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ARTICLE

B cell adaptor for PI3-kinase (BCAP) modulates CD8+ effector and memory T cell differentiation

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CD8+ T cells respond to signals via the T cell receptor (TCR), costimulatory molecules, and immunoregulatory cytokines by developing into diverse populations of effector and memory cells. The relative strength of phosphoinositide 3-kinase (PI3K) signaling early in the T cell response can dramatically influence downstream effector and memory T cell differentiation. We show that initial PI3K signaling during T cell activation results in up-regulation of the signaling scaffold B cell adaptor for PI3K (BCAP), which further potentiates PI3K signaling and promotes the accumulation of CD8+ T cells with a terminally differentiated effector phenotype. Accordingly, BCAP-deficient CD8+ T cells have attenuated clonal expansion and altered effector and memory T cell development following infection with *Listeria monocytogenes*. Thus, induction of BCAP serves as a positive feedback circuit to enhance PI3K signaling in activated CD8+ T cells, thereby acting as a molecular checkpoint regulating effector and memory T cell development.

Introduction

Infection with intracellular pathogens provokes strong CD8+ cytotoxic T cell responses that help eliminate the pathogen and provide protection from reinfection. This is accomplished through the robust proliferative expansion of pathogen-specific CD8+ T cells such that they increase from a small number of naive precursors to represent up to 30–40% of total CD8+ T cells at the peak of the response. Following pathogen clearance, ∼90% of these effector cells undergo programed cell death, and the remaining cells form a stable, long-lived memory cell pool. Importantly, pioneering work over the last decade has demonstrated that this CD8+ T cell response is not uniform but instead is composed of several distinct populations of effector and memory cells that fulfill specialized roles in pathogen elimination and protective immunity (Cui and Kaech, 2010). These include distinct effector cell populations such as terminal effector cells (TECs) and memory precursor effector cells (MPECs) that differ in their effector potential and their contribution to the long-lived memory cell pool. Additionally, specialized memory cell populations have been described, such as effector memory T cells (T_{EM}), which recirculate through nonlymphoid tissues, have limited proliferative potential, and display immediate effector function upon restimulation, and central memory T cells (T_{CM}), which recirculate through secondary lymphoid organs and retain extensive proliferative capacity, thereby acting as a reservoir of antigen-specific cells that can be rapidly expanded upon secondary infection. Additionally, recently characterized tissue-resident memory T cells do not recirculate in the blood and lymph but instead establish long-term residency in nonlymphoid tissues where they provide a potent first line of defense against reinfection (Gebhardt and Mackay, 2012).

Although the signals that regulate the phenotypic and functional diversity of effector and memory CD8+ T cells are complex, it is clear that the extent of signaling from the TCR, costimulatory receptors, and immunomodulatory cytokines can dramatically impact CD8+ effector and memory T cell generation (Chang et al., 2014; Kim and Harty, 2014). For instance, during acute infection, strong IL-12 signaling in responding CD8+ T cells favors the development of TECs and T_{EM} cells at the expense of MPECs and T_{CM} cells (Joshi et al., 2007). Similarly, TEC and T_{EM} differentiation is also favored by strong TCR and costimulatory receptor signaling, whereas weaker signals favor T_{CM} cell formation (Chang et al., 2014). Downstream of these external signals, several transcription factor networks help control effector and memory CD8+ T cell differentiation (Gray et al., 2014). These include the t-box transcription factors T-bet and eomesodermin (Eomes); E proteins and their inhibitors Id2 and Id3; and other transcription factors such as Foxo1, Zeb-2, TCF-1, and Blimp-1. Collectively, these data highlight the concept that extrinsic signals from the immune environment direct the differentiation of CD8+ effector and memory T cell populations through induction of distinct transcriptional regulators that control cell phenotype and function. However, the molecular basis by which activated CD8+ cells

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integrate and interpret these signals during effector and memory differentiation is still poorly understood.

During CD8⁺ T cell activation, engagement of the TCR and costimulatory and cytokine receptors results in the activation of multiple signaling pathways, which ultimately lead to the changes in gene expression and cell behavior associated with different effector and memory T cell differentiation. Of these, activation of the phosphoinositide 3-kinase (PI3K) signaling cascade has dramatic consequences on T cell responses by influencing cell proliferation and survival, metabolic reprogramming, cellular migration and tissue tropism, acquisition of effector function, and generation of long-lived memory cells (Han et al., 2012; Kim and Suresh, 2013). Class I PI3Ks catalyze the phosphorylation of $PI(4,5)P_2$ to generate the second messenger $PI(3,4,5)P_3$, which subsequently acts to recruit proteins containing pleckstrin homology domains to the plasma membrane and initiates signaling cascades that proceed through the Akt/mTor and phospholipase $C\gamma/Ca^{2+}$/RAS pathways. PI3K signaling during T cell activation is essential for the proper clonal expansion of antigen-specific T cells (Shi et al., 1997). Additionally, strong PI3K-dependent Akt activation favors the generation of short-lived TECs at the expense of the longer-lived MPECs (Rao et al., 2010, 2012; Kim and Suresh, 2013), and manipulation of PI3K signaling can be used to alter the balance of effector and memory T cell generation (Macintyre et al., 2011; Kim et al., 2012). The influence of PI3K signaling on T cell differentiation is due in part to the ability of Akt to regulate expression of cytokine and chemokine receptors such as the IL-7R and CCR7 via phosphorylation and inhibition of the key transcriptional regulator Foxo1 (Fabre et al., 2008; Haxhinasto et al., 2008). Additionally, PI3K signaling promotes cell proliferation and metabolic reprogramming of cells based on Akt-mediated phosphorylation of glycogen synthase kinase 3β (GSK3β), and by activation of mTOR signaling complexes TORC1 and TORC2. Importantly, recent data have highlighted the concept that PI3K signaling in lymphocytes should not be viewed as a binary on/off switch but instead acts more as a rheostat in which graded amounts of PI3K signaling result in vastly different cellular responses (So and Fruman, 2012). This highlights the need to understand how PI3K activity is controlled by multiple inputs during T cell activation in order to pharmacologically promote or inhibit different types of T cell responses.

Despite the dramatic impact of PI3K signaling on T cell responses, the mechanisms by which PI3K is activated during T cell activation and differentiation are still poorly defined. Of the class I PI3Ks, p110δ appears to play the dominant role in T cell activation/differentiation (So and Fruman, 2012). In resting T cells, p110δ exists in a catalytically inactive state and is bound by the regulatory p85 subunit. The p85/p110δ complex is recruited to sites of active signaling via interaction between SH2 domains in p85 and phosphorylated tyrosines contained within YxxM motifs in signaling adaptors. This leads to a conformational change in p85, resulting in increased catalytic activity of the associated p110δ. In T cells, the costimulatory receptors CD28 and ICOS both contain YxxM motifs in their cytoplasmic tails, and, thereby, both can activate PI3K signaling (Arimura et al., 2002; Parry et al., 2003). Surprisingly, however, mutation of the YxxM motif in either CD28 or ICOS had a minimal impact on the clonal expansion of antigen-specific T cells in vivo (Gigoux et al., 2009; Pagán et al., 2012). Additionally, signaling through the IL-2 receptor can activate PI3K via phosphorylation of the adaptor protein shc (Gu et al., 2000). Finally, cross-linking of the TCR-associated CD3 complex also activates PI3K, although the mechanisms underlying this activation are poorly defined. The signaling adaptor TRIM was identified as a YxxM-containing protein that associates with the CD3 complex, is phosphorylated by Src-family kinases upon TCR engagement, and can bind p85 and potentiate PI3K activation in Jurkat T cells (Bruyns et al., 1998). However, TRIM-deficient mice showed normal T cell development and function in vivo, and Akt phosphorylation following α-CD3 cross-linking was actually enhanced in the absence of TRIM (Kölsch et al., 2006). Other adaptors implicated in PI3K activation following TCR/CD3 stimulation include SLP-76, LAT, and TIM-3 (Lee et al., 2011; Shim et al., 2011). Additionally, activation of PI3K by ZAP-70 or Src-family kinases has been proposed (Pleiman et al., 1994; Moon et al., 2005), as has direct activation of p110δ by Ras (Rodriguez-Viciana et al., 1994), but the importance of these mechanisms of CD3-mediated PI3K activation for T cell proliferation and differentiation in vivo is not clear.

As its name implies, the B cell adaptor for PI3K (BCAP) protein was originally identified as a B cell–expressed protein that can recruit and activate PI3K signaling when phosphorylated on its four YxxM motif tyrosines after BCR ligation (Okada et al., 2000). Additionally, BCAP is highly expressed in several myeloid cell populations, where it regulates PI3K activation downstream of Toll-like receptor signaling (Ni et al., 2012; Troutman et al., 2012). BCAP is a relatively large ∼91-kD protein, and in addition to its YxxM sequences, it contains numerous potential protein–protein interaction domains, including an N-terminal DBB domain, an ankyrin repeat region, a coiled coil domain, and several C-terminal proline-rich regions. However, BCAP does not contain any obvious enzymatic domains. Therefore, BCAP has the potential to act as a signaling adaptor that can integrate different inputs and facilitate and coordinate activation of multiple downstream signaling pathways including PI3K. Although not expressed in naive T cells (Yamazaki et al., 2002), we found that BCAP is rapidly up-regulated in CD8⁺ T cells upon activation in a PI3K-dependent manner. In the absence of BCAP, activated CD8⁺ T cells showed impaired CD3-dependent PI3K activation and blunted clonal expansion associated with altered effector and memory T cell differentiation in vivo. Thus, we have identified BCAP as a critical signaling hub in T cells that is up-regulated upon initial activation and helps coordinate CD8⁺ T cell responses through activation of PI3K signaling.

