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Abstract: C-Cbl-associated protein (CAP), also known as Sorbin and SH3 domain-containing protein 1 (Sorbs1) or ponsin, an adaptor protein of the insulin-signalling pathway, mediates anti-viral and anti-cytotoxic protection in acute viral heart disease. In the present study we describe a novel protective immuno-modulatory function of CAP in inflammation. Among the three members of the Sorbs family of adapter molecules, which include CAP (Sorbs1), ArgBP2 (Sorbs2), and Vinexin (Sorbs3), CAP consistently down-regulated the expression of pro-inflammatory cytokines in mouse fibroblasts, cardiomyocytes, and myeloid-derived leukocytes, after Toll-like receptor (TLR) stimulation. Upon the same TLR stimulation, ArgBP2 partially down-regulated pro-inflammatory cytokine production in mouse fibroblasts and cardiomyocytes, while Vinexin rather promoted their production. Mechanistically, CAP limited pro-inflammatory cytokine expression by suppressing the phosphorylation of Inhibitor of kappa B (I B) kinase (I)- and I - and their downstream NF- B-dependent signalling pathway. Molecular affinity between CAP and I - / I - was necessary to block the NF- B pathway. The CAP-dependent inhibitory mechanism - in vivo exclusively IL-6 inhibition - was confirmed after collecting blood from mice with systemic inflammation induced by lipopolysaccharide (LPS) and in the heart tissue collected from mice infected with the cardiotropic Coxsackievirus B3 (CVB3). Taken together, CAP down-regulates pro-inflammatory cytokines by interfering with the normal function of the NF- B pathway. The promotion of CAP production could support the development of new strategies aiming to limit excessive and detrimental activation of the immune system.

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ABSTRACT

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Among the three members of the Sorbs family of adapter molecules, which include CAP (Sorbs1), ArgBP2 (Sorbs2), and Vinexin (Sorbs3), CAP consistently down-regulated the expression of pro-inflammatory cytokines in mouse fibroblasts, cardiomyocytes, and myeloid-derived leukocytes, after Toll-like receptor (TLR) stimulation. Upon the same TLR stimulation, ArgBP2 partially down-regulated pro-inflammatory cytokine production in mouse fibroblasts and cardiomyocytes, while Vinexin rather promoted their production. Mechanistically, CAP limited pro-inflammatory cytokine expression by suppressing the phosphorylation of Inhibitor of kappa B (IκB) kinase (Iκκ)-α and Iκκ-β and their downstream NF-κB-dependent signalling pathway. Molecular affinity between CAP and Iκκ-α/ Iκκ-β was necessary to block the NF-κB pathway. The CAP-dependent inhibitory mechanism - in vivo exclusively IL-6 inhibition - was confirmed after collecting blood from mice with systemic inflammation induced by lipopolysaccharide (LPS) and in the heart tissue collected from mice infected with the cardiotropic Coxsackievirus B3 (CVB3).

Taken together, CAP down-regulates pro-inflammatory cytokines by interfering with the normal function of the NF-κB pathway. The promotion of CAP production could support the development of new strategies aiming to limit excessive and detrimental activation of the immune system.

1. Introduction

Infections trigger the innate immune system through Toll-like receptors (TLRs) and RIG-I-like receptors (RLRs), promoting antigen presenting cells (APC) to produce pro-inflammatory cytokines, such as IL-6 and TNF-α, which in turn induce acute phase proteins [1,2]. Extensive activation of pro-inflammatory innate pathways leads to self-protein release, tissue damage, and ultimately autoimmunity or general hyperinflammatory conditions, such as cytokine-release syndrome (CRS) [3–6].

The sorbin homology (SoHo) family of adapter and scaffold proteins consists of three proteins: CAP, also known as Sorbin and SH3 domain containing 1 (Sorbs1), ArgBP2, also known as Sorbs2, and Vinexin, also known as Sorbs3 [7]. All Sorbs proteins are highly expressed in heart tissue, skeletal muscle, adipose tissue, and cells of the immune system, functioning as SH3-domain-mediated adaptors of scaffolding molecules. CAP, together with nectin and afadin, composes the NAP cell-cell adhesion system [8]. CAP modulates cell adhesion, migration, cytoskeleton reorganisation, membrane trafficking, and intracellular signalling [7,9,10]. A protective anti-viral function of CAP in Coxsackievirus B3 (CVB3)-induced myocarditis has been also discovered [11]. CAP promotes type I interferon production and at the same time limits cytotoxic cytokine release, tuning a balanced and non-detrimental anti-viral response. These data point toward a potential regulatory role of CAP in the innate immune system. In LPS-induced sepsis, regulation of ArgBP2 by the micro RNA miR-21-3p contributes to cardiac dysfunction in

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affected mice, suggesting that ArgBP2 may have a detrimental function in heart inflammation [12]. On the other side, the Vinexin isoform Vinexin-β has been shown to promote inflammation by inducing NF-κB activation and cytokine production in a mouse model of myocardial infarction and also in a mouse model of atherosclerosis [13,14].

Pathogen- and damage-associated molecular patterns (PAMPs and DAMPs, respectively) are recognized by different surface and cytoplasmic pattern recognition receptors (PPRs), such as TLRs and RLRs. Downstream of these receptors, the NF-κB pathway is activated and regulates the expression of pro-inflammatory cytokines, such as *Il-6, Tnf-α*, and *Ip-10* [15]. The adapter protein MyD88 promotes phosphorylation of the kinases IRAK1, IRAK2, and IRAK4 upon TLR stimulation (except for TLR3), which then dissociate from MyD88 and associate with the ubiquitin ligase TRAF6 [16]. The complex made up of TAK1, TAB1, TAB2, and TAB3 is activated by the free lysine polyubiquitin chain and phosphorylates IκB kinase (Iκκ)-β [17]. The Iκκ complex, which is composed of Iκκ-α, Iκκ-β, and Iκκ-γ, phosphorylates the inhibitory κB (IκB) protein, which undergoes proteosomal degradation thereby promoting nuclear translocation and phosphorylation of NF-κB, which activates the transcription of pro-inflammatory cytokines [18].

Since the role of CAP in inflammation has never been elucidated, in this study we figured out how proteins of the Sorbs family of adapter proteins regulate NF-κB-dependent production of pro-inflammatory cytokines by using different primary cells and a cardiac cell line. Furthermore, we confirmed the anti-inflammatory function of CAP in vivo.

