

PCNA-interacting peptides reduce Akt phosphorylation and TLR-mediated cytokine secretion suggesting a role of PCNA in cellular signaling



Camilla Olaisen¹, Rebekka Müller¹, Aina Nedal, Marit Otterlei*

Department of Cancer Research and Molecular Medicine, Norwegian University of Science and Technology, 7489 Trondheim, Norway

ARTICLE INFO

Article history:

Received 3 March 2015

Accepted 12 March 2015

Available online 20 March 2015

Keywords:

PCNA

TLR

PI3K/Akt

MAPK

APIM

ABSTRACT

Proliferating cell nuclear antigen (PCNA), commonly known as a nuclear protein essential for regulation of DNA replication, DNA repair, and epigenetics, has recently been associated with multiple cytosolic functions. Many proteins containing one of the two known PCNA-interacting motifs, the AlkB homologue 2 PCNA interacting motif (APIM) and the PCNA-interacting peptide (PIP)-box, are considered to be mainly cytosolic. APIM is found in more than 20 kinases and/or associated proteins including several direct or indirect members of the mitogen-activated protein kinase (MAPK) and PI3K/Akt pathways. Mass spectrometry analysis of PCNA-pull downs verified that many cytosolic proteins involved in the MAPK and PI3K/Akt pathways are in complex with PCNA. Furthermore, treatment of cells with a PCNA-interacting APIM-containing peptide (APIM-peptide) reduced Akt phosphorylation in human peripheral blood monocytes and a human keratinocyte cell line (HaCaT). Additionally, the APIM-peptide strongly reduced the cytokine secretion from monocytes stimulated with toll like receptor (TLR) ligands and potentiated the effects of MAPK and PI3K/Akt inhibitors. Interestingly, the protein level of the APIM-containing PKR/RIG-1 activator protein (PACT) was initially strongly reduced in HaCaT cells stimulated with APIM-peptide in combination with the TLR ligand polyinosinic–polycytidylic acid (polyIC). Our results suggest that PCNA has a platform role in cytosol affecting cellular signaling.

© 2015 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

Proliferating cell nuclear antigen (PCNA) is an essential coordinator of DNA replication and associated processes, including DNA repair and chromatin/epigenetic maintenance. During these events, PCNA acts as a DNA sliding clamp/binding platform recruiting and binding other proteins to ensure genome stability [1,2]. Recently, PCNA has been suggested to act as a binding platform also independent of chromatin and outside the nucleus. For example, in mature non-proliferating neutrophils, PCNA is exclusively located in the cytosol where it serves as a binding platform for procaspases, inhibits their activation and apoptosis, and thereby regulates neutrophil survival [3]. We have shown that high

levels of cytosolic PCNA are found in multiple myeloma cells, and that targeting PCNA with APIM-peptide induces rapid caspase activation (caspases 3,7, 8 and 9) followed by apoptosis in multiple myeloma, but not in healthy primary cells [4]. Recently, cytosolic PCNA was shown to bind and stabilize procaspase-9 in a neuroblastoma cell line, and interestingly S-nitrosylation of PCNA blocked this interaction suggesting that the binding is regulated by posttranslational modifications (PTMs) [5]. Furthermore, PCNA on the cell surface of cancer cells interacts with the natural cytotoxicity receptor NKp44 on activated natural killer cells and inhibits their cytotoxic function. This may be a mechanism for cancer cells to evade antitumor immunity [6,7]. In addition, proteomic analyses have identified several putative PCNA-binding proteins participating in glycolysis [8,9].

Two PCNA-interacting motifs have until now been identified: the PCNA-interacting peptide (PIP)-box and the AlkB homologue 2 PCNA-interacting motif (APIM) [10,11]. We found that overexpression of the APIM-peptide, or treatment of cells with an APIM-containing cell penetrating peptide, reduced growth rate and increased apoptosis when cancer cells were exposed to chemotherapeutics and/or were stressed, but not, or far less, under normal conditions. Furthermore, a peptide with an impaired APIM-sequence, mutAPIM-peptide, had less effect, thus the APIM–PCNA interaction is required for these activities [4,11].

Abbreviations: APIM, AlkB homologue 2 PCNA-interacting motif; Co-IP, co-immunoprecipitation; IFN, interferon; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MST, microscale thermophoresis measurement; PACT, PKR activator protein; PCNA, proliferating cell nuclear antigen; PIP-box, PCNA-interacting peptide box; polyIC, polyinosinic–polycytidylic acid; PTM, posttranslational modification; TLR, toll like receptor.

* Corresponding author at: Department of Cancer Research and Molecular Medicine, Faculty of Medicine, Norwegian University of Science and Technology, NO-7489 Trondheim, Norway. Tel.: +47 72573075; fax: +47 72576400.

E-mail address: marit.otterlei@ntnu.no (M. Otterlei).

¹ C.O. and R.M. contributed equally to this work.

We have recently shown that APIM-peptide increases the anti-cancer activity of melphalan, mitomycin C, and bleomycin in three different animal models [4,12].

More than 200 different proteins contain APIM and several of these proteins are involved in cellular signaling, regulating proliferation and apoptosis, e.g. three direct members of the PI3K/Akt pathway (p110- α , p110- γ , and PI3K-C2 β), proteins regulating PTEN (MAST3 and PLK3), and proteins of the mitogen-activated protein kinase (MAPK) pathways (ERK8, MK2, MK5, MST4, SOS1, NF1, and TAO1 and 2) [13–20] (see pink proteins in Fig. 1). Thus, cytosolic PCNA may have a platform/organizer role similar to nuclear PCNA, and APIM-PCNA interactions might be important in cellular stress responses others than genotoxic stress.

Toll-like receptors (TLRs) are important for the innate immune system and recognize pathogen-associated molecular patterns. TLR-signaling is closely linked to the MAPK, PI3K/Akt, mTOR, and Wnt signaling pathways, and together these pathways regulate the expression and secretion of cytokines needed to induce an immune response. TLR4 on the cell surface of immune cells recognizes among others lipopolysaccharide (LPS) from Gram-negative bacteria and TLR3 recognizes dsRNA from viruses and the synthetic dsRNA analog polyinosinic-polycytidylic acid (polyIC). In addition to TLR3, cytosolic receptors including RIG-1 and PKR also recognize dsRNA and mediate antiviral responses via signaling pathways common with the TLR signaling pathways [14,21]. Upon ligand binding, TLRs recruit the main adaptor proteins MyD88 and/or TRIF leading to activated TRAF6, which activates NF κ B, several MAPKs, and Akt [13,22–25]. The MyD88-dependent pathway is used by all TLRs except TLR3. The adaptor TRIF is recruited by TLR3 and TLR4 and induces in addition type I interferons (IFNs) via the transcription factor IRF3. It has been shown that PI3K/Akt activity is required for full activation of IRF3 in the TRIF-dependent pathway during TLR3 and 4 signaling; however, the exact mechanisms are not clear [26,27]. Arrows in Fig. 1 illustrate some of the published connections between APIM-containing proteins (pink), TLR signaling, and the MAPK, PI3K/Akt, and Wnt pathways. Our

objective was to investigate a possible role of APIM-PCNA interactions in cellular signaling using TLR-signaling and cytokine response as a reporter system.

Our data show that isolated PCNA-complexes contain many proteins, including APIM-proteins participating in the MAPK, PI3K/Akt, and TLR-pathways. Targeting PCNA with APIM-peptide affects cellular signaling, cell proliferation, and response to TLR stimuli in a spontaneously transformed keratinocyte cell line (HaCaT) and in primary cells (monocytes). Our data suggest a platform/scaffolding role of PCNA in the cytosol important in cellular signaling.

2. Materials and methods

2.1. Peptides

APIM-peptide (Ac-MDRWLVKWKKKRRKIRRRRRRRRRR [4]), mutAPIM-peptide (Ac-MDRALVKWKKKRRKIRRRRRRRRRR [4]), R11 (Ac-RRRRRRRRRRR), and p21 (Ac-MDQTSMRDFYWKKKRIRRRRRRRRRR) were purchased from Innovagen, Sweden.

2.2. Cell line

A spontaneously transformed human keratinocyte cell line, HaCaT, was cultured in DMEM (Sigma-Aldrich) supplemented with 10% fetal bovine serum, 2 mM glutamine (Sigma-Aldrich), 2.5 μ g/ml amphotericin B (Sigma-Aldrich) and 100 μ g/ml gentamicin (Invitrogen). The cells were cultured at 37 °C in a humidified atmosphere of 5% CO₂.

