B-CELL ADAPTER FOR PHOSPHOINOSITIDE 3-KINASE IS A SIGNALING ADAPTER IN THE TOLL-LIKE RECEPTOR/INTERLEUKIN-1 RECEPTOR SUPERFAMILY

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DEDICATION

To my grandparents, David and Carolyn Erkeneff, and my mother, Tambla Jean Habiger, for their unfailing support, which made possible my achievements.

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by

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Toll-like receptor (TLR)/Interleukin-1 receptor (IL1R) superfamily members share signaling components and (with the exception of TLR3) depend on the adapter myeloid differentiation primary response gene 88 (MyD88) for engagement of downstream pathways. Signals from the receptor to the adapter are transmitted through homotypic interaction of TIR (Toll-Interleukin-1 receptor) homology domains found in all TLR/IL1R family members and their adapters. The present work defines a novel TLR/IL1R signaling adapter, termed BCAP (B-cell adapter for PI3K), which was identified based on the presence of a cryptic N-terminal

TIR domain. I show here that BCAP (B-cell adapter for PI3K) contains a functional TIR domain enabling its participation in the TLR signaling pathway. Through its TIR domain, BCAP associates with the TLR/IL1R signaling adapter MyD88, as well as the TLR signaling adapter toll-interleukin 1 receptor domain containing adapter protein (TIRAP). Importantly, BCAP plays an obligate role in linking TLRs to activation of phosphoinositide 3-kinase (PI3K) through recruitment of PI3K to the signaling complex and relief of inhibitory influences on PI3K activity. Importantly, BCAP selectively mediates TLR signaling towards the PI3K branch without affecting signaling to NFκB nor MAP kinases. In this capacity, BCAP inhibits secretion of inflammatory cytokines and regulates susceptibility to inflammatory colitis. Because the TLR/IL1R family shares signaling components, BCAP may also function in IL1R family signaling. To test this hypothesis, T cells were chosen as a model cell type responding to IL1R family signals. T helper cells utilize IL18 and IL1 (which engage the IL18R or the IL1R respectively, both IL1R family members) cytokines provided by myeloid cells to achieve optimal Th1 and Th17 effector capacities. I show here that BCAP intrinsically regulates differentiation of naïve T cells towards Th1 and Th17 effector lineages by participation in the IL1R family signaling pathways. Further, BCAP intrinsically regulates both T cell proliferation and survival during priming. The significance of this work lies in the revelation of a TLR signaling adapter serving as a node connecting TLRs to PI3K. Further, the findings here will increase the understanding of key signaling pathways involved in disease and inflammation.

TABLE OF CONTENTS

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TABLE OF FIGURES

LIST OF DEFINITIONS

AP-1, activator protein-1

BANK1, B-cell scaffold protein with ankyrin repeats 1

BCAP, B-cell adapter for PI3K (also known as PIK3AP1)

CCL, chemokine (C-C motif) ligand

CXCL, chemokine (C-X-C motif) ligand

DC, dendritic cell

DSS, dextran sulfate sodium

ERK, extracellular signal regulated protein kinases

IFN, interferon

IκBα, NF-κB inhibitor, α

IKK, inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase

IL, interleukin

IRAK, interleukin-1 receptor-associated kinase

IRF, interferon regulatory factors

JNK, c-JUN NH2 terminal kinases

LPS, lipopolysaccharide

LRR, leucine-rich repeat

MAP, mitogen activated protein

miRNA, microRNA

MyD88, myeloid differentiation primary response gene 88

NEMO, NF-κB essential modulator

NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells

NK, natural killer

PDPK1, 3-phosphoinositide dependent protein kinase-1

PI3K, phosphoinositide 3-kinase

PTEN, phosphatase and tensin homolog

PtIns, phosphatidylinositol

RAC1, ras-related C3 botulinum toxin substrate 1

RIP, receptor interacting protein

SARM, sterile alpha and TIR motif containing

SHIP1, Src homology-2-domain containing inositol 5-phosphatase 1

SIGIRR, single immunoglobulin and toll-interleukin 1 receptor domain

STAT, signal transducer and activator of transcription

TAB, TAK1 binding protein

TAK1, TGF-β activated kinase 1

TBK1, TANK-binding kinase 1

TIG, transcription factor-Ig

TIR, toll-Interleukin-1 receptor

TIRAP, toll-interleukin 1 receptor domain containing adapter protein

TLR, Toll-Interleukin-1 receptor; Tnf, tumor necrosis factor

TPL2, tumor progression locus 2

TRADD, TNFRSF1A-associated via death domain

TRAF, TNF receptor associated factor

TRAM, TRIF-related adapter molecule

TRIF, TIR-domain-containing adapter-inducing interferon-β

UBC13, ubiquitin conjugating enzyme

UEV1A, ubiquitin-conjugating enzyme E2 variant 1.

CHAPTER ONE

Introduction

Part of the writing presented in this chapter was previously published as an invited perspective in *Cell Cycle*, volume 11, issue 19, pages 3559-3567 (Troutman et al., 2012a).

Overview

In 1989, Dr. Charles Janeway proposed the existence of a family of receptors that impart upon a cell the ability to respond to conserved components of microbial organisms (Janeway, 1989). These receptors, termed pattern recognition receptors, were proposed to signal in response to lipopolysaccharide (LPS) and viral components to induce expression of co-stimulatory molecules important to a developing clonal immune response (Janeway, 1989). This hypothesis led to the eventual discovery and characterization of an important group of pattern recognition receptors called Toll-like receptors (TLRs). TLRs are a family of pattern recognition receptors that recognize conserved molecular structures/products from a wide variety of microbes. Following recognition of ligands, TLRs recruit signaling adapters to initiate a pro-inflammatory signaling cascade culminating in activation of several transcription factor families, including NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), AP-1 (activator protein-1), and IRFs (interferon regulatory factors). Additionally, TLR signals lead to activation of PI3K (phosphoinositide 3-kinase), affecting

many aspects of the cellular response, including cell survival, proliferation and regulation of the pro-inflammatory response. The focus of my dissertation research has been on the identification and characterization of BCAP (B-cell adapter for PI3K, or PIK3AP1) as a TLR signaling adapter, crucial for linking TLRs to PI3K activation. Further, BCAP participates downstream of IL1R family members, which belong to the more broad TLR/IL1R superfamily. These findings will allow new questions of the importance of PI3K activation downstream of TLRs and could potentially be extended to the TLR/IL1R superfamily.

TLRs

TLRs are a major family of pattern recognition receptors that play a critical role in innate host defense as well as in initiation of adaptive immune responses. Since the discovery of the first human TLR (Medzhitov et al., 1997), there have been significant advances in the field of TLR biology, both in identification of the receptors, their diverse ligands and the signaling pathways that lead to responses necessary for protection of the host. There are currently ten described TLRs in humans and twelve in mice, ligands for most of which have been identified. This family of TLRs can be broadly divided into two classes, depending upon the cellular location where they engage their respective ligands. TLR1, TLR2, TLR4, TLR5, and TLR6 engage their respective ligands from the cells surface, while TLR3, TLR7, TLR8, and TLR9 bind their cognate ligands in endosomes. Initiation of TLR signaling occurs when the Leu-rich repeat ectodomains of the TLR engage and complex with their respective ligand. A subset of the human TLRs have crystallographically elucidated complexes of their Leu-rich

repeat (LRR) ectodomains and their respective ligands, showing the remarkable plasticity of the LRR recognition platform for unrelated pathogen molecules (Botos et al., 2011; Lu and Sun, 2012). For a more detailed review of TLRs and their ligands as well as the importance of their cellular signaling location, please refer to other reviews (Barton and Kagan, 2009; Engel and Barton, 2010; Takeuchi and Akira, 2010).

Ligand binding by TLRs initiates recruitment of TLR signaling adapters through homotypic interaction of the cytosolic Toll-Interleukin-1 receptor (TIR) homology domain found at the carboxyl terminus of all TLRs as well as the carboxyl terminus of the cytosolic adaptors. Until recently, there were only five known TLR signaling adaptors; MyD88, TRIF, TRAM, TIRAP and SARM (Couillault et al., 2004; Fitzgerald et al., 2001; Fitzgerald et al., 2003; Hirotani et al., 2005; Hoebe et al., 2003; Horng et al., 2002; Horng et al., 2001; Kawai et al., 1999; Medzhitov et al., 1998; Mink et al., 2001; Muzio et al., 1998; O'Neill and Bowie, 2007; Oshiumi et al., 2003a; Oshiumi et al., 2003b; Takeuchi et al., 2000; Yamamoto et al., 2003a; Yamamoto et al., 2002a; Yamamoto et al., 2003b; Yamamoto et al., 2002b). All TLRs, except TLR3, utilize MyD88 for signal transduction upon ligand binding, whereas TLR3 signals through the adaptor TRIF. TLR4 uniquely utilizes both a MyD88-dependent and MyD88–independent pathway whereby TRIF is the critical signaling adaptor for the MyD88 independent node. TIRAP and TRAM serve as shuttling adaptors for TLR2 and TLR4 with TIRAP mediating recruitment of MyD88 and TRAM mediating recruitment of TRIF. SARM is the fifth member of the TLR signaling adaptor family. SARM was initially implicated in suppressing TRIF dependent signaling through TLR3 and TLR4 in human cells

(Carty et al., 2006; Kenny and O'Neill, 2008). Studies using SARM deficient mice demonstrated SARM is mainly expressed in the brain and SARM deficient macrophages were functionally normal when stimulated with TLR ligands (Kenny and O'Neill, 2008; Kim et al., 2007). Therefore, it is proposed that could play different roles between the two species (Kenny and O'Neill, 2008). Indeed, SARM deficient mice were found to be highly susceptible to infection with West Nile virus and displayed enhanced viral replication in the brain, decreased activation of microglia, and ultimately, increased mortality (Szretter et al., 2009).

Upon binding to their respective ligands, TLRs initiate a signaling cascade resulting in activation of the responding cell. The outcome of TLR activation depends on both the TLR that is activated as well as the type of responding cell. For instance, in macrophages and neutrophils, TLR activation enhances phagocytosis and increases the oxidative burst that facilitates rapid uptake and killing of microbes. In addition, resident macrophages responding to TLR ligands secrete chemokines that recruit additional neutrophils and monocytes to the site of infection. Activation of TLRs in dendritic cells (DCs) leads to their migration to the draining lymph nodes, allowing priming of pathogen specific T cells. Additionally, TLR stimulation of DCs causes their maturation, including increased expression of co-stimulatory molecules important for activation of naïve T cells. Further, TLR ligands induce macrophages and DCs to secrete pro-inflammatory cytokines important for activation and differentiation of naïve T cells (an event critical for development of adaptive immunity), regulating the host acute phase response thereby further mediating the immediate clearance

of pathogens, as well as contributing to the repair response by providing critical tissue remodeling and regeneration factors (Iwasaki and Medzhitov, 2010). B cells also respond to TLR ligands, and this response is includes increasing expression of co-stimulatory molecules, proliferating, inducing class switch recombination machinery, and differentiating into antibody producing cells (Pasare and Medzhitov, 2005; Rawlings et al., 2012). Thus TLRs induce multiple cellular outcomes that vary depending upon the cell type responding to the stimuli.

Although TLR activation is important for host defense, an exaggerated innate immune response with very high circulating levels of pro-inflammatory cytokines can lead to septic shock and death of the host. In addition, chronic activation of TLRs can also lead to development of autoimmune diseases such as systemic lupus erythematous and inflammatory bowel disease in genetically pre-disposed individuals. Consequently, it is important for responding cells to regulate the TLR signaling pathway to contain inflammation. Several negative regulators of TLR signaling, including IRAK3 (also known as IRAKM), SIGIRR and A20, to name just a few, have been previously identified and described (Boone et al., 2004; Kobayashi et al., 2002; Turer et al., 2008; Wald et al., 2003). In addition, many studies implicate phosphoinositide 3-kinases (PI3K) and their substrate AKT in regulation of TLR signaling (Androulidaki et al., 2009; Fukao and Koyasu, 2003; Fukao et al., 2002; Hazeki et al., 2007; Ruse and Knaus, 2006). The PI3K pathway integrally regulates important cellular processes including cell survival and proliferation. Although we have a thorough understanding of the signaling pathways and transcription factors involved in induction of a

pro-inflammatory response, the molecular players and events involved in TLR mediated PI3K are, by comparison, poorly understood.

Pathways of Activation by TLRs

TLR signaling leads to multiple outcomes (cell differentiation, induction of inflammatory/regulatory genes, cell-surface expression of co-stimulatory molecules, cellular proliferation, antibody class-switching production, etc.) dependent upon the cell type responding to the stimuli. These outcomes are a direct result of activation of several transcription factors including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), activator protein-1 (AP-1), and interferon regulatory factors (IRFs). All TLRs activate NF-κB and AP-1, while activation of IRF3 and IRF7 is regulated by differential usage of the signaling adapters as well as the cellular compartment where the signaling originates. The role of these different transcription factors in driving the pro-inflammatory response is discussed below.

NF-κB

Activation of NF-κB is a major outcome of TLR signaling and contributes to most of the pro-inflammatory response induced by TLR ligands. Activation of NF-κB is a multi-step process beginning with recruitment of the proximal TLR adaptors MyD88 and/or TRIF to the TLR membrane complex. Based on the initial choice of adapter proteins, the TLR signaling network is broadly classified as the MyD88-dependent pathway or the TRIF-dependent

pathway, though both lead to activation of NF-κB (Takeuchi and Akira, 2010). The MyD88-dependent pathway is engaged by all known TLRs, with the exception of TLR3, while the TRIF-dependent pathway is used by both TLR3 and TLR4. Uniquely, TLR4 activates both the MyD88 and TRIF-dependent signaling pathways (Takeuchi and Akira, 2010). The MyD88-dependent pathway (activated at the plasma membrane) is important for early activation of NF-κB and the TRIF-dependent pathway (activated upon internalization of the receptor complex into endosomes) is involved in late phase activation of NF-κB in response to TLR4 stimulation (Covert et al., 2005). Activation of both of these pathways is important for induction of pro-inflammatory cytokine secretion downstream of TLR4 (Takeuchi and Akira, 2010).

Following recruitment of MyD88 to TLRs through their TIR domains, MyD88 via its death domain engages a complex of IRAK4/IRAK1/IRAK2 through interaction with death domains found in these adaptors. A structural understanding has emerged about the assembly of this critical complex, termed the 'Myddosome' (Gay et al., 2011). The IRAK-containing complex then engages and activates TRAF6, an E3 ubiquitin ligase. TRAF6, along with the E2 ubiquitin ligases UBC13 (also known as UBE2N) and UEV1A (also known as UBE2V1), catalyzes the formation of Lys 63 linked poly-ubiquitin chains upon itself as well as free ubiquitin chains (Jiang and Chen, 2012). TAK1 phosphorylates IKKβ, which is part of a complex with IKKα and NEMO. The IKK complex then phosphorylates IκBα (which enforces sequestration of NF-κB outside of the nucleus). Phosphorylated IκBα is targeted for

ubiquitin mediated proteasome degradation, freeing NF-κB to translocate to the nucleus and act upon target genes (Flannery and Bowie, 2010).

The TRIF-dependent pathway also leads to NF-κB activation. This pathway includes a complex of TRADD and RIP-1, which mediate cleavage of the zymogens capsase-8 and caspase-10 into their active forms, which in turn activate NF-κB (Cusson-Hermance et al., 2005; Ermolaeva et al., 2008; Meylan et al., 2004; Takahashi et al., 2006; Takeuchi and Akira, 2010). TRIF also leads to NF-κB activation through interaction with TRAF6. Activation of both of these pathways is important for induction of pro-inflammatory cytokine secretion downstream of TLR4 (Takeuchi and Akira, 2010). NF-κB activation driven by the TLR3 mediated TRIF-dependent pathway is not very robust at driving inflammatory cytokine secretion and instead leads to activation of IRF3 and a subsequent interferon response, which is discussed in greater detail below.

AP-1

Signaling through TLRs also leads to activation of the AP-1 family of transcription factors, which are activated by mitogen activated protein (MAP) kinases. MAP kinases are composed of a sequential three-component system involving activation of a MAP 3-kinase, which phosphorylates a MAP 2-kinase, which in turn phosphorylates a MAP kinase (Chang and Karin, 2001; Dong et al., 2002; Li et al., 2010; Symons et al., 2006). The mammalian MAP kinase family consists of four groups: extracellular signal regulated protein kinases (ERK1/2), c-JUN NH_2 terminal kinases (JNK1/2/3), p38 kinases (p38α/β/γ/δ), and ERK5

(Chang and Karin, 2001; Li et al., 2010). Both the MyD88 dependent and independent pathways activate MAP kinases and AP-1, and the two pathways converge upon TRAF6. Synthesis of Lys 63 linked poly-ubiquitin chains by TRAF6 mediates recruitment of TAB2 and TAB3, which regulate the kinase TAK1, thus permitting TAK1 activity (Jiang and Chen, 2012). TAK1 undergoes a requisite lysine-63 linked ubiquitination upon interaction with the E3 ligase TRAF6 and TAB1/2/3 (Takeuchi and Akira, 2010). TAK1, a MAP 3-kinase, transduces signals leading to the activation of JNK and p38 (Huang et al., 2009; Sato et al., 2005; Shim et al., 2005; Symons et al., 2006). Activation of ERK1/2 proceeds through the activity of TPL2 (also known as MAP3K8 or COT), which is regulated through its interaction with NF-κB p105 (Li et al., 2010; Symons et al., 2006). Upon TLR activation, TPL2 is released from p105 and activates MEK1 and MEK2, which then activate ERK1/2. However, ERK1/2 activation can proceed independent of TPL2 in some cell types, suggesting the existence of an alternate pathway (Banerjee and Gerondakis, 2007; Symons et al., 2006). Activation of MAP kinases then leads to activation of the AP-1 family of transcription factors which further feeds into regulation of the pro-inflammatory response, including inducing inflammatory cytokines and regulating susceptibility to endotoxin shock (Chang and Karin, 2001; Dong et al., 2002; Huang et al., 2009; Li et al., 2010; Symons et al., 2006).

IRFs

Another consequence of TLR activation is the production of type I interferons, which are critical for induction of anti-viral immunity. The induction of type I interferons depends upon

activation of the IRF family of transcription factors (Tamura et al., 2008). This family includes nine members in both mice and humans, the most important of which are IRF3, IRF5 and IRF7 (Tamura et al., 2008). Upon activation, IRFs form homo- or hetero-dimers and translocate to the nucleus for binding to target DNA sequences. Activation of IRF transcription factors through TLR stimulation is accomplished through both the MyD88-dependent and TRIF-dependent signaling pathways upon ligand binding (Thompson et al., 2011). The MyD88-dependent pathway leads to IRF7 activation upon stimulation of TLR7, TLR8 or TLR9 (Honda et al., 2005). Ligand binding leads to an interaction of IRF7 with MyD88 through its death domain (Honda et al., 2004). This pathway further depends upon IRAK1 and IKKα as cells lacking expression of these proteins are defective for IRF7 activation and subsequent IFN production (Honda et al., 2004; Hoshino et al., 2006; Uematsu et al., 2005). Likewise stimulation of TLR7, TLR8, or TLR9 leads to phosphorylation of IRF5, however the biochemical mechanism mediating IRF5 activation is incompletely understood.

Stimulation of TLR3 or TLR4, through the TRIF-dependent pathway, induces phosphorylation of IRF3 leading to IFNβ induction. TRIF-mediated activation of IRF3 depends upon upstream activation of TRAF3. Upon TLR4 stimulation, TRAF3 is modified by Lys-63 linked ubiquitination, which is required for subsequent activation TANK-binding kinase 1 (TBK1) and IKKε (Kayagaki et al., 2007; Tseng et al., 2010). Together, TBK1 and IKKε then mediate phosphorylation of IRF3 (Barbalat et al., 2011; Häcker et al., 2011). Consequently, cells deficient in TRAF3, TBK1, or IKKε are severely compromised in their

ability to activate IRF3 and induce IFNβ upon TLR3 stimulation (Barbalat et al., 2011; Hacker et al., 2006; Häcker et al., 2011; Oganesyan et al., 2006; Tseng et al., 2010).

PI3K

Phosphoinositide 3-kinases (PI3K) are a family of lipid kinases consisting of three classes (class I, class II and class III) based upon substrate specificity as well as several structural characteristics (Bunney and Katan, 2010; Fruman et al., 1998; Katso et al., 2001; Vadas et al., 2011; Vanhaesebroeck et al., 2010). Class I PI3K are further sub-divided into class IA and class IB depending upon which regulatory subunit they engage (Vadas et al., 2011). The class IA PI3K, which plays a major role in cells of the immune system, consists of p110α, p110β, and p110δ and are all regulated by either p85α or p85β (Fruman and Cantley, 2002). The class IB PI3K, p110γ, instead binds the regulatory subunits p101 or p87. A detailed description of PI3K classes and their roles in regulating cellular responses can be found elsewhere (Bunney and Katan, 2010; Fruman et al., 1998; Katso et al., 2001; Vanhaesebroeck et al., 2010). One mechanism mediating class I PI3K activation occurs through recruitment of the PI3K complex through the SH2 domain of the regulatory p85 subunit to phosphorylated tyrosine residues (Vanhaesebroeck et al., 2010). Engagement of p85 to phosphorylated tyrosines releases the inhibitory pressure p85 places upon the p100 PI3K subunit. Further, recruitment of the PI3K complex to phosphorylated tyrosines brings PI3K to the lipid membrane and in close proximity with its lipid substrate. Upon activation, PI3K phosphorylates phosphatidylinositol-4,5-*bis*phosphate ($PtdIns(4,5)P_2$) to yield

phosphatidylinositol-4,5-*tris*phosphate (PtdIns(3,4,5)P_3) (Bunney and Katan, 2010; Fruman et al., 1998; Katso et al., 2001; Vanhaesebroeck et al., 2010). Increased concentrations of PtdIns(3,4,5)P_3 at the membrane leads to recruitment of PDPK1 (3-phosphoinositide dependent protein kinase-1) and AKT (also known as PKB). PDPK1 phosphorylates the serine/threonine kinsase AKT which consists of three isoforms derived from three separate genes(Bunney and Katan, 2010; Fruman et al., 1998; Katso et al., 2001; Vanhaesebroeck et al., 2010). The kinase AKT then acts upon its extensive complement of targets.

The precise role PI3K pathway plays in regulation of the cell or host response to pathogenic insult is not completely clear. Studies using pharmacological inhibitors of PI3K activation have yielded controversial results. Some studies suggested PI3K contributes to NF-κB activation, and thus the inflammatory response, while other studies advocated a role for PI3K in inhibiting the inflammatory response (Fukao and Koyasu, 2003; Hazeki et al., 2007; Ruse and Knaus, 2006). These discrepancies may be incompletely explained by usage of different pharmacological inhibitors as well as non-standard dosages.

Conversely, genetic evidence suggests the PI3K pathway acts as a critical negative regulator of the pro-inflammatory response. Dendritic cells from p85α deficient mice produced more Il12 upon TLR stimulation, correlating with greater p38 activity *in vitro*, leading to increased resistance to *Leishmania major* infection with an enhanced Th1 effector phenotype *in vivo* (Fukao et al., 2002). Intriguingly, thioglycollate macrophages have

increased ERK1/2 and JNK activity, but not p38 activity, and increased message levels of TNF and IL-6, suggesting a cell type specific role for p85 in regulating p38 MAP kinase activation (Luyendyk et al., 2008).

Pdpk1, which functions through PI3K to coordinate activation of multiple downstream effectors, including a critical role in Akt phosphorylation, suppressed production of IL-6 and TNF from macrophages upon stimulation through TLR2 or TLR4 (Chaurasia et al., 2010). Pdpk1 deficient macrophages had enhanced levels of phosphorylated IκBα, but in contrast to p85α deficient DCs, had normal levels of MAP kinase activity (Chaurasia et al., 2010). As a result, mice with myeloid specific deletion of Pdpk1 *in vivo* contain higher levels of serum TNF upon systemic administration of LPS. Consequently, Pdpk1 deficient mice exhibited increased end organ pathology and succumbed more rapidly to systemic LPS challenge (Chaurasia et al., 2010).

Furthermore, macrophages from Akt1 deficient mice had increased inflammatory responses to TLR4 stimulation, including enhanced production of TNF, IL-6 and CCL3 (also known as MIP1α) (Androulidaki et al., 2009). Mechanistically, the authors found that signals provided by Akt1 were critical for induction of several miRNA species, including miRNA-155. Importantly, miRNA-155 was found to directly target the 3' UTR of an important negative regulator of PI3K, SHIP1 (Src homology-2-domain containing inositol 5-phosphatase 1), blocking its expression (Androulidaki et al., 2009; O'Connell et al., 2009). Importantly, detection of phosphorylated Akt in macrophages stimulated through TLR4 is

largely abrogated in Akt1 deficient cells but not Akt2 deficient cells, suggesting that Akt1 is the most critical isoform utilized by the TLR-PI3K signaling axis (Bauerfeld et al., 2012).

Phosphatase and tensin homolog (PTEN), a 3-phosphatase counterbalancing PI3K by catalyzing conversion of PtdIns(3,4,5)P_3 back into PtdIns(4,5)P_2, plays a positive role in inducing inflammation in TLR stimulated cells. PTEN deficient macrophages secreted decreased quantities of TNF and IL-6 upon stimulation with TLR ligands (Cao et al., 2004; Kuroda et al., 2008; Luyendyk et al., 2008). Consequently, in a pneumococcal pneumonia model, mice deficient for PTEN in the myeloid compartment have less TNF, IL6 and CXCL1 (also known as KC), but more IL-10 in their bronchiolar lavage fluid (Schabbauer et al., 2010). This phenotype corresponded with increased phagocytosis and elimination of intracellular bacteria by alveolar macrophages infected *in vitro* with *Streptococcus pneumonia* (Schabbauer et al., 2010). In contrast, Kuroda *et al* found a requisite role for PTEN in elimination of *Leishmania* parasites *in vivo* in a manner suggested to be dependent upon TNF mediated induction of nitric oxide (Kuroda et al., 2008). This discrepancy details the instructive role of inflammatory cytokines in an autocrine/paracrine fashion for eliciting the effector capacity of local immune cells.

Another important regulatory step imposed upon the class I PI3K pathway is through lipid hydrolysis of PtdIns(3,4,5)P_3 by the phosphatase SHIP1, which acts as a 5' phosphatase, mediating PtdIns(3,4)P_2 conversion from PtdIns(3,4,5)P_3. The role SHIP1 plays in TLR stimulated cells is currently unclear as conflicting results exist in the literature. In some reports, macrophages from SHIP1 deficient mice secreted less TNF and IL-6 and had a

corresponding increase in PI3K activity through Akt phosphorylation (Fang et al., 2004; Keck et al., 2010). Paradoxically, in a series of reports from another group, SHIP1 blocked TNF and IL-6 secretion upon TLR3 or TLR4 stimulation, but induced IL-12 and expression of surface co-stimulatory molecules upon TLR stimulation, suggesting a more complex role for SHIP1 in downstream TLR signaling (Antignano et al., 2010a; Antignano et al., 2010b; Sly et al., 2009; Sly et al., 2004). Consequently, SHIP1 deficient DCs were functionally incompetent for priming Th1 effector T cells (Antignano et al., 2010a; Antignano et al., 2010b). These conflicting reports warrant further and more detailed investigation into the molecular mechanism of SHIP1 mediated control of the TLR pathway. Indeed, the ability of SHIP1 to suppress inflammation was found to be independent of its phosphatase activity (An et al., 2005). Further work remains to fully understand the role SHIP1 plays in TLR signaling in a manner dependent and independent of its role in suppressing PI3K activity.

Linking TLR Signaling to PI3K Activation

The mechanism for TLR-mediated activation of the PI3K pathway has been explored and a direct linkage of TLRs themselves, through phosphorylated tyrosine residues, is suggested to mediate activation of PI3K. Phosphorylation of tyrosine residues at the carboxyl terminal end of TLR2 was required for recruitment of PI3K-p85 subunit and subsequent activation by RAC1 (Arbibe et al., 2000). Similarly, TLR3 and TLR8 had phosphorylated tyrosine residues upon ligand engagement, which led to the recruitment of PI3K-p85 subunit (Rajagopal et al., 2008; Sarkar et al., 2004). However, these studies were performed in the context of

overexpression and the detailed biochemistry as well as genetic confirmatory studies are yet to be performed. In addition to recruitment to TLRs themselves, roles for adaptor-mediated recruitment of PI3K to the TLR signaling complex have also been described. MyD88 itself contains a YXXM motif within its TIR domain and was found to associate with p85, however, truncation and mutagenesis studies demonstrated that neither the YXXM motif of MyD88, nor its TIR domain, was required for permissive association with p85, suggesting the existence of other mechanisms promoting complex assembly (Laird et al., 2009). The adaptor TIRAP was also found to mediate Akt phosphorylation post-stimulation with the TLR2 ligand MALP2 (Santos-Sierra et al., 2009). Receptor-interacting protein 1 (RIP1) deficient splenocytes were impaired in their ability to phosphorylate Akt upon LPS or CpG stimulation compared to control cells (Vivarelli et al., 2004). MyD88 deficient macrophages were also found to be defective for Akt phosphorylation through several TLR ligands, as reported by us and others (Bauerfeld et al., 2012; Laird et al., 2009; Troutman et al., 2012b). Similarly, TRIF signals were required by macrophages for Akt phosphorylation upon LPS stimulation, and thus likely serves as the upstream adaptor allowing RIP mediated PI3K activation (Bauerfeld et al., 2012). Together, these data suggest that TLR adaptors may serve to recruit a previously unknown downstream target mediating PI3K activation.

The IL1R Family

TLRs belong to a larger superfamily of receptors including IL1R family. Unlike TLRs, IL1R members bind their respective ligands using extracellular immunoglobulin domains and not

LRR domains (Casanova et al., 2011). However, like TLRs, IL1R family members contain conserved cytosolic TIR domain and critically depend on the MyD88 for signal transmission (Dinarello, 2009; Garlanda et al., 2013; Gay and Keith, 1991; Medzhitov et al., 1998; O'Neill, 2008a). Because of the shared adapter usage, signaling pathways employed by IL1R family members are similar to those employed by all MyD88-dependent TLRs (O'Neill, 2008a).

The IL1R family is composed of 11 known receptors: IL1RI/IL1R1, IL1RII/IL1R2, IL1RAcP/IL1R3, ST2/Fit-1/IL1R4, IL18Rα/IL1R5, IL1Rrp2/IL1R6, IL18Rβ/IL1R7, and SIGIRR/IL1R8, TIGIRR2/IL1RAPL/IL1R9, and TIGIRR1/IL1R10 (Garlanda et al., 2013). Together, these receptors and decoy receptors govern signaling in response to eight cytokines: IL1α/IL1F1, IL1β/IL1F2, IL18/IL1F4, IL33/IL1F11, IL36α/IL1F6, IL36β/IL1F7 IL36γ/IL1F8, and IL37/IL1F7. In addition, the IL1R family can be engaged by three receptor antagonists: IL1Ra/IL1F3, IL36Ra/IL1F5, and IL38/IL1F10 (Dinarello, 2009; Garlanda et al., 2013; O'Neill, 2008a). Signaling in response to the IL1 family cytokines occurs through complexes of the receptor chains. The IL1 receptor signals in response to IL1α, IL1β, and binds its antagonist, IL1Ra (Dinarello, 2009; Eisenberg et al., 1991; Garlanda et al., 2013). There are two described IL1 receptor complexes, the type I IL1 receptor, composed of IL1RI and IL1RAcP, and the type II IL1 receptor, composed of the decoy receptor IL1R2, which lacks the requisite cytosolic TIR domain for signal transduction, and the IL1RAcP (Colotta et al., 1993; Garlanda et al., 2013; Re et al., 1996; Stylianou et al., 1992). The IL18 receptor signals in response to IL18 and is composed of IL18Rα and IL18Rβ (Dinarello, 2009;

Garlanda et al., 2013). In addition, IL18Rα binds to IL37, potentially functioning in an anti-inflammatory capacity (Bufler et al., 2002; Garlanda et al., 2013; McNamee et al., 2011; Nold et al., 2010; Pan et al., 2001; Sims and Smith, 2010). The IL33 receptor is composed of ST2 and the co-receptor IL1RAcP, binding to IL33 (Garlanda et al., 2013; Liu et al., 2013; Schmitz et al., 2005; Sims and Smith, 2010). The IL36 receptor, composed of IL1Rrp2 and IL1RAcP, is responsible for binding to IL36α, IL36β, IL36γ and the antagonist IL36Ra (Debets et al., 2001; Garlanda et al., 2013; Towne et al., 2004). The receptor chain SIGIRR can inhibit signaling in response to many IL1 family cytokines (as well as TLR ligands), including IL1, IL18, IL33, and IL36 (Bulek et al., 2009; Garlanda et al., 2009; Garlanda et al., 2004; Wald et al., 2003). Finally, the receptor chains TIGIRR1 and TIGIRR2, considered atypical members of the family, were identified based upon sequence homology and as of yet have no known function (Born et al., 2000; Garlanda et al., 2013).

IL1R family and T cells

IL1 (once known as pyrexin, endogenous pyrogen, lymphocyte activation factor, catabolin, and hemopoietin-1) was the first described cytokine and has many biological activities (Dinarello, 1994; Gabay et al., 2010). Recent evidence has strongly linked activity of IL1 to the differentiation of Th17 cells (Sims and Smith, 2010). In the mouse, Th17 cells differentiate in response to the combination of IL6 and TGFβ through activities of the transcription factor RORγt (Korn et al., 2009). In human cells, TGFβ is not required for Th17 development; instead IL1β, IL6, and IL23 play key roles (Acosta-Rodriguez et al., 2007;

Wilson et al., 2007). In the absence of IL6 *in vivo*, IL17 competent T cells are still detected in the periphery. However, in IL1R1-deficient mice, as well as MyD88-deficient mice, Th17 cells are absent (Hu et al., 2011; Kryczek et al., 2007). This effect was seen also found to be true using in vitro systems (Chung et al., 2009; Hu et al., 2011). In part, this effect is attributed to a role for IL1 in relieving a IL2 mediated blockade of Th17 differentiation, in part through overcoming the inhibitory effect of IL2 on induction of the Th17 transcription factor RORγt (Kryczek et al., 2007). Consequently, a role for IL1R signaling intrinsic to T cells has been linked to multiple sclerosis models, (Chung et al., 2009; Sims and Smith, 2010; Sutton et al., 2006). Furthermore, an axis of IL1 mediated induction of IL17 and Th17 cells has been shown to play a key role in inducing a mouse model of arthritis (Nakae et al., 2003).

