP) mRNA by quantitative realtime PCR

During realtime PCR, changes in fluorescence were caused by the Taq-polymerase degrading the probe that contains a fluorescent dye (FAM used for IL-18BP, VIC for GAPDH) and a quencher (TAMRA). Primers and probe for IL-18BP were designed using Primer Express (Applied Biosystems) according to AF110798: forward, 5'-ACCTCCACCCGGCAGCTG-3'; reverse, 5'-CTCTGGCACAGCTGCTACC-3'; probe, 5'-CACCACGCGGGA ACGTGGGA-3'. Amplification of contaminating genomic DNA was avoided by selecting an amplicon that crosses an exon/intron boundary. For GAPDH (4310884E), pre-developed assay reagents were used (Applied Biosystems). Quantitative realtime PCR of cDNA was performed on 7500 FAST Realtime PCR System (Applied Biosystems): One initial step at 95°C for 20 s was followed by 40 cycles at 95°C for 3 s and 60°C for 30 s. Detection, calculation of threshold cycles (Ct values), and data analysis was performed by Sequence Detector software. mRNA was quantified by use of cloned cDNA standards for IL-18BP and GAPDH, respectively. IL-18BP mRNA expression was normalized to that of GAPDH.

Detection of phosphorylated interferon regulatory factor (pIRF)-3, phosphorylated signal transducer and activator of transcription (pSTAT)-1, pSTAT3, phosphorylated eukaryotic initiation factor 2 (p-eIF2a)-z, IRF7, p56 and IDO by immunoblot analysis

Total lysates with intracellular proteins were obtained by treatment of cells with lysis buffer (150 mM NaCl, 1 mM CaCl2, 25 mM Tris–Cl, pH 7.4) containing 1% Triton-X-100, protease inhibitor cocktail (Roche), DTT, Na3VO4, PMSF (each 1 mM), and NaF (20 mM). Where indicated (see Figure 6D), nuclear lysates were isolated as previously described (33) and used to detect nuclear p65, IRF7 and pIRF3. Proteins were separated on 10% SDS–PAGE gels and transferred onto a Hybond-C Extramembrane (GE Healthcare, Freiburg, Germany). pSTAT1 and pIRF3 were detected by primary rabbit polyclonal antibodies whereas p-eIF2a and pSTAT3 were detected by primary rabbit monoclonal antibodies (Cell Signaling, Frankfurt, Germany). IDO and IFN-IF7 were detected by primary mouse monoclonal antibodies purchased from Millipore (Schwalbach, Germany) and Santa Cruz Biotechnology Inc. (Heidelberg, Germany), respectively. p56 was detected using a goat polyclonal antibody obtained from Santa Cruz Biotechnology Inc. Detection was achieved by horseradish peroxidase-labeled secondary antibodies (Bio-Rad, Munich, Germany), and a chemiluminescence detection kit (GE Healthcare) according to manufacturer’s instructions.

For determination of transcription factor activation (pSTAT1, pSTAT3, pIRF3, p65, IRF7), cells were stimulated with U1-snRNA as described above. However, in that case, nuclear acid/lysozyme-2000 complexes were not removed after four hours. Instead, protein lysates were generated for up to 6 h after onset of transfection for time-course analyses.

Detection of TLR3 by immunofluorescence

A549 cells were seeded on cover slips in 24-well plates. For analysis, cells were washed twice with PBS and fixed in ice-cold methanol for 20 min. After two washes with
PBS, non-specific binding sites were blocked using 0.1% Triton-X-100/1% BSA (Sigma-Aldrich) in PBS for 30 min at room temperature. Primary and the secondary antibodies were diluted in this buffer. For staining, cells were incubated with a murine monoclonal anti-human TLR3 (BioLegend, San Diego, CA, USA) antibody for 1 h at room temperature. After two washes with PBS, cells were incubated with an anti-mouse secondary antibody (Cy3, Sigma-Aldrich) for 30 min at room temperature. After two washes, PBS washes nuclei were stained with DAPI and washed thrice with PBS. Finally, samples were covered with fluoromount (Biozol, Eching, Germany) and analyzed using a confocal fluorescence microscope (LSM 510 Meta, Zeiss, Jena, Germany).

