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A role for the Ankyrin repeat containing protein Ankrd17 in Nod1- and Nod2-mediated inflammatory responses

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ABSTRACT

Innate immune responses induced by the pattern-recognition receptors Nod1 and Nod2 play pivotal roles to combat infection and to instruct adaptive immunity. Here we identify Ankrd17 as a novel binding partner of Nod2 and show that its N-terminal domain mediates Nod2 binding. Knock-down and overexpression analysis revealed that Ankrd17 is functionally involved in Nod2- and Nod1-mediated responses in human myeloid and epithelial cells. In HeLa cells Ankrd17 contributed to pro-inflammatory responses induced by *Shigella flexneri*, however not to type I interferon responses induced by Sendai virus. In conclusion, this reveals a novel function for Ankrd17 in anti-bacterial innate immune pathways.

Structured summary of protein interactions:
NOD2 and **Ankrd17** colocalize by fluorescence microscopy (View interaction)

1. Introduction

Innate immune recognition of microbes is mediated by multiple germ-line encoded receptors of the host, termed pattern recognition receptors (PRRs) [1]. Important intracellular PRRs are the members of the nucleotide-binding domain and leucine-rich repeat containing (NLR) family. One of the best studied examples of NLR–PRRs being Nod1 and Nod2. Both proteins are activated by bacterial peptidoglyan (PGN) subunits and induce NF-κB and MAPK pathways in host cells that result in the production of pro-inflammatory cytokines and antimicrobial peptides [2].

Nod2 gained particular attention as it was the first susceptibility gene linked to early onset of the inflammatory bowel disease Crohn's disease. Beside loss-of function mutations in Nod2 that are associated with Crohn's disease, gain-of function mutations in Nod2 have been genetically linked to Blau Syndrome, a rare hereditary autoinflammatory disease [3].

The molecular details of the core signal transduction cascade used by Nod1 and Nod2 are pretty well defined. After activation of Nod1/2 by PGN, the kinase RIP2 is recruited and multiple ubiquitination events mediated by the baculovirus inhibitor of apoptosis proteins XIAP, cIAP1 and cIAP2 and likely other ubiquitin ligases trigger activation and recruitment of the TAK1 kinase complex that results in phosphorylation of MAPKKs and the IKK complex, ultimately inducing NF-κB and MAPK activation [4–6].

Tight regulation of Nod2 activity is achieved in part by interaction with negative regulatory proteins including Erbin, SHIP-1, JNKBP1, CAD, Beta-PIX, CD147 and AAMP [7–14] and by proteins that enhance Nod2-mediated responses such as the chaperons Hsp90 and SGT-1 [15,16] and FRMPD2 [17].

Ankrd17 was first identified in a gene-trapping approach as a marker for early liver development in the mouse [18]. It belongs to the family of ankyrin repeat-containing proteins and displays two distinct arrays of 15 and 10 ankyrin repeats in its amino-terminal region. Ankyrin repeats serve as protein–protein interaction modules and are involved in diverse biological processes including the regulation of the pro-inflammatory NF-κB signaling cascade [19].

Ankrd17 has been shown to contribute to DNA replication in the S-phase of the cell cycle in human cells [20]. In contrast, Ankrd17 knock-out mice die early in embryogenesis, displaying profound defects in the vascular development without obvious cell-division defects [21]. Notably, a function of Ankrd17 in cell-autonomous anti-viral innate immune regulation has recently been reported [22], underscoring a role of Ankrd7 in innate immunity.

Here we revealed a novel role of Ankrd17 in Nod1- and Nod2-mediated innate immune responses.

2. Materials and methods

2.1. Cell lines

HEK293T and HeLa cells were cultured in DMEM (Biochrom), and THP1 and THP1 blue (Invivogen, France) in VLE RPMI 1640

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(Biochrom) each containing 10% heat-inactivated FBS, 2 mM glutamine, and 100 U/ml each penicillin and streptomycin in 5% CO_2 at 37 °C. THP1 blue cells were additionally selected with 100 μg/ml Zeocin (Invivogen). All cell-lines were continuously tested for absence of mycoplasma contamination by PCR.