2.3. Isolation and stimulation of peripheral blood monocytes

Peripheral blood mononuclear cells were isolated from A+ buffy coats (Blood Bank, St. Olav's University Hospital, Norway) by density gradient centrifugation (Lymphoprep; Axis-Shield PoC, Norway). Mononucleated cells were seeded at 4 million cells/ml in serum-free RPMI 1640 supplemented with glutamine and gentamicin. After

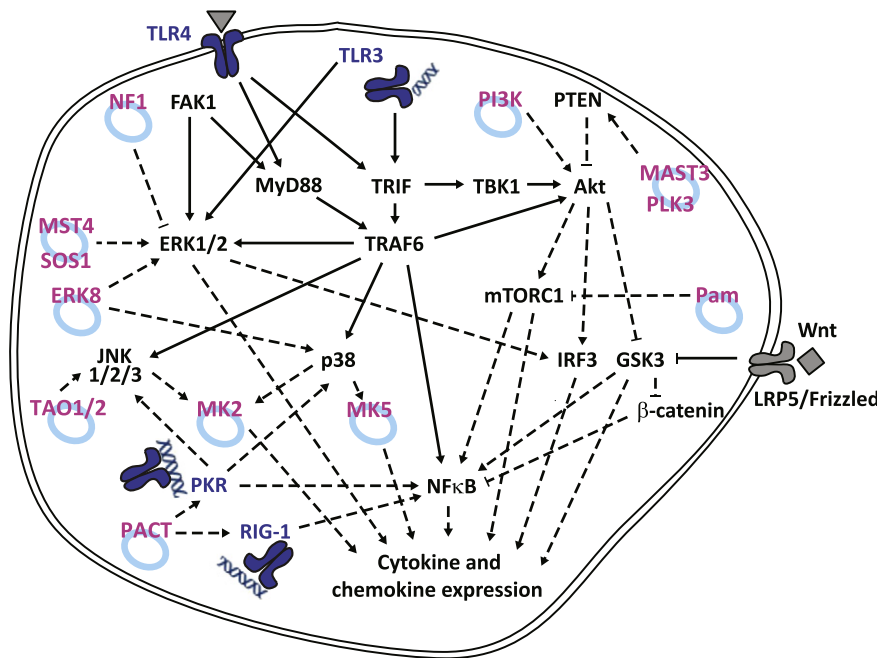


Fig. 1. A potential role for cytosolic PCNA in cellular signaling. Schematic overview of known connections between the pattern recognition receptors TLRs, PKR and RIG-1 (illustrated in dark blue) and the PI3K/Akt, MAPK and NF κ B signaling pathways [13–27,32,37–41,57,58,68,69,71,78,79]. Proteins containing the APIM-sequence associated with these pathways are illustrated in pink [11]. PCNA are illustrated as light blue circles interacting with these proteins. Dashed lines mean pathways possibly deregulated by addition of APIM-peptide and inhibition of the APIM-proteins-PCNA interactions.

60–90 min the adherent cell population was washed before cultured in medium with 10% heat-inactivated human serum (Blood Bank, St. Olav's University Hospital). APIM-peptide, mutAPIM-peptide, R11-peptide, p38 inhibitor (SB203580, 10 μ M [Sigma-Aldrich]), PI3K inhibitor (LY294002, 5 and 10 μ M [Sigma-Aldrich]), and Akt inhibitor (Akt inhibitor XVIII SC66, 1 μ g/ml [Sigma-Aldrich]) were added in serum-free medium and incubated for 5 min before LPS (10 ng/ml [Sigma-Aldrich]), polyIC (40 μ g/ml [GE Healthcare]), PAM3Cys (10 ng/ml [Invivogen]) or R848 (10 μ g/ml [Invivogen]) were added in serum-containing medium. The cells were incubated for 4 h before the supernatants were harvested and frozen prior to further cytokine analysis by the 27-plex assay (Bio-Plex Pro™ Human cytokine 27-plex assay). All together monocytes from nine different blood donors were used in the different experiments. The levels of cytokines and phosphorylation of Akt varied as expected between the donors; however, the trends were very reproducible. Number of donors included in each figure, and number of reproduced experiments are given in the figure legends.

2.4. Flow cytometry

Peripheral blood monocytes were seeded and stimulated as described above. For measurement of the apoptotic cell population, the cells were stained with annexin V-Pacific Blue (Invitrogen) according to the manufacturer's instructions. Cells were analyzed by a FACSAria and the FACSDiva software (BD Biosciences).

2.5. Preparation of cell extracts and western analysis

Mononucleated cells were seeded and stimulated as described above. HaCaT cells were treated with APIM-peptide (12 μ M) and polyIC (2 μ g/ml) for 4 h. The cells were harvested, the cell pellet was resuspended in 1 \times packed cell volume of buffer 1 (10 mM Tris-HCl pH 8.0, 200 mM KCl) and diluted in the same volume (packed cell volume + buffer 1) of buffer 2 (10 mM Tris-HCl pH 8.0, 200 mM KCl, 10 mM EGTA, 10 mM MgCl₂, 40% glycerol, 0.5% NP40, 1 mM DTT, 1% phosphatase inhibitor cocktails 1 and 3 [Sigma-Aldrich], 2% Complete EDTA-free protease inhibitor [Roche], and 2 μ l/ml Omnicleave [Epicentre Technologies]). After incubation for 1.5 h at 4 °C, the cell extracts were centrifuged at 14,000 rpm for 10 min. Supernatants were collected and separated on 10% Bis-Tris gels (NuPAGE, Invitrogen). After gel electrophoresis, the polyvinylidene fluoride membranes (Immobilon, Millipore) were blocked in 50% Odyssey blocking buffer (LI-COR Bioscience) in TBS. The primary antibodies against (α) P-Akt (monoclonal, Cell Signalling), PACT (monoclonal, Santa Cruz Biotechnology Inc.), and β -tubulin (polyclonal, Abcam), as well as the fluorescently labeled secondary antibodies, goat α -rabbit 680RD and goat α -mouse 800CW (LI-COR Bioscience) were diluted in 20% Odyssey blocking buffer in TBST (TBS with 0.1% Tween 20). The proteins were visualized with the Odyssey infrared imaging system (LI-COR Bioscience) and quantified using Odyssey Image Studio V2. Protein levels were compared to the protein level in untreated cells, which was set to 100%. β -Tubulin was used as reference for data normalization.

2.6. Co-immunoprecipitation

HaCaT cells were cross-linked with 0.25% formaldehyde in PBS for 20 min at room temperature. This cross-linking method does not induce massive crosslinking as enzymatically active protein-complexes capable of base excision repair were recovered using the same method [28]. The cross-linking was stopped by addition of glycine (final concentration 0.125 M). After addition of lysis buffers 1 and 2 as described above, cross-linked extract was sonicated and incubated for 1.5 h at 4 °C before centrifugation at 14,000 rpm for 10 min. Extract was dialysed at 4 °C overnight in dialysis buffer (10 mM Tris-HCl pH 8.0, 200 mM KCl, 2 mM EDTA, 10% glycerol, 1 mM DTT). α -PCNA (PC10)

or mock (α -Tom20) antibodies (Santa Cruz biotechnology Inc.) were covalently linked to Dynabeads protein A (Life Technologies) with BS³ (Thermo Scientific) according to the manufacturer's instructions. Cell extract (2 mg) was subjected to immunoprecipitation with 60 μ l α -PCNA or mock beads under constant rotation at 4 °C overnight. The beads were washed three times in 10 mM Tris-HCl pH 8.0, 500 mM KCl and once in 10 mM Tris-HCl pH 8.0 and 50 mM KCl. Tubes were changed twice between washes. The samples were added 1 \times LDS loading buffer containing 1 mM DTT and incubated at 90 °C for 30 min in order to reverse the cross-links. Proteins were separated by electrophoresis for 10 min. The gel-lanes were sliced from the gel and subjected to in-gel tryptic digestion as described [29]. The peptide mixture after digestion was analyzed on an Orbitrap Elite mass spectrometer (Orbitrap-MS) equipped with an Easy nLC 1000 UHPLC system (Thermo Fisher Scientific). The generated data were analyzed with the Proteome Discoverer 1.4 software using both Mascot and Sequest HT search engines against the Swissprot and Uniprot *Homo sapiens* protein databases (accessed on 2014-08-20). The false discovery rate (FDR) was set to 1% for proteins and peptides, with a minimum length of 7 amino acids. Listed proteins in Table 1 and Supplementary Table 1 are detected solely in the α -PCNA in the second biological replica. Only selected proteins are listed, and they represent 15% of identified proteins. In the first biological replica, we detected the same proteins, but some of these proteins were also detected with low score in the mock-IP, less than 50% compared to in α -PCNA IP. In the second replica, we changed the tubes twice before elution as described above.

2.7. Immunofluorescence and confocal imaging

HaCaT cells and peripheral blood monocytes were grown on glass bottom dishes and stained as described using α -PCNA (1:1000) and Alexa fluor 532 goat α -mouse (Invitrogen) [30]. The nuclei were stained with DRAQ5 prior to imaging according to the manufacturer's manual (eBioscience). The fluorescent images were acquired using a Zeiss LSM 510 Meta laser scanning microscope equipped with a Plan-Apochromate 63 \times /1.4 oil immersion objective in 2% FCS in PBS at RT using the Zeiss LSM 510 software. The stained cells were excited at λ = 543 nm and detected at λ \geq 560–615 for Alexa fluor 532. DRAQ5 was excited at λ = 633 nm and detected at λ > 650 nm. The thickness of the slice was 1 μ m. All images were acquired with

Table 1

Proteins involved in MAPK, PI3K/Akt, and TLR signaling detected by Orbitrap-mass spectrometry in PCNA pull downs.