Luciferase reporter assays
An IFN-β promoter fragment was cloned into pGL3-Basic (Promega, Mannheim, Germany) and entitled pGL3-IFNB. Position of cloned DNA: nt −1 to nt −282 (282 nt). The following primers were used: (F): 5'-GACT CAGAATTCTCAGG1CGTTTC-3', (R): 5'-GAAAG CTTCCCTTCTCCATGGAATG-3'. In order to blunt the pIRES binding site (PRDII region) in the IFN-β promoter (34), site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene, Amsterdam, Netherlands). The following primer was used: pGL3-mutIFNB: 5'-ATGAAATGACATAGGA TTCTCTTTTTCTCCATGGAATG3'. Identity of mutants was confirmed by sequencing. For each reaction conducted in 6-well plates 0.25 or 1 µg (as indicated) of the respective plasmids was transfected into A549 cells using lipofectamine-2000™ (Invitrogen) according to manufacturer's instructions. As a control for transfection efficiency 0.1 µg pRL-TK (Promega) coding for Renilla luciferase was cotransfected. After 4 h of transfection, culture medium was changed and cells were rested for 20 h. Thereafter, cells were transfected with U1-snRNA as described above. After a further 24 h stimulation period, A549 cells were harvested and luciferase activity was determined using the dual reporter gene system (Promega) and an automated chemoluminescence detector (Berthold, Bad Wildbad, Germany) according to manufacturers’ instructions. For analysis, luciferase activity was normalized to that of Renilla-TK. For data analyses, unstimulated control of the same transfected plasmid was set as 1.

Detection of IFN-γ, IL-10, CXCL10/IP-10, TNF-α and IFN-β by enzyme linked immunosorbent assay (ELISA)
Concentrations of IFN-γ, TNF-α, IP-10/CXCL10 (Pharmingen/BD Biosciences, Heidelberg, Germany), IL-10 (Diaclone/Hölz Diagnostics, Cologne, Germany), IFN-β (PBL InterferonSource/R&D Systems, Wiesbaden Germany) in cell-free cell culture supernatants were determined by ELISA according to the manufacturers’ instructions.

Statistics
Data are shown as mean ± SD (for A549 cells and for DLD-1) or as mean ± SEM (for PBMC). Data are presented as pg/ml, or fold-induction. If not stated otherwise, raw data (A549 cells, DLD-1 cells and PBMC) were analyzed by one-way ANOVA with post-hoc Bonferroni correction (GraphPad 4.0).

RESULTS
IFN-β, IDO and IP-10 gene expression and activation of IRF3 by misplaced U1-snRNA
Activation of innate immunity by RNA converges at initiation of IRF3/7 signaling, a process that is supposed to be accomplished mainly by action of RIG-I-like receptors and by endosomal TLRs, specifically TLR3, TLR7 and TLR8 (16–21). In order to characterize activation of A549 cells by misplaced U1-snRNA, endosomal delivery was achieved by transfection (28,29). Thereafter, expression of prototypic IFR3-dependent genes was determined, namely IFN-β, IDO and IP-10 (35–38). As shown in Figure 1, mRNA expression of all three genes was induced by U1-snRNA in dose- (A) and time-dependent (B) manner. mRNA induction translated into secretion of IFN-β (C) and IP-10 (D) as well as expression of cellular IDO protein (E). Notably, U1ctr (see ‘Materials and Methods’ section) was unable to mediate significant induction of any of these three parameters (Figure 1BCD) indicating that residual RIG-I activating 5’triphosphates hypothetically present in in vitro-transcribed and SAP-treated U1-snRNA preparations are negligible in this experimental setup. As expected, induction of these genes was paralleled by robust phosphorylation/activation of IRF3 which was obviously absent in the U1ctr stimulatory control (Figure 1F). Sensing of U1-snRNA might also result in activation of IRF3-related IRF7, targeting the same ISRE DNA element, alike all IRFs (17,18,20). However, IRF7 activation by exposure of A549 cells to U1-snRNA (6 h) was barely, if at all, detectable and thus must be regarded as of minor relevance for direct action of U1-snRNA compared to the strong, robust, and fast activation of IRF3 in these same experiments (data not shown). IRF5 is another IRF family member also capable of inducing IRF3 by the RNA sensors RIG-I or TLR7/8. Characteristically, IRF5 is not activated by U1-snRNA compared to the strong, robust, and fast activation of IRF3 in these same experiments (data not shown). IRF5 is another IRF family member also capable of inducing IFN-β after its activation by the RNA sensors RIG-I or TLR7/8. Characteristically, IRF5 is not activated by U1-snRNA (20,39). Since RIG-I and TLR7/8 are unlikely to mediate activation of A549 cells by misplaced U1-snRNA (see below), a role for IRF5 in this context can be excluded.