Results

BCAP is up-regulated in activated T cells

BCAP is constitutively expressed in B cells, NK cells, and myeloid cells but has not previously been found in T cells (Yamazaki et al., 2002; MacFarlane et al., 2008; Ni et al., 2012). However, analysis of publically available data from the Immgen Consortium showed that BCAP (gene name *Pik3ap1*) is rapidly up-regulated in OVA-specific CD8⁺ T cells responding to infection with recombinant *Listeria monocytogenes* (LM) OVA or vesicular sto-

matitis virus–OVA (Best et al., 2013). Indeed, following LM-OVA infection, increased *Pik3ap1* expression was detected as early as 12–24 h after infection, reached a peak by 6 d after infection, and was maintained in long-lived memory cells to at least 100 d after infection (Fig. 1 A). By intracellular staining with a BCAP-specific monoclonal antibody, we confirmed that although expression could not be detected in naive CD8$^+$ T cells directly ex vivo, BCAP was detectably expressed within 1 d of stimulation with plate-bound anti-CD3/anti-CD28 in vitro and further up-regulated by day 2 (Fig. 1 B). Moreover, analysis of BCAP expression in CFSE-labeled CD8$^+$ T cells 1 d after stimulation revealed that BCAP could be detected in activated CD25$^+$ cells even before initiation of cell division and thus is poised to influence early events in the clonal expansion and functional differentiation of CD8$^+$ T cells (Fig. 1 C). Similarly, activated CD4$^+$ T cells also up-regulated BCAP, and expression was higher when cells were cultured in Th1-polarizing conditions vs. Th2-polarizing conditions (Fig. 1 D). We also observed BCAP expression in human effector/memory CD8$^+$ T cells, particularly in CD45RA$^-$CCR7$^-$ T_{EM} cells and in terminally differentiated CD45RA$^+$CCR7$^-$ T_{EM}RA cells (Fig. 1 E).

Similar to what has been observed in macrophages and B cells, Western blot analysis of activated CD8$^+$ T cells showed two dominant BCAP isoforms, a full-length ∼97-kD isoform and a short ∼64-kD isoform that lacks the N-terminal domain (Fig. 2 A). Additionally, as in activated B cells, BCAP was tyrosine phosphorylated in activated CD8$^+$ T cells, and coimmunoprecipitation showed association with the p85 regulatory subunit of PI3K (Fig. 2, B and C). Thus, rapid induction of BCAP in activated CD8$^+$ T cells may influence PI3K activation/signaling during T cell clonal expansion and effector/memory T cell differentiation.

BCAP expression in CD8$^+$ T cells is modulated by PI3K signaling, T-bet, and IRF4

To identify factors that regulate BCAP induction upon T cell activation, we mined publically available epigenetic and chromatin immunoprecipitation (ChIP) sequencing data to assess changes in chromatin structure and transcription factor binding that occur at the *Pi3kap1* locus during T cell activation. Consistent with rapid BCAP up-regulation, CD8$^+$ T cell activation and differentiation into effector cells were associated with opening of the *Pik3ap1* locus at several sites identified by ATAC-seq analysis, and these sites were further decorated with $H3K27_{AC}$ histone modifications, indicative of active enhancers (Fig. 3 A). This was particularly evident in the large intron between exons 2 and 3 of the *Pi3kap1* gene. Interestingly, in naive CD8$^+$ T cells the transcription factor Foxo1 is bound to multiple sites in the *Pi3kap1* locus, and these overlap with several of the ATAC-seq peaks identified in this population. PI3K signaling in CD8$^+$ T cell results in the Akt-mediated phosphorylation of Foxo1, leading to its nuclear exclusion and changes in expression of Foxo1-regulated genes. Thus, we hypothesized that induction of BCAP depends on PI3K-dependent inactivation of Foxo1, and that BCAP can therefore act in a positive feedback loop to amplify PI3K signaling during T cell activation. Indeed, we found that blocking PI3K signaling using the pan class I PI3K inhibitor ZSTK474 potently inhibited BCAP induction during CD8$^+$ T cell activation in vitro, while having only minimal effects on cell proliferation or expression of other activation markers such as CD69 (Fig. 3 B). Additionally, RNA sequencing (RNA-seq) analysis of activated CD8$^+$ T cells expressing a constitutively activated allele of Foxo1 showed significantly diminished up-regulation of the *Pik3ap1* mRNA compared with control WT cells (Fig. 3 C). Elevated expression of BCAP in TH1 vs. TH2 polarized cells (Fig. 1 D) suggests that in addition to Foxo1, lineage-specific factors help control the level of BCAP expression in effector T cells. Differentiation of both TH1 cells and effector CD8$^+$ T cells relies on expression of the transcription factor T-bet, and ChIP-seq analyses identified several active enhancers in the *Pik3ap1* locus that were bound by T-bet in TH1 cells (Fig. 3 A; GEO accession no. GSE33802). Similarly, the transcription factor Irf4, which helps control effector and memory T cell differentiation, is also bound at the *Pik3ap1* locus in activated T cells (GEO accession no. GSE49930), and consistent with a role for these transcription factors in promoting BCAP expression, activated T cells lacking either of these proteins showed impaired *Pik3ap1* induction in RNA-seq analysis (Fig. 3 D; GEO accession nos. GSE68056 and GSE49929). Taken together, these data suggest that optimal induction of BCAP expression during T cell activation occurs in a multistep process that requires both the PI3K-dependent inhibition of Foxo1 and the cooperative functions of the effector transcription factors T-bet and Irf4.

BCAP potentiates PI3K signaling in response to CD3 engagement in activated T cells

During B cell activation, BCAP serves as a critical adaptor protein that links engagement of the BCR to activation of PI3K (Okada et al., 2000). In T cells, the molecular pathways linking the T cell receptor to activation of PI3K are poorly defined, and thus, we hypothesized that BCAP may function analogously in activated T cells to amplify PI3K signaling downstream of antigen receptor engagement. To test this, we assessed PI3K activation by measuring Akt and S6 phosphorylation in activated CD8$^+$ T cells from WT or BCAP-deficient mice following restimulation with plate-bound anti-CD3. Consistent with BCAP expression only in activated T cells, there was no difference in the extent of PI3K-dependent S6 phosphorylation in anti-CD3–stimulated naive CD8$^+$ T cells from WT and BCAP-deficient mice (now shown). By contrast, in activated CD8$^+$ T cells, phosphorylation of Akt and S6 in response to anti-CD3 stimulation was significantly decreased in BCAP-deficient cells (Fig. 4, A and B). Importantly, no defect was observed in IL-2–mediated Akt–S6 phosphorylation in BCAP-deficient cells, indicating that loss of BCAP does not lead to a general defect in PI3K signaling (not shown).

To determine if BCAP modulates signaling during CD8$^+$ T cell activation in vivo, we cotransferred OVA-specific TCR transgenic OT-I cells from WT (CD45.1$^+$) or BCAP-deficient (CD45.2$^+$) OT-I mice into intact CD45.1$^+$/.2$^+$ recipients and infected these mice 24 h later with recombinant LM-OVA. To further facilitate identification of the transferred cells and to correlate cell signaling events with different stages of cell division, the donor OT-I cells were also CFSE labeled before transfer. At 54 h after infection, cells were harvested from the spleens of the recipient animals, and S6 phosphorylation in each donor T cell population was assessed by flow cytometry directly ex vivo (without any further in vitro restimulation). At this time point, proliferation was ob-