Protein	Function	Reference
MST4*	Activates ERK pathway.	[31]
NF1*	Negative regulator of Ras.	[20]
Pam*	Ubiquitin ligase involved in degradation of TSC2/tuberin in mTOR pathway.	[32]
LRP5	Cell surface co-receptor of Wnt/ β -catenin signaling, which activates mTORC2.	[33,34]
ERK2	Essential component of MAPK/ERK kinase signal transduction pathway. Regulates cytokine production.	[13]
mTOR	Central role in regulating cell growth, metabolism, and immune function.	[35]
FAK1	Activation of PI3K/Akt, MEK/ERK1/2, and MyD88 pathways. Regulates NF κ B activation and cytokine production.	[36–39]
GSK3 β	Component of Akt, ERK, mTOR, and Wnt pathways. Regulatory function in the immune response.	[40–42]
PKN2	Inhibits Akt kinase activity.	[43]
STK10	Negative regulator of MEK1/MAP3K1 and NF κ B pathway.	[44,45]
PAK1	Activates MEK1. Phosphorylated by Akt. Mast cell degranulation.	[46–48]
NEMO	Activation of the transcription factor NF κ B.	[49]
CASP-1	Cleaves IL-1 β , releasing the mature cytokine.	[50]

*Proteins containing the APIM-sequence.

consecutive scans. No image processing was performed, except contrast and intensity adjustments.

2.8. IFN- β ELISA

Monocytes were stimulated as described above. IFN- β was measured in supernatants with the VeriKine-HS™ Human IFN- β Serum ELISA kit (Pestka Biomedical Laboratories) according to the manufacturer's instructions with the following adaptations: the amount of supernatant was doubled from 50 to 100 μ l; sample buffer was reduced from 50 to 25 μ l; and antibody solution was reduced from 50 to 25 μ l (reducing assay diluent, keeping the same volume of antibody concentrate and diluent additive). The OD was measured at 450 nm.

2.9. Cell survival assay

HaCaT cells were seeded into 96-well plates and APIM-peptide (12 μ M) and polyIC (2 μ g/ml) were added alone or in combination at the indicated concentrations. Cells were exposed continuously and harvested on days one and three using the MTT assay as described [11]. The average from six wells was used to calculate cell survival.

2.10. In vitro interactions by Microscale Thermophoresis Measurements (MST)

PCNA was labeled using the RED-Maleimide Labeling Kit (NanoTemper Technologies). The labeling reaction was performed according to the manufacturer's instructions in the supplied labeling buffer applying concentration of 20 μ M protein at RT. The ligands (APIM-, mutAPIM-, R11- and p21-peptides) were dissolved in supplemented MST buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM MgCl₂) with 2% fetal bovine serum and incubated for 10 min in RT. A series of 16 1:1 dilutions was prepared in the MST buffer producing ligand concentrations ranging from 500 μ M to 15 nM. Each ligand dilution was diluted 1:1 with fluorescent labeled PCNA (diluted in MST buffer to 100 nM) and further incubated for 10 min. Thermophoresis was measured using Monolith NT.115 instrument (20% MST power and 100% LED power) and Monolith NT Standard Treated Capillaries (both NanoTemper Technologies, GmbH). K_d was calculated using the mass action equation by the software NT Analysis 1.5.41 (NanoTemper Technologies) using fluorescence data from triplicate experiments. Fluorescence changes caused by binding event were confirmed by denaturation with 8 M guanidine chloride.

3. Results

3.1. PCNA is in complex with several cytosolic proteins involved in PI3K/Akt and MAPK signaling

We wanted to investigate PCNA-complexes for cytosolic proteins because more than 200 of the proteins containing either APIM or the PIP-box are considered to be mainly cytosolic (<http://tare.medisin.ntnu.no/pcna/index.php>). PCNA is found both in the nucleus and cytosol of HaCaT cells (Fig. 2A), and we therefore performed co-immunoprecipitation (co-IP) experiments on extracts from these cells. As suspected, several proteins involved in cellular signaling were detected specifically in the PCNA co-IP (selected proteins are shown in Table 1). This includes the APIM-containing proteins MST4, NF1, and Pam involved in regulation of ERK and Akt-signaling [20,31] (APIM-containing proteins are in pink in Fig. 1A and marked with* in Table 1).

In addition, we detected among others LRP5, ERK2, mTOR, FAK1, GSK3- β , PKN2, STK10, and PAK1; all proteins involved in regulating MAPK, NF κ B and/or PI3K/Akt pathways [36–39,43–50] (some of these proteins are included in Fig. 1). Endothelial-cell FAK was recently shown to be important for DNA-damage induced NF κ B activation and

the production of cytokines from endothelial cells [39], and caspase-1 and NEMO, two other proteins found in the PCNA-complexes are involved in cleaving of the IL-1 β precursor and NF κ B activation, respectively [49,50]. In addition to the selected proteins listed in Table 1, we detected classical PCNA-binding proteins such as replication factor C and flap endonuclease 1 (data not shown). Many of the proteins pulled down are not APIM- or PIP-box-containing proteins (Table 1 and additional proteins shown in Supplementary Table S1); however, many of the cytosolic proteins found in the PCNA-complexes are involved in cellular signaling supporting a role for PCNA in these processes.

3.2. Targeting PCNA with APIM-peptide in HaCaT cells affects cell growth and Akt phosphorylation

Many of the proteins detected in the PCNA-complexes are proteins important in the MAPK and PI3K/Akt pathways, and thereby TLR and apoptosis signaling. Human keratinocytes respond to polyIC via the known dsRNA sensing receptors TLR3, PKR, RIG-I, and MDA5 [51–54], and TLR3 is reported to mediate cell death and release of IL-1 β in keratinocytes in a caspase-4 dependent manner [55]. TLR3 uses the TRIF/TRAF6 pathways to activate MAPKs, Akt, NF κ B, and IRF3 [13, 22–27]. RIG-I and PKR trigger activation of p38 and NF κ B, and NF κ B is reported to be regulated via Akt/mTOR [56]. Thus, to explore the role of PCNA in cellular signaling we examined how APIM-peptides affected growth of HaCaT cells stimulated with polyIC. We found that treatment of cells with a cell penetrating APIM-peptide [4], as well as overexpressed APIM-peptide (APIM-EYFP) [11] increased the growth inhibitory effects of polyIC (Fig. 2B and C). After 4 h, treatment with APIM-peptide strongly reduced Akt-phosphorylation in both unstimulated and polyIC-stimulated cells (Fig. 2D, left panel), and the protein level of the dsRNA-binding APIM-containing PKR/RIG-1 activator protein (PACT) [57,58] was also reduced (Fig. 2E, left panel). However, after 24 h, APIM-peptide and polyIC, both alone and in combination, increased the levels of both phosphorylated Akt and PACT after (Fig. 2D and E, right panels). The effects of the APIM-peptide were more pronounced in polyIC-stimulated cells, similar to what was previously seen with cells treated with various chemotherapeutics [4,11,12] suggesting that the effects of the APIM-peptide are correlated with the cellular stress responses.

3.3. Targeting PCNA with APIM-peptide in monocytes reduces Akt-phosphorylation and cytokine secretion after TLR stimulation

Because different cell lines have different sensitivity to the APIM-peptide [4], we next used Western analysis to examine how the APIM-peptide affected the PI3K/Akt pathway in primary monocytes isolated from healthy blood donors. Human primary monocytes contain substantial amounts of PCNA in the cytosol (Fig. 3A). LPS- and polyIC-induced Akt-phosphorylation, as well as the basal Akt-phosphorylation level in monocytes was reduced by treatment with APIM-peptide (Fig. 3B). Importantly, APIM-peptide alone and in combination with LPS or polyIC did not induce apoptosis-levels that could explain the reduction in Akt-phosphorylation (Fig. 3C). We next tested whether APIM-peptide could affect cytokine secretion from monocytes stimulated with different TLR ligands. By measuring the concentration of 27 different cytokines in the cell culture supernatant, we found that secretion of many LPS-induced cytokines was efficiently reduced by addition of APIM-peptide (Fig. 4A, black bars). Background levels of cytokines not increased, or increased less than two fold by LPS addition (defined as “no induction”), were also reduced in some cases (Fig. 4A, gray bars). Similarly, treatment with APIM-peptide strongly reduced the polyIC-induced secretion of CXCL10, CCL2, and CCL4 (Fig. 4B). We found similar reductions in cytokine secretions after stimulation with the TLR7/8 ligand R848 and the TLR2 ligand PAM3Cys (Supplementary Fig. S1). In general, fewer cytokines were induced in monocytes