In order to prove that intact U1-snRNA is actually responsible for activation of A549 cells under those conditions, RNA was degraded by pre-incubation with RNase A/T1 (Figure 2AC) or benzonase (Figure 2BD). Figure 2 demonstrates that RNA degradation nullified U1-snRNA stimulatory activity detected by IDO and IFN-β mRNA expression (AB) and phosphorylation of IRF3 (CD). Notably, activation of A549 cells by U1-snRNA was absent in cells exposed to U1-snRNA without transfection as detected by IRF3 phosphorylation (Figure 2E) and induction of IDO and IFNβ (data not shown). Integrity of transfected radiolabeled U1-snRNA was analyzed by gel electrophoresis. Figure 2F demonstrates that no significant degradation of U1-snRNA was detectable 4 h after its transfection into A549 cells.
Activation of A549 cells under the influence of misplaced U1-snRNA is suppressed by pre-incubation with bafilomycin A1, is not mediated by PKR, and cannot be mimicked by the TLR7/8 ligand resiquimod/R-848

In order to gain insight into the RNA sensing machinery potentially available in A549 cells, PCR analysis was performed and proved mRNA expression of TLR3, TLR7, TLR8, PKR, RIG-I and MDA5 in this cell type. In addition, mRNA expression of TLR4 and TLR5 is shown (Figure 3A). Consequently, we sought to assess the contribution of these sensors concerning cellular activation by U1-snRNA. As already alluded to, MDA5 is supposed to be activated by extended stretches of double-stranded RNA (23) and thus appears unlikely to contribute to U1-snRNA immunoregulatory activity.

Bafilomycin A1 is a highly specific pharmacological inhibitor of the vacuolar-type H⁺-ATPase thereby potently blocking endosomal acidification and subsequent endosomal TLR signaling. Accordingly, this compound has been successfully employed to identify activation of endosomal TLR signaling (40–43). As shown in Figure 3B, pre-incubation with bafilomycin A1 entirely prevented U1-snRNA-induced activation of IRF3. In contrast, phosphorylation of IRF3 was not influenced by blockage of translation using cycloheximide, which is...
in accord with direct action of RNA sensors on IRF3 function. Impaired IRF3 signaling was associated with striking reduction of IDO, IFN-\(\beta\) and IP-10 mRNA expression (Figure 3C) and potent suppression of IFN-\(\beta\) (Figure 3D) and IP-10 (Figure 3E) secretion. These data indicate that endosomal TLR signaling likely plays a key role for cellular activation under the influence of misplaced U1-snRNA. In a further attempt to characterize mechanisms driving stimulatory properties of U1-snRNA, expression of IDO and IFN-\(\beta\) mRNA was investigated in response to TLR7/8 activation. For that purpose A549 cells were treated with U1-snRNA or resiquimod/R-848, a membrane permeable agent activating TLR7/TLR8 (44), as well as LPS (activating TLR4) and flagellin (activating TLR5) by using the standard protocol for transfection of U1-snRNA. As shown in Figure 4A, neither LPS nor flagellin nor resiquimod were able to mediate expression of IDO and IFN-\(\beta\) under those experimental conditions. Analogous data were obtained with regard to activation of IRF3 (Figure 4B). In contrast to U1-snRNA, R-848 is membrane permeable and thus supposed to reach TLR7/8 in the endosomal compartment.