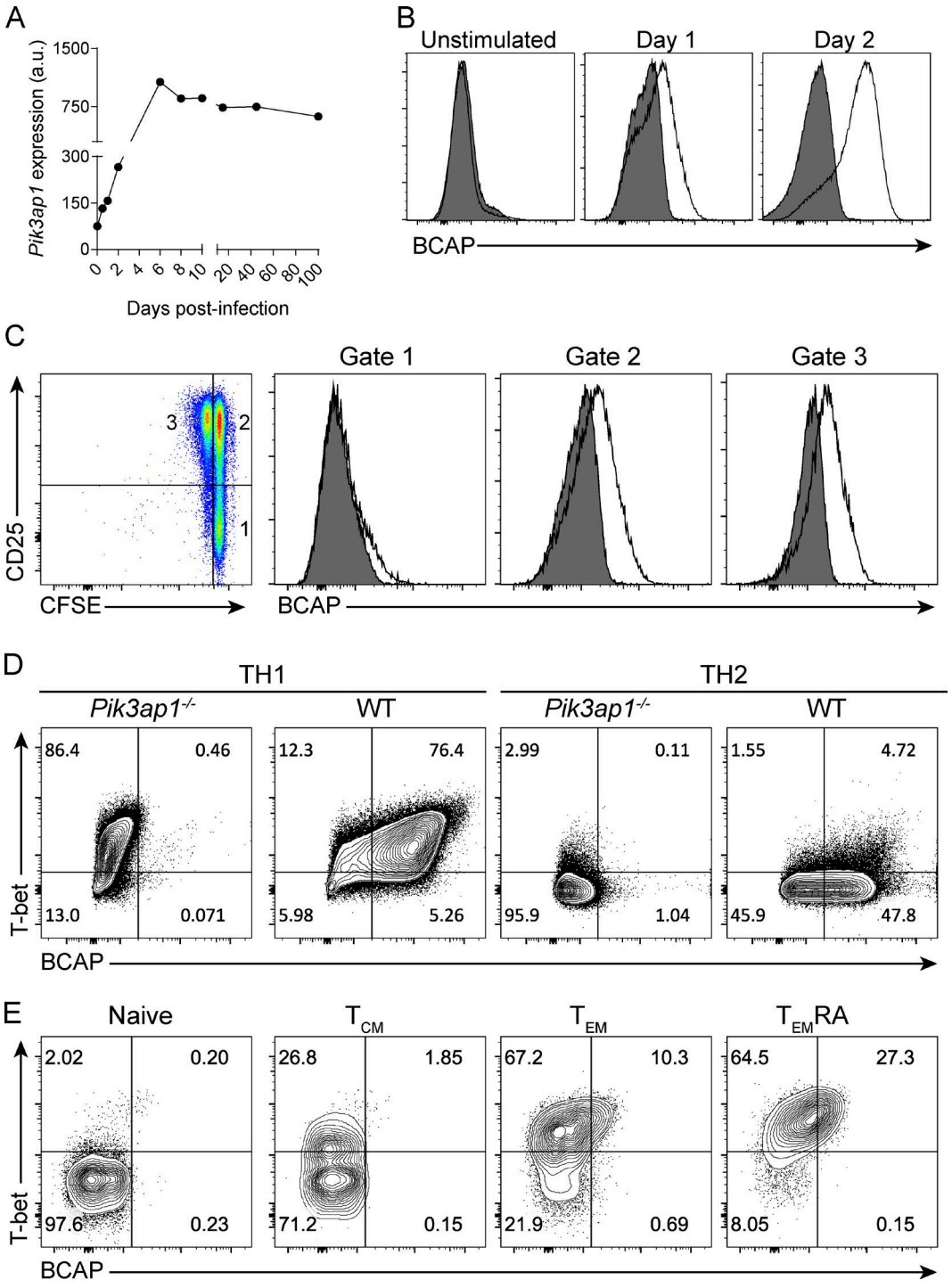

Figure 1. **BCAP is up-regulated in activated CD8⁺ T cells. (A)** Expression of *Pik3ap1* mRNA by splenic CD8⁺ OT-I T cells at the indicated times following infection with LM-OVA. Data are from the Immunological Genome Project. **(B)** Flow cytometry analysis of BCAP expression by CD8⁺ T cells from WT (open histograms) or *Pik3ap1*$^{-/-}$ (filled histograms) mice at the indicated times following stimulation with plate-bound anti-CD3/anti-CD28. Data are representative of more than five independent experiments. **(C)** Flow cytometry analysis of CFSE dilution and CD25 expression by WT CD8⁺ T cells activated for 29 h with plate-bound anti-CD3/anti-CD28 + IL-2 (left) and BCAP expression by WT (open histograms) or *Pik3ap1*$^{-/-}$ (filled histograms) cells in each of the indicated gates. **(D)** Flow cytometry analysis of BCAP and T-bet expression by WT or *Pik3ap1*$^{-/-}$ CD4⁺ T cells activated and polarized under TH1 or TH2 conditions as indicated. **(E)** Flow cytometry analysis of BCAP and T-bet expression by gated naive, T_{CM}, T_{EM}, and T_{EM}RA CD8⁺ T cells from human peripheral blood as indicated. **(C–E)** Data are representative of three independent experiments.

served in both WT and BCAP$^{-/-}$ OT-I cells, indicating that both populations had undergone antigen-driven activation. However, in each recipient mouse, the fraction of cells with high levels of S6 phosphorylation was greater in WT cells, demonstrating that loss of BCAP impairs antigen-dependent signaling in CD8⁺ T cells in vivo (Fig. 4, C and D).

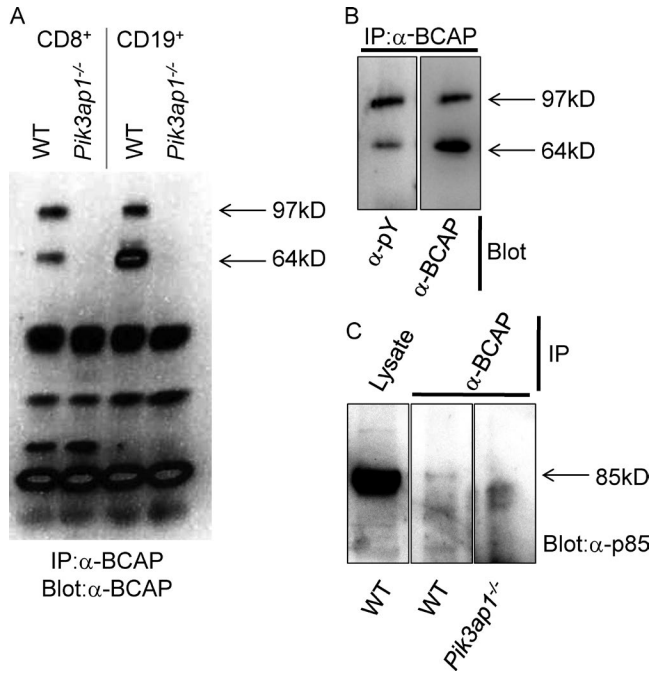

Figure 2. **BCAP is phosphorylated and associated with PI3K in activated T cells. (A)** Immunoprecipitation (IP) and Western blot analysis of BCAP expression by WT or *Pik3ap1*$^{-/-}$ CD8$^+$ T cells activated for 72 h with plate-bound anti-CD3/anti-CD28 + IL-2 or splenic CD19$^+$ B cells directly ex vivo as indicated. Data are representative of three independent experiments. **(B)** Western blot analysis using anti-phosphotyrosine (4G10) was performed on BCAP immunoprecipitate from WT CD8$^+$ T cells that had been stimulated with anti-CD3/anti-CD28 + IL-2 for 48 h and rested for 12 h (left). The blot was then stripped and reprobed with an anti-BCAP antibody (right). **(C)** Western blot analysis of the p85 subunit of PI3K on BCAP immunoprecipitate from WT or *Pik3ap1*$^{-/-}$ CD8$^+$ T cells that had been stimulated with anti-CD3/anti-CD28 + IL-2 for 48 h and rested for 12 h as indicated. Data are from the same blot with intervening lanes omitted for clarity. **(B and C)** Data are representative of two independent experiments.

BCAP modulates effector and memory T cell differentiation

The magnitude of PI3K signaling during CD8$^+$ T cell activation is a critical factor that modulates their clonal expansion and differentiation into specific effector and memory cell populations. Importantly, differences in signals delivered in the initial phases of T cell activation can result in dramatic alterations in T cell phenotype and function in both the effector and memory phases of the response (Yang et al., 2011). Therefore, to analyze the cell intrinsic function of BCAP throughout the course of the CD8$^+$ T cell response to infection, we cotransferred WT and BCAP-deficient OT-I$^+$ cells into congenically marked recipients and subsequently infected with LM-OVA as described above. We then assessed the clonal expansion, tissue localization, phenotype, and function of each donor population at various times after infection. Examining cells at the peak of the effector response on day 7 after infection, we found that accumulation of BCAP-deficient OT-I cells was reduced approximately three- to fivefold in the spleen compared with WT OT-I cells (Fig. 5, A and B). More strikingly, formation of long-lived memory cells is severely impaired in the absence of BCAP, and contraction of BCAP$^{-/-}$ OT-I cells between days 7 and 42 after infection was ~10-fold greater than that observed for WT cells. Examining OT-I cells accumulating in the

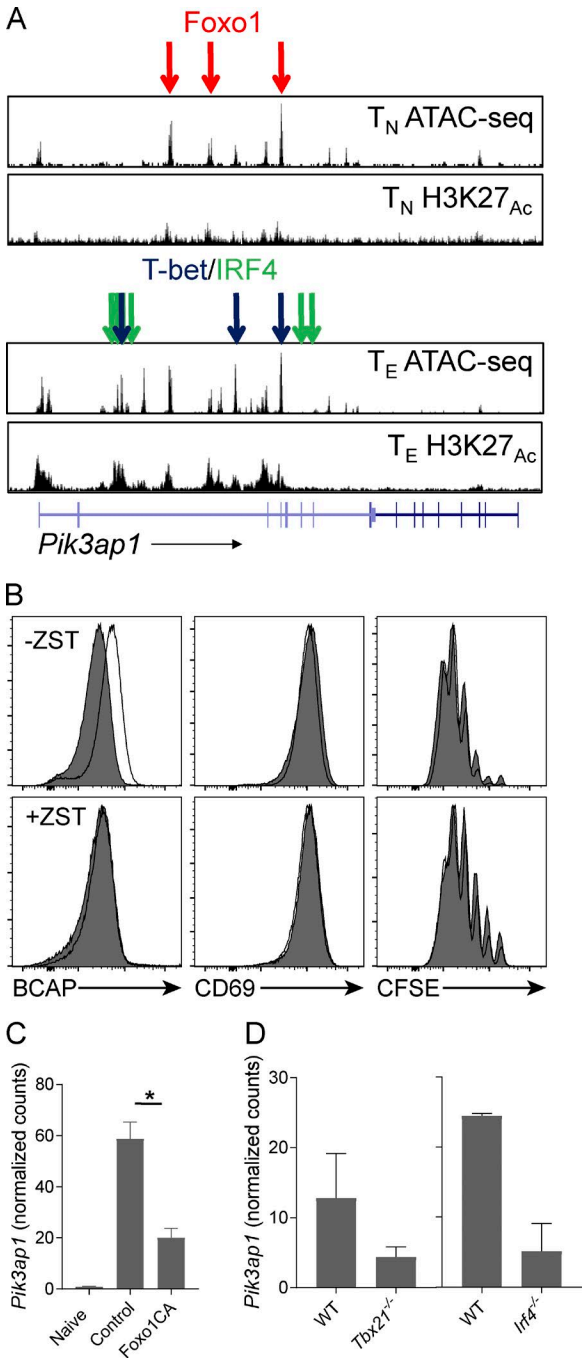

Figure 3. **Control of BCAP expression in CD8$^+$ T cells. (A)** Distribution of ATAC-seq peaks and H3K27$_{Ac}$ histone modifications in naive (T_N) and effector (T_E) CD8$^+$ T cells were mapped to the Pik3ap1 locus in the University of California at Santa Cruz (UCSC) genome browser. Foxo1, T-bet, and IRF4 binding sites were further mapped to the *Pik3ap1* locus based on publicly available ChIP-seq data. Data are from GEO under accession nos. GSE95237, GSE46943, GSE49930, and GSE33802. **(B)** Flow cytometry analysis of BCAP expression by CD8$^+$ T cells from WT (open histograms) or *Pik3ap1*$^{-/-}$ (filled histograms) mice, which were stimulated with plate-bound anti-CD3/anti-CD28 + IL-2 for 48 h with or without the class I PI3K inhibitor ZSTK474 as indicated. Data are representative of four independent experiments. **(C)** Expression of *Pik3ap1* mRNA in naive CD8+ T cells or in activated CD8$^+$ T cells from WT mice or Foxo1CA mice as determined by RNA-seq. Cells were from three mice per group. *, adjusted P ≤ 0.05 as determined by a linear model. **(D)** Expression of *Pik3ap1* mRNA (mean ± SD) in activated CD8$^+$ T cells from WT, *Tbx21*$^{-/-}$, or *Irf4*$^{-/-}$ mice as determined by RNA-seq. Data are from GEO under accession nos. GSE68056 and GSE49929.