IL18, originally called interferon-gamma inducing factor, plays a key role in promoting induction of Th1 cells (Garlanda et al., 2013; Okamura et al., 1995; Robinson et al., 1997; Robinson and O'Garra, 2002; Sims and Smith, 2010). Further, the effect of IL18 on induction of IFNγ production by T cells can occur independent of stimulation through the TCR, but dependent upon co-stimulation with IL12 (Dinarello et al., 2013). Treatment of T cells with IL12 potently induced expression of the IL18 receptor on T cells, explaining the need for concurrent IL12 to impart the biological activity of IL18 upon responding cells (Smith, 2011; Yoshimoto et al., 1998). Furthermore, cooperation of IL1 family cytokines with other cytokines, through combined activity of STATs has been suggested to explain the

synergistic role IL1 family cytokines have in potentiating helper T cell lineage commitment (Guo et al., 2009).

Search for New TLR/IL1R Family Adapters

Together with our collaborator, Dr. J. Fernado Bazan, Dr. Chandrashekhar Pasare proposed the existence of other TIR domain-containing signaling adapters used by the TLR/IL1R superfamily. The TIR domain is evolutionarily ancient and can be found throughout the phylogenetic tree, including in bacteria (Koonin and Aravind, 2002; Turner, 2003). The crystal structure of a TIR domain from the TIR-like protein PdTLP of the bacteria *Paracoccus denitrificans* shows that while the sequence has been divergent (only 20% similarity with mammalian counterparts), the domain architecture is well conserved (Chan et al., 2009). Furthermore, TIR domain containing-proteins from pathogenic bacteria retain their ability to functionally interact with TLR signaling adapters, thus serving as virulence factors by inhibiting host MyD88 and TRIF (Cirl et al., 2008; Yadav et al., 2010). Due to the sequence divergence, traditional methodology employing recognition through sequence similarity was suggested to allow potential candidates to evade detection. Therefore, Dr. J. Fernando Bazan employed a computational approach using "sensitive methods utilized in structure prediction, fold recognition and modeling" to search the human proteome for unrecognized TIR adaptors cryptically located in other proteins. This method identified two candidates, BCAP (B-cell adaptor for PI3K, also known as PIK3AP1) and BANK1 (B-cell scaffold protein with ankyrin repeats 1), paralogous to each other and orthologs of the

Drosophila DOF protein (also known as stumps) (Figure 1-1) (Troutman et al., 2012b). In contrast to existent TLR signaling adapters and TLRs themselves, the TIR domains of BCAP and BANK1 were located at the N-terminus. Further sequence assessment suggested the TIR domains were closely followed by a transcription factor-Ig (TIG) domain found in many transcription factors such as NF-κB, p53 and STATs (Figure 1-1) (Rudolph and Gergen, 2001).

B-cell adapter for PI3K

B-cell adapter for PI3K, abbreviated here as BCAP, is also known by its NCBI preferred name of phosphoinositide-3-kinase adaptor protein 1 (PIK3AP1, gene ID 118788). BCAP is found on mouse chromosome 19 in a region syntenic with the human BCAP locus on chromosome 10 (Figure 1-2). Expression of BCAP is detected by immunoblotting to occur in at least four isoforms (Figure 3-24), including splice variants and potentially post-translational cleavage events (Figure 3-10) (Okada et al., 2000; Troutman et al., 2012b). BCAP was initially characterized in the lab of Tomohiro Kurosaki, Ph.D. In a series of manuscripts, they found that BCAP is utilized by chicken B cells, downstream of the B-cell receptor as well as CD19, to mediate activation of PI3K (Inabe and Kurosaki, 2002; Okada et al., 2000). Importantly, the ability of BCAP to mediate activation of PI3K in these cells critically depends on the phosphorylation of four tyrosine residues within BCAP by Syk and Btk for BCR stimulation, or Lyn for CD19 stimulation (Inabe and Kurosaki, 2002; Okada et al., 2000). These finding prompted the creation of a targeted deletion mouse whereby exon eight was replaced with a selection cassette (Yamazaki et al., 2002). Unexpectedly, this B

cells from this BCAP-deficient mouse have no defect in their ability to activate PI3K and phosphorylate Akt upon stimulation through the BCR yet have an impairment in calcium fluxing (Yamazaki et al., 2002). This observation was later reconciled with data from chicken B cells to include a redundant role for both BCAP and CD19 in mediating activation of PI3K upon BCR stimulation (Aiba et al., 2008). Instead, the investigators found a developmental reduction in mature B cells and a deficiency in B1 B cells. Consequently, BCAP-deficient mice were unable to mount a normal antibody response upon immunization with the T-independent type II antigen, TNP-Ficoll. Conversely, these mice were found to elicit a normal antibody response to the hapten component of the T-dependent antigen, TNP-KLH (Yamazaki et al., 2002). Furthermore, and of keen interest to us as we developed our hypotheses, BCAP-deficient B cells were found to be hypo-proliferative in response to BCR ligation, CD40 ligation, and importantly, LPS stimulation (Yamazaki and Kurosaki, 2003; Yamazaki et al., 2002).

Work from the lab of Dr. Kerry Campbell reveals an important role for the regulation of NK-cell development and function (MacFarlane et al., 2008). They found that BCAP is expressed by NK-cells and that expression of BCAP by NK-cells is required for Akt phosphorylation upon cross-linking the NK1.1 receptor. Further, they found increased numbers of splenic NK-cells, as well as an altered NK-cell receptor repertoire in cells from BCAP-deficient mice. Finally, BCAP-deficient NK-cells were found to have increased cytotoxic function and INF-γ production. Thus, perhaps somewhat surprisingly, NK-cells use BCAP in a negative regulatory fashion, in contrast to how B cells utilize BCAP.

BCAP expression is not limited to just B cells and NK-cells, however. It has been known since the initial characterization of BCAP, that macrophage cell lines also highly express BCAP (Okada et al., 2000). Furthermore, searching the Immunological Genome Consortium database reveals that BCAP is expressed ubiquitously by all queried immune cell populations, and at the highest levels by B cells and myeloid cells (Figure 1-3). Due to the broad expression pattern of BCAP, its role in mediating B cell proliferation upon LPS stimulation, and the putative TIR domain predicted to exist at its N-terminal region, we embarked upon a project to test its role as a TLR signaling adapter. Results from this approach led us to suspect that BCAP plays a role intrinsic to T cells, perhaps through signaling through IL1R family members.

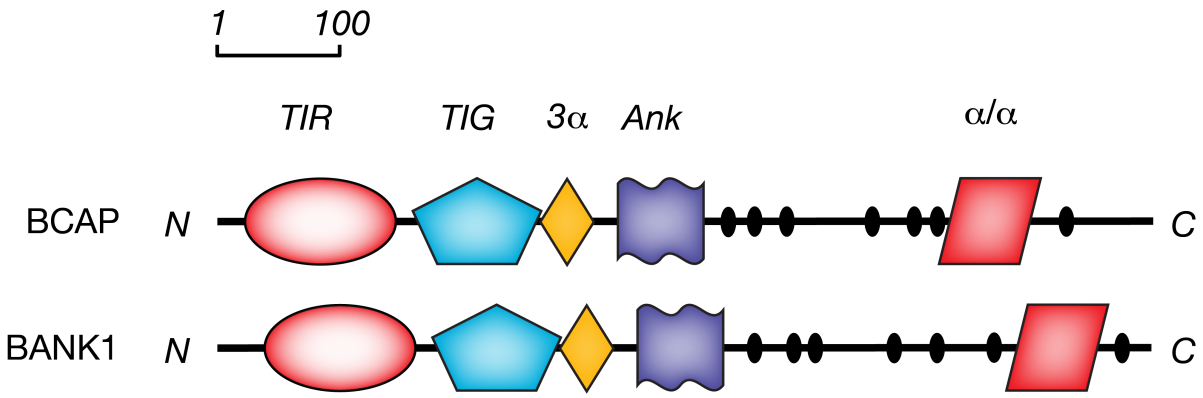

Figure 1-1. Domain architecture of human BCAP and BANK1 adaptors

The five component domains of BCAP and BANK1 chains (805 and 785 amino acids, respectively) are drawn schematically for the N terminal TIR module (red oval), and the closely packed TIG (teal pentagon) and 3-α-helix (yellowish diamond) structures; a short 3 Ankyrin repeat region (ANK, wavy purple) is separated by a nonconserved and unstructured linker with a C terminal α-helical module (red parallelogram). Black dots mark prospective phosphotyrosine sites that are largely located in the unstructured linker.

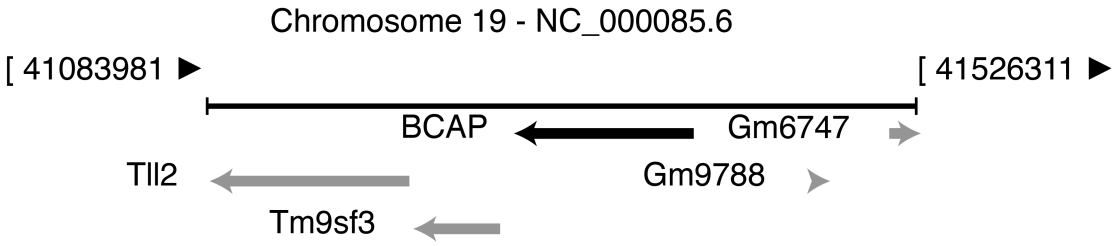

Figure 1-2. Gene neighbors of mouse BCAP on chromosome 19

Chromosome map of the mouse BCAP locus, located on chromosome 19. Tll2; tolloid-like 2. Tm9sf3; transmembrane 9 superfamily member 3. BCAP, B-cell adapter for PI3K. Gm9788, developmental pluripotency associated 2 pseudogene. Gm6747; predicted gene 6747. Data adapter from NCBI, Gene ID: 83490.

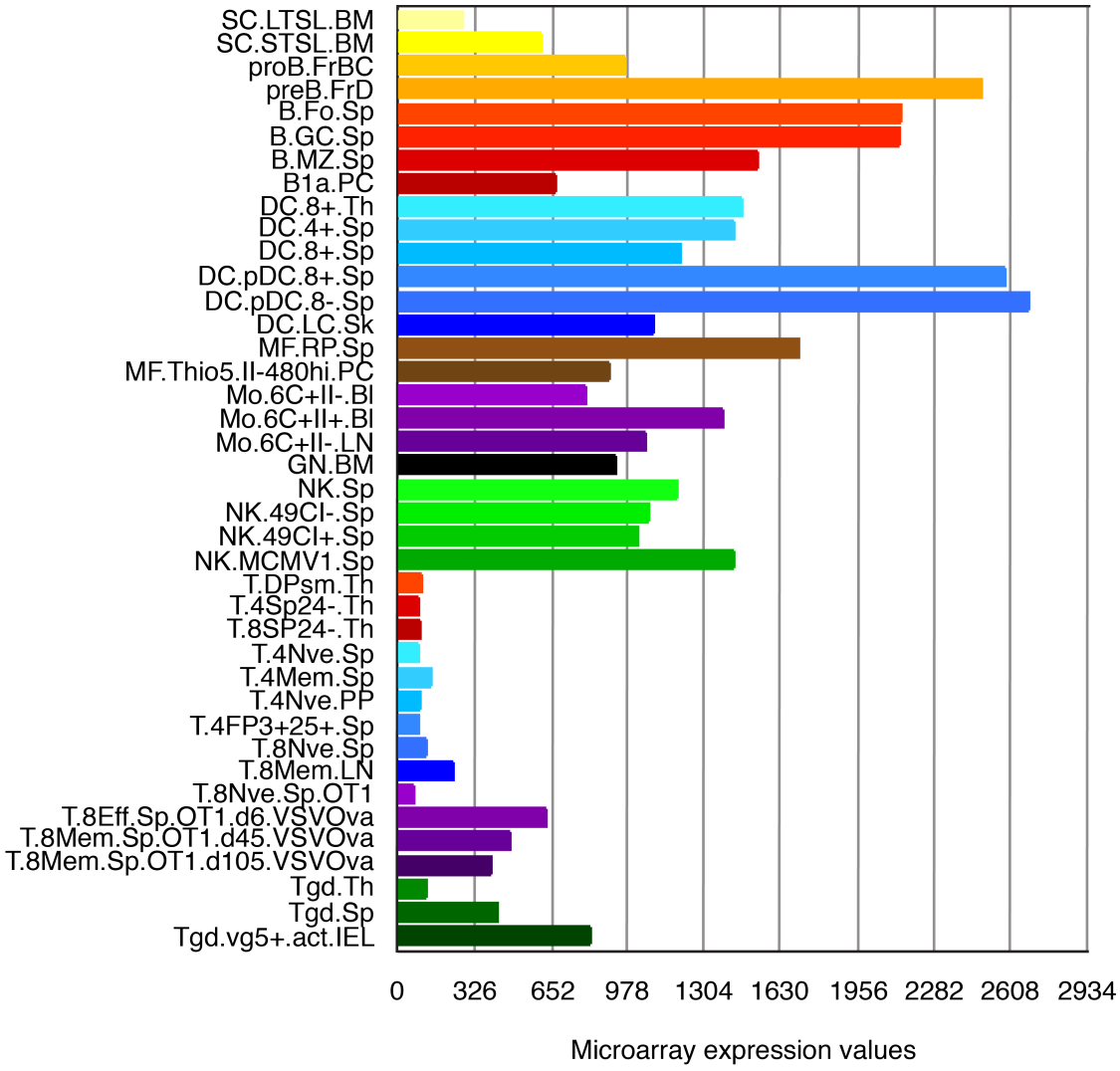

Figure 1-3. Expression pattern of BCAP in key populations of immune cells

Microarray expression data of BCAP in the FACS purified cell populations. Data adapted from the Immunological Genome Project (http://www.immgen.org/) Gene Skyline data browser. Abbreviations are defined in order from top to bottom as follows with the tissue source listed in parentheses: SC.LTSL.BM, long-term repopulating hematopoietic stem cell (bone marrow); SC.STSL.BM, short-term repopulating hematopoietic stem cells (bone marrow); proB.FrBC fraction B/C pro-B cells; preB.FrD fraction D pre-B cells; B.Fo.Sp, follicular B cells (spleen); B.GC.Sp, germinal center B cells (spleen); B.MZ.Sp, marginal zone B cells (spleen); B1a.PC, B-1a B cells, (peritoneal cavity); DC.8+.Th, CD8 DCs (thymus); DC.pDC.8+.Sp, CD8 positive plasmacytoid DCs (spleen); DC.pDC.8-.Sp, CD8

negative plasmacytoid DCs (spleen); DC.LC.Sk, epidermal/Langerhans DCs (skin); MF.RP.Sp, red-pulp macrophages (spleen); MF.Thio5.II-480hi.PC, 5 day Thioglycollate elicited peritoneal macrophages (peritoneal cavity); Mo.6C+II-.Bl, MHC-II negative classical monocytes (blood); Mo.6C+II+.Bl, MHC-II positive classical monocytes (blood); Mo.6C+II-.LN, MHC-II negative classical monocytes (lymph nodes); GN.BM, neutrophils (bone marrow); NK.Sp, natural killer cells (spleen); NK.49CI-.Sp, Ly49C/l negative natural killer cells (spleen); NK.49CI+.Sp, Ly49C/l positive natural killer cells (spleen); NK.MCMV1.Sp, day 1 post-infection with MCMV natural killer cells (spleen); T.DPsm.Th, small resting double-positive thymocytes (thymus); T.4Sp24-.Th, mature CD4 single-positive thymocytes (thymus); T.8SP24-.Th, mature CD8 single-positive thymocytes (thymus); T.4Nve.Sp, naïve CD4 positive T cells (spleen); T.4Mem.Sp, memory CD4 positive T cells (spleen); T.4Nve.PP, naïve CD4 positive T cells (Peyers patches); T.4FP3+25+.Sp, regulatory CD25 positive Tregs (spleen); T.8Nve.Sp, naïve CD8 positive T cells (spleen); T.8Mem.LN, memory CD8 positive T cells (lymph nodes); T.8Nve.Sp.OT1, OT1 transgenic naïve T cells (spleen); T.8Eff.Sp.OT1.d6.VSVOva, OT1 transgenic effector T cells 6 days post-infection with VSV-Ova (spleen); T.8Mem.Sp.OT1.d45.VSVOva, OT1 transgenic memory T cells 45 days post-infection with VSV-Ova (spleen); T.8Mem.Sp.OT1.d105.VSVOva, OT1 transgenic memory T cells 105 days post-infection with VSV-Ova (spleen); Tgd.Th, double-negative gamma delta TCR thymocytes (thymus); Tgd.Sp, double-negative gamma delta T cells (spleen); Tgd.vg5+.act.IEL, activated Vγ5 positive gamma delta T cells (intra-epithelial lymphocytes).

Summary of the Dissertation

Interaction and recruitment of TIR domain-containing signaling adapters to the cytosolic TIR domain of TLR/IL1R superfamily members is key for assembly of the signaling complex (Kawai and Akira, 2006; Medzhitov, 2009; O'Neill and Bowie, 2007). Usage of these TIR domain-containing signaling adapters ultimately leads to a robust cellular program playing a crucial role in orchestration of a developing immune response. Thus, identification of all TIR domain-containing signaling adapters, and robust characterization of their mechanistic role(s), will prove invaluable towards our enhanced understanding of the inflammatory process. Here, I provide evidence that BCAP is a proximal TIR domain-containing TLR/IL1R superfamily signaling adapter. In this role, BCAP serves to link TLRs to activation of PI3K, a previously poorly understood component of the TLR signaling network. Through this role, BCAP functions in macrophages to suppress production of inflammatory cytokines. In agreement with this finding, BCAP-deficient mice are highly susceptible to an acute inflammatory colitis model. Finally, I have further linked BCAP to regulation of T helper cell priming through intrinsic participation in signaling downstream of the IL1R family. Together, these efforts have revealed an important new signaling component of the TLR/IL1R superfamily. These findings will permit further assessment of the precise role branches of these signaling pathways play in an immune response.

CHAPTER TWO

Methodology

Computational Screens

Using methods described earlier (Bazan and de Sauvage, 2009), BCAP, BANK and Dof alignments were structurally aligned against proteins in the PDB using HHPRED resulting in a series of top matches to TIR domain structures (Z scores of 43.2 to 26, with high probabilities ranging from 94.8 to 58), strongly suggestive of common ancestry and function.

Mice

BCAP KO mice were previously generated and were bred and maintained on C57BL/6 background at the animal facility of UT Southwestern Medical Center. Control C57BL/6 mice were obtained from UT Southwestern Mouse Breeding Core Facility. Mice were maintained in specific pathogen free conditions. Mice were used between 6 to 12 weeks of age and all mouse experiments were done as per protocols approved by the Institutional animal care and use committee (ICAUC) at UT Southwestern Medical Center.

Infections

For infection experiments, one million CFU of *Salmonella typhimurium* χ4550 were injected into mice by the intraperitoneal route. Three days after infection, mice were euthanized and tissues harvested for analysis.

NF-κB Reporter Assay

293T cells were transfected using linear Polyethyleneimine (Polysciences). Cells were transfected with the NF-κB reporter vector pBIIX-luc and pCMV-Renilla-luc. Cells were co-transfected as described with CD4/TLR4 (Medzhitov et al., 1997; Medzhitov et al., 1998), Flag-MyD88, HA-TIRAP, or HA-TLR9 and the indicated BCAP constructs. Empty cloning vectors were used to equalize total plasmid mass transfected. Twenty hours later, cells were lysed in TNT lysis buffer (20mM TRIS, pH 8.0, 150 mM NaCl, 1% Triton X-100) and lysates were assayed for luciferase activity using the Dual-Glo luciferase kit (Promega). Data is reported relative to Renilla luciferase counts and normalized to cells transfected with only the empty vector.

Immunoblotting

RAW264.7 cells lines or BMDM were stimulated with 100 ng/ml LPS, 100 ng/ml Pam3CSK4, or 1 μM CpG as indicated, washed twice with ice-cold PBS and lysed with TNT lysis buffer containing Complete Protease Inhibitor Cocktail (Roche), 1 mM sodium

orthovandate, and 20 mM glycerol 2-phosphate. For the TNF-α stimulation experiment, cells were stimulated with 10 ng/ml mTNF-α (R&D) and lysed directly in SDS-sample buffer. Lysates were resolved on SDS-Page gels followed by transfer to PVDF membranes (Millipore). Membranes were analyzed by immunoblotting using specific antibodies. Stained membranes were developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and exposed to film (Kodak).

Immunoprecipitations

For co-immunoprecipitation experiments, 293T cells were transiently transfected with the indicated combinations of Flag-BCAP mutants and HA-MyD88 or HA-TIRAP or empty vectors. Thirty-six hours later, cells were lysed with TNT lysis buffer containing protease inhibitors. Lysates were quantified by BCA assay and 1 mg was incubated with anti-HA (Covance, clone 16B12) overnight and complexes were pulled down using protein G sepharose beads (GE Healthcare). Precipitates were resolved on SDS-Page gels and transferred to PVDF membranes. Membranes were analyzed by immunoblotting for anti-Flag (Clone M2) or anti-HA for detection of co-precipitating proteins.

Dextran Sulfate Sodium Colitis Model

Age matched B6 or BCAP KO mice were treated for 7 days with 2.5% 36 to 50 kDa dextran sulfate sodium salt (DSS, MP Biomedicals) ad libitum in their drinking water. Mass was monitored by weighing daily. Following 7 days of treatment, mice were euthanized and

colons was removed from the cecum to the anus, photographed, fixed with 4% paraformaldehyde and submitted to the UT Southwestern Molecular Pathology Core for paraffin embedding, sectioning, and hematoxylin and eosin staining.

Antibodies and Reagents

Antibodies used were: purified anti-IL-6, biotin-conjugated anti-IL-6, purified anti-IL-12 p40/p70, biotin-conjugated anti-IL-12 p40/p70, anti-IL-17A, biotin-conjugated anti-IL-17A, purified anti-IFN-γ, biotin-conjugated anti-IFN-γ, purified anti-TNF-α, biotin-conjugated anti-TNF- (BD Biosciences), anti-phosphorylated Akt (Cell Signaling #4051 and #4060) anti-Akt (Cell Signaling #9272), anti-phosphorylated ERK1/2 (Cell Signaling #9101), anti-ERK1/2 (Cell Signaling #9102), anti-phosphorylated IκBα (Cell Signaling #2859), anti-IκBα (Cell Signaling #9242), anti-phosphorylated JNK (Cell Signaling #9251), anti-JNK (Cell Signaling #9252), anti-MyD88 (R&D #AF3109), anti-phosphorylated PI3K p85 (Cell Signaling #4228), anti-PI3K p85 (Cells Signaling #4257), polyclonal anti-MyD88 (R&D) polyclonal anti-BCAP (R&D), anti-Flag M2 (Sigma Aldrich), anti-HA (Santa Cruz, clone 12CA5), anti-HA (Covance, clone 16B12), FITC-conjugated anti-TCRβ, FITC-conjugated anti-TNF-α, PE-conjugated anti-CD11b, PE-conjugated anti-CD44, PerCP-conjugated anti-CD4, biotin-conjugated anti-CD62L (BD Biosciences), and biotin-conjugated anti-Ly-6c (eBioscience). Secondary antibodies and reagents used were HRP-conjugated streptavidin (Jackson), Pacific Blue-conjugated streptavidin (Invitrogen), APC-conjugated streptavidin

(eBioscience), HRP-conjugated goat anti-mouse, HRP-conjugated donkey anti-goat, and HRP-conjugated donkey anti-rabbit (Jackson). TLR ligands used were Ultrapure LPS, MALP-2, Pam3CSK4 (Invivogen), and CpG ODN 1826, TCCATGACGTTCCTGACGTT, with phosphorothioate linkages (W.M. Keck Facility).

Bone Marrow Derived Macrophages

Bone marrow derived macrophages (BMDM) were obtained using standard techniques. In brief, marrow cells were isolated from the femurs and tibias of mice. Cells were cultured overnight in tissue culture treated dishes to remove stromal cells in the presence of conditioned L929 culture supernatant as a source of M-CSF. Non-adherent cells were collected and two to three million cells cultured for an additional five to seven days with conditioned L929 supernatant in non-tissue culture treated dishes. Following differentiation, BMDM were collected and plated for use in experiments. BMDM were stimulated, unless indicated otherwise, for 20 hours with 100 ng/ml LPS, 100 ng/ml Pam3CSK4, 100 ng/ml MALP-2, or 1 μM CpG.

Cloning

BCAP and mutants were cloned into host vectors following PCR amplification using an I.M.A.G.E Consortium clone as the template (clone ID 40047744). The BCAP-TIR mutant

corresponded with amino acids 1-321 and the BCAPΔTIR mutant corresponded with amino acids 180-812. Clones were verified by sequencing.

ELISA

Cytokines were quantified by the sandwich ELISA method using coating and detecting antibodies following standard techniques. HRP-conjugated streptavidin was used to detect bound biotin conjugated antibodies.

Flow Cytometry and Intracellular Staining

Erythrocyte lysed cell suspensions were stained with antibodies and samples run on a BD LSRIITM. In some experiments, samples were run on a BD FACSCalibur™. Intracellular staining was performed following ex vivo restimulation for 5 hours with heat-killed *Salmonella typhimurium* in the presence of brefeldin A. Following stimulation, cells were surface stained, then intracellular stained using a cell fixation and permeabilization buffer set according to the manufacturers protocol (Biolegend #421403). Samples were run on a BD FACSCalibur™. Flow cytometry data was analyzed using FlowJo software (Tree Star).

In Vitro Priming of Naïve CD4 T Cells

CD4 T cells were purified from the spleen and lymph nodes by negative selection using the following hybridoma supernatants: anti-CD8 (TIB-105 and TIB-150), anti-CD11b (TIB-128)

anti-B220 (TIB-146 and TIB-164) anti-NK1.1 (HB191), and anti-MHC-II (Y3JP). Cell suspensions were depleted of CD8 T cells, B cells, NK cells, and myeloid cells by coupling antibody labeled cells to magnetic beads bound to goat anti-rat IgG, goat anti-mouse IgG, and goat anti-mouse IgM (Qiagen). Next, enriched CD4 T cells were labeled with biotinylated anti-CD62L and anti-biotin microbeads (Miltenyl). Labeled cells were passed through an AutoMACs, yielding purified naïve CD4 T cells (greater than 95 percent purity was typical). Splenic dendritic cells were enriched from B6 mice bearing B16 melanoma tumors secreting Flt3l by negative selection. In brief, splenocytes were labeled with the hybridoma supernatants anti-CD90 (Y19) and anti-NK1.1 (HB191). Cell suspensions were depleted of T cells, B cells, and NK cells by coupling labeled cells to magnetic beads bound to goat anti-rat IgG, goat anti-mouse IgG, and goat anti-mouse IgM. DCs were further enriched by positive selection using biotinylated anti-CD11c and anti-biotin microbeads, followed by passing through an AutoMACs twice. For in vitro priming, naive CD4 T cells were cultured with splenic dendritic cells at a 5:1 ratio for 5 days with 100 ng/ml LPS, 100 ng/ml Pam3CSK4, or 1 μM CpG and 3 ng/ml anti-CD3ε followed by assessment of cytokine secretion in culture supernatants by ELISA. In some experiments, T cells were co-cultured with DCs that had been irradiated with 15 Gy and the addition of exogenous cytokines. After 72 hours co-cultures using irradiated cells were pulsed with 0.5 μCi ^{3}H-thymidine (Perkin Elmer) for an additional 16 hours and thymidine uptake was monitored as a readout of cell proliferation using a MicroBeta liquid scintillation counter (Perkin Elmer).

In Vitro Polarization of Naïve CD4 T Cells into Th17 cells

Naïve T cells were purified as above and cultured at 300,000 cells per well of flat-bottom 96-well plates having been pre-coated for 4 hours at 37C with 50 μL of a PBS solution containing 5 μg/ml anti-CD3ε and 5 μg/ml anti-CD28. Naïve T cells were cultured with 10 μg/ml anti-IFNγ, 10 μg/ml anti-IL4, and 50 U/ml IL2 for the "basal" neutralized condition. To induce polarization to the Th17 lineage, 20 ng/ml IL6 and 5 ng/ml TGFβ was added to the neutralization cocktail. IL1β was used in some wells at 10 ng/ml. All cytokines were from Peprotech except IL2, which came from Biolegend. Neutralizing antibodies were from Biolegend. Cells were cultured for 96 hours at which point supernatants were saved for assessment of cytokine secretion by ELISA and cells were restimulated with PMA and Ionomycin in the presence of brefeldin A to assess intracellular cytokine secretion. Intracellular staining was performed using the Biolegend FoxP3 buffer set according to the manufactures protocol.

shRNA Knockdown

BCAP specific shRNA constructs were from Sigma Aldrich (clones used were: NM_031376.1-880s1c1, NM_031376.1-1464s1c1, NM_031376.1-1538s1c1 and NM_031376.1-1743s1c1) and Lentiviruses were packaged using 293T cells. RAW264.7 macrophages were infected with the viral particles and selected for resistance to 2 μg/ml puromycin. Efficiency of BCAP silencing was monitored by immunoblotting. Cells were

stimulated, unless indicated otherwise, for 20 hours with 100 ng/ml LPS, 100 ng/ml Pam3CSK4, or 1 μM CpG.

Transcriptome Analysis

BCAP-sufficient or BCAP-deficient macrophages were stimulated with 1 μM CpG (ODN1826) for 2 hours, 8 hours, or left unstimulated. After stimulation, RNA was extracted from the cells using the TRIzol method (Invitrogen) and cleaned up by passing through a Qiagen miRNeasy column. cDNA libraries were prepared from purified RNA (performed in the lab of Dr. Edward Wakeland by Ms. Chaoying Liang) and sequenced using the HiSeq 2000 system (Illumina). Data was assembled and quantified using CLC-Biosystems Genomic Workbench in the lab of Dr. Wakeland. Our collaborator, Dr. Igor Dozmorov, performed the differential gene expression analysis with required restrictions of minimal 2-fold change across all data sets and a RPKM expression values of 2 in at least one condition. The list of differentially expressed genes was subjected to hierarchical clustering using the Gene Pattern server (http://www.broadinstitute.org/cancer/software/genepattern/) (de Hoon et al., 2004; Eisen et al., 1998; Reich et al., 2006). Following clustering, data was displayed as a globally normalized heat map using Gene Pattern.

Vaccinations

Vaccine emulsions were prepared using Incomplete Freund's Adjuvant (Sigma Aldrich) diluted 1:1 in a solution of PBS containing 500 micrograms per milliliter ovalbumin and 50 micrograms per milliliter of *E. coli* LPS (Sigma Aldrich). Solution was emulsified using a stationary Dremel^{TM} tool immersed into the solution for one to two minutes at medium speed. Mice were vaccinated in the rear foot pads with 50 microliters of the above emulsion, yielding a targeted dose of 25 micrograms of ovalbumin and 2.5 micrograms of LPS per footpad. After 7 days, mice were humanely euthanized and draining popliteal and inguinal lymph nodes collected. CD4+ T cells were enriched by negative selection from single cell suspensions using mixtures of hybridoma supernatants and BioMag beads as described in detail above, purities for this procedure (based on flow cytometry staining for CD4) are expected to range from 85 to 95 percent. After enrichment, purified CD4 T cells were cultured at a 5:1 ration with TLR2/4 double-deficient dendritic cells and soluble ovalbumin (at the indicated concentrations) for 48 to 72 hours to assess the recall response. Where appropriate, culture supernatants were harvested prior to pulsing cultured cells with 0.5 micro Curie ^{3}H-thymidine and chased 16 hours later to assess incorporation as a measure of proliferation using a Perkin Elmer β-harvester.

CHAPTER THREE

Role for B-cell Adapter for PI3K as a Signaling Adapter Linking Toll-Like Receptors to Serine/Threonine Kinases PI3K/Akt

Part of the writing presented in this chapter was previously published as in *Proceedings of the National Academy of Sciences*, volume 109, issue 1, pages 273-278 (Troutman et al., 2012b).

Introduction

Toll-like receptors (TLRs) are a family of transmembrane receptors that sense the presence of evolutionarily conserved microbial motifs such as lipopolysaccharide, lipoproteins and nucleic acids (Takeuchi and Akira, 2010). Ligation of TLRs by their cognate ligands initiates a signaling cascade culminating in the production of pro-inflammatory mediators (Takeuchi and Akira, 2010). Intracellular adapters for TLRs critically rely on homotypic Toll-Interleukin-1 receptor (TIR) module interactions for signal propagation (O'Neill and Bowie, 2007). All known signaling adapters for TLRs use TIR domains for engaging their respective receptors. Upon recognition of ligands, TLRs recruit their adapters MyD88 and/or TRIF, which then activate downstream components of the signaling pathway, ultimately leading to activation of transcription factors such as NF-κB, AP-1, IRF-3, IRF-7, etc. (Takeuchi and

Akira, 2010). TLR activation in cells of the innate immune system plays a major role in host defense by contributing to enhanced phagocytosis (Blander and Medzhitov, 2004) and increased oxidative burst (West et al., 2011). TLR activation also leads to synthesis and secretion of pro-inflammatory cytokines and chemokines (Kawai and Akira, 2010). These cytokines and chemokines play a major role in recruiting additional cells to the site of infection as well as in shaping the nature of adaptive immune responses.

Uncontrolled activation of the TLR signaling pathway leads to damaging inflammation as seen in sepsis and chronic autoimmune diseases. Furthermore, chronic exposure to endogenous TLR ligands is implicated in the pathogenesis of atherosclerosis (Moore and Tabas, 2011) and susceptibility to tumor metastasis (Kim and Karin, 2011; Kim et al., 2009). Many regulatory checks have evolved to counter-balance the potentially damaging consequences of TLR ligation. Regulation of TLRs occurs at several levels in the signaling pathway and is mediated by numerous proteins, including; SIGIRR, A20, IRAK-M, and TANK (Boone et al., 2004; Kawagoe et al., 2009; Kobayashi et al., 2002; Turer et al., 2008; Wald et al., 2003). Further, TLRs can be counter-regulated by secreted factors including IL-10, agonists of TAM receptors, and type I interferon, all of which can be induced by TLR signaling (Lee and Kim, 2007; O'Neill, 2008b; Rothlin et al., 2007).

