INFLAMMASOMES

The signaling adaptor BCAP inhibits NLRP3 and NLRC4 inflammasome activation in macrophages through interactions with Flightless-1

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B cell adaptor for phosphoinositide 3-kinase (PI3K) (BCAP) is a signaling adaptor that activates the PI3K pathway downstream of B cell receptor signaling in B cells and Toll-like receptor (TLR) signaling in macrophages. BCAP binds to the regulatory p85 subunit of class I PI3K and is a large, multidomain protein. We used proteomic analysis to identify other BCAP-interacting proteins in macrophages and found that BCAP specifically associated with the caspase-1 pseudosubstrate inhibitor Flightless-1 and its binding partner leucine-rich repeat flightless-interacting protein 2. Because these proteins inhibit the NLRP3 inflammasome, we investigated the role of BCAP in inflammasome function. Independent of its effects on TLR priming, BCAP inhibited NLRP3- and NLRC4-induced caspase-1 activation, cell death, and IL-1β release from macrophages. Accordingly, caspase-1–dependent clearance of a *Yersinia pseudotuberculosis* mutant was enhanced in BCAP-deficient mice. Mechanistically, BCAP delayed the recruitment and activation of pro–caspase-1 within the NLRP3/ASC preinflammasome through its association with Flightless-1. Thus, BCAP is a multifunctional signaling adaptor that inhibits key pathogen-sensing pathways in macrophages.

INTRODUCTION

B cell adaptor for phosphoinositide 3-kinase (PI3K) (BCAP) is a signaling adaptor expressed in several immune lineages, including macrophages, B cells, and natural killer (NK) cells. First identified in B cells, BCAP is phosphorylated on four YxxM tyrosines after B cell receptor (BCR) ligation, which promotes the binding and activation of class I phosphoinositide 3-kinase (PI3K) (*1*). In B cells, BCR-induced BCAP tyrosine phosphorylation depends on immunoreceptor tyrosine-based activation motif (ITAM)–mediated activation of Syk and Btk and is important for B cell proliferation and survival (*2*, *3*). In NK cells, BCAP is phosphorylated and activates PI3K after signaling by the NK cell–activating receptor NKR-P1C, and lack of BCAP alters NK cell development (*4*). In T cells, BCAP participates in both T cell receptor and Toll/interleukin-1 receptor (IL-1R) PI3K activation (*5*, *6*). Thus, BCAP acts as a signaling adaptor linking ITAM signaling and PI3K activation in B cells, T cells, and NK cells (*4*–*7*).

In addition to its function downstream of ITAM-containing receptor complexes in lymphocytes, BCAP signals downstream of Toll-like receptors (TLRs) in macrophages (*8*, *9*). TLR ligation leads to activation of several signaling pathways, including the nuclear factor κB, mitogen-activated protein kinase, and PI3K pathways. BCAP is specifically required for optimal TLR-induced PI3K pathway activation in macrophages (*8*, *9*). Because PI3K is a potent inhibitory signal that keeps TLR responses in check, BCAP-deficient macrophages display increased TLR-induced TNF (tumor necrosis factor), IL-6, and IL-12 p40 production compared to wild-type (WT) macrophages, which depends on the four YxxM tyrosines in BCAP (*8*). TLRs do not contain ITAM sequences nor are they known to associate with ITAM-containing adaptors; thus, BCAP activation in macrophages is fundamentally distinct from that in lymphocytes. BCAP is a large protein of ~800 amino acids with several protein-protein interaction domains, including three proline-rich sequences, a coiled-coil domain, ankyrin repeats, and a homodimerization domain in addition to the YxxM tyrosines (*1*, *10*). In addition, the N-terminal domain of BCAP has been defined as a Toll/IL-1R homology (TIR) domain important for limiting TLR responses (*9*, *11*, *12*). Because BCAP is a large multidomain signaling adaptor, we reasoned that BCAP may interact with other signaling pathways in addition to the TLR pathway in macrophages.

Inflammasomes are multiprotein cytoplasmic complexes that concentrate and activate the cysteine protease caspase-1, resulting in the processing and release of IL-1β and IL-18 and the caspase-1–dependent cell death known as pyroptosis (*13*, *14*). Inflammasome activation contributes to clearance of a variety of pathogens through the recruitment and activation of immune cells and the depletion of an intracellular niche for pathogen replication. However, aberrant or prolonged inflammasome activation can be pathogenic, as is seen in sterile inflammatory disorders such as gout and pseudogout and in autoinflammatory diseases caused by autosomal dominant activating mutations in inflammasome components, such as cryopyrin-associated periodic syndromes with mutations in *NLRP3* and familial Mediterranean fever with mutations in *MEFV* (*15*, *16*).

Structural studies of inflammasome components have revealed a nucleation-dependent polymerization mechanism for inflammasome assembly (*17*, *18*). Sensor proteins such as nucleotide-binding domain, leucine-rich repeat-containing, and pyrin domain-containing 3 (NLRP3) recruit the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC), which in turn recruits and activates pro–caspase-1 through proximity-induced autoproteolysis. ASC polymerizes in a prion-like manner that allows for the assembly of a single inflammasome focus containing NLR, ASC, and caspase-1, which acts as a platform for IL-1β and IL-18 processing. Therefore, the sequential nature of inflammasome assembly provides opportunities for host and pathogen inhibition. Inflammasomes serve to restrict a

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variety of pathogens, as is underscored by the numerous inflammasome inhibitors encoded by bacteria and viruses (*13*, *19*–*21*). Poxviruses and other virus families encode direct competitive inhibitors of caspase-1 proteolytic activity, as do pathogenic *Yersinia* species. Enteropathogenic *Escherichia coli* and viruses such as Kaposi's sarcoma-associated herpesvirus and Myxoma virus encode proteins that interfere with earlier events in the assembly of the inflammasome complex (*19*, *21*). Therefore, pathogens have multiple strategies to evade inflammasome-mediated immune responses, which highlights the importance of inflammasome activation for proper pathogen control. Whereas many pathogen-derived proteins that interfere with inflammasome activation are defined, relatively few host proteins that dampen this process to control potentially pathogenic inflammation are described. Similar to the pathogen-encoded inflammasome inhibitors, host inflammasome inhibitors either directly inhibit the proteolytic activity of caspase-1 or interfere with inflammasome assembly (*22*–*24*).

One host inflammasome inhibitor is Flightless-1 (*24*, *25*), a ubiquitously expressed protein with an N-terminal leucine-rich repeat (LRR) domain and C-terminal gelsolin repeats, which regulates actin remodeling and cell migration (*26*, *27*). Flightless-1 also contains a caspase-1 pseudosubstrate site, which inhibits caspase-1 activity (*24*). Here, we show that BCAP interacted with Flightless-1 and its binding partner LRR Flightless-1–interacting protein 2 (LRRFIP2) in macrophages. Therefore, we investigated whether BCAP inhibited inflammasome activation through its association with Flightless-1.

RESULTS

BCAP interacts with the endogenous caspase-1 inhibitor Flightless-1

To identify novel pathways that may be regulated by BCAP in macrophages, we performed an unbiased screen for BCAP-interacting proteins using immunoprecipitation coupled with tandem mass spectrometry (MS/MS). We immunoprecipitated BCAP using a specific monoclonal antibody from lysates of WT macrophages and BCAP$^{-/-}$ macrophages. Identified proteins were filtered using the following criteria: at least five unique peptides identified in all three anti-BCAP immunoprecipitates from WT macrophages, zero or one unique peptide in negative-control immunoprecipitate from BCAP$^{-/-}$ macrophages, and low nonspecific interactions as determined by the CRAPome database (<10 average spectral counts) (*28*). This analysis yielded 14 proteins, many of which were proteins that bind to and/or regulate the actin cytoskeleton, including several myosins, gelsolin, and components of the Arp2/3 complex (Fig. 1A).

We also noted in our list of specific BCAP interactors the presence of two proteins that have defined functions in inhibiting NLRP3 inflammasomes, Flightless-1 (encoded by *Flii*) and LRRFIP2 (encoded by *Lrrfip2*; Fig. 1A). Flightless-1 is a ubiquitously expressed protein that contains both a gelsolin domain, which participates in actin capping, and an LRR domain, for protein-protein interactions. LRRFIP2 is predominantly expressed in myeloid cells and binds to Flightless-1 in macrophages where, together, they inhibit NLRP3 inflammasome activation through the ability of Flightless-1 to directly inhibit caspase-1 activity (*24*, *25*). To quantitatively assess the presence of BCAP, Flightless-1, and LRRFIP2 in our immunoprecipitates, we performed precursor area quantification on selected peptides that we found to be abundant and to exhibit reproducible elution profiles. We quantified these peptides derived from each protein in WT and BCAP$^{-/-}$ macrophages, showing a strong enrichment in immunoprecipitates from WT macrophages (Fig. 1B). We then confirmed the interaction of BCAP, Flightless-1, and LRRFIP2 in macrophages by coimmunoprecipitation and Western blot (Fig. 1C). Together, these results demonstrated that BCAP interacts with the NLRP3 inflammasome inhibitors Flightless-1 and LRRFIP2 in macrophages.

BCAP interacts with the NLRP3 inflammasome in the presence of Flightless-1 and LRRFIP2

Flightless-1 binds to pro–caspase-1 in the forming NLRP3 inflammasome, which is mediated by LRRFIP2 (*25*). To directly test whether BCAP is also a part of this complex, we cotransfected NLRP3, ASC, and a pro–caspase-1 catalytic site mutant into human embryonic kidney (HEK) 293T cells along with BCAP, Flightless-1, and LRRFIP2. Immunoprecipitation of BCAP using a C-terminal Flag-tag also precipitated Flightless-1 (Fig. 2A), as we found in macrophages. BCAP also coimmunoprecipitated with pro–caspase-1, which was in a larger complex containing NLRP3 and ASC (Fig. 2A). We next determined whether this association required the presence of LRRFIP2. The ability of BCAP and Flightless-1 to coimmunoprecipitate did not require LRRFIP2; however, pro–caspase-1 association with this complex required LRRFIP2 expression (Fig. 2B). The interaction of BCAP and Flightless-1 with pro–caspase-1 also required NLRP3 expression, unlike the association of BCAP and Flightless-1 (Fig. 2B). Thus, BCAP interacts with the NLRP3 inflammasome in the presence of both Flightless-1 and LRRFIP2.

