B-cell adaptor for PI3K (BCAP) negatively regulates Toll-like receptor signaling through activation of PI3K

Minjian Ni[a], Alexander W. MacFarlane IV[b], Michelle Toft[c], Clifford A. Lowell[c], Kerry S. Campbell[b], and Jessica A. Hamerman[a,1]

[a]Immunology Program, Benaroya Research Institute, Seattle, WA 98101; [b]Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, PA 19111; and [c]Department of Laboratory Medicine, University of California, San Francisco, CA 94143

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Toll-like receptors (TLRs) recognize pathogens and their components, thereby initiating immune responses to infectious organisms. TLR ligation leads to the activation of NF-κB and MAPKs through well-defined pathways, but it has remained unclear how TLR signaling activates PI3K, which provides an inhibitory pathway limiting TLR responses. Here, we show that the signaling adapter B-cell adaptor for PI3K (BCAP) links TLR signaling to PI3K activation. BCAP-deficient macrophages and mice are hyperresponsive to TLR agonists and have reduced PI3K activation. The ability of BCAP to inhibit TLR responses requires its capacity to bind PI3K. BCAP is constitutively phosphorylated and associated with the p85 subunit of PI3K in macrophages. This tyrosine-phosphorylated BCAP is transiently enriched in the membrane fraction in response to LPS treatment, suggesting a model whereby TLR signaling causes the phosphorylation of the small amount of BCAP that is associated with membranes in the resting state or the translocation of phosphorylated BCAP from the cytoplasm to the membrane. This accumulation of tyrosine-phosphorylated BCAP at the membrane with its associated PI3K would then allow for the catalysis of Ptd Ins P2 to Ptd Ins P3 and downstream PI3K-dependent signals. Therefore, BCAP is an essential activator of the PI3K pathway downstream of TLR signaling, providing a brake to limit potentially pathogenic excessive TLR responses.

inflammatory response | innate immunity

Toll-like receptors (TLRs) recognize microbial components derived from a wide range of bacteria, viruses, parasites, and fungi (1). Ligand binding to TLRs causes the recruitment of the cytoplasmic adapter proteins MyD88 and/or TIR domain-containing adapter-inducing IFN-β (TRIF), resulting in a signal transduction cascade leading to the activation of the MAPK and NF-κB pathways (1). A key consequence of TLR signaling is the transcriptional regulation of proinflammatory genes, such as IL-12 p40, IL-6, and TNF. Although TLR-induced proinflammatory cytokine production is important for host defense in clearing pathogens, hyperproduction of inflammatory cytokines is associated with the pathogenesis of inflammatory and autoimmune diseases (2). Therefore, it is important to understand mechanisms that the immune system uses to control the magnitude of TLR responses.

In addition to the activation of MAPK and NF-κB pathways, TLR ligation leads to activation of the PI3K pathway (3). Although some evidence using pharmacological PI3K inhibition indicates that PI3K activation can promote TLR responses (4–6), the preponderance of studies with macrophages or dendritic cells from mice with genetically or pharmacologically altered PI3K activation indicates that PI3K activation negatively regulates TLR-induced proinflammatory cytokine production (7–12). In support of a negative role for PI3K in TLR signaling, genetic deficiency in the p85α regulatory subunit of PI3K results in increased LPS-induced IL-12 production in dendritic cells (8). Additionally, genetic deficiency in lipid phosphatases that counter the activity of PI3K, such as SRC homology 2 domain-containing inositol-5-phosphatase (SHIP) or phosphatase and tensin homolog (PTEN), results in decreased TLR responses (9, 11). Although it is clear that PI3K activity is an important negative regulator of TLR responses, the exact mechanisms by which PI3K is activated after TLR ligation are still unclear.

The PI3Ks are a family of lipid kinases that phosphorylate the 3′-hydroxyl group of phosphatidylinositol and phosphoinositides. The product of PI3K, Ptd Ins P3 (PIP3), recruits and activates a number of signaling proteins at the plasma membrane, including Akt/PKB, Bruton's tyrosine kinase, PDK, atypical PKCs, and phospholipase Cγ (13). The PI3K family is classified according to sequence homology and substrate specificity into three different types: class I, class II, and class III. Class Ia PI3Ks consist of a catalytic subunit (p110) and a regulatory subunit encoded by at least three distinct genes (p85α, p85β, and p55γ). p85α is the most abundantly expressed regulatory isoform of PI3K, and it is found constitutively associated with one of the three catalytic subunits (p110α, p110β, and p110δ) in the cytoplasm in an inactive PI3K complex (13). The p85α subunit contains two SH2 domains that bind with high affinity to phosphotyrosines within YxxM sequences found in a variety of proteins. The SH2-dependent binding to p-YxxM motifs within docking proteins causes a conformational change in p85 resulting in increased catalytic activity of the associated p110 and recruitment of PI3K to the plasma membrane, where it can access its substrate, Ptd Ins P2 (PIP2) (14). Therefore, binding of p85 to p-YxxM motifs is critical for initiating downstream signaling.