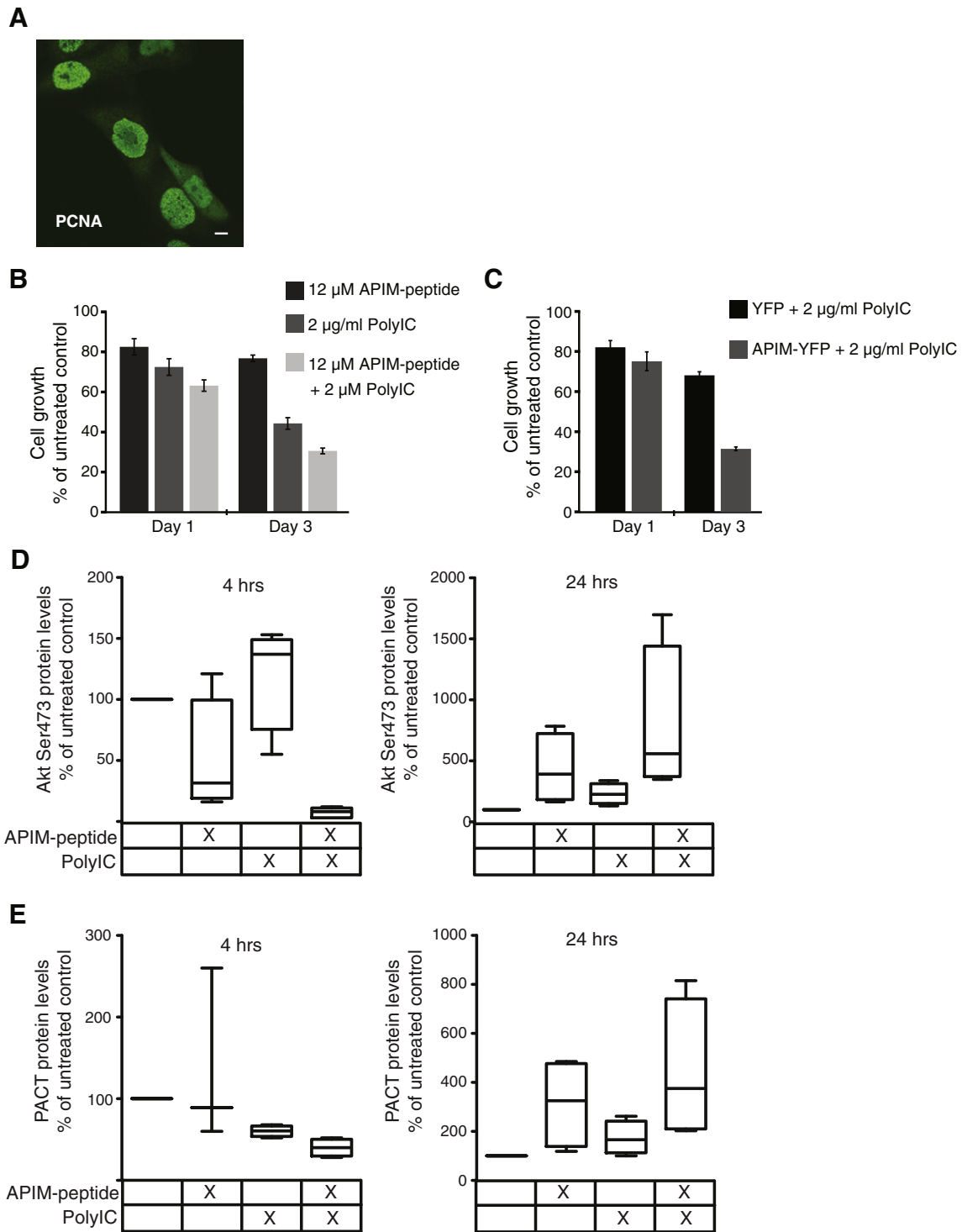


Fig. 2. APIM-peptide affects Akt phosphorylation and PACT protein levels in polyIC-stimulated HaCaT cells. (A) Confocal fluorescence image of immunofluorescently stained PCNA in HaCaT cells. Bar, 5 μ m. (B) Cell growth (MTT-assay) of polyIC-stimulated (2 μ g/ml) HaCaT cells treated with APIM-peptide (12 μ M) or (C) expressing APIM-EYFP. Data given are mean \pm SD from six parallel wells from one representative experiment. (D) Quantification of Akt Ser473 phosphorylation and (E) PACT protein levels in HaCaT cells treated with APIM-peptide (12 μ M), polyIC (2 μ g/ml), and combination of APIM-peptide and polyIC for 4 h (left panels) or 24 h (right panels) by Western blot analysis. Protein levels were corrected for loading differences (β -tubulin) and normalized to untreated control cells, which was set to 100. Data are from 3–5 independent experiments. Whisker plots presenting median, minimum, and maximum values are shown.

stimulated with polyIC compared to LPS, R848, and Pam3Cys (black bars, Figs. 4 and S1). However, our results show that targeting PCNA with APIM-containing peptides in monocytes affected both Akt-phosphorylation and cytokine secretion after TLR stimulation.

APIM-peptide treatment strongly decreased both the polyIC-induced and the basal CXCL10 secretion from monocytes (Fig. 4B, black bar and Fig. 4A, gray bar, respectively). Induction of CXCL10 expression is a secondary event and requires the initial secretion of IFN- β . We

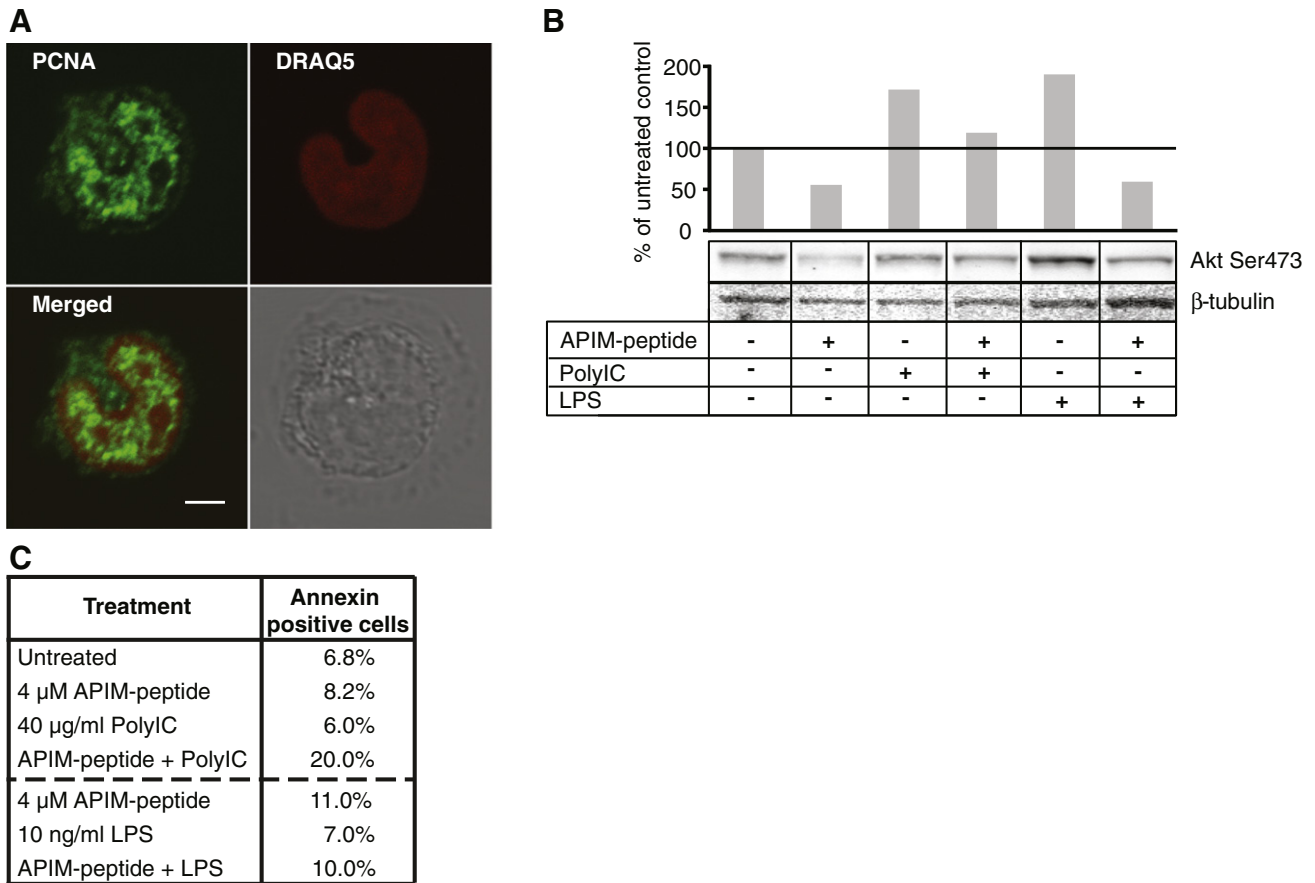


Fig. 3. APIM-peptide reduces Akt phosphorylation in monocytes. (A) Confocal fluorescence image of immunofluorescently stained PCNA (green) in freshly isolated peripheral blood monocytes. DRAQ5 was used for nuclear staining (red). Bar, 5 μ m. (B) Western blot analysis of Akt Ser473 phosphorylation in peripheral blood monocytes stimulated with LPS (10 ng/ml) or polyIC (40 μ g/ml) alone and in combination with APIM-peptide (4 μ M) for 4 h. The upper panel shows the quantification of phosphorylated Akt corrected for loading differences (β -tubulin) and normalized to untreated control cells, which were set to 100 (black horizontal line). Data are from one representative donor out of three. (C) Annexin measurements of peripheral blood monocytes treated with polyIC (40 μ g/ml) or LPS (10 ng/ml) alone and in combination with APIM-peptide (4 μ M) for 4 h. Results are from two different blood donors (separated by dashed line).

therefore measured the IFN- β levels in supernatants from polyIC-stimulated monocytes. Consistent with the reduced CXCL10 secretion, APIM-peptide treatment decreased the IFN- β secretion (Fig. 4C), supporting that APIM-peptide affects TLR3/PKR/RIG-1 signaling and not, or not only, IFN- β /STAT/IRF9 signaling.

We and others have previously shown that the APIM-peptide interacts with PCNA [4,59]. A mutation in APIM (see Materials and methods) was found to reduce the ability of APIM-EYFP to co-localize with PCNA [11], and the corresponding cell penetrating peptide, mutAPIM-peptide, did not sensitize cells to chemotherapeutics [4]. Microscale thermophoresis measurement (MST) showed that the mutAPIM-peptide has lower affinity for PCNA than the wild type APIM-peptide (Fig. 4D). The p21-peptide containing the canonical PIP-box [2] and the cell-penetrating part of the peptide, R11, were included as positive and negative controls, respectively. In agreement with this, we found that the mutAPIM-peptide had little to no effect on the secretion of cytokines after LPS and polyIC stimulation of monocytes (Fig. 4E). Thus, our results support that the cytokine reducing activities observed are mediated via direct binding of APIM to PCNA.