We next investigated which domain of BCAP was required for its ability to bind to Flightless-1. BCAP has a variety of sequence motifs, including an N-terminal TIR domain, ankyrin repeats, a "DBB" domain shared with the drosophila protein Dof and the mammalian protein B cell scaffold protein with ankyrin repeats 1 (BANK1), and proline-rich regions and coiled coil sequences in its C-terminal region, in addition to four tyrosines present in YxxM motifs required for p85 PI3K binding (Fig. 2C) (*1*). To examine which BCAP region binds to Flightless-1, we constructed a panel of Flag-tagged BCAP deletion mutants and examined their ability to immunoprecipitate Flightless-1 when transfected into HEK293T cells. As shown in Fig. 2D, only the BCAP mutant lacking the N-terminal domain was unable to coimmunoprecipitate Flightless-1. BCAP's four YxxM tyrosines, which are required for inhibition of TLR responses but not located in the N-terminal domain (*8*), were not necessary for BCAP to coimmunoprecipitate Flightless-1 (fig. S1). Thus, BCAP uses its N-terminal domain but does not require sequences for PI3K binding/activation, for Flightless-1 association.

BCAP inhibits NLRP3 inflammasome activation independent of lipopolysaccharide priming

Because we found that BCAP interacts with Flightless-1 and LRRFIP2, which together inhibit NLRP3 inflammasomes, we reasoned that BCAP may promote Flightless-1 function. We lipopolysaccharide (LPS)–primed WT and BCAP-deficient macrophages and assessed NLRP3 inflammasome activity after treatment with the NLRP3-activating *Streptomyces hygroscopicus* toxin nigericin. BCAP$^{-/-}$ macrophages had increased measures of inflammasome activation, including increased caspase-1 activation, cell death, and IL-1β secretion, compared to WT macrophages (Fig. 3A). This nigericin-induced cell death was due to pyroptosis because we detected no death of *Casp1/11*$^{-/-}$ macrophages. In addition to increased active caspase-1 as measured

A

		BCAP IP			
		WT		BCAP$^{-/-}$	
Gene	Protein	Unique	Total	Unique	Total
Myo5a	Myosin VA	39	79	0	0
Pik3ap1	BCAP	21	68	1	3
Myo1e	Myosin IE	17	32	0	0
Flii	Flightless-1	16	30	0	0
Tpm4	Tropomysin α4 chain	14	33	0	0
Gsn	Gelsolin	13	22	0	0
Tpm1	Tropomysin α1 chain	13	33	0	0
Tmod3	Tropomodulin13	12	30	0	0
Fbn1	Fibrillin-1	12	16	0	0
Lrrfip2	LRRFIP2	11	24	0	0
Actr3	ARP3	11	22	0	0
Capzb	CapZ subunit β	10	27	0	0
Arpc2	Arp2/3 complex subunit 2	9	16	0	0
Capza2	CapZ subunit α2	8	14	0	0
Lcp1	Plastin 2	8	13	0	0

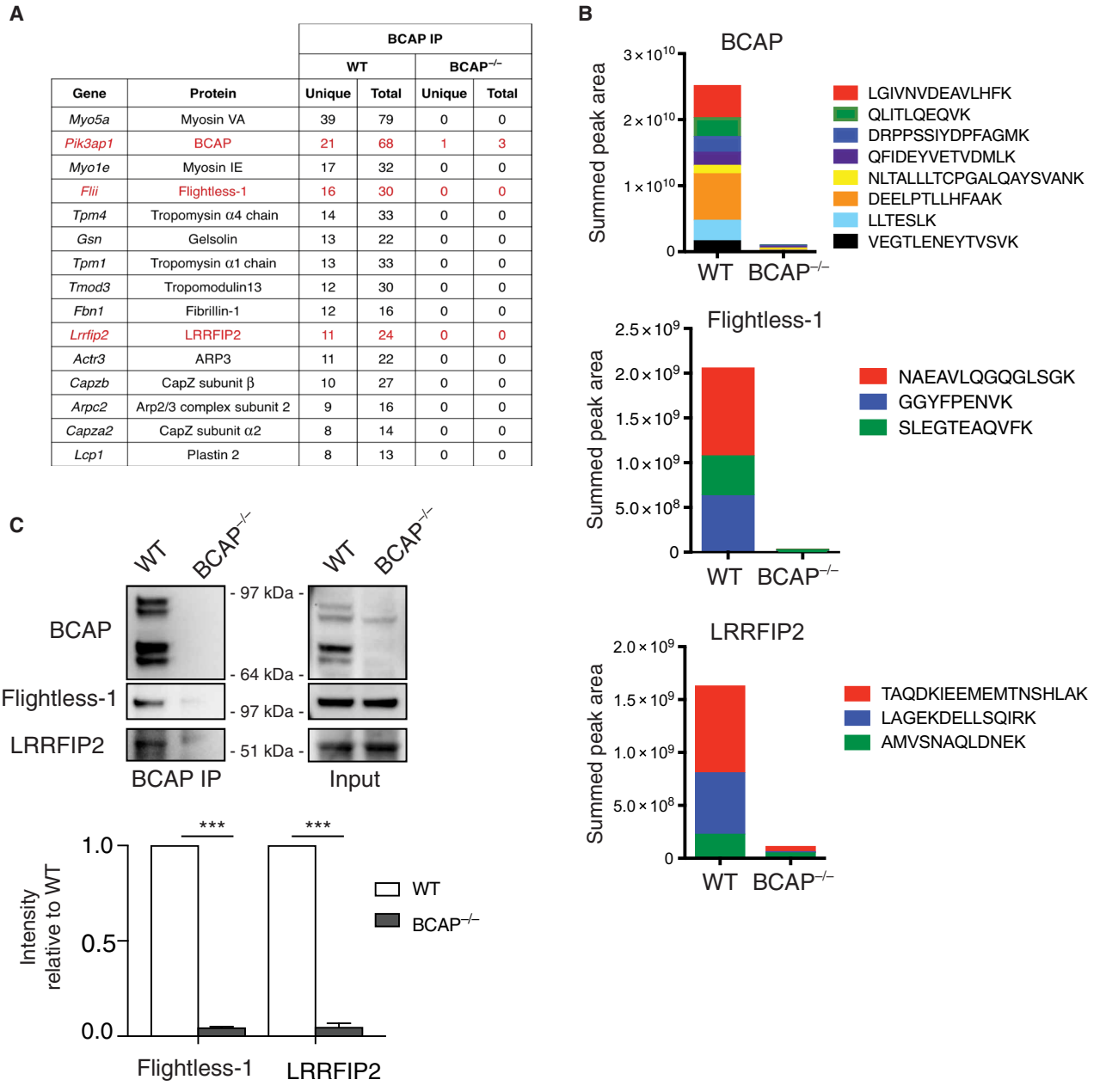

Fig. 1. BCAP interacts with Flightless-1 and LRRFIP2 in macrophages. (**A** and **B**) MS analysis of proteins associated with BCAP in lysates from WT or BCAP$^{-/-}$ bone marrow–derived macrophages (BMDMs) immunoprecipitated (IP) for BCAP. Tabulated data (A) are the average numbers of unique and total peptides from each protein. Mean peak area quantification (B) for each BCAP, Flightless-1, and LRRFIP2 peptide isolated is representative of three independent experiments. The intensity of each peptide was summed to generate an overall protein quantification score (peak area). (**C**) Western blot analysis of the indicated proteins in WT or BCAP$^{-/-}$ macrophages lysates immunoprecipitated for BCAP. Blots (top) are representative of three independent experiments. Quantified band intensity values (bottom) of Flightless-1 and LRRFIP2 are means ± SEM pooled from all experiments. ***$P < 0.001$ by two-sided unpaired *t* test.

by fluorescence microscopy, BCAP$^{-/-}$ macrophages also had increased caspase-1 (Fig. 3B) and gasdermin D (Fig. 3C) cleavage compared to WT macrophages. To assess the kinetics of NLRP3 inflammasome activation in the absence of BCAP, we performed a time course of cell death using live-cell fluorescence microscopy to assess uptake of the membrane-impermeant fluorescent probe SYTOX Green. BCAP-deficient macrophages had significantly increased cell death compared to WT macrophages as early as 12 min after nigericin treatment, which was sustained until close to maximal cell death at ~30 min (Fig. 3D). Thus, the absence of BCAP led to increased NLRP3 inflammasome function in macrophages, suggesting that in WT macrophages, BCAP serves to inhibit inflammasome activation.

LPS priming of macrophages increases NLRP3 inflammasome activation by inducing the expression of NLRP3 and pro–IL-1β. Because we have previously shown that BCAP inhibits LPS-induced TNF, IL-6, and IL-12 p40 production in macrophages due to a reduction in PI3K activation (*8*), we directly examined the expression of components of the NLRP3 inflammasome to determine whether