Figure 2. Biological activity of U1-snRNA is blunted by digestion using RNases A/T1 or benzonase and is dependent on U1-snRNA transfection. U1-snRNA was digested by RNases A/T1 (A and C) or benzonase (B and D) as outlined in the 'Materials and Methods' section. A549 cells were either kept as mock-transfected control or stimulated with intact or digested U1-snRNA (each at 0.1 \(\mu\)g/ml). (A and B) After 24 h, cells were harvested and mRNA coding for IDO and IFN-\(\beta\) was determined by RT-PCR. For each experimental setup, one representative of five independently performed experiments is shown. (C and D) After 6 h, cells were harvested and cellular pIRF3 content was determined by immunoblot analysis. For each experimental setup, one representative of three independently performed experiments is shown. (E) A549 cells were kept as unstimulated control or exposed to U1-snRNA (0.1 \(\mu\)g/ml) without transfection. In addition, cells were either kept as mock-transfected control or stimulated by standard transfection with U1-snRNA (0.1 \(\mu\)g/ml). After 2 h, cells were harvested and cellular pIRF3 content was determined by immunoblot analysis. For each experimental setup, one representative of three independently performed experiments is shown. (F) \(^{32}\)P-labeled U1-snRNA (0.1 \(\mu\)g/ml) was transfected by standard protocol into A549 cells. After 4 h, total cellular RNA was isolated. Thereafter, integrity of transfected U1-snRNA was assessed by gel electrophoresis and subsequent analysis using a PhosphoImager. Left lane (ivt), 1000 cpm of \textit{in vitro} transcribed \(^{32}\)P-labeled U1-snRNA (not transfected into cells); middle lane, 1000 cpm of total RNA isolated 4 h after transfection of \(^{32}\)P-labeled U1-snRNA into A549 cells; right lane \(^{32}\)P-labeled GAPDH probe (184 nt) serving as size-control (U1-snRNA: 164 nt).
without the need of transfection by liposomes. Notably, alike U1-snRNA, R-848 failed to mediate IRF3 activation as well as IDO and IFN-β expression when exposed to cells directly without transfection (data not shown). R-848 was however active on human PBMC that were stimulated with the compound as positive control for its biological activity. In fact, we confirmed previous data (45) on robust activation of PBMC by R-848 (10 μM) as detected by IFN-γ secretion (63.5 ± 42.1 pg/ml for unstimulated control versus 16 520 ± 2848 pg/ml for R-848 activation of PBMC, 24 h incubation, n = 3, p < 0.01 analyzed by unpaired Student’s t-test on raw data). These results argue against a function of endosomal TLR7/8 yet substantiating a key role of TLR3 recognizing double-stranded RNA for activation of A549 cells by misplaced U1-snRNA. As a further negative control, cells were transfected with total human RNA (derived from A549 cells). As shown in Figure 4AB, transfection of total RNA was unable to activate A549 cells as detected by IDO or IFN-β mRNA expression and IRF3 phosphorylation, respectively. These data agree with a recent report indicating that human total RNA preparations are unable to mediate endosomal TLR activation, specifically concerning the RNA sensors TLR3 and TLR7/8 (46).

Since data presented herein supported involvement of TLR3 in activation of A549 cells by misplaced U1-snRNA, immunofluorescence analysis visualizing TLR3 in A549 cells was performed (Figure 4C). In accord with previous data (47), confocal microscopy revealed that TLR3 primarily displays intracellular location with an obvious granular staining pattern that furthermore confirms its vesicular occurrence. Notably, staining of TLR3 at the membrane was not detected and agrees with the observation that exposure to U1-snRNA without liposome-aided endosomal delivery failed to activate IRF3 (Figure 2E) as well as IDO and IFN-β expression (data not shown) in A549 cells.

To assess the role of PKR in U1-snRNA-induced activation of A549 cells, stimulation of this kinase was verified by analysis of eIF2α phosphorylation, a well-known PKR primary substrate (17). Interestingly, activation of A549 cells by misplaced U1-snRNA did not result in...
phosphorylation of eIF2α (Figure 4D). As a positive control, cells were exposed to poly(I-C) (PIC). Poly(I-C) is supposed to activate both, PKR (17) and TLR3 (17,19). As expected, transfection of poly(I-C) not only stimulated PKR, as detected by eIF2α phosphorylation, but moreover induced activation of IRF3 as well as expression of IDO and IFN-β mRNA (Figure 4E). These data indicate that PKR is unlikely to be involved in activation of A549 cells under the influence of U1-snRNA.

**Induction of IFN-β by U1-snRNA is dependent on the ISRE-like PRDIII IFN-β promoter element**

In order to substantiate that misplaced U1-snRNA is in fact able to mediate gene expression by activating the IRF pathway, IFN-β promoter analysis was performed. As shown in Figure 5 and in accordance with aforementioned data on IFN-β mRNA expression and protein secretion, misplaced U1-snRNA upregulated IFN-β promoter activity which was suppressed by pre-incubation of cells with bafilomycin A1 (A). Notably, mutation of the PRDIII element, an ISRE-like site previously shown as crucial for induction by IRF3 (34), suppressed activation of the IFN-β promoter by U1-snRNA (Figure 5B). These results demonstrate that misplaced U1-snRNA mediates IFN-β production by action on the PRDIII promoter element, most likely via IRF3.