3.4. APIM-peptide potentiates the effect of MAPK and PI3K inhibitors on cytokine secretion from monocytes

Because MAPK and PI3K/Akt pathways are important for TLR signaling and cytokine secretion [13,22–26], and many APIM-proteins

participate in these pathways, we next tested if the effect of APIM-peptide on cytokine production from LPS- and polyIC-stimulated monocytes was enhanced in combinations with inhibitors of p38 MAPK (SB203580) and class I PI3K (LY294002). The p38 inhibitor reduced cytokine secretion as a single agent, and in combination with APIM-peptide a further reduction of many cytokines was detected, both after LPS and polyIC stimulation (Fig. 5A and B, respectively). The PI3K inhibitor had less effect on cytokine secretion both alone and in combination with APIM-peptide, but still the combination showed an additive effect on the secretion of some cytokines; largest effects were seen for IFN γ after LPS, and CCL2–4 after polyIC stimulation (Fig. 5C and D, respectively). These results further support a role for PCNA in regulation of both these pathways. We also tested direct inhibition of Akt using the inhibitor SC66 [60], which efficiently reduced Akt phosphorylation in JJN-3 cells (C. Olaisen and M. Otterlei, unpublished data). SC66 efficiently reduced the secretion of cytokines from LPS- and polyIC-stimulated monocytes with between 50–99% (Supplementary Fig. S2), supporting a vital role for Akt in regulation of cytokine secretion. SC66 may induce apoptosis, but at the dose used in our study (1 μ g/ml) we detected only 13% apoptotic cells in the SC66-treated versus 8% in the control cells after 24 h. The strong cytokine inhibitory activity of SC66 made it difficult to quantify any combinatory effects with the APIM-peptide; however, a further reduction (~30–80%) in CXCL8 (polyIC only), CCL4 (polyIC only), and TNF (LPS and PolyIC) was detected. We also examined the effect of APIM-peptide on

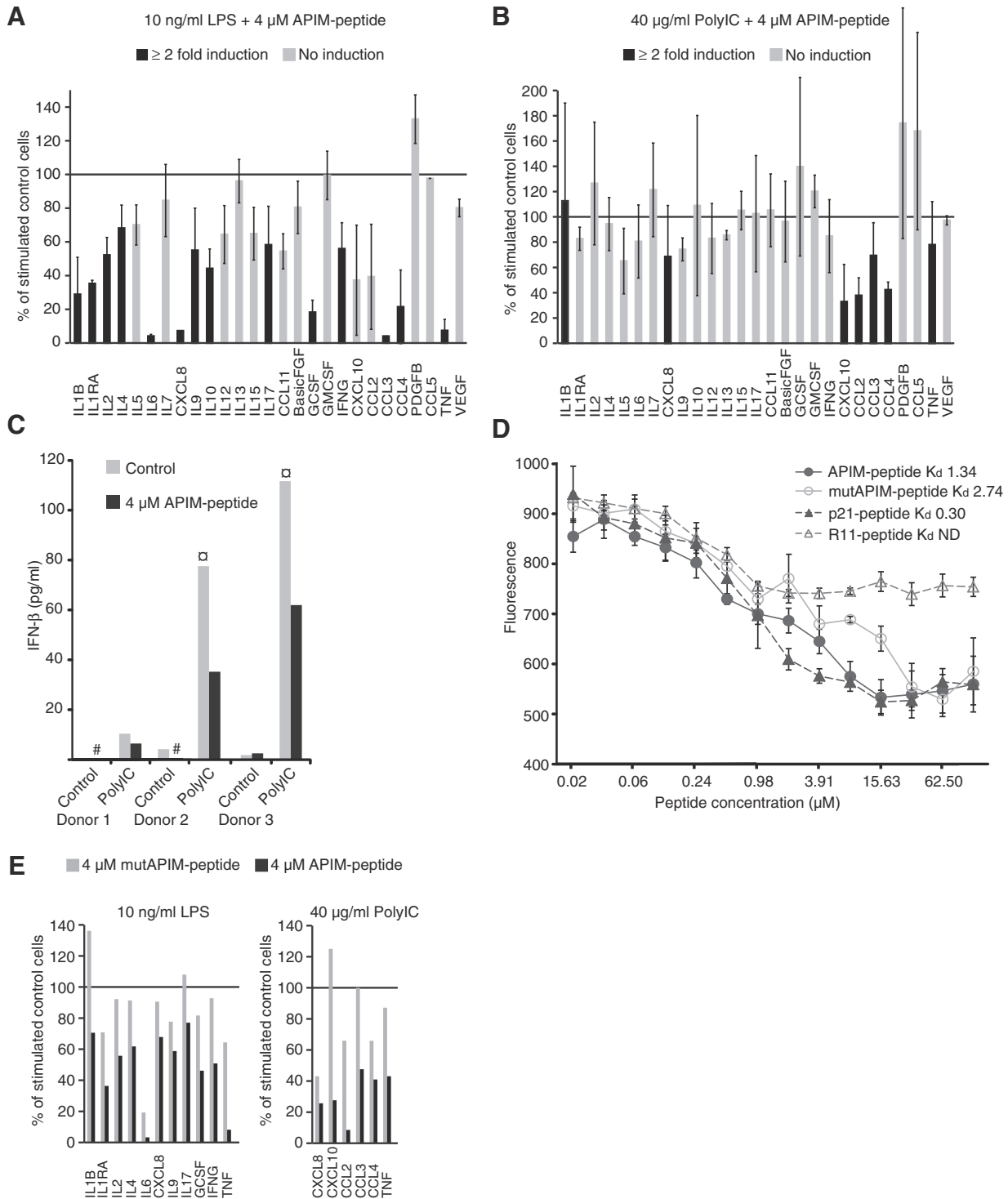


Fig. 4. APIM-peptide reduces cytokine secretion from monocytes after TLR stimulation. (A–B) Multiplex analysis of cytokine levels produced by peripheral blood monocytes after 4 h treatment with APIM-peptide (4 μM) in combination with LPS (10 ng/ml) (A) or polyIC stimulation (40 μg/ml) (B). Cytokine levels are normalized to cytokine levels from monocytes stimulated with TLR ligand alone, which were set to 100 (black horizontal line). A two-fold or higher increase in cytokine level after TLR stimulation was defined as cytokine induction, and shown in black bars. Effects on cytokines that are not induced are shown with gray bars. Data are given as mean ± SD from three different donors. □ Means value over linear range. (C) ELISA of IFN-β levels produced by peripheral blood monocytes from three donors is shown. Isolated monocytes were stimulated with polyIC (40 μg/ml) alone and in combination with APIM-peptide (4 μM) for 4 h. # Under linear range; □ over linear range. (D) In vitro interactions between PCNA and APIM-, mutAPIM-, p21- and R11- peptides measured with MST. K_d -values calculated with the NT Analysis 1.5.41 software are given in the figure. N.D. = not detectable, i.e. no specific interaction. (E) Multiplex analysis of LPS (10 ng/ml) and polyIC (40 μg/ml) induced cytokine levels from peripheral blood monocytes after 4 h treatment in present of 4 μM APIM-peptide or 4 μM mutAPIM-peptide. Measured cytokine levels were normalized to cytokine levels from monocytes stimulated with TLR ligand alone. A two-fold or higher increase in cytokine level after TLR stimulation was defined as cytokine induction, and shown with black bars. Effects on cytokines that are not induced are shown with gray bars. Representative data from one out of two independent experiments from two different blood donors are shown.