2.2. Plasmids and reagents

Flag-tagged Ankrd17 encoding expression vector [20] was a gift from Xin Ye (Chinese Academy of Sciences, Beijing). Myc-Ankrd17 and the indicated fragments of Ankrd17 were obtained by subcloning this Ankrd17 coding sequence by PCR-cloning into a pCDNA3.1 vector containing an N-terminal triple myc-tag using the following primer (5′–3′): Ankrd17 FL fl_fwd: GCGCGGTACCTATGGAGAAGGCGACGGTTCC, Ankrd17_rev_n: GCGCGCGGCCGCTCAGCCAAGCTGGTTCATATGCA , Ankrd17 n-terminal: Ankrd17_fl_fwd and Ankrd17_752_rev: GCGCGCGGCCGCTACAGTGCTTGAACTGGTACACGAG. Ankrd17 c-terminal: Ankrd17_752_fwd: GCGCGGTACCACCCATGGTTGTTCCACCTCAG and Ankrd17_rev_n.

Plasmids encoding FLAG-Nod1 and FLAG-Nod2 are described in [10,23]. A TLR2 expression vector (pUNO1-hTLR2) was obtained from Invivogen. Sendai virus (hen egg allantoid fluid) was obtained from Charles River Laboratories. For stimulation 160 hemagglutination units (HAU)/ml were used for the HeLa cells. TriDAP, MDP, LPS, Pam3CSK4 and TNF were obtained from Invivogen.

2.3. Indirect immunoflourescence microscopy

Cells were first transfected with 1 μg myc-tagged Ankrd17 using Lipofectamine 2000 (Invitrogen). After overnight incubation, cells were transfected with 1 μg FLAG-Nod2 plasmid using Lipofectamine 2000 (Invitrogen). After 16–24 h incubation cells were fixed and processed as described in [24].

Antibodies used: mouse anti-myc antibody 9E10 (Sigma), rabbit anti-mouse Alexa-Fluor 546 (Invitrogen), mouse anti-Nod2 6F6 [25], rabbit anti-rat Alexa-Fluor 488 (Invitrogen). DNA was stained using DAPI. Images were acquired on an Olympus Cell-R microscope and processed using ImageJ.

2.4. siRNA treatment

siRNA-based knockdown in HeLa and THP1 cells was performed as described in [26]. siRNAs used: Ankrd17_52 described in [20] synthesized by Qiagen, Ankrd17_69: SI04361371 (Qiagen) and All-Stars negative control (Qiagen).

2.5. Bacterial infection and gentamycin protection assays

For the infection *with Shigella flexneri*, HeLa cells were seeded in 24-well plates. Bacterial infection of the cells was performed using the *S. flexneri* strain M90T *afaE* as described previously [27]. Gentamycin (100 μg/ml) was added to the cells 30 min after addition of the bacteria. As control a non-invasive derivative (BS176 *afaE*) was used [28].

2.6. Co-immunoprecipitation and immunoblot analysis

Immunoprecipitations and immunoblots were conducted essentially as described previously [10]. Cells were transiently transfected with Ankrd17 constructs for 24 h and subsequently with Nod1 and Nod2 expression plasmids for another 24 h. Protein complexes were immunoprecipitated using 9E10-agarose (Santa Cruz). Antibodies used: mouse anti-FLAG M2 (Stratagene), rabbit anti-myc (Santa Cruz), rabbit anti-p38 (Cell Signaling) and mouse anti-p-p38 (Cell Signaling), HRP-conjugated goat anti-mouse IgG (Bio-Rad) and HRP-conjugated goat anti-rabbit IgG (Bio-Rad).

2.7. Luciferase reporter assays

Activation of inflammatory pathways was measured using a modification of the luciferase reporter assay described previously [29]. The cells were stimulated immediately after transfection as indicated. After 16 h of incubation, the cells were lysed, and the luciferase activity was measured. Luciferase activity was normalized to β-galactosidase activity. The means and standard deviations were calculated from triplets.

2.8. RNA preparation and RT-PCR

Total RNA was extracted from cells using the RNeasy kit (Qiagen). 1 μg of RNA was reverse transcribed using the First-Strand cDNA synthesis kit (Fermentas).

For quantitative PCR analyses, 50 ng cDNA was analyzed in a total volume of 25 μl using the iQ SYBR Green Supermix (Bio-Rad), according to the manufacturer's protocol. All quantitative PCR reactions were run on a Bio-Rad iQ5 cycler, and data were evaluated by the iQ5 system software (version 2.0) using the ΔΔCT method.