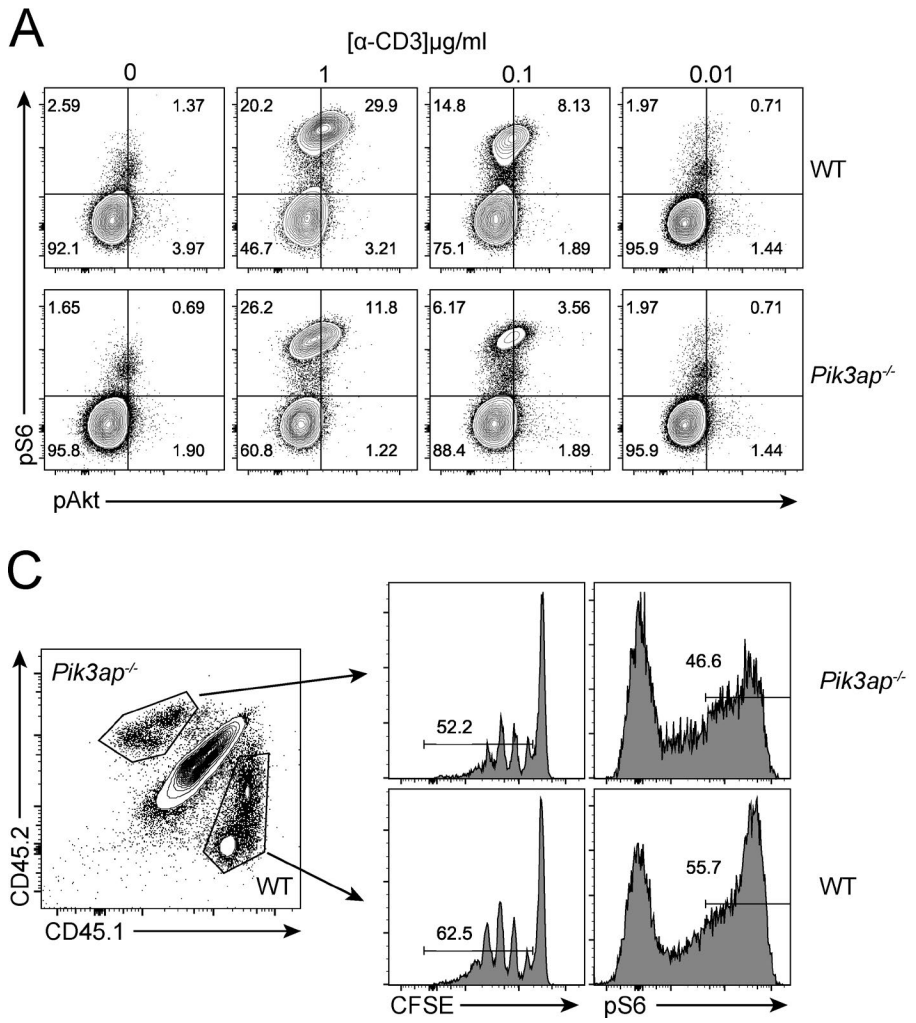

Figure 4. **BCAP potentiates PI3K signaling in activated CD8⁺ T cells. (A)** CD8⁺ T cells from WT or *Pik3ap1*$^{-/-}$ mice were activated for 48 h with plate-bound anti-CD3/anti-CD28 + IL-2 and then rested overnight in media alone before restimulation for 90 min with the indicated concentrations of plate-bound anti-CD3. Flow cytometry analysis of Akt and S6 phosphorylation in restimulated cells as indicated. Data are representative of five independent experiments. **(B)** Graphical analysis of Akt and S6 phosphorylation by WT and *Pik3ap1*$^{-/-}$ CD8⁺ T cells (mean ± SEM) in response to different concentrations of anti-CD3. Data are compiled from four independent experiments. Overall statistical significance is determined by two-way paired ANOVA, and multiple comparisons were performed using Sidak's multiple comparisons test. *, P ≤ 0.05. **(C)** CFSE-labeled CD8⁺ T cells from WT (CD45.1⁺) or *Pik3ap1*$^{-/-}$ (CD45.2⁺) OT-I mice were cotransferred into CD45.1⁺CD45.2⁺ recipient animals, which were subsequently infected with LM-OVA. Flow cytometry analysis of CD45.1/CD45.2 expression, CFSE dilution, and S6 phosphorylation by gated splenic CD8⁺ T cells as indicated 54 h after infection. **(D)** Graphical analysis of S6 phosphorylation in gated WT and *Pik3ap1*$^{-/-}$ splenic OT-I T cells. Linked points represent values from cotransferred WT and *Pi3kap1*$^{-/-}$ OT-I T cells recovered from individual recipient mice. **(C and D)** Data are representative of 10 mice analyzed in two independent experiments. Statistical significance was determined by paired Student's *t* test. *, P ≤ 0.05.

lungs as a representative nonlymphoid tissue, this phenotype was even more dramatic, with an ∼100-fold difference in the number of WT and BCAP-deficient OT-I cells present at this site at day 42 after infection.

Effector CD8⁺ T cells can be divided based on differential expression of the markers KLRG1 and CD127 into distinct populations with varying effector function, proliferative potential, and ability to form long-lived memory cells. Most extreme among these are the KLRG1⁺CD127^{lo} TECs, which are potent effector cells but have little potential for long-term memory formation, and KLRG1⁻CD127⁺ MPECs, which are relatively poor effector cells but highly proliferative and give rise to long-lived memory cells capable of robust clonal expansion and effector differentiation upon secondary infection (Joshi et al., 2007). Additionally, KLRG1⁺CD127⁺ double-positive effector cells (DPECs) and KLRG1⁻CD127⁻ early effector cells (EECs) have been described, but their functional significance and contributions to pathogen clearance and memory cell formation are not well defined. Consistent with the notion that strong PI3K signaling favors differentiation of TECs (Rao et al., 2010), at day 7 after infection we observed a significant reduction in the frequency of KLRG1⁺CD127⁻ cells among BCAP-deficient OT-I cells compared with WT OT-I cells in both spleen and lungs (Fig. 5, C and D; and not shown). Similarly, the frequency of EECs was reduced among BCAP-deficient OT-I cells, whereas the proportion of DPECs was significantly increased in the BCAP-deficient population, and no difference in the frequency of MPECs was observed. Consistent with decreased TEC formation, BCAP-deficient OT-I cells also showed diminished