2. Materials and methods

2.1. Mice

$CAP^{-/-}$ C57Bl/6 mice, also known as $Sorbs1^{-/-}$ mice, were previously described in references [10,11]. All animal experiments were conducted in accordance with the Animal Care Committee of the University of Zurich. Lipopolysaccharides (LPS, Sigma-Aldrich) at a concentration of 30 mg/kg body weight was intraperitoneally injected into $CAP^{+/+}$ and $CAP^{-/-}$ C57Bl/6 mice. Two hours later blood was collected from the tail vein into serum tubes for cytokine measurement by ELISA. Viral myocarditis was induced as previously described [11].

2.2. Cells, viruses, and plasmids

Mouse embryonic fibroblasts (MEF) were isolated from embryos at day 13.5 of gestation. Bone marrow-derived dendritic cells (BMDC) and bone marrow-derived macrophages (BMM) were cultivated as previously described [6,19]. For mouse neonatal fibroblasts (MNF), the ventricles of 1-day old newborns were prepared as previously described [11,20]. Briefly, heart ventricles were minced in small pieces and then digested with trypsin (0.8 mg/ml). Ventricular cell suspensions were plated for two hours at 37 °C to allow the MNF to attach to the plate. Cells in suspension were removed and MNF were washed several times with warm PBS before using them for experiments. HL-1 cells were maintained in Claycomb medium (Sigma) and expanded in 75 cm^2 flasks pre-coated with 5 μg/ml fibronectin (Sigma) and 0.02% gelatin (Sigma) as previously described [21]. HEK-Blue-hTLR4 cells with the inducible SEAP (secreted embryonic alkaline phosphatase) reporter gene and the HEK-Blue Detection medium were purchased from Invivogen and used according to the manufacturer's protocol. The cardiovirulent CVB3 (Gauntt strain) and the vesicular stomatitis virus (VSV) have been described previously [11].

2.3. siRNA and plasmid transfection

For knockdown experiments, CAP (catalog number SI01429106), ArgBP2 (catalog number SI00872221) and Vinexin (catalog number SI01417206) siRNAs were purchased from Qiagen. The AllStars Neg. Control siRNA (catalog number 001027281, Qiagen) was used as siMock control, as previously described [21]. For transfection, 1×10^5 cells were plated in 12-well plate with 1 ml supplemented Claycomb medium. Cells were transfected with 25 pmol siRNA and RNAiMAX (Invitrogen) according to the manufacturer's protocol.

The FLAG-tagged CAP-WT and the CAP-ΔSH3 vectors, as well as the empty control vector, were previously described [11]. Transfection of 10^6 HEK cells was performed with the calcium phosphate precipitation method as previously described [21].

2.4. Quantitative RT-PCR (qRT-PCR)

To measure cytokine expression at the RNA level, RNA was isolated with Trizol Reagent (Invitrogen) according to the manufacturer's protocol. Reverse transcription was performed with 1 μg RNA. The $2^{-\Delta\Delta Ct}$ method was used for qRT-PCR gene expression analysis. Genes of interest were compared with the housekeeping gene GAPDH. Used primers have been previously described [20].

2.5. Cytokine measurement

For cytokine concentration measurements, two different methods were used. A screen of ten cytokines was performed with the Cytometry Bead Array (CBA) LEGENDplex Mouse Inflammation Panel (BioLegend), which included IL-1β, IL-6, IL-10, IL-12p70, IL-17A, IL-23, IFN-γ, TNF-α, GM-CSF, and MCP-1. The CBA were analyzed on a BD FACSCanto II. Commercially available ELISA Development Kits (PeproTech) were used to measure IL-6, TNF-α, and CCL5 (RANTES) after specific cell stimulations.

2.6. Western blot analysis and immunoprecipitation

Cell lysates were separated using NuPAGE Novex Bis-Tris gel (Invitrogen), transferred on PVDF membrane (Roche Diagnostics), and then immunoblotted with specific antibodies. Bands were visualized with a Licor scanner. For immunoprecipitation, Dynabeads Protein G (Invitrogen) were used according to the manufacturer's instructions. FLAG-tagged proteins were immunoprecipitated using anti-DDK Tag (L5) Affinity Gel (BioLegend).

2.7. Immunofluorescence staining

HeLa cells previously transfected with the FLAG-tagged CAP-WT vector were first fixed with 4% paraformaldehyde and then permeabilized with 0.2% Triton X-100. Blocking was performed with PBS containing 1% BSA and 0.2% Triton X-100. Anti-NF-κB p65 antibodies (dilution 1:250) and anti-FLAG antibodies (dilution 1:200) were incubated overnight at 4 °C. Secondary anti-rabbit AlexaFluor488 antibodies for NF-κB p65 and secondary anti-goat PE antibodies for FLAG were incubated at room temperature. Vectashield counting solution with DAPI was used to cover the slides and stain the nuclei. Cells were observed with a Leica fluorescent microscope.

2.8. Reagents

High molecular weight (HMW) Polyinosinic-Polycytidylic acid (PolyIC), low molecular weight (LMW) PolyIC, and the imidazoquinoline R848 were purchased from Invivogen. LPS and CpGs were purchased from Sigma. Antibodies to Iκκ-α/β, phosphorylated-Iκκ-α/β (p-Iκκ-α/β), Iκκ-α, Iκκ-β, Iκκ-γ, NF-κB p65, NF-B p-p65 (detects phosphorylation at serine 536 (Ser536)), p38, p-p38, ERK1/2, p-ERK1/2, MyD88, and IRAK-M were purchased from Cell Signaling Technology. Antibodies to IκB-α and GAPDH were purchased from Santa Cruz Biotechnology. Antibodies to FLAG were purchased from Sigma-Aldrich.