For amplification of fragments, the following primers were used (5′-3′): Ankrd17_fwd: AATGTTGCCACCACTCTTCC, Ankrd17_rev: TGCAGCTGTGCATTCTTTTC, IL8_fwd: ATGACTTCCAAGCTGGCCGTGGCT, IL8_rev: TCTCAGCCCTCTTCAAAAACTTCTC, IFN-beta_fwd: ACTGCCTCAAGGACAGGATG, IFN-beta_rev: GGCCTTCAGGTAATGCAGAA [30]. Primer for Nod1, Nod2 and GAPDH are described in [31].

2.9. Measurement of cytokines

CCL5, IL-8 and IL-6 was measured in the supernatants using the corresponding ELISA kits (Duoset, R&D Systems) according to the manufacturer's conditions.

2.10. Statistical analysis

The data were analyzed using two-sided Student's *t* test with Microsoft Excel. The differences were regarded as significant (*) when $P < 0.05$ and highly significant (**) when $P < 0.005$.

3. Results

3.1. The N-terminal part of Ankrd17 binds to Nod2

We recently conducted a yeast-two hybrid screen using human Nod2 as bait. This work identified several candidates as Nod2 binding proteins, including AAMP and Erbin that we and others verified as Nod2-binding partners with regulatory functions in Nod2 signaling [7,10]. Among the candidates of the initial screen was also an N-terminal fragment of the human Ankrd17 protein that covers most of the first N-terminal ankyrin repeat region of this protein (Fig. 1A).

In order to validate Ankrd17 as a binding partner of Nod2, we conducted co-immunoprecipitation experiments in HEK293T cells co-expressing FLAG-tagged Nod1 or Nod2 together with myc-tagged Ankrd17 or a C-terminal truncation mutant thereof. We observed that both Ankrd17 as well as the N-terminal fragment of Ankrd17 (aa1-752), covering the region identified in the Y2H screen, showed interaction with Nod2 but no strong binding to Nod1 (Fig. 1B).

We found ectopically expressed Ankrd17 to be localized mainly in the cytoplasm of HeLa cells. Ankrd17 showed a partial co-localization with ectopically expressed Nod2 in particular at sites near the plasma membrane (Fig. 1C).