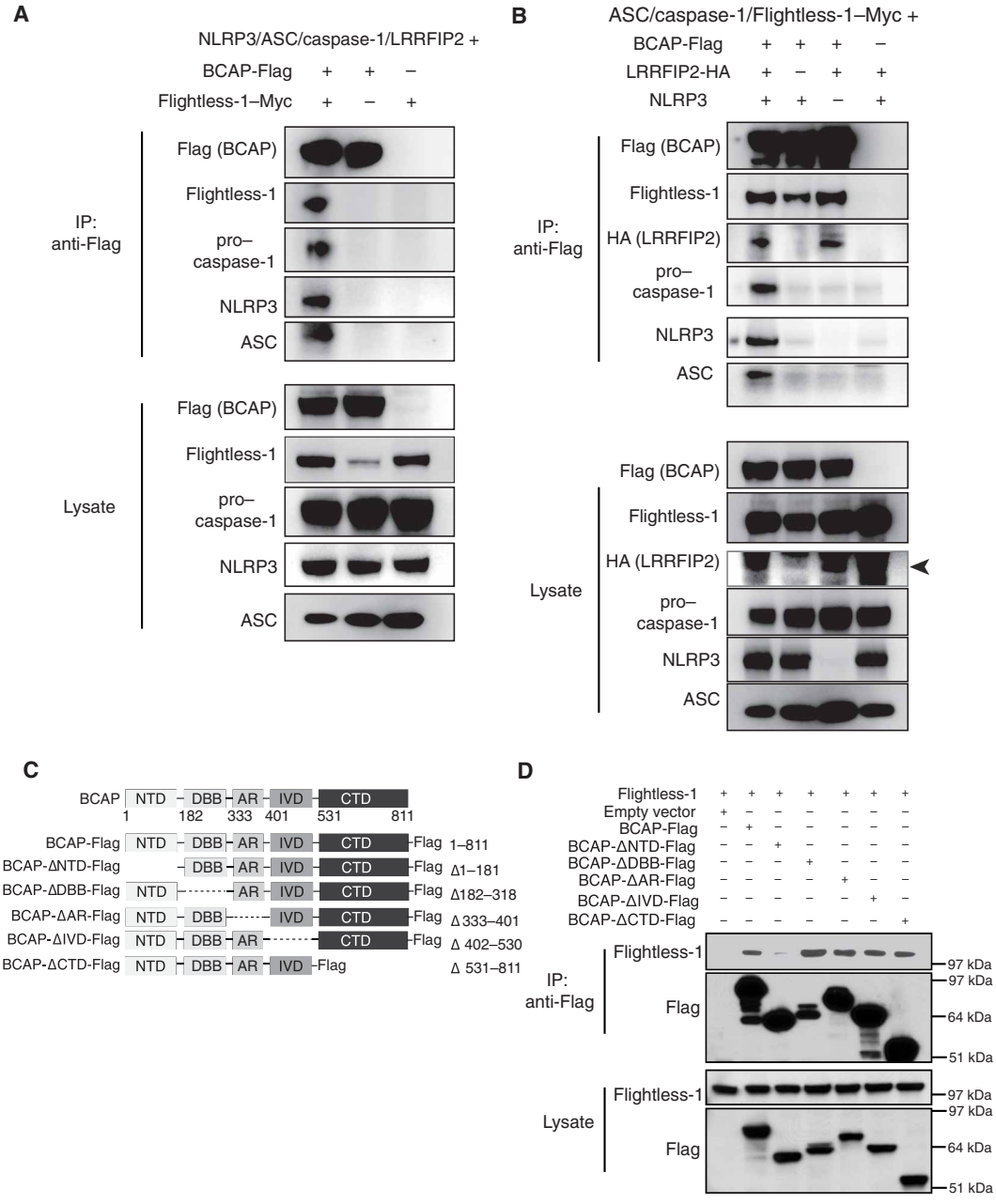

Fig. 2. BCAP interacts with NLRP3 inflammasome components through its N-terminal domain. (**A** and **B**) Western blot analysis of the indicated proteins in lysates from HEK293T cells cotransfected with plasmids encoding BCAP-Flag, Flightless-1, LRRFIP2–hemagglutinin (HA), NLRP3, ASC, and pro–caspase-1–red fluorescent protein (RFP) as indicated and immunoprecipitated for Flag. Black arrowhead in (B) indicates LRRFIP2 band. (**C**) Flag-tagged BCAP deletion mutants used in (D). (**D**) Western blot analysis of HEK293T cells cotransfected with plasmids encoding BCAP-Flag deletion mutants and Flightless-1. Lysates were immunoprecipitated for Flag and immunoblotted with anti-Flag and anti–Flightless-1 antibodies. Data are representative of three independent experiments. NTD, N-terminal domain; AR, ankyrin repeats; IVD, intervening domain; CTD, C-terminal domain.

the increased NLRP3 inflammasome activation in BCAP$^{-/-}$ macrophages was due to increased LPS priming. We found no difference between WT and BCAP$^{-/-}$ macrophages in NLRP3, ASC, pro–caspase-1, or pro–IL-1β protein by Western blot at concentrations of LPS used for priming in our experiments at either 4 or 16 hours (Fig. 3E and fig. S2A). Although we did not find increased NLRP3 or pro–IL-1β protein in BCAP$^{-/-}$ macrophages, we did find increased LPS-induced TNF secretion consistent with our previous work (fig. S2B). Quantitative polymerase chain reaction (PCR) analysis also showed no difference in the induction of mRNA encoding NLRP3 and pro–IL-1β in response to LPS between WT and BCAP$^{-/-}$ macrophages (fig. S2C).

Although differences in LPS priming of NLRP3 inflammasome components measured did not explain the increased NLRP3 activation in BCAP$^{-/-}$ macrophages, it was possible that BCAP$^{-/-}$ macrophages differentially secreted a soluble factor that could affect NLRP3 inflammasome function. We first measured IL-10 production and found no difference in IL-10 secretion between LPS-treated WT and

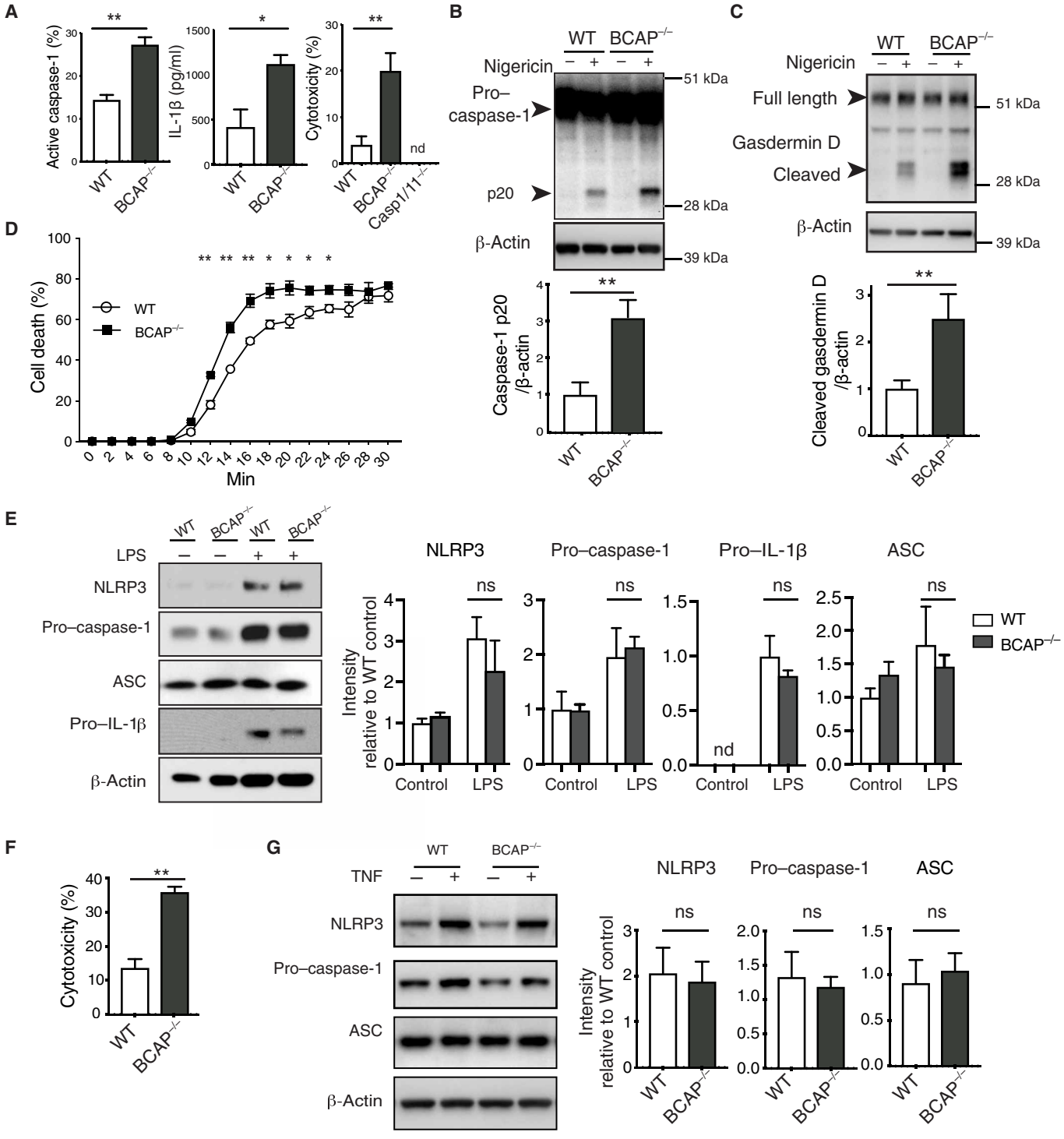

Fig. 3. Increased NLRP3 inflammasome activation in the absence of BCAP. (**A**) Immunofluorescence staining for active caspase-1 (left), enzyme-linked immunosorbent assay (ELISA) analysis of IL-1β secretion (middle), and lactate dehydrogenase (LDH) assay of cell death (right) in BMDMs primed with LPS for 4 hours and then treated with nigericin for 30 min. Data are means + SEM pooled from three experiments. (**B** and **C**) Western blot analysis for pro–caspase-1 (B) and gasdermin D (C) cleavage in cell lysates of LPS-primed nigericin-treated BMDMs. Blots (top) are representative of three independent experiments, and band intensity values (bottom) are means + SEM pooled from experiments. (**D**) Live-cell imaging of cell death in LPS-primed nigericin-treated BMDMs. Data are means ± SEM of three independent experiments. (**E**) Western blot analysis of indicated proteins in lysates from cells treated for 16 hours with LPS. Blots (left) are representative of three experiments, and quantified band intensity values are means + SEM pooled from all experiments. (**F**) LDH assay of cell death in BMDMs primed with TNF for 6 hours and treated with nigericin for 30 min. Data are means ± SEM of three independent experiments. (**G**) Western blot analysis of indicated proteins from lysates of BMDMs primed with TNF for 6 hours. Blots (left) are representative of three independent experiments, and quantified band intensity values (right) are means + SEM pooled from all experiments. nd, not detected; ns, $P > 0.05$. *$P < 0.05$, **$P < 0.01$, by two-sided unpaired *t* test.

BCAP$^{-/-}$ macrophages, consistent with our previous work (fig. S2B) (*8*). Next, we took a broader approach, assessing whether supernatants from LPS-primed BCAP$^{-/-}$ macrophages increased NLRP3 inflammasome function when transferred onto WT macrophages. Macrophages were LPS-primed for 4 hours, washed, and then cultured for an additional 2 hours in supernatants from either LPS-primed WT or BCAP$^{-/-}$ macrophages before treatment with nigericin. Culturing WT macrophages with supernatants from LPS-primed BCAP$^{-/-}$ macrophages did not increase nigericin-induced cell death or IL-1β release (fig. S2D). Conversely, culturing BCAP$^{-/-}$ macrophages with supernatants from LPS-primed WT macrophages did not decrease these measures (fig. S2D). We conclude that the difference in NLRP3 inflammasome function between LPS-primed WT and BCAP$^{-/-}$ macrophages is not due to differential secretion of a soluble factor and is therefore cell intrinsic.