Because the binding of p85 PI3K to p-YxxM sequences is critical for activation of PI3K, we hypothesized that a YxxM-containing signaling adapter would link TLR signaling to PI3K activation. We noted that the YxxM-containing B-cell adaptor for PI3K (BCAP), encoded by the *pik3ap1* gene, is highly expressed in macrophages (15). BCAP was originally identified as a B cell-expressed adapter protein that can recruit PI3K p85 when tyrosine-phosphorylated on its four YxxM motifs after B-cell receptor (BCR) ligation and is critical for BCR-induced Akt phosphorylation (15). BCAP serves a similar role in immunoreceptor tyrosine-based activation motif (ITAM)-mediated Akt phosphorylation in natural killer (NK) cells (16). Here, we show that BCAP is a critical link between TLR ligation and PI3K activation in macrophages. Thus, in addition to its role in activating PI3K downstream of BCR cross-linking in B cells, our findings show that BCAP regulates PI3K activation from a distinct signaling pathway downstream of TLRs, resulting in a unique inhibitory role for BCAP in regulating macrophage function.

Results

Increased TLR-Induced Inflammatory Cytokine Production by BCAP-Deficient Macrophages. Because the PI3K pathway can negatively regulate IL-12 production in dendritic cells (8), we first examined IL-12 p40 production by BCAP-deficient macrophages. Bone

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[1]To whom correspondence should be addressed. E-mail: jhamerman@benaroyaresearch.org.

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marrow (BM)-derived macrophages from WT and BCAP-deficient mice were stimulated with TLR4, TLR7, and TLR9 agonists to induce cytokine production. BCAP-deficient macrophages secreted a significantly higher amount of IL-12 p40 when stimulated with all three TLR ligands tested, indicating that BCAP negatively regulates IL-12 p40 expression in BM-derived macrophages (Fig. 1*A*). We also examined whether BCAP negatively regulates the production of other proinflammatory cytokines. Secretion of both IL-6 and TNF was higher in BCAP-deficient macrophages than in WT macrophages (Fig. 1*A*). Therefore, BCAP negatively regulates not only IL-12 p40 secretion but IL-6 and TNF secretion downstream of TLR ligation in macrophages.

TLR signaling also induces the secretion of the antiinflammatory cytokine IL-10, which can decrease inflammatory cytokine production in an autocrine and paracrine manner (17). We therefore asked whether the increased IL-12 p40, IL-6, and TNF secretion from TLR-activated BCAP-deficient macrophages was attributable to decreased IL-10 secretion. However, IL-10 secretion was not lower in LPS-treated BCAP-deficient macrophages than in WT macrophages (Fig. 1*B*). We also investigated whether BCAP negatively regulates TLR responses in vivo. BCAP-deficient mice produced significantly more IL-12 p40 in response to in vivo injection of LPS than WT mice over a 6-h time course (Fig. 1*C*). Therefore, BCAP inhibits TLR responses both in vitro and in vivo.

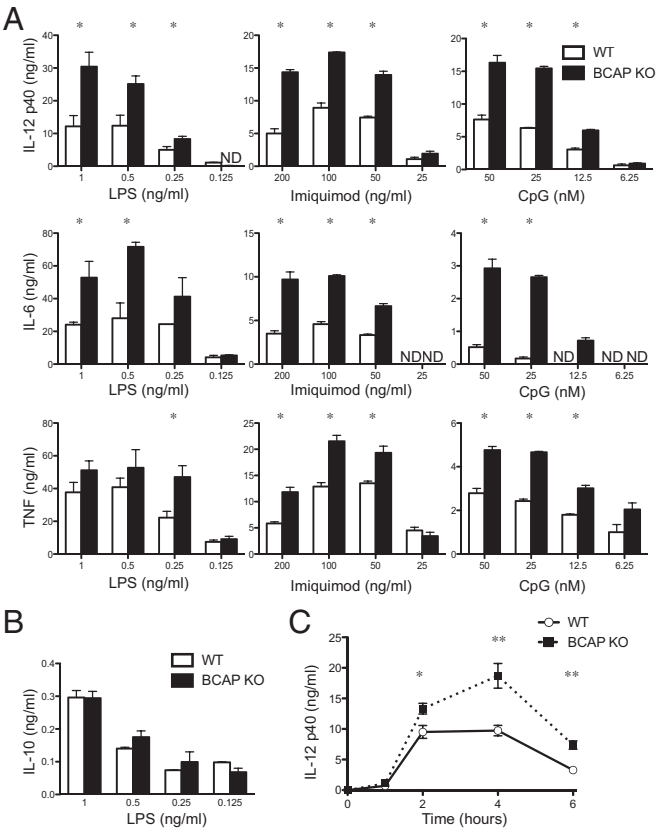

Fig. 1. BCAP negatively regulates TLR-induced inflammatory cytokine production in vitro and in vivo. (*A* and *B*) BM-derived macrophages from WT or BCAP-deficient (BCAP KO) mice were incubated with the indicated concentrations of LPS (TLR4), Imiquimod (TLR7), or CpG DNA (TLR9) for 16 h. Supernatants were collected, and the amounts of IL-12 p40, IL-6, and TNF (*A*) or IL-10 (*B*) were measured by ELISA. Data are representative of four independent experiments and are expressed as the mean ± SD of triplicate wells. $*P < 0.05$. ND, not detected. (*C*) WT or BCAP-deficient mice were injected i.p. with 1 μg/g of LPS, and plasma IL-12 p40 concentrations at 1, 2, 4, and 6 h postinjection were measured. Data are from one of three independent experiments and are expressed as the mean ± SD with $n = 4$ mice per group, with each mouse assayed in triplicate. $*P < 0.05$; $**P < 0.01$.

