The adaptor protein c-Cbl-associated protein (CAP) limits pro-inflammatory cytokine expression by inhibiting the NF-B pathway

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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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The adaptor protein c-Cbl-associated protein (CAP) limits pro-inflammatory cytokine expression by inhibiting the NF-κB pathway

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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, while 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 (IκB)α and IκBβ and their downstream NF-κB-dependent signalling pathway. Molecular affinity between CAP and IκBα/ IκBβ 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.

\textbf{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...
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 (PRRs), 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 (IκK)-β [17]. The IκK complex, which is composed of IκK-α, IκK-β, and IκK-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 Clamyd medium (Sigma) and expanded in 75 cm² 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 Invitrogen. HEK cells was performed with the calcium phosphate precipitation method as previously described [21].

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 × 10⁵ 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-ASH3 vectors, as well as the empty control vector, were previously described [11]. Transfection of 10⁶ 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−ΔΔCt 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 (PolyI:C), low molecular weight (LMW) PolyI,C, and the imidazooquinoline R848 were purchased from Invivogen. LPS and CpGs were purchased from Sigma. Antibodies to Iκκα/β, phosphorylated-Iκκα/β (p-Iκκα/β), Iκκα, Iκκβ, Iκκγ, NF-κB p65, NF-β 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 Iκκκα and GAPDH were purchased from Santa Cruz Biotechnology. Antibodies to FLAG were purchased from Sigma-Aldrich.
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 transflecting 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-

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. +$P < 0.05$, ++$P < 0.01$, +++$P < 0.001$ for siArgBP2 vs. siVinexin.

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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-xB pathway

The major regulator of Il-6 expression is the transcription factor NF-xB [15,22], which translocates into the nucleus following phosphorylation and subsequent proteosomal degradation of the inhibitory xB (IxB) 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-xB 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-xB pathway before and after TLR4 stimulation. The NF-xB 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-xB- and MAP Kinase-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-xB p65 and ERK1/2 phosphorylation when compared to mock-transfected HEK293 (Fig. 3E). Taken together, these results indicate that CAP inhibits both the NF-xB and the MAP Kinase signalling pathways in different cells after various stimuli.

To demonstrate that CAP limits NF-xB 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-xB 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-xB p65 in the nucleus, while untransfected cells showed increased NF-xB p65 fluorescence in the nucleus, demonstrating that CAP limits NF-xB p65 activation and nuclear translocation (Fig. 4).

3.4. CAP binds to Ixα-α and Ixα-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-xB 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 Ixα-α and Ixα-β copurified 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, co-immunoprecipitated with Ixα-α and Ixα-β, suggesting that its SH3 domains are necessary for this interaction (Fig. 5A and Suppl. Fig. 3B).

The complex formation of CAP with Ixα-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 Ixα-β into HEK-hTLR4 cells, activation of NF-xB was reduced in LPS-stimulated CAP/ixα-β-co-transfected cells when compared to LPS-stimulated cells transfected only with Ixα-β or with an empty control vector (Fig. 5B), suggesting that CAP can directly dampen the NF-xB pathway by limiting the effect of Ixα-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
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-κκB activation (Suppl. Fig. 4).

The NF-κκB pathway is activated in response to TLRs and RLRs agonists. Other pathways, such as the MAP Kinase pathway, selectively enhance the accessibility of NF-κκB to specific promoters, contributing to Il-6, Il-8, and Il-12 gene expression [24]. Similarly, other transcription regulators of the Iκκ family, such as IκBNS and IκBζ, selectively modulate binding of NF-κκB 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-κκB 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-κκB, but rather it may interact with proteins upstream of NF-κκB. 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. 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.
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.
inflammatory cytokine expression upon TLR activation [18]. It is known that upon TLR activation, the catalytic Iκκα and Iκκβ kinases and the regulatory protein Iκκγ bind together to make the Iκκ complex [26]. However, it has been recently described in hepatocarcinogenesis and inflammation that Iκκγ works in an Iκκα/Iκκβ-independent manner [27]. A possible explanation for the lack of interaction between CAP and Iκκγ may be the inhibition of the interaction between Iκκα/Iκκβ and Iκκγ, as demonstrated by using amino acid sequence NEMO-binding domain (NBD) [28]. It is likely that CAP, by binding to Iκκα/Iκκβ, may mask the binding domains used by Iκκγ to complex with Iκκα/Iκκβ, thus dampening the pro-inflammatory NF-xB pathway and repressing IL-6 production.

CAP-dependent inhibition of IL-6 was not exclusively observed after stimulation of 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 Iκκα and Iκκβ upon LPS stimulation in MEF and upon co-stimulation with LPS and IFN-γ in HL-1 cells, suggesting that CAP controls the NF-xB 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-xB, 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 cytokotic 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.
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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