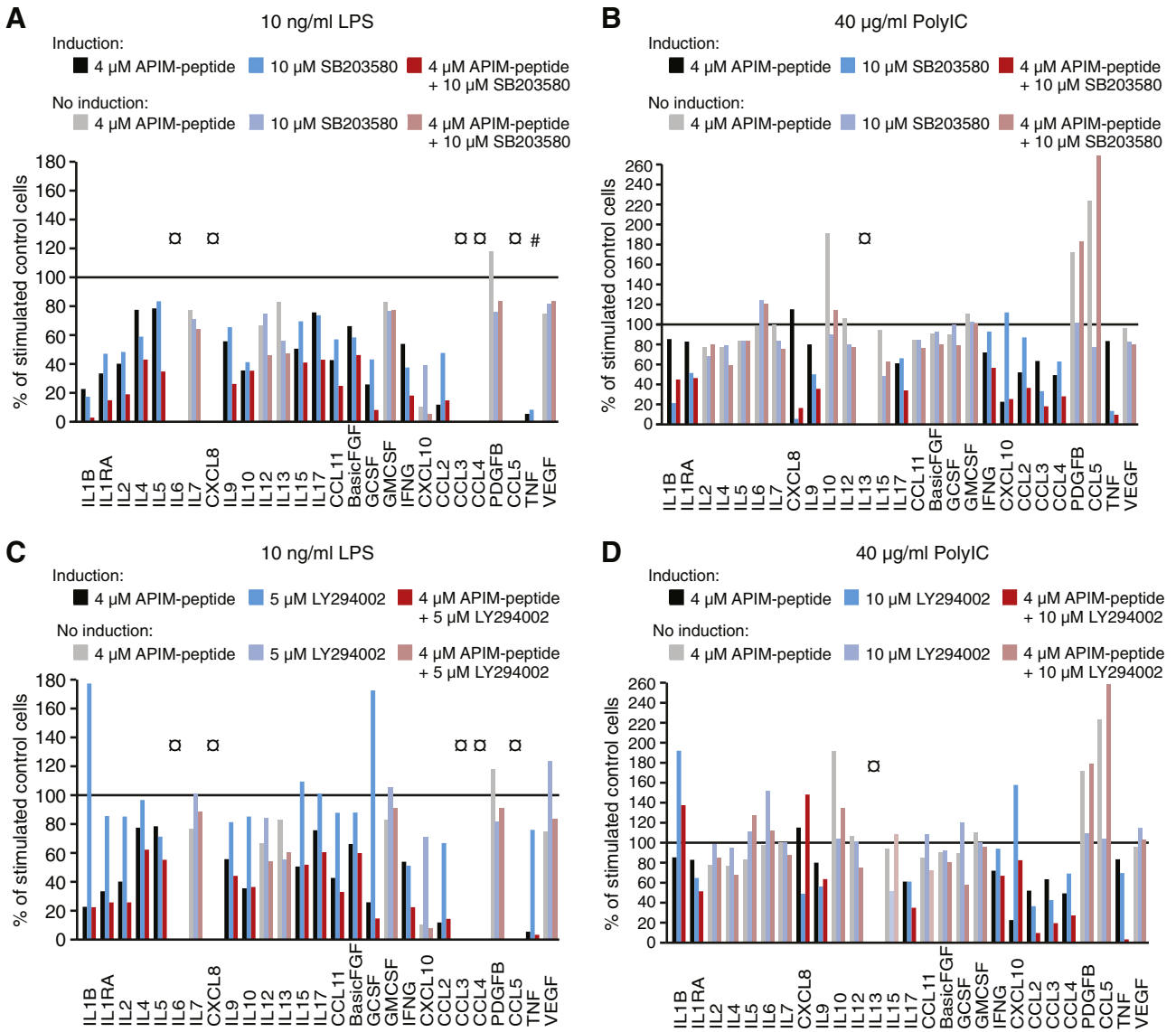


Fig. 5. Combinations of APIM-peptide with specific inhibitors of MAPK and PI3K/AKT further reduce cytokine secretion from monocytes. (A–D). Multiplex analysis of cytokine levels produced by peripheral blood monocytes stimulated with LPS (10 ng/ml) (A and C, same donor) and polyIC (40 µg/ml) (B and D, same donor) after 4 h. (A and B) Stimulated-monocytes were treated with APIM-peptide (4 µM), SB203580 (10 µM) (p38 MAPK inhibitor), and combination of APIM-peptide and SB203580. (C and D) Stimulated-monocytes were treated with APIM-peptide (4 µM), LY294002 (5 and 10 µM) (PI3K class I inhibitor), and combination of APIM-peptide and LY294002. Measured cytokine levels were normalized to cytokine levels from monocytes stimulated with TLR ligand alone, which were set to 100 (black horizontal line). A two-fold or higher increase in cytokine level after TLR stimulation was defined as cytokine induction and shown with black bars. Effects on cytokines that are not induced are shown with gray bars. # Value is too low to be visible in the column diagram; □ Cytokine measurement was under linear range. Representative data from one out of two independent experiments from two different blood donors are given.

LPS-induced NFκB activation in monocytes. We found a decrease in NFκB activation after treatment with APIM-peptide in all experiments performed. The reduction of p65 (RelA) activation after treatment with LPS (10 ng/ml) in combination with APIM-peptide versus LPS only was relatively small in the three blood donors tested, 21, 29, and 35% respectively (data not shown).

Taken together, our data support that targeting PCNA with APIM-peptide has the ability to affect regulation of signaling pathways, including the PI3K/Akt and MAPK pathways, two pathways with extensive crosstalk [61,62]. APIM-peptide blocks interactions of APIM-containing proteins with PCNA, but likely does not directly inhibit any kinase activity. Impairing the platform-role of PCNA likely compromises signaling and pathway crosstalk, resulting in reduced cytokine secretion. These effects are enhanced when kinase inhibitors are combined with APIM-peptide.

4. Discussion

PCNA has an essential role in coordination of DNA replication and cellular response to genotoxic stress [2]. Recently, PCNA has also been linked to regulation of apoptosis [3,4], glycolysis [9], and natural killer-cell cytotoxicity [63], all processes localized in cytosol. Here we show that PCNA-complexes pulled down from unstimulated HaCaT cells contain many proteins involved in cellular signaling. Many of these do not contain any of the two known PCNA-interacting motifs. However, their presence in PCNA-complexes is not necessarily due to direct interactions with PCNA. For example, presence of caspase-1 in PCNA complexes could be via indirect interaction of p21 as p21 directly interacts with several caspases [64] and with PCNA via its PIP-box [2]. We were not able to deplete the extracts for PCNA, possibly because the epitope on PCNA recognized by the antibody used was not available in all

contexts. Therefore, we have not identified the complete PCNA interactome using this specific approach.

PCNA has more than 400 potential interaction partners and the regulation of different PCNA–protein complexes at a given time is likely regulated at many levels [2,11,65]. The type of proteins that until now are verified to have functional APIM–PCNA interactions, are proteins important in the cellular response to genotoxic and/or replicative stress, and it is shown that stress-induced PTMs on PCNA are important for their interaction with PCNA [4,11,30,59,65,66]. PTMs likely regulate which binding motif has highest affinity at a given time because the APIM and PIP-box sequences have overlapping binding regions on PCNA [4,59]. Our MST analysis indicates that the PIP-box-peptide has higher affinity for naked recombinant PCNA than the APIM-peptide. Because stress levels and PTMs on PCNA likely are important for which proteins are in complex with PCNA at a given time, the protein repertoire co-IPed with PCNA from different cell types are expected to be different. Preliminary data from similarly performed co-IP experiments in extracts from JJN3 support this (C. Olaisen and M. Otterlei, unpublished results). APIM-peptide reduced Akt phosphorylation and PACT protein levels after 4 h, but an increase was seen after 24 h. This effect of the APIM-peptide is also likely cell type specific because APIM-peptide treatment resulted in a prolonged reduction in Akt-phosphorylation in the multiple myeloma cell line JJN-3 (>24 h, C. Olaisen, M. Otterlei; unpublished data), and JJN-3 cells are also much more sensitive to APIM-peptide than HaCaT cells [4]. Further studies of the PCNA interactome in several different cell types under different conditions are required for exploring the role of PCNA in cellular signaling.

We hypothesize that the APIM-peptide inhibits binding between PCNA and APIM-containing proteins, such as PACT, and that this affects various signaling pathways. These effects could be due to reduced protein stability of APIM-containing proteins when not interacting with PCNA, e.g. as detected here for PACT and previously shown for procaspases [3,5]. A platform role of PCNA could also facilitate interaction between several proteins within PCNA-complexes. Nuclear PCNA-complexes are dynamic and regulated by PTMs during genotoxic stress [2] and we hypothesize similar regulation of PCNA-interactions in cytosol in agreement with recent data [5].

In spite of donor variations both in cytokine secretion after TLR stimulation and in response to the different inhibitors, the overall results show that the APIM-peptide as well as p38, PI3K, and Akt inhibitors reduce the cytokine secretion. The APIM-peptide contains an Arg-rich cell-penetrating peptide sequence that mediates its cellular import [4]. It is reported that a different cationic cell-penetrating peptide reduced the secretion of TNF, IL-6, and IL-8 in LPS-stimulated THP-1 cells similar to a MK2 inhibitor peptide fused to the same cell-penetrating peptide, indicating that cell-penetrating peptides themselves can alter cellular functions [67]. Our data suggest that APIM-peptide reduces cytokine secretion via APIM-binding to PCNA because mutAPIM-peptide with reduced ability to bind PCNA had less effect. In line with this, the same mutation in the APIM-sequence in the DNA repair protein XPA and the translesion synthesis protein TFII-I, reduced the ability of these proteins to interact with PCNA and their functionality in DNA repair and translesion synthesis, respectively, was also impaired [30,66]. The small observed effects of mutAPIM-peptide could be a combination of that this peptide had some, but weaker, ability to interact with PCNA and the potential non-PCNA dependent effects due to cationic cell penetrating part of the peptide.

In accordance with a vital role of Akt in cytokine production, it was shown by others that Akt was phosphorylated in response to LPS and polyIC, and that this plays a vital role in the full activation of IRF3 [26,68]. A selective inhibitor of PI3K p110- α and γ reduced LPS-induced Akt-phosphorylation in the human monocytic cell line THP-1 and IL-6 and TNF secretion from primary peripheral blood monocytes [69]. Additionally, PI3K p110- γ was recently found to directly interact with Rab8a and regulate Akt-signaling induced by TLR4 [70].

Two members of the class I PI3Ks (p110- α and p110- γ) and in addition the class II member PI3K-C2 β contain the APIM-sequence and their putative interaction with PCNA could thus potentially be inhibited by APIM-peptide. Both p110- α (contain APIM) and β are found to be in a complex with PCNA [71]. Also, a putative reversed PIP-box sequence found in Akt and a peptide from PI3K p85 α (regulatory domain) were found to bind to PCNA in vitro [72].