**Induction of STAT signaling under the influence of misplaced U1-snRNA**

In order to assess activation of the STAT-signaling pathway by U1-snRNA in A549 cells, immunoblot analysis of phosphorylated STAT molecules was performed.
As shown in Figure 6, STAT1 (A) and STAT3 (B) signaling was engaged by misplaced U1-snRNA. Interestingly, compared to IRF3, activation of both parameters was delayed by at least 1 h. Recently, we could demonstrate that activation of STAT1 holds a pivotal position concerning induction of the prototypic IFN-γ-inducible gene IL-18BP (48,49). Accordingly, misplaced U1-snRNA mediated significant upregulation of IL-18BP indicative of enhanced biological activity of STAT1 under those conditions (Figure 6C). In contrast to STAT1, activation of nuclear factor-κB (NF-κB), as detected by p65 translocation to the nucleus, was not obvious after 2 h of exposure to U1-snRNA. As expected, p65 translocation was achieved by the combination of IL-1β and TNFα serving as positive control for NF-κB and negative control for IRF3 dependent signaling. Those data also demonstrate translocation of pIRF3 to the nuclear compartment under the influence of U1-snRNA (Figure 6D).

**Activation of DLD-1 colon epithelial/carcinoma cells by misplaced U1-snRNA**

In order to demonstrate that activation by misplaced U1-snRNA can be observed in different cell types, key findings detected in A549 cells were verified in DLD-1 cells. As shown in Figure 7, misplaced U1-snRNA in fact was able to mediate activation of IRF3 (A), which was associated with expression of IDO (BC), IFN-β (B), and IP-10 (DE). In accord with data on A549 cells, U1-snRNA effects, as detected by IRF3 activation and IP-10 release, were potently suppressed in the presence of bafilomycin A1 (Figure 7AE) or after pre-treatment with benzonase (Figure 7D).

**Misplaced U1-snRNA mediates anti-inflammatory effects as detected in A549 cell/PBMC co-culture experiments**

In order to assess the overall regulatory potential of U1-snRNA-activated A549 cells on inflammatory cytokine production, co-cultivation with freshly isolated human PBMC was performed in presence and absence of PHA. Notably, pre-activation of A549 cells by U1-snRNA potently enhanced release of IL-10 by PBMC under the influence of PHA (Figure 8A). In contrast, PHA-induced release of TNFα was significantly suppressed by misplaced U1-snRNA (Figure 8B), implying an anti-inflammatory effect mediated by U1-snRNA. In the absence of PHA, IL-10 and TNFα remained undetectable in co-cultures (Figure 8AB). Notably, when cultured without PBMC, A549 cells exposed to misplaced U1-snRNA did not display secretion of IL-10, irrespective of the presence or absence of PHA (data not shown).
Activation of A549 cells by misplaced U2-snRNA

U2-snRNA, in similarity to U1-snRNA, consists of several stretches of double-stranded RNA (50). Thus, for proof of principle and further control, cellular response to U2-snRNA transfection was investigated herein. Notably, as detected by IRF3 activation, misplaced U2- and U1-snRNA showed similar stimulatory activity (Figure 9).

DISCUSSION

Using human lung A549 epithelial cells we demonstrate for the first time that endosomal delivery of U1-snRNA mediates activation of the transcription factor IRF3, a process associated with expression of IRF3-inducible immunoregulatory parameters such as IFN-β, IDO and IP-10/CXCL10. We also detected activation of A549 cells by misplaced U2- and U1-snRNA showed similar stimulatory activity (Figure 9).