BCAP Deficiency Minimally Affects TLR-Induced MAPK Activation and IκBα Degradation. To investigate the molecular mechanism by which BCAP inhibits TLR-induced cytokine production, we assessed how BCAP deficiency influences MAPK and NF-κB signaling downstream of TLR ligation. The kinetics and magnitude of phosphorylation of all three MAPKs were similar in BCAP-deficient and WT macrophages after stimulation with LPS (Fig. 2*A*). In some experiments, there was a slight increase in ERK phosphorylation in BCAP-deficient macrophages in comparison to WT macrophages; however, these differences were subtle and not consistent. In WT and BCAP-deficient macrophages, IκBα, the cytoplasmic inhibitor of NF-κB, was degraded with similar kinetics and was present in similar amounts (Fig. 2*B*). These data suggested that the hyperresponsiveness to TLR stimulation in BCAP-deficient macrophages did not result from increased NF-κB or MAPK activation.

Reduced Akt Phosphorylation and PI3K Activity in BCAP-Deficient Macrophages. Because BCAP has four YxxM motifs and is associated with PI3K activation in B cells, we hypothesized that deficiency in BCAP would reduce TLR-induced Akt phosphorylation, which is dependent on PI3K activation (18). Therefore, we compared LPS-induced Akt phosphorylation in WT and BCAP-deficient macrophages. Indeed, we found that LPS-induced phosphorylation of Akt was dramatically reduced in BCAP-deficient macrophages, at both Ser473 and Thr308 residues (Fig. 3*A*), indicating a reduced capacity for PI3K to stimulate downstream Akt activation in the absence of BCAP. Interestingly, basal Akt phosphorylation at both sites was also reduced in BCAP-deficient macrophages, suggesting that BCAP functions to regulate PI3K activation in resting macrophages in addition to those activated by TLR ligation. BCAP-deficient macrophages were not defective in Akt phosphorylation in response to TNF (Fig. S1), suggesting that BCAP specifically participates in the activation of PI3K downstream of TLR signaling.

Because BCAP-deficient macrophages had reduced Akt activation in response to TLR ligation, we reasoned that the PI3K inhibitor wortmannin would have a reduced capacity to enhance TLR-induced cytokine responses by BCAP-deficient macrophages compared with WT macrophages. To test this hypothesis, we measured the increase in TLR-induced cytokine production after pretreatment of macrophages with varying doses of wortmannin. In WT macrophages, CpG DNA-stimulated IL-12 p40 and IL-6 secretion increased when cells were pretreated with wortmannin in a dose-dependent manner (Fig. 3 *B* and *C*), consistent with published data demonstrating the inhibitory impact of PI3K on TLR responses (8, 19–21). In contrast to WT macrophages, however, pretreatment of BCAP-deficient macrophages with wortmannin had little effect on IL-12 p40 or IL-6 secretion (Fig. 3 *B* and *C*), suggesting that the amount of PI3K activity suppressing TLR function is minimal in cells lacking BCAP. Together with the reduced TLR-induced Akt phosphorylation, these results demonstrate that BCAP-deficient macrophages have a defect in TLR-induced PI3K activation.

BCAP Is Constitutively Tyrosine-Phosphorylated and Associated with PI3K in Macrophages. When immunoprecipitated from resting macrophages, BCAP was found as two doublets, which correspond to the bands designated 1/2 and 3/4 by Kurosaki and colleagues (15) (Fig. 4*A*). The 1/2 and 3/4 bands of BCAP are thought to be generated by alternative splicing of the BCAP mRNA (15). Interestingly, we never observed bands 5 and 6 seen in B cells, which run at over 100 kDa. Previous studies showed that BCAP is inducibly tyrosine-phosphorylated in B cells on BCR ligation, resulting in association with the p85 subunit of PI3K (15). Surprisingly, BCAP was constitutively tyrosine-phosphorylated and associated with PI3K p85 in resting macrophages, and the magnitude of tyrosine phosphorylation did not