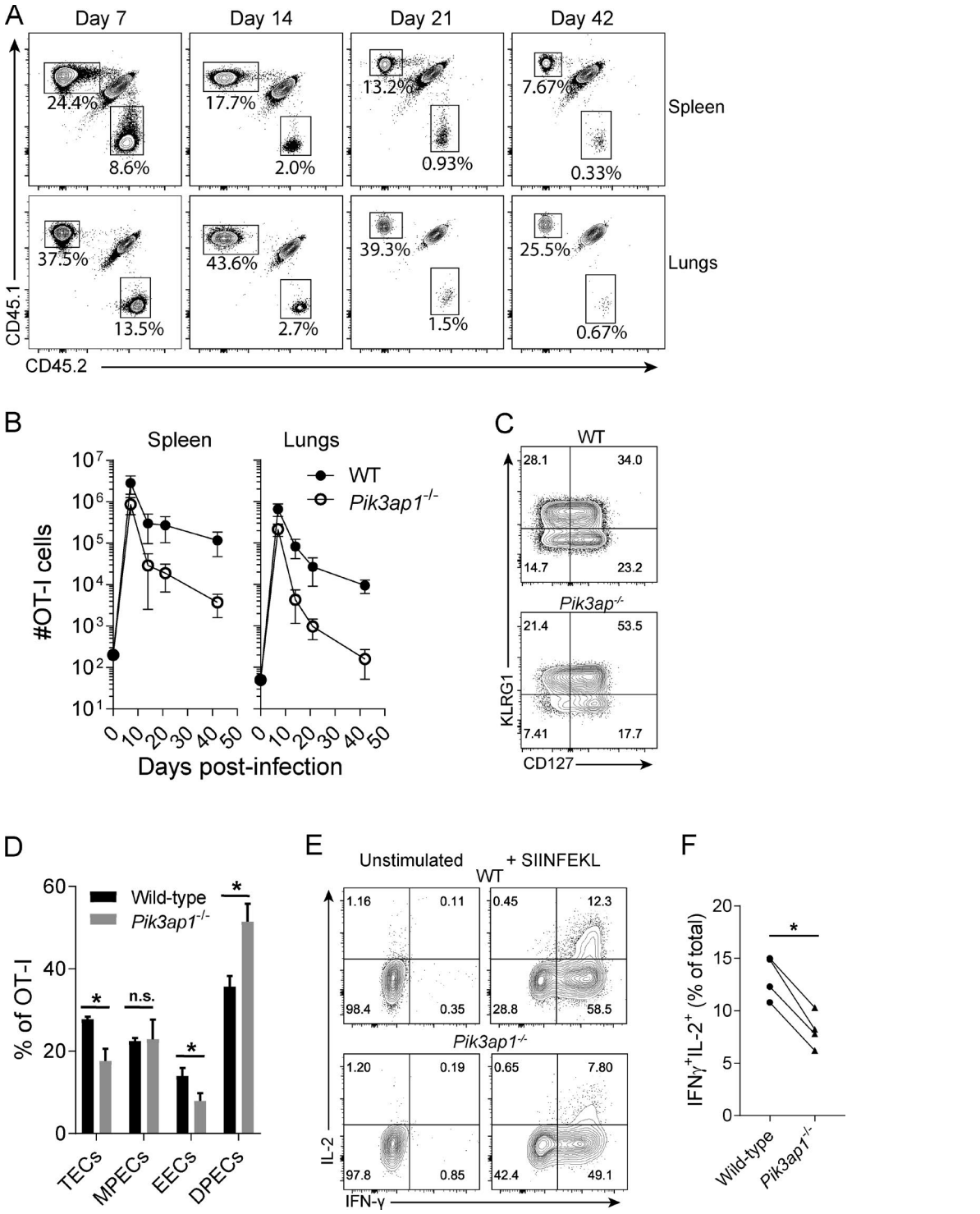

Figure 5. **Loss of BCAP alters CD8⁺ T cell responses in vivo. (A)** CD8⁺ T cells from WT (CD45.1⁺) or *Pik3ap1*$^{-/-}$ (CD45.2⁺) OT-I mice were cotransferred into CD45.1⁺CD45.2⁺ recipient animals, which were subsequently infected with LM-OVA. Flow cytometry analysis of CD45.1 and CD45.2 expression by gated CD8⁺CD44^{hi} effector/memory T cells from the spleens and lungs of the recipient animals at the indicated times after infection. Data are representative of eight mice per time point that were analyzed in two independent experiments. **(B)** Graphical analysis indicating the number of WT and *Pik3ap1*$^{-/-}$ CD8⁺ T cells (mean ± SD) recovered from the spleens and lungs of recipient mice at the indicated times after infection. *n* = 4 mice analyzed per group in a single experiment, representative of two individual experiments. **(C)** Flow cytometry analysis of KLRG1 and CD127 expression by gated WT and *Pi3kap1*$^{-/-}$ OT-I T cells as indicated in spleens of recipient mice at day 7 after infection. Data are representative of eight mice per genotype that were analyzed in two independent experiments. **(D)** Graphical analysis of the frequency of WT and *Pi3kap1*$^{-/-}$ splenic OT-I T cells (mean ± SD) with the indicated phenotype at day 7 after infection. *n* = 4 mice analyzed in a single experiment, representative of two individual experiments. Overall statistical significance is determined by two-way paired ANOVA, and multiple comparisons were performed using Sidak's multiple comparisons test. *, P ≤ 0.05. **(E)** Flow cytometry analysis of IFN-γ and IL-2 production by gated splenic WT and *Pi3kap1*$^{-/-}$ OT-I T cells with or without SIINFEKL peptide restimulation as indicated. Data are representative of eight mice that were analyzed

production of the proinflammatory cytokines IFN-γ and IL-2 following in vitro restimulation with the antigenic SIINFEKL peptide (Fig. 5, E and F).

Memory CD8⁺ T cells can also be divided into multiple functionally distinct populations that work in concert to protect from reinfection (Cui and Kaech, 2010). T_{CM} cells recirculate through secondary lymphoid organs and act as a reservoir of antigen-specific cells that can be rapidly expanded upon secondary infection, whereas T_{EM} cells recirculate through nonlymphoid tissues and have limited proliferative potential but display immediate effector function upon restimulation. Additionally, a subset of T_{EM} cells retains effector cell characteristics such as high KLRG1 expression and low expression of CXCR3 and CD27, and these effector-like T_{EM} cells provide potent protective immunity (Olson et al., 2013). To determine how BCAP influences the development and persistence of these different memory cell populations, we examined CD62L and KLRG1 expression by WT and BCAP-deficient OT-I T cells in the spleens of mice at different times after infection with LM-OVA. As expected, at day 7 after infection, very few CD62L⁺ cells were seen in either population, and the vast majority of cells are KLRG1⁺ effector T cells. However, as early as day 14 after infection, KLRG1 expression was significantly lower among BCAP-deficient OT-I cells, and by day 21 after infection this was accompanied by a significant increase in the frequency of CD62L⁺ T_{CM} cells (Fig. 6, A and B). Nevertheless, the absolute number of BCAP-deficient OT-1 cells in each subset was substantially decreased relative to WT by day 21 after infection (Fig. 6 C). Consistent with impaired differentiation of effector-like T_{EM} cells in the absence of BCAP, at days 21 and 42 the remaining BCAP-deficient KLRG1⁺ memory cells showed increased expression of the markers CD27 and CXCR3 and therefore display a less terminally differentiated phenotype relative to their WT counterparts (Fig. 6 D and data not shown). Thus, loss of BCAP-dependent signaling results in altered memory cell differentiation and maintenance. However, BCAP-deficient memory cells did not display any significant defects in TNF-α, IFN-γ, or IL-2 production at any of the memory time points examined (data not shown).

The differentiation and function of different CD8⁺ effector and memory T cell populations is driven by their expression of distinct combinations of key transcription factors, and among these the balance of the two T box transcription factors T-bet and Eomes plays a central role. Although partially redundant, T-bet and Eomes also have specialized functions. Whereas T-bet promotes the differentiation and function of CD8⁺ TECs, high levels of Eomes are associated with memory cell development and particularly with the differentiation of CD62L⁺ T_{CM} cells (Banerjee et al., 2010). Therefore, we also assessed T-bet and Eomes expression by WT and BCAP-deficient OT-I T cells at different times after infection (Fig. 7). At day 7 after infection, WT and BCAP-deficient cells expressed equivalent levels of T-bet; however, levels of Eomes were higher in cells lacking BCAP. Moreover, consistent with their increased T_{CM} phenotype, at day 21 after infection, Eomes expression was further increased in BCAP-deficient OT-I cells, which also displayed a small but significant decrease in expression of T-bet. Taken together, these data demonstrate that BCAP has a key role in the differentiation and function of effector and memory CD8⁺ T cell responses and that this is due in part to altered Eomes and T-bet expression.

BCAP potentiates effector differentiation to low-affinity TCR ligands

Due to its ability to potentiate PI3K signaling downstream of TCR engagement, we hypothesized that loss of BCAP would have even more dramatic consequences on T cell activation and effector differentiation upon stimulation with low-affinity TCR ligands. To test this, we cotransferred naive WT and BCAP-deficient OT-I cells into congenically marked recipients as above and infected them with a series of LM strains expressing OVA variants with decreasing affinities for the OT-I TCR based on substitution of the N4 residue of the SIINFEKL peptide (Zehn et al., 2009). We then assessed the activation, clonal expansion, and effector differentiation of responding WT and BCAP-deficient OT-I cells. Infection with all of the LM-OVA variants led to activation of both WT and BCAP-deficient OT-I cells as assessed by CD44 up-regulation, and decreasing ligand affinity did not significantly impact the relative clonal expansion of the WT and BCAP-deficient OT-I cells (not shown). However, BCAP-deficient cells responding to the lowest-affinity T4 and V4 OVA-variants showed significantly increased proportions of KLRG1⁻CD62L⁺ cells at day 7 after infection (Fig. 8, A and B). Thus, by enhancing PI3K signaling, BCAP potentiates effector T cell differentiation in response to low-affinity ligands.

Discussion

The ability of CD8⁺ T cells to undergo robust clonal expansion and differentiation into specialized populations of effector and memory cells underlies adaptive immune responses to a wide range of intracellular pathogens. However, the signals that direct these processes and the mechanisms by which they are interpreted by responding CD8⁺ T cells remain incomplete. Here, we have demonstrated that the signaling adaptor BCAP is rapidly up-regulated in responding CD8⁺ T cells and that this molecule acts as a key regulator of CD8⁺ T cell activation and effector/memory differentiation.

Although BCAP is constitutively expressed in naive B cells, NK cells, and multiple myeloid cell populations, we and others have not detected expression in naive T cells (Okada et al., 2000; Yamazaki et al., 2002). However, BCAP was rapidly up-regulated upon stimulation both in vitro and in vivo, and through analysis of transcription factor binding to the *Pik3ap1* locus in publicly available ChIP-seq data, we identified several signaling and transcriptional pathways that appear to regulate BCAP expression in responding T cells. Because up-regulation of BCAP

in two independent experiments. **(F)** Graphical analysis of the frequency of WT and *Pi3kap1*$^{-/-}$ splenic OT-I T cells coproducing IFN-γ and IL-2 following SIINFEKL peptide restimulation. Linked points represent values from cotransferred WT and *Pi3kap1*$^{-/-}$ OT-I T cells recovered from individual recipient mice. $n = 4$ mice analyzed in a single experiment, representative of two individual experiments. Statistical significance is determined by paired Student's *t* test. *, $P \le 0.05$.