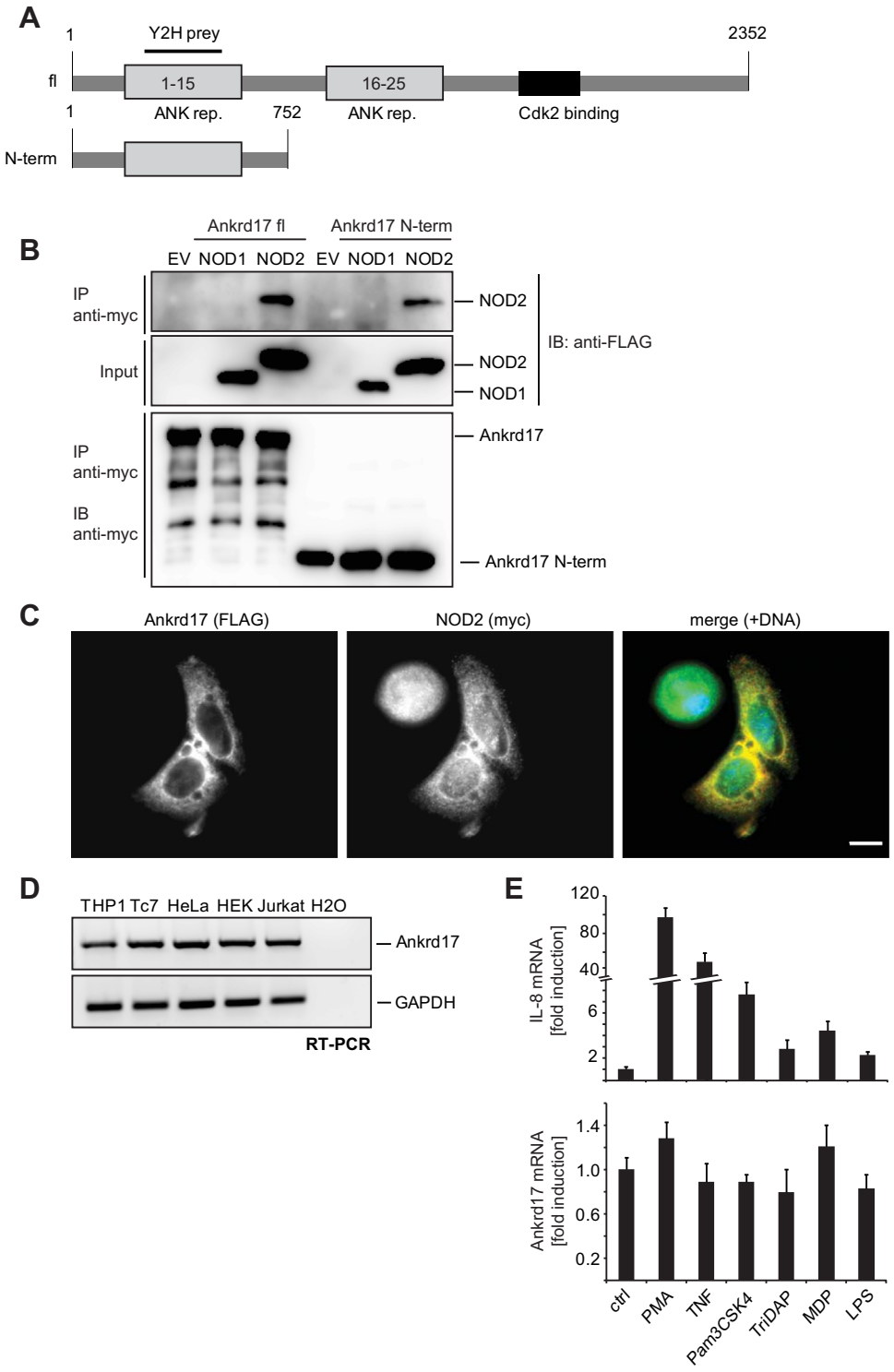

Fig. 1. Ankrd17 interacts with Nod2. (A) Schematic representation of the Ankrd17 proteins used. Ankyrin repeats are represented by gray boxes and are indicated by numbers. (B) Co-immunoprecipitation of ectopically expressed Ankrd17 with Nod1 and Nod2 in HEK293T cells. HEK293T cells were transiently transfected with FLAG-Nod1 and FLAG-Nod2 together with myc-Ankrd17 or a amino-terminal fragment (N-term) of Ankrd17. Ankrd17 constructs were immunoprecipitated (IP) by anti-myc antibody and co-precipitated Nod2 and Nod1 detected by immunoblot (IB), EV: empty vector. (C) Indirect immunofluorescence microscopy of HeLa cells transiently transfected with FLAG-Ankrd17 (red) and myc-Nod2 (green). DNA is stained in blue by DAPI. Bar = 10 μm. (D) Relative expression of Ankrd17 mRNA in different human cell lines. RT-PCR products with primers specific for Ankrd17 and GAPDH (as control) are shown. (E) Ankrd17 and IL-8 mRNA levels in THP1 cells treated for 6 h with PMA (100 nM), TNF (50 ng/ml), Pam3CSK4 (100 ng/ml), TriDAP (10 μg/ml), MDP (10 μM) or LPS (100 ng/ml). Quantitative PCR data, normalized for GAPDH expression relative to non-treated cells (CTL) set to 1 is shown as mean + S.D. (n = 3).

Ankrd17 was expressed in all human cell lines we tested, including myeloid THP1 cells that are known to also express functional Nod2 [31] (Fig. 1D). Ankrd17 mRNA expression was not significantly changed by differentiation of myeloid THP1 cells into macrophage-like cells by phorbol 12-myristate 13-acetate (PMA) or by stimulation of these cells with the TLR2 and TLR4 ligands

or Nod1/2 elicitors although all these treatments led to the expected strong induction of IL-8 mRNA (Fig. 1E).

3.2. Ankrd17 affects Nod1- and Nod2-mediated pro-inflammatory responses

Next, in order to elucidate if Ankrd17 contributed to Nod2-mediated signaling, we overexpressed Ankrd17 in HEK293T human embryonic kidney cells (HEK293T) together with Nod1 and Nod2. Nod1/2 were activated by TriDAP and MDP and Nod1/2-mediated NF-κB activation was measured by luciferase gene-reporter assays. We observed that Ankrd17 significantly increased both Nod1- and Nod2- but not TLR2-mediated NF-κB activation in a dose-dependent manner (Fig. 2A).