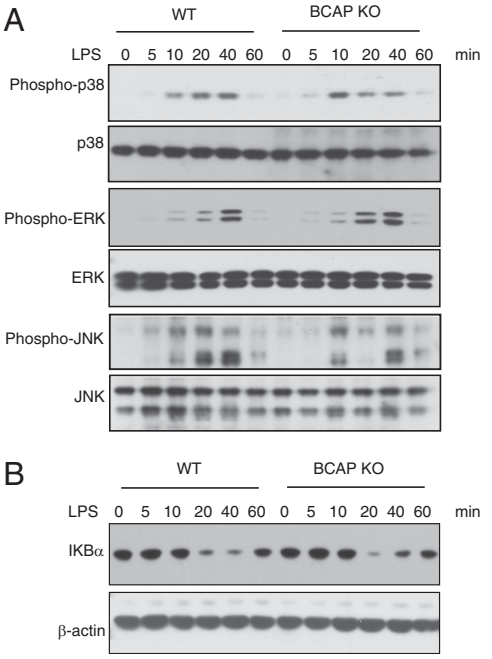

Fig. 2. BCAP deficiency has minimal effects on TLR-induced MAPK activation and IκBα degradation. BM-derived macrophages were stimulated with 1 ng/mL LPS for the indicated times, after which cells were lysed. Cytoplasmic extracts were analyzed by Western blot using antibodies specific for p38 MAPK, p42/44 ERK, and JNK or for phosphorylated versions of these proteins (*A*) or for IκBα with β-actin as a loading control (*B*). Data are representative of three independent experiments.

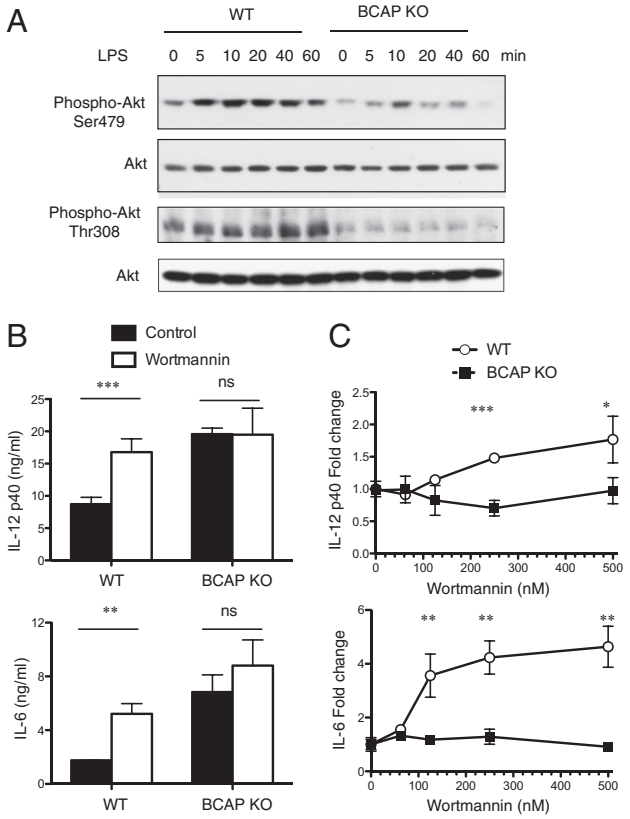

Fig. 3. PI3K activity is reduced in BCAP-deficient macrophages. (*A*) Macrophages were stimulated with 1 ng/mL LPS for the indicated time and then lysed. Cytoplasmic extracts were analyzed by Western blot using antibodies specific for phospho-Akt (Ser473 or Thr308) or Akt. (*B* and *C*) Macrophages were stimulated for 16 h with CpG DNA (25 nM) after 30 min of pretreatment with vehicle control or wortmannin. (*B*) Secretion of IL-12 p40 and IL-6 was measured by ELISA after treatment with 250 nM wortmannin. (*C*) Results are expressed as fold change compared with CpG DNA-treated, no wortmannin control, and they show the mean ± SD of triplicate wells. Data are representative of five (*A*) and three (*B* and *C*) experiments. ns, not significant; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

increase with LPS stimulation at any time point examined, from 5 to 30 min (Fig. 4 *A* and *B*). As previously reported in B cells and NK cells, all four BCAP bands were tyrosine-phosphorylated (15, 16). Consistent with the constitutive tyrosine phosphorylation of BCAP, we also observed the association of PI3K p85 subunit in resting macrophages, and this association also did not increase with LPS stimulation (Fig. 4 *A* and *B*). These data show that BCAP is tyrosine-phosphorylated and associated with PI3K in the absence of TLR ligation, indicating that upstream signals causing BCAP tyrosine phosphorylation and PI3K recruitment are active in resting macrophages.

Neither CSF1 Receptor nor Syk Signaling Is Required for BCAP Tyrosine Phosphorylation and PI3K Association in Macrophages. Because the BM-derived macrophages used in these immunoprecipitation studies are grown in macrophage (M)-CSF, whose receptor, CSF1R, can mediate PI3K activation, we examined whether CSF1R signaling may induce BCAP phosphorylation and PI3K association. To test this hypothesis, we starved macrophages for 3 h in media without M-CSF or serum and measured BCAP tyrosine phosphorylation and PI3K association. Identical to macrophages grown continuously in M-CSF, M-CSF–starved macrophages showed constitutive BCAP tyrosine phosphorylation and PI3K association that did not change with LPS treatment (Fig. S2*A*). Therefore, we conclude that the constitutive tyrosine phosphorylation of BCAP is not mediated by CSF1R signaling.

In B cells, the Syk tyrosine kinase is required for BCAP tyrosine phosphorylation after BCR cross-linking (15). Therefore, we examined whether Syk was required for BCAP phosphorylation in macrophages. Surprisingly, the tyrosine phosphorylation of BCAP was not reduced but was dramatically increased in resting macrophages in the absence of Syk (Fig. S2*B*). This increased phosphorylation was limited to bands 3/4 and was accompanied by increased PI3K association. Thus, signaling through Syk tyrosine kinase is not required for BCAP tyrosine phosphorylation and PI3K association in macrophages.