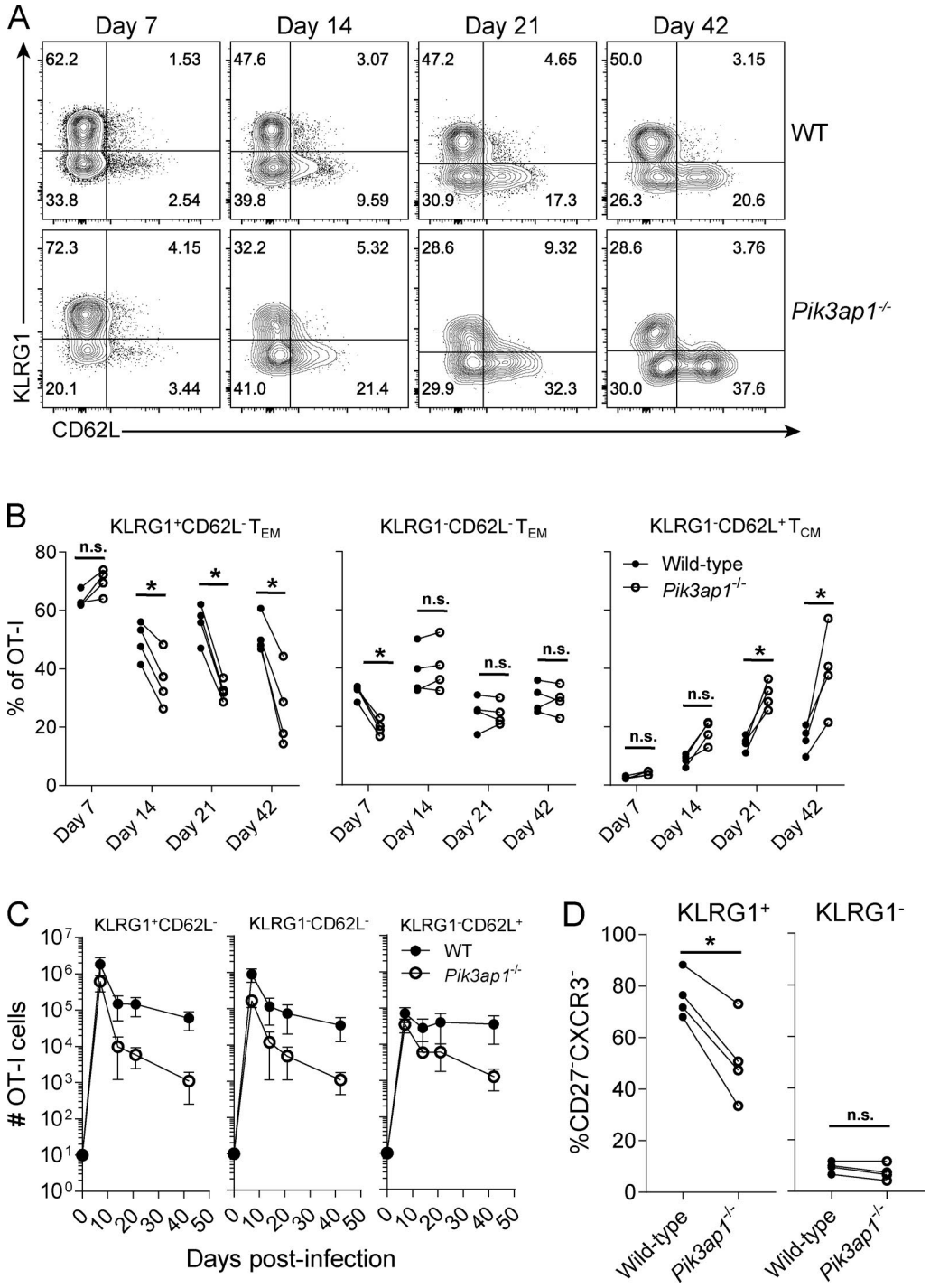

Figure 6. **Altered CD8⁺ memory T cell development in the absence of BCAP. (A)** Flow cytometry analysis of KLRG1 and CD62L expression by splenic WT and *Pi3kap1*$^{-/-}$ OT-I T cells gated as in Fig. 4 A at the indicated times after LM-OVA infection. Data are representative of eight mice per time point that were analyzed in two independent experiments. **(B)** Graphical analysis of the frequencies of splenic WT and *Pi3kap1*$^{-/-}$ OT-I T cells with the indicated KLRG1^{+}T$_{EM}$, KLRG1^{-}T$_{EM}$, and T$_{CM}$ phenotypes at each of the indicated times after LM-OVA infection. *n* = 4 mice analyzed per time point in a single experiment, representative of two individual experiments. Overall statistical significance is determined by two-way paired ANOVA, and multiple comparisons were performed using Sidak's multiple comparisons test. *, P ≤ 0.05. **(C)** Graphical analysis indicating the number of WT and *Pik3ap1*$^{-/-}$ CD8⁺ T cells (mean ± SD) of the indicated phenotypes recovered from the spleens of recipient mice at the indicated times after infection. Data are representative of eight mice per time point that were analyzed in two independent experiments. **(D)** Graphical analysis of the frequencies of CD27^{+}CXCR3^{+} cells among gated KLRG1^{+} or KLRG1^{-} splenic WT and *Pi3kap1*$^{-/-}$ OT-I T cells 21 d after LM-OVA infection. Linked points represent values from cotransferred WT and *Pik3ap1*$^{-/-}$ OT-I T cells recovered from individual recipient mice. Statistical significance was determined by paired Student's *t* test. Data are representative of eight mice per genotype per time point that were analyzed in two independent experiments. *, P ≤ 0.05.

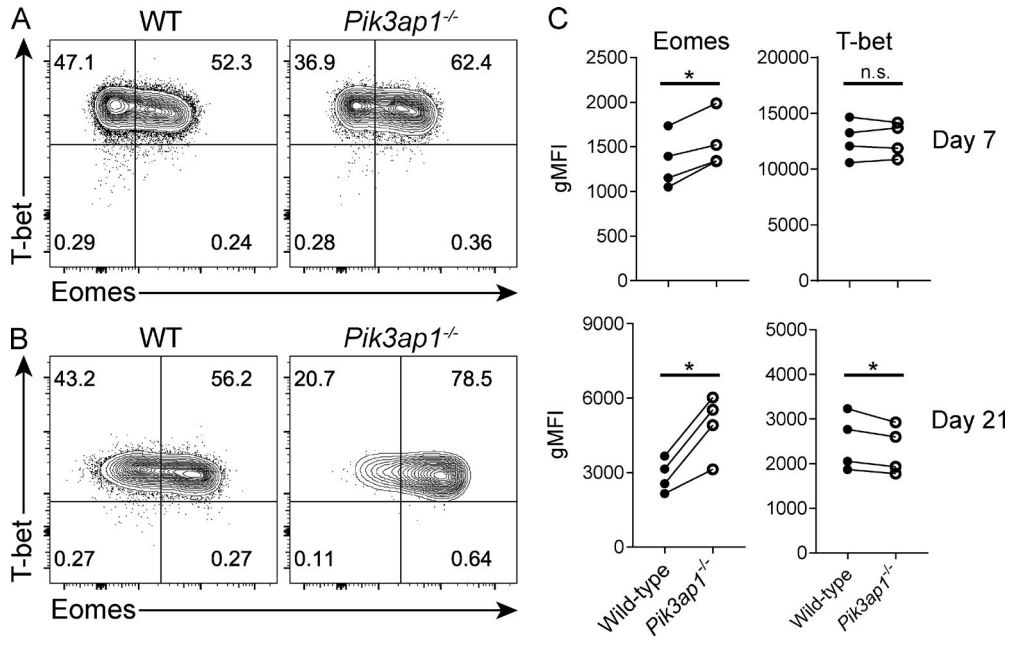

Figure 7. **Decreased T-bet and increased Eomes expression in BCAP-deficient memory CD8⁺ T cells. (A and B)** Flow cytometry analysis of T-bet and Eomes expression by splenic WT and *Pi3kap1*$^{-/-}$ OT-I T cells gated as in Fig. 4 A on days (A) 7 or (B) 21 after LM-OVA infection. Data are representative of eight mice per time point that were analyzed in two independent experiments. **(C)** Graphical analysis of the geometric mean fluorescence intensity of T-bet and Eomes expression by WT and *Pi3kap1*$^{-/-}$ OT-I T cells at days 7 and 21 after LM-OVA infection as indicated. Linked points represent values from cotransferred WT and *Pi3kap1*$^{-/-}$ OT-I T cells recovered from individual recipient mice. *n* = 4 mice analyzed per time point in a single experiment, representative of two individual experiments. Statistical significance is determined by paired Student's *t* test. *, P ≤ 0.05.

was blocked by inhibition of class I PI3K signaling, we propose that BCAP induction acts as a mechanism by which PI3K signaling is maintained or amplified following activation. Additionally, RNA-seq analysis of T-bet– or Irf4-deficient CD8⁺ T cells showed impaired induction of *Pik3ap1* expression in the absence of these transcription factors. T-bet is required for differentiation and function of effector CD8⁺ T cells, and high expression of T-bet helps direct cells to the KLRG1⁺CD127⁻ TEC fate (Dominguez et al., 2015). Thus, induction of BCAP expression and consequent potentiation of PI3K signaling may be one mechanism by which T-bet contributes to TEC differentiation. Like BCAP, IRF4 is rapidly up-regulated upon T cell activation, and IRF4 acts in conjunction with Batf to drive CD8⁺ T cell expansion and effector differentiation (Man et al., 2013; Yao et al., 2013; Kurachi et al., 2014). However, unlike T-bet expression, we have found that IRF4 induction is independent of PI3K signaling (not shown). There-