PI3K are a family of serine/threonine kinases that phosphorylate variants of PIP2, creating variants of PIP3, ultimately leading to activation of the downstream kinases, PDK1 and AKT (also called PKB) (Katso et al., 2001). The PI3K pathway plays a major role in the immune system, including promoting cell survival, proliferation and protein synthesis (Katso

et al., 2001). TLR signaling, in addition to promoting activation of transcription factors including NF-κB, MAP kinases, and IRFs, also leads to activation of PI3K (Hazeki et al., 2007; Ruse and Knaus, 2006), however, its role is less well understood (Hazeki et al., 2007). Examinations of the role of the PI3K pathway in TLR signaling have utilized chemical inhibitors of PI3K, as well as genetic modification and mRNA silencing. Studies using chemical inhibitors (i.e. wortmannin and/or LY294.002) have inconclusively demonstrated that TLR mediated PI3K activation can be either pro- or anti-inflammatory, potentially due to differences in cell-types or off-target affects of the inhibitor. Growing genetic evidence, however, suggests that the PI3K pathway is involved in limiting the inflammatory response by TLRs (Androulidaki et al., 2009; Fukao et al., 2002; Hazeki et al., 2007).

We explored the possibility that uncharacterized TIR domains existed within the human genome and undertook a comprehensive computational screen to "predict" the existence of novel TIR-domain containing proteins. This approach suggested the existence of a conserved TIR folds at the amino terminal ends of the B cell adapter for PI3K (BCAP, also known as PIK3AP1) and its paralog BANK1. BCAP has previously been demonstrated to serve as a signaling adapter linking the BCR and CD19 to activation of PI3K (Inabe and Kurosaki, 2002; Okada et al., 2000). Here, we ascribe a novel role to BCAP in linking TLRs to PI3K through a previously unknown TIR domain. We show that BCAP regulates activation of NF-κB dependent on its TIR domain and associates with the TLR signaling adapters MyD88 and TIRAP. In addition, BCAP is critically required for TLR mediated activation of PI3K/Akt. Thus, we have identified a novel role for BCAP in regulation of

inflammatory responses through its role as a proximal TIR domain-containing adapter in the TLR signaling pathway.

Results

BCAP contains a novel and functional amino-terminal TIR domain

Using sensitive fold recognition methods (Bazan and de Sauvage, 2009), we screened the human genome for novel TIR-domain proteins and discovered that BCAP, a protein previously reported to be downstream of B cell receptor signaling (Okada et al., 2000; Yamazaki et al., 2002), has a cryptic TIR domain in its N-terminal region (Figure 3-1). In order to functionally confirm the presence of a novel TIR domain in BCAP, we cloned mutants by truncating the C-terminus (referred to here as BCAP-TIR) or by truncating the N-terminus (referred to as BCAPΔTIR) (Figure 3-2). In reporter assays, TLR adapter proteins, such as MyD88 and TIRAP, minimally truncated to only their respective TIR modules and lacking their relevant activation domains, act as dominant negative repressors of NF-κB reporters downstream of TLRs and TLR signaling adapters (Fitzgerald et al., 2001; Horng and Medzhitov, 2001; Yamamoto et al., 2002b). Using NF-κB reporter assays, we confirmed that the TIR domain of BCAP, just like the TIR domains of MyD88 and TIRAP (Horng et al., 2001; Horng and Medzhitov, 2001), represses activation of a NF-κB reporter downstream of a constitutively active TLR4 (CD4/TLR4) (Medzhitov et al., 1997), MyD88 and TIRAP (Figure 3-3). Importantly, in contrast to the other known TLR signaling adapters,

overexpression of BCAP did not lead to activation of NF-κB (Figure 3-4). Therefore, we tested whether full length BCAP synergizes with other TLR signaling adapters to induce NF-κB activation but surprisingly found that full length BCAP represses CD4/TLR4- and TIRAP-mediated activation of NF-κB (Figure 3-5). Similarly, full length BCAP represses activation of NF-κB through CpG dependent activation of TLR9 (Figure 3-6). BCAPΔTIR does not have the ability to inhibit NF-κB activation induced by TLR signaling adapters, suggesting that the inhibitory activity of BCAP is dependent on the presence of its previously uncharacterized TIR domain (Figure 3-7).

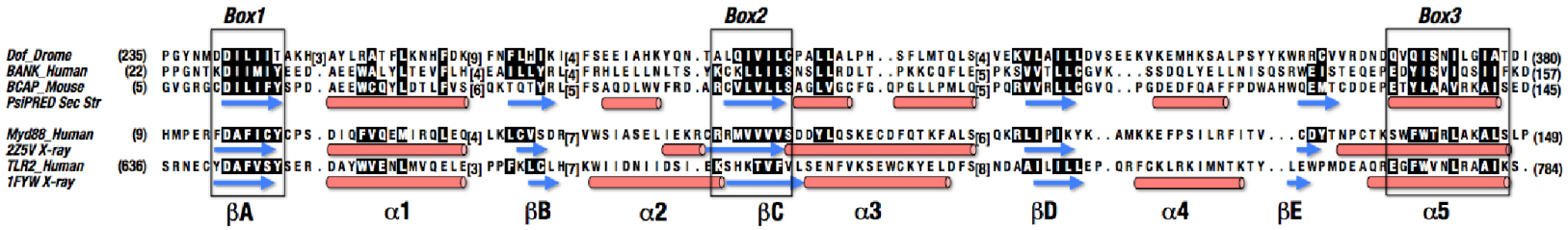

Figure 3-1. BCAP contains a cryptic amino-terminal TIR domain

Modular architecture of BCAP and TIR domain relationships. The mouse BCAP TIR domain is aligned to a second human paralog, BANK, and its Drosophila ortholog, Dof (Battersby et al., 2003), and in turn, superposed using HHPRED (Soding, 2005) on the human Myd88 and TLR2 TIR domain structures (PDB IDs 2Z5V and 1FYW, respectively), highlighting the register of PsiPRED (McGuffin et al., 2000) and X-ray-defined α-helices (red cylinders) and β-strands (blue arrows) that comprise the conserved TIR fold (Rock et al., 1998); conserved residues are noted by reverse lettering and Box 1-3 regions are diagnostic of TIR domains (Rock et al., 1998). The figure and figure legend were created by J. Fernando Bazan, Ph.D. Identification, alignment and analysis performed by J. Fernando Bazan, Ph.D.

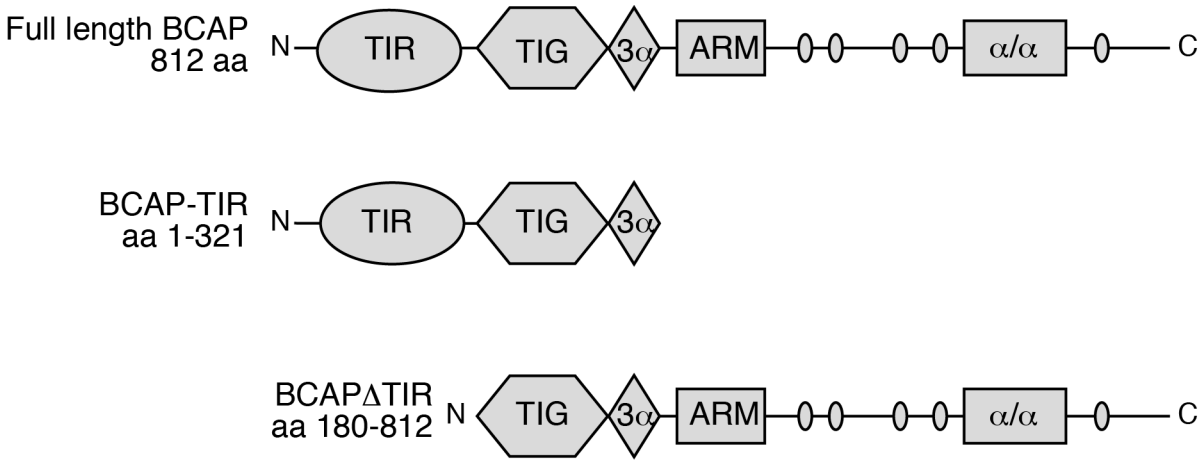

Figure 3-2. Truncated constructs of BCAP

Diagrammatic representation of BCAP clones used in this study.

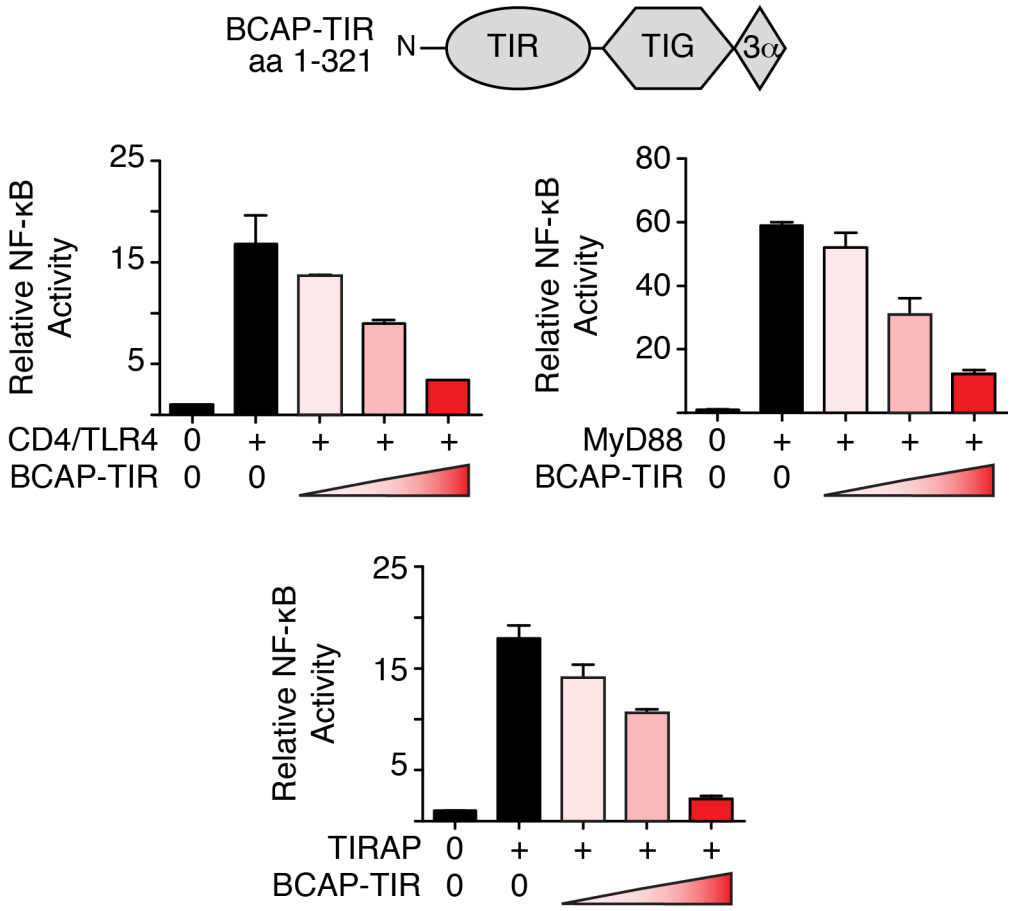

Figure 3-3. BCAP-TIR represses NF-κB reporter activity

NF-κB reporter assay using 293T cells co-transfected with the indicated constructs, a NF-κB luciferase reporter, and a Renilla luciferase reporter as an internal control. Reporter activity was quantified in cellular lysates the next day by assessment of firefly luciferase activity relative to Renilla luciferase activity.

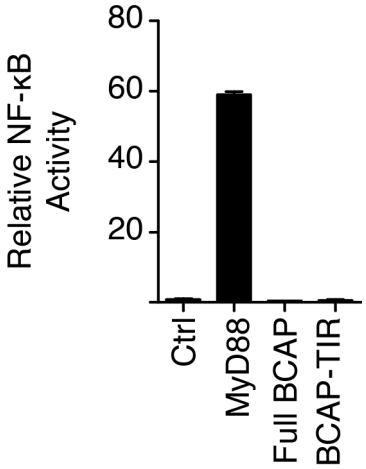

Figure 3-4. Full-length BCAP does not lead to NF-κB reporter activation

NF-κB reporter assay using 293T cells co-transfected with the indicated constructs, a NF-κB luciferase reporter, and a Renilla luciferase reporter as an internal control. Reporter activity was quantified in cellular lysates the next day by assessment of firefly luciferase activity relative to Renilla luciferase activity.

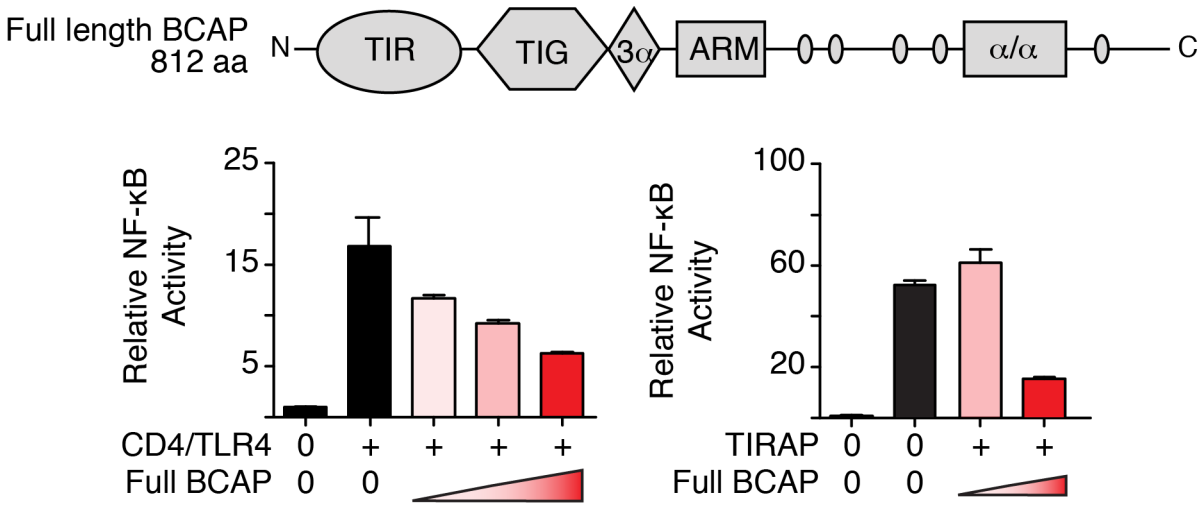

Figure 3-5. Full-length BCAP represses NF-κB reporter activation in the TLR pathway

NF-κB reporter assay using 293T cells co-transfected with the indicated constructs, a NF-κB luciferase reporter, and a Renilla luciferase reporter as an internal control. Reporter activity was quantified in cellular lysates the next day by assessment of firefly luciferase activity relative to Renilla luciferase activity.

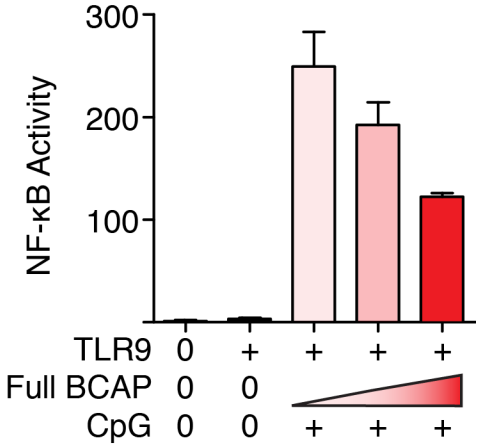

Figure 3-6. BCAP represses CpG-dependent activation of NF-κB by TLR9

NF-κB reporter assay using 293T cells co-transfected with the indicated constructs, a NF-κB luciferase reporter, and a Renilla luciferase reporter as an internal control. After 18 hours of transfection, cells were stimulated with 1 μM CpG as indicated and reporter activity was quantified in cellular lysates the next day by assessment of firefly luciferase activity relative to Renilla luciferase activity.

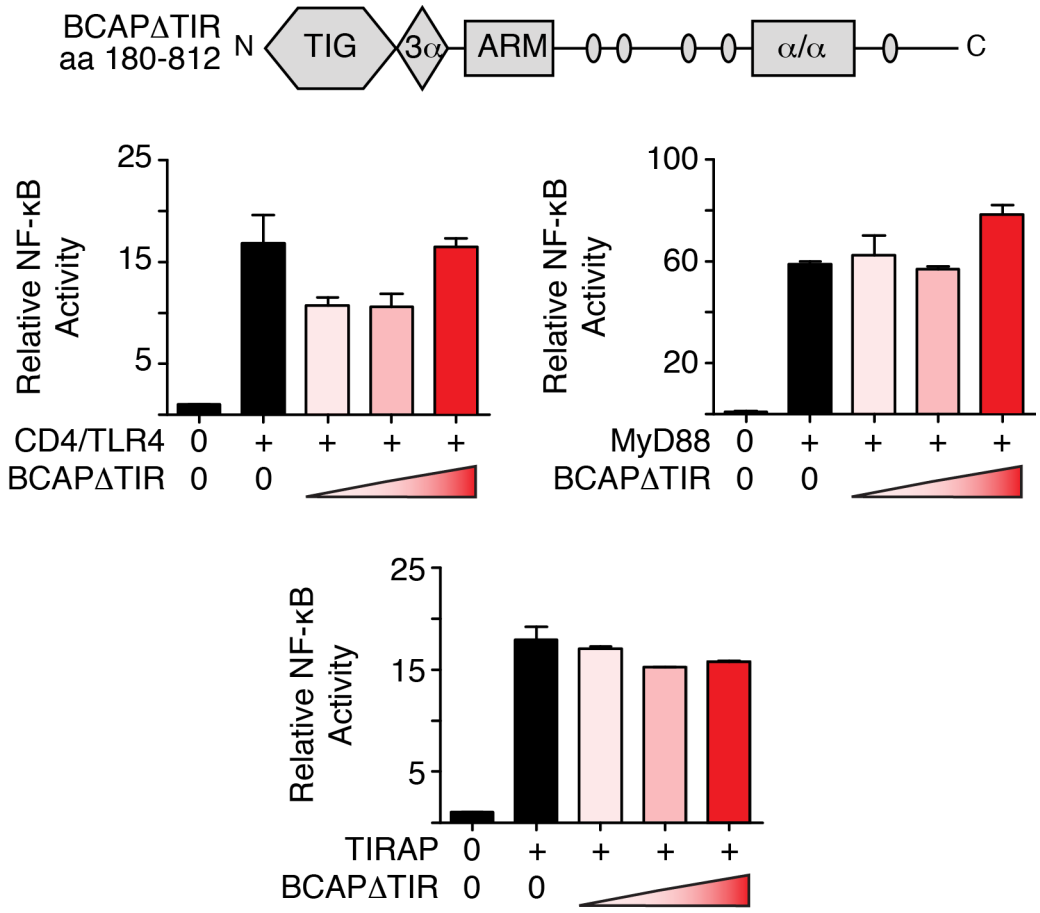

Figure 3-7. Repression of NF-κB reporter activity by BCAP is mediated through the TIR domain

NF-κB reporter assay using 293T cells co-transfected with the indicated constructs, a NF-κB luciferase reporter, and a Renilla luciferase reporter as an internal control. Reporter activity was quantified in cellular lysates the next day by assessment of firefly luciferase activity relative to Renilla luciferase activity.

BCAP associates with TLR adapters

Because activation of TLRs depends on homotypic interaction of the cytosolic TIR domains of TLRs and the TIR domains of TLR signaling adapters, we tested whether epitope tagged BCAP mutants can associate with components of the TLR signaling pathway. These experiments demonstrate that BCAP-TIR, but not BCAPΔTIR, associates with MyD88 (Figure 3-8) and TIRAP (Figure 3-9 and Figure 3-10) suggesting that the TIR domain of BCAP can associate with TLR signaling adapters. Furthermore, these experiments demonstrate the requirement of the TIR domain of BCAP for mediating its participation in the immune complex. Similarly, overexpression studies also suggest that full-length BCAP can associate with the TLR signaling adapters MyD88 and TIRAP (Figure 3-11). This experiment also demonstrates that in the absence of transient expression of BCAP in the 293T cells, a detectable immune complex is still formed with the endogenous level of BCAP expressed by these cells. However, if full-length BCAP contains an amino-terminal epitope tag, formation of an immune complex with HA-TIRAP (Figure 3-10), HA-MyD88 (data not shown), or GFP-MyD88 (data not shown) was undetectable, suggesting that a critical interaction epitope is being masked by the presence of the Flag-tag. Interestingly, co-expression of full-length BCAP reproducibly led to a decreased expression of MyD88 or TIRAP (Figure 3-10 and data not shown). The reason for this reproducible observation

remains unknown, yet was found to not be mediated by ubiquitin mediated degradation (data no shown).

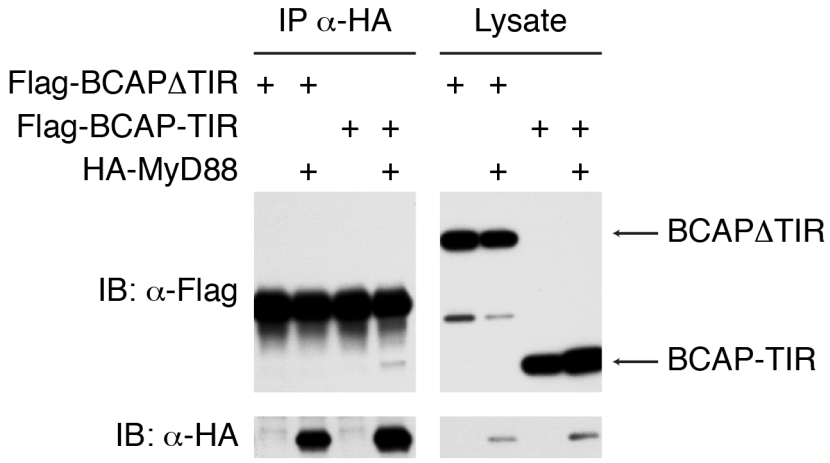

Figure 3-8. The TIR domain of BCAP is both necessary and sufficient for forming an immune complex with MyD88

293T cells were transiently transfected with the indicated constructs, or the relevant empty vectors for 36 hours followed by subjecting cellular lysates to immunoprecipitation with anti-HA. Precipitates containing HA-tagged MyD88 were assessed for co-association of Flag-tagged BCAP mutants as indicated. BCAP negatively regulates TLR responses in macrophages.

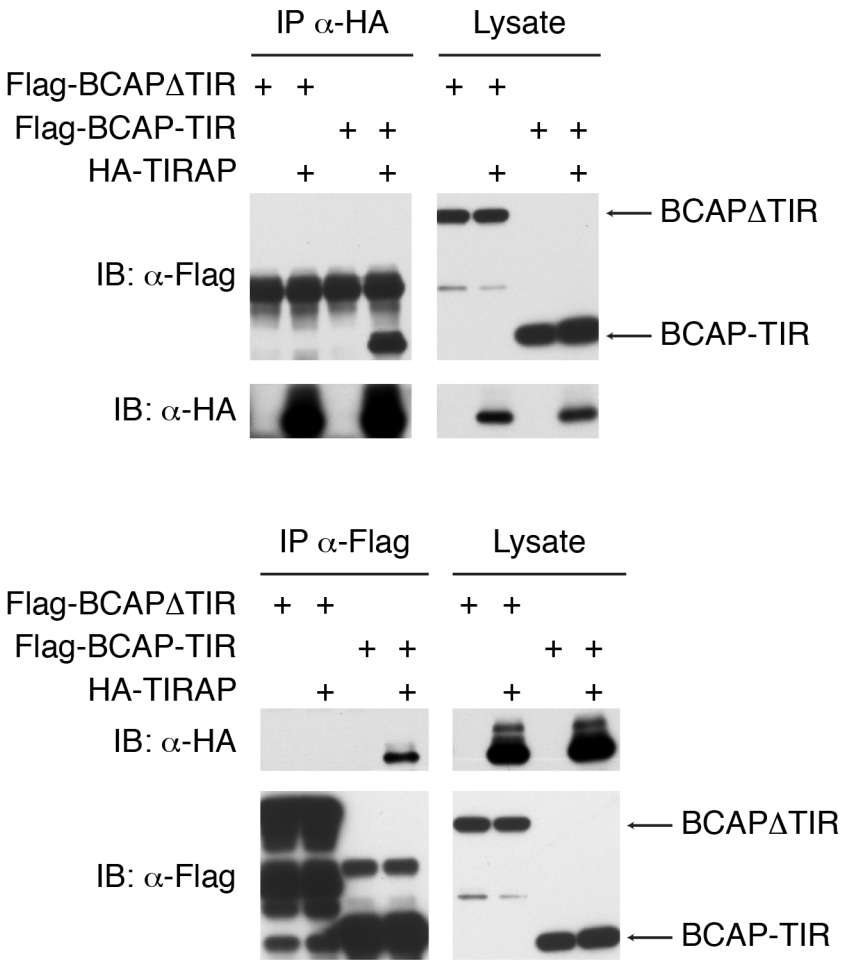

Figure 3-9. The TIR domain of BCAP is both necessary and sufficient for forming an immune complex with TIRAP

293T cells were transiently transfected with the indicated constructs, or the relevant empty vectors for 36 hours followed by subjecting cellular lysates to immunoprecipitation with anti-HA (top). Precipitates containing HA-tagged TIRAP were assessed for co-association of Flag-tagged BCAP mutants as indicated. The reciprocal immunoprecipitation experiment was performed by precipitating with (bottom) anti-Flag followed by assessment for co-precipitation of HA-tagged TIRAP.

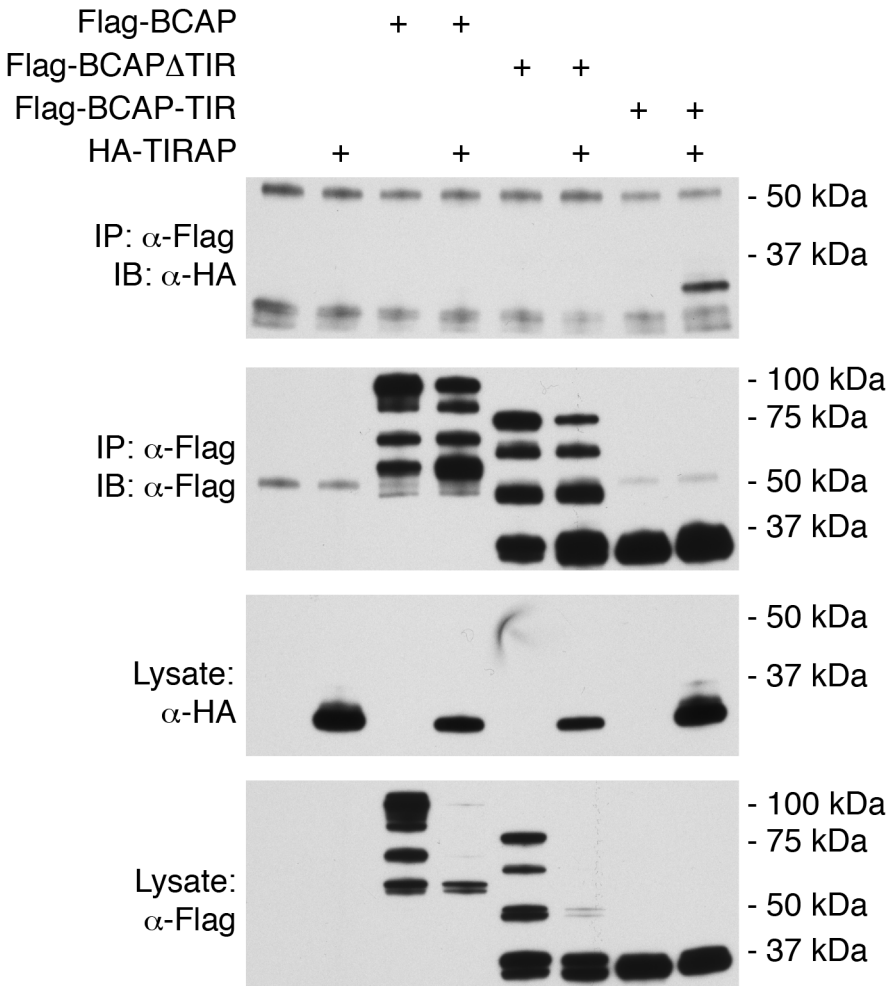

Figure 3-10. The TIR domain of BCAP is both necessary and sufficient for forming an immune complex with TIRAP

293T cells were transiently transfected with the indicated constructs, or the relevant empty vectors for 36 hours followed by subjecting cellular lysates to immunoprecipitation with anti-Flag. Precipitates containing Flag-tagged BCAP or BCAP mutants were assessed for co-association of HA-tagged TIRAP as indicated. This experiment demonstrated the inability of full-length Flag-tagged BCAP to form detectable immune complexes with HA-tagged TIRAP, perhaps due to epitope masking. This experiment further clearly demonstrates multiple post-translational cleavage products of BCAP generated by transient expression of the clone.

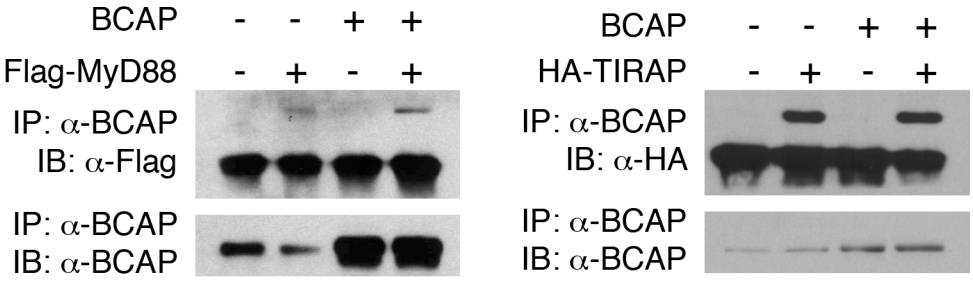

Figure 3-11. Full-length BCAP associates with TLR adapters

293T cells were transiently transfected with the indicated constructs, or the relevant empty vectors for 36 hours followed by subjecting cellular lysates to immunoprecipitation with polyclonal goat anti-BCAP (R&D). Precipitates containing were assessed for co-association of Flag-tagged MyD88 (left) or HA-tagged TIRAP (right) as indicated. Association of TLR adapters with full-length BCAP is not masked in the absence of the Flag-epitope tag encoded at the amino-terminal end of BCAP.

BCAP negatively regulates TLR responses in macrophages

BCAP is expressed in B cells, macrophages and NK cells (MacFarlane et al., 2008; Okada et al., 2000). In B cells, BCAP undergoes tyrosine phosphorylation upon stimulation of the B cell receptor or CD19 (Inabe and Kurosaki, 2002; Okada et al., 2000). To investigate whether BCAP is phosphorylated following TLR activation, we assayed the phosphorylation status of BCAP in TLR stimulated B cells and macrophages. We found that BCAP undergoes a rapid and transient phosphorylation in both RAW264.7 macrophages and primary murine B cells upon stimulation with TLR ligands (data not shown). To further examine the function of BCAP in TLR signaling, we created *BCAP* deficient RAW264.7 macrophage cells by using shRNA mediated gene silencing. Several shRNA clones induced varying amounts of *BCAP* silencing and one particular clone (880) had no detectable expression of BCAP protein (Figure 3-12). We stimulated the *BCAP*-silenced RAW264.7 cells and the relevant controls using ligands for TLR4, TLR2 and TLR9. These experiments revealed that *BCAP*-silenced RAW macrophages, in comparison to control RAW macrophages, secreted higher quantities of IL-6 in response to TLR ligands (Figure 3-13). *BCAP*-silenced RAW macrophages are sensitive to activation by TLR ligands at doses where control RAW macrophages fail to secrete detectable IL-6 (Figure 3-14). Consistent with *BCAP*-silenced cells, bone marrow derived macrophages (BMDM) derived from BCAP deficient mice made significantly higher quantities of the pro-inflammatory cytokines IL-6, IL-12 (Figure 3-15, Figure 3-16, and Figure 3-17) and TNF-α (Figure 3-18) in response to TLR ligands. Thioglycollate induced peritoneal macrophages from BCAP deficient mice, when compared to cells from WT mice,

also secreted significantly higher levels of IL-6 in response to TLR stimulation (Figure 3-19). Importantly, BCAP expression and function by human macrophages was evolutionarily conserved because human THP-1 macrophages silenced for *BCAP* secreted increase TNF in response to LPS stimulation (Figure 3-20).

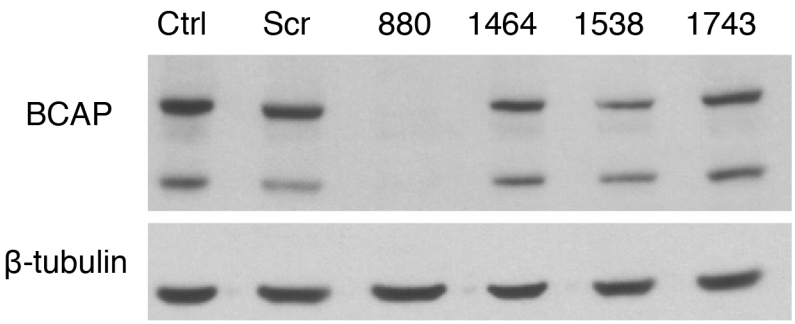

Figure 3-12. Efficiency of *BCAP* silencing in the RAW264.7 macrophage cell line

BCAP was silenced in the RAW264.7 macrophage cell line using shRNA and efficiency was monitored by immunoblotting. Ctrl; untransduced control cells. Scr; scrambled sequence. 880, 1464, 1538, and 1743; shRNA silencing at the corresponding nucleic acid positions of *BCAP*.

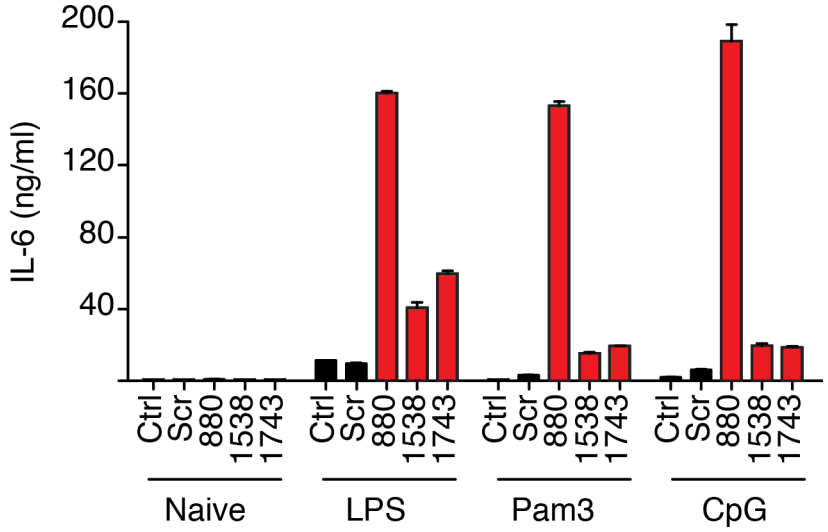

Figure 3-13. *BCAP*-silenced RAW264.7 cells secrete increased IL6 in response to TLR stimulation

BCAP silenced RAW264.7 cells were stimulated for 20 hours with 100 ng/ml LPS, 100 ng/ml Pam3CSK4 (Pam3), or 1 μM CpG and secreted IL-6 was quantified by ELISA. Data represent four independent experiments.