We and others previously found that BCAP does not inhibit TNF signaling in macrophages (*8*, *9*); thus, we also examined NLRP3 inflammasome activation after TNF priming. BCAP deficiency also increased nigericin-mediated cytotoxicity in macrophages after TNF priming without affecting NLRP3, pro–caspase-1, or ASC expression (Fig. 3, F and G). Thus, we conclude that the effect of BCAP on NLRP3-induced caspase-1 activation is independent of priming and, therefore, BCAP must inhibit inflammasome activation through a distinct cell-intrinsic mechanism, likely through interactions with Flightless-1.

BCAP inhibits NLRP3 inflammasome activation during *Yersinia pseudotuberculosis* infection in vitro and in vivo

To ask whether BCAP also inhibits inflammasome activation during bacterial infection, we infected LPS-primed WT and BCAP$^{-/-}$ macrophages with a *Y. pseudotuberculosis* (*Ypstb*) mutant that efficiently activates the NLRP3 inflammasome. This *Ypstb*Δ mutant has no Yop effectors to translocate into the host cytoplasm that interfere with inflammasome activation (*29*–*31*). Similar to when we activated the NLRP3 inflammasome with nigericin, during infection with *Ypstb*Δ, BCAP$^{-/-}$ macrophages had increased active caspase-1, IL-1β secretion, and caspase-1/11–dependent cell death compared to WT macrophages (Fig. 4A and fig. S3). Caspase-1 cleavage, as measured by Western blot, was also increased during *Ypstb*Δ infection in BCAP$^{-/-}$ macrophages compared to WT macrophages (Fig. 4B). Therefore, BCAP inhibited inflammasome activation not only during toxin-induced NLRP3 inflammasome activation but also during bacterial infection with *Ypstb*Δ.

To examine whether BCAP inhibits inflammasome activation in vivo, we examined the clearance of a *Ypstb* mutant solely lacking YopM, a *Ypstb* caspase-1 inhibitor. Clearance of *YpstbΔYopM* is attenuated compared to WT *Ypstb*, and this attenuation depends on caspase-1 (*29*). We infected WT and BCAP$^{-/-}$ mice intraperitoneally with *YpstbΔYopM* and measured colony-forming units (CFU) in the spleen 4 days after infection. BCAP$^{-/-}$ mice had significantly increased clearance of *YpstbΔYopM*, with ~2 logs fewer bacteria in the spleen than WT mice (Fig. 4C). Eleven of 20 BCAP$^{-/-}$ mice had completely cleared the infection by day 4, whereas only 2 of 20 WT mice had performed so. To exclude increased TLR signaling, rather than increased caspase-1 activation, in BCAP$^{-/-}$ mice that led to the improved bacterial clearance, we infected WT and BCAP$^{-/-}$ mice with WT *Ypstb* that express YopM, clearance of which is caspase-1 independent (*29*). We observed no difference in bacterial clearance between WT and BCAP$^{-/-}$ mice infected with WT *Ypstb* (Fig. 4D). Together, these results suggest that during *YpstbΔYopM* infection, increased caspase-1 activation, rather than enhanced TLR signaling, is responsible for enhanced clearance of caspase-1–dependent *YpstbΔYopM* bacteria by BCAP$^{-/-}$ mice compared to WT mice.

BCAP inhibits the NAIP5/NLRC4 and NAIP2/NLRC4 inflammasomes

We next examined whether BCAP plays a role in the inhibition of inflammasomes in addition to NLRP3. First, we assessed inflammasome activation after infection with *Salmonella* Typhimurium (*S.* Typhimurium), which is principally sensed by NLRC4 inflammasomes and does not require LPS priming (*32*). We observed increased caspase-1 activation in *S.* Typhimurium–infected unprimed BCAP$^{-/-}$ macrophages compared to unprimed WT macrophages as measured by fluorescence microscopy, cell death, and pro–caspase-1 cleavage (Fig. 5, A and B). Cell lysis was not detected during infection of *Casp1/11*$^{-/-}$ macrophages with *S. Typhimurium*, which, together with the observed cleavage of pro–caspase-1, indicates pyroptotic cell death during this infection. We also assessed IL-1β release from *S.* Typhimurium–infected macrophages that had been LPS-primed to induce high amounts of pro–IL-1β protein. LPS-primed BCAP$^{-/-}$ macrophages secreted significantly more IL-1β than LPS-primed WT macrophages (Fig. 5C), consistent with increased NLRC4 inflammasome activation.

In addition, we observed increased cell death in unprimed BCAP$^{-/-}$ macrophages upon administration of FlaTox (Fig. 5D), a direct nucleotide-binding domain leucine-rich repeat family, apoptosis inhibitory protein 5 (NAIP5)/NLRC4 activator that consists of *Legionella pneumophila* flagellin fused with the N-terminal domain of *Bacillus anthracis* lethal factor (LFn), which facilitates cytosolic delivery in combination with the anthrax protective antigen (PA) channel in the absence of TLR activation (*33*). Because components of the FlaTox we used were generated in *E. coli* and could have LPS contamination (*33*), we also used a mammalian-expressed NLRC4-activating agent RodTox, composed of the inner rod protein (PrgJ) from *S.* Typhimurium SPI-1 type III secretion system fused to LFn combined with mammalian-expressed PA, which requires both NAIP2 and NLRC4 for caspase-1 activation (*34*). Kinetic analysis of cell death with mammalian-expressed RodTox showed significantly increased cell death of BCAP$^{-/-}$ macrophages compared to WT macrophages at all time points where cell death was detected (Fig. 5E). BCAP deficiency did not affect NLRC4 expression in macrophages (fig. S4). Together, these data strongly support a role for BCAP in suppressing caspase-1 activation downstream of NLRC4.

BCAP plays a role in inhibiting both NLRP3 and NLRC4 inflammasomes, which suggests that BCAP may play a general role in the inhibition of inflammasome function. We therefore examined whether BCAP inhibits the DNA-sensing AIM2 (absent in melanoma 2) inflammasome (*35*). We transfected unprimed WT and BCAP$^{-/-}$ macrophages with calf thymus DNA and measured cell death. Unexpectedly, cell lysis of WT and BCAP$^{-/-}$ macrophages was similar after DNA transfection (Fig. 5F). Therefore, BCAP does not inhibit all caspase-1–activating inflammasomes.

In addition to caspase-1, caspase-11 is also an inflammasome effector caspase that induces pyroptosis (*36*). We therefore investigated whether BCAP inhibits the noncanonical caspase-11 inflammasome that senses cytosolic LPS. To activate caspase-11, we delivered LPS to the cytoplasm of macrophages using the noncatalytic B subunit

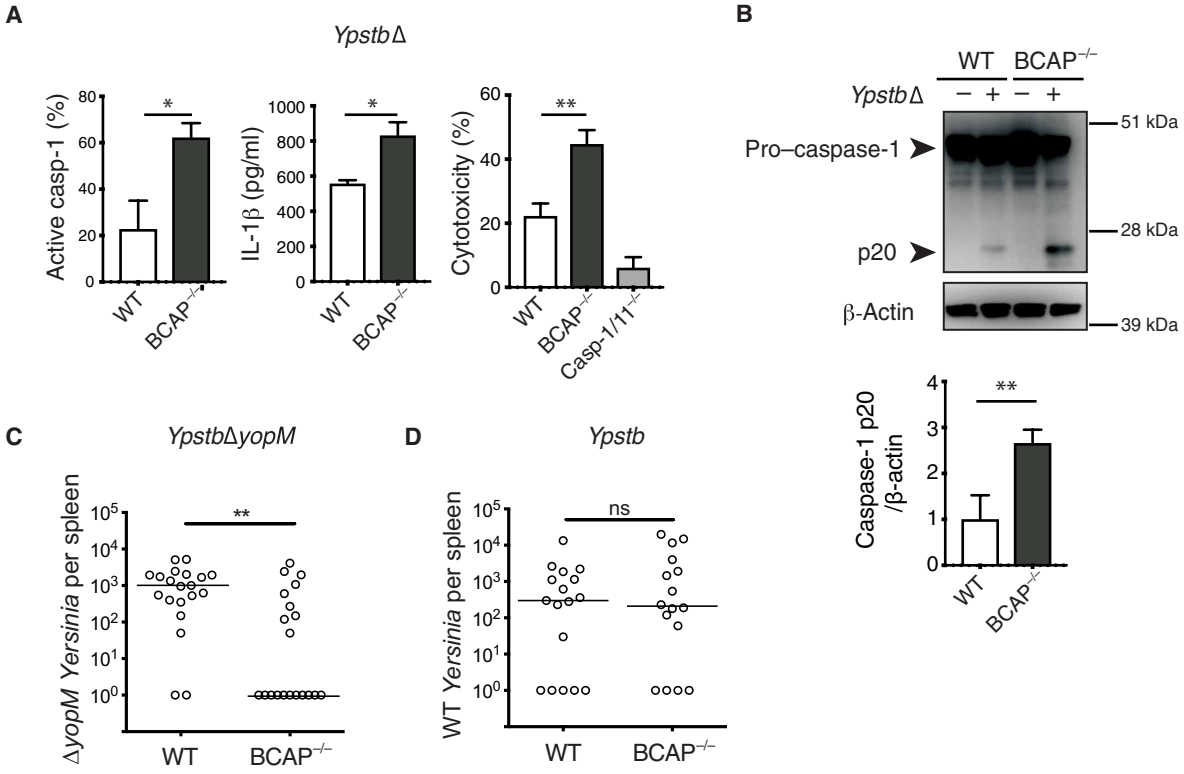

Fig. 4. Increased *Yersinia*-mediated inflammasome activation in the absence of BCAP. (**A**) Immunofluorescence staining for active caspase-1 (casp-1) (left), ELISA analysis of IL-1β secretion (middle), and LDH assay of cell death (right) in BMDMs primed with LPS overnight and then infected with *Ypstb*Δ for 90 min. Data are means + SEM pooled from four independent experiments. (**B**) Western blot analysis of pro–caspase-1 cleavage in lysates of BMDMs primed with LPS overnight and then infected with *Ypstb*Δ for 90 min. Blots (top) are representative of three independent experiments, and quantified band intensity values (bottom) are means + SEM pooled from all experiments. (**C** and **D**) Bacterial burden in the spleens of WT and BCAP$^{-/-}$ mice on day 4 after infection with *YpstbΔYopM* (C) or *Ypstb* (D). Data and median values (line) of at least 16 mice are from two independent experiments. *$P < 0.05$, **$P < 0.01$, by two-sided unpaired *t* test (A and B) and Mann-Whitney test (C and D).

of cholera toxin (*37*). Under these conditions, caspase-11–mediated cell lysis of LPS-primed WT and BCAP$^{-/-}$ macrophages was similar (Fig. 5G). These data demonstrate that BCAP does not inhibit noncanonical caspase-11 inflammasomes. Thus, BCAP inhibits NLRP3 and NLRC4 inflammasomes but not AIM2 inflammasomes or noncanonical caspase-11 inflammasomes.