Tyrosine-Phosphorylated BCAP Is Enriched on Membrane Fractions After LPS Treatment. YxxM-containing adapter proteins often function not only to bind and activate PI3K but to bring active PI3K to membranes where its substrate, PIP2, is found. In B cells, Okada et al. (15) found BCAP predominantly in the cytoplasm by immunofluorescence analysis and by biochemical separation of membrane and cytosolic cellular fractions followed by Western blot, with a small minority in the membrane fraction. We hypothesized that TLR signaling may cause the accumulation of tyrosine-phosphorylated BCAP at membranes where it may access PIP2. To test this hypothesis, we separated cytosolic and membrane fractions of macrophages before and after LPS treatment, as shown by the enrichment for cadherins in the membrane fraction and GAPDH in the cytosolic fraction (Fig. 4*C*, *Bottom*). We immunoprecipitated BCAP from both cytosolic and membrane fractions, and we performed Western blotting for phosphotyrosine and BCAP. As in B cells, the majority of BCAP protein was found in the cytosolic fraction, with a small but detectable amount of BCAP in the membrane fraction as seen when blotting for BCAP (Fig. 4*C*). Both the amount of tyrosine-phosphorylated BCAP and the total amount of BCAP in the membrane fraction were transiently increased 5 min after LPS treatment and returned to baseline by 10 min. Interestingly, this

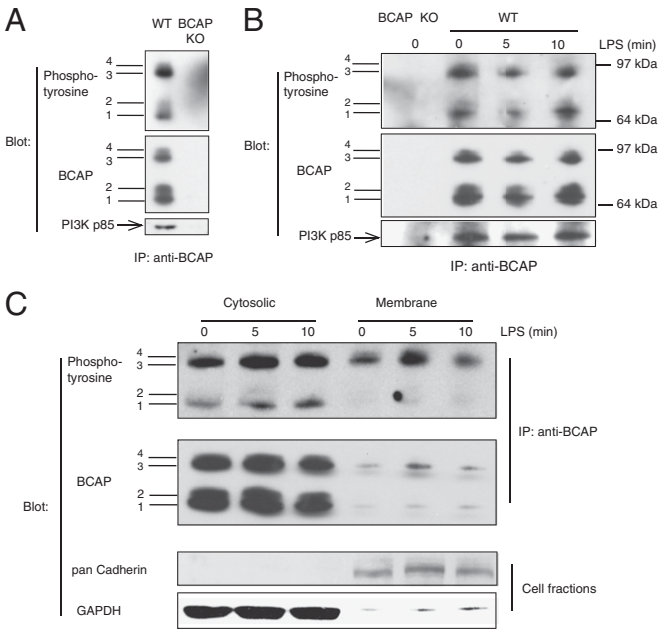

Fig. 4. BCAP is constitutively tyrosine-phosphorylated and associated with p85 PI3K in macrophages. (*A*) WT or BCAP-deficient macrophages were lysed and immunoprecipitated with anti-BCAP monoclonal antibody, separated by SDS-PAGE and immunoblotted for phosphotyrosine or BCAP. (*B*) Macrophages were treated with 1 ng/mL LPS for the indicated times and then lysed and analyzed as in *A*. (*C*) WT macrophages were treated with 1 ng/mL LPS for the indicated times, and cytosolic or membrane protein fractions were then extracted and subjected to immunoprecipitation with anti-BCAP monoclonal antibody and analyzed as in *A*. Cell fractions were also analyzed by Western blot for pan-Cadherin or GAPDH to examine the extraction efficiency. Data are representative of eight (*A*), six (*B*), and three (*C*) experiments.

increased tyrosine phosphorylation of BCAP was mainly the larger species seen in bands 3/4. This difference in tyrosine phosphorylation of BCAP isoforms in membrane and cytoplasmic fractions, along with the similarly low amount of cytosolic protein (GAPDH) in our membrane fractions, leads us to believe that the increase in tyrosine-phosphorylated and total BCAP in the membrane fractions was not attributable to contamination from cytosolic fractions. These data show that TLR signaling leads to the accumulation of the tyrosine-phosphorylated form of BCAP at membranes, presumably bringing its associated PI3K.

Reconstitution of BCAP into BCAP-Deficient Macrophages Reduces TLR Responses. To investigate whether there was a developmental deficit attributable to BCAP deficiency, we introduced BCAP and empty control constructs into WT or BCAP-deficient BM-derived macrophages through retroviral transduction. After retroviral transduction, the macrophages were activated with CpG DNA and proinflammatory cytokine production was assessed by flow cytometry, gating on the GFP-expressing retrovirally transduced cells. BCAP-deficient macrophages transduced with a control vector showed an increased percentage of cells producing IL-12 p40 and TNF compared with control virus-transduced WT macrophages, consistent with the increased cytokine secretion measured by ELISA (Fig. 5*A*). In contrast, reintroduction of BCAP into BCAP-deficient macrophages caused a reduction in the percentage of cytokine-producing cells to an amount equivalent to that of WT macrophages transduced with control vector (Fig. 5 *A* and *B*). These data suggest that there is not an early developmental defect attributable to the lack of BCAP or an effect of targeting the BCAP gene on another gene required for normal development or TLR responses. Additionally, BCAP-deficient macrophages had normal morphology and uniformly expressed F4/80, suggesting they do not have a developmental defect.