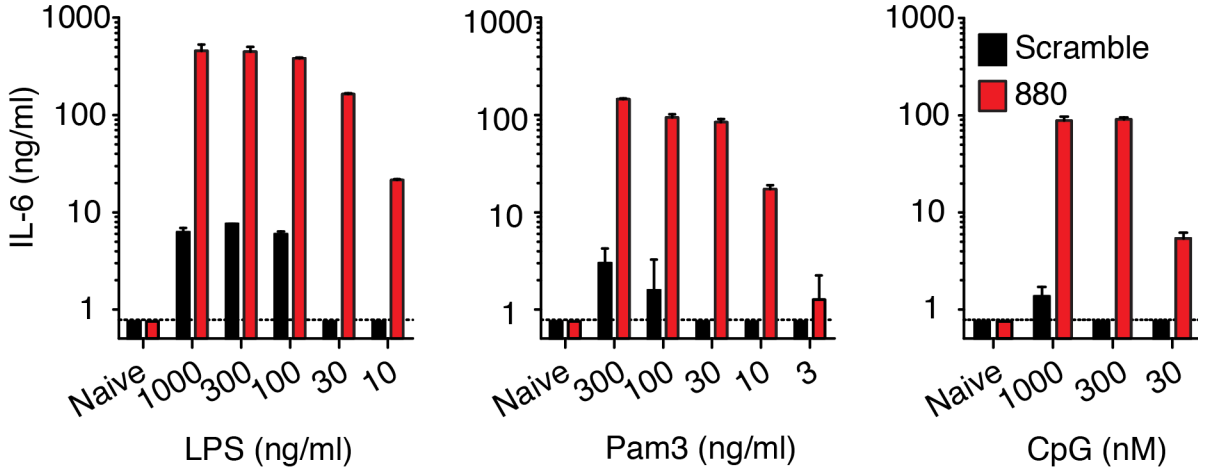

Figure 3-14. *BCAP*-silenced RAW264.7 cells have an increased IL6 secretion response to titration of TLR ligands

BCAP silenced RAW264.7 cells were stimulated for 20 hours with LPS, Pam3CSK4 (Pam3), or CpG at the indicated concentrations and secreted IL-6 was quantified by ELISA.

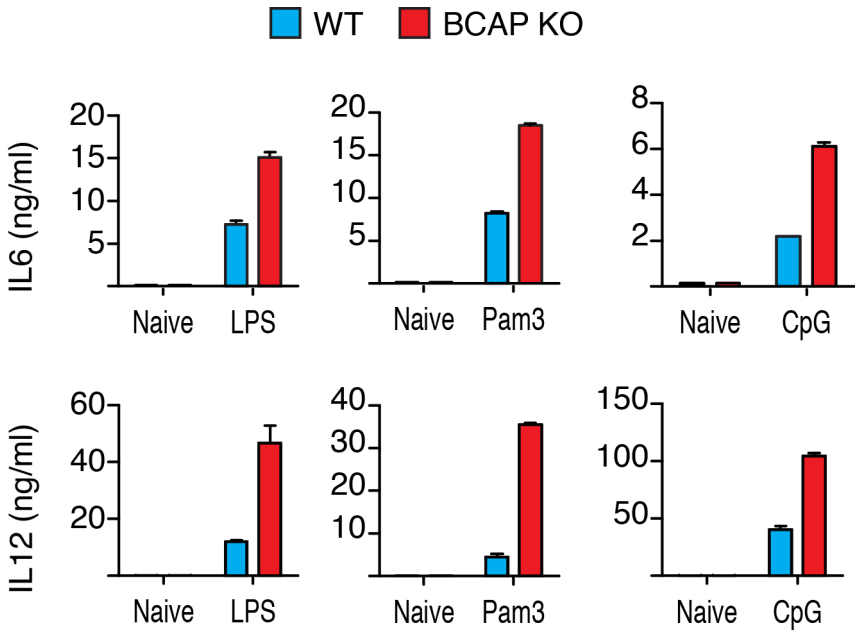

Figure 3-15. BCAP-deficient BMDMs secrete more IL6 and IL12 in response to TLR stimulation

BMDMs from WT or BCAP KO mice were stimulated for 20 hours with 100 ng/ml LPS, 100 ng/ml Pam3CSK4 (Pam3), or 1 μM CpG and secreted IL-6 or IL-12 p40/p70 was analyzed by ELISA. Data represent three or more independent experiments and are presented as the mean ± s.d.

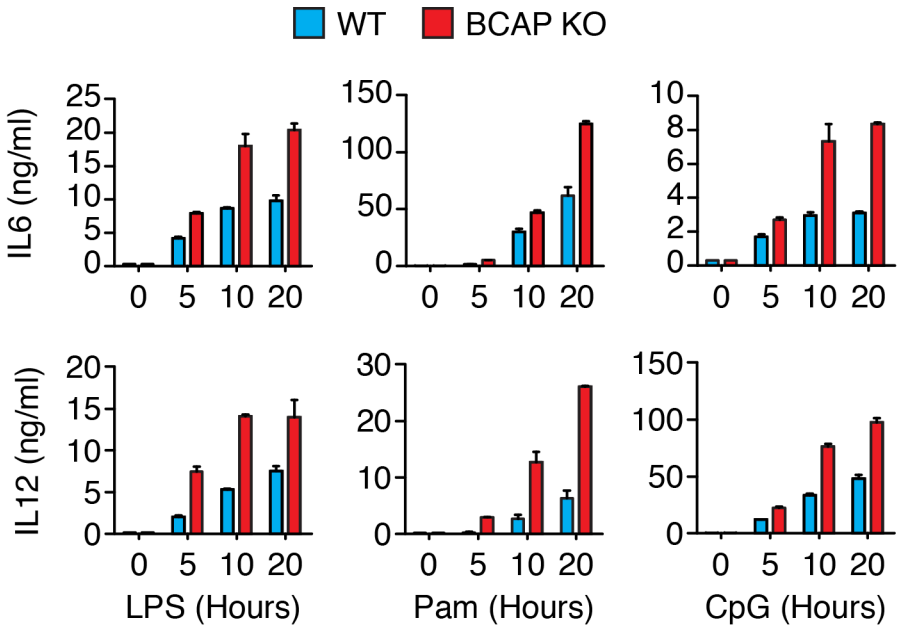

Figure 3-16. BCAP-deficient BMDMs secrete more IL6 and IL12 in response to a temporal TLR stimulation

BMDMs from WT or BCAP KO mice were stimulated for the indicated times with 100 ng/ml LPS, 100 ng/ml Pam3CSK4 (Pam3), or 1 μM CpG and secreted IL-6 or IL-12 p40/p70 was analyzed by ELISA. Data represent three or more independent experiments and are presented as the mean ± s.d.

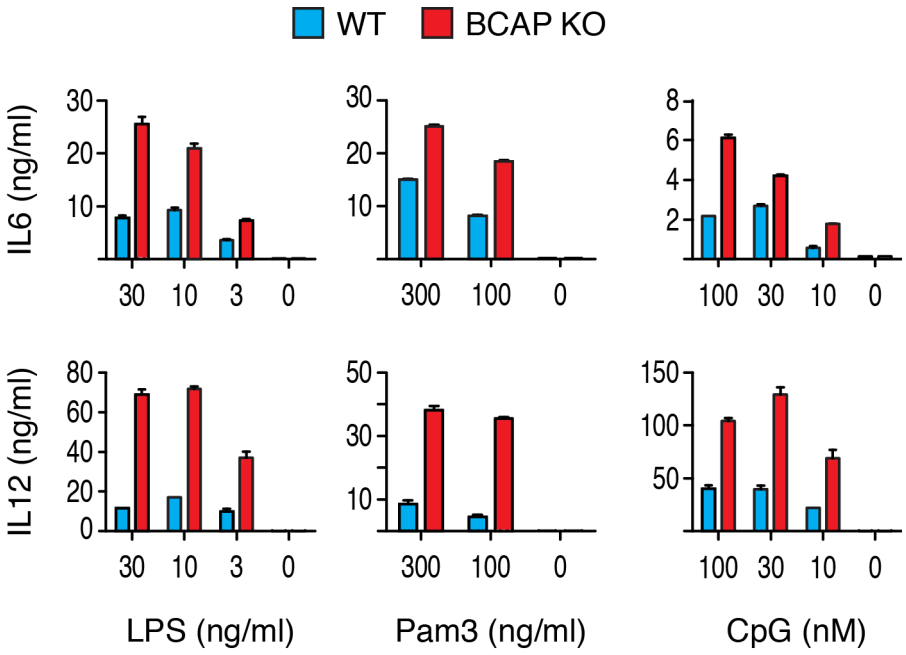

Figure 3-17. BCAP-deficient BMDMs secrete more IL6 and IL12 in response to a dose-response TLR stimulation

BMDMs from WT or BCAP KO mice were stimulated for 20 hours with LPS, Pam3CSK4 (Pam3), or CpG at the indicated concentrations and secreted IL-6 or IL-12 p40/p70 was analyzed by ELISA. Data represent three or more independent experiments and are presented as the mean ± s.d.

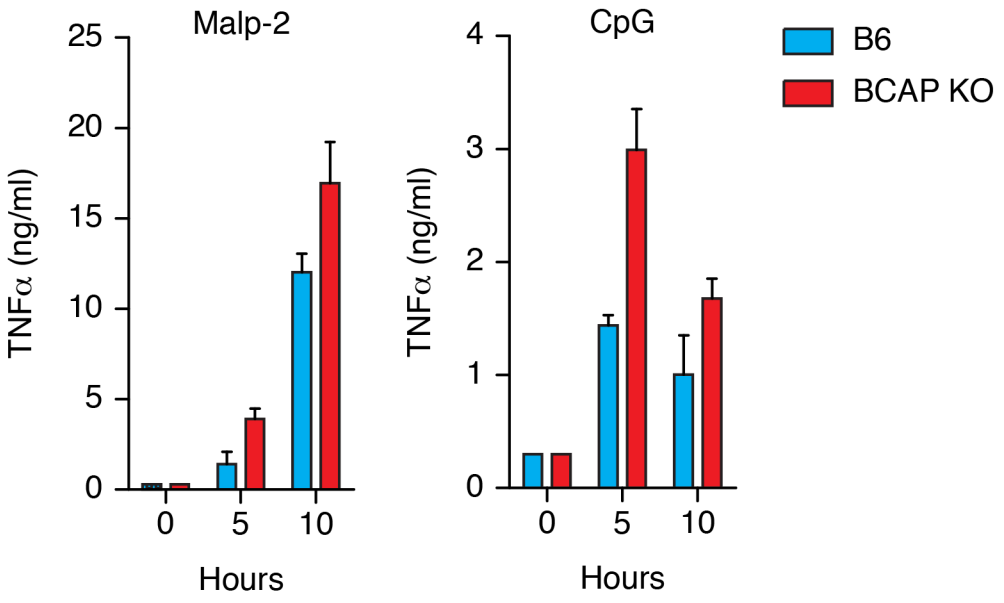

Figure 3-18. BCAP-deficient BMDMs secrete more TNFα in response to TLR stimulation

BMDMs were stimulated as indicated with 100 ng/ml MALP-2 or 1 μM CpG as indicated. Secreted TNFα was quantified by ELISA.

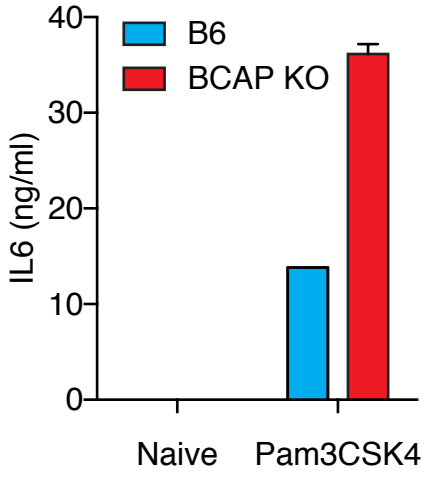

Figure 3-19. BCAP-deficient thioglycollate-elicited peritoneal macrophages secrete more IL6 in response to TLR stimulation

Thioglycollate-elicited peritoneal macrophages were isolated from mice and treated with 100 ng/ml PAM3CSK4 overnight, followed by assessment of secreted IL6 in the culture supernatant by ELISA.

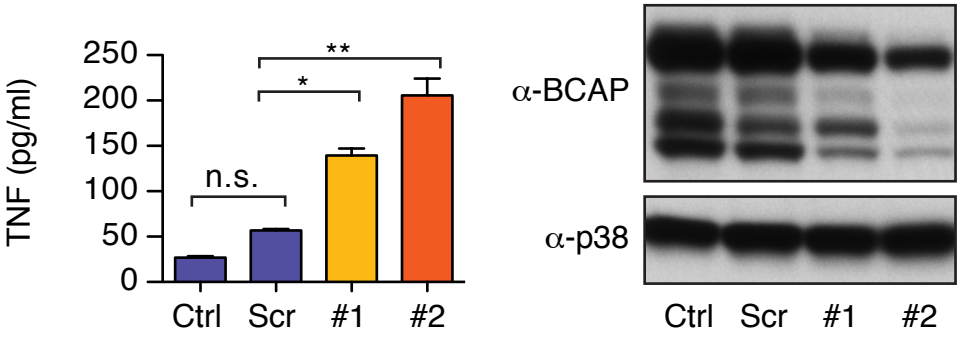

Figure 3-20. *BCAP*-silenced THP-1 human macrophages secrete increased TNF in response to LPS stimulation

BCAP silenced THP-1 cells were differentiated for 24-hours with 50 micrograms/milliliter PMA to differentiate cells into macrophages. THP-1 macrophages were then washed and stimulated for 20 hours with 300 ng/ml LPS and culture supernatants were assayed for secreted TNF by ELISA. Bars denote mean +/- SEM from biological replicates. * denotes $p < 0.05$ and ** denotes $p < 0.01$ as determined by one-way ANOVA and Bonferonni post test. (Right panel) BCAP silenced or control THP-1 cells were assayed for silencing efficiency by immunoblotting. Lysates were run on a SDS-PAGE gel followed by immunoblotting with anti-BCAP antibody or anti-p38 antibody as a loading control. Ctrl, unmanipulated cells; Scr, scramble transduced cells; #1, BCAP shRNA construct number 769; #2, BCAP shRNA construct number 1028.

BCAP does not regulate the TLR inflammatory response through NF-κB or MAP kinase signaling, nor through regulation of signals involved in tolerance

The above data clearly indicated that BCAP functions to regulate the inflammatory cytokine response in cells stimulated with TLR ligands. Several characterized TLR regulators have also been found to play critical roles in the ability of cells to become refractory towards a second stimulation. This response is termed TLR tolerance (Kawai and Akira, 2006; Kobayashi et al., 2002). However, when BMDMs from BCAP deficient mice were tested for a response to secondary stimulation by TLR ligands, they refractory, as were WT BMDMs, suggesting that BCAP is dispensable for the induction of TLR tolerance in macrophages (Figure 3-21). Likewise, BCAP deficient BMDMs had an equivalent capacity to induce message levels of *Irak3* and *Tnfaip3*, the genes encoding IRAK-M and A20 (Figure 3-22). Examination of NF-κB and MAP kinase activation upon TLR stimulation of BCAP silenced macrophages or BCAP deficient BMDMs demonstrated early modest enhancements in phosphorylation of IκBα and sustained activation of, JNK and ERK, when compared to control cells (Figure 3-23 and Figure 3-24). Together, these data suggests that BCAP is important for regulating the initial burst of pro-inflammatory cytokine production, in a manner independent of A20, and induction of other negative regulators, including IRAK-M, might then contribute to tolerance towards secondary stimulation by TLR ligands.

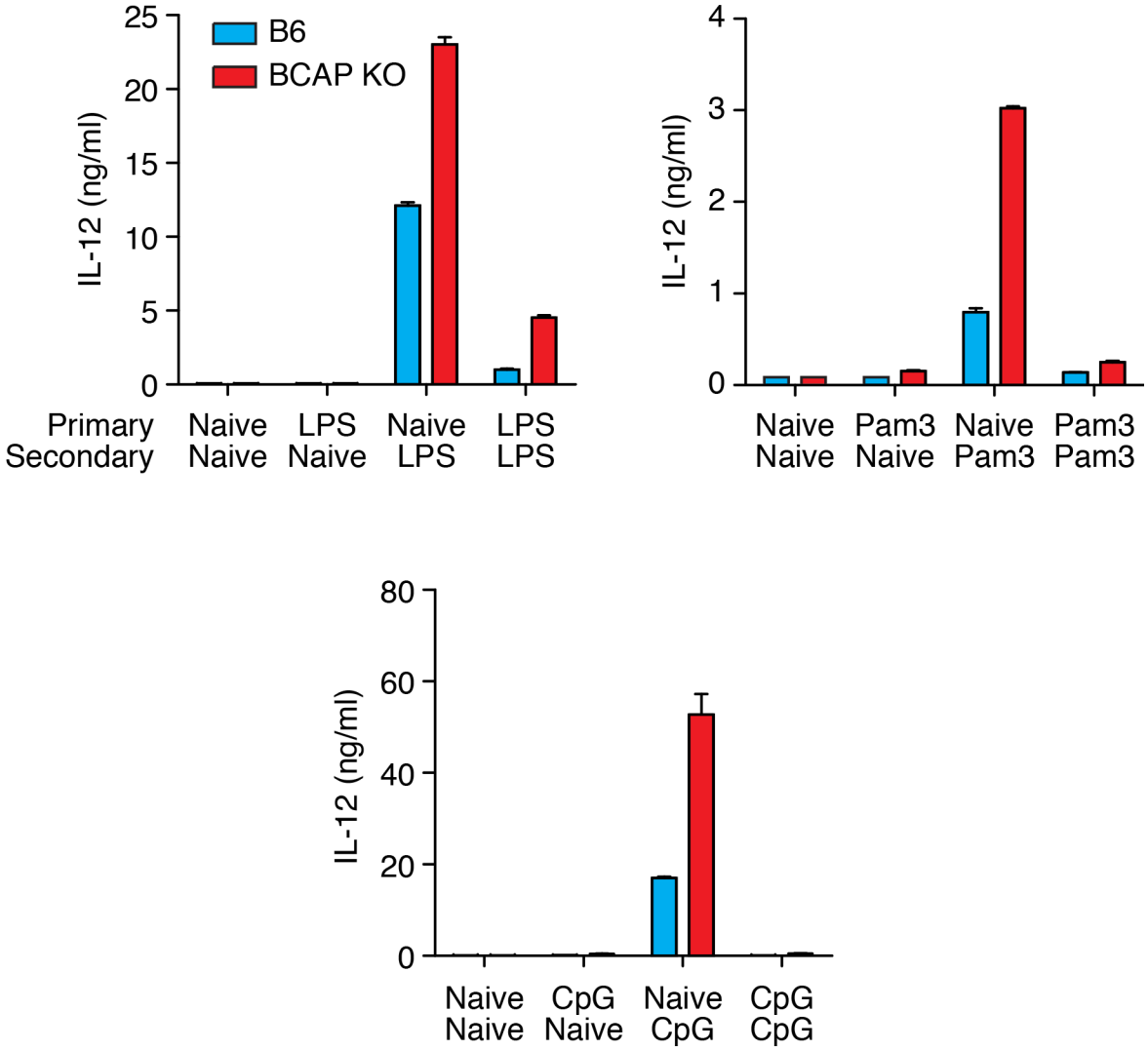

Figure 3-21. BCAP-deficient BMDMS are refractory to secondary TLR stimulation

BMDMs were stimulated for 24 hours with 100 ng/ml LPS, 100 ng/ml Pam3CSK4 (Pam3), or 1 μM CpG, washed twice with PBS, and re-stimulated for 20 hrs. with LPS, Pam3CSK4, or CpG as indicated. Secreted IL-12 p40/p70 after secondary stimulation was quantified by ELISA. Data represent three independent experiments and are presented as the mean ± s.d.

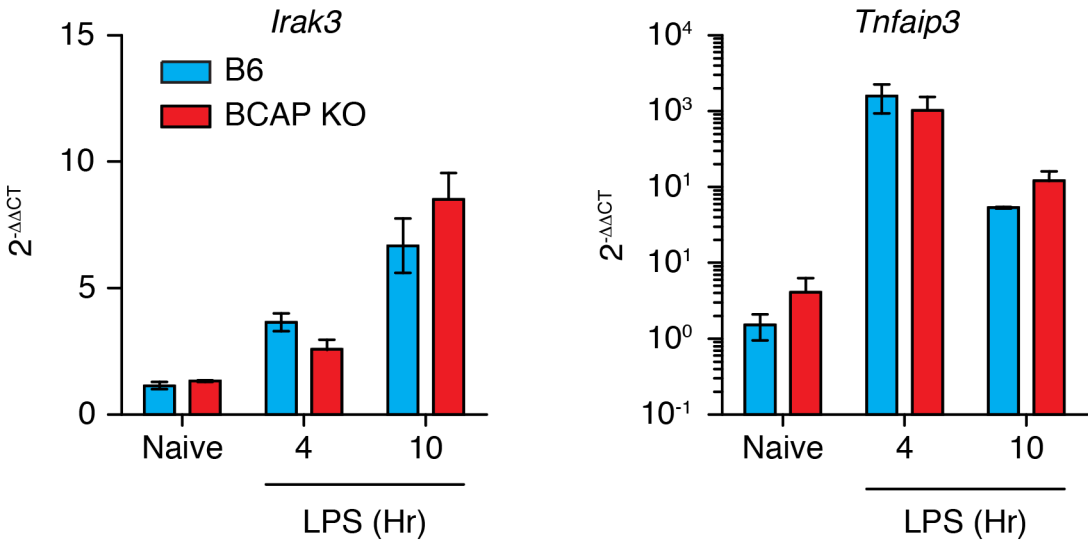

Figure 3-22. BCAP-deficient BMDMs induce normal levels of *Irak3* and *Tnfaip3* upon LPS stimulation

Expression of *Irak3* or *Tnfaip3* relative to *Actin* as measured by quantitative rtPCR on cDNA from BMDM stimulated as indicated with 100 ng/ml LPS. Data represent the mean ± s.d. of samples from macrophage preparations of two independent mice.

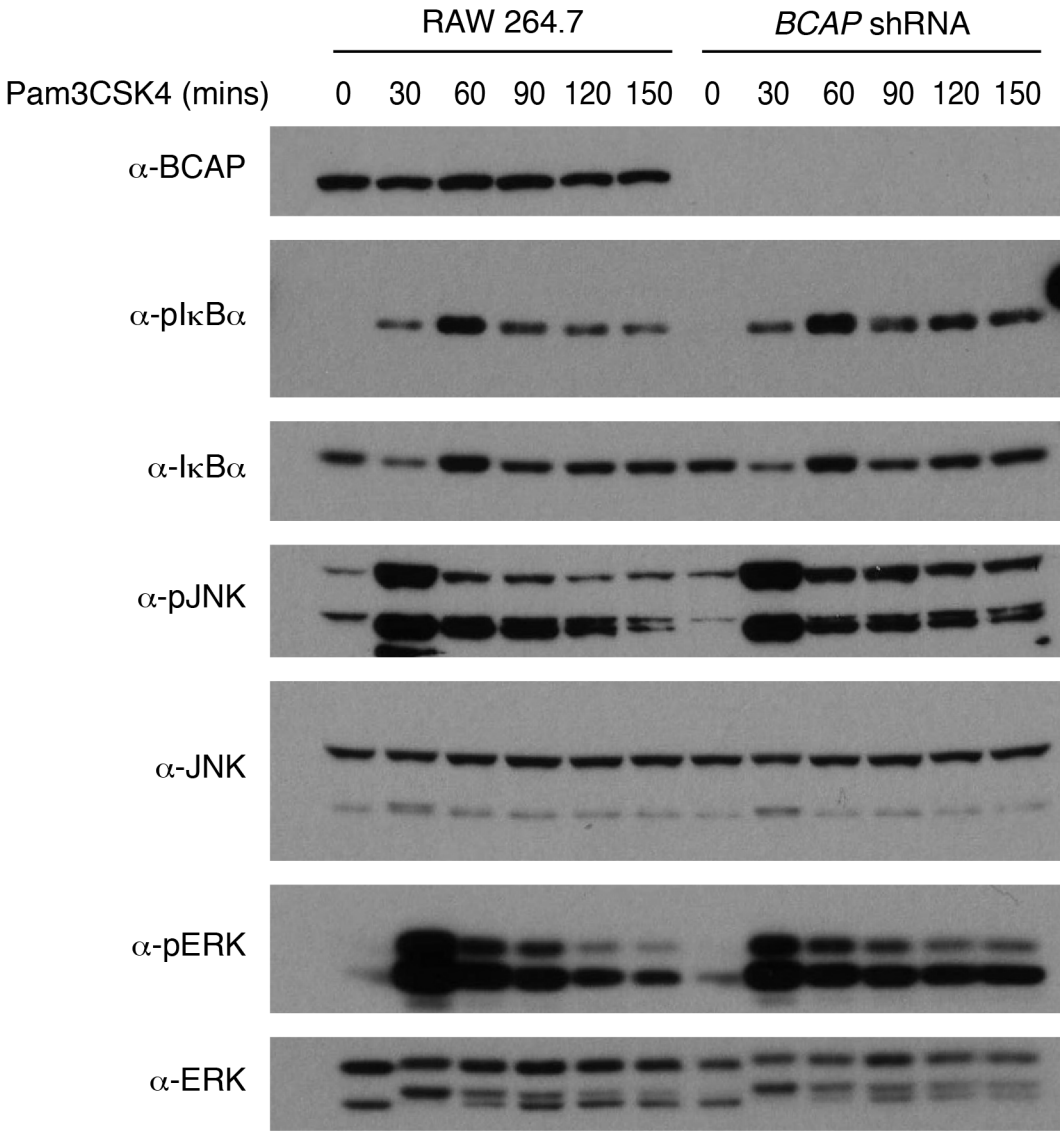

Figure 3-23. *BCAP*-silenced RAW264.7 cells have marginal changes in signaling to IκBα, JNK, and ERK during TLR stimulation

Control and BCAP silenced RAW264.7 cells were stimulated with 100 ng/ml Pam3CSK4 and cell lysates were immunoblotted for phosphorylation of IκBα, JNK, and ERK.

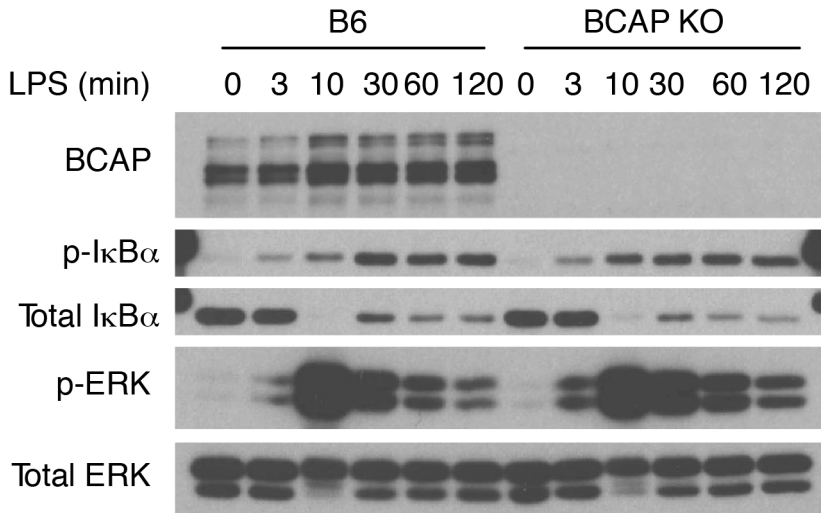

Figure 3-24. BCAP-deficient BMDMs have marginal changes in signaling to IκBα, and ERK during TLR stimulation

Lysates from B6 or BCAP KO BMDMs stimulated with 100 ng/ml LPS as indicated and immunoblotted for phosphorylation of IκBα or ERK.

BCAP links TLRs to PI3K/Akt

Since BCAP has previously been demonstrated to play a role in activating PI3K downstream of the B cell receptor (Okada et al., 2000), we hypothesized that BCAP may also play a role in PI3K activation upon TLR stimulation. There are contradictory reports regarding the role of PI3K in activation of NF-κB, following TLR activation(Hazeki et al., 2007; Ruse and Knaus, 2006). Pharmacological drugs that inhibit PI3K implicate the PI3K pathway in both enhancing and inhibiting TLR mediated NF-κB and MAP kinase activation (Hazeki et al., 2007; Ruse and Knaus, 2006). Genetic evidence, however, suggests that PI3K activation is involved in dampening the pro-inflammatory profile of both macrophages and dendritic cells (Androulidaki et al., 2009; Fukao et al., 2002; Schabbauer et al., 2010). To investigate if BCAP links TLR signaling and PI3K activation, we examined the phosphorylation status of the downstream kinase, Akt following activation of TLR2, TLR4 and TLR9. TLR activation induced phosphorylation of Akt at serine 473 in WT cells, (Figure 3-25 and Figure 3-26), however, BCAP deficient cells had decreased phosphorylation of Akt upon activation of TLR4 and TLR9 (Figure 3-25). The defect in Akt phosphorylation by TLR stimulated BCAP deficient cells was further enhanced during serum deprivation (Figure 3-26).

BCAP contains several C-terminal tyrosine residues known to be phosphorylated upon stimulation of B cells through the BCR (Inabe and Kurosaki, 2002; Okada et al., 2000). In macrophages, we found a non-reproducible phosphorylation of BCAP that is some experiments demonstrated constitutive phosphorylation of BCAP, while in others

demonstrated a TLR ligand dependent tyrosine phosphorylation of BCAP (data not shown). While in my hands, BCAP phosphorylation in macrophages was evident, the dependence of TLR signals to induce phosphorylation is unclear. However, another research group published that in macrophages, TLR signals are required to induce tyrosine phosphorylation of BCAP (Ni et al., 2012).

Activation of PI3K proceeds when the Src homology 2 (SH2) domain of the p85 subunit is recruited to phosphorylated tyrosine motifs (Vanhaesebroeck et al., 2010). Since BCAP is able to undergo tyrosine phosphorylation, we proposed that PI3K may be recruited to BCAP during TLR stimulation. Indeed, we found that stimulation of BMDMs with LPS led to enhanced co-precipitation of PI3K-p85α with BCAP (Figure 3-27). This suggests that upon TLR stimulation, BCAP undergoes tyrosine phosphorylation which leads to the recruitment of PI3K to BCAP. Upon engagement with tyrosine-phosphorylated BCAP, the p85 subunit of PI3K releases the p110 subunit of PI3K, which is in proximity with the cellular membrane and its substrate.

Analysis of MyD88 deficient macrophages demonstrated that activation of Akt also depends on MyD88 (Figure 3-28), in agreement with previous reports. Furthermore, just as seen with BCAP, LPS stimulation induces formation of an immune complex containing MyD88 and PI3K-p85α (Figure 3-29). Activation of BMDMs using TNF-α led to comparable phosphorylation of Akt in both WT and BCAP deficient cells suggesting that BCAP plays a specific role in TLR mediated activation of Akt (Figure 3-30). These data

clearly demonstrate that BCAP participates in TLR signaling, potentially downstream of MyD88, and is a critical link between TLR signaling and activation of the PI3K-Akt pathway.

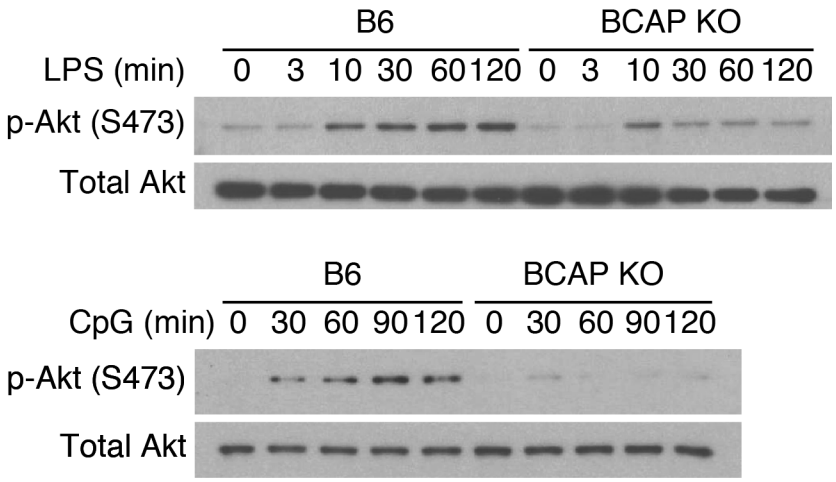

Figure 3-25. BCAP-deficient BMDMs are defective for activation of Akt in response to TLR stimulation

Lysates from B6 or BCAP KO BMDMs stimulated with 100 ng/ml LPS or 1 μM CpG as indicated, were analyzed for S473 phosphorylation of Akt by immunoblotting.

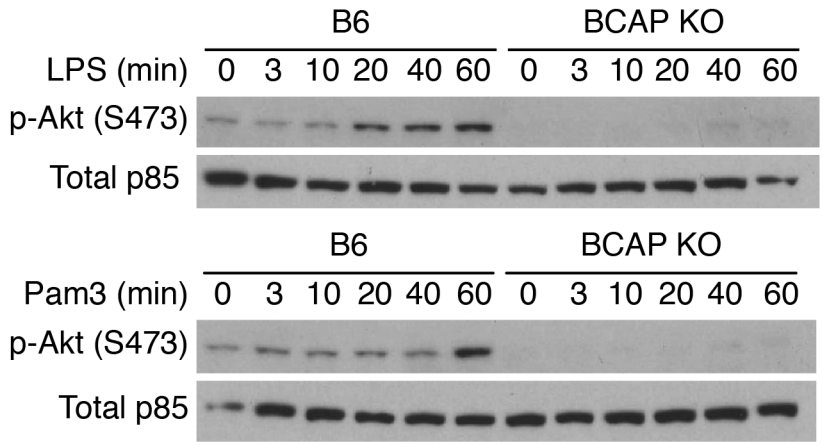

Figure 3-26. Serum deprivation further augments the dependency of BMDMs on BCAP for activation of Akt upon TLR stimulation

BMDMs from B6 or BCAP KO BMDMs were serum deprived for 4 hours with media containing 1% FCS. BMDM were then stimulated using 100 ng/ml LPS or 100 ng/ml Pam3CSK4 as indicated and lysates subjected to immunoblotting to detect phosphorylation of Akt.

