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PI3Kσ Regulates the Magnitude of CD8+ T Cell Responses after Challenge with Listeria monocytogenes

Verity Q. Pearce, Hicham Bouabe, Amy R. MacQueen, Valentina Carbonaro, and Klaus Okkenhaug

PI3Ks regulate diverse immune cell functions by transmitting intracellular signals from Ag, costimulatory receptors, and cytokine receptors to control cell division, differentiation, survival, and migration. In this study, we report the effect of inhibiting the p110δ subunit of PI3Kσ on CD8+ T cell responses to infection with the intracellular bacteria Listeria monocytogenes. A strong dependency on PI3Kσ for IFN-γ production by CD8+ T cells in vitro was not recapitulated after Listeria infection in vivo. Inactivation of PI3Kσ resulted in enhanced bacterial elimination by the innate immune system. However, the magnitudes of the primary and secondary CD8+ T cell responses were reduced. Moreover, PI3Kσ activity was required for CD8+ T cells to provide help to other responding CD8+ cells. These findings identify PI3Kσ as a key regulator of CD8+ T cell responses that integrates extrinsic cues, including those from other responding cells, to determine the collective behavior of CD8+ T cell populations responding to infection. The Journal of Immunology, 2015, 195: 000–000.

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D8+ T cells respond to Ags presented by MHC class I molecules and provide protective immunity against intracellular pathogens and cancer. Following infection, low frequencies of naive CD8+ T cells whose TCRs recognize specific peptide Ags undergo massive proliferative expansion during which responding cells differentiate to become CTLs (1, 2). CTLs kill target cells, often leading to elimination of pathogens, after which most CTLs die to restore immune homeostasis. However, a small proportion of CTLs survive following primary infection and establish themselves in the lymph nodes and bone marrow as long-term memory T cells (Tmem) (1–3). Robust recall responses by Tmem contribute to enhanced protection upon pathogen re-encounter (1–3). Thus, dynamic regulation of the size and function of CD8+ T cell populations responding to infection is required for appropriate control of immunity.

In CD8+ T cells, the PI3K pathway is activated by TCR, cytokine receptor, and costimulatory signaling (4). Despite this, the function of the PI3K pathway in regulating primary and secondary immune responses to infections is poorly elucidated. The class I PI3Ks, of which there are four types (PI3Kα, PI3Kβ, PI3Kδ, and PI3Kγ), are a subfamily of evolutionarily conserved kinases that catalyze the phosphorylation of phosphatidylinositol(4,5)-biphosphate to generate the second messenger signaling molecule phosphatidylinositol(3,4,5)-trisphosphate [PtdIns(3,4,5)P3] (5). PI3Kδ (which is a heterodimer of a p110δ catalytic subunit in complex with an SH2-containing regulatory subunit p85α or p85β) is predominantly responsible for activating the Akt signaling pathway in CD8+ T cells, but it can also make a minor contribution to TCR-induced Akt activation in naive T cells (10–13). In comparison with PI3Kδ and PI3Kγ, the contribution of PI3Kα and PI3Kβ to PI3K signaling in T cells is thought to be minimal (4, 5, 14). PtdIns(3,4,5)P3 acts as a tether for intracellular proteins with pleckstrin homology domains on the cytosolic surface of the lipid bilayer. Key among these are the serine-threonine kinases Pdk1 and Akt. The activity of Akt is regulated by phosphorylation by Pdk1 at Thr308, an event that is dependent on PtdIns(3,4,5)P3 binding to the pleckstrin homology domains of both Akt and Pdk1 (15–17). A major role for Akt is to phosphorylate transcription factors (TFs) of the Foxo family, sequestering them in the cytosol where they are degraded (5, 18). Consequently, PI3K signaling can negatively regulate Foxo TF target genes, such as Klf-2, Sell, Ccr7, Il7r, and Slp (8, 9, 19–21). By suppressing the expression of these Foxo target genes, PI3K can help T cells prepare for lymph node exit and the initiation of an immune response in the peripheral tissues. To what extent the failure to regulate these trafficking events affects systemic immune responses remains to be fully elucidated.