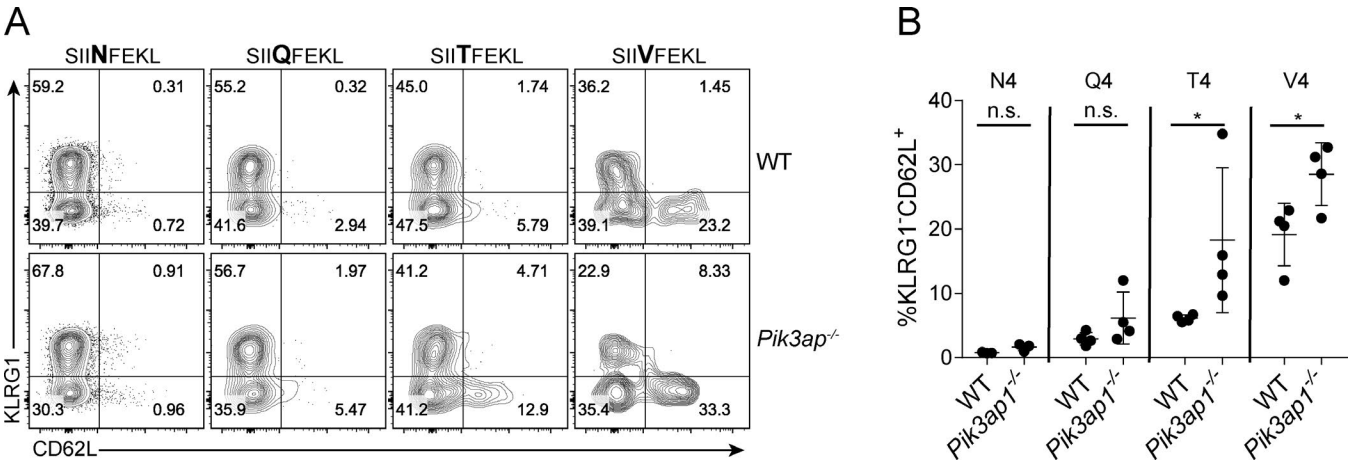

Figure 8. **BCAP promotes effector cell differentiation to low-affinity TCR ligands. (A)** Flow cytometry analysis of KLRG1 and CD62L expression by splenic WT and *Pi3kap1*$^{-/-}$ OT-I T cells gated as in Fig. 4 A on day 7 after infection with LM-OVA strains carrying the indicated antigenic peptide variants. Data are representative of eight mice that were analyzed in two independent experiments. **(B)** Graphical analysis of the frequency of KLRG1-CD62L⁺ T_{CM} cells among gated splenic WT and *Pi3kap1*$^{-/-}$ OT-I T cells (mean ± SD) on day 7 after infection with the indicated LM-OVA variants. Points represent values from cells recovered from individual recipient mice. *n* = 4 mice analyzed per group in a single experiment, representative of two individual experiments. Overall statistical significance is determined by two-way paired ANOVA, and multiple comparisons were performed using Sidak's multiple comparisons test. *, P ≤ 0.05.

fore, IRF4 and PI3K/Foxo1/T-bet appear to represent separate pathways that converge to regulate BCAP expression. Moreover, induction of Irf4 in the absence of PI3K signaling did not lead to substantial BCAP expression, suggesting that PI3K signaling may result in epigenetic remodeling of the *Pik3ap1* locus that allows IRF4 to bind and activate expression in effector/memory T cells.

The relevant molecular pathways that link TCR engagement to PI3K activation have not been well-defined. Our identification of BCAP as a novel signaling adaptor that potentiates PI3K signaling in activated T cells helps clarify this issue. However, it is important to note that although less efficient, CD3-dependent PI3K activation does occur in the absence of BCAP, and thus, TCR likely engages multiple downstream pathways that turn on PI3K signaling. Consistent with this, CD3 cross-linking results in PI3K activation even in naive T cells that do not express BCAP. Nonetheless, because the magnitude of PI3K signaling during activation is a key determinant of T cell fate (Rao et al., 2012), it is likely that disrupting this circuit contributes to the altered effector and memory T cell differentiation we observed in BCAP-deficient OT-I T cells. BCAP-dependent activation of PI3K requires phosphorylation of the tyrosines found in one or more of its four YxxM motifs. During B cell stimulation, BCAP is phosphorylated by the tyrosine kinases Syk and the Src-family kinase Btk (Okada et al., 2000), neither of which are highly expressed in T cells. However, the tyrosine kinase Zap-70 is closely related to Syk and has an analogous function in TCR signaling, and therefore, it is possible that BCAP phosphorylation in activated T cells is mediated in part by Zap-70 activation. Similarly, the Btk-related tyrosine kinase Itk is highly expressed in T cells and may contribute to BCAP phosphorylation. Interestingly, Itk and Btk contain Ph domains that bind to the PI3K product $PI(3,4,5)P_3$, and this binding is required for their activation. Thus, $PI(3,4,5)P_3$-mediated activation of Itk and subsequent phosphorylation of BCAP may help locally amplify PI3K signaling in specific membrane domains such as the immunological synapse that are required for full T cell activation (Le Floc'h et al., 2013). Finally, Nck1 and Nck2 are adaptor proteins that recruit BCAP to the BCR complex through direct interactions with the Igα chain of the BCR and allow for BCAP phosphorylation (Castello et al., 2013). Both Nck1 and Nck2 are expressed in T cells and can associate with the CD3ε chain and with SLP-76 after TCR cross-linking (Wunderlich et al., 1999; Gil et al., 2002), and these may also contribute to BCAP activation and subsequent PI3K signaling in activated T cells.

In vivo, T cell activation occurs in multiple phases during which T cells display different behaviors within secondary lymphoid tissues. Importantly, up-regulation of BCAP occurs during a critical temporal window in vivo during which T cells continue to interact with antigen-bearing dendritic cells in secondary lymphoid organs, initiate robust proliferative expansion, and begin to undergo differentiation into functionally specialized populations of effector and memory cells (Mempel et al., 2004; Gérard et al., 2013). Thus, BCAP is poised to impact each of these processes in activated $CD8^+$ T cells. Indeed, the fact that BCAP is clearly detected in activated T cells even before initiation of cell division raises the possibility that BCAP may participate in the asymmetric division of PI3K activity that helps regulate effector and memory T cell differentiation (Lin et al., 2015). Although loss of BCAP resulted in only a modest defect in the clonal expansion of OT-I cells responding during LM-OVA infection, their differentiation into $CD127^-$ TECs and EECs was impaired, with a corresponding increase in the accumulation of $CD127^+$ DPECs. Indeed, expression of CD127 (encoded by the *Il7r* gene) is directly regulated by PI3K signaling via Foxo1 (Kerdiles et al., 2009), and the accumulation of $CD127^+$ T cells is therefore likely a consequence of diminished PI3K activation in BCAP-deficient cells. Moreover, in response to low-affinity TCR ligands, we found that BCAP-deficient cells failed to fully acquire effector phenotype at the peak of the response and instead showed increased expression of the T_{CM} marker CD62L.

Although activation of PI3K via its YxxM motifs is the only function that has been ascribed to BCAP thus far, the presence of multiple protein–protein interaction domains in BCAP strongly suggests that it has additional functions as a signaling adaptor. For instance, in macrophages, we have found that BCAP regulates inflammasome activation, and this function is completely independent of its YxxM motifs and ability to activate PI3K (unpublished data). In this regard, it is useful to compare the phenotype of BCAP-deficient T cells with that observed in $CD8^+$ T cells with severe defects in PI3K signaling due to loss of the catalytic p100δ subunit (Gracias et al., 2016). Similar to what we observed with BCAP-deficient cells, OT-I cells lacking p100δ displayed a three- to fourfold decrease in clonal expansion in adoptive hosts following infection with LM-OVA or an OVA-expressing strain of influenza virus. However, in contrast to the further loss of BCAP-deficient memory cells, no decrease in memory cell formation/maintenance was observed with p100δ-deficient cells. Thus, rather than simply controlling the magnitude of PI3K signaling, BCAP may coordinate PI3K with other key signaling pathways engaged by TCR and cytokine and costimulatory receptor engagement to help dictate cell expansion, differentiation, and maintenance. In that regard, cross-talk between PI3K signaling and other key signaling nodes that control the proliferation, development, and survival of effector T cells has been observed, and these include the Wnt/β-catenin, NF-kB, and JAK/STAT pathways (Kim and Suresh, 2013).

Recently, a subset of effector-like $KLRG1^+CD27^-$ cells was shown to persist into the memory phase following pathogen clearance, and these cells could provide potent protection from reinfection upon transfer to naive hosts (Olson et al., 2013). Importantly, compared with other memory cell populations, these cells express relatively low levels of receptors for the cytokines IL-7 and IL-15 that support memory cell survival in vivo, and thus, it is not clear how these effector-like cells are maintained following pathogen clearance. Although in response to high-affinity peptide stimulation, BCAP-deficient T cells showed no impairment in induction of KLRG1, the rapid decline in the abundance of $KLRG1^+$ memory cells in the absence of BCAP demonstrates that BCAP-dependent signaling is important for maintenance of these cells into the memory pool. One intriguing possibility is that rather than competing for homeostatic cytokines, effector-like memory cells are maintained by low-level TCR signaling in response to self-peptide–MHC complexes, and that this tonic signaling is potentiated/amplified by BCAP-mediated signal coordination. Like $KLRG1^+CD27^-$ memory cells in mouse, human

$CD8^+CD45RA^+CCR7^-$ $T_{EM}RA$ cells show potent effector function upon restimulation, are poorly proliferative, and have a terminally differentiated phenotype (Geginat et al., 2003). Notably, in our analysis of human $CD8^+$ T cell subsets, $T_{EM}RA$ cells showed the highest level of BCAP expression. Analogously, BCAP expression and function may be therefore be required for the development and maintenance of $CD8^+$ $T_{EM}RA$ cells in humans, an important memory subset with roles in diverse processes including protection from viral infection following vaccination, graft rejection, and delayed healing of bone fractures (Miller et al., 2008; Betjes et al., 2012; Reinke et al., 2013).