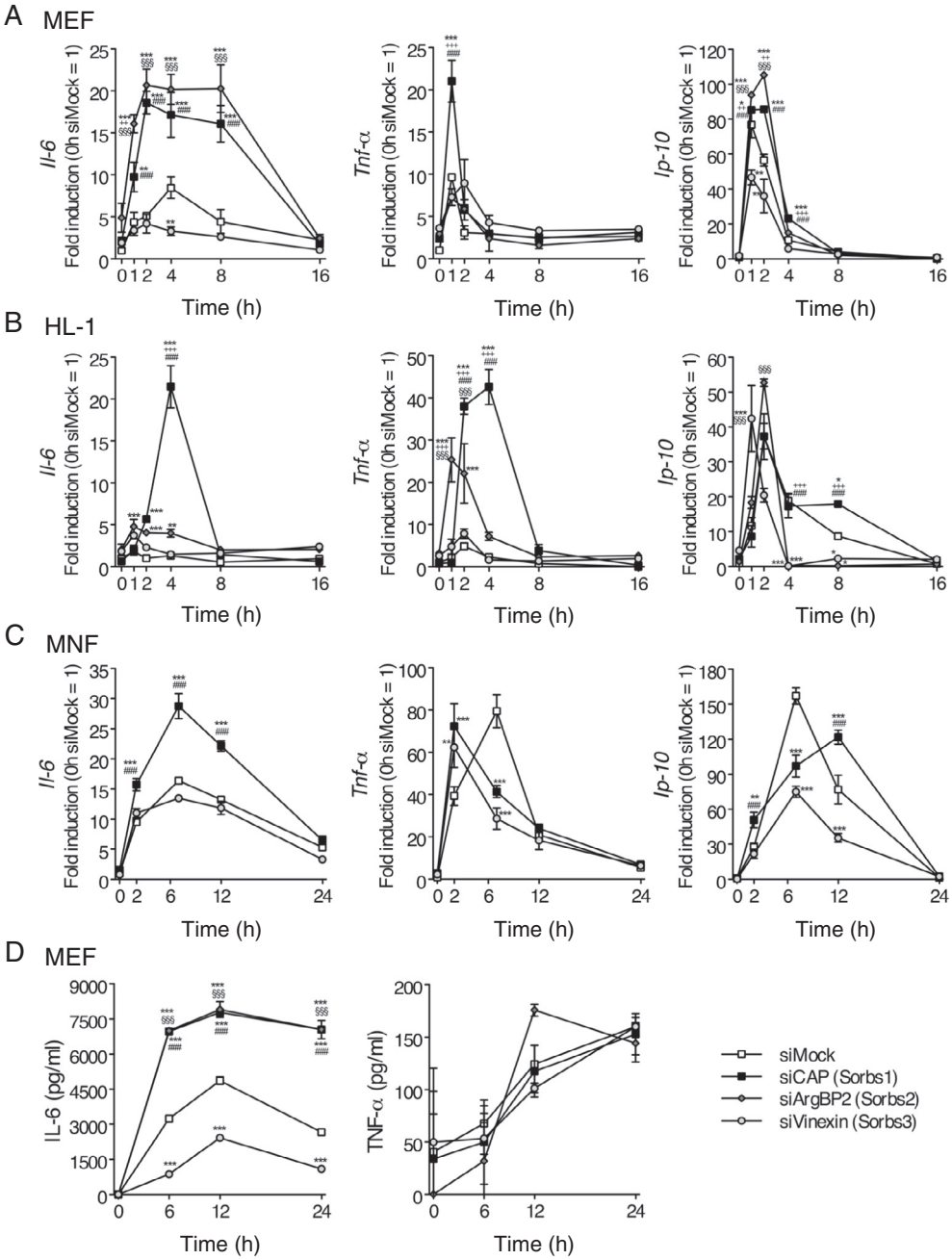

Fig. 1. CAP and partially ArgBP2, but not Vinexin, inhibit *Il-6* expression in knocked down cells. (A) CAP, ArgBP2, and Vinexin knockdown with siRNA in MEF stimulated with LPS for the indicated times. Transcription of the pro-inflammatory cytokines *Il-6*, *Tnf-α*, and *Ip-10* was determined by real-time RT-PCR. (B-C) CAP, ArgBP2, and Vinexin knockdown with siRNA in HL-1 cells (B), and CAP and Vinexin knockdown with siRNA in MNF (C) stimulated with LPS and IFN-γ (HL-1 cells) or with LPS (MNF) for the indicated times. Transcription of the pro-inflammatory cytokines *Il-6*, *Tnf-α*, and *Ip-10* was determined by real-time RT-PCR. (D) CAP, ArgBP2, and Vinexin knockdown with siRNA in MEF stimulated with LPS for the indicated times. Concentrations of IL-6 and TNF-α in supernatants collected at the indicated times was determined by ELISA. From A to D: means ± s.d. and values measured form one out of three independent experiments performed in duplicates are shown. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ for siCAP vs. siMock, siArgBP2 vs. siMock, or siVinexin vs. siMock. $^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.001$ for siCAP vs. siArgBP2. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ for siCAP vs. siVinexin. $^{\S}P < 0.05$, $^{\S\S}P < 0.01$, $^{\S\S\S}P < 0.001$ for siArgBP2 vs. siVinexin.

2.9. Statistics

Data were analyzed with 2-way ANOVA and Bonferroni post hoc testing. Statistical analysis was conducted using the Prism 6 software (GraphPad Software). All data were expressed as mean ± s.d. Differences were considered statistically significant for $P < 0.05$.

3. Results

3.1. CAP (Sorbs1) and ArgBP2 (Sorbs2), but not Sorbs3 (Vinexin), repress IL-6 and partially TNF-α expression in vitro

To identify which members of the Sorbs family may regulate inflammatory cytokine expression, each Sorbs member was knocked down by transfecting specific small-interfering RNA (siRNA) in MEF, HL-1 cardiomyocytes, and MNF. Knockdown efficiency is shown in Supplementary Fig. 1A and B. LPS stimulation was used to activate the TLR/NF-κB pathway. In MEF, CAP and ArgBP2 knockdown resulted in increased *Il-6* and *Ip-10* RNA expression, while *Tnf-α* RNA levels were significantly higher only upon CAP knockdown (Fig. 1A). Vinexin, on the contrary, showed opposite results. Indeed, upon Vinexin knockdown, *Il-6* and *Ip-10* RNA expression was significantly reduced compared to siMock-treated MEF (Fig. 1A). Induction of cytokine expression in HL-1 cardiomyocytes, as previously shown, was only achieved after co-stimulation with LPS and IFN-γ [21]. LPS alone or IFN-γ alone were not effective, since HL-1 cardiomyocytes did not express any detectable inflammatory cytokine [21]. In transfected HL-1 cardiomyocytes co-stimulated with LPS and IFN-γ, *Il-6* and *Tnf-α* RNA expression was increased after CAP and ArgBP2 knockdown, while Vinexin showed similar levels as the siMock-transfected cells (Fig. 1B). *Ip-10* RNA expression in HL-1 cells was inconsistently down- and up-modulated after CAP, ArgBP2, and Vinexin knockdown (Fig. 1B). In LPS-stimulated MNF cells, *Il-6* expression was higher only after CAP knockdown, while *Tnf-α* and *Ip-10* RNA expression was inconsistently down- and up-

regulated after CAP and Vinexin knockdown (Fig. 1C). Taken together, these results indicate that CAP dampens the expression of *Il-6* and *Tnf-α* in all tested cells, ArgBP2 inhibits *Il-6* and partially *Tnf-α* expression, while Vinexin rather promotes *Il-6* and *Ip*-10 expression in MEF and MNF without affecting *Tnf-α* expression.