In order to address the function of endogenous Ankrd17 we used siRNA mediated knock-down of Ankrd17 in THP1 cells stably expressing Secreted Alkaline Phosphatase (SEAP) under the control of a NF-κB responsive promoter (THP1 blue) as these cells do respond well to the Nod1 and Nod2 elicitors TriDAP and MDP [31]. 48 h after transfection with two Ankrd17 specific or a non-targeting siRNA as control, the cells were treated with TriDAP to activate Nod1, MDP to trigger Nod2 and LPS to activate TLR4-mediated responses, respectively. Subsequently, NF-κB activation was measured by quantification of SEAP in the supernatant. In accordance with the results obtained when overexpressing Ankrd17 in HEK293T cells, treatment with both Ankrd17 specific siRNA duplexes resulted in a reduction of both Nod1- and Nod2-mediated NF-κB responses. In sharp contrast, LPS-induced NF-κB activation

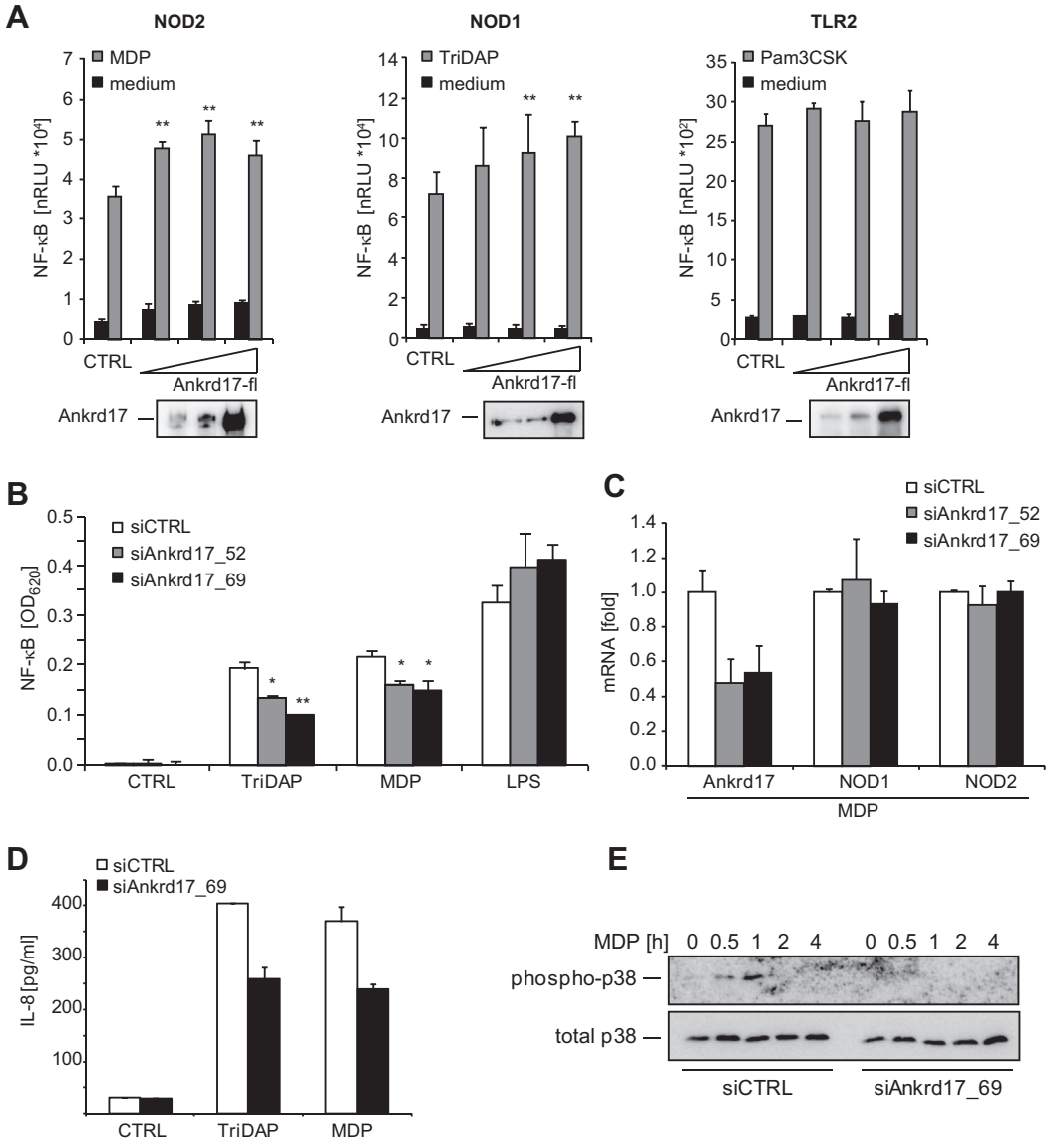

Fig. 2. Ankrd17 affects Nod1- and Nod2-induced pro-inflammatory responses. (A) Luciferase NF-κB gene-reporter assays in HEK293T cells. Three different amounts (5, 10 and 20 ng) of expression plasmids encoding Ankrd17 were transiently expressed in HEK293T cells together with Nod2 (left panel), Nod1 (middle panel) or TLR2 (right panel) together with the reporter system. Cells were treated with TriDAP, MDP or Pam3CSK4 where indicated (gray bars) or left untreated (black bars). Normalized mean luciferase activity + S.D. ($n = 6$) from three independent experiments (one representative experiment out of two for TLR2) is shown ($^{**}P < 0.005$, $^{*}P < 0.05$). Expression of the Ankrd17 protein was controlled by immunoblotting (lower panels). (B) Knock-down of Ankrd17 in PMA differentiated THP1 blue cells. THP1 blue cells were transfected with a Ankrd17 specific siRNA or a non-targeting siRNA duplex as control for 48 h. Cells were stimulated with 10 μg/ml TriDAP, 10 μM MDP or 100 ng/ml LPS for 16 h and SEAP activity as proxy for NF-κB activation was determined in the supernatant. Mean activity + S.D. ($n = 3$) representative of three independent experiments is shown ($^{**}P < 0.005$, $^{*}P < 0.05$). (C) Relative levels of Anrkd17, Nod1 and Nod2 mRNA in the MDP stimulated cells from (C) are shown by qPCR as mean + S.D. ($n = 3$). (D) Knock-down