$CD8^+$ T cells mediate immunity to a wide range of intracellular pathogens and tumors, and inappropriate $CD8^+$ T cell responses can contribute to development of chronic infections, cancers, and autoimmune disease. Thus, there is an urgent need to continue defining the signals and molecular pathways that regulate expansion, diversification, and maintenance of effector and memory $CD8^+$ T cells in order to harvest the power of $CD8^+$ T cells in vaccination strategies and cellular therapies. Our identification of BCAP as a key regulator of PI3K signaling and effector/memory T cell development helps define a new molecular pathway by which these processes are controlled, thus providing new opportunities for therapeutic manipulation of $CD8^+$ T cells in a host of immune-mediated diseases.

Materials and methods

Mice

C57BL/6 (B6) and $CD45.1^+$ B6 congenic mice were purchased from the Jackson Laboratory and subsequently intercrossed to produce $CD45.1^+/CD45.2^+$ mice used as recipients in adoptive transfer experiments. BCAP-deficient mice lacking the *Pik3ap1* gene have been described (Yamazaki et al., 2002) and were backcrossed to C57BL/6 mice for nine generations. OT-I TCR transgenic mice on the B6.CD45.1 background were provided by Dr. Mike Bevan (University of Washington, Seattle, WA) and crossed to BCAP-deficient mice to produce B6.CD45.2 BCAP-deficient OT-I animals. Mice carrying a stop-floxed allele of the human *FOXO1* gene lacking all Akt phosphorylation sites knocked into the *Rosa26* locus have been described (Ouyang et al., 2012) and were crossed to mice expressing Cre under the distal-Lck promoter to generate animals in which all T cells express this constitutively active Foxo1 protein. All mice were bred and maintained at the Benaroya Research Institute, and all experiments were approved by the Institutional Animal Care and Use Committee of the Benaroya Research Institute.

Human cells

Human peripheral blood mononuclear cells were obtained from healthy donors participating in the Benaroya Research Institute Immune Mediated Disease Registry. Informed consent was obtained from all subjects according to Institutional Review Board–approved protocols at Benaroya Research Institute.

Cell culture and activation

T cells were activated and cultured in RPMI-1640 medium containing 10% FCS, 50 U/ml Penicillin, 50 µg/ml Streptomycin, 50 µg/ml Gentamycin, 1 nM Sodium Pyruvate, 10 µM Hepes, 2 mM L-glutamine, and 50 µM 2-Mercaptoethanol (complete RPMI). Purified naive T cells were stimulated in 24-well plates (3–5 × 10^6 cells/well) precoated with the indicated concentrations of anti-CD3 (2C11; Bio-XCell) and anti-CD28 (37.51; Bio-XCell) in PBS in complete medium containing 50–100 U/ml recombinant murine IL-2 (eBioscience) for the indicated times. In some experiments, the class I PI3K inhibitor ZSTK474 (Sigma) was added as indicated. For restimulation experiments, cells were stimulated for 2 d with 1 µg/ml plate-bound anti-CD3/anti-CD28 + IL-2, then removed from stimulus and placed in complete media (without IL-2) overnight before restimulation with plate-bound anti-CD3 as indicated. For polarization of $CD4^+$ T cells into TH1 and TH2 cells, total CD4+ T cells were activated with plate-bound anti-CD3 and anti-CD28 in media containing 200 U/ml IL-2 supplemented with 10 ng/ml IL-12 (eBioscience) and 20 µg/ml anti–IL-4 (11B11; Bio-XCell) for TH1 polarization or 100 ng/ml IL-4 (eBioscience) and 40 µg/ml anti–IFN-γ (XMG1.2; Bio-XCell) for TH2 polarization.

Flow cytometry and cell sorting

Cells were isolated from the spleen by mashing the tissue between glass slides followed by red blood cell lysis with ACK lysis solution (Gibco). Cell isolation from the lungs was performed as previously described (Sheih et al., 2017). Cell surface staining was performed with the following directly conjugated anti-murine antibodies (all antibodies were from BioLegend unless otherwise specified): anti-CD8 (53–6.7), anti-CD44 (IM7), anti-CD62L (MEL-14), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD127 (A7R34), and anti-KLRG1 (2F1). For intracellular staining, cells were surface stained and then permeabilized with FixPerm buffer (eBioscience). Cells were then washed and stained with antibodies against T-bet (4B10) and Eomes (Dan11mag; eBioscience). Intracellular staining for BCAP was performed with an unlabeled anti-BCAP antibody (4L8E6, purified from hybridoma) followed by PE- or APC-conjugated anti-mouse IgG1 secondary antibodies (A85-1). For intracellular cytokine staining, splenocytes were stimulated with 1 µM SIINFEKL peptide in the presence of 10 µg/ml monensin in 1 ml of complete media for 6 h at 37°C, 5% CO_2. Following stimulation, cells were harvested, surface stained, permeabilized with BD Fix/Perm, and stained with anti–IFN-γ (XMG1.2) and anti–IL-2 (JES6-5H4). To assess Akt and S6 phosphorylation, cells were stimulated with the indicated concentrations of plate-bound anti-CD3 for 90 min, harvested, and immediately fixed in Cytofix/Cytoperm buffer (BD Bioscience). After incubation for 30 min at room temperature, the cells were washed, resuspended in 400 µl 90% methanol, and incubated on ice for 30 min. After an additional wash, cells were stained for surface and intracellular antigens (including anti-pS6 [S235/236, D57.2.2E; Cell Signaling] and anti-pAKT [S473, D9E; Cell Signaling]) for 45 min at room temperature in the dark. To assess S6 phosphorylation directly ex vivo, spleens were immediately disrupted using glass slides into Cytofix/Cytoperm buffer (BD Bioscience), and staining proceeded as described above. Human cells were surface stained with the following directly conjugated antibodies: anti-CD4 (RPA-T4), anti-CD8 (SK1), anti-CXCR3 (1C6), anti-CD45RA (HI100), and anti-CCR7 (GO43H7). Following surface

staining, cells were permeabilized with FixPerm buffer (eBioscience), washed, and stained with antibodies against T-bet (4B10) and BCAP (4L8E6) as described above. Data were acquired on either LSRII or FACsCanto flow cytometers (BD Bioscience). For cell sorting, $CD8^+$ T cells were enriched from the spleens of WT and BCAP-deficient mice by magnetic selection using anti-CD8 microbeads from Miltenyi, and $CD8^+CD44^{lo}$ naive T cells were sorted on a FACsAria high-speed cell sorter (BD Bioscience).

Adoptive transfer and infection

$CD8^+$ T cells from the spleens of B6.CD45.1$^+$ and BCAP-deficient B6.CD45.2$^+$ donor mice were isolated by magnetic selection using anti-CD8 microbeads (Miltenyi). Except as indicated, 5,000 WT and 5,000 BCAP-deficient OT-I T cells were cotransferred into CD45.1$^+$/CD45.2$^+$ recipient mice by retro-orbital injection, and the recipient animals were infected the following day by retro-orbital injection of 10^4 CFU of LM-OVA as described previously (Koch et al., 2012). For ex vivo analysis of S6 phosphorylation, infection was as described, except that to facilitate identification of the transferred OT-I T cells, 5×10^5 CFSE-labeled WT and 5×10^5 CFSE-labeled BCAP-deficient OT-I cells were cotransferred into the recipient mice before infection.

Immunoprecipitation and Western blotting

B cells and $CD8^+$ T cells were isolated from WT and BCAP-deficient mice by magnetic selection using anti-CD19 or anti-CD8 microbeads, respectively (Miltenyi). Splenic B cells were immediately lysed in lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.25% sodium deoxycholate, 1 mM PMSF, 1 mM sodium orthovanadate and protease inhibitor (mammalian protease inhibitor mixture; Sigma), and sodium orthovanadate (1 mM; Sigma). $CD8^+$ T cells were lysed in the same buffer following in vitro activation with plate-bound anti-CD3 and anti-CD28 (1 µg/ml each) + 100 U/ml rmIL-2 for 48–72 h as described above. Lysates were either used directly or sequentially incubated with the 4L8E6 anti-BCAP monoclonal antibody and protein A-agarose, and they were then eluted with 1× SDS sample buffer (Invitrogen). Samples were separated by Tris-bis SDS/PAGE gels (Invitrogen); transferred to polyvinylidene difluoride (Millipore) membrane; probed with the 4L8E6 anti-BCAP antibody, the 4G10 anti-pY antibody (Millipore), or a polyclonal anti-PI3K p85 antibody (Cell Signaling) as indicated; and detected with the Immobilon chemiluminescence system (Millipore).

Statistical analyses

Comparisons between groups were analyzed using paired Student's *t* tests or two-way ANOVA with Sidak's multiple comparison tests as appropriate and as indicated in the figure legends and were performed using GraphPad Prism Software. Statistical significance was established at the level of $P \leq 0.05$.

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Author contributions: M.D. Singh, D.J. Campbell, and J.A. Hamerman conceived of the project and designed the experiments. M.D. Singh, D.J. Campbell, J.M. Sullivan, and M. Ni performed experiments. D.J. Campbell and M.D. Singh wrote the manuscript with editorial input from J.A. Hamerman.

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