BCAP Negatively Regulates TLR-Induced Cytokine Production Through Binding to p85 Subunit of PI3K. Because tyrosine-phosphorylated BCAP recruits the PI3K p85 subunit to four YxxM motifs within BCAP to activate the PI3K pathway in B cells (15, 22), we hypothesized that the YxxM motifs of BCAP are required to inhibit TLR responses. To test this hypothesis, we transduced BCAP-deficient macrophages with a retrovirus encoding a mutant BCAP protein in which the four YxxM tyrosine residues were mutated to phenylalanines to prevent PI3K p85 binding and compared this with transduction of the WT BCAP protein or control retrovirus (23). The YxxM mutant BCAP had a greatly reduced ability to inhibit cytokine production in response to CpG DNA compared with the WT BCAP protein (Fig. 5 *C* and *D*). Interestingly, the YxxM mutant still diminished the TLR responses to a small degree compared with responses of macrophages transduced with empty vector. These data show that the ability of BCAP to inhibit TLR signaling in macrophages was mediated predominantly by PI3K recruitment and activation.

Discussion

Although TLR-mediated proinflammatory cytokine production during infection is necessary for the host to eradicate pathogens successfully, these cytokines can be detrimental if not tightly regulated. One mechanism by which TLR responses are kept in check is through activation of the PI3K pathway (3, 24). Here, we show that the signaling adapter BCAP is required for optimal signaling through the PI3K pathway in response to TLR ligation, identifying a link between TLR signaling and PI3K activation in controlling inflammatory responses. By its ability to activate PI3K, BCAP serves as a negative regulator of TLR responses both in vitro and in vivo.

BCAP functions in B and NK cells to bind and activate PI3K, leading to Akt phosphorylation (16, 25). Here, we show several pieces of evidence that the ability of BCAP to negatively regulate TLR responses depends on the PI3K pathway. We detected severely reduced LPS-induced Akt phosphorylation in BCAP-deficient macrophages compared with WT macrophages, consistent with reduced PI3K activity in the absence of BCAP. Furthermore, the effect of PI3K inhibition with wortmannin is reduced in BCAP-deficient macrophages compared with its effect in WT macrophages. This indicates that there is less TLR-induced PI3K activity in BCAP-deficient macrophages, consistent with the reduced TLR-induced Akt phosphorylation in these cells. Additionally, reconstitution of BCAP-deficient macrophages with a BCAP mutant that cannot associate with PI3K p85 because of the substitution of phenylalanines for the tyrosines in the four PI3K binding motifs found in BCAP had a significantly reduced ability to inhibit TLR responses in comparison to reconstitution with the WT protein. Therefore, we conclude that BCAP inhibits TLR responses through activation of the PI3K pathway and that BCAP is a critical link between TLR signaling and the PI3K signaling pathway in macrophages.

Unlike in B cells, where BCAP tyrosine phosphorylation and PI3K association are induced by receptor cross-linking (15, 22), BCAP was constitutively tyrosine-phosphorylated and associated with PI3K p85 in resting macrophages. In addition to TLR-induced Akt phosphorylation, basal Akt phosphorylation in the absence of activation was reduced in macrophages lacking BCAP. This suggests that BCAP-associated PI3K is also important for the basal PI3K activity in macrophages. Interestingly, the total amount of BCAP tyrosine phosphorylation and PI3K association did not change with LPS treatment, even though LPS-induced Akt phosphorylation was reduced in the absence of BCAP. The basal and LPS-induced tyrosine phosphorylation of BCAP, and its PI3K association, did not depend on signaling by M-CSF through the CSF1R, showing that cross-talk between CSF1R and BCAP does not influence BCAP function. Additionally, BCAP tyrosine phosphorylation and PI3K association did not depend on the Syk tyrosine kinase, which is required for BCR-induced BCAP activation in B cells (15). Taken together, these data suggest that the ability of BCAP to control PI3K activity in macrophages is not