At the protein level, IL-6 measured in the supernatant of stimulated MEF was useful to confirm that CAP and ArgBP2 knockdown resulted in higher levels of IL-6 when compared to their siMock controls, while TNF-α production was comparable among both groups (Fig. 1D). Vinexin, on the contrary, slightly promoted IL-6 production (Fig. 1D). Summarizing, IL-6 is reduced by CAP and ArgBP2, while Vinexin promotes it. TNF-α production, on the contrary, is not affected by CAP, ArgBP2, or Vinexin.

3.2. Acute phase cytokines are dampened by CAP in vitro upon various TLRs and RLRs stimuli

To better understand how CAP modulates cytokine production at the protein level, we investigated its function in cells belonging to the immune system. Taking advantage of CAP knockout mice, BMDC were cultivated from bone marrow cells as described in the materials and methods section. Mature $CAP^{+/+}$ and $CAP^{-/-}$ BMDC were then stimulated for 24 h with two different concentrations of LPS. Cytokines were evaluated in collected cell-free supernatants with a multiplex array. Among ten pro- and anti-inflammatory cytokines tested, only IL-1β and IL-6 were significantly increased in $CAP^{-/-}$ BMDC at both tested LPS concentrations (Fig. 2A).

To further understand the function of CAP upon PRRs activation, also MEF and BMM cells were harvested from $CAP^{+/+}$ and $CAP^{-/-}$ mice. Upon stimulation with various TLRs and RLRs agonists, IL-6 was consistently over-expressed in $CAP^{-/-}$ cells when compared to $CAP^{+/+}$ cells (Fig. 2B and Suppl. Fig. 2A). Similarly, upon infection of BMDC with CVB3 and vesicular stomatitis virus (VSV), which are two RNA viruses, IL-6 production was significantly higher in $CAP^{-/-}$-infected cells compared to $CAP^{+/+}$-infected cells. TNF-α production was significantly increased in $CAP^{-/-}$ MEF only upon PolyIC stimulation and in $CAP^{-/-}$ BMDC only after CVB3 infection when compared to their $CAP^{+/+}$ counterparts (Fig. 2C and E). Significantly higher CCL5 expression was also observed in $CAP^{-/-}$ BMDC just after VSV infection compared to $CAP^{+/+}$ BMDC (Suppl. Fig. 2B and C). These results indicate that CAP consistently down-regulates IL-6 but just partially controls the expression of other acute phase cytokines.

3.3. CAP hampers the NF-κB pathway

The major regulator of *Il-6* expression is the transcription factor NF-κB [15,22], which translocates into the nucleus following phosphorylation and subsequent proteasomal degradation of the inhibitory κB (IκB) protein [23]. Therefore, we aimed to figure out whether CAP and ArgBP2 may influence the inflammatory signalling pathway triggered by TLR4 stimulation that eventually leads to NF-κB activation. To this end, we transfected MEF and HL-1 cells with CAP siRNA, ArgBP2 siRNA, or mock siRNA, and then measured phosphorylation of proteins of the canonical NF-κB pathway before and after TLR4 stimulation. The NF-κB pathway was consistently up-regulated in the absence of CAP, but only partially in the absence of ArgBP2, suggesting a predominant role of CAP as limiting factor of pro-inflammatory TLR signalling (Fig. 3A, B and Suppl. Fig. 3A).

Given the observed differences in TLRs- and RLRs-induced pro-inflammatory cytokines in the presence or absence of CAP, we next aimed to confirm the inhibitory role of CAP also in CAP-knockout cells. $CAP^{+/+}$ and $CAP^{-/-}$ BMDC were stimulated in vitro with LPS, while $CAP^{+/+}$ and $CAP^{-/-}$ MEF were infected with CVB3. In both cases, increased or extended phosphorylation of NF-κB- and MAP Kinasae-related proteins was observed in $CAP^{-/-}$ cells compared to $CAP^{+/+}$ cells (Fig. 3C and D). In addition, HEK293 cells, which were first transfected with a CAP-expressing vector and then stimulated with transfected high molecular weight (HMW)-PolyIC or low molecular weight (LMW)-PolyIC, showed reduced NF-κB p65 and ERK1/2 phosphorylation when compared to mock-transfected HEK293 (Fig. 3E). Taken together, these results indicate that CAP inhibits both the NF-κB and the MAP Kinase signalling pathways in different cells after various stimuli.

To demonstrate that CAP limits NF-κB p65 activation and nuclear translocation, HeLa cells were first transfected with FLAG-tagged CAP plasmid and then stimulated for 30 min with PolyIC for detection of NF-κB p65 by immunofluorescence. In some HeLa cells the CAP plasmid was not transfected, therefore these untransfected cells represented the internal control. After PolyIC stimulation, CAP-transfected HeLa cells did not overexpress NF-κB p65 in the nucleus, while untransfected cells showed increased NF-κB p65 fluorescence in the nucleus, demonstrating that CAP limits NF-κB p65 activation and nuclear translocation (Fig. 4).

3.4. CAP binds to Iκκ-α and Iκκ-β

It is known that CAP binds to the RLR receptor MDA5 and to MyD88 after CVB3 infection, upregulating the phosphorylation of IRF3 in viral myocarditis [11]. On the other side, it is not yet known whether CAP aggregates with any protein of the NF-κB pathway upon LPS stimulation. Therefore, we transfected HEK293 and HeLa cells with a FLAG-tagged vector encoding human CAP. After pulling down CAP, we observed that only Iκκ-α and Iκκ-β complexed with CAP before and after stimulation (Fig. 5A and Suppl. Fig. 3B). In addition, we used an expression vector encoding CAP that lacked its SH3 domains. Interestingly, only full-length CAP, but not CAP depleted of its SH3 domains, co-immunoprecipitated with Iκκ-α and Iκκ-β, suggesting that its SH3 domains are necessary for this interaction (Fig. 5A and Suppl. Fig. 3B).