whereas accumulation of U2-snRNA in apoptotic bodies has never been reported (8–13). As already alluded to in the ‘Materials and Methods’ section, we like to emphasize that, on a cellular basis, the amount of U1-snRNA delivered per A549 cell (for transfection of 0.1 μg/ml) is approximately in the range of the endogenous U1-snRNA pool. Mutational analysis of the IFN-β promoter furthermore demonstrated that induction of the gene was most likely mediated via IRF3. Several lines of evidence indicate that TLR3 sensing double-stranded RNA likely plays a crucial role for cellular activation in the context of misplaced U1-snRNA. First, gene expression of IFN-β, IDO and IP-10/CXCL10 was suppressed by pre-incubation of cells with bafilomycin A 1 suggesting that an endosomal RNA sensing principle is key to activation by U1-snRNA. Among those, TLR7/8 sensing single-stranded RNAs are obvious candidates (52). However, the TLR7/8 ligand resiquimod/R-848 failed to mimic U1-snRNA action on A549 cells thus precluding a major function of this receptor complex. A recent report actually suggests a minor role for TLR3 sensing double-stranded RNA compared to U1-snRNA action on A549 cells thus precluding a major function of this receptor complex. A recent report actually suggests a minor role for TLR3 sensing double-stranded RNA compared to U1-snRNA action on A549 cells thus precluding a major function of this receptor complex. A recent report actually suggests a minor role for TLR3 sensing double-stranded RNA compared to U1-snRNA action on A549 cells thus precluding a major function of this receptor complex.
single-stranded segment at the very 5'-end of U1-snRNA that is in fact specifically cut off during apoptosis (54). The remaining 2'-O-methylated nucleoside in U1-snRNA is a modified adenine located in the single-stranded loop B of U1-snRNA (53,55). Collectively, these data imply that at least two of the single-stranded segments of endogenous U1-snRNA are unable to activate TLR7/8 due to nucleoside modifications. Cellular activation by U1-snRNA was furthermore independent of 5'-triphosphate ends. Consequently, a role for cytosolic RIG-I in detecting U1-snRNA appears unlikely (22). These observations were complemented by lack of PKR activation as detected by eIF2α phosphorylation (17) and thus altogether suggest a prominent role for TLR3 in the cellular response towards misplaced U1-snRNA. In this context, it is noteworthy that TLR3 activation does not exclusively rely on uninterrupted double-stranded RNA but apparently tolerates mismatches (56). Thus, according to their structures (57) U1-snRNA [at stem-loop I and III: each 9 bp, at stem-loop II: 5 and 8 bp, at stem-loop IV: 6 bp (+3 bp)] and U2-snRNA [at stem-loop IV: 11 bp, at stem-loop III: 8 bp (+4 bp)] show regions with double-stranded RNA that may be able to fold into a three-dimensional fit recognized by TLR3. Recent analysis using synthetic RNA duplexes revealed that 21 bp are sufficient for activation of TLR3 (58). Notably, this rigid model does not necessarily mirror activation of TLR3 by endogenous RNA populations.

Compared to IRF3, activation of STAT1/3 was delayed and possibly related to production of intermediate factors including IFN-β (59,60). In concurrence with activation of STAT1 signaling, induction of anti-inflammatory IL-18BP (48,49) was evident under the influence of U1-snRNA. As a negative control, A549 cells were transfected with human total RNA, a regime which in agreement with a previous report (46) failed to show stimulatory capabilities. As already alluded to, extensive nucleoside modifications impair recognition of endogenous mammalian...
RNA by the TLR system. Precisely, the presence of N6-methyladenosine and 2-thiouridine prevents activation of TLR3 by endogenous RNA populations (46). Notably, U1-snRNA neither contains N6-methyladenosine nor 2-thiouridine, the latter occurring uniquely in tRNA (61,62). As mentioned before, 2'-O-methylated nucleosides have been associated with suppression of TLR7/8 activation by single-stranded RNA (46,53). Effects of 2'-O-methylated nucleosides on TLR3 activation have, to the best of our knowledge, not been specifically addressed. However, since the three 2'-O-methylated nucleosides of endogenous U1-snRNA are located in single-stranded regions of the molecule (at the very 5'-end and in loop B) (53,55) it appears highly unlikely that those are able to interfere with TLR3 activation. Thus, endogenous U1-snRNA is supposed to be capable of activating TLR3.

Immunoregulatory effects of U1-snRNA were not restricted to lung A549 cells but were also observed in DLD-1 colon carcinoma cells. Activation of IRF3 as well expression of IDO, IFNβ and IP-10 were ditto hallmarks of activation in DLD-1 cells clearly demonstrating that this pathway also applies to cultured colon carcinoma cells. However, it became apparent that, compared to A549 cells, DLD-1 cells reacted somewhat less towards transfection with U1-snRNA. It might be that uptake of liposomes by DLD-1 cells is less effective. Notably, TLR3 is detectable in DLD-1 cells (data not shown). However, it might also be that TLR3 expression levels or its functionality are diminished in DLD-1 cells, compared to A549 cells. In fact, moderate/controlled responsiveness towards TLR ligands might be key to epithelial biology in the gut, an environment constantly exposed to myriads of microbes and their debris. The basis for this interesting difference in sensitivity between both cell types is currently under investigation.