of Ankrd17 in PMA differentiated THP1 cells. THP1 cells were transfected with the Ankrd17 specific siRNA (#69) or a non-targeting siRNA duplex as control for 48 h. Cells were stimulated with TriDAP or MDP and IL-8 was determined by ELISA (mean + S.D. $n = 3$). Data representative of two independent experiments is shown. (E) Immunoblotting for phospho-p38 and p38 as loading control in cells treated as in (D). Cells were stimulated with MDP for up to 4 h and harvested at the indicated times.

was not decreased by the Ankrd17 siRNAs (Fig. 2B). The Ankrd17 specific siRNA duplexes efficiently reduced Ankrd17 although did not affect Nod1 or Nod2 expression (Fig. 2C). SiRNA-mediated Ankrd17 knock-down also reduced MDP and TriDAP mediated IL-8 secretion in differentiated THP1 cells, that did not contain the reporter (Fig. 2D). Also Nod2-mediated p38 activation [32] was strongly reduced in cells treated with the Ankrd17 specific siRNA 30–60 min after stimulation (Fig. 2E).

3.3. Ankrd17 affects bacterial induced pro-inflammatory cytokine release but not viral induced type I interferon responses

To corroborate a role of Ankrd17 in Nod-mediated innate immune responses we next tested the influence of Ankrd17 knock-down on the cytokine response of HeLa cells after infection with the invasive Gram-negative pathogen *S. flexneri*, a well defined trigger of Nod1- and Nod2-mediated signaling [10,33]. To this end, we infected HeLa cells that had been treated for 48 h with either of two Ankrd17-specific siRNAs or a non-targeting siRNA as control with the invasive *S. flexneri* strain M90T *afaE*. Reduction of endogenous Ankrd17 mRNA levels by either siRNA duplex resulted in significantly impaired IL-8 secretion compared to cells treated with a non-targeting control siRNA (Fig. 3A). In contrast, infection of the cells with the non invasive, *S. flexneri* strain BS176 *afaE* did not trigger any relevant IL-8 release (Fig. 3A). Similar results were observed for IL-6 secretion, although IL-6 production was less affected by lack of Ankrd17 (data not shown). Of note, reduced expression of Ankrd17 did not affect bacterial invasion and propagation up to 6 h post infection as shown by gentamycin protection assays to monitor viable intracellular bacteria (Fig. 3B).