The complex formation of CAP with Iκκ-β was further analyzed in HEK cells over-expressing human TLR4 (HEK-hTLR4). After transfection or co-transfection of vectors expressing full-length CAP and Iκκ-β into HEK-hTLR4 cells, activation of NF-κB was reduced in LPS-stimulated CAP/Iκκ-β-co-transfected cells when compared to LPS-stimulated cells transfected only with Iκκ-β or with an empty control vector (Fig. 5B), suggesting that CAP can directly dampen the NF-κB pathway by limiting the effect of Iκκ-β.

3.5. CAP represses IL-6 in mice challenged with LPS and in the heart of CVB3-infected mice

To confirm the CAP-dependent up-regulation of IL-6 in vivo, we challenged $CAP^{+/+}$ and $CAP^{-/-}$ C57Bl/6 mice with LPS or with the CVB3 virus. After LPS-induced septic shock, only IL-6 was significantly increased in sera of $CAP^{-/-}$ mice compared to $CAP^{+/+}$ control mice (Fig. 6A). Since CVB3 has a particular tropism for heart tissue, hearts from CVB3-infected mice were analyzed for cytokine expression at the RNA level by quantitative real-time PCR. Ten days after CVB3 infection, which corresponds to the inflammatory phase of viral myocarditis in susceptible C57Bl/6 mice, hearts were collected and processed to analyze RNA expression. *Il-6*, but not *Il-1β*, *Tnf-α* or *Ip-10* was significantly increased in heart tissue of infected $CAP^{-/-}$ mice compared to $CAP^{+/+}$ control mice (Fig. 6B). It is worth to notice that C57Bl/6 mice infected with the CVB3 virus do not develop autoimmune myocarditis, meaning that the increased *Il-6* expression observed in the heart at day 10 was not caused by heart-specific autoimmune cells. Taken together, these results demonstrate that CAP suppresses IL-6 in innate immune responses in vivo, supporting the immunomodulatory function of CAP observed in vitro.

4. Discussion

In this study we showed for the first time the role of CAP in inflammation. In addition, we observed that ArgBP2 and Vinexin partially

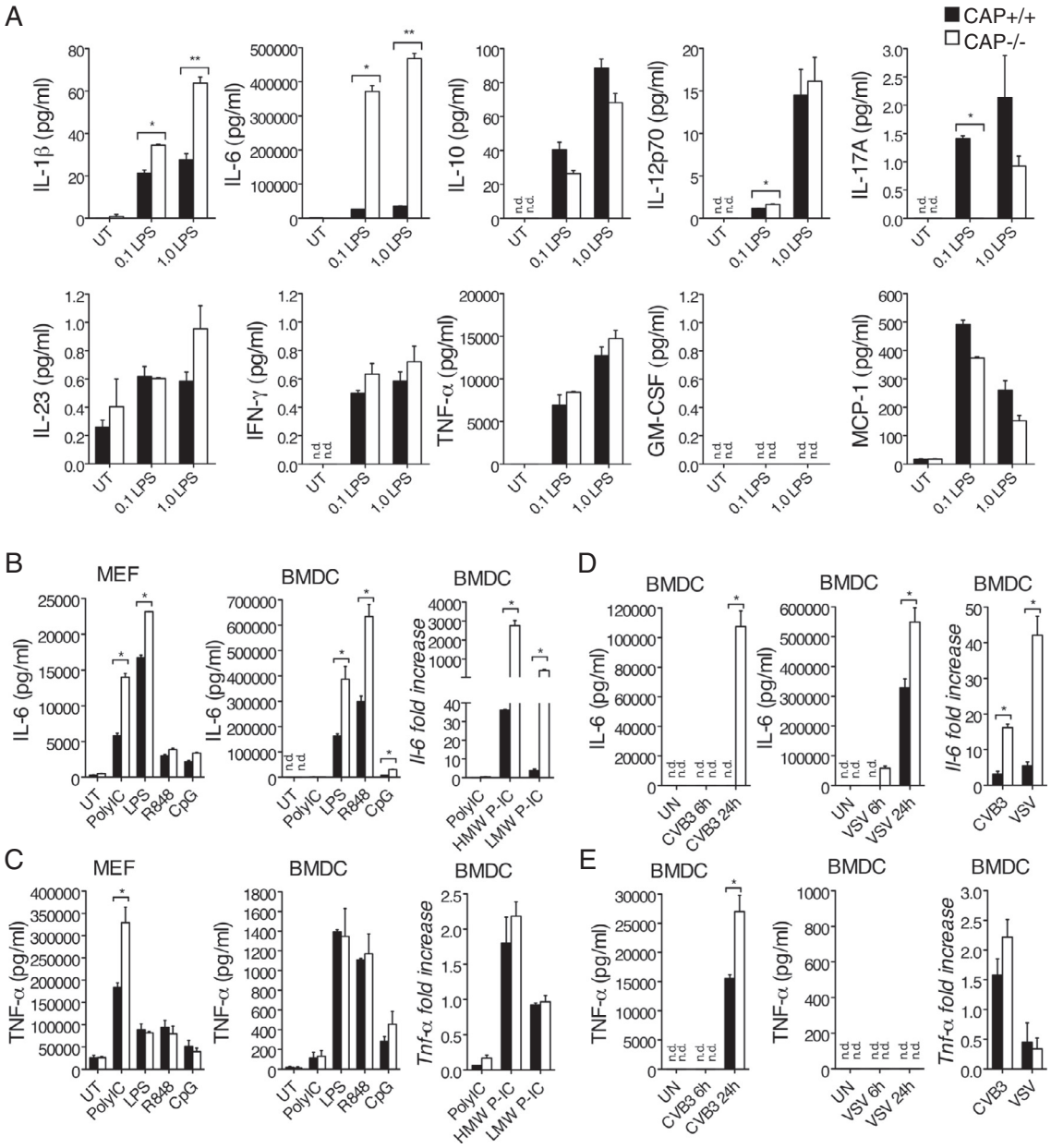

Fig. 2. CAP inhibits the pro-inflammatory cytokine IL-6 in vitro. (A) Ten pro- and anti-inflammatory cytokines were measured by a Cytometric Bead Array (CBA) in $CAP^{+/+}$ (black squares) and $CAP^{-/-}$ (white squares) BMDC after stimulation with 0.1 μg/ml LPS or 1 μg/ml LPS. Supernatants were collected after 24 h. (B-C) IL-6 (B) and TNF-α (C) were measured by ELISA in $CAP^{+/+}$ (black bars) and $CAP^{-/-}$ (white bars) MEF and BMDC after stimulation with 10 ng/ml PolyIC, 1 μg/ml LPS, 2.5 μg/ml R848, 1 μg/ml CpG, or left untreated for TLR activation or transfected with 1 ng/ml HMW PolyIC or 1 ng/ml LMW PolyIC for RLRs activation. Supernatants were collected after 24 h, while cells were harvested after 6 h for RNA extraction and measurement by qRT-PCR. (D-E) CVB3 and VSV, both at a concentration of 1 MOI, were used for direct in vitro infection of BMDC. After 6 h and 24 h, IL-6 (D) and TNF-α (E) were analyzed in supernatants by ELISA and at the RNA level by qRT-PCR. UT, untreated. UN, uninfected. n.d., not detected. Data are representative for three experiments performed in triplicates (mean, s.d.). $^{*}P < 0.05$, $^{**}P < 0.01$.