BCAP inhibits the entry of pro–caspase-1 into inflammasome foci

To mechanistically determine how BCAP inhibits caspase-1 activation, we examined the formation of NLRP3/ASC/caspase-1 inflammasome foci during infection with *Ypstb*Δ. Upon detection of *Ypstb*Δ infection, NLRP3 undergoes a conformational change that results in self-oligomerization into a single focus. This conformational change leads to NLRP3 recruitment of the adaptor protein ASC to the preinflammasome focus. ASC then oligomerizes and recruits pro–caspase-1, resulting in increased local concentrations of pro–caspase-1 and enzymatic activation. We used immunofluorescence to assess whether BCAP deficiency affects the kinetics of NLRP3 inflammasome formation (*29*). The kinetics of formation of NLRP3 and ASC foci between WT and BCAP$^{-/-}$ macrophages during infection with *Ypstb*Δ were similar (Fig. 6, A to D), reinforcing our finding that differences between WT and BCAP$^{-/-}$ macrophages are not due to differences in NLRP3 or ASC expression after LPS priming. In contrast to NLRP3 and ASC foci formation, there was a significant increase in the recruitment of total and active caspase-1 to inflammasome foci in BCAP$^{-/-}$ macrophages compared to WT macrophages at both 1 and 2 hours after infection (Fig. 6, E and F).

These data show that in the absence of BCAP, pro–caspase-1 is more quickly recruited to and activated within NLRP3 inflammasome foci. To assess this more directly, we costained for ASC and active caspase-1 after *Ypstb*Δ infection. About 70% of the WT macrophages with ASC foci also had active caspase-1 in the same focus at 1 hour after infection, whereas >95% of BCAP$^{-/-}$ macrophages with ASC foci had active caspase-1 in the same focus (Fig. 6, G and H). Thus, in the absence of BCAP, entry and activation of caspase-1 into the NLRP3 inflammasome occurred more rapidly, showing that BCAP acts to delay the recruitment of pro–caspase-1 to the NLRP3 inflammasome, where it becomes activated by autoproteolysis.

BCAP is required for Flightless-1 inhibition of NLRP3 inflammasome activation

Our proteomics data suggested that BCAP may inhibit NLRP3 inflammasome function by associating with Flightless-1. Flightless-1 directly inhibits caspase-1 proteolytic activity through its pseudosubstrate site, but whether it affects recruitment of NLRP3 and ASC into forming NLRP3 inflammasomes has not been addressed. Thus, we knocked down Flightless-1 in macrophages and assessed formation of NLRP3/ASC/caspase-1 foci. Short hairpin RNA (shRNA)–mediated knockdown (KD) of Flightless-1 efficiently reduced Flightless-1

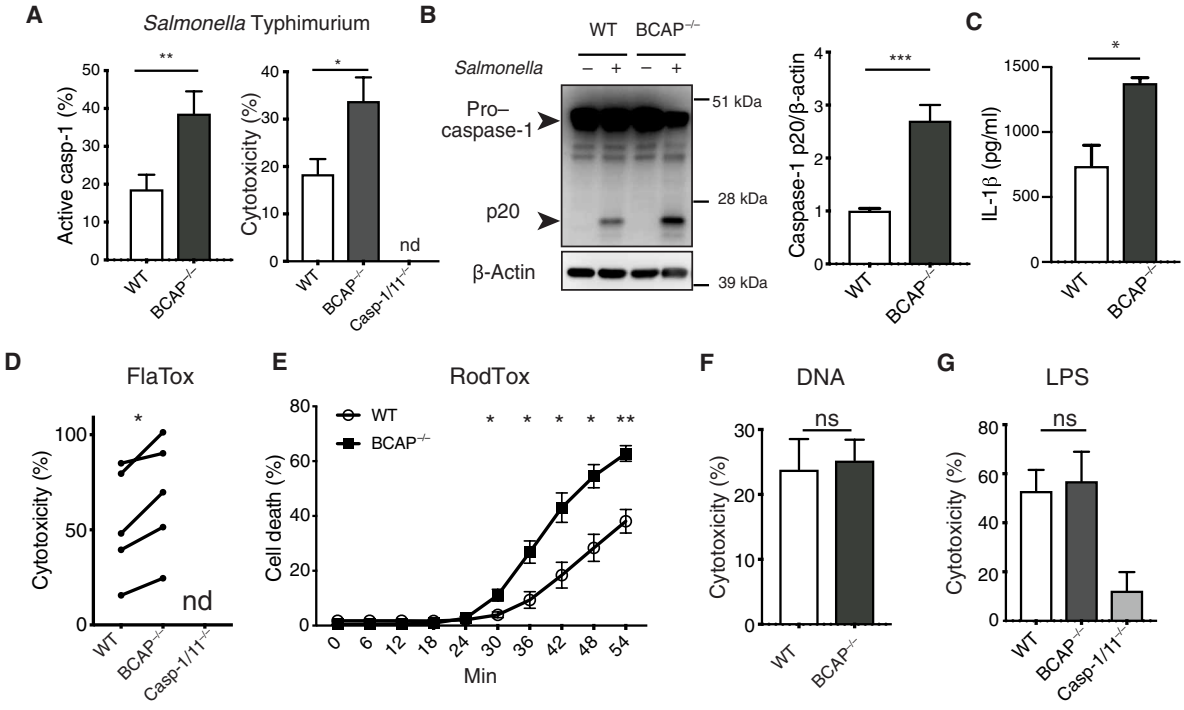

Fig. 5. BCAP inhibits NLRC4 inflammasomes. (**A**) Immunofluorescence staining for active caspase-1 (left) and LDH assay of cell death (right) in unprimed BMDMs infected with *S.* Typhimurium for 30 min. Data are means + SEM pooled from three independent experiments. (**B**) Western blot analysis of pro–caspase-1 cleavage in lysates of BMDMs infected with *S.* Typhimurium for 30 min. Blots (left) are representative of three independent experiments, and quantified band intensity values are means + SEM pooled from all experiments. (**C**) ELISA analysis of IL-1β release from LPS-primed BMDMs infected with *S.* Typhimurium for 30 min. Data are means + SEM pooled from three independent experiments. (**D**) LDH assay of cell death in unprimed BMDMs treated with FlaTox for 20 min. Data are individual means from five independent experiments (connected with lines). (**E**) Live-cell imaging to measure cell death in BMDMs treated with RodTox. Data are means ± SEM of three independent experiments. (**F**) LDH assay of cell death in unprimed BMDMs transfected with calf thymus DNA for 3 hours. Data are means + SEM pooled from three independent experiments. (**G**) LDH assay of cell death in unprimed BMDMs treated with LPS and cholera toxin B for 3.5 hours. Data are means ± SEM of three independent experiments. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, by two-sided unpaired (A to C and E to G) and paired *t* test (D).

protein in both WT and BCAP$^{-/-}$ macrophages compared to the nontarget (NT) control (Fig. 7A and fig. S5A). Similar to the effect of BCAP deficiency in macrophages, Flightless-1 KD caused increased recruitment of active caspase-1, but not NLRP3 or ASC, to foci after *Ypstb*Δ infection (Fig. 7B and fig. S5B). These data suggest that BCAP and Flightless-1 both affect inflammasome maturation at the stage of pro–caspase-1 recruitment and activation.

To determine whether BCAP requires Flightless-1 to inhibit inflammasome activation, we knocked down Flightless-1 in both WT and BCAP$^{-/-}$ macrophages and infected with *Ypstb*Δ to assess NLRP3 inflammasome activation (Fig. 7C). Whereas Flightless-1 KD in WT macrophages statistically significantly increased *Ypstb*Δ-induced caspase-1 activation, Flightless-1 KD had no effect in BCAP$^{-/-}$ macrophages, proving that the interaction between BCAP and Flightless-1 is required to inhibit inflammasome activation during *Ypstb*Δ infection. We found a similar result with nigericin-induced caspase-1 activation using two distinct shRNAs; Flightless-1 KD significantly increased caspase-1 activation in WT macrophages but not in BCAP$^{-/-}$ macrophages (Fig. 7D and fig. S5C).

We next assessed what role BCAP serves in Flightless-1 inhibition of NLRP3 inflammasomes. Flightless-1 was not constitutively associated with pro–caspase-1 in macrophages in the LPS-primed state but was inducibly associated with pro–caspase-1 after activation of NLRP3 with nigericin, confirming the data of Jin *et al.* (*25*). BCAP was required not only for Flightless-1 inhibition of NLRP3 inflammasomes but also for Flightless-1 to associate with pro–caspase-1 (Fig. 7E). These data reinforced our findings that BCAP was required for Flightless-1 function in inflammasome inhibition (Fig. 7, C and D).

We initially found that BCAP and Flightless-1 were associated in resting macrophages (Fig. 1). We asked whether BCAP remained associated with Flightless-1 after LPS priming and NLPR3 inflammasome activation. BCAP and Flightless-1 were associated in LPS-primed macrophages. However, nigericin treatment caused the dissociation of BCAP and Flightless-1 (Fig. 7F), at a time when Flightless-1 is bound to pro–caspase-1 (Fig. 7E). Our data suggest a model in which BCAP facilitates the delivery of Flightless-1 and LRRFIP2 to the forming NLRP3 inflammasome, and then, BCAP is subsequently released, whereas Flightless-1 remains associated with pro–caspase-1 (fig. S6).