Foxo TFs have also been proposed to differentially regulate the expression of the transcription factors T-bet and eomesoderm (Eomes), favoring the formation of CD8+ Tmem by repressing the expression of T-bet and promoting that of Eomes (22). Similarly, the inhibition of mammalian target of rapamycin (mTOR) has been shown to favor the formation of CD8+ Tmem via differential regulation of T-bet and Eomes (23, 24). The relationship between PI3K and mTOR is complex (5). Although PI3K and Akt contribute to mTOR activity in some cell systems, in CD8+ T cells other sig-

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Abbreviations used in this article: EdU, 5-ethynyl-2’-deoxyuridine; Eomes, eomesoderm; FAM-VAD-FMK, FAM–L-valyl–L-alanyl–L-aspartic acid(methyl ester)–fluorescent protein; GzmB, granzyme B; Lm-OVA, Listeria monocytogenes expressing OVA; MPEC, memory precursor effector cell; mTOR, mammalian target of rapamycin; PtdIns(3,4,5)P3, phosphatidylinositol(3,4,5)-trisphosphate; SLEC, short-lived effector cell; Teff, effector T cell; Tet, Kb MHC tetramer loaded with SIINFEKL peptide; TF, transcription factor; Tmem, memory T cell; WT, wild-type; YFP, yellow fluorescent protein.
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naling pathways may contribute more strongly to mTOR activation (7, 8, 25, 26). In turn, mTOR can phosphorylate Akt at Ser273 in a PI3K-independent fashion (22). It is therefore important to appreciate that the PI3Ks regulate signaling proteins in addition to Akt, mTOR, and Foxo TFs and, moreover, that mTOR can be controlled by signaling pathways other than PI3K (5).

The contribution of PI3Kδ to T cell development and function has been studied using p110δD910A mice (in which the p110δ subunit of PI3Kδ is inactivated by a point mutation in the catalytic domain), p110γ−/− mice, p85-deficient mice, and/or small molecule inhibitors such as IC87114 that selectively inhibit PI3Kδ (5, 27–29). We have found that CD8+ T cells from p110δD910A mice, or wild-type (WT) T cells stimulated in the presence of IC87114, respond poorly to stimulation with Ag in vitro (6). In particular, IFN-γ production was profoundly diminished in p110δD910A CD8+ T cells and could be suppressed in WT T cells using very low concentrations of IC87114 (6). We and others have also found that the production of cytokotoxic proteins produced in CTL granules as granzyme B (GzmB) and perforin were impaired in vitro, albeit to different extents, when PI3Kδ was inhibited (30, 31). However, mice that both the p85α and p85β regulatory subunits in T cells respond normally to infection with mouse hepatitis virus despite impaired PI3K signaling (32). Moreover, we have recently shown that tumors are killed more effectively by the immune system in p110δD910A mice than in WT mice (33). Enhanced killing of tumors was the consequence of inhibition of p110δ within regulatory T cells, which helped unleash more potent CD8+ T cell–dependent antitumor responses (33). These findings imply that CD8+ T cell function is not entirely abrogated with inactivation of PI3Kδ (33). These findings imply that CD8+ T cell function is not entirely abrogated with inactivation of PI3Kδ (33). These findings imply that CD8+ T cell function is not entirely abrogated with inactivation of PI3Kδ (33). These findings imply that CD8+ T cell function is not entirely abrogated with inactivation of PI3Kδ (33). 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plate (Nunc). For the last 90 min of each time point, cultures were incubated with 10 μg/ml frefeldin A (eBioscience, 15406-51) at 37˚C then stained for surface markers, fixed, and permeabilized as previously described before analysis on an LSFRFortessa flow cytometer.

Adoptive cell transfers

OVA-specific CD8+ T cells obtained from the lymph nodes of WT OT1 or p110D910A OT1 mice and purified by magnetic sorting as described above. The number of OT1 cells was calculated based on purity determined by flow cytometry, and indicated numbers or OT1 cells (usually 500) were injected i.v. into the lateral tail vein of mice 18 h prior to infection with Ln-OVA.

5-Ethynyl-2’-deoxyuridine labeling of lymphocytes in vivo

WT and p110D910A mice were infected i.v. with ~5 × 10^6 CFU DeacLm-OVA. 5-Ethynyl-2’-deoxyuridine (EdU, 250 μg; Life Technologies), dissolved in PBS, was injected i.v. 24 h before culling the mice and collection of organs. Splenocytes were then subjected to cell surface immunostaining, and the incorporation of EdU by DNA was detected by intracellular fluorogenic “click” reaction using a Click-iT Plus EdU Alexa Fluor 647 flow cytometry assay kit (Life Technologies), according to the manufacturer’s protocol. Briefly, fixed and permeabilized cells were incubated with copper and Alexa Fluor 647 dye–labeled picolyl azide at room temperature for 30–45 min. Copper catalyzes a covalent reaction between picolyl azide and alkyne found in the ethynyl moiety of EdU.