modulate inflammatory responses. We found that CAP specifically limited the expression of the pro-inflammatory cytokine IL-6 and partially TNF-α in mouse myeloid-derived cells, fibroblasts, and cardiac cells in vitro and exclusively IL-6 in vivo. CAP down-regulated IL-6 by binding to Iκκ proteins and then, after inhibiting their downstream signalling pathway, inhibited NF-κB activation (Suppl. Fig. 4).

The NF-κB pathway is activated in response to TLRs and RLRs agonists. Other pathways, such as the MAP Kinase pathway, selectively enhance the accessibility of NF-κB to specific promoters, contributing to *Il-6*, *Il-8*, and *Il-12* gene expression [24]. Similarly, other transcription regulators of the IκB family, such as IκBNS and IκBς, selectively modulate binding of NF-κB to the promoters of *Il-6* and *Il-12* [25]. In our study we found a general reduction of IL-6 production in the presence of CAP and in part in the presence of ArgBP2, which reflected the lower levels of phosphorylated NF-κB p65. Nevertheless, we observed that induction of several other pro-inflammatory cytokines was almost independent of CAP. We therefore exclude that CAP directly influences the transcriptional function of NF-κB, but rather it may interact with proteins upstream of NF-κB. CAP has been shown to be an adaptor protein modulating membrane trafficking, intracellular signalling, and cytoskeleton [7,9]. In our pull-down experiments, we observed that CAP co-precipitated with Iκκ-α and Iκκ-β, but not with other proteins that usually aggregate with them, such as Iκκ-γ. Impaired or depleted Iκκ-α and Iκκ-β function leads to a general reduction of pro-