DISCUSSION

Inflammasome activation is a potent inflammatory defense required for clearance of pathogens, yet excessive inflammasome activation leads to pathological inflammation and autoinflammatory syndromes. Therefore, tight regulation of this pathway is required to resist infection yet prevent excess collateral damage. In this study, we showed that the macrophage signaling adaptor protein BCAP dampened NLRP3 and NLRC4 inflammasome activation through interaction with the endogenous caspase-1 inhibitor Flightless-1. Together with

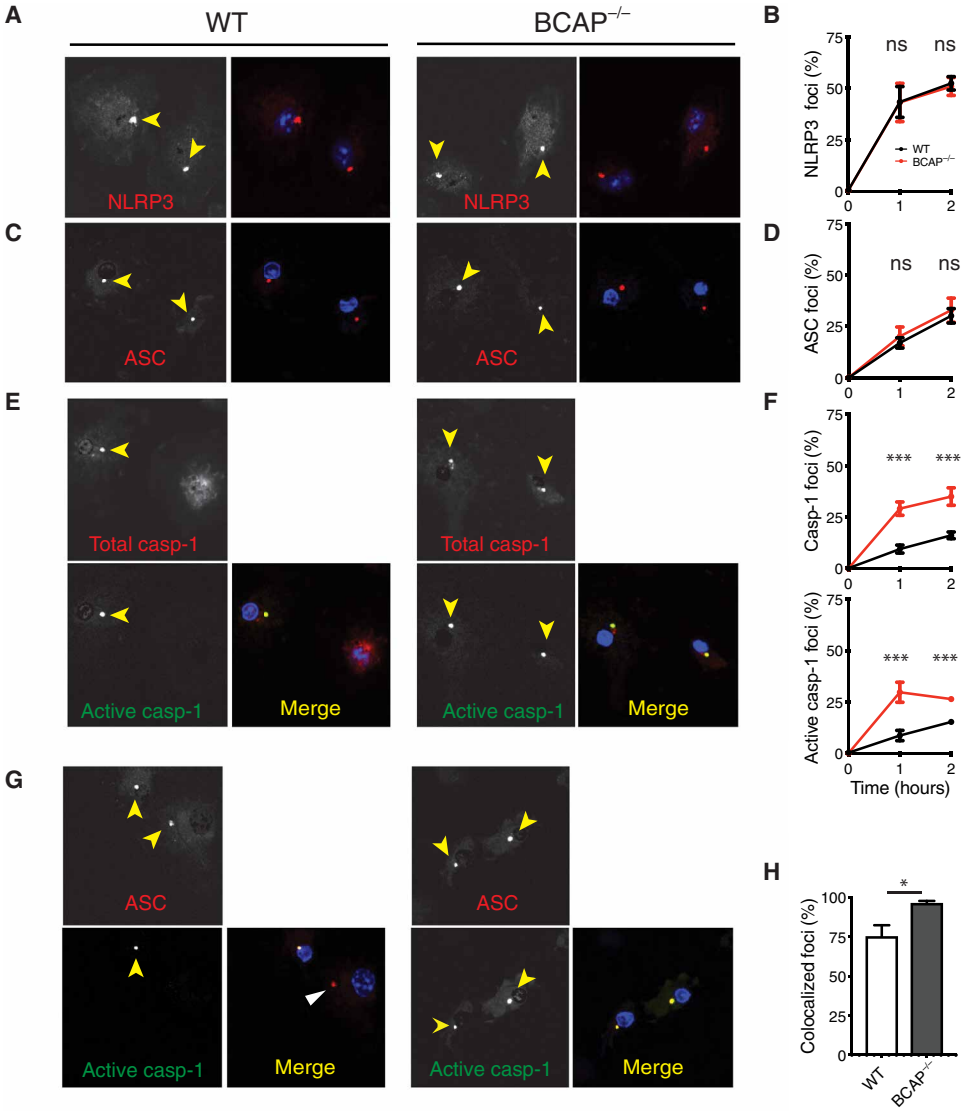

Fig. 6. BCAP delays caspase-1 recruitment into NLRP3 inflammasome foci. (**A** to **F**) Immunofluorescence analysis of NLRP3, ASC, or caspase-1 foci formation in BMDMs primed with LPS overnight and then infected with *Ypstb*Δ. Yellow arrowheads indicate foci. Images (A, C, and E) are representative of three independent experiments, and quantification of the percentage of cells with foci of the indicated protein (B, D, and F) is means ± SEM from all experiments. (**G** and **H**) Immunofluorescence analysis of active caspase-1 and ASC in BMDMs primed with LPS overnight and then infected with *Ypstb*Δ for 90 min. White arrowhead indicates preinflammasome focus containing ASC but not active caspase-1. Images (G) are representative of three independent experiments, and quantification of the percentage of cells with ASC foci with active caspase-1 (H) is means ± SEM from all experiments. **P* < 0.05, ****P* < 0.001, by two-sided unpaired *t* test.

our previous work showing that BCAP can inhibit TLR signaling in macrophages by activating PI3K, we have identified BCAP as a multifunctional signaling adaptor tuning macrophage innate immunity.

Together, BCAP and Flightless-1 inhibit pro–caspase-1 recruitment to NLRP3 preinflammasomes to limit inflammasome activation. BCAP-deficient macrophages have similar formation of NLRP3 and ASC containing preinflammasome foci as WT macrophages yet have increased total and active caspase-1–containing foci and increased measures of caspase-1 activation similar to cells with Flightless-1 KD (*24*, *25*). We confirmed that Flightless-1 was not complexed with pro–caspase-1 in resting macrophages, but that early after activation of NLRP3 inflammasomes, Flightless-1 interacted with pro–caspase-1 (*25*). We found that the ability of Flightless-1 to bind to pro–caspase-1 and inhibit its function required BCAP. Thus, we propose that BCAP facilitates binding of Flightless-1 to pro–caspase-1, which delays pro–caspase-1 entry into the forming NLRP3 inflammasome focus (fig. S6). Whereas BCAP and Flightless-1 interacted without coexpression of NLRP3, ASC, and pro–caspase-1, the ability of BCAP and Flightless-1 to form a complex with pro–caspase-1 required NLRP3 and ASC, as well as the Flightless-1 binding partner LRRFIP2. These findings support a model in which NLRP3 inflammasome activation signals for BCAP to facilitate Flightless-1/pro–caspase-1 interactions. Our data suggest that this complex is then targeted to NLRP3-containing inflammasome foci by LRRFIP2 and BCAP is released. Identification of the signals that lead to BCAP release from the Flightless-1/pro–caspase-1 complex is an important area of future work.

We found here that BCAP does not inhibit formation of all inflammasomes. Of those we tested, BCAP inhibited NLRP3, NAIP5/NLRC4, and NAIP2/NLRC4 inflammasomes but not AIM2 inflammasomes or noncanonical caspase-11 inflammasomes. We found that the BCAP-facilitated complex between Flightless-1 and pro–caspase-1 required the expression of the Flightless-1 binding partner LRRFIP2, which is required for Flightless-1 to associate with and inhibit only the NLRP3 inflammasome (*25*). Given that BCAP required both Flightless-1 and LRRFIP2 to associate with NLRP3 inflammasomes but that LRRFIP2 only associates with NLRP3 (*25*), we hypothesize that there may be an analogous protein to LRRFIP2 that allows BCAP and Flightless-1 to inhibit NLRC4 inflammasomes. This adaptor would likely bind to NLRC4 and Flightless-1 similar to how LRRFIP2 binds to NLRP3 and Flightless-1.

In contrast to NLRP3 and NLRC4 inflammasomes, BCAP did not inhibit AIM2 inflammasome function. This may suggest that there are no adaptor proteins in macrophages to bridge AIM2 to Flightless-1. Similarly, BCAP did not inhibit noncanonical caspase-11 inflammasomes. Although Flightless-1 was previously identified as a protein that interacts with caspase-11, Flightless-1 did not have the capacity to inhibit caspase-11 activity (*24*). This may explain why BCAP deficiency has no effect on noncanonical

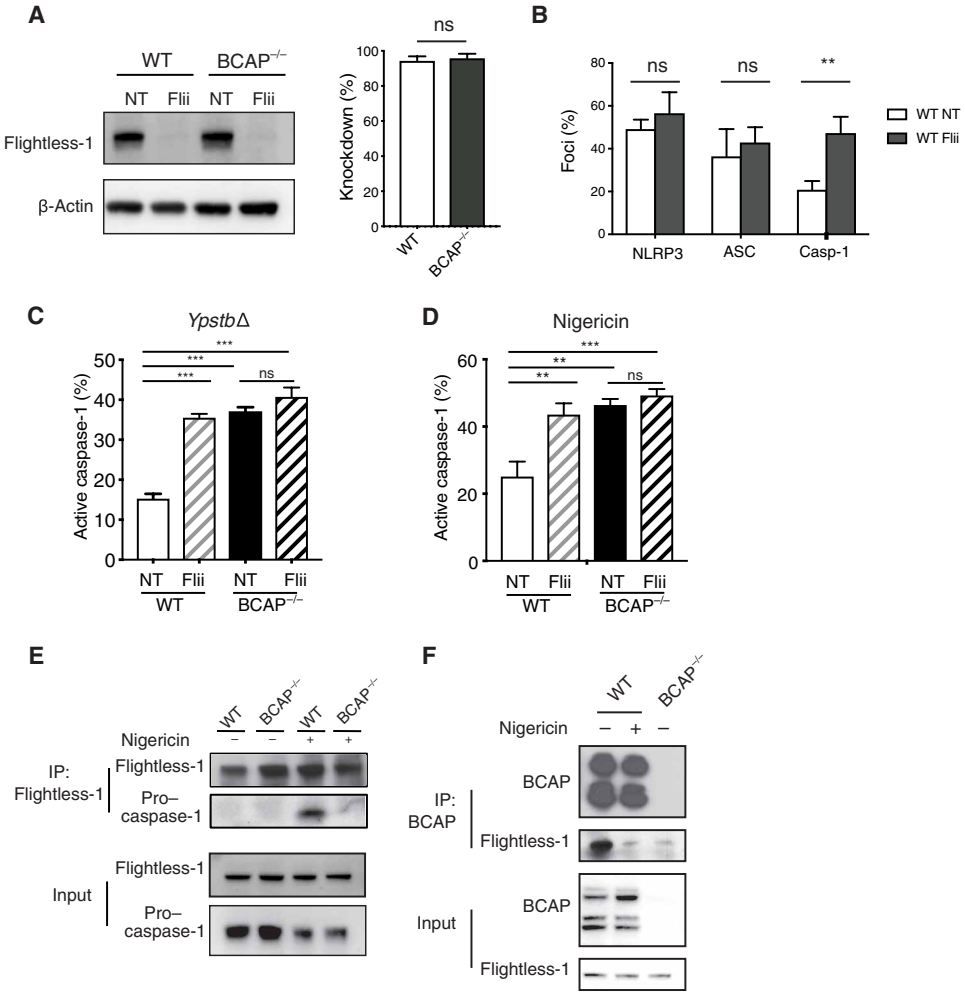

Fig. 7. BCAP inhibition of NLRP3 inflammasome activation requires Flightless-1. (**A**) Western blot analysis of Flightless-1 in BMDMs transduced with lentiviruses encoding shRNA#1 (Flii) or NT shRNA. Blots (left) are representative of three independent experiments, and quantified KD efficiency values (right) are means + SEM from three independent experiments. (**B**) Immunofluorescence analysis of NLRP3, ASC, and active caspase-1 in WT BMDM transduced with NT or Flii-specific shRNA#1, primed with LPS overnight, and then infected with *Ypstb*Δ. Data are means + SEM from three independent experiments. (**C** and **D**) Immunofluorescence analysis of active caspase-1 in WT and BCAP$^{-/-}$ BMDM transduced with NT shRNA or Flii-specific shRNA#1 and were either LPS-primed overnight and infected with *Ypstb*Δ for 60 min (C) or LPS-primed for 4 hours and treated with nigericin for 30 min (D). Data are means + SEM of three independent experiments. (**E** and **F**) Western blot analysis of proteins immunoprecipitated with antibodies to Flightless-1 (E) or BCAP (F) in lysates of WT or BCAP$^{-/-}$ BMDM primed with LPS for 4 hours and then treated with nigericin for 15 min. Blots are representative of three independent experiments. ns, not significant. $^{**}P < 0.01$, $^{***}P < 0.001$, by two-sided unpaired *t* test (A and B) or one-way analysis of variance (ANOVA) with Tukey's post hoc test (C and D).