Recently, it was reported that Ankrd17 has an influence in Rig-I mediated type I interferon responses in colon epithelial cells [22]. To investigate if Ankrd17 might affect this pathway also in HeLa cells, we infected HeLa cells with Sendai virus (SeV), a ssRNA virus that activates TLR3 and Rig-I pathways. Efficient knock-down of Ankrd17 mRNA as shown by quantitative PCR (Fig. 3C, left panel) influenced SeV-induced interferon-beta mRNA levels only marginally at 6 h (data not shown) and 16 h post infection (Fig. 3C). In the same experiment we also measured the release of CCL5 (RANTES), a cytokine that is strongly induced by SeV in HeLa cells. In accordance with the qPCR data, we found no significant effect of Ankrd17 knock-down on SeV-induced CCL5 levels at 6 h (data not shown) or 16 h post infection (Fig. 3D).

4. Discussion

We report here on a novel role of Ankrd17 in Nod1- and Nod2-mediated innate immune pathways suggesting a more general involvement of Ankrd17 in innate immune signaling.

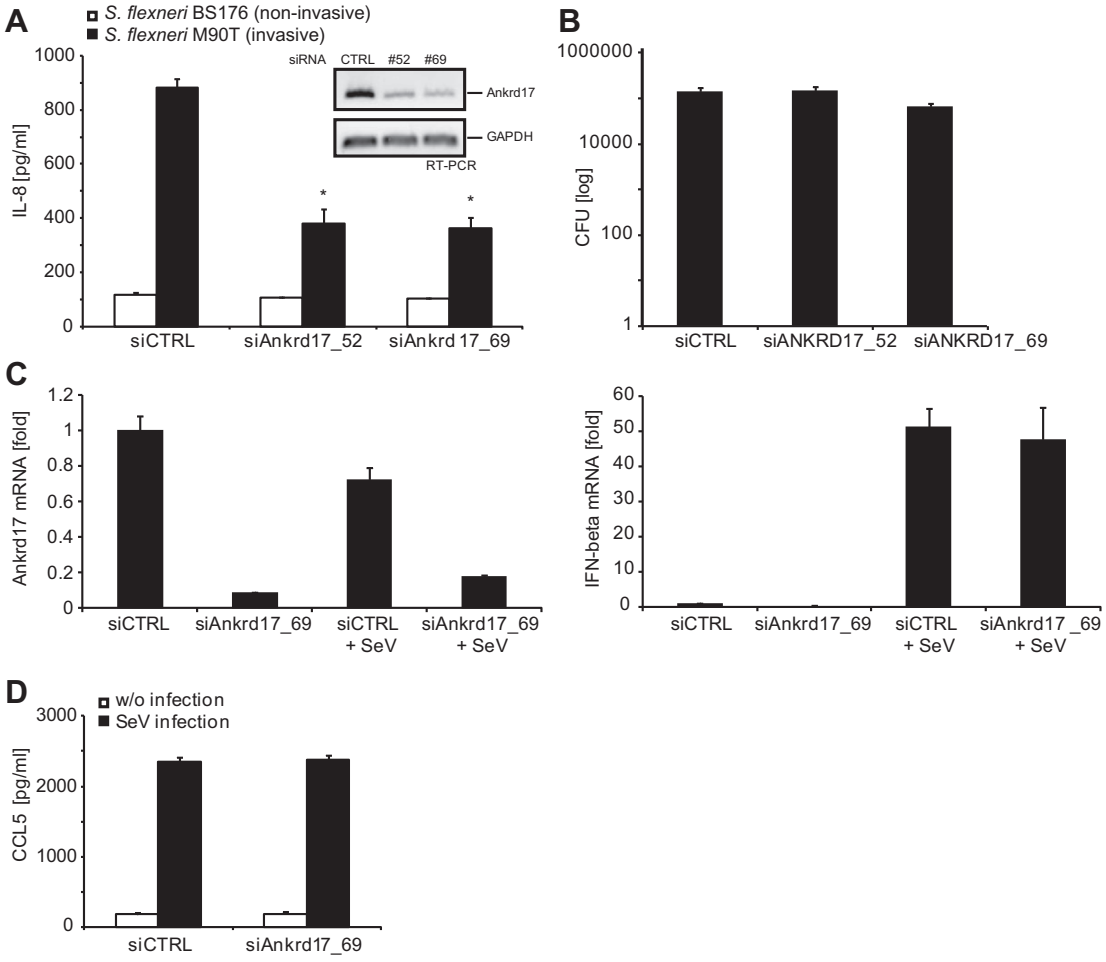

Fig. 3. Ankrd17 specifically contributes to bacterial induced responses in HeLa cells. (A) HeLa cells were treated with the indicated siRNA duplex for 48 h and infected with *S. flexneri* M90T afaE (invasive) or *S. flexneri* BS167 afaE (non-invasive) for 6 h. IL-8 secretion was determined by ELISA (mean + S.D., $n = 3$, $^{*}P < 0.05$). Knock-down of Ankrd17 was verified by end-point RT-PCR in the same cells using GAPDH as control (inlay). Data are representative for at least four independent experiments. (B) Gentamycin protection assay to monitor bacterial uptake and propagation of *Shigella* in HeLa cells treated as in (A). (C) HeLa cells treated with control siRNA or Ankrd17 siRNA (#69) for 48 h were infected with SeV overnight. Subsequently interferon-beta and Ankrd17 mRNA levels were determined by quantitative PCR. Fold induction relative to non-infected cells treated with control siRNA set to 1 is shown as mean + S.D. ($n = 3$). (D) ELISA to determine CCL5 secretion in the supernatant of the cells shown in (C) 16 h post infection with SeV (mean + S.D., $n = 3$). Data are representative for at least two independent experiments.