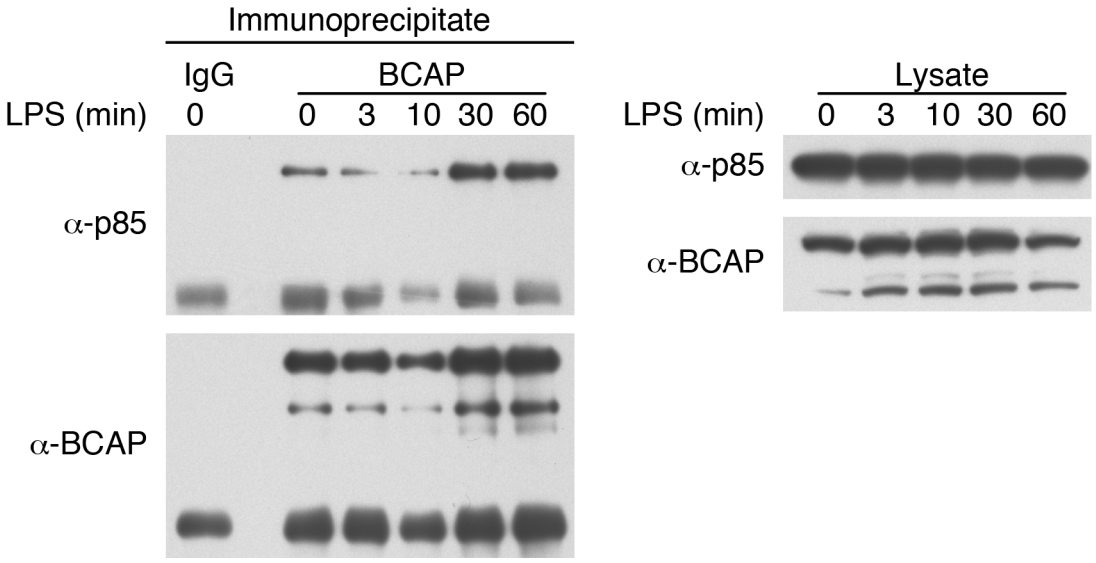

Figure 3-27. LPS stimulation leads to recruitment of the p85α subunit of PI3K to BCAP

B6 BMDMs were stimulated as indicated with 100 ng/ml LPS and lysed. Cleared lysates were subjected to immunoprecipitation with goat polyclonal anti-BCAP antibody. Precipitates were probed by immunoblotting for co-precipitation of p85α.

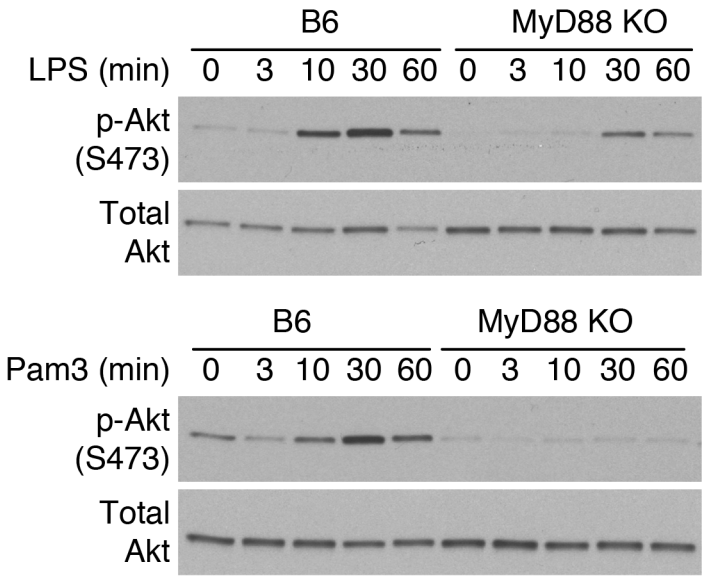

Figure 3-28. Activation of Akt by BMDMs depends on MyD88, suggesting that BCAP is dependent upon MyD88 derived signaling for mediating PI3K activation

B6 or MyD88 KO BMDMs were stimulated using 100 ng/ml LPS or 100 ng/ml Pam3CSK4 as indicated and lysates subjected to immunoblotting. Data represent three independent experiments.

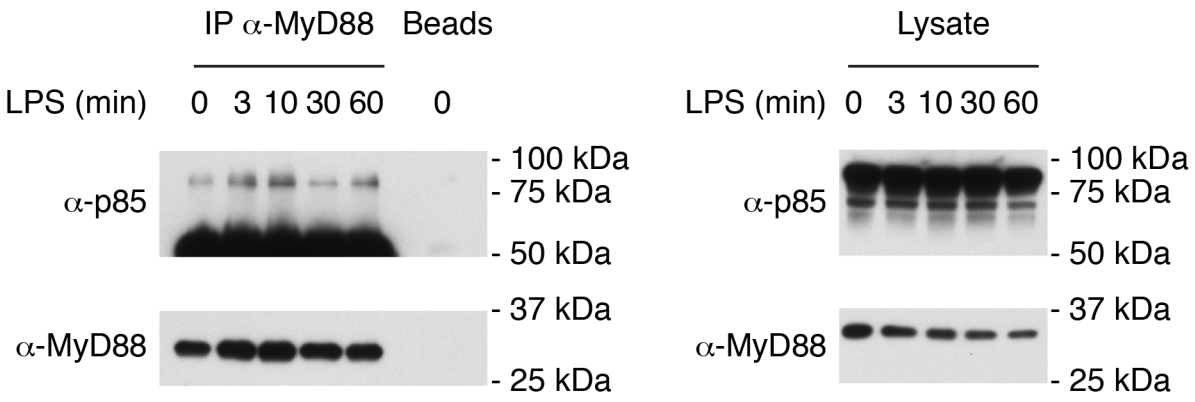

Figure 3-29. LPS stimulation leads to the recruitment of the p85α subunit of PI3K to MyD88

B6 BMDMs were stimulated as indicated with 100 ng/ml LPS and lysed. Cleared lysates were subjected to immunoprecipitation with goat polyclonal anti-MyD88 antibody (R&D). Precipitates were probed by immunoblotting for co-precipitation of p85α.

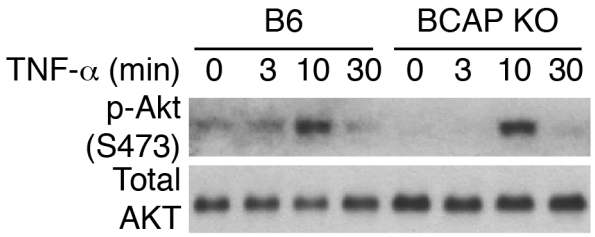

Figure 3-30. Phosphorylation of Akt in response to TNF stimulation does not require BCAP

B6 or BCAP KO BMDMs were stimulated using 10 ng/ml of mTNF-α for the indicated periods of time. Lysates were analyzed for phosphorylation of Akt. As in other experiments with TLR ligands, WT BMDMs have constitutively phosphorylated Akt at the 0- and 3-minute time points.

Transcriptional profiling of BCAP-deficient cells

To more completely reveal the manner of control BCAP imparts upon cells stimulated with TLR ligands, we compared the transcriptome of BCAP-sufficient and BCAP-deficient macrophages left unstimulated or stimulated for two or eight hours in the presence of 1 μM CpG. This approach revealed a large group of genes utilizing BCAP-derived signals to control gene expression in macrophages (Figure 3-31). Importantly, *Pik3ap* (the gene encoding BCAP) was one of the most significantly lowest expressed genes in BCAP-deficient cells (Figure 3-33) while genes neighboring BCAP (Figure 1-2) were not found to be differentially regulated in BCAP-deficient cells.

These findings suggested that important cellular processes discriminately controlled by BCAP mediated signaling had a critical role in regulation of the inflammatory response by macrophages. By using Ingenuity Pathway Analysis as well as combined usage of the DAVID Bioinformatics Resource and the KEGG PATHWAY database, we discovered a significant role for BCAP in regulation of several biological pathways. In confirmation of our previous data, BCAP-deficient cells had increased RNA levels for many inflammatory cytokines, including *Il6*, *Il12a*, *IL1b*, and *Il1a* (Figure 3-32 and Figure 3-34). We also discovered other gene networks that could play dominant roles in BCAP-mediated regulation of inflammation and colitis susceptibility. One network included a group of CXC chemokines that were overrepresented upon TLR stimulation of BCAP-deficient macrophages (Figure 3-32 and Figure 3-34).

BCAP-deficient cells were also defective for induction of dual specificity phosphatases (DUSPs), transcriptionally regulated phosphatases that block signal transduction of MAP kinases (Caunt and Keyse, 2012). Abundant literature provides evidence for regulatory roles of DUSP family members in suppressing the inflammatory response mediated by TLR stimulation (Caunt and Keyse, 2012; Jeffrey et al., 2007). A regulatory circuit composed of PI3K mediated Dusp1 induction, leading to increased production of IL10 has previously been described, and supports our proposed model (Gunzl et al., 2010). Importantly, we found that BCAP controls expression of several Dusp family members, including *Dusp1*, *Dusp4*, and *Dusp7* (Figure 3-35 and Figure 3-37) as well as *Il10* a finding we were unable to confirm by assessment of secreted IL10 (Figure 3-33) in culture supernatants of TLR stimulated macrophages (not shown).

A third transcriptional program found to be regulated by BCAP contained genes enriched in the PPARγ pathway, including *Pparg*, *Olr1*, *Nr1h3*, and *Slc27a1* (Figure 3-35 and Figure 3-36). Importantly, PPARγ has been previously linked to control of inflammation, and mice deficient in PPARγ are susceptible to diabetes and atherosclerosis (Ogawa et al., 2005; Olefsky and Glass, 2010). Expression of chemokines and chemokine receptors has also been linked to the activity of PPARγ in macrophages (Nguyen et al., 2012), suggesting an interconnecting linkage of BCAP regulated transcriptional programs.

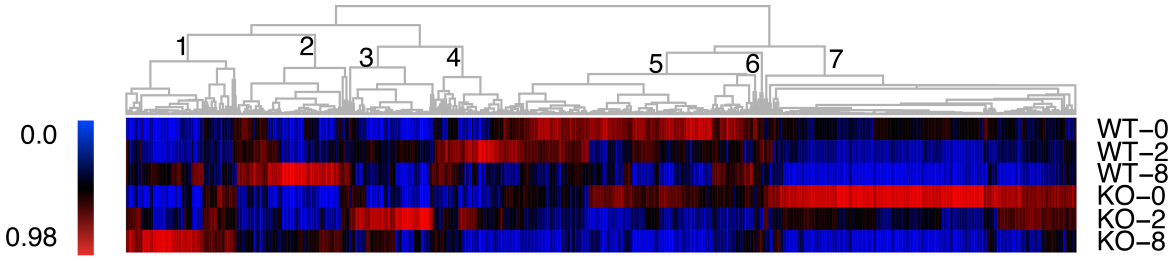

Figure 3-31. Transcriptional profiling of TLR9 stimulated BCAP-sufficient and BCAP-deficient macrophages

BCAP-sufficient (WT) or BCAP-deficient (KO) macrophages were stimulated with 1 μM CpG (ODN 1826) for 2 hours, 8 hours, or left unstimulated, as indicated. Genes found to be regulated differentially between WT or KO macrophages were normalized individually for each gene by multiplication of individual RPKM values by a scale factor S so that the sum of the squares of the values for each gene is 1.0. Normalized data was then subjected to hierarchical clustering by Pearson correlation. The clustered data is presented as a heat map using the indicated scale intensity. Cluster numbers above the heat map are presented in a zoomed in scale in the following figures. Data normalization and normalization, as well as heat map generation, was performed using GenePattern (de Hoon et al., 2004; Eisen et al., 1998; Reich et al., 2006).

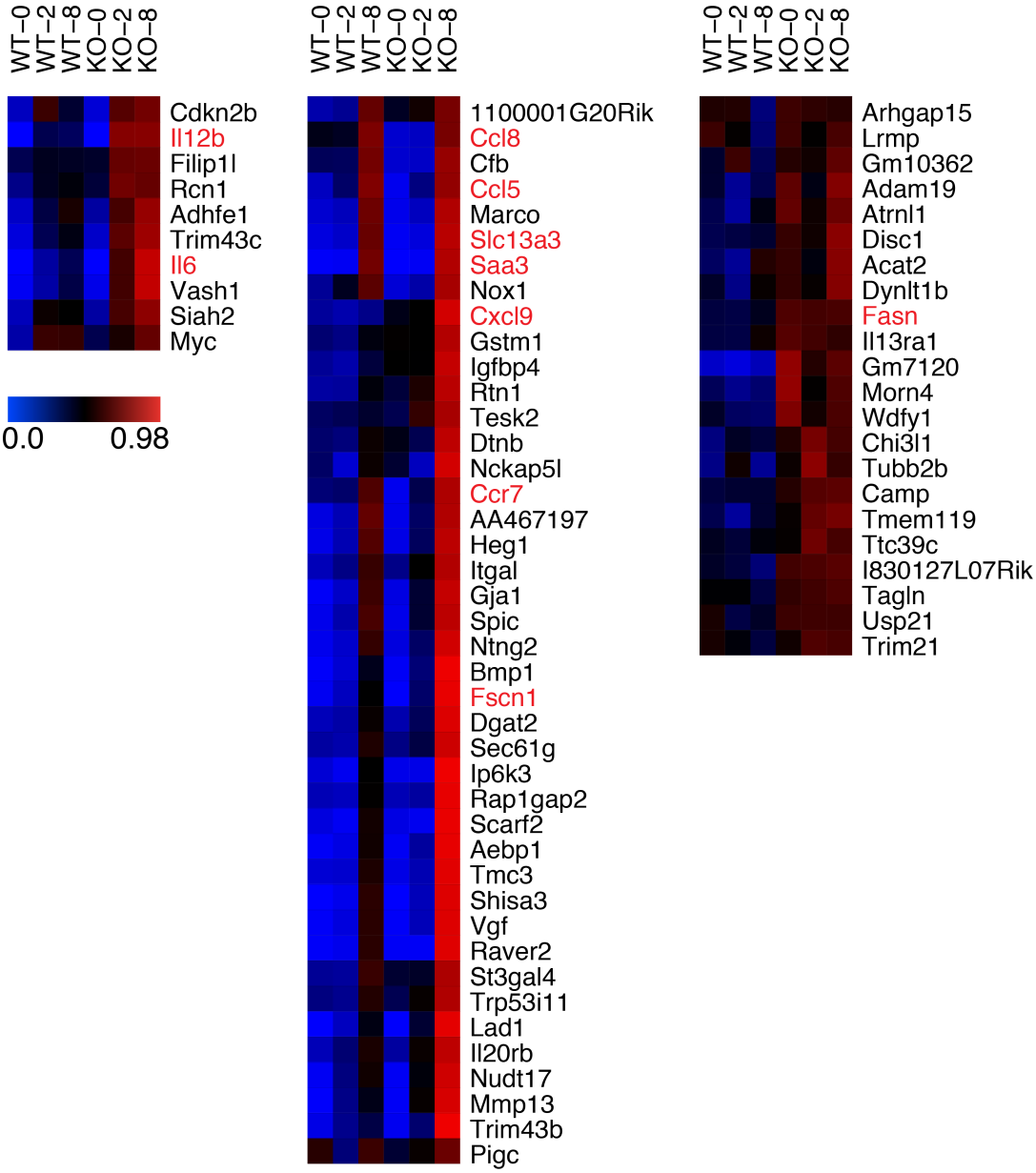

Figure 3-32. Genes in cluster 1

BCAP-sufficient (WT) or BCAP-deficient (KO) macrophages were stimulated with 1 μM CpG (ODN 1826) for 2 hours, 8 hours, or left unstimulated, as indicated. Genes of interest are highlighted in red.

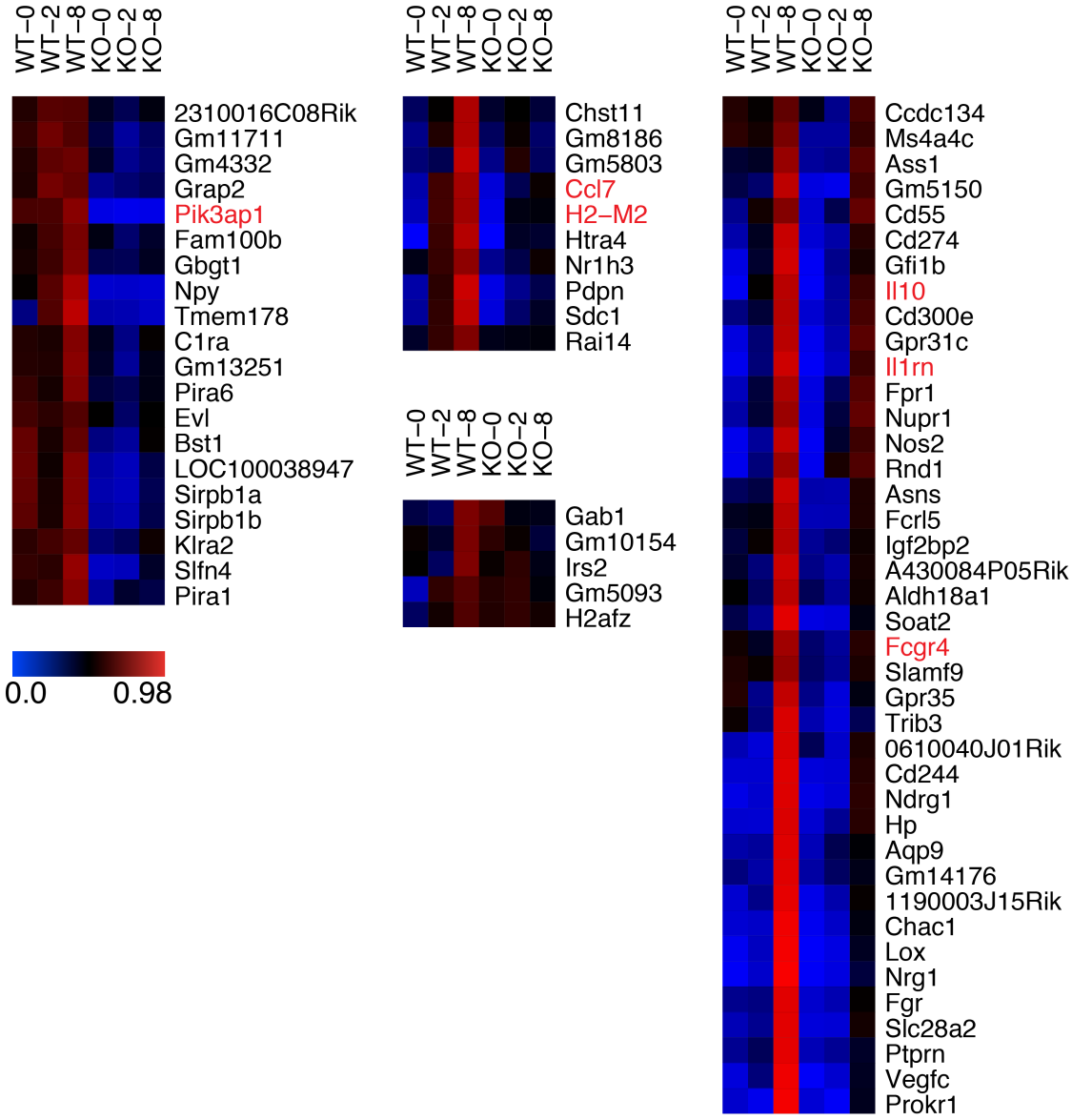

Figure 3-33. Genes in cluster 2

BCAP-sufficient (WT) or BCAP-deficient (KO) macrophages were stimulated with 1 μM CpG (ODN 1826) for 2 hours, 8 hours, or left unstimulated, as indicated. Genes of interest are highlighted in red.

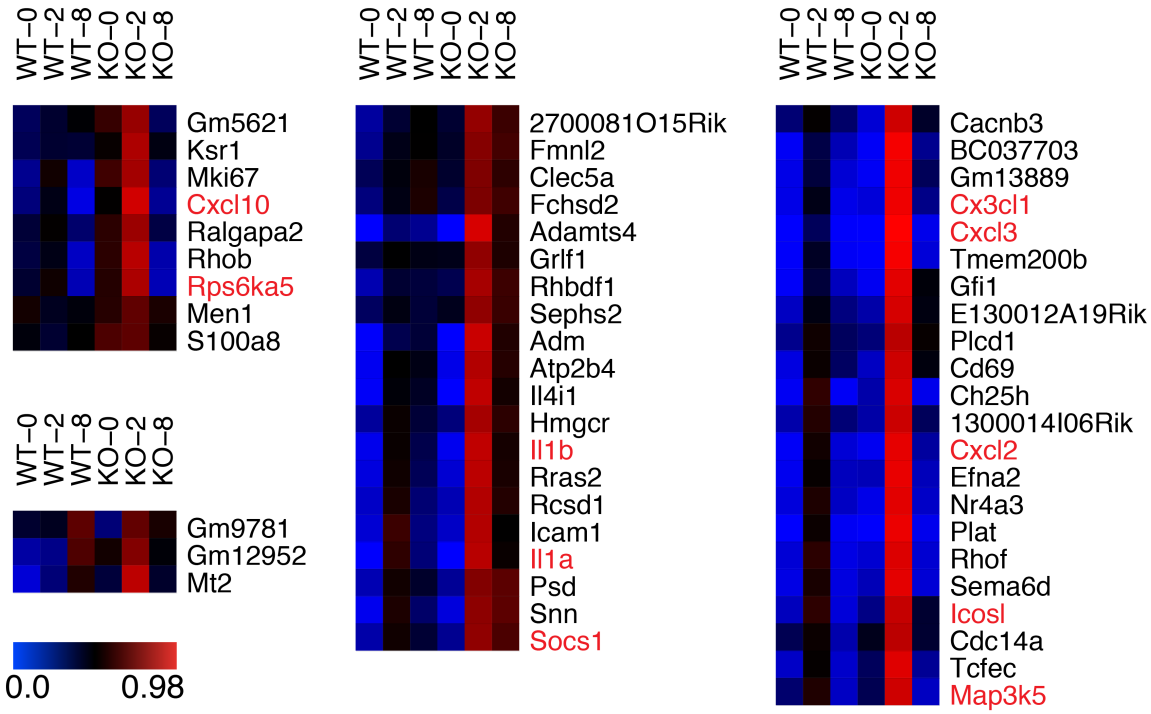

Figure 3-34. Genes in cluster 3

BCAP-sufficient (WT) or BCAP-deficient (KO) macrophages were stimulated with 1 μM CpG (ODN 1826) for 2 hours, 8 hours, or left unstimulated, as indicated. Genes of interest are highlighted in red.

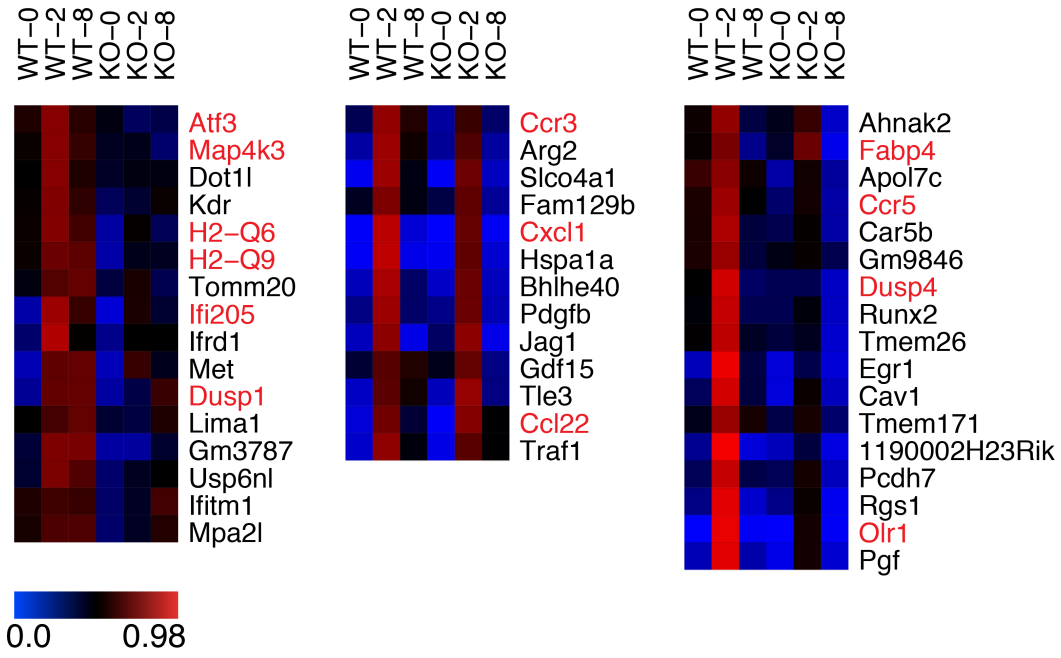

Figure 3-35. Genes in cluster 4

BCAP-sufficient (WT) or BCAP-deficient (KO) macrophages were stimulated with 1 μM CpG (ODN 1826) for 2 hours, 8 hours, or left unstimulated, as indicated. Genes of interest are highlighted in red.

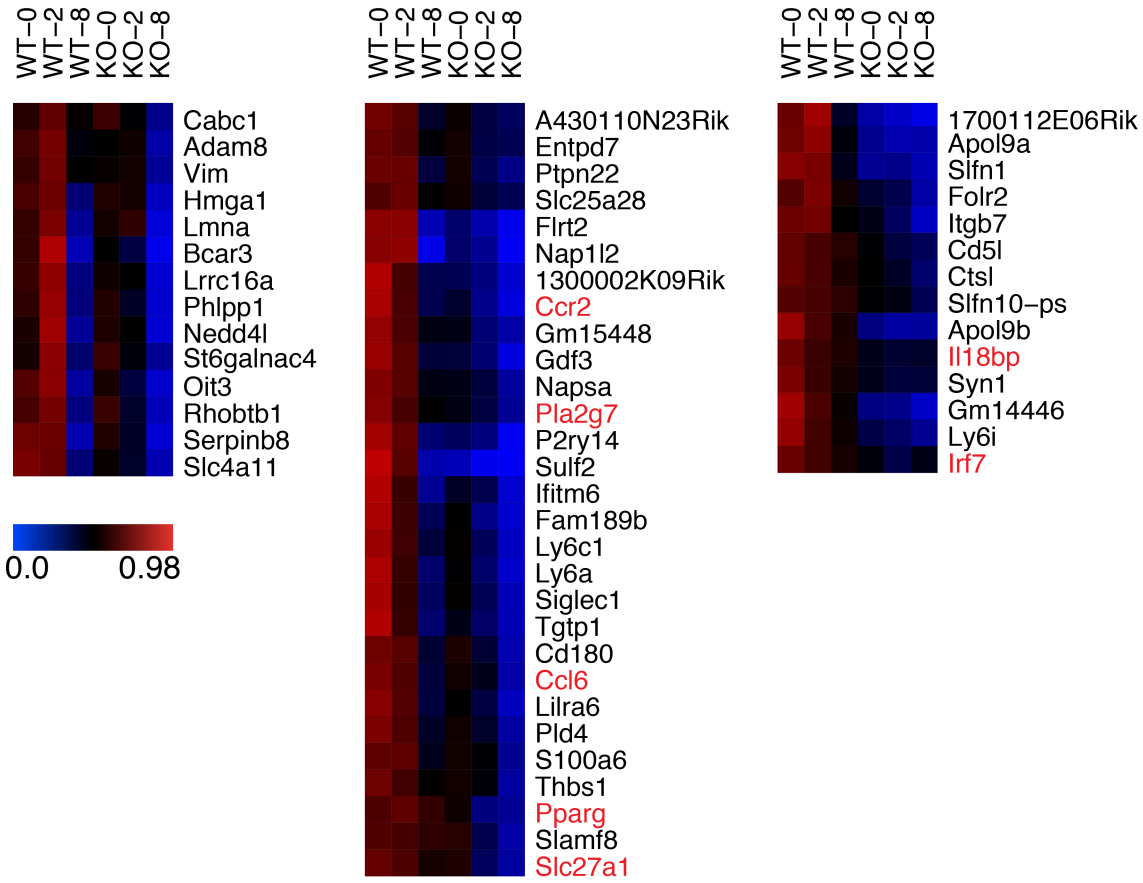

Figure 3-36. Genes in cluster 5

BCAP-sufficient (WT) or BCAP-deficient (KO) macrophages were stimulated with 1 μM CpG (ODN 1826) for 2 hours, 8 hours, or left unstimulated, as indicated. Genes of interest are highlighted in red.

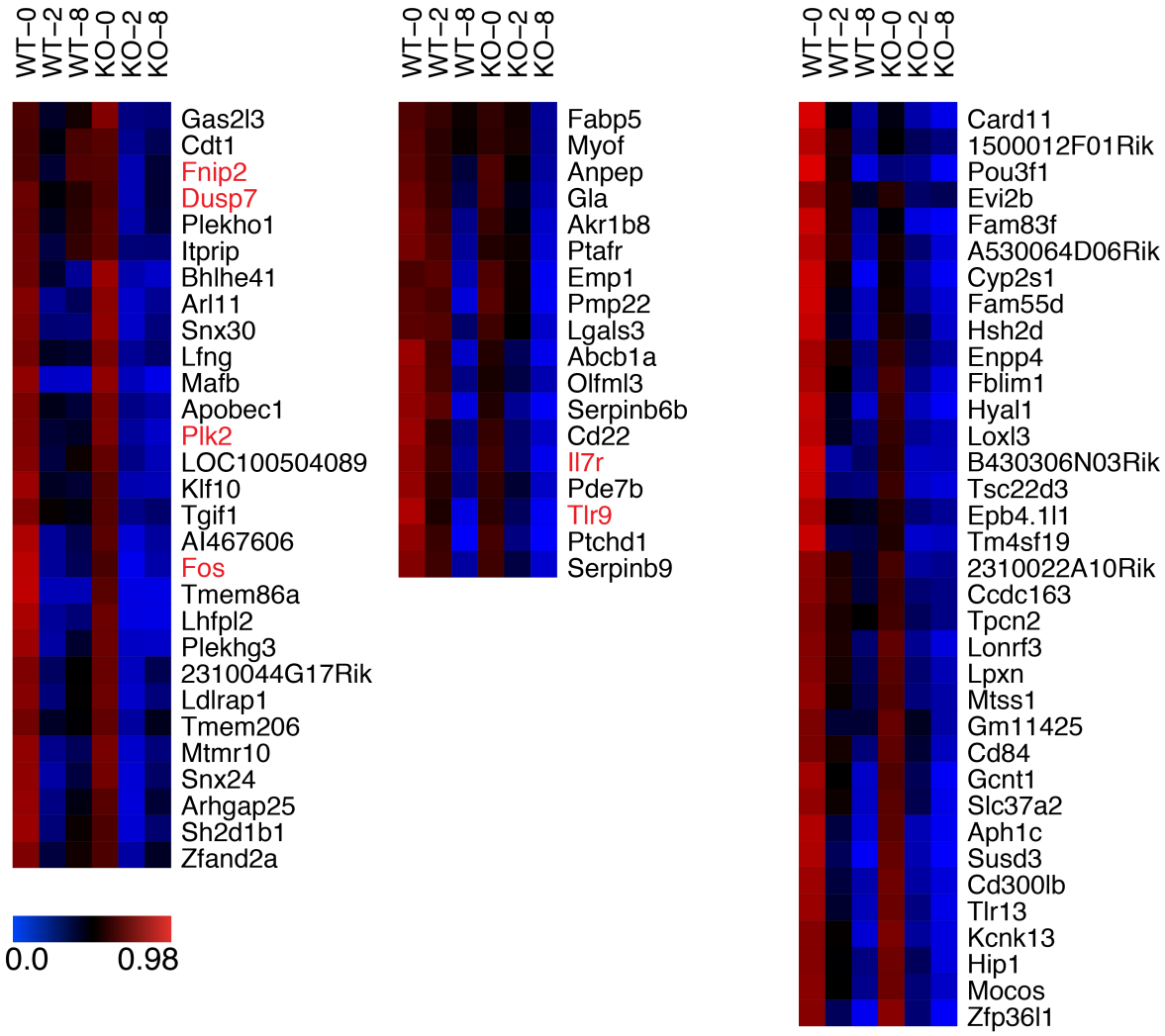

Figure 3-37. Genes in cluster 5 continued

BCAP-sufficient (WT) or BCAP-deficient (KO) macrophages were stimulated with 1 μM CpG (ODN 1826) for 2 hours, 8 hours, or left unstimulated, as indicated. Genes of interest are highlighted in red.

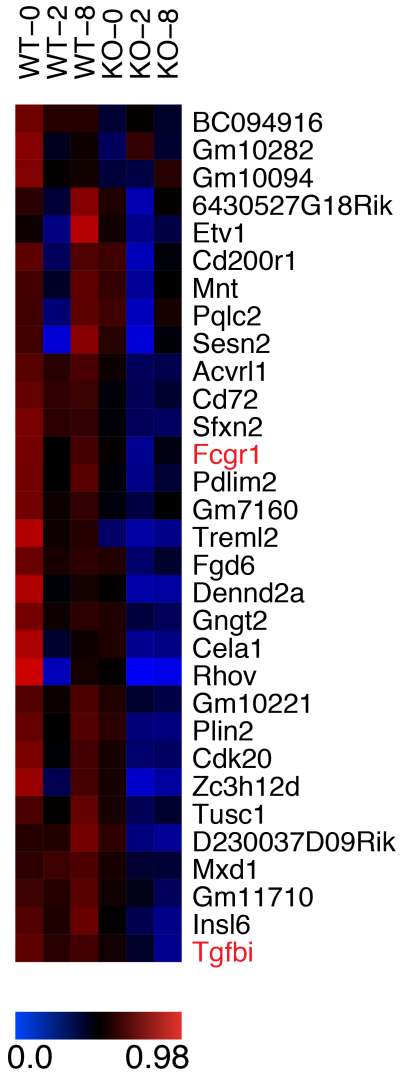

Figure 3-38. Genes in cluster 6

BCAP-sufficient (WT) or BCAP-deficient (KO) macrophages were stimulated with 1 μM CpG (ODN 1826) for 2 hours, 8 hours, or left unstimulated, as indicated. Genes of interest are highlighted in red.