caspase-11 inflammasome–mediated cell death. Thus, we speculate that the reasons why BCAP inhibits certain inflammasomes, but not others, may depend on Flightless-1 specificity for caspase-1 over caspase-11, as well as the availability of Flightless-1 binding partners to assist in recruitment of the Flightless-1/BCAP/pro–caspase-1 complex. The discovery of the protein or proteins that bridge Flightless-1 to NLRC4, allowing for BCAP inhibition, is under investigation.

We and others first defined a role for BCAP in limiting pathogen sensing through the TLR family of pattern recognition receptors (*8*, *9*). BCAP inhibits TLR-induced inflammatory cytokine production, specifically TNF, IL-6, and IL-12 p40 production, through its ability to bind the p85 subunit of class I PI3K and thereby activate PI3K catalytic activity (*8*). Therefore, upon finding that NLPR3 inflammasome function was increased in BCAP-deficient macrophages compared to WT cells, we examined whether this was due to increased LPS-dependent priming, which transcriptionally induces NLRP3 and pro–IL-1β. We showed that high-dose LPS induced similar expression of NLRP3 and pro–IL-1β mRNA and protein in WT and BCAP-deficient macrophages, even under conditions where BCAP-deficient cells produced more TNF than WT macrophages. In addition, WT and BCAP-deficient macrophages did not differ in the expression of ASC, pro–caspase-1, Flightless-1, or LRRFIP2. Consistent with our previous findings, LPS-induced IL-10 production was also similar between WT and BCAP-deficient macrophages. Therefore, BCAP inhibits some LPS-induced responses through its ability to activate PI3K (*8*, *9*). However, BCAP is not able to inhibit the expression of all LPS-induced genes, including *Nlrp3* and *Il1b*.

Furthermore, supporting that the ability of BCAP to inhibit NLRP3 inflammasomes is not due to effects on LPS priming, BCAP-deficient macrophages also showed increased NLRP3 inflammasome activation after TNF priming, which is a signaling pathway not inhibited by BCAP (*8*, *9*). In addition, we showed that the kinetics NLRP3 and ASC foci formation are not different between WT and BCAP$^{-/-}$ macrophages, supporting our conclusion that increased NLRP3 inflammasome activation in BCAP$^{-/-}$ macrophages was not due to increased NLRP3 expression. We also demonstrated that soluble factors produced in response to LPS priming are not responsible for the enhanced NLRP3 inflammasome activation of BCAP$^{-/-}$ macrophages. Our finding that BCAP inhibited NAIP5/NLRC4 and NAIP2/NLRC4 inflammasomes induced by *S.* Typhimurium infection or by direct cytosolic delivery of the NAIP5/NLRC4 activator FlaTox (*33*) and the NAIP2/NLRC4 activator RodTox (*34*) in the absence of LPS priming also supports a direct role for BCAP in inhibiting inflammasome activation. Furthermore, distinguishing the role of BCAP in limiting activation of the TLR pathway through PI3K and the NLRP3 and NLRC4 inflammasomes through Flightless-1 is our finding that mutation of the four YxxM tyrosines in BCAP required for PI3K p85 binding had no effect on BCAP binding to Flightless-1. The N-terminal domain of BCAP that contains a TIR domain is both required for Flightless-1 interaction and for inhibition

of TLR signaling (*9*), supporting the key role of this BCAP domain in limiting innate immune signaling.

Flightless-1 not only binds and inhibits pro–caspase-1 activation but also regulates actin remodeling through its gelsolin domains (*26*). Although we have not directly assessed how BCAP- and Flightless-1–mediated actin cytoskeletal rearrangements may limit inflammasome activity, this is an intriguing possibility, given the emerging information on how actin polymerization may regulate and be sensed by various inflammasomes (*38*–*42*). We found that in addition to Flightless-1 and LRRFIP2, BCAP and Flightless-1 were also associated with other actin-binding proteins in macrophages, including members of the ARP2/3 complex and several myosins. Thus, the ability of BCAP and Flightless-1 to allow for sensing of aberrant actin dynamics will be the focus of future studies.

Our results reveal a role for BCAP in the inhibition of NLRP3 and NLRC4 inflammasome function, as BCAP and Flightless-1 work cooperatively to delay pro–caspase-1 recruitment and activation to forming inflammasomes containing NLRP3 and ASC. This delay in entry of pro–caspase-1 into NLRP3 inflammasomes may allow pro–caspase-1 to be directed to sites within the cell where it can be activated and cleave substrates distinct from the prototypical caspase-1 targets, pro–IL-1β, pro–IL-18, and gasdermin D. These targets may include subunits of the NADPH (reduced form of nicotinamide adenine dinucleotide phosphate) oxidase on phagosomal membranes (*43*) or metabolic proteins (*44*), pathways targeted by caspase-1 to regulate the key macrophage cellular processes, phagosomal acidification, and glycolysis, respectively (*45*). It is interesting to speculate that pro–caspase-1 association with Flightless-1 and the actin cytoskeleton may facilitate some of these alternative functions of caspase-1 in macrophages.

Our data show that BCAP and Flightless-1 are a critical hub for limiting inflammasome activity in macrophages. Inhibition of inflammasome activation is a novel function for BCAP, which had previously only been shown to stimulate PI3K-dependent signaling pathways in hematopoietic cells, which include the TLR pathway in macrophages, ITAM-dependent pathways in B cells, NK cells, and T cells, and the IL-1R pathway in T cells. Our work highlights how inhibition is intrinsically integrated into innate sensing pathways to produce the appropriate amount of inflammation, key to both protective and pathogenic immune responses.

MATERIALS AND METHODS

Mice

WT C57BL/6J mice were bred at the Benaroya Research Institute or purchased from the Jackson Laboratories. BCAP$^{-/-}$ mice lacking the *Pik3ap1* gene were back-crossed to C57BL/6J mice for nine generations (*3*). Caspase-1/11$^{-/-}$ mice (*46*) were provided by R. Flavell (Yale University, New Haven, CT). Caspase-11$^{-/-}$ mice (*47*) were provided by M. Bevan (University of Washington, Seattle, WA). All experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at the Benaroya Research Institute and the University of Washington.

Cell culture

BMDMs were generated from bone marrow from femurs and/or tibias of mice by culture for 7 days at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS), 5 mM Hepes, L-glutamine (0.2 mg/ml), 0.05 mM 2-mercaptoethanol (2-ME), gentamicin sulfate (50 mg/ml), and penicillin and streptomycin (10,000 U/ml) with 30% L cell–conditioned medium or 10% CMG12-14 supernatant as a source of macrophage colony-stimulating factor. Macrophages were then harvested using phosphate-buffered saline (PBS) containing 1 mM EDTA, suspended in antibiotic-free DMEM supplemented with 5% FBS. Macrophages were activated with *Salmonella minnesota* R595 LPS (100 ng/ml; List Biological Laboratories) 4 hours before nigericin treatment or 16 hours before bacterial infection, unless otherwise noted.

MS-based proteomics

BMDMs (10^8 cells per condition) were lysed with 1 ml of radioimmunoprecipitation assay buffer [150 mM NaCl, 50 mM tris (pH 7.4), 1 mM EDTA (pH 8.0), 1% Triton X-100, 0.25% sodium deoxycholate, and protease and phosphatase inhibitors]. Immunoprecipitation was performed by incubation of lysates with 2 μg of anti-BCAP antibody (4L8E6) (*4*) overnight (4°C), followed by subsequent enrichment using protein A agarose beads (Calbiochem). The resulting protein complexes were washed several times in lysis buffer and twice in 50 mM ammonium bicarbonate buffer and were subsequently reduced, alkylated, and digested into peptides using trypsin (V5280, Promega). Peptides were loaded onto a 3-cm self-packed C18 capillary precolumn (5 μM; Reprosil, Dr. Maisch). After a 10-min rinse (0.1% formic acid), the precolumn was connected to a 25-cm self-packed C18 (3 μM; Reliasil, Orochem) analytical capillary column (inner diameter, 50 μm; outer diameter, 360 μm) with an integrated electrospray tip (~1-μm orifice). Online peptide separation followed by MS analyses was performed on a two-dimensional nano–liquid chromatography system (nanoACQUITY UPLC system, Waters Corporation). Peptides were eluted using a 150-min gradient with solvents A [H_2O/formic acid, 99.9:1 (v/v)] and B [acetonitrile/formic acid, 99.9:1 (v/v)]: 10 min from 0 to 10% B, 105 min from 10 to 40% B, 15 min from 40 to 80% B, and 20 min with 100% A. Eluted peptides were directly electrosprayed into an Orbitrap Q Exactive MS (Thermo Fisher Scientific) equipped with a high-energy collision cell [high-energy C-trap dissociation (HCD)].

The MS was operated in a data-dependent mode to automatically switch between MS and MS/MS acquisitions. Each full scan [mass/charge ratio (*m*/*z*) from 300 to 1500] was acquired in the Orbitrap analyzer (resolution, 70,000), followed by MS/MS analyses on the top 20 most intense precursor ions that had charge states greater than one. The HCD MS/MS scans were acquired using the Orbitrap system (resolution, 17,500) at a normalized collision energy of 28%. The precursor isolation width was set at 2 *m*/*z* for each MS/MS scan, and the maximum ion accumulation times were as follows: MS (100 ms) and MS/MS (100 ms). MS/MS data files were searched using the CoMEt algorithm (*48*), and the data were further processed using the Institute for Systems Biology's Trans Proteomic Pipeline (*49*). Static modification of cysteine (carbamidomethylation; 57.02 Da) was used in the search. For precursor area quantification of LRRFIP2, PIK3AP1, and FLII peptides, the data files were imported into the Skyline software package (*50*), whereupon the retention time windows for each peptide was determined on the basis of coelution the MS/MS identification. For each peptide, the relative intensity of the precursor ions was quantified using this retention time window.