Detection of apoptosis

Splenocytes isolated from WT and p110D910A mice 5 d after i.v. infection with ~5 × 10^6 CFU DeacLm-OVA were incubated (at ~3–5 × 10^6 cells/well in a 96-well plate) in RPMI 1640, 5% FCS, antibiotics, and 0.75 μM FAM–L-valyl–L-alanyl–L-aspartic acid (methyl ester)–fluoromethyl ketone (FAM-VAD-FMK, Intracellular Technologies) for 3 h at 37˚C. Cells were then immunostained and analyzed by a flow cytometer. Dead/necrotic cells (positive for viability dye) were gated out and the percentage of Tet’CD8+ cells labeled with FAM-VAD-FMK was determined.

Results

PI3Kδ is required for Akt phosphorylation and IFN-γ production by p110D910A T cells stimulated in vitro

To test the role of PI3Kδ in transducing signals downstream of the TCR and CD28, we stimulated CD8+ T cells purified from WT and p110D910A mice and monitored phosphorylation of key signaling proteins. Phosphorylation of Akt, Foxo, S6, and p70S6K was profoundly diminished in p110D910A CD8+ T cells (Fig. 1A), indicating that p110δ is the predominant PI3K isoform contributing to Akt phosphorylation in CD8+ T cells. However, some compensatory signaling was evident, because phosphorylation of Foxo, S6, and Erk was detected in p110D910A T cells. Moreover, assessment

FIGURE 1. Inhibition of PI3Kδ leads to defective signaling and CD8+ T cell differentiation in vitro. PI3Kδ is essential for Akt and Foxo phosphorylation in CD8+ cells. Serine–threonine kinase signaling in p110D910A CD8+ T cells. (A) Purified CD8+ T cells from WT and p110D910A mice were stimulated with biotinylated Abs against CD3, CD8, and CD28 and crosslinked with streptavidin. Western blots of the cell lysates were probed with the indicated phospho-specific Abs. Abs against Akt and β-actin were used as loading controls. Experiment is representative of two repeats. (B) Expression of IFN-γ, YFP, T-bet, Eomes, and GzmB by YETI or p110D910A YETI CD8+ T cells activated with anti-CD3, anti-CD28, and IL-12, as determined by flow cytometry. The plots are representative of CD8+ T cells 48 h after stimulation. Similar results were obtained in three independent experiments.
of the response to stimulation by PMA demonstrated that whereas the S6K and Erk pathways can be rescued by diacylglycerol-dependent signaling pathways in p110<sub>D910A</sub> cells, Akt and Foxo phosphorylation cannot. Hence, compensatory activation of the Ras-Erk pathway can contribute to mTOR activity independently of PI3K and Akt, consistent with previous results (7, 26). T cells from YETI mice express yellow fluorescent protein (YFP) when the Irf5 gene is actively being transcribed (39). To examine the role of P13Kδ in promoting IFN-γ production more closely, we used cells from YETI mice to monitor the proportion of CD8<sup>+</sup> T cells able to produce IFN-γ after stimulation with anti-CD3, anti-CD28, and IL-12. We found that P13Kδ inactivation reduced YFP and IFN-γ expression in CD8<sup>+</sup> T cells (Fig. 1B). These experiments also showed that during in vitro activation of CD8<sup>+</sup> T cells, T-bet, Eomes, and GzmB expression were lower in p110<sub>D910A</sub>-T cells (Fig. 1B).

**P13Kδ regulates the magnitude of primary and secondary responses to L. monocytogenes infection**

To track CD8<sup>+</sup> T cells responding to infection, we infected mice with a transgenic strain of *L. monocytogenes* that expresses chicken OVA and detected OVA-specific CD8<sup>+</sup> T cells using MHC class I H-<sub>2</sub>b tetramers loaded with OVA<sub>257–264</sub> (SIINFEKL) peptide. The proportions of CD8<sup>+</sup> T cells in the blood that stained positive with the tetramer (Tet<sup>+</sup> CD8<sup>+</sup>) were reduced in p110<sub>D910A</sub> mice (Fig. 2A). This reduction in the proportion of Tet<sup>+</sup>CD8<sup>+</sup> T cells was apparent both at the peak of response as well as among the Tmem that persisted and could be detected before and after subsequent challenges (Fig. 2A). Nevertheless, the p110<sub>D910A</sub> mice did show evidence of CD8<sup>+</sup> Tmem formation, as their secondary responses were more rapid and achieved greater peak magnitude than during their primary response (Fig. 2A). Diminished CD8<sup>+</sup> T cell responses could either be due to a developmental defect that occurs as a result of a congenital lack of p110<sub>α</sub> activity in p110<sub>D910A</sub> mice, or due to an acute requirement for P13Kδ activity during T cell activation in vivo. To distinguish between these, we treated WT mice with IC87114 and infected them with Lm-OVA. IC87114 caused a similar defect in the primary response to that seen with the p110<sub>D910A</sub> mice, demonstrating that acute P13Kδ activity is required for appropriate CD8<sup>+</sup> T cell response kinetics (Fig. 2B).