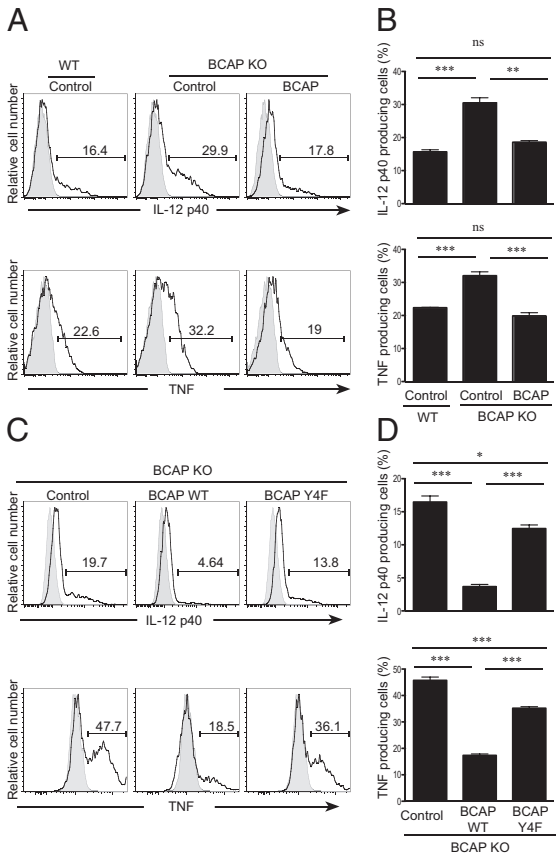

Fig. 5. BCAP negatively regulates TLR-induced cytokine production through binding to p85 subunit of PI3K. (*A* and *B*) WT or BCAP-deficient macrophages were transduced with an empty vector (control) retrovirus or retrovirus encoding WT BCAP. The macrophages were activated with CpG DNA (25 nM) in the presence of Brefeldin A for 6 h, and cytokine production was assessed by flow cytometry. Transduced cells were gated based on GFP fluorescence, and the percentage of IL-12 p40 or TNF-producing cells was determined. (*C* and *D*) BM-derived macrophages from BCAP-deficient mice were transduced with an empty vector (Control) retrovirus or retrovirus encoding WT BCAP (BCAP WT) or a BCAP tyrosine mutant (BCAP Y4F). Cytokine production was measured as in *A*. Gray histograms show staining of unstimulated cells. Data are representative of four independent experiments and are expressed as the mean ± SD of triplicate wells. ns, not significant; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

solely through its ability to associate with the p85 regulatory subunit, distinct from how BCAP functions in B cells.

Unlike many other YxxM-containing proteins that activate PI3K, BCAP is not an integral membrane or membrane-associated protein. We found BCAP mainly in the cytosolic fraction in macrophages, as has been shown in B cells (15). In response to LPS treatment, the amount of tyrosine-phosphorylated BCAP increased transiently in the membrane fraction but not in the cytosolic fraction of macrophages. Our experiments cannot distinguish whether BCAP that is already present in the membrane fraction is inducibly tyrosine-phosphorylated after LPS treatment or whether phosphorylated BCAP is translocated from the cytoplasm to membranes in response to LPS. We propose that the function of BCAP during TLR signaling is to enrich active PI3K at membranes where it can access its substrate PIP2, thereby increasing PIP3 production and downstream signaling. This may allow for BCAP to regulate PI3K activity in two distinct steps: first, by binding p85/p110 heterodimers, thereby increasing catalytic activity, and, second, by interacting with other proteins to move to the plasma membrane or other intracellular membranes. Interestingly, we only found enrichment at the membrane of tyrosine-phosphorylated BCAP isoforms 3/4, suggesting that these isoforms may be responsible for regulating TLR responses. Identification of the signaling pathway that directs the enrichment of tyrosine-phosphorylated BCAP in the membrane fraction after TLR ligation will be the subject of future work.

The molecular mechanisms underlying PI3K negative regulation of cytokine expression are complex. One recent study suggests that PI3K regulates IL-12 production in dendritic cells through both mammalian target of rapamycin (mTOR) and glycogen synthase kinase 3b (GSK3b) pathways (12). PI3K-generated PIP3 activates Akt, which phosphorylates and causes the degradation of GSK3b. GSK3b positively regulates LPS-induced IL-12 p70 directly through transcriptional induction and indirectly through inhibition of IL-10 production. Therefore, when PI3K activity is reduced, GSK3b levels remain high, causing more IL-12 and less IL-10 to be produced. Akt also activates the mTOR pathway, which regulates IL-12 production only indirectly through induction of IL-10, again causing less IL-10 to be produced when PI3K activity is reduced. It is not likely that IL-10 is involved in BCAP-mediated suppression of IL-12 p40 secretion by macrophages because there is no decrease in TLR-induced IL-10 secretion by BCAP-deficient macrophages. We therefore favor a direct form of inhibition of IL-12 p40 secretion by BCAP-induced PI3K activity, possibly through regulation of GSK3b degradation.

Although we believe the principal role of BCAP is to activate PI3K, BCAP is not the only pathway through which PI3K gets activated in macrophages. In the absence of BCAP, we saw a small amount of basal and LPS-induced Akt phosphorylation. Therefore, other pathways exist downstream of TLRs leading to PI3K activation. TLRs have been reported to interact directly or indirectly through MyD88 with the p85 subunit to activate PI3K (5, 26), suggesting one BCAP-independent pathway leading to PI3K activation downstream of TLR ligation. Alternatively, the activation of PI3K described in these reports may depend on BCAP. Further work is required to define better whether BCAP is activated directly or indirectly by TLRs. Additionally, BCAP may have other functions during TLR signaling than activation of PI3K. BCAP is a large protein with many regions of unknown function. Our reconstitution assay with the BCAP Y→F mutant showed a small but consistent ability to inhibit TLR responses, although this was much lower than the inhibition achieved with the WT protein. This suggests that there is a function of BCAP independent of PI3K binding that can affect the magnitude of TLR responses.