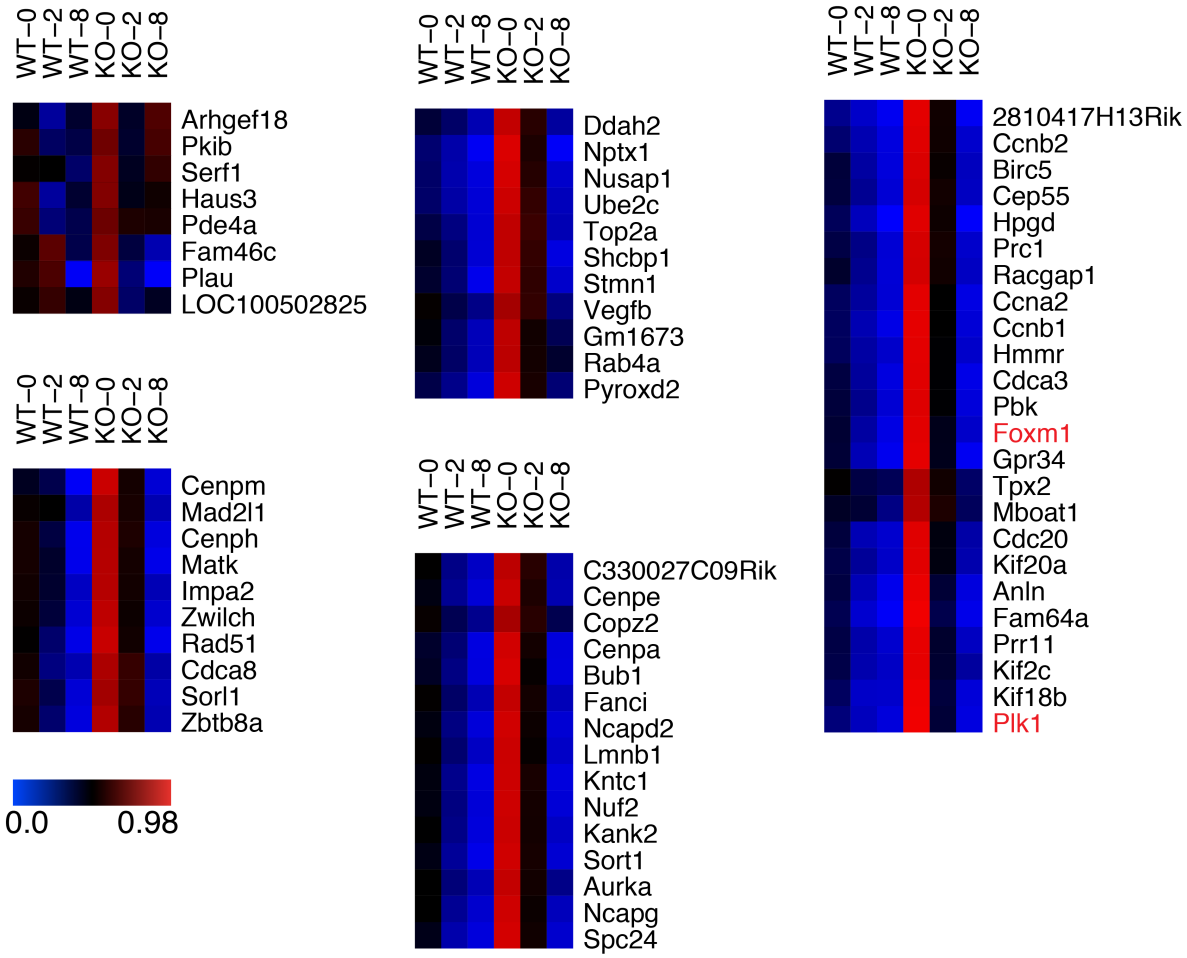

Figure 3-39. Genes in cluster 7

BCAP-sufficient (WT) or BCAP-deficient (KO) macrophages were stimulated with 1 μM CpG (ODN 1826) for 2 hours, 8 hours, or left unstimulated, as indicated. Genes of interest are highlighted in red.

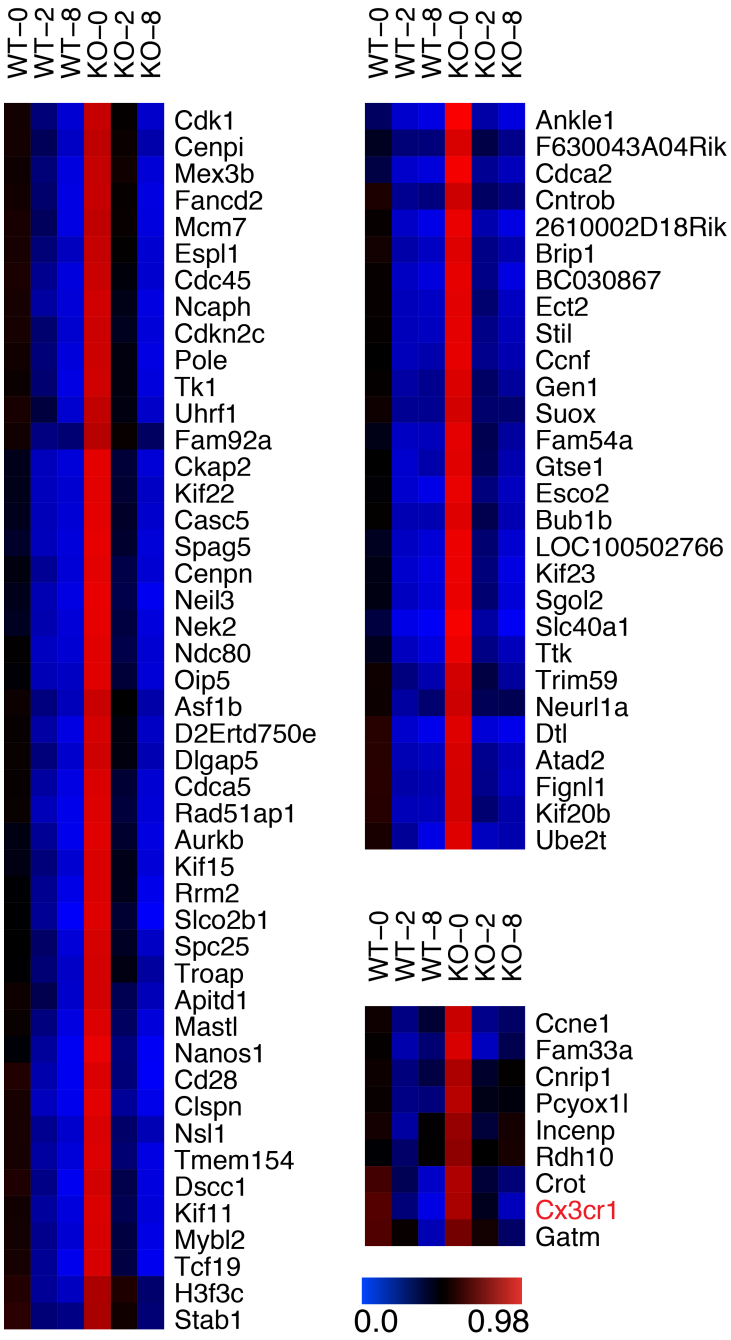

Figure 3-40. Genes in cluster 7 continued 1

BCAP-sufficient (WT) or BCAP-deficient (KO) macrophages were stimulated with 1 μM CpG (ODN 1826) for 2 hours, 8 hours, or left unstimulated, as indicated. Genes of interest are highlighted in red.

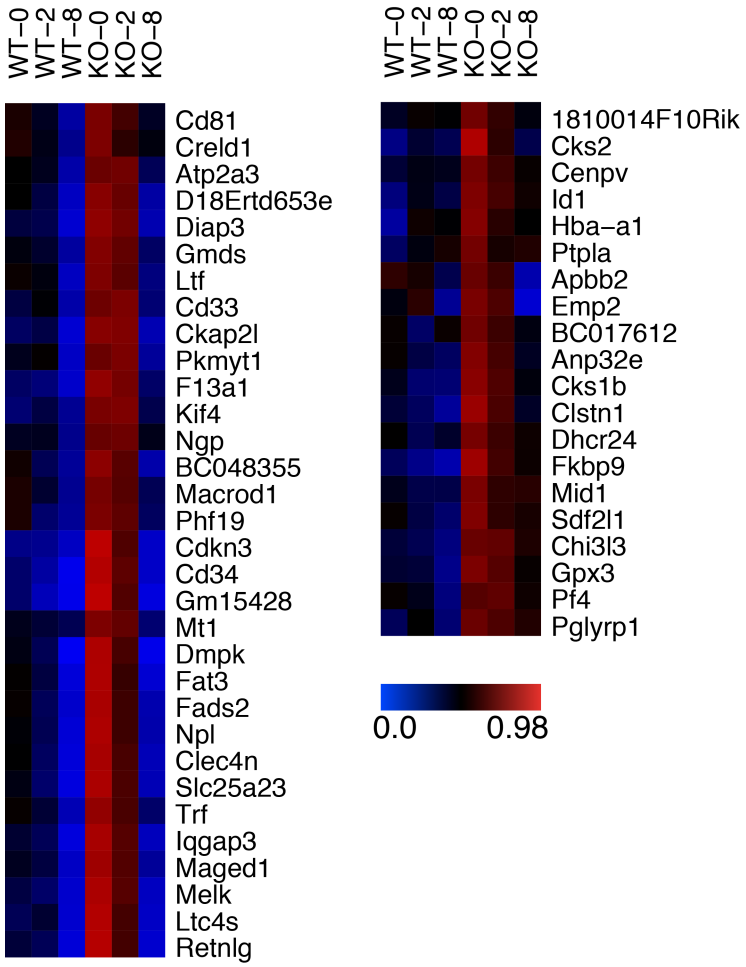

Figure 3-41. Genes in cluster 7 continued 2

BCAP-sufficient (WT) or BCAP-deficient (KO) macrophages were stimulated with 1 μM CpG (ODN 1826) for 2 hours, 8 hours, or left unstimulated, as indicated.

BCAP regulates the inflammatory response in vivo

To understand the significance of BCAP in regulating pro-inflammatory responses *in vivo*, we challenged cohorts of WT and BCAP deficient mice with live *Salmonella typhimurium* by the intraperitoneal route. Examination of these mice 72 hours after infection revealed significantly enhanced recruitment of inflammatory myeloid cells in the spleens of BCAP deficient mice when compared to WT mice (Figure 3-42). BCAP deficient mice also recruited higher proportions of TNF-α producing DCs (Serbina et al., 2003) and $CD11b^{+}$ myeloid cells into their spleens following intraperitoneal infection by *S. typhimurium* (Figure 3-43). Since these data demonstrate increased inflammation in BCAP deficient mice, we investigated if such profound activation of the innate immune system influences the activation status of CD4 T cells. Examination of proportions of naïve and memory CD4 T cells in the spleens of resting mice demonstrated that BCAP deficient mice had higher proportions of $CD44^{Hi}$ $CD62L^{Lo}$ effector/memory phenotype cells compared to age matched control mice (Figure 3-44). Together, these results demonstrate that BCAP plays a critical role in regulating inflammation following early activation of TLRs, and the absence of BCAP causes exaggerated responses in the innate immune system, consequently leading to higher activation of CD4 T cells.

To further investigate the role of BCAP in regulating inflammatory responses *in vivo*, we subjected cohorts of WT and BCAP deficient mice to dextran sodium sulfate (DSS) mediated colitis. These experiments revealed a more severe form of colitis in BCAP deficient mice as demonstrated by increased weight loss (Figure 3-45) and increased colon shortening

(Figure 3-46) following 7 days of DSS treatment in the drinking water. Similarly, histological examination of colons from DSS treated mice showed that BCAP deficient mice had higher crypt ablation and effacement of epithelial cells (Figure 3-47) when compared to WT controls. There was also increased neutrophil recruitment into the tissues of BCAP deficient mice. These data demonstrate a very important role for BCAP in regulating inflammation *in vivo*.

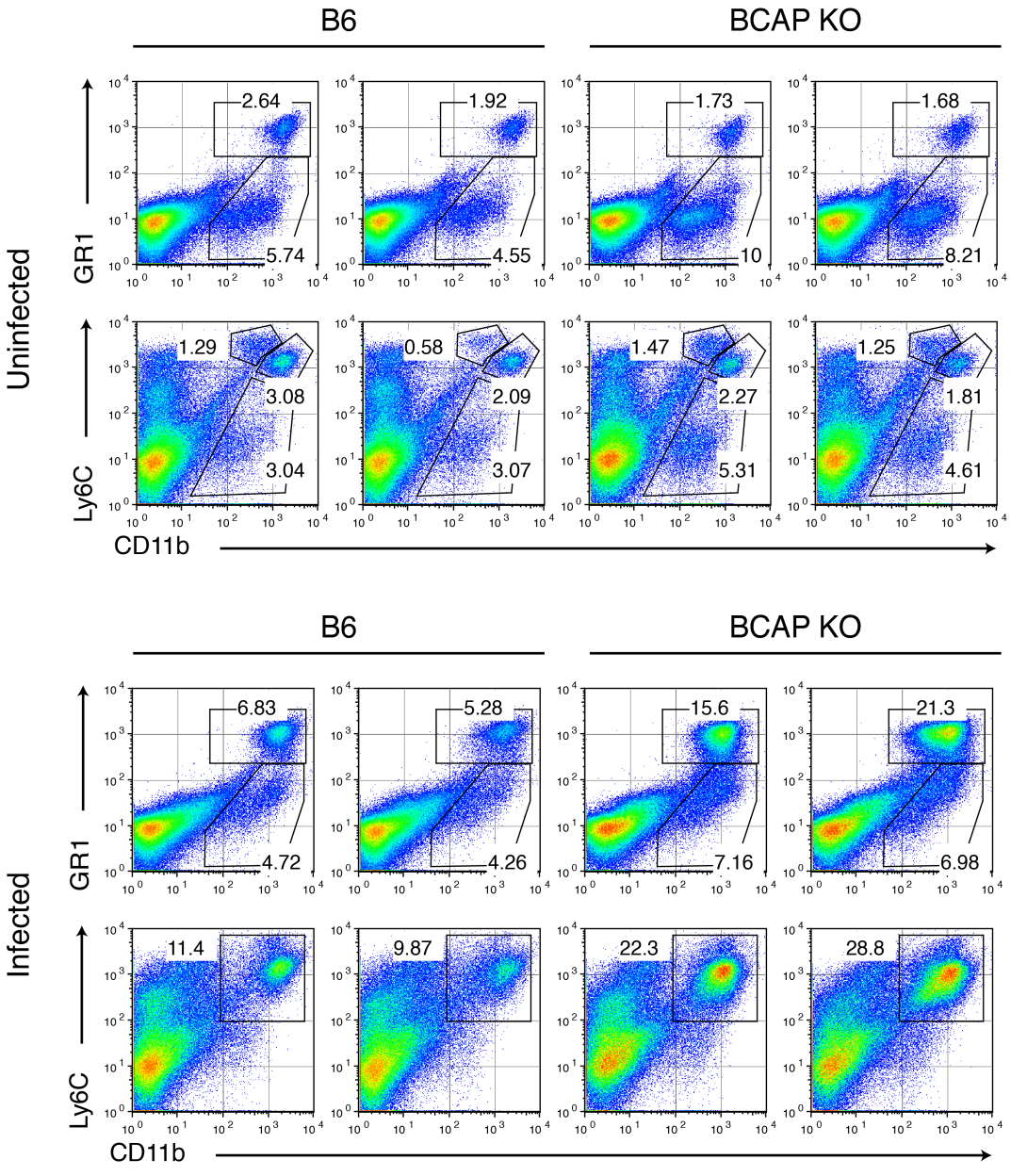

Figure 3-42. Infection with *Salmonella typhimurium* leads to enhanced recruitment of inflammatory myeloid cells in BCAP-deficient mice

B6 or BCAP KO mice were infected with $1*10^6$ CFU *Salmonella typhimurium* by the intraperitoneal route and 3 days later splenocytes were analyzed by flow cytometry for recruitment and expansion of $CD11b^+$ $GR1^+$ or $CD11b^+$ $Ly6C^+$ inflammatory myeloid cells.

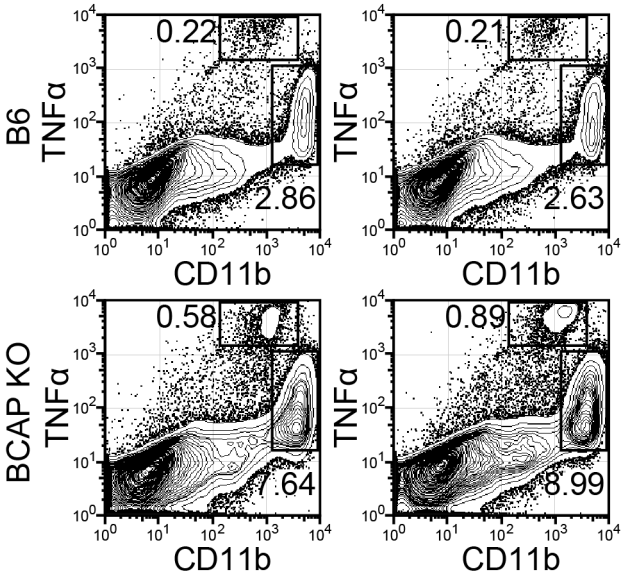

Figure 3-43. Enhanced recruitment of TNF competent cells to the spleens of BCAP-deficient mice

Splenocytes from two independent mice infected as in Figure 3-42 were stimulated ex vivo with heat-killed *S. typhimurium* for 4 hours in the presence of brefeldin A and TNF-α production by $CD11b^{Int}$ DCs and $CD11b^{Hi}$ cells was monitored by intracellular staining.

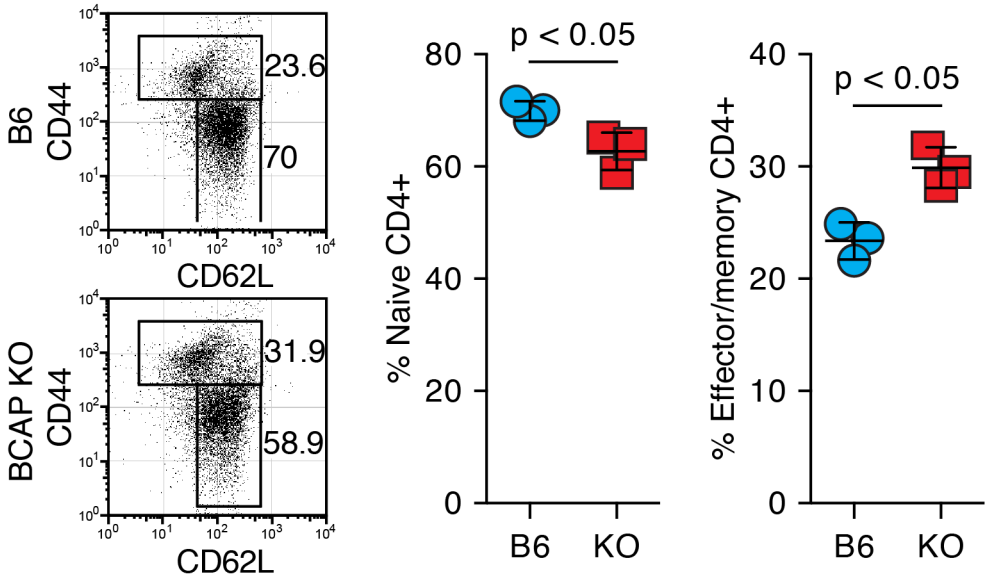

Figure 3-44. BCAP-deficient mice have augmented naïve/effector T helper cell ratios under steady state conditions

Proportions of naïve ($CD62L^{Hi}$ $CD44^{Lo}$) and effector/memory ($CD62L^{Lo}$ $CD44^{Hi}$) $CD4^{+}$ $TCR\beta^{+}$ T cells were analyzed in the spleens of age matched naïve B6 or BCAP KO mice. Representative flow cytometry plots are shown at left and data from 3 independent mice are shown at right. Errors denote s.d.

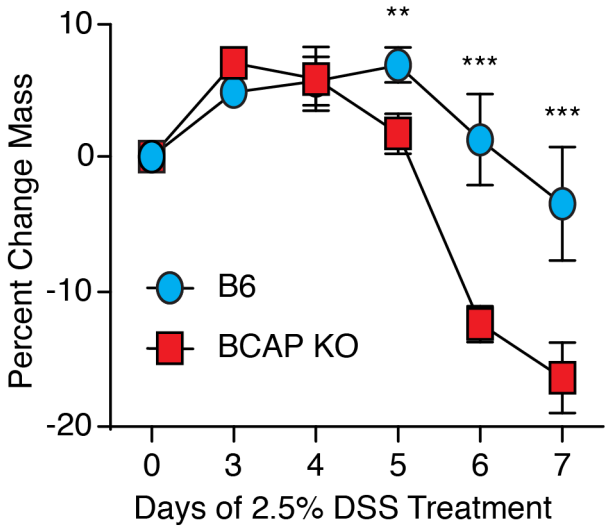

Figure 3-45. BCAP regulates susceptibility to an acute inflammatory colitis disease model

Cohorts of B6 or BCAP KO mice were restricted to 2.5% DSS in the drinking water for 7 days. DSS treated mice were monitored daily for weight loss. Data represent mean and s.d. of five mice. **, p<0.01, ***, p<0.001 as determined by Two way repeated measures ANOVA and subsequent Bonferonni post-test. Data represent three independent experiments.

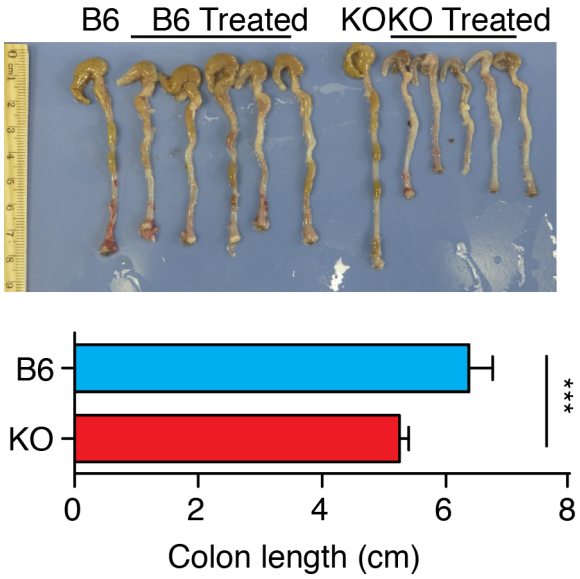

Figure 3-46. BCAP regulates colon length during acute inflammatory colitis

Cohorts of B6 or BCAP KO mice were restricted to 2.5% DSS in the drinking water for 7 days. Following treatment, mice were euthanized and colons harvested, photographed and lengths measured (lower panel). ***, p<0.001 as determined by T-test. Errors denote s.d. Data represent three independent experiments.

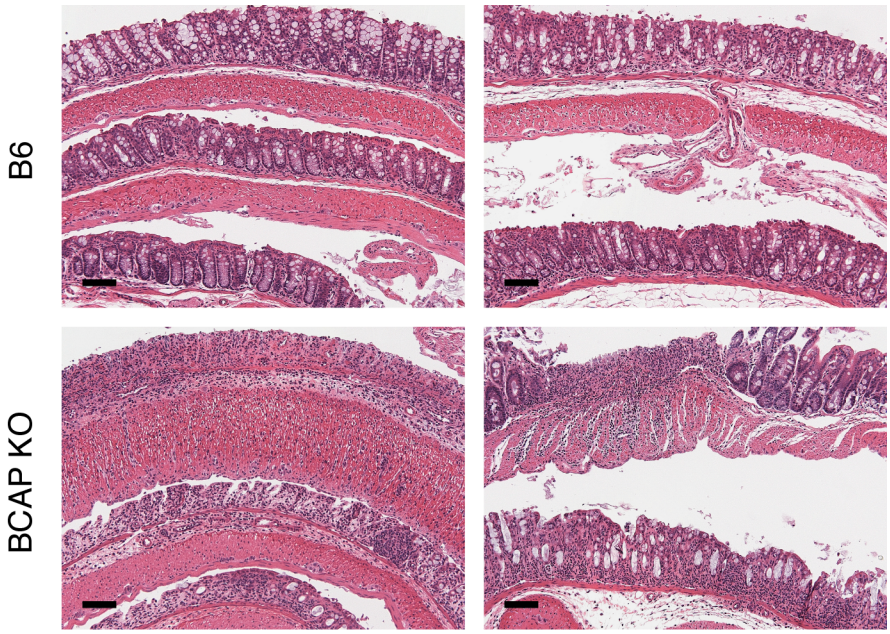

Figure 3-47. BCAP regulates colonic damage during acute inflammatory colitis

Cohorts of B6 or BCAP KO mice were restricted to 2.5% DSS in the drinking water for 7 days. Following treatment, mice were euthanized and tissue sections from DSS treated mice were stained with hematoxylin and eosin to monitor colon damage histologically. Data represent three independent experiments. Scale bars are 100 μm.

Discussion

Ligation of TLRs with their cognate ligand leads to a cytosolic signaling cascade beginning with the recruitment of the proximal adapters MyD88 and/or TRIF. MyD88 and TRIF's recruitment to TLRs depends on homotypic interactions of the respective TIR domains found within the TLRs as well as the adapters (Lee and Kim, 2007; O'Neill and Bowie, 2007). Although the molecules and events that connect TLRs to NF-κB and MAP kinases are well understood, the mechanism of how TLRs activate PI3K is not known. We demonstrate here that an adapter molecule shown to participate in BCR signaling and B cell survival (Okada et al., 2000; Yamazaki and Kurosaki, 2003; Yamazaki et al., 2002) contains a cryptic but functional TIR domain and plays a major role in connecting the TLR signaling pathway to PI3K/Akt activation. The interaction of BCAP with TLR signaling machinery not only interferes with NF-κB and MAP kinase activation, by potentially disrupting the interaction between TLRs and known signaling adapters such as MyD88 and TIRAP, but also leads to TLR-mediated activation of the PI3K-Akt pathway. As a consequence, BCAP mediated regulation of TLR signaling serves to limit production of the pro-inflammatory cytokines IL-6, IL-12, and TNF-α as well as limit inflammatory responses *in vivo*. Regulation of TLR signaling is critical for limiting inflammation in response to microbial stimuli as well as limiting adverse affects associated with signaling in response to endogenous TLR ligands, such as DNA complexes (Christensen et al., 2006) and fatty acids (Stewart et al., 2010) that could potentially exacerbate inflammatory conditions such as lupus and atherosclerosis.

Many regulatory steps limiting the outcome of TLR ligation have been defined including the regulation of post-translational modifications, signaling intermediates, chromatin modifications, transcriptional control, RNA stability, and secreted inhibitory factors. An additional player in the control of TLR mediated inflammation is PI3K (Hazeki et al., 2007; Ruse and Knaus, 2006). However, contradictory results addressing its role have led to confusion in the field. In experiments using chemical inhibitors, both positive and negative roles have been attributed to PI3K activity upon TLR stimulation (Hazeki et al., 2007; Ruse and Knaus, 2006). Recently, genetic tools have helped clarify this confusion and suggest that PI3K is involved in the negative regulation of TLR signaling outcomes. Using mice deficient for the p85 subunit of PI3K it was demonstrated that PI3K regulates IL-12 production by DCs (Fukao et al., 2002). Akt1 deficient mice have defects in regulating several miRNAs that subsequently regulate repression of inflammation (Androulidaki et al., 2009). Conversely, cells deficient for the tumor suppressor phosphatase and tensin homolog (PTEN), which regulates PI3K by converting PIP3 back into PIP2, and subsequently have increased activity of effectors downstream of PI3K including Akt, produce less TNF-α and KC (Schabbauer et al., 2010). Clearly, PI3K is functionally important for shaping the cellular response to TLR ligation.

The mechanism of PI3K activation by TLRs is not well understood. An initial study suggested that YXXM motifs in TLRs mediate the recruitment of PI3K to the signaling complex, which is in turn activated by RAC-1, but these assays were performed in the context of over-expression systems in cell lines and have yet to be confirmed genetically

(Arbibe et al., 2000). Another study suggests that the TLR signaling adapter TIRAP (or MAL) links TLR2 signaling to PI3K activation (Santos-Sierra et al., 2009). We have shown here that the adapter BCAP is specifically required for TLR mediated PI3K activation. It is important to note here that MyD88 deficient cells, as shown here as well as by a previous study (Laird et al., 2009), are also defective in activation of Akt suggesting that BCAP functions downstream of MyD88. Importantly, BCAP deficient cells still maintain equivalent activation of Akt upon stimulation with TNF-α, an early secreted effector produced as a consequence of TLR stimulation. This, along with the early kinetic differences of Akt activation in BCAP deficient cells compared to control cells supports the hypothesis that BCAP acts as a proximal adapter in the TLR pathway and the resulting phenotype is not due to an off-target secondary effect. Together, our data suggest a complex including known TLR adapters and BCAP is required for subsequent TLR mediated activation of PI3K. It is also possible that BCAP directly interacts with TLRs and the nature of endogenous association of BCAP with either MyD88, TIRAP or TLRs, especially following TLR stimulation, requires further investigation. Functionally, BCAP could also be working by competitively inhibiting recruitment of MyD88 and/or TIRAP to the TLRs, as evidenced by our studies in 293T cells, where overexpression of full length BCAP inhibits TLR4 and TLR9 mediated activation of NF-κB.

Improperly controlled signaling through TLRs contributes to aspects of several inflammatory diseases. Mice genetically deficient for A20 develop severe systemic inflammation, cachexia and succumb to an early death, a process alleviated in the absence of

MyD88 or upon deletion of the intestinal flora with broad spectrum antibiotics (Turer et al., 2008). Another TLR regulator, single immunoglobulin IL-1 receptor-related molecule (SIGIRR), regulates intestinal inflammation and susceptibility to DSS induced colitis (Xiao et al., 2007) as well as regulating susceptibility to lupus (Lech et al., 2008). Control of inflammatory cytokines is critical for regulation of systemic inflammation as well as shaping the adaptive immune response. As a result, priming of CD4 T cells by BCAP deficient dendritic cells results in increased proliferation and enhanced priming of these T cells towards the Th1 and Th17 lineages. Similarly, BCAP deficient mice have enhanced proportions of effector/memory CD4 T cells, concurrent with our *in vitro* observation. Consequently, in mice deficient for BCAP, we find a substantial increase in numbers of inflammatory myeloid cells following a systemic infection. BCAP deficient mice also display an enhanced susceptibility to DSS-induced colitis, corresponding with enhanced weight loss as well as enhanced intestinal damage. It is probable that the increased susceptibility of BCAP deficient mice to DSS colitis is due to a greater inflammatory response to intestinal bacteria breaching the intestinal barrier following DSS treatment. It is probable that the increased susceptibility of BCAP deficient mice to DSS colitis is due to a greater inflammatory response to intestinal bacteria breaching the intestinal barrier following DSS treatment. Although the *in vivo* data of greater numbers of inflammatory cells and greater inflammation of the colon are consistent with our finding that BCAP is a negative regulator of TLR signaling pathway, it is plausible that there might be additional mechanisms by which BCAP could be regulating these in vivo outcomes.

Finally, our studies show that BCAP is a unique TIR-domain containing adapter molecule that forms a critical link between TLRs and activation of PI3K. Early in signal transduction, BCAP may engage MyD88 via its TIR domain and potentially reduce the availability of MyD88 for activation of NF-κB, thereby affecting the canonical pathway of TLR signaling. BCAP also mediates activation of PI3K downstream of TLRs and, as has been demonstrated before, PI3K activation has regulatory effects on the outcome of TLR signaling, including limiting cytokine secretion and inflammation. As the classical MyD88 dependent pathway is still functional in BCAP deficient cells, utilization of cells deficient in BCAP will enable further genetic studies addressing the role of PI3K specifically through the TLR pathway. Future studies using BCAP deficient mice or mice with cell-type specific BCAP deletion will further expand our understanding of the biological processes regulated by BCAP.

CHAPTER FOUR

Intrinsic Expression of BCAP in T-Helper Cells Regulates their Differentiation and Effector Function via Signals through the IL1R Family

Introduction

The IL1R/TLR superfamily is composed of transmembrane receptors signaling in response to IL1 family cytokines and TLR ligands, respectively (Garlanda et al., 2013; Kawai and Akira, 2010; Sims and Smith, 2010). In this capacity, these receptors transmit signals from the extracellular environment through a common cytosolic domain crucially involved in recruitment of information relaying signaling adapters (Kenny and O'Neill, 2008; O'Neill and Bowie, 2007; Xu et al., 2000). The shared domain of the IL1R/TLR superfamily, the Toll-Interleukin-1 Receptor homology domain (TIR), mediates the ligand dependent recruitment of the signaling adapter through homotypic recruitment of a shared TIR domain found within the cytosolic portion of the receptor itself (Kenny and O'Neill, 2008; O'Neill and Bowie, 2007; Xu et al., 2000). Therefore, signals relayed from IL1R/TLR superfamily members are analogous in usage of shared components culminating in activation of subsequent factors and transcription factors common between the various members. The first known adapter, myeloid differentiation factor 88 (MyD88) is utilized by all TLRs with the exceptions of

TLR4, which makes use of a MyD88-dependent pathway and a MyD88-independent pathway through the adapter TRIF, as well as TLR3, which proximally depends upon TRIF and is completely independent of MyD88. In contrast to the TLR members of the superfamily, IL1R family members all depend upon recruitment of MyD88 for subsequent signal transmission (Garlanda et al., 2013).

Expression of TLRs is broadly restricted to myeloid cells, B cells, and in some cases, specialized epithelial cells. This restriction is thought to promote a separation of the potentially dangerous outcomes mediated by TLR sensing of pathogen associated products to cells specialized in handling/responding in a manner most beneficial to the host. In contrast, many cell types of the host respond to cytokine cues provided by IL1 family members (Dinarello, 1994; Gabay et al., 2010; Garlanda et al., 2013). In T cells, cues coming from IL1 provide important mitogenic signals allowing for enhancement of the proliferative capacity (Dinarello, 1994; Gabay et al., 2010; Garlanda et al., 2013). Further, IL1 plays an integral role in promoting the differentiation of naïve T cells into the Th17 lineage (Acosta-Rodriguez et al., 2007; Chung et al., 2009; Hu et al., 2011; Sutton et al., 2006). The related cytokine, IL18 acts in a similar fashion to elicit optimal responses from IFNγ producing cell types, including NK cells, cytotoxic CD8 T cells, as well as Th1 cells (Dinarello et al., 2013; Okamura et al., 1995; Robinson et al., 1997; Robinson and O'Garra, 2002; Smith, 2011; Yoshimoto et al., 1998).

We previously defined an obligate role for the signaling adapter BCAP as novel TIR-domain containing TLR signaling adapter mediation activation of the PI3K pathway in

macrophages stimulated with TLR ligands (Troutman et al., 2012a; Troutman et al., 2012b). Here, we extend our previous findings to implicate the adapter BCAP in as an important signaling adapter broadly used by members of the IL1R/TLR superfamily, including the IL1R and the IL18R. In this capacity, BCAP functions to augment the mitogenic capacity of IL1 and IL18 on enhancement of Th17 and Th1 cells during priming. This outcome was partially mediated through regulation of intrinsic survival of CD4 T cells undergoing differentiation. Consequently, BCAP deficient T cells were defective in their ability to commit towards Th17 effector cells. The significance of our finding has broad implications into dissection of how instructive cues provided by IL1 family members to T cells direct and promote their commitment to effector lineages.