P13Kδ affects the expression of various receptors, including CD62L and CCR7, which facilitate trafficking of T cells to the lymph nodes (8, 9). Consistent with this, p110<sub>D910A</sub>-Tet<sup>+</sup>CD8<sup>+</sup> T cells in the lymph nodes still expressed high levels of CD62L. 8 d postinfection (Supplemental Fig. 1). Nevertheless, most p110<sub>D910A</sub>-Tet<sup>+</sup>CD8<sup>+</sup> T cells in the spleen and blood effectively downregulated the expression of CD62L (Supplemental Fig. 1), indicating partially redundant pathways for regulation of lymphoid-homing molecules in CD8<sup>+</sup> T cells.

To address whether the reduced proportion of T cells in the blood reflected their altered distribution, we enumerated Tet<sup>+</sup>CD8<sup>+</sup> T cells in the spleen, lymph nodes, and bone marrow (Fig. 2C–E). In p110<sub>D910A</sub> mice the numbers of Tet<sup>+</sup>CD8<sup>+</sup> T cells were reduced at the peak of the primary response (8 d postinfection) in all organs examined (Fig. 2C). However, the number of Tet<sup>+</sup>CD8<sup>+</sup> T cells detected 47 d postinfection were reduced in the spleen, similar in the blood and lymph nodes, but increased in the bone marrow (Fig. 2D). During secondary responses (5 d after second challenge), Tet<sup>+</sup>CD8<sup>+</sup> T cell numbers were again reduced in all tissues examined (Fig. 2E). These data indicate that p110<sub>D910A</sub> cells produce near normal numbers of Tmem, but that the p110<sub>D910A</sub> Tmem, similar to their naive precursors, produce an effector T cell (Teff) response of reduced magnitude upon acute challenge with Lm-OVA.

*p110<sub>D910A</sub> mice generate normal numbers of memory precursor effector cells in the lymph nodes and bone marrow and mount intact long-term memory T cell responses*

During the primary immune response, responding CD8<sup>+</sup> T cells can be classified as short-lived effector cells (SLECs) or as memory precursor effector cells (MPECs) based on the differential expression of KLRG1 and CD127 (41). SLECs (KLRG1<sup>high</sup>, CD127<sup>low</sup>) are responsible for the immediate clearance of bacteria and most will die following resolution of the infection. Some of the MPECs (KLRG1<sup>low</sup>, CD127<sup>high</sup>, in contrast, will form long-lived memory T cells. In p110<sub>D910A</sub> mice, we found a significant reduction in SLECs in all tissues analyzed 8 d following infection (Fig. 3A, 3B). There were also fewer MPECs in the blood and spleen of p110<sub>D910A</sub> mice (Fig. 3A, 3C). However, the numbers of MPECs in the lymph nodes and bone marrow were similar in WT and p110<sub>D910A</sub> mice (Fig. 3A, 3C). Because the lymph nodes and bone marrow are thought to be the primary site for the generation and maintenance of memory T cells (3, 42), these results further support the notion that P13Kδ inhibition preferentially impairs the generation of effector cells without having a strong impact on the generation of Tmem.

**Long-term memory responses and aging-related loss of CD8<sup>+</sup> T cell function**

Memory T cell responses can provide life-long protection against specific Ags (3). With the aim to examine the role of P13Kδ in the long-term persistence and function of memory CD8<sup>+</sup> T cells, we started by comparing immune responses in young and old mice. Mice can live for 2–3 y, but mortality from age-related decline increases in frequency from ~18 mo of age (43). We therefore compared immune responses in mice that were 2–4 mo of age (young) to mice that were 15–18 mo of age (old). The old mice mounted reduced primary and secondary immune responses when compared with young mice (compare Fig. 3D and 3E). However, old p110<sub>D910A</sub> mice mounted primary and secondary CD8<sup>+</sup> T cell responses that were similar in magnitude to old WT mice (Fig. 3E). Moreover, both WT and p110<sub>D910A</sub> mice that had been infected when young and then reinfected a year later mounted recall responses of similar magnitudes despite the impaired primary response observed in young p110<sub>D910A</sub> mice (Fig. 3F). Hence, the impaired primary and secondary CD8<sup>+</sup> T cell responses in young p110<sub>D910A</sub> mice give rise to memory T cells that persist into old age to elicit recall responses that are similar in magnitude to those raised by old WT mice.