Since U1-snRNA enriches together with U1-RNP in apoptotic bodies (8–13), the pathophysiological context of the current study is in particular related to the immunobiology of apoptosis. Intracellular degradation of engulfed apoptotic cells/bodies is linked to the endosomal pathway (63), a compartment supposed to harbor TLR3 expression (19,52). In agreement with previous data (47), we demonstrate intracellular but not membrane expression of TLR3 in A549 cells. Endosomal delivery of U1-snRNA by cationic liposomes was used to investigate immunoregulation by U1-snRNA in relation to phagocytosis. In fact, silent phagocytosis of apoptotic cells/bodies has been linked to immunosuppression and inhibition of inflammation (14,15,64,65). Interestingly, among the IRF3-inducible parameters upregulated by U1-snRNA were IDO and IFN-β. IDO is regarded a most relevant mediator of immunosuppression (66). Although the role of type I interferons in inflammation is not discussed uniformly (67), anti-inflammatory/therapeutic properties of IFNβ have been identified in the context of rheumatoid arthritis (68), inflammatory

Figure 8. Misplaced U1-snRNA mediates anti-inflammatory effects as detected in A549 cell/PBMC co-culture experiments. A549 cells were either kept as mock-transfected control or stimulated with U1-snRNA (0.1 μg/ml) or U1 ctr (0.003 μg/ml), respectively. After removal of liposomes at 4 h, PBMC were seeded into transwell inserts (9 x 10⁶ cells per insert) and placed on top A549 cells for co-culturing as described in the ‘Materials and Methods’ section. Where indicated, PBMC were stimulated with PHA (1 μg/ml). After 72 h, co-culture supernatants were harvested and production of IL-10 (A) and TNF-α (B) was assessed by ELISA analysis. Data are expressed as means±SEM with n = 4 (A) and 6 (B), respectively; **P < 0.01 compared to unstimulated A549 cells, #P < 0.05 and ##P < 0.01 compared to PHA-stimulated co-cultures with mock-transfected A549 cells.

Figure 9. Activation of A549 cells by misplaced U2-snRNA. A549 cells were either kept as mock-transfected control or were stimulated with either U1-snRNA or U2-snRNA (both at 0.1 μg/ml) or with U1 ctr or U2 ctr (both at 0.003 μg/ml). After 6 h, cellular pIRF3 content was determined by immunoblot analysis. One representative of three independently performed experiments is shown.
bowl diseases (69,70) and multiple sclerosis where this cytokine is approved for therapy (71). Notably, protection by activation of TLR9 as seen in experimental colitis is mediated by endogenous IFN-β (72). In that context, it is moreover interesting to note that a therapeutic effect of TLR3 has been proposed for experimental colitis (73) and asthma (74).

Upregulation of anti-inflammatory mediators such as transforming growth factor (TGF)-β (75) and IL-10 with coinciding suppression of pro-inflammatory TNFα (76,77) appears crucial for avoiding inflammation associated with apoptosis. By using a transwell-cocultivation model of A549 cells and PBMC, we demonstrate herein that exposure of A549 cells to misplaced U1-snRNA redirects PHA activation of PBMC thereby establishing an anti-inflammatory cytokine milieu comparable with that previously observed in the context of apoptosis. Given the key role of TNFα (78) and IL-10 (79) for immunoregulation, present data suggest an overall anti-inflammatory effect of misplaced U1-snRNA concerning cytokine production as detected in A549/PBMC cocultures.

Phagocytosis of apoptotic cells/bodies by epithelial cells (26,27) not only serves to simply remove cell remnants but beyond that appears to stabilize tissue homeostasis and limit pathology by supporting an anti-inflammatory/protective cytokine milieu (14,15,64,65). Current knowledge on mechanisms of immunomodulation by efferocytosis is incomplete. By studying A549 lung epithelial cells, we characterized misplaced U1-snRNA as a potential signal that may reprogram tissue behavior at host/environment interfaces during epithelial efferocytosis. The latter being a crucial protective process that when defective potentially favors a status of dysregulation with accompanied inflammation as seen in patients with cystic fibrosis, asthma or chronic obstructive pulmonary disease (80).

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