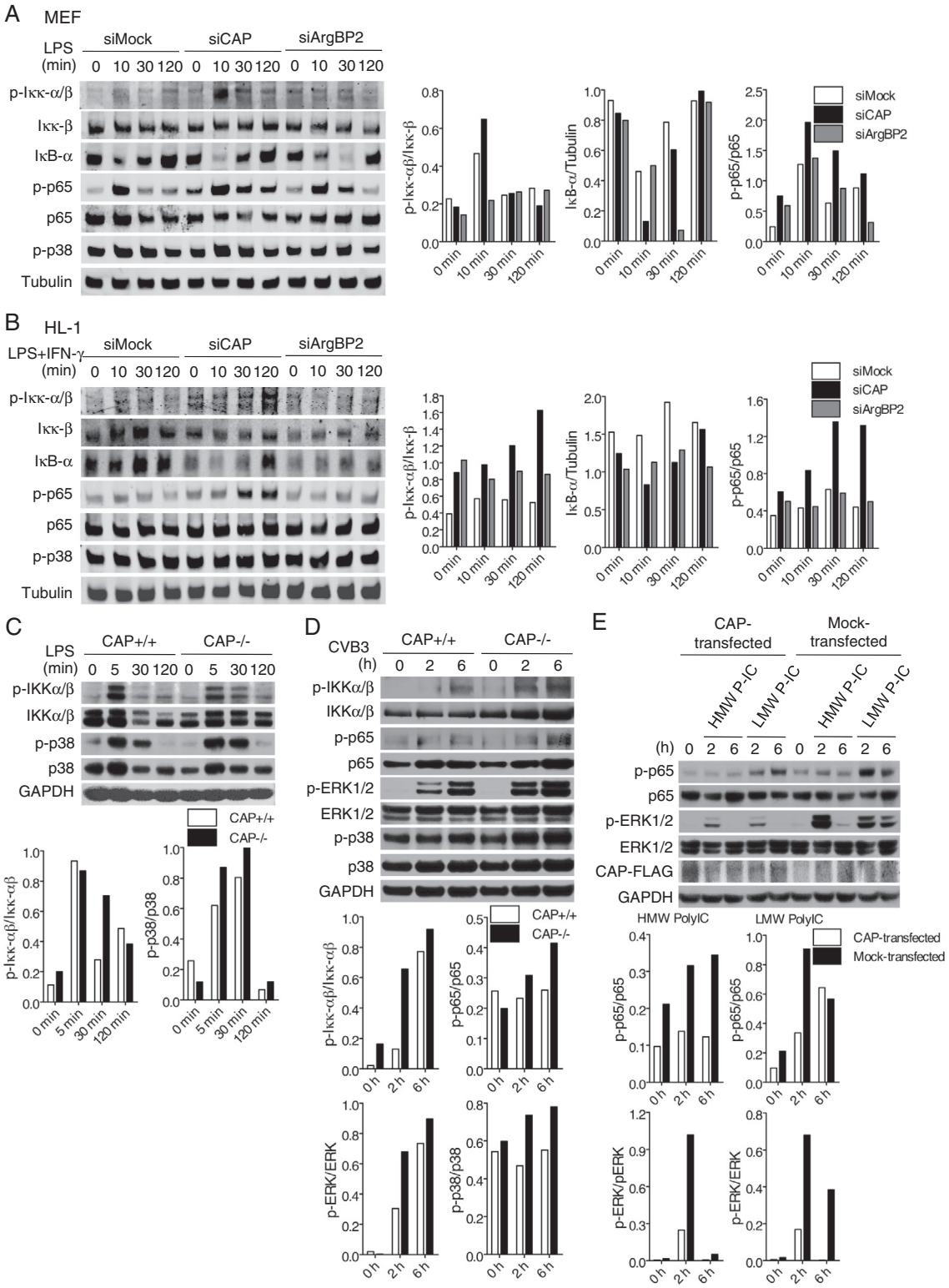

Fig. 3. CAP reduces phosphorylation of members of the NF-κB pathway. (A-B) Total cell lysates from Mock-, CAP-, or ArgBP2-knocked down MEF (A) and HL-1 cells (B) stimulated with LPS alone or co-stimulated with LPS and IFN-γ, respectively, for 10, 30, and 120 min. The NF-κB pathway was analyzed by immunoblotting with the indicated antibodies. (C) Immunoblot analysis of phosphorylated (p)-Iκκ-α/β and total Iκκ-α/β, as well as p-p38 and total p38, in $CAP^{+/+}$ and $CAP^{-/-}$ MEF stimulated with 1 μg/ml LPS for the indicated times. GAPDH was used as an internal loading control. (D) Immunoblot analysis of p-Iκκ-α/β, total Iκκ-α/β, p-p65, total p65, p-ERK1/2, total ERK1/2, p-p38, and total p38 in $CAP^{+/+}$ and $CAP^{-/-}$ MEF infected with 1 MOI CVB3 for the indicated times. GAPDH was used as an internal loading control. (E) Immunoblot of p-p65, total p65, p-ERK1/2, and total ERK1/2 in HEK293 cells transfected with FLAG-tagged CAP-encoding plasmid or control mock plasmid 24 h before stimulation with 1 ng/ml transfected HMW PolyIC or LMW PolyIC for the indicated times. CAP-FLAG was used as plasmid transfection control, GAPDH as internal loading control. Data are representative of one out of two experiments.

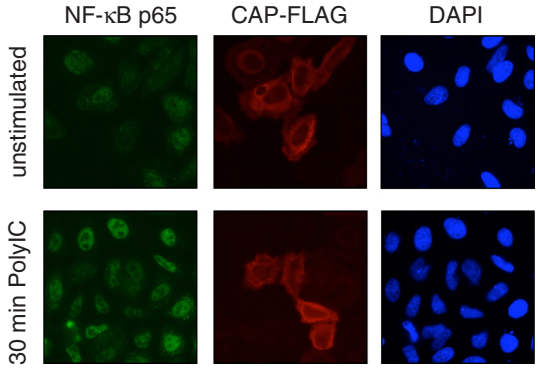

Fig. 4. CAP limits nuclear translocation of NF-κB p65 after stimulation. Immunofluorescence staining of HeLa cells transfected with the FLAG-tagged CAP-WT vector. Untransfected cells were used as an internal control for cells that did not express CAP. Cells were left unstimulated or were stimulated with PolyIC for 30 min and then stained with antibodies detecting total NF-κB (green fluorescence) and antibodies detecting FLAG-tagged CAP (red fluorescence). DAPI was used to stain the nuclei. Magnification 320×.

inflammatory cytokine expression upon TLR activation [18]. It is known that upon TLR activation, the catalytic Iκκ-α and Iκκ-β kinases and the regulatory protein Iκκ-γ bind together to make the Iκκ complex [26]. However, it has been recently described in hepatocarcinogenesis and inflammation that Iκκ-γ works in an Iκκ-α/Iκκ-β-independent manner [27]. A possible explanation for the lack of interaction between CAP and Iκκ-γ may be the inhibition of the interaction between Iκκ-α/Iκκ-β and Iκκ-γ, as demonstrated by using amino acid sequence NEMO-binding domain (NBD) [28]. It is likely that CAP, by binding to Iκκ-α/Iκκ-β, may mask the binding domains used by Iκκ-γ to complex with Iκκ-α/Iκκ-β, thus dampening the pro-inflammatory NF-κB pathway and repressing IL-6 production.

CAP-dependent inhibition of IL-6 was not exclusively observed after stimulation of membrane-bound TLRs. Also upon stimulation of the cytosolic RLRs receptors MDA5 und RIG-I, which sense intracellular HMW and LMW PolyIC, respectively, IL-6 was significantly increased in the absence of CAP. In addition, after viral infection with CVB3 and VSV, whose RNAs typically sense MDA5 and RIG-I, respectively, IL-6 was significantly increased in CAP-deficient cells. We previously observed that CAP binds to MyD88 and MDA5 after viral infection of HeLa cells with CVB3 [11]. In the present study CAP complexed with Iκκ-α and Iκκ-β upon LPS stimulation in MEF and upon co-stimulation with LPS and IFN-γ in HL-1 cells, suggesting that CAP controls the NF-κB pathway in different ways. Indeed, when membrane bound TLR4 is sensed, CAP inhibits excessive IL-6-mediated inflammation, dampening the production of detrimental acute phase proteins, while when cytosolic RLRs are sensed, CAP simultaneously dampens IL-6 expression while promoting anti-viral protection by increasing type I interferon production.

Some variations were observed in the cells used to elucidate the function of CAP. In MEF, for example, CAP, but not ArgBP2 and Vinexin, consistently regulated the expression of *Il-6*, *Tnf-α*, and *Ip-10* while in HL-1 cells, both CAP and ArgBP2, but not Vinexin, controlled *Il-6*, *Tnf-α*, and *Ip-10* expression. On the other side, in cells belonging to the immune system, such as dendritic cells, CAP regulated the expression of *Il-6*, and just marginally the expression of *Tnf-α* and *Ccl5*. Lower expression of *Il-6* in MEF in comparison with BMM upon LPS stimulation has been explained after measuring different kinetics of NF-κB, ERK, p38, and JNK phosphorylation between MEF and BMM [29]. Although we also found different kinetics for protein phosphorylation in the present study, CAP constantly reduced IL-6 production in all cells used, while TNF-α and CCL5 were just partially affected.

It has been recently demonstrated in mice with viral myocarditis that CAP protects the heart from detrimental anti-viral cytotoxic responses and increases the survival rate of infected CAP$^{+/+}$ mice [11]. In the present study we showed that CAP repressed IL-6 expression in a mouse model of LPS-induced shock and in a mouse model of CVB3-induced viral myocarditis, which does not develop the autoimmune phase, but only the initial innate inflammatory phase of the disease [30]. The expression of other cytokines was independent of CAP, suggesting that CAP specifically down-regulates IL-6 production in vivo.