Here, we show that the signaling adapter BCAP is required for optimal PI3K activation downstream of TLR ligation, identifying an elusive link between TLR signaling and PI3K activation in controlling inflammatory responses. Although BCAP serves to activate PI3K both in B cells downstream of BCR signaling and in macrophages downstream of TLR signaling, this BCAP-mediated PI3K activation has opposite effects, promoting B-cell activation and inhibiting macrophage activation, thus revealing cell type-specific roles of BCAP in regulation of cellular activation pathways. The negative regulation of TLR responses by BCAP may be beneficial to the host in limiting macrophage responses to infection and in preventing chronic inflammatory or autoimmune disorders. Therefore, BCAP provides a potential new target for designing therapeutic approaches to treat inflammation or sepsis.

Methods

Mice. We purchased C57BL/6 mice from Charles River Laboratories. BCAP-deficient mice lacking the *pik3ap1* gene (25) were back-crossed to C57BL/6 mice for nine generations. $Syk^{-/-}$ fetal liver chimeras were generated as previously described (27). All experiments and animal care procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committees at Benaroya Research Institute; Fox Chase Cancer Center; and the University of California, San Francisco.

Macrophage Preparation. BM-derived macrophages were made as described (28).

TLR Stimulation and Cytokine Measurement. For ELISA, day 5 BM-derived macrophages were plated in a 96-well tissue plate (5×10^4 cells per well) overnight. Titrations of TLR stimuli were added for 16 h as follows: *Salmonella minnesota* R595 LPS (List Biological Laboratories), CpG DNA (ODN1826; Invivogen), and Imiquimod (Invivogen). The concentration of TNF, IL-6, IL-12 p40, and IL-10 in supernatants from triplicate wells was measured with ELISA (eBioscience). For measurement of intracellular TNF and IL-12 p40 by flow cytometry, 2×10^5 macrophages were plated in 24-well non–TC-treated plates overnight and then stimulated for 6 h with CpG DNA in the presence of Brefeldin A (10 μg/mL) for the final 2 h. Macrophages were blocked with 2.4G2 for 10 min and fixed in 4% paraformaldehyde (wt/vol), followed by permeabilization using Perm/Wash buffer (BD Bioscience). Staining for intracellular TNF and IL-12 p40 was performed using Pacific blue-labeled anti-TNF antibody and AlexFluor 647-labeled anti–IL-12 p40 (eBioscience). Cells were analyzed by flow cytometry using an LSR2 (BD Bioscience) and analyzed with FlowJo software (TreeStar).

Retroviral Transduction of Macrophages. Generation of VSVg-pseudotyped retroviruses and infection of macrophages were as described (28). Retroviral constructs were in the pMSCVIRES vector, in which the cDNA is followed by an IRES-eGFP to identify infected cells, and included control empty vector, BCAP WT, and a mutant BCAP in which the four tyrosines in the pI3K p85 binding motifs have been changed to phenylalanine (23).

Immunoprecipitation and Western Blot Analysis. Macrophages were activated with 1 ng/mL LPS or recombinant mouse TNF (Peprotech) and lysed at the indicated times in lysis buffer containing 1% Triton X-100, protease inhibitors (mammalian protease inhibitor mixture; Sigma), and sodium orthovanadate (1 mM; Sigma). For immunoprecipitation, day 6 macrophages 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 PMSF, 1 mM sodium orthovanadate and protease inhibitor (Sigma)]. Lysates were sequentially incubated with anti-BCAP monoclonal antibody (16) and protein A-agarose, and they were then eluted with 1× SDS sample buffer (Invitrogen). For membrane protein extraction, macrophages were removed from plates, resuspended in media, and stimulated with 1 ng/mL LPS for indicated times. Cytosolic and transmembrane proteins were extracted with a ProteoExtract Transmembrane protein extraction kit (Novagen). Cells were resuspended in Extraction Buffer 1 for cytosolic protein fraction extraction, and Extraction Buffer 2A was used subsequently to extract the membrane protein fraction. BCAP was immunoprecipitated from both fractions as described above. Lysates or immunoprecipitates were separated by Tris·bis SDS/PAGE gels (Invitrogen), transferred to PVDF (Millipore) membrane, and detected by the indicated antibodies and the Immobilon chemiluminescence system (Millipore). Antibodies for Western blotting used were specific for phosphorylated and nonphosphorylated Akt, p38 MAPK, p42/44 ERK and JNK, PI3K p85, IκBα, and pan-Cadherin (all from Cell Signaling); phosphotyrosine (4G10; Millipore); and anti-GAPDH (Trevigen).

In Vivo LPS Treatment. Mice were injected with 1 μg/g of *Escherichia coli* 055:B5 LPS (Sigma) in PBS. At the indicated times, a sample of blood was taken and plasma IL-12 p40 was determined by ELISA.

Statistical Analysis. The Student's unpaired *t* test was used in Figs. 1 and 3, and one-way ANOVA with Bonferroni's multiple comparison test was used in Fig. 4 as determined using Prism 5 software (GraphPad Software).

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