The MAPK pathways are also important for expression and secretion of inflammatory cytokines downstream of TLRs. MK2 and MK5, kinases downstream of p38, contain APIM. MK2 affects cytokine production by promoting stability and translation of cytokine mRNAs, including TNF, IL-6, IL-8, IL-10, and IFN- β [73–77]. Interestingly, ERK8, which also contains APIM, is reported to interact with and stabilize PCNA [78]. Additional APIM-containing proteins involved in MAPK signaling are SOS1, MST4, NF1, and TAO 1 and 2 (pink proteins in Fig. 1A). We found that MST4 and NF1 were co-IPed with PCNA from HaCaT cells (Table 1). Thus, the data supporting a role for PCNA in cellular signaling is increasing.

5. Conclusions

We found that several proteins involved in cellular signaling were pulled down with PCNA from a keratinocyte cell line (HaCaT). Inhibiting potential PCNA–protein interactions with an APIM-peptide reduced PI3K/Akt signaling both in HaCaT cells and in primary monocytes.

Our data show that APIM-peptide reduces cytokine secretion from TLR-stimulated monocytes. We therefore add the possibility that APIM-mediated PCNA-interactions also play a role in the cellular stress response to pathogen- and damage-associated molecular patterns, similar to its importance in the cellular response to genotoxic stress [11,30,59,65,66]. Our results suggest that targeting PCNA's protein interactions via APIM may represent a novel approach for treatments of inflammatory disorders.

The APIM-peptide could reduce cytokine secretion from tumor cells and tumor microenvironment. Because inflammation and cytokines can be a driver for many cancers, the observed reduction might be a useful add-on effect of the APIM-peptide in addition to its reported inhibitory effect of DNA repair [11,30].

Conflict of interest

No conflict of interest to declare.

Acknowledgments

We especially want to thank Liv Ryan for performing the multiplex analysis, and Nina B. Liabakk and Siri Bachke for technical assistance. We thank Professor Terje Espevik for valuable discussions. The fluorescence imaging was performed at the Cellular & Molecular Imaging Core Facility, Norwegian University of Science and Technology (NTNU). The mass spectrometry analysis was performed at the Proteomics and Metabolomics Core Facility (PROMEC), NTNU.

This work was supported by grants from NTNU, Trondheim, Norway, The Cancer Fund at St. Olav's Hospital, Trondheim, Norway, The Norwegian Cancer Society and The Liaison Committee between the Central Norway Regional Health Authority (RHA) and NTNU. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.cellsig.2015.03.009>.