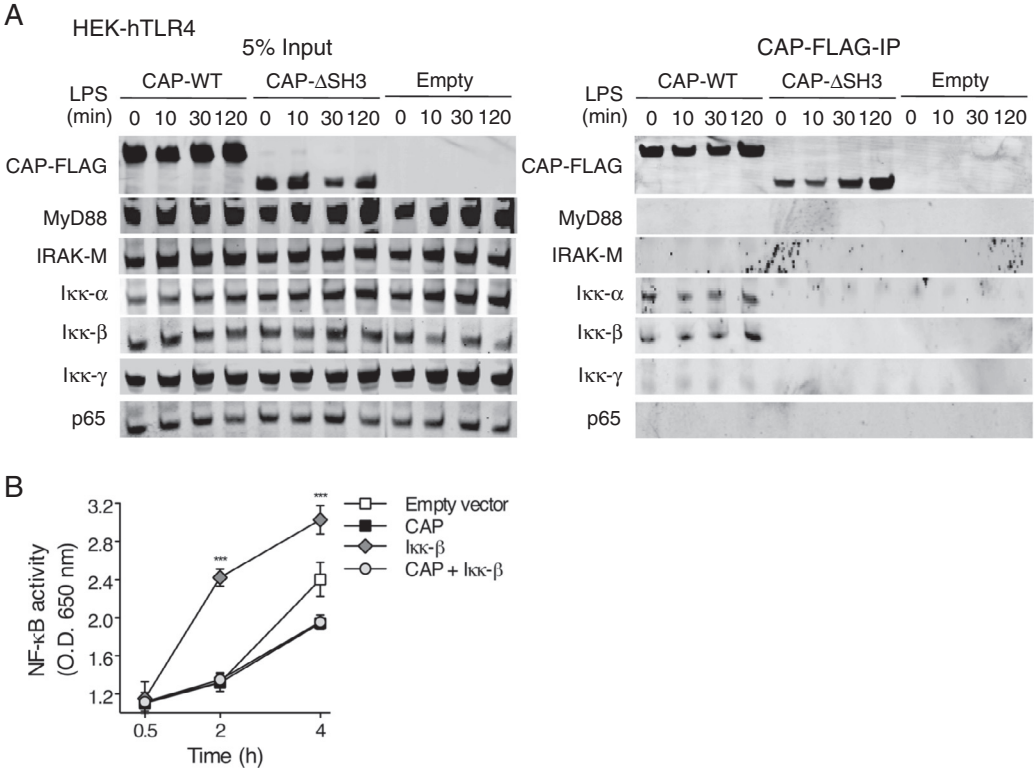

Fig. 5. CAP binds to Iκκ-α and Iκκ-β. (A) HEK-hTLR4 cells were transfected with FLAG-tagged CAP-WT or CAP-ΔSH3 vectors, or an empty control vector. Physical interactions of CAP or of its mutated form CAP-ΔSH3 with proteins of the NF-κB pathway were analyzed by immunoprecipitation and immunoblotting. One out of two independent experiments are shown. (B) HEK-hTLR4 cells were transfected with a vector expressing CAP (black squares), with a vector expressing Iκκ-β (grey rhombus), or co-transfected with both vectors expressing CAP and Iκκ-β (grey circles). An empty vector was used as control (white squares). Cells were stimulated with LPS for 0.5, 2, and 4 h. NF-κB activity was determined by measuring the O.D. value of the reporter gene. Data are representative of one out of two experiments. ***$P < 0.001$ for CAP/Iκκ-β-co-transfected cells vs. Iκκ-β-transfected cells.

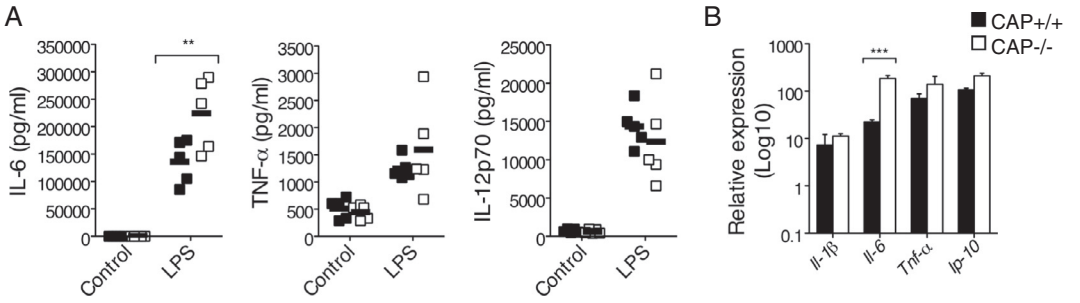

Fig. 6. CAP limits IL-6 in vivo. (A) $CAP^{+/+}$ (n = 5) and $CAP^{-/-}$ (n = 5) mice were injected intraperitoneally with LPS to induce septic shock (30 mg/kg body weight). Two hours after LPS challenge, blood was collected and analyzed by ELISA. Data are representative for two independent experiments. $^{**}P < 0.01$ (B) Quantitative RT-PCR analysis of various pro-inflammatory cytokines in the heart of CVB3-infected $CAP^{+/+}$ (n = 5) and $CAP^{-/-}$ (n = 5) mice 10 days after infection. Data are representative for two independent experiments (mean, s.d.). $^{***}P < 0.001$.

These findings indicate the potential of CAP in suppressing excessive IL-6-dependent inflammatory responses that can lead to adverse clinical outcomes in patients with pre-existing immunological disorders. Indeed, IL-6 and other pro-inflammatory cytokines that induce acute phase proteins play a major role in hyperinflammatory conditions, such as in cytokine-release syndrome (CRS). Typical hallmarks of CRS have been observed in leukemic patients treated with chimeric antigen receptor-modified T (CAR-T) cells, that showed increased IL-6 production, and in patients with respiratory virus infections, such as in influenza virus and corona virus diseases [4,5,31]. Currently, the suggested therapeutic option to reduce IL-6 is the anti-IL-6 receptor antibody Tocilizumab [31,32]. Other therapeutic strategies to avoid CRS may be based on preventive modulation of the immune system. In this case, CAP could be used as a therapeutic agent to prevent hyperinflammatory conditions.

Taken together, we demonstrate a novel function of CAP as a modulator of the immune system. Our data show that CAP specifically interacts with proteins of the NF-κB signalling pathway and hampers IL-6 production. By enhancing CAP production, innate immune responses could be balanced towards a favourable clinical outcome, limiting inflammation and reducing adverse organ damages.

CRediT authorship contribution statement

Daria Vdovenko: Data curation, Investigation, Formal analysis, Writing - review & editing. **Marta Bachmann:** Investigation. **Winandus J. Wijnen:** Investigation. **Michael O. Hottiger:** Funding acquisition. **Urs Eriksson:** Writing - review & editing, Funding acquisition. **Alan Valaperti:** Conceptualization, Data curation, Investigation, Formal analysis, Project administration, Writing - original draft, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2020.106822.

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