**P13Kδ activity within CD8<sup>+</sup> T cell populations is required for optimal expansion**

The reduced primary and recall T cell response in young mice could be a consequence of impaired signaling within CD8<sup>+</sup> T cells or due to factors extrinsic to the responding T cell populations, such as differences in bacterial load, Ag presentation, or cytokine production by macrophages and dendritic cells and/or paracrine help from other responding or bystander T cell populations. We recovered reduced numbers of *L. monocytogenes* colonies from the spleen of p110<sub>D910A</sub> mice compared with WT mice during the first 4 d postinfection (Fig. 4A). Because p110<sub>D910A</sub> spleens are smaller than WT spleens, we also measured CFU in the liver and found again the counts to be significantly lower in p110<sub>D910A</sub> hosts than in WT hosts, similar to what we found in the spleen (Fig. 4B, 4C). Because *Listeria* clearance happens before *Listeria*-specific CD8<sup>+</sup> T cells can be detected, these findings suggest that P13Kδ inhibition can enhance bacterial clearance by the innate immune system.

We therefore sought to determine whether P13Kδ plays a cell-intrinsic role in regulating the expansion of CD8<sup>+</sup> T cells following...
FIGURE 2. Defective primary and secondary CD8+ T cell responses in p110<sup>D910A</sup> mice. (A) WT or p110<sup>D910A</sup> mice were infected with Lm-OVA on days 0, 62, and 115 (indicated with arrows) with 10<sup>4</sup> CFU Lm-OVA. The graph shows the percentage of CD8+ T cells in blood samples that were Tet+. n = 5 or 6 per group. Statistical analyses: two-way ANOVA of days 0–15, p = 0.002; days 63–110, p < 0.001; days 116–183, p = 0.0013. Results are representative of two independent experiments. (B) Mice treated with IC87114 via the food from days 21 to 10 and infected on day 0 with Lm-OVA. The number of Tet+ CD8+ T cells in the blood were enumerated by flow cytometry; n = 8/group. Statistical analyses: two-way ANOVA, p = 0.049. Results are representative of two independent experiments. (C–E) Number of Tet+CD8+ T cells recovered from blood (cells per µl), spleen (cells per spleen), lymph (Figure legend continues)
infection in the presence of WT innate immune cells. We started by injecting different numbers of CD45.1 WT or CD45.2 p110δ<sup>D910A</sup> T cells expressing the OT1 TCR transgene into WT recipient mice expressing CD45.2 and CD45.1 Ags before infecting recipient mice with Lm-OVA. When 200,000, 1,000, or 500 OT1 T cells were injected, the magnitude of expansion by p110δ<sup>D910A</sup> OT1 T cells was reduced compared with WT OT1 T cells (Fig. 5, left panel). Interestingly, however, when 100 OT1 T cells were injected, the difference between the expansion of WT and p110δ<sup>D910A</sup> T cell responses was less noticeable (Fig. 5, left panel), indicating that the function of PI3Kδ is dependent on the size of the responding population.

Consistent with previously published results (44), transfer of 200,000 OT1 T cells quenched endogenous T cell responses and this was similar for both genotypes, indicating that the p110δ<sup>D910A</sup> T cells could compete effectively with endogenous WT T cells for access to APCs (Fig. 5, right panel). When 500 OT1 T cells were injected, the magnitude of the donor and recipient Tet<sup>+</sup>CD8<sup>+</sup> T cell response was most evident in the blood and spleen (Fig. 6D). We associated with long-term memory (Fig. 6E). Thus, p110δ<sup>D910A</sup> OT1 T cells in lymph nodes and bone marrow, which are organs associated with long-term memory (Fig. 6E). Thus, p110δ<sup>D910A</sup> CD8<sup>+</sup> T cell populations mediate protective responses to infection, but components of this defect are rescued by provision of WT OT1 T cells. These findings suggest a role for PI3