Results

BCAP regulates secretion of Th1/Th17 effector cytokines

In chapter three, we described and characterized the adapter BCAP as a crucial TIR-domain containing TLR signaling adapter. Furthermore, we found that BCAP-deficient mice had increased numbers and percentages of effector/memory phenotype T helper cells relative to naïve T cells, indicative of increased antigen experienced cells. Therefore, we proposed that through heightened innate immune activation we would observe an enhanced adaptive immune response in BCAP-deficient mice challenge with a model vaccination. To test this hypothesis, we vaccinated wild type and BCAP-deficient mice with ovalbumin and lipopolysaccharide (LPS) emulsified in incomplete Freund's adjuvant (IFA). After seven

days, we purified CD4 T cells from the draining popliteal and inguinal lymph nodes and assessed the recall capacity of antigen-experienced cells to restimulation with ovalbumin. Surprisingly, BCAP-deficient T cells were defective both in their ability to proliferate and secrete IL17 upon recall (Figure 4-1 and Figure 4-2). As discussed in depth in chapter three, we previously demonstrated a crucial role for BCAP in regulation of inflammation *in vivo*. Because an important mediator of T cell priming is provided by instructive inflammatory cytokines, we initially predicted that the T helper responses to the vaccine antigen (ovalbumin) would be enhanced in mice deficient for BCAP. The opposite result suggested to us that perhaps BCAP was playing a crucial role intrinsic to T helper cells.

It was possible that confounding cell population(s), involved directly or indirectly, in the observed requirement of BCAP *in vivo* for T cell responses to vaccination could be playing a key role. To exclude this possibility, we employed an *in vitro* co-culture system to induce naïve T cell priming. In this manner, we restricted the deficiency of BCAP to highly purified naïve T cells existing in wells co-cultured with highly purified wild type (and thus BCAP-sufficient) dendritic cells (DCs). By adding low concentrations of anti-CD3 into the co-culture, we mimicked TCR signaling in the pool of co-cultured T cells through DC interaction with the soluble antibody on the Fc receptors of the DC. By provision of TLR ligands into the culture system, we effectively mimicked priming conditions *in vivo* by inducing the necessary co-stimulatory surface molecules and secreted cytokines required to promote T cell differentiation into effector T helper cells. As expected, naïve T cells failed to produce Th1 or Th17 effector cytokines when co-cultured in the absence of TLR ligands

(Figure 4-3). Provision of a TLR2 ligand or a TLR9 ligand potently induced both Th1 and Th17 effector cytokines. Interestingly, we found that BCAP-deficient T cells were highly defective in the ability to secrete IFNγ and IL17A, supporting the hypothesis that intrinsic BCAP expression by T cells is required during T cell priming by DCs (Figure 4-3).

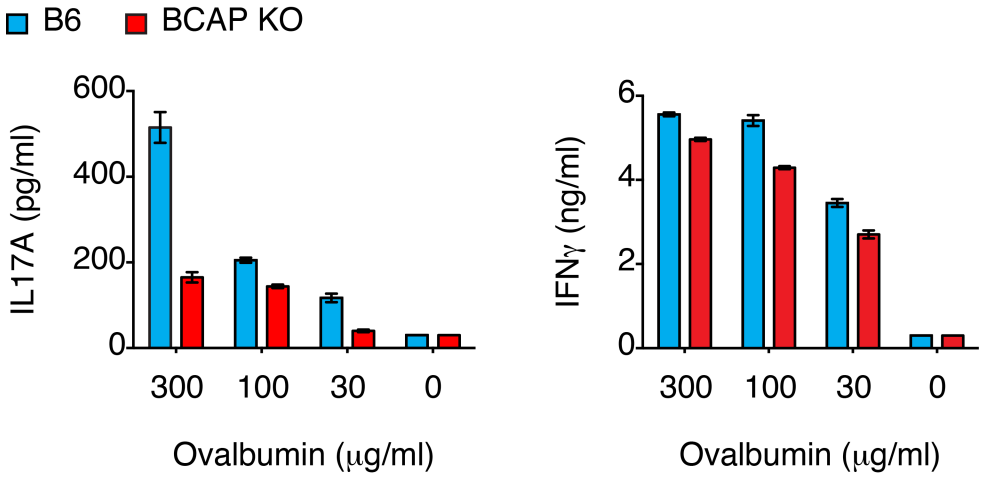

Figure 4-1. BCAP controls secretion of T helper cell effector cytokines upon recall

Cohorts of BCAP-sufficient (B6) or BCAP-deficient (KO) mice were vaccinated in the hind leg footpads with LPS and ovalbumin emulsified in IFA. After 7 days, purified $CD4^+$ T cells from the draining lymph nodes were recalled with the indicated concentration of soluble ovalbumin delivered using 5:1 ratio of T cells to DCs. After 48 hours, culture supernatants were harvested and secreted IL17A or IFNγ was measured by ELISA. Data represent 4 independent experiments.

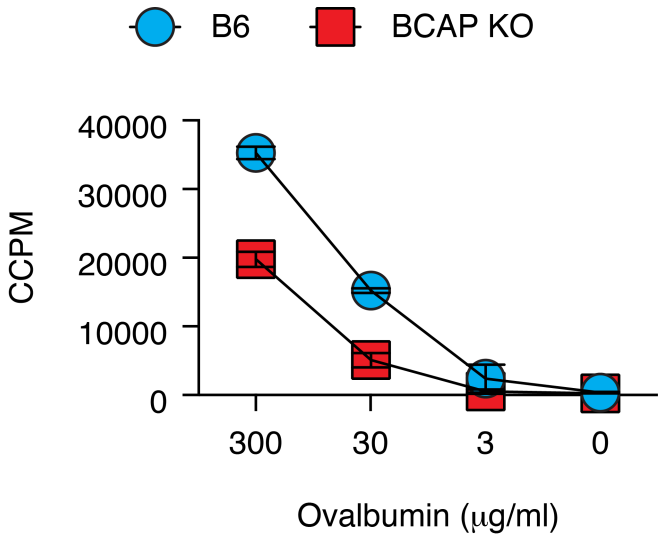

Figure 4-2. BCAP participates in proliferation of recalled T helper cells

Cohorts of BCAP-sufficient (B6) or BCAP-deficient (KO) mice were vaccinated in the hind leg footpads with LPS and ovalbumin emulsified in IFA. After 7 days, pooled purified $CD4^+$ T cells from the draining lymph nodes were recalled with the indicated concentration of soluble ovalbumin delivered using 5:1 ratio of T cells to DCs. After 48 hours, cells were pulsed with 0.5 μCi ^{3}H-thymidine and chased 16-hours later to assess proliferation. Data represent 4 independent experiments.

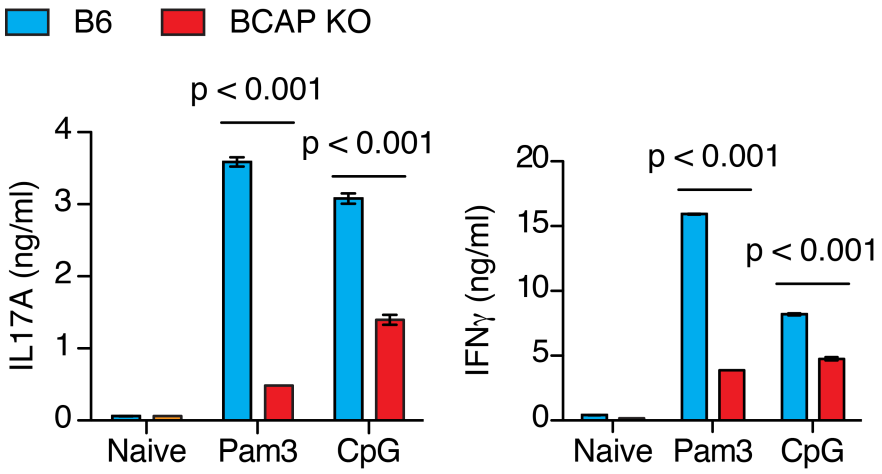

Figure 4-3. BCAP intrinsically promotes priming of naive T helper cells

Purified naïve CD4+ T cells of the indicated genotypes were cultured at a 5:1 ratio with wild type DCs and 3 ng/ml of anti-CD3ε to mimic TCR ligation. TLR ligands (100ng/ml Pam3CKS4 or 1 μM CpG) were added to the co-cultures to induce DC provided co-stimulatory molecules and cytokines necessary to drive T cell differentiation. After 4 days of culture, supernatants were harvested and secreted IL17A or IFNγ was assessed by ELISA. Statistical assessment was performed using two-way ANOVA and subsequent Bonferonni post-test. Data represent multiple experiments.

BCAP functions in the IL1R family signaling pathway

Due to the functional conservation of the cytosolic signaling domains of TLRs and IL1R family members (Dinarello, 2009; Garlanda et al., 2013), we predicted that the functional requirement for BCAP in regulating Th1 and Th17 differentiation came through signaling via IL1R family members. To test the involvement of BCAP in IL1R family signaling for optimal differentiation of naïve T cells, we adopted a traditional *in vitro* polarization approach. Upon culture of purified naïve CD4 T cells in neutralized conditions, we found as expected undetectable Th17 effector cytokine secretion. By adding IL6 and TGFβ into the mono-culture, we polarized the T cells into IL17A producing cells (Figure 4-4). This process was independent of BCAP expression in the T cells, suggesting that BCAP plays no role in promoting Th17 commitment through signals provided by IL2, IL12, IL6 nor TGFβ. Addition of IL1β into the Th17 polarization cocktail promoted a robust enhancement in the development of IL17 competent T cells, as expected. This enhancement was significantly reduced however in BCAP-deficient T cells (Figure 4-4). Notably, assessment of the mean fluorescent intensity of intracellular IL17A also revealed a reduced intensity of the intracellular IL17 stain in BCAP-deficient cells treated with IL1β. Finally, BCAP-deficient cells secreted less IL17 when IL1β was added to the Th17 cytokine cocktail (Figure 4-4). These results suggested strongly that BCAP signaling through the IL1R in T cells is required for optimal effector commitment or effector function.

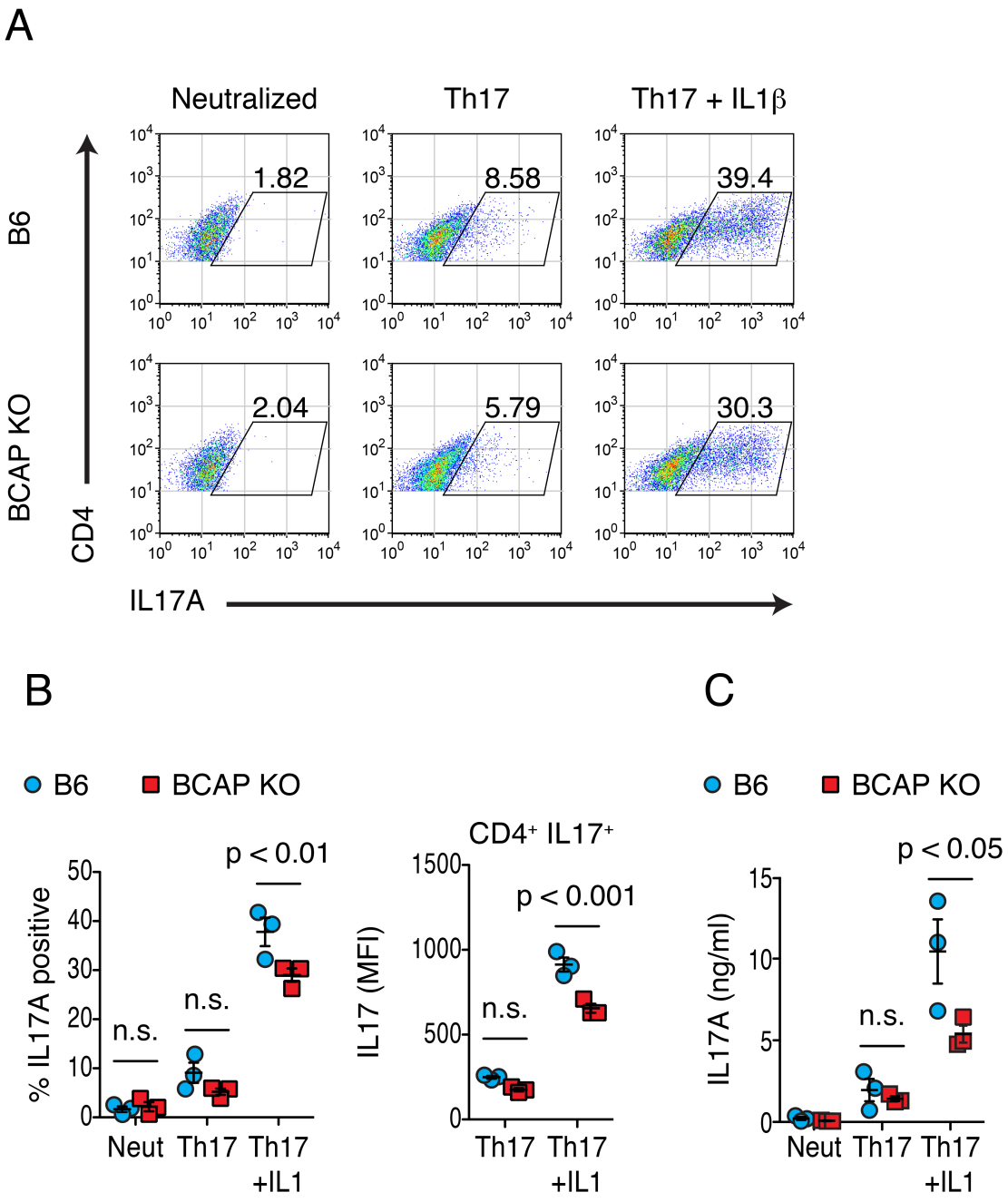

Figure 4-4. BCAP intrinsically regulated Th17 polarization dependent on IL1β

Purified naive CD4+ T cells were stimulated with plate bound anti-CD3/CD28 and Th17 priming conditions (10 μg/ml anti-IFNγ and anti-IL4, 50 U/ml IL2, 20 ng/ml IL6, and 5 ng/ml TGFβ) with or without 10 ng/ml IL1β as indicated. After 4 days, supernatants were saved for ELISA assessment (**C**) and cells were restimulated with PMA/ionomycin in the presence of brefeldin A to assess for intracellular IL17A (**A** and **B**). Symbols in **B** and **C** represent samples from biological replicates (discrete mice). **A** displays a representative flow cytometry plot from the same experiment. **B**, the left panel plots cells that were double positive for both CD4 and IL17 base on gating while the right panel displays the mean fluorescent intensity (MFI) of the cells existing in the same gate. Significance was determined by two-way ANOVA and subsequent Bonferonni test (n.s. is not significant). Data are representative of multiple experiments.

BCAP regulates Th1/Th17 differentiation through regulating proliferation/survival

Priming of naïve T helper cells occurs through a series of discrete steps. Upon stimulation, T cells must engage pro-survival signals and undergo proliferation, which is followed by licensing of effector cytokine secreting ability through the activity of lineage specific transcription factors (Mueller et al., 2013). This led us to question at what step signaling by BCAP is required for the effector licensing of T cells. To test this, we assessed the ability of BCAP-sufficient and BCAP-deficient T cells to survive and proliferate during priming. Upon co-culture of naïve T cells with DCs, we found TLR ligands enhanced the survival of naïve T cells when primed with co-cultured DCs (Figure 4-5), as expected. Further, TLR ligands enhanced the dilution of CFSE in the co-cultured T cells, indicative of cell proliferation (Figure 4-5), as expected. TLR ligands had a reduced protective effect on BCAP-deficient T cells, an effect evident after three days of culture with PAM3CSK4 and five days of culture with LPS or CpG (Figure 4-5). Further, we observed that viable BCAP-deficient T cells were significantly reduced in their ability to divide and accumulate into the $CFSE^{lo}$ population cells (Figure 4-5). This result suggested to us that BCAP expression in naïve CD4 T cells is necessary for survival and proliferation of cells during priming.

To further test if BCAP signaling through the IL1R was responsible for the proliferative defect in BCAP-deficient T cells, we co-cultured purified naïve T cells with irradiated DCs. In agreement with the previous experiment, BCAP-deficient cells were defective in their ability to proliferate in response to Th1 or Th17 polarizing conditions supplemented with IL18 or IL1β respectively (Figure 4-6). These results suggested that

BCAP signaling via the IL1R family members during priming of naïve T helper cells leads to optimal survival/proliferation of the differentiating cells. Importantly, no role for BCAP was revealed in cells polarized toward Th1 conditions in the absence of IL18 Figure 4-6, nor in cells polarized towards the Th17 lineage in the absence of IL1β (Figure 4-4 and Figure 4-6) This finding suggested that BCAP-intrinsic signaling in T helper cells undergoing priming was specific to IL1R family members.

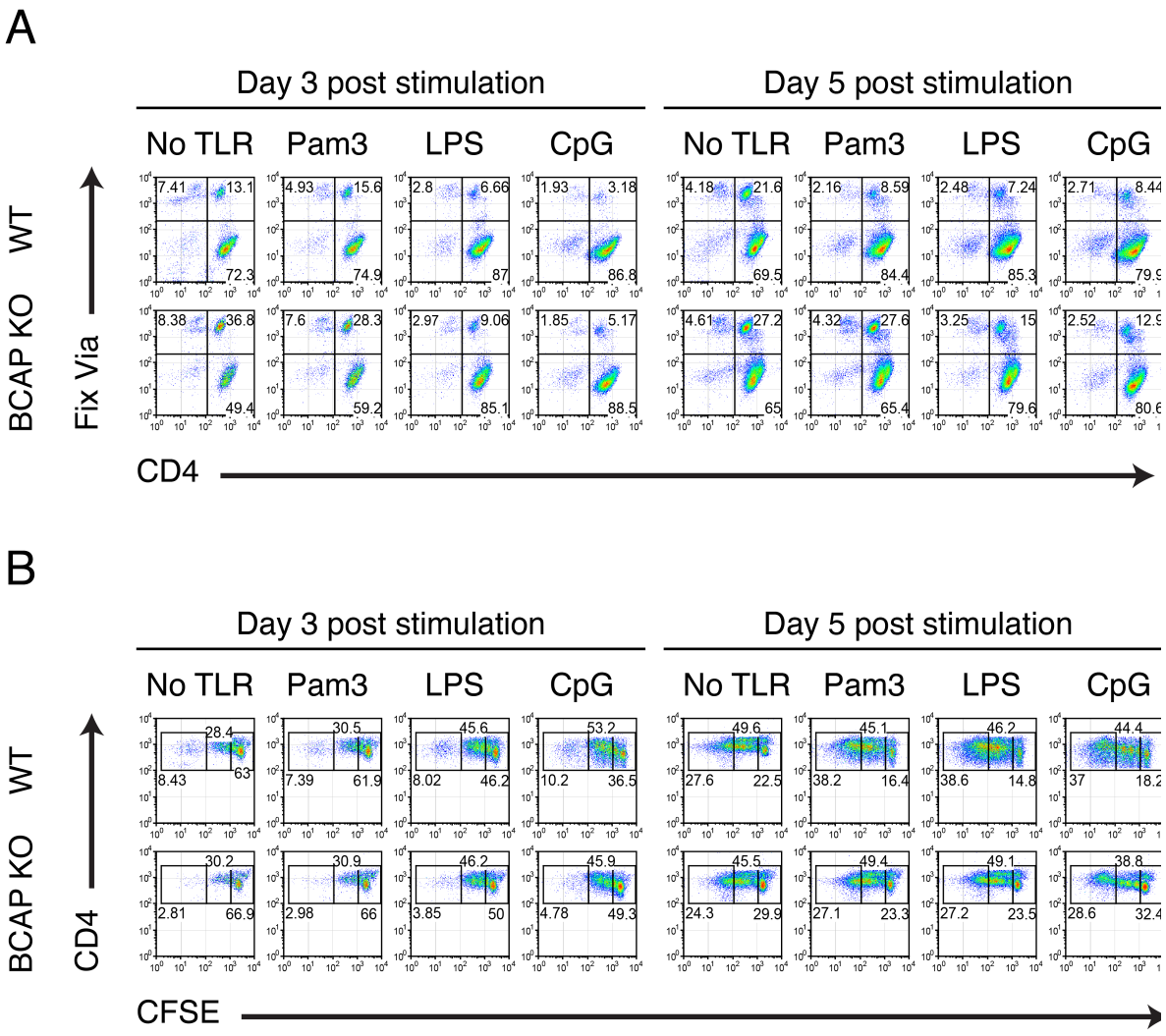

Figure 4-5. BCAP promotes survival and expansion of CD4+ T cells

Purified naïve CD4 T cells labeled with CFSE (1 μM) were culture at a 5:1 ratio with wild type DCs in the presence of 3 ng/ml anti-CD3ε, without the addition of a TLR ligand or with 100 ng/ml Pam3CSK4, 100 ng/ml LPS, or 1 μM CpG as indicated. After 3 or 5 days of culture, cells were labeled with a viability dye and assessed by flow cytometry. In panel **A**, cells labeled with the viability dye are considered dead while cells staining positive for CD4 and negative for the viability dye are considered to be viable $CD4^+$ T cells. In panel **B**, cells were gated on the viable CD4+ cells from panel **A** and dilution of CFSE was assessed as an indicator of cell division.

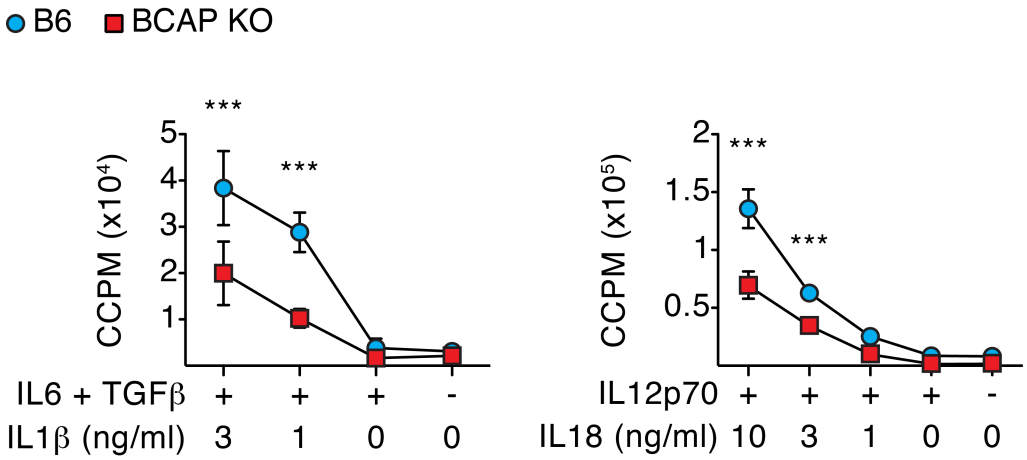

Figure 4-6. BCAP regulates T cell proliferation in response to IL1β and IL18

Naïve CD4 T cells of the indicated genotype were cultured with irradiated (15 Gy) purified splenic DCs (5:1 ratio) in the presence of anti-CD3ε. Cultures were supplemented with (left) IL6 and TGFβ with or without IL1β as indicated or (right) IL12p70 with or without IL18 as indicated. After 3 days, cells were stimulated overnight with 0.5 μCi ^{3}H-thymidine to monitor proliferation. Significance determined by two-way ANOVA and subsequent Bonferonni test. ***, $p < 0.001$.

T cell intrinsic expression of BCAP is required for Th17 commitment in vivo

Our above results suggested that BCAP functions *in vitro* through IL1R family members to promote Th1/Th17 commitment. To confirm our observations were not an artifact of *in vitro* culturing, we used a newly generated strain of mice permitting deletion of BCAP in T cells. We obtained BCAP-fl/+ embryonic stem cells from the KOMP repository (https://www.komp.org/), which were engineered to introduce loxP sites flanking exons 4 through 7 of *BCAP* (Figure 4-7). Through crossing the generated mice to a strain expressing the flp recombinase under control of the *actin* promoter, we were able to remove the selection cassette and derive mice with a "wild type" allele with loxP sites flanking critical exons of *BCAP*. Further crossing of the derived mice to LCK-Cre mice gave us BCAP-fl/fl control mice and BCAP-fl/fl LCK-Cre. This technology permitted expression of Cre recombinase, and subsequent deletion of the BCAP gene, in T cells developing in the thymus (Hennet et al., 1995; Orban et al., 1992; Salmena et al., 2003). Mice of these strains had no obvious developmental abnormalities and reproduced at expected Mendelian ratios.

To confirm the *in vivo* significance of our findings, we vaccinated cohorts of BCAP-fl/fl control mice and BCAP-fl/fl LCK-Cre with ovalbumin and LPS emulsified in IFA as before. After seven days, we harvested the draining lymph node and purified CD4 T cells. The purified CD4 T cells were subjected to an *ex vivo* recall assay by culturing cells with APCs (TLR2/4 DKO DCs) and titrating concentrations of ovalbumin. As seen using whole body BCAP-deficient mice, we observed a defect IL17A production and proliferation upon recall of T cells deficient from BCAP-fl/fl LCK-Cre mice (Figure 4-8 and Figure 4-9). With

these conditions, the requirement for BCAP-intrinsic expression in T cells for potentiation of normal IFNγ secretion remains unclear.

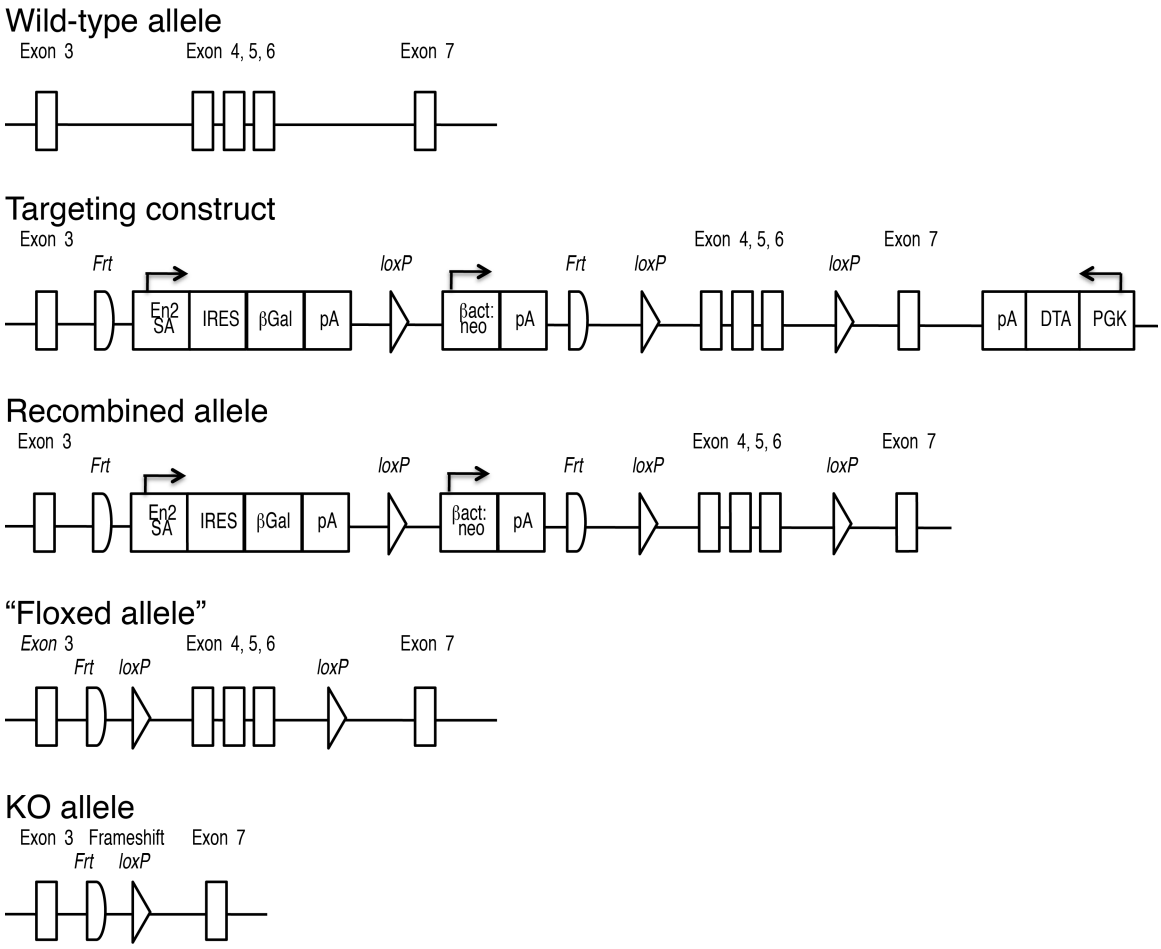

Figure 4-7. Construct and strategy for generating BCAP flox mice

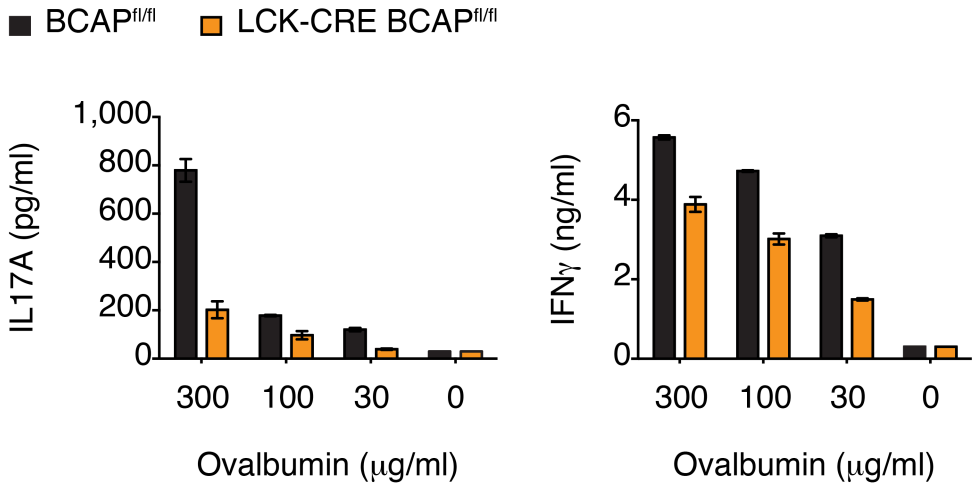

Figure 4-8. T cell intrinsic expression of BCAP promotes an antigen specific Th17 response

Cohorts of BCAP-fl/fl control mice or BCAP-fl/fl LCK-Cre were vaccinated as before with LPS and ovalbumin emulsified. After 7 days, CD4+ T cells were purified from the draining popliteal and inguinal lymph nodes and recalled in the presence of soluble ovalbumin using a 5:1 ratio of DCs. After 72 hours, culture supernatants were harvested and cytokine secretion assessed by ELISA.

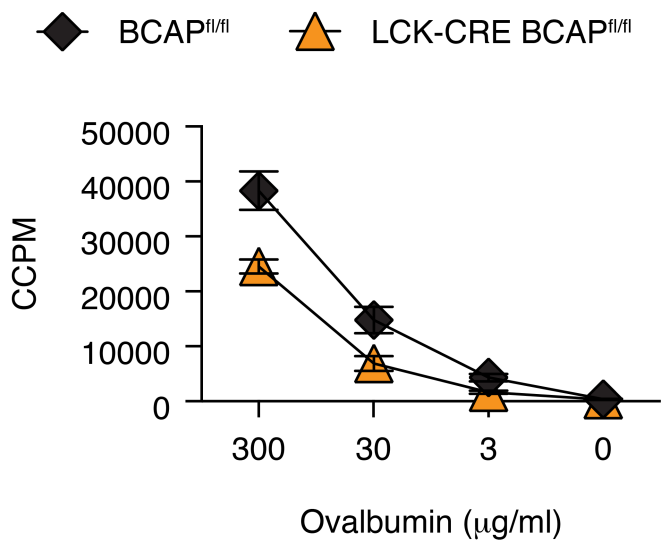

Figure 4-9. Expansion of antigen specific T helper cells depends on their intrinsic expression BCAP

Cohorts of BCAP-fl/fl control mice or BCAP-fl/fl LCK-Cre were vaccinated as before with LPS and ovalbumin emulsified. After 7 days, CD4+ T cells were purified from the draining popliteal and inguinal lymph nodes and recalled in the presence of soluble ovalbumin using a 5:1 ratio of DCs. After 72 hours, cells were pulsed with 0.5 μCi of ^{3}H-thymidine for 16 hours, followed by assessment on a β-harvester to monitor proliferation.

.

Discussion

The role for IL1 and IL18 in potentiation of Th1 and Th17 responses is well established (Acosta-Rodriguez et al., 2007; Casanova et al., 2011; Chung et al., 2009; Dinarello, 2009; Garlanda et al., 2013; Guo et al., 2009; Hu et al., 2011; Robinson et al., 1997; Sims and Smith, 2010; Smith, 2011; Sutton et al., 2006; Yoshimoto et al., 1998). The precise mechanisms controlling how IL1R family signals contribute to T helper cell differentiation are not clearly understood. Here we show that the TLR signaling adapter BCAP also functions downstream of members of the IL1R family. In this respect, BCAP signals promote the survival and expansion of naïve CD4 T cells during priming and lineage commitment (Figure 4-10). Through *in vitro* polarization experiments, we find that BCAP signals were restricted to the IL1 receptor and did not play a detectable role through TCR signaling, nor signals provided by IL2, IL6 nor TGFβ. Consequently, BCAP enhanced the effector capacity of Th17 cells by increasing the numbers of IL17 competent cells, as well as increasing on a per cell basis the ability to produce IL17A via the cytokine IL1β. In preliminary data, we find supporting evidence that BCAP functions in a similar manner to promote Th1 effector cytokine production via the cytokine IL18. Additionally, when co-cultured with DCs, naïve T cells were highly dependent upon BCAP for lineage commitment to Th1 and Th17 cells when cultured in the presence of a TLR2 or a TLR9 agonist. *In vivo*, the dependence upon BCAP for Th17 priming was confirmed using a vaccination model and was further found to depend upon T cell intrinsic expression of BCAP by the employment of newly T cell-specific BCAP-deficient mice.

Signals downstream of the IL1 receptor critically depend on the adapter MyD88 (Dinarello, 2009; Garlanda et al., 2013; Medzhitov et al., 1998). Furthermore, during *in vitro* priming experiments with DCs, MyD88 deficient T cells and IL1R1-deficient T cells are unable to commit to IL17 producing cells. (Chung et al., 2009; Hu et al., 2011). This indicates a crucial and requisite role for IL1 receptor signaling intrinsic to T cell in promoting Th17 development.