How Ankrd17 contributes at the molecular level to Nod1 and Nod2 signaling remains elusive. As Nod1-mediated responses were affected by Ankrd17, but Ankrd17 did not bind to Nod1 in our assays suggests that Ankrd17 might regulate other factors needed for Nod1 and Nod2 mediated responses. Still, as Ankrd17 did not affect TLR2 and TLR4-mediated NF-κB activation such a factor would be specific for the Nod1/2 pathway. The presence of anykrin repeats in Ankrd17, which serve as interaction modules of the IKK kinase complex makes it tempting to speculate that Ankrd17 might serve to bridge IKK to other factors involved in Nod1/2 signaling. Candidates for such proteins include Rip2, as well as the ubiquitin ligases XIAP and cIAP1,2 that have recently been reported to be critical for Rip2-induced NF-κB activation [4,5].

A recent paper showed that Ankrd17 also contributes to innate immune responses by regulating the viral dsRNA receptor Rig-I [22]. The authors of this study show that Ankrd17 binds to the RLRs Rig-I and MDA5 and their adaptor proteins and provide evidence that Ankrd17 enhances Rig-I and MDA5-mediated responses by promoting the interaction of RLRs with VISA. Of note, the interaction with Rig-I was mapped to the second ankyrin-repeat region, whereas as 1–752 of Ankrd17 mediate the interaction with Nod2. Wang et al. showed that knock-down of Ankrd17 in colon epithelial cells impairs Sendai virus-induced type I interferon responses [22]. We however, observed that Ankrd17 only marginally at best, contributed to Sendai virus induced type I interferon responses in HeLa cells. This discrepancy might be explained by the cell type used, i.e. that HeLa cells, in contrast to colon cell lines express other RNA sensing PRRs including TLR3, that might bypass Rig-I mediated signaling. Of note, according to database entries and our own unpublished results, Ankrd17 is expressed as at least two isoforms. So differential expression of these could also well explain the apparently diverse contributions of Ankrd17 to anti-viral and anti-bacterial innate immunity in different cell-lines. Future studies will help to define the molecular details of the involvement of Ankrd17 in these processes.

In conclusion, our data puts Ankrd17 on the growing list of proteins that contribute to Nod1 and Nod2 signaling. Targeting Ankrd17 might thus be an option for the development of new strategies to counteract diseases related to misfunctional Nod2, such as Blau Syndrome and Crohn's disease.

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