Bacterial growth, in vitro infection, and inflammasome activation

Y. pseudotuberculosis YPIII serogroup O:3 (*Ypstb*) and its mutant *Ypstb*Δ (*yopJ*-, *yopO*-, *yopE*-, *yopH*-, *yopK*-, and *yopM*-) (*31*) were

grown overnight at 25°C in LB and then diluted 1:40 into LB with 20 mM $MgCl_2$ and 20 mM $Na_2C_2O_4$, grown at 25°C for 1 hour and then 37°C for 2 hours. Bacteria were then washed in PBS, added to a multiplicity of infection of 10, and spun onto macrophages for 3 min at 150*g*. NLRP3 was induced with 5 μM nigericin (Sigma-Aldrich). *S.* Typhimurium was grown in LB overnight at 37°C, diluted 1:40, and grown for 3 hours in LB containing 0.3 M NaCl. NAIP5/NLRC4 was induced with FlaTox, consisting of LFn-FlaA (5 μg/ml) and PA (10 μg/ml; List Biological Laboratories). Recombinant LFn-FlaA was expressed and purified as previously described (*33*). NAIP2/NLRC4 was induced by RodTox, consisting of mammalian-expressed LFn-PrgJ (20 ng/ml) and PA (4 μg/ml) as previously described (*34*). To activate the AIM2 inflammasome, macrophages were transfected with calf thymus DNA (Sigma-Aldrich) complexed with Lipofectamine 2000 (Invitrogen). To induce caspase-11 activation, LPS (2 μg/ml; *S. minnesota* R595, List Biological Laboratories) was mixed with cholera toxin B subunit (20 μg/ml; Sigma-Aldrich) in Opti-MEM (Gibco), added to macrophages primed overnight with LPS after removal of culture medium, and spun at 1000*g* for 5 min (*37*). CytoTox 96 kit (Promega) was used to determine LDH release. Experiments measuring IL-1β release, caspase-1 activation, or inflammasome assembly were performed in the presence of 5 mM glycine to reduce cell lysis (*51*). IL-1β in supernatants was quantified by ELISA (R&D Systems).

Real-time cell death analysis

BMDMs were seeded on 96-well (RodTox stimulation) or 24-well tissue culture plates (nigericin stimulation). Before live-cell imaging, medium was replaced with fresh macrophage medium containing 1:5000 SYTOX Green dye (Life Technologies) and Hoechst 33342 nucleic acid stain (Molecular Probes). Cells were incubated at 37°C for 20 min before adding 5 μM nigericin or RodTox (20 ng/ml). After the stimuli treatment, cells were immediately visualized for SYTOX Green uptake (cell death) and Hoechst nuclei staining (total cell number) using Cytation 3 Imaging reader (BioTek). Images were taken every 2 min (nigericin) or 6 min (RodTox) for up to 30 min (nigericin) or 54 min (RodTox). Cell death percentage was calculated as the number of SYTOX Green–positive cells divided by the Hoechst-positive cell number.

Fluorescence microscopy

Caspase-1 activation and active caspase-1 foci formation was determined by FAM-YVAD-FMK (ImmunoChemistry Technologies) staining of macrophages infected on glass coverslips. DNA was stained with Hoescht 33342. Cells were washed with PBS and subsequently fixed with Cytofix (BD Biosciences). Immunofluorescence staining was performed after fixation in Perm/Wash (BD Biosciences) with antibodies specific to NLRP3 (sc-66846, Santa Cruz Biotechnology), ASC (ADI-905-173-100, Enzo Life Sciences), or pro–caspase-1 (sc-514, Santa Cruz Biotechnology) with Alexa Fluor–conjugated secondary antibodies (Invitrogen). Coverslips were mounted with ProLong (Molecular Probes) and examined using confocal microscope (Leica SP8X) at the W. M. Keck Microscopy Center. Caspase-1 activation or foci formation was enumerated by counting the fraction of positive cells in four separate fields for each coverslip. Caspase-1 and ASC foci colocalization was enumerated by counting the fraction of positive cells in eight separate fields from duplicate coverslips.

Plasmid construction and transfection

Recombinant vectors encoding mouse Flightless-1 with a C-terminal myc tag and LRRFIP2 with a C-terminal HA tag were constructed by PCR-based amplification of complementary DNA from mouse BMDMs. Truncated mutants of BCAP were generated by PCR-based mutation and amplification of WT BCAP expression vector (*3*) using Q5 Site-Directed Mutagenesis Kit (E0554S, New England Biolabs). Expression plasmids encoding mouse ASC (pUNO1-mASC, InvivoGen), NLRP3 (puno1ha-mnalp3, InvivoGen), RFP–caspase-1 (C285A) (*43*), LRRFIP2, BCAP, and Flightless-1 were transfected into HEK293T cells with TransIT-LT1 transfection reagent (MIR 2300, Mirus Bio) according to the manufacturer's instructions.

Immunoprecipitation and immunoblot analysis

Macrophages were lysed at the indicated times in lysis buffer containing 1% Triton X-100, protease inhibitors (P8340, Sigma-Aldrich), and sodium orthovanadate (1 mM; Sigma-Aldrich). For immunoprecipitation, macrophages or HEK293T cells were lysed using lysis buffer [50 mM tris-HCl, (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.25% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and protease inhibitor (Sigma-Aldrich)]. Whole-cell lysates were incubated with 1.5 μg of anti-Flag (2 hours at 4°C), anti-BCAP, or anti–Flightless-1 antibodies (overnight at 4°C), followed by protein A agarose (30 min at 4°C), extensively washed with lysis buffer, and then eluted with boiling in 1× lithium dodecyl sulfate sample buffer (Invitrogen). Lysates or immunoprecipitates were separated by SDS–polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane, and detected by the indicated antibodies and Immobilon chemiluminescence system (Millipore). The ImageJ software was used to quantify immunoblot bands. For immunoprecipitation blots, the relative intensity was calculated by dividing the intensity of the immunoprecipitated protein by the intensity of same protein in lysates. Relative intensity was calculated by normalizing to WT. For all other experiments, the intensity of the protein of interest was first normalized to β-actin expression, and then relative intensity was calculated by normalizing to WT BMDMs. Antibodies used were Flightless-1 (sc-55583, Santa Cruz Biotechnology), LRRFIP2 (sc-100025, Santa Cruz Biotechnology), caspase-1 p20 (AG-20B-0042B-C100, AdipoGen Life Sciences), human caspase-1 (sc-622, Santa Cruz Biotechnology), NLRP3 (15101S, Cell Signaling Technology), ASC (sc-22514-R, Santa Cruz Biotechnology), IL-1β (AF-401-NA, R&D Systems), BCAP (4LE86) (*4*), Flag M2 (F3165, Sigma-Aldrich), and c-Myc (sc-40, Santa Cruz Biotechnology).

Lentivirus-mediated shRNA KD

HEK293T cells were cotransfected with packaging plasmid (psPAX2) and envelope plasmid (VSVg) together with a plasmid expressing shRNA specific for mouse Fli1 (TRCN0000324345 for shRNA#1 or TRCN000032413 for shRNA#2, Sigma-Aldrich) or NT control shRNA (Sigma-Aldrich). We transfected HEK293T cells in a 10-cm dish and harvested the virus-containing culture medium 48 hours after changing the transfection medium to DMEM containing 10% FBS. The collected medium was filtered through 0.45-μm filters. The filtered virus were diluted in DMEM containing 10% FBS and used to infect bone marrow cells after 1 day of culture. Twenty-four hours after infection, the medium was replaced with fresh macrophage medium containing puromycin (5 μg/ml). After 72 hours of puromycin selection, the macrophages were replated for further experiments.

In vivo infection

Y. pseudotuberculosis YPIII *yopM-* (*29*) or *Y. pseudotuberculosis* YPIII cultures were grown overnight in LB and enumerated by Coulter counter (Multisizer 4, Beckman Coulter). *Yersinia* was diluted in PBS for intraperitoneal delivery of 2000 CFU (*Y. pseudotuberculosis* YPIII *yopM-*) or 1000 CFU (*Y. pseudotuberculosis* YPIII) in 200 μl to 6- to 8-week-old mice. Mice were euthanized on day 4, and spleens were homogenized and plated for CFU.

Statistical analysis

Statistics were calculated using GraphPad Prism, and tests used for individual experiments are listed in each figure legend.

SUPPLEMENTARY MATERIALS

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Fig. S1. BCAP YxxM tyrosines are not required for association with Flightless-1.

Fig. S2. No difference in the expression of NLRP3 inflammasome components or soluble factors induced by LPS between WT and BCAP$^{-/-}$ macrophages.

Fig. S3. BCAP$^{-/-}$ macrophages have increased caspase-1 activation after *YpstbΔ* infection.

Fig. S4. No difference in NLRC4 or AIM2 expression between WT and BCAP$^{-/-}$ macrophages.

Fig. S5. BCAP inhibition of inflammasome activation requires Flightless-1.

Fig. S6. Model of NLRP3 inflammasome inhibition by BCAP and Flightless-1.

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The signaling adaptor BCAP inhibits NLRP3 and NLRC4 inflammasome activation in macrophages through interactions with Flightless-1

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BCAPing inflammasome activation

The PI3K adaptor protein BCAP limits cellular responses to TLR stimulation and IL-1β. Using proteomics analysis, Carpentier *et al*. showed that BCAP interacted with Flightless-1 and its binding partner leucine-rich repeat Flightless-1–interacting protein 2 (LRRFIP2), which promoted an association between BCAP and the inflammasome component NLRP3. In macrophages, BCAP reduced the abundance of active caspase-1, maturation of the cytokine IL-1 β, and cell death after exposure to the toxin nigericin or bacterial infection. Kinetic analyses determined that BCAP delayed recruitment of pro–caspase-1 to intracellular inflammasome foci and loss of BCAP promoted bacterial clearance in mice. These data identify a distinct role for this PI3K adaptor in preventing excessive, potentially harmful activation of NLRP3 and NLRC4 inflammasomes.