Further dissection of the IL1 receptor-signaling pathway revealed that the kinase activity of IRAK4 was required for Th17 cells in the EAE model (Staschke et al., 2009). Mechanistically, this study revealed that signals provided by IRAK4 lead to induction of the IL23R, a key player in Th17 induction (Korn et al., 2009; Staschke et al., 2009). In contrast, BCAP plays a supporting role in promoting differentiation of Th17 cells because BCAP-deficient T cells were not completely abrogated in their ability to produce IL17. This observation is consistent with the hypothesis that BCAP functions independent of IRAK4 dependent NFκB and MAP kinase activation signals in the TLR pathway and could be extended to include the IL1R family as well. Thus, it will be necessary to test if IL1 signals on BCAP-deficient T cells are still competent for inducing potent induction of Th17 promoting transcripts, including the transcription factor *RORc*, the *IL23R*, and *IRF4*.

Further, the role for BCAP in inducing activation of PI3K in T cells stimulated with IL1 family members remains unknown. Given the role for BCAP in activation of PI3K downstream of TLRs, the BCR, and CD19, it seems likely that BCAP will function in a similar capacity downstream of IL1R family members as well (Aiba et al., 2008; Inabe and

Kurosaki, 2002; Ni et al., 2012; Okada et al., 2000; Troutman et al., 2012a; Troutman et al., 2012b). This reasoning is in line with our data demonstrating that IL1 and IL18 signals promote the survival and proliferation of T cells in a manner dependent upon BCAP because cell survival and proliferation are closely linked to the activity of PI3K (Fruman and Cantley, 2002; Katso et al., 2001; Vanhaesebroeck et al., 2010).

The data provided here strongly support a model wherein DC provided IL1 and IL18 act directly on antigen specific naïve CD4 T cells, through intrinsic expression of BCAP, to promote both the survival and expansion of the differentiating cells, culminating in increased effector cytokine production by lineage specific T helper cells (Figure 4-10). The significance of these findings could have implications for several human diseases, including multiple sclerosis and the inflammatory bowel disease spectrum. A well-established link exists between IL1 and IL17 for the pathogenesis of mice using the EAE model (Casanova et al., 2011; Chung et al., 2009; Dinarello, 2009; Garlanda et al., 2013; Staschke et al., 2009; Sutton et al., 2006). Considering this, we have proposed to test the intrinsic participation of BCAP in T cells for driving the EAE disease model. In this setting, we would predict that T cell-intrinsic expression of BCAP promotes the expansion of auto-reactive Th17 cells, leading to exacerbated disease. Indeed, in our first preliminary experiment, we found that mice with a T cell-intrinsic BCAP deficiency had significantly less disease at early time-points, then displayed similar disease scores during intermediate time-points, but had reduced disease again at the later time-points. This result, though exciting and promising, was with a small cohort of mice and will require validation with a larger sample size. However, if BCAP

signaling in T cells does push the development of auto-reactive T cells, the implications could have a significant impact on the development and targeting of therapies to patients with multiple sclerosis.

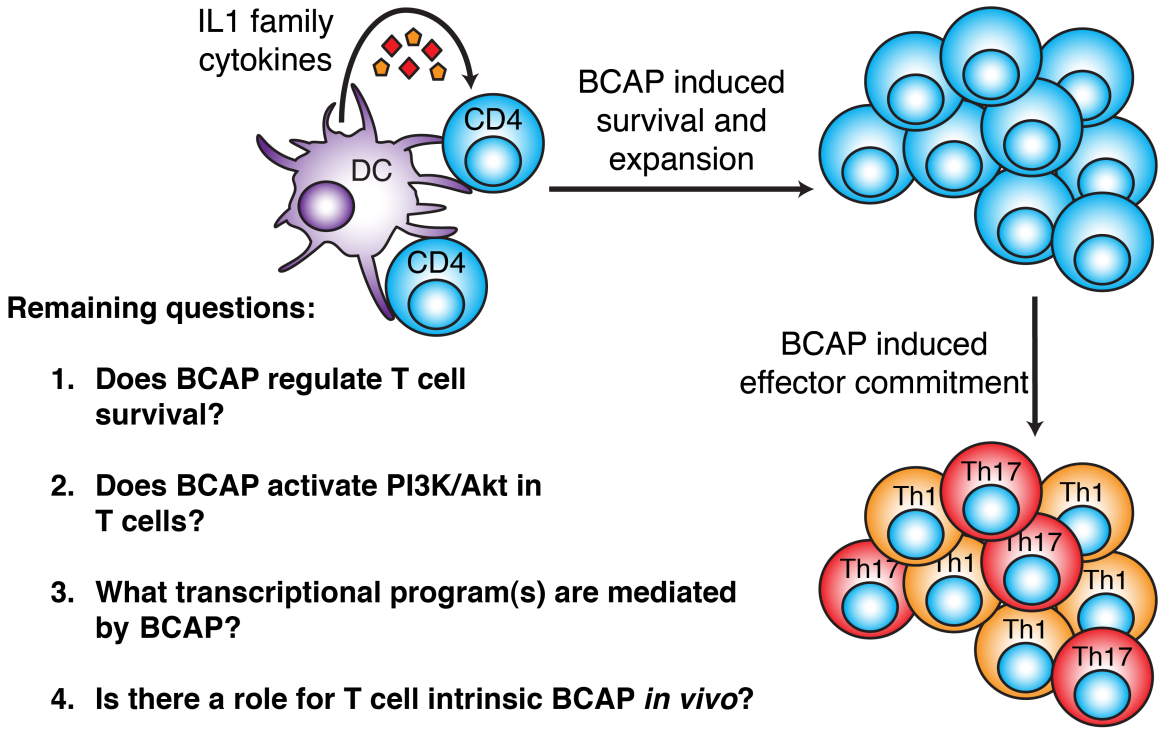

Figure 4-10. A model for how BCAP promotes T helper cell differentiation

CHAPTER FIVE

Discussion

Part of the writing presented in this chapter was previously published as an invited perspective in *Cell Cycle*, volume 11, issue 19, pages 3559-3567 (Troutman et al., 2012a).

Overview

The TLR/IL1R family plays a crucial role in providing instructive signals to responding cells. Macrophages and dendritic cells responding to ligation of TLRs respond by inducing a complex and coordinated response leading to instruction and control of a developing or ongoing immune response. Similarly, ligation of IL1R family members by T cells leads to enhanced proliferation and survival, leading to enhanced T-helper cell lineage commitment and concurrent production of lineage cytokines. However, understanding of the precise roles the related TLR/IL1R family imparts upon responding cells remains unclear.

The data I have presented here provides strong evidence for including BCAP as a key proximal signaling adapter functioning downstream of TLRs and IL1R family members. Importantly, the data support a model in which TLR signaling divided into two discrete signaling pathways. The first pathway is composed of the Myddosome (Gay et al., 2011; Lin et al., 2010) and branches again upon IRAK mediated activation of TRAF-6, ultimately

leading to activation of NF-κB and AP-1 family transcription factors (Takeuchi and Akira, 2010; Troutman et al., 2012a). The second branch of the TLR signaling pathway leads to the activation of PI3K. Previously, the players and mechanisms controlling TLR mediated activation of PI3K were unclear (Fukao and Koyasu, 2003; Ruse and Knaus, 2006; Troutman et al., 2012a). I have shown here, in chapter three, that BCAP serves as a requisite upstream component for TLR mediated activation of PI3K in macrophages (Troutman et al., 2012b). These findings have been further extended, in chapter four, to include BCAP as a key signaling adapter functioning in the broad TLR/IL1R superfamily. Given both the biological importance and conservation through evolution of the TLR/IL1R signaling system, the research presented here could prove crucial in the design and discovery of new drugs and vaccines.

BCAP as a Link Between TLRs and PI3K

TLR signaling is initiated upon recruitment of signaling adapters homotypically interacting through TIR domains found in both the cytosolic region of the TLR as well as the C-terminus of the TLR signaling adapter. Previously, five TLR signaling adapters had been described: MyD88, TRIF, TIRAP, TRAM, and SARM (Couillault et al., 2004; Fitzgerald et al., 2001; Fitzgerald et al., 2003; Hirotani et al., 2005; Hoebe et al., 2003; Horng et al., 2002; Horng et al., 2001; Kawai et al., 1999; Medzhitov et al., 1998; Mink et al., 2001; Muzio et al., 1998; O'Neill and Bowie, 2007; Oshiumi et al., 2003a; Oshiumi et al., 2003b; Takeuchi et al., 2000; Yamamoto et al., 2003a; Yamamoto et al., 2002a; Yamamoto et al., 2003b; Yamamoto et al.,

2002b). Through computational searching of the human genome database, our collaborator, Fernando J. Bazan, Ph.D., found the existence of a putative TIR domain at the N-terminus of BCAP, as well as the homologous BANK1 and the paralogous fly protein, DOF (Figure 1-1). As the presence of a TIR domain strongly suggests participation in the TLR/IL1R signaling systems, it was expected to find that BCAP functions as a TLR signaling adapter.

Previous work implicated BCAP as positively regulating B cell signaling through the B-cell receptor and CD19 via activation of PI3K. The ability of BCAP to mediate PI3K activation through the B-cell receptor or CD19 depended upon phosphorylation of critical tyrosine residues in YXXM motifs within BCAP (Aiba et al., 2008; Inabe and Kurosaki, 2002; Okada et al., 2000). In addition to B cells, BCAP is expressed by many other immune cells types, including macrophages, NK cells, and T cells (Figure 1-3) (MacFarlane et al., 2008; Matsumura et al., 2010; Ni et al., 2012; Okada et al., 2000; Song et al., 2011; Troutman et al., 2012a). As described in detail in chapter three, by using BCAP-deficient mice, BCAP was shown to act as a negative regulator of inflammation through TLRs and NK cell receptors respectively, as shown by us and others (MacFarlane et al., 2008; Matsumura et al., 2010; Ni et al., 2012; Troutman et al., 2012b). Indeed, BCAP sufficiency was critical in mediating PI3K/Akt activation upon stimulation of cells with TLR ligands (Ni et al., 2012; Song et al., 2011; Troutman et al., 2012b). BCAP mechanistically connects TLRs to PI3K through a cryptic TIR domain at the N-terminus of BCAP, which is utilized for recruitment to the TLR signaling apparatus (Figure 5-1) (Troutman et al., 2012b). Consequently,

macrophages deficient for BCAP produced increased quantities of proinflammatory cytokines and BCAP deficient mice were more susceptible to LPS toxicity assays as well as a model of inflammatory colitis (Matsumura et al., 2010; Ni et al., 2012; Troutman et al., 2012b). PI3K/Akt activation in macrophages also depended upon the TLR adaptors MyD88 and TIRAP, thus, it is possible that BCAP acts downstream of these two adaptors and is recruited to the TLR signaling adaptors and not the TLR itself, though this remains to be tested. In support of that idea, BCAP interacted with both MyD88 and TIRAP dependent upon the TIR domain of BCAP (Troutman et al., 2012b). Alternatively, the more classical TLR adaptors may mediate recruitment of an unknown intermediate factor required for permissive tyrosine phosphorylation of BCAP, and thus mediating its downstream capacity for PI3K/Akt activation.

Previous work showed that tyrosine phosphorylation of BCAP is critical for recruitment of PI3K. Mutation of four critical tyrosine residues to phenylalanine in BCAP abrogated the ability of PI3K to be activated upon stimulation of B cells through their receptor (Aiba et al., 2008; Inabe and Kurosaki, 2002; Okada et al., 2000). Similarly, BCAP undergoes phosphorylation upon TLR stimulation, which mediated recruitment of PI3K to BCAP in a kinetic fashion upon TLR stimulation (Ni et al., 2012). We have had limited success in replicating this observation reproducibly as in some experiments we found constitutive BCAP phosphorylation that was not inducible above background levels. However, we did observe in some experiments a TLR ligand induced tyrosine phosphorylation of BCAP, suggesting inherent technical concerns in our experiments.

Critically, as is the case for BCAP mediated PI3K activation through BCR stimulation, tyrosine phosphorylation of BCAP is required for optimal restriction of inflammatory cytokine production, suggesting the requirement of tyrosine phosphorylation of BCAP for TLR mediated PI3K activation (Ni et al., 2012). Thus, BCAP phosphorylation appears required for signal transmission to PI3K upon TLR stimulation.

At present, the tyrosine kinase mediating BCAP phosphorylation in TLR stimulated cells remains unknown. Notably, the SRC family kinase Lyn is implicated in phosphorylation of BCAP in B cells stimulated through their receptor or CD19 crosslinking (Inabe and Kurosaki, 2002). Further, Lyn also is implicated in mediating the linkage of TLRs to PI3K activation, with a consequential increase in IL6 and TNF production by cells genetically deficient for Lyn (Keck et al., 2010). Thus, it is plausible to speculate that Lyn serves as the tyrosine kinase imparting permissive phosphorylation upon BCAP and subsequent induction of TLR mediated PI3K activation. This hypothesis is further supported by the overlapping phenotype of BCAP deficient and Lyn deficient macrophages stimulated with TLR agonists (Keck et al., 2010; Ni et al., 2012; Troutman et al., 2012b). However, this hypothesis remains to be experimentally tested.

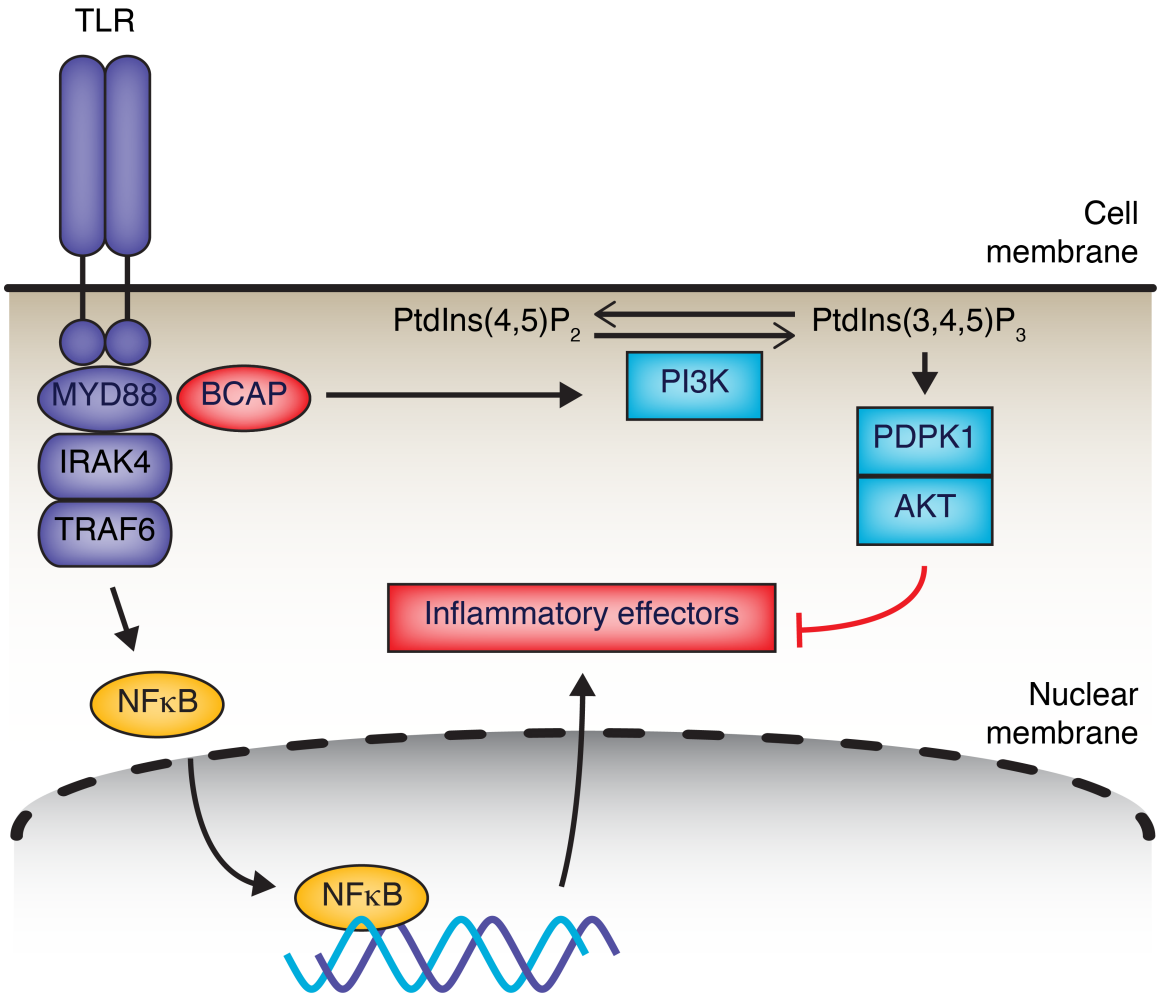

Figure 5-1. BCAP links TLRs to PI3K

TLR ligation initiates a signaling cascade leading to activation of NFκB. Activation of NFκB by TLRs is mediated through recruitment of signaling adaptors, including MyD88. The MyD88-dependent pathway mediates assembly of a ternary complex of IRAK proteins, which engages and activates TRAF6, ultimately leading to activation of NFκB. In addition to activation of NFκB, MyD88-dependent TLR signaling also leads to activation of PI3K.

Activation of PI3K by TLRs critically requires BCAP. Upon stimulation, tyrosine phosphorylated BCAP recruits the PI3K complex, leading to its activation. Activation of the PI3K signaling axis represents a major branch of TLR mediated signaling and contributes to a variety of cellular outcomes, including regulation of the inflammatory response, cell proliferation and cell survival.

Inherent difficulties in studying PI3K

During TLR stimulation, BCAP-deficient cells were restricted in their inability to activate phosphoinositide3-kinases (PI3K) and had no defect in acute activation of neither NFκB nor MAP kinases. The significance of this observation lies in the inherent difficulty of study of the PI3K pathway. PI3K signaling is crucial for regulation of cell proliferation and survival, control of protein synthesis, and regulation of growth and glucose metabolism (Bunney and Katan, 2010; Vanhaesebroeck et al., 2010). However, the multifaceted role of PI3K necessarily depends on a wide array of upstream receptors controlling PI3K activation (Bunney and Katan, 2010; Vanhaesebroeck et al., 2010). Perturbation of the PI3K pathway, either genetically or by pharmacological methods is therefore frequently associated with confounding off-target effects uncontrolled within the experimental system. Thus, interpretations of studies addressing the precise role of PI3K in regulation of TLR signaling are complicated by the limited availability of experimental tools, leading to contradictory reports as to the functional role of PI3K within a response system, including within the field of TLR biology (Fang et al., 2004; Fruman and Cantley, 2002; Fukao and Koyasu, 2003; Guha and Mackman, 2002; Hazeki et al., 2007; Luyendyk et al., 2008; Park et al., 1997; Utsugi et al., 2009).

Through pharmacological inhibition of PI3K, numerous studies demonstrate contradictory phenotypes as to the role PI3K plays during a cellular response to TLR stimulation (Fukao and Koyasu, 2003; Gunzl and Schabbauer, 2008; Hazeki et al., 2007; Ruse and Knaus, 2006). This suggests that pharmacological inhibitors may have dosage-dependent effects and/or off target effects. In support of this, an analog of LY294002, a broadly used inhibitor of PI3K, which does not affect PI3K activity, still functionally repressed the TLR mediated signaling and activity (Kim et al., 2005).

Another complication of studying the role of PI3K in TLR signaling lies in the ubiquitous and widespread utilization of PI3K by various cellular signaling processes. Thus, disrupting a cell's ability to utilize PI3K in response to TLR stimulation also disrupts the same cell's ability to utilize PI3K in response to co-existent stimuli, including growth factors, cytokines, and chemokines. In experimental systems testing for the role of PI3K (through pharmacological or genetic disruption of the pathway) in response to TLR ligation, the effects measured also include the disruptive departure from homeostasis necessarily invoked by disturbing the cells normal ability to utilize PI3K. Furthermore, experimental systems depending on unperturbed cells to act as the control condition and establish basal homeostasis necessarily ignore the possibility of significant interacting cross-talk between signaling systems co-dependent upon PI3K signaling.

Overcoming complications in revealing the role of PI3K in TLR signaling

Disrupting a cell's ability to utilize a signaling pathway downstream of only one family of receptors has been immensely useful in enhancing our understanding of various signaling systems employed during a biological response. In cell culture systems, signaling pathways can be discretely studied by experimenter-controlled application of targeted stimuli. This point can best be contextually illustrated through describing MyD88-dependent signaling, which as introduced earlier, impart a cell's responsiveness to both TLR stimulation and IL1R family member stimulation. In the field of TLR biology, whereby most TLRs utilize the signaling adapter MyD88, substantial progress has been made through selective stimulation of cells with purified ligands, thus restricting the responding cell to stimulation of only the targeted TLR, and subsequently MyD88-dependent events through the same TLR. This approach can become confounded during later events in which cells begin releasing IL1 family members, which also signal in a MyD88-dependent manner. However, because regulation of IL1 family cytokines further depends upon signals provided by the inflammasomes, experimenters have ignored the effect of autocrine IL1 family cytokine feedback because in the absence of an additional inflammasome ligand, release of IL1 family cytokines occurs in very limited concentrations. Thus, TLR signaling using in vitro culture systems can be temporally assessed with confidence over time frames spanning hours. These same assumptions however cannot be made in vivo when using whole-body MyD88-deficient mice. The biological importance of IL1 family members in the settings of vaccination, infection, chronic inflammation, and autoimmune disease is well established.

Thus, employment of MyD88-deficient mice as a tool to understand the role of TLRs in in vivo model systems necessarily requires consideration of IL1 family members in the response as well. To more specifically assess the role of TLRs or IL1R family members in a biological response, restriction of MyD88-deficiency to controlled cell populations has been used. These approaches have been useful in delineating the contributions of IL1 family members during T cell activation as well as revealing the importance of intrinsic TLR signaling by DCs for inducing T cell activation (Hu et al., 2011; Pasare and Medzhitov, 2003, 2004, 2005). Questions concerning intrinsic usage of TLRs/IL1R family members continue to be a well-studied and fruitful area of immunology.

Using the example of MyD88 above, a needed tool to address the usage of PI3K during TLR signaling events is a manner to selectively disrupt PI3K activity only downstream of TLRs. This can be done through employment of MyD88-deficient cells (Laird et al., 2009; Troutman et al., 2012a; Troutman et al., 2012b). However, this leads to disruption of all known signaling pathways downstream of MyD88-dependent TLRs. Thus, the relative contribution of PI3K in the signaling pathway cannot be revealed in systems restricting MyD88 signaling. Our finding that BCAP-deficient cells were restricted to their inability to activate PI3K with no defect in acute activation of neither NFκB nor MAP kinases represents a significant advance towards this problem. Thus, BCAP is the proximal branching point splitting TLR signaling towards activation of PI3K (**Error! Reference source not found.**). This finding promotes an idea whereby BCAP-deficient cells can be

used as a tool to further define the relative contributions of TLR-specific mediated PI3K signaling during a cellular response.

One question of importance which would be served through usage of BCAP-deficient cells would be the relative contribution of discrete TLR signaling branches to the transcriptional response. Through usage of BCAP-deficient cells, the contribution of TLR mediated PI3K signaling to the transcriptional response can be revealed. Furthermore, if cells with a targeted disruption of TRAF6 were also used, the relative contribution of TLR mediated NFκB and MAP kinase activation to the transcriptional response could also be revealed. Through mathematical comparison of these two data sets (and appropriate controls), an investigator can assess the proportion of the transcriptional response controlled by these signaling molecules, and thus the transcriptional controls imparted by these signaling branches. Furthermore, through employment of mathematical modeling, data of this type might also suggest the existence of other undescribed signaling pathways playing a significant role in the final transcriptional program induced by TLR signaling.

Patterns of control imposed upon the TLR/IL1R family by BCAP

Stimulation of macrophages with TLR ligands induces an inflammatory signaling cascade ultimately leading to induction of thousands of genes and secretion of inflammatory cytokines. Work presented here demonstrate that BCAP functions in macrophages to link TLRs to activation of PI3K, and in this role, BCAP functions to block levels of secreted cytokines (IL6, IL12, and TNF), which through the preliminary RNA sequencing

experiment, occurs through transcriptional regulation. The precise mechanism for this finding remains unknown. Prior studies found that phosphorylation and inactivation of glycogen synthase kinase 3β (GSK3β) by Akt activity leads to enhanced secretion of the regulatory cytokine IL10 (Beurel et al., 2010; Hazeki et al., 2007; Martin et al., 2005; Ohtani et al., 2008). Thus, GSK3β represses IL10 production in a manner counter-regulated by activation of PI3K. This initially led to the hypothesis that BCAP may function to suppress TLR mediated inflammation utilizing an axis composed of PI3K inhibition of GSK3β leading to enhanced IL10. The simplicity of this model was attractive as it was already established in the field and readily testable through available reagents. Surprisingly, culture supernatants from BCAP-deficient macrophages stimulated with TLR ligands had no reproducible differences in levels of IL10 although expression analysis did reveal control of IL10 message levels by BCAP. Further, activity of GSK3β was similar between BCAP-sufficient and BCAP-deficient cells stimulated with TLR ligands, seemingly invalidating the hypothesis. However, these experiments were preliminary and bear repetition. Thus, the mechanistic role BCAP-mediated activation of PI3K during TLR stimulation remains unknown.

Future experiments will be required to more fully understand the role of BCAP in TLR signaling. One intriguing observation is that BCAP is expressed as multiple isoforms (Okada et al., 2000; Troutman et al., 2012b; Yamazaki et al., 2002). The contribution of these various isoforms however remains unknown. One possibility is that the isoforms take advantage of the adapter characteristic of the TIR domain and ankyrin repeat domains within

BCAP. Thus, a BCAP isoform not containing the TIR domain would be unexpected to participate in TLR signaling. In this scenario, one might expect then that the relative activation of PI3K would be significantly reduced if sufficient TIR restricted BCAP were expressed. Indeed, according to the NCBI (http://www.ncbi.nlm.nih.gov/gene/83490) and UniPROT (http://www.uniprot.org/uniprot/Q9EQ32) databases, BCAP transcripts are expected to exist not encoding the TIR domain. The biological significance is not known.

There is also a need to further assess contribution of BCAP to TLR signaling in other cells types. We have reproducibly found that DC expression of BCAP is less important for control of inflammatory cytokine production than what has been reported for macrophages. The explanation for this observation is not known. One could speculate that perhaps the phenotypic differences existing between BCAP-deficient macrophages and DCs could lie in the relative contribution of PI3K activity to these cell types. Macrophages are long-lived tissue resident cells responsible for surveying and maintaining tissue sterility. Consequently, one might expect that signals through PI3K relay important and requisite survival signals for macrophages. Conversely, DCs upon activation are rapidly recruited through draining lymphatics to the T cells zones of lymphoid tissues. Here, after upon interaction with T cells, they are cleared by apoptosis. Thus, DCs may not need TLR mediated PI3K signaling as they are destined to die anyways. One crucial flaw in this argument lies in the observation that DCs with a genetic deletion for p85 produce enhanced inflammatory cytokines upon stimulation with TLR ligands (Fukao and Koyasu, 2003; Fukao et al., 2002).

In an attempt to reveal the potential mechanisms whereby BCAP asserts control on the TLR pathway, we performed a preliminary transcriptome analysis on macrophages stimulated through TLR9. With this approach and the help of our bioinformatics collaborators, we found 1001 genes differentially regulated by BCAP at 2-hours post-stimulation and 1026 genes differentially regulated by BCAP at 8-hours post-stimulation. These findings suggested that important cellular processes discriminately controlled by BCAP mediated signaling had a critical role in regulation of the inflammatory response by macrophages. By using Ingenuity Pathway Analysis as well as combined usage of the DAVID Bioinformatics Resource and the KEGG PATHWAY database, we discovered a significant role for BCAP in regulation of several biological pathways. We also discovered other gene networks that could play dominant roles in BCAP-mediated regulation of inflammation and colitis susceptibility, which could in part explain the susceptibility of BCAP-deficient mice to the DSS-colitis model. One network included a group of CXC chemokines that were overrepresented upon TLR stimulation of BCAP-deficient macrophages. This suggests that during barrier damage in the DSS-induced colitis model, myeloid cells responding to mislocalized commensal bacteria could utilize BCAP to limit chemokines production. This hypothesis could account for the exaggerated recruitment of inflammatory neutrophils and other innate immune cells observed in BCAP-deficient mice.

Further, BCAP-deficient cells were also defective for induction of dual specificity phosphatases (DUSPs), transcriptionally regulated phosphatases that block signal transduction of MAP kinases (Caunt and Keyse, 2012). In the IL10-deficient model of colitis,

mice doubly deficient in IL10 and Dusp1 have increased disease scores and tissue concentrations of multiple inflammatory cytokines (Matta et al., 2012). Therefore, BCAP could be regulating DSS-colitis susceptibility through providing the upstream transcriptional signals involved in induction of *Dusp* genes.

A third transcriptional program found to be regulated by BCAP contained genes enriched in the PPARγ pathway. In humans, a series of clinical trials found that a subset of IBD patients responded beneficially to agonistic stimulation of PPARγ (Milestone et al., 2008; Pedersen and Brynskov, 2010; Wada et al., 2001). Furthermore, PPARγ is established as a key regulator controlling conversion of inflammatory M1 macrophages to regulator M2 macrophages (Novak and Koh, 2013; Sica and Mantovani, 2012). These data suggest that BCAP expression may be crucial for controlling a PPARγ dependent conversion of inflammatory M1 macrophages to the M2 phenotype. As M2 macrophages play an important role in the tissue regenerative response upon damage (Novak and Koh, 2013; Olefsky and Glass, 2010; Pull et al., 2005; Sica and Mantovani, 2012), we further question if susceptibility of BCAP-deficient animals to regulate colitis could in part be due to an inability to restore the epithelial barrier during the course of the disease.

Furthermore, PPARγ has been previously linked to control of inflammation, and mice deficient in PPARγ are susceptible to diabetes and atherosclerosis (Ogawa et al., 2005; Olefsky and Glass, 2010). This suggests that BCAP could be involved in regulation of obesity-induced diabetes. In an intriguing and promising preliminary experiment, we indeed

found that BCAP-deficient mice were resistant to weight gain when given a high-fat diet. Furthermore, BCAP-deficient mice were observed to have increased mortality associated with the treatment. Though preliminary and requiring reproduction in a more controlled setting with a larger cohort of mice, these data suggest a promising direction for future exploration.

Dissecting the Role of the IL1R Family in Regulating Naïve T-Helper Cell Differentiation

Given the important role of helper T cells in protection from infectious diseases, as well as their contribution to auto-immune diseases, understanding of the pathways regulating these cells is crucial. The role for IL1R family signaling in regulation of CD4 T cell differentiation is well established (Acosta-Rodriguez et al., 2007; Chung et al., 2009; Gabay et al., 2010; Garlanda et al., 2013; Guo et al., 2009; Hu et al., 2011; Robinson et al., 1997; Smith, 2011; Staschke et al., 2009; Sutton et al., 2006). However, the contributions of the various signaling pathways remain elusive. In addition to participation in the TLR signaling pathway, data presented here also links BCAP to signaling downstream of the IL1R family. In this capacity, BCAP plays a clear role in promoting the survival and expansion of T cells during priming. It seems likely, give previous data (Inabe and Kurosaki, 2002; Ni et al., 2012; Okada et al., 2000; Troutman et al., 2012a; Troutman et al., 2012b), that BCAP will function to connect

the IL1R family to activation of PI3K in these T cells, yet this will require experimental validation. Furthermore, other factors acting further downstream of BCAP during stimulation of differentiating T cells remains to be explored. A clear role for the PI3K signaling axis has been established for many aspects of T cell biology, including Th17 differentiation, but the precise manner in which the pathway is utilized is not clear (Chang et al., 2013; Delgoffe et al., 2011; Kurebayashi et al., 2012; Pierau et al., 2009; Wan et al., 2011; Ward, 2004). If BCAP is shown to function as PI3K activating adapter through the IL1R family, then just as with TLRs, BCAP-deficient T cells will be an important tool for further dissecting the mechanistic contribution IL1 family cytokines have on T cell biology.

One hypothesis we currently favor is a putative role for FOXO transcription factor family members downstream of BCAP provided signals for promotion of T cell differentiation. To support this idea, FOXO1 was found to promote inflammatory cytokine production by LPS stimulated cells (Brown et al., 2011; Fan et al., 2010; Su et al., 2009). Mechanistically, FOXO1 is regulated by an inhibitory signal provided by Akt. Thus, this finding can be extended to suggest that BCAP signaling downstream of TLRs promotes the activation of PI3K/Akt, which in turn leads to blockade of FOXO1 activity, leading to reduced transcriptional activity of FOXO1 and less inflammatory transcript production. Given this data, it is possible that BCAP signaling downstream of IL1R family members could also lead to inhibited FOXO1 activity. In T cells, FOXO transcription factors are known to play a multitude of roles, including regulation of *Il7ra* message levels (FOXO1), inhibition of cell cycling (FOXO3), and the development of natural regulatory T cells

(FOXO1 and FOXO3) (Hedrick et al., 2012). Given the important role for FOXO family transcription factors in T cells, as well as the link of TLR signaling to FOXO1 family inactivation, it could prove fruitful to expand future studies on the role of BCAP signaling downstream of IL1R family members to include assessing FOXO activity.

Overall Conclusions

The goal for studying TLR/IL1R signaling is to reveal the how these receptors initiate and control an inflammatory response. Insights into these pathways have proven instrumental to our understanding of both beneficial immune responses as well as debilitating auto-immune diseases. The work presented here provides clarity into the mechanistic way in which TLRs mediate activation of the PI3K pathway. A great body of research has led to an in depth understanding of the biochemical pathways regulating activation of NFκB, MAP kinases, and IRFs during TLR signaling. Comparatively little was known, however, about the mechanism of TLR mediated PI3K activation. Further, the role PI3K plays during TLR also requires additional research. Our finding that BCAP functions as critical TIR-domain containing TLR signaling adapter responsible for linking TLRs to activation of PI3K will serve as a valuable tool towards enhancing our understanding of this pathway. In addition, these studies further place BCAP as a signaling adapter downstream of IL1R family members, providing further insight into the mechanisms controlling the differentiation of Th17 cells.

Together, our findings have enhanced knowledge immediately applicable to human health, including vaccine design, inflammatory colitis, multiple sclerosis, as well as metabolic inflammatory diseases. The focus of future studies on the contribution of BCAP using *in vivo* model systems pertinent to these aforementioned areas will greatly enhance both our understanding of the immune process as well as suggest avenues for tailored design of therapeutics and vaccines.

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