References

- [1] G.-L. Moldovan, B. Pfander, S. Jentsch, *Cell* 129 (2007) 665–679.
- [2] N. Mailand, I. Gibbs-Seymour, S. Bekker-Jensen, *Nat. Rev. Mol. Cell Biol.* 14 (2013) 269–282.
- [3] V. Witko-Sarsant, J. Mocek, D. Bouayad, N. Tamassia, J.A. Ribeil, C. Candalh, N. Davezac, N. Reuter, L. Mouthon, O. Hermine, M. Pederzoli-Ribeil, M.A. Cassatella, *J. Exp. Med.* 207 (2010) 2631–2645.
- [4] R. Müller, K. Misund, T. Holien, S. Bachke, K.M. Gilljam, T.K. Våtsveen, T.B. Rø, E. Bellacchio, A. Sundan, M. Otterlei, *PLoS ONE* 8 (2013) e70430.
- [5] L. Yin, Y. Xie, S. Yin, X. Lv, J. Zhang, Z. Gu, H. Sun, S. Liu, *PLoS ONE* 10 (2015) e0117546.
- [6] B. Rosental, M. Brusilovsky, U. Hadad, D. Oz, M.Y. Appel, F. Afergan, R. Yossef, I.A. Rosenberg, A. Aharoni, A. Cerwenka, K.S. Campbell, A. Braiman, A. Porgador, *J. Immunol.* 187 (2011) 5693–5702.
- [7] N.C. Horton, S.O. Mathew, P.A. Mathew, *PLoS ONE* 8 (2013) e59552.
- [8] S.N. Naryzhny, *Cell. Mol. Life Sci.* 65 (2008) 3789–3808.
- [9] S.N. Naryzhny, H. Lee, *FEBS Lett.* 584 (2010) 4292–4298.
- [10] E. Warbrick, *Bioessays* 20 (1998) 195–199.
- [11] K.M. Gilljam, E. Feyzi, P.A. Aas, M.M. Sousa, R. Muller, C.B. Vagbo, T.C. Catterall, N.B. Liabakk, G. Slupphaug, F. Drablog, H.E. Krokan, M. Otterlei, *J. Cell Biol.* 186 (2009) 645–654.
- [12] O.A. Gederas, C.D. Søgaard, T. Viset, S. Bachke, P. Bruheim, C.-J. Arum, M. Otterlei, *Transl. Oncol.* 7 (2014) 812–823.
- [13] J.S.C. Arthur, S.C. Ley, *Nat. Rev. Immunol.* 13 (2013) 679–692.
- [14] M.A. Garcia, E.F. Meurs, M. Esteban, *Biochimie* 89 (2007) 799–811.
- [15] M. Valiente, A. Andres-Pons, B. Gomar, J. Torres, A. Gil, C. Tapparel, S.E. Antonarakis, R. Pulido, *J. Biol. Chem.* 280 (2005) 28936–28943.
- [16] S. Pierre, A.S. Bats, X. Coumoul, *Biochem. Pharmacol.* 82 (2011) 1049–1056.
- [17] D. Xu, Y. Yao, X. Jiang, L. Lu, W. Dai, *J. Biol. Chem.* 285 (2010) 39935–39942.
- [18] P. Ling, T.J. Lu, C.J. Yuan, M.D. Lai, *Cell. Signal.* 20 (2008) 1237–1247.
- [19] J. Chia, K.M. Tham, D.J. Gill, E.A. Bard-Chapeau, F.A. Bard, *ELife* 3 (2014) e01828.
- [20] G. Bollag, D.W. Clapp, S. Shih, F. Adler, Y.Y. Zhang, P. Thompson, B.J. Lange, M.H. Freedman, F. McCormick, T. Jacks, K. Shannon, *Nat. Genet.* 12 (1996) 144–148.
- [21] Y.M. Loo, M. Gale Jr., *Immunity* 34 (2011) 680–692.
- [22] W.L. Yang, J. Wang, C.H. Chan, S.W. Lee, A.D. Campos, B. Lamothe, L. Hur, B.C. Grabiner, X. Lin, B.G. Darnay, H.K. Lin, *Science* 325 (2009) 1134–1138.
- [23] P.B. Jakus, N. Kalman, C. Antus, B. Radnai, Z. Tucsek, F. Gallyas Jr., B. Sumegi, B. Veres, *J. Nutr. Biochem.* 24 (2013) 819–823.
- [24] H. Kumar, T. Kawai, S. Akira, *Int. Rev. Immunol.* 30 (2011) 16–34.
- [25] T. Kawai, S. Akira, *Nat. Immunol.* 11 (2010) 373–384.
- [26] S.M. Joung, Z.Y. Park, S. Rani, O. Takeuchi, S. Akira, J.Y. Lee, *J. Immunol.* 186 (2011) 499–507.
- [27] S.N. Sarkar, K.L. Peters, C.P. Elco, S. Sakamoto, S. Pal, G.C. Sen, *Nat. Struct. Mol. Biol.* 11 (2004) 1060–1067.
- [28] M. Akbari, K. Solvang-Garten, A. Hanssen-Bauer, N.V. Lieske, H.S. Pettersen, G.K. Pettersen, D.M. Wilson III, H.E. Krokan, M. Otterlei, *DNA Repair (Amst)* 9 (2010) 785–795.
- [29] A. Shevchenko, M. Wilm, O. Vorm, M. Mann, *Anal. Chem.* 68 (1996) 850–858.
- [30] K.M. Gilljam, R. Muller, N.B. Liabakk, M. Otterlei, *PLoS ONE* 7 (2012) e49199.
- [31] J.L. Lin, H.C. Chen, H.I. Fang, D. Robinson, H.J. Kung, H.M. Shih, *Oncogene* 20 (2001) 6559–6569.
- [32] S. Han, R.M. Witt, T.M. Santos, C. Polizzano, B.L. Sabatini, V. Ramesh, *Cell. Signal.* 20 (2008) 1084–1091.
- [33] J. Mao, J. Wang, B. Liu, W. Pan, G.H. Farr, Flynn C. III, H. Yuan, S. Takada, D. Kimelman, L. Li, D. Wu, *Mol. Cell* 7 (2001) 801–809.
- [34] E. Esen, J. Chen, C.M. Karner, A.L. Okunade, B.W. Patterson, F. Long, *Cell Metab.* 17 (2013) 745–755.
- [35] H. Yang, X. Wang, Y. Zhang, H. Liu, J. Liao, K. Shao, Y. Chu, G. Liu, *J. Cell. Physiol.* 229 (2014) 17–26.
- [36] H. Xia, R.S. Nho, J. Kahm, J. Kleidon, C.A. Henke, *J. Biol. Chem.* 279 (2004) 33024–33034.
- [37] Y. Pylayeva, K.M. Gillen, W. Gerald, H.E. Beggs, L.F. Reichardt, F.G. Giancotti, *J. Clin. Invest.* 119 (2009) 252–266.
- [38] N. Semaan, G. Alsaleh, J.E. Gottenberg, D. Wachsmann, J. Sibilia, *J. Immunol.* 180 (2008) 3485–3491.
- [39] B. Tavora, L.E. Reynolds, S. Batista, F. Demircioglu, I. Fernandez, T. Lechertier, D.M. Lees, P.P. Wong, A. Alexopoulos, G. Elia, A. Clear, A. Ledoux, J. Hunter, N. Perkins, J.G. Gribben, K.M. Hodivala-Dilke, *Nature* 514 (2014) 112–116.
- [40] M. Martin, K. Rehani, R.S. Jope, S.M. Michalek, *Nat. Immunol.* 6 (2005) 777–784.
- [41] E. Beurel, S.M. Michalek, R.S. Jope, *Trends Immunol.* 31 (2010) 24–31.
- [42] J.E. Forde, T.C. Dale, *Cell. Mol. Life Sci.* 64 (2007) 1930–1944.
- [43] H. Koh, K.H. Lee, D. Kim, S. Kim, J.W. Kim, J. Chung, *J. Biol. Chem.* 275 (2000) 34451–34458.
- [44] L. Tao, S. Wadsworth, J. Mercer, C. Mueller, K. Lynn, J. Siekierka, A. August, *Biochem. J.* 363 (2002) 175–182.
- [45] K. Fukumura, Y. Yamashita, M. Kawazu, E. Sai, S. Fujiwara, N. Nakamura, K. Takeuchi, M. Ando, K. Miyazono, T. Ueno, K. Ozawa, H. Mano, *Oncol. Rep.* 30 (2013) 1542–1548.
- [46] J.K. Slack-Davis, S.T. Eblen, M. Zecevic, S.A. Boerner, A. Tarcsafalvi, H.B. Diaz, M.S. Marshall, M.J. Weber, J.T. Parsons, A.D. Catling, *J. Cell Biol.* 162 (2003) 281–291.
- [47] G.L. Zhou, Y. Zhuo, C.C. King, B.H. Fryer, G.M. Bokoch, J. Field, *Mol. Cell. Biol.* 23 (2003) 8058–8069.
- [48] J.D. Allen, Z.M. Jaffer, S.J. Park, S. Burgin, C. Hofmann, M.A. Sells, S. Chen, E. Derr-Yellin, E.G. Michels, A. McDaniel, W.K. Bessler, D.A. Ingram, S.J. Atkinson, J.B. Travers, J. Chernoff, D.W. Clapp, *Blood* 113 (2009) 2695–2705.
- [49] C. Scheidereit, *Oncogene* 25 (2006) 6685–6705.
- [50] T. Strowig, J. Henao-Mejia, E. Elinav, R. Flavell, *Nature* 481 (2012) 278–286.
- [51] B.N. Kalali, G. Kollisch, J. Mages, T. Muller, S. Bauer, H. Wagner, J. Ring, R. Lang, M. Mempel, M. Ollert, *J. Immunol.* 181 (2008) 2694–2704.
- [52] K. Kimura, Y. Matsuzaki, Y. Nishikawa, H. Kitamura, E. Akasaka, D. Rokunohe, H. Nakano, T. Imaizumi, K. Satoh, D. Sawamura, *J. Dermatol. Sci.* 66 (2012) 64–70.
- [53] A. Weber, Z. Kirejczyk, R. Besch, S. Potthoff, M. Leverkus, G. Hacker, *Cell Death Differ.* 17 (2010) 942–951.
- [54] G. Kollisch, B.N. Kalali, V. Voelcker, R. Wallich, H. Behrendt, J. Ring, S. Bauer, T. Jakob, M. Mempel, M. Ollert, *Immunology* 114 (2005) 531–541.
- [55] O. Grimstad, H. Husebye, T. Espevik, *J. Dermatol. Sci.* 72 (2013) 45–53.
- [56] H.C. Dan, M.J. Cooper, P.C. Cogswell, J.A. Duncan, J.P. Ting, A.S. Baldwin, *Genes Dev.* 22 (2008) 1490–1500.
- [57] R.C. Patel, G.C. Sen, *EMBO J.* 17 (1998) 4379–4390.
- [58] K.H. Kok, P.Y. Lui, M.H. Ng, K.L. Siu, S.W. Au, D.Y. Jin, *Cell Host Microbe* 9 (2011) 299–309.
- [59] A. Bacquin, C. Pouvelle, N. Siaud, M. Perderiset, S. Salomé-Desnoulez, C. Tellier-Lebegue, B. Lopez, J.-B. Charbonnier, P.L. Kannouche, *Nucleic Acids Res.* 14 (2013) 6501–6513.
- [60] H. Jo, P.K. Lo, Y.T. Li, F. Loison, S. Green, J. Wang, L.E. Silberstein, K.Q. Ye, H.X. Chen, H.B.R. Luo, *Proc. Natl. Acad. Sci. U. S. A.* 108 (2011) 6486–6491.
- [61] T. Steinbrunn, T. Stuhmer, C. Sayehli, M. Chatterjee, H. Einsele, R.C. Bargou, *Br. J. Haematol.* 159 (2012) 430–440.
- [62] M.C. Mendoza, E.E. Er, J. Blenis, *Trends Biochem. Sci.* 36 (2011) 320–328.
- [63] M. Brusilovsky, B. Rosental, U. Hadad, K. Campbell, A. Porgador, *J. Immunol.* 188 (2012).
- [64] T. Abbas, A. Dutta, *Cancer* 9 (2009) 400–414.
- [65] A. Ciccia, A.V. Nimonkar, Y. Hu, I. Hajdu, Y.J. Achar, L. Izhar, S.A. Petit, B. Adamson, J.C. Yoon, S.C. Kowalczykowski, D.M. Livingston, L. Haracska, S.J. Elledge, *Mol. Cell* 47 (2012) 396–409.
- [66] F.J. Fattah, K. Hara, K.R. Fattah, C. Yang, N. Wu, R. Warrington, D.J. Chen, P. Zhou, D.A. Boothman, H. Yu, *PLoS Genet.* 10 (2014) e1004419.
- [67] J.L. Brugnano, B.K. Chan, B.L. Seal, A. Panitch, *J. Control. Release* 155 (2011) 128–133.
- [68] Y.H. Ou, M. Torres, R. Ram, E. Formstecher, C. Roland, T. Cheng, R. Brekken, R. Wurz, A. Tasker, T. Polverino, S.L. Tan, M.A. White, *Mol. Cell* 41 (2011) 458–470.
- [69] N.M. Dagia, G. Agarwal, D.V. Kamath, A. Chetrapal-Kunwar, R.D. Gupta, M.G. Jadhav, S.S. Dadarkar, J. Trivedi, A.A. Kulkarni-Almeida, F. Kharas, L.C. Fonseca, S. Kumar, M.R. Bhande, *Am. J. Physiol. Cell Physiol.* 298 (2010) C929–C941.
- [70] L. Luo, A.A. Wall, J.C. Yeo, N.D. Condon, S.J. Norwood, S. Schoenwaelder, K.W. Chen, S. Jackson, B.J. Jenkins, E.L. Hartland, K. Schroder, B.M. Collins, M.J. Sweet, J.L. Stow, *Nat. Commun.* 5 (2014) 4407.
- [71] M. Marques, A. Kumar, A.M. Poveda, S. Zuluaga, C. Hernandez, S. Jackson, P. Pasero, A.C. Carrera, *Proc. Natl. Acad. Sci. U. S. A.* 106 (2009) 7525–7530.
- [72] A.M. Pedley, M.A. Lill, V.J. Davisson, *PLoS ONE* 9 (2014) e102481.
- [73] A. Kotlyarov, A. Neininger, C. Schubert, R. Eckert, C. Birchmeier, H.D. Volk, M. Gaestel, *Nat. Cell Biol.* 1 (1999) 94–97.
- [74] A. Neininger, D. Kontoyiannis, A. Kotlyarov, R. Winzen, R. Eckert, H.D. Volk, H. Holtmann, G. Kollias, M. Gaestel, *J. Biol. Chem.* 277 (2002) 3065–3068.
- [75] R. Winzen, M. Kracht, B. Ritter, A. Wilhelm, C.Y. Chen, A.B. Shyu, M. Muller, M. Gaestel, K. Resch, H. Holtmann, *EMBO J.* 18 (1999) 4969–4980.
- [76] C. Ehling, N. Ronkina, O. Bohmer, U. Albrecht, K.A. Bode, K.S. Lang, A. Kotlyarov, D. Radzioch, M. Gaestel, D. Haussinger, J.G. Bode, *J. Biol. Chem.* 286 (2011) 24113–24124.
- [77] I.B. Johnsen, T.T. Nguyen, B. Bergstrom, E. Lien, M.W. Anthonsen, *Cytokine* 57 (2012) 337–346.
- [78] A.L. Groehler, D.A. Lannigan, *J. Cell Biol.* 190 (2010) 575–586.
- [79] C. Labbe, P. Goyette, C. Lefebvre, C. Stevens, T. Green, M.K. Tello-Ruiz, Z. Cao, A.L. Landry, J. Stempak, V. Annesse, A. Latiano, S.R. Brant, R.H. Duerr, K.D. Taylor, J.H. Cho, A.H. Steinhart, M.J. Daly, M.S. Silverberg, R.J. Xavier, J.D. Rioux, *Genes Immun.* 9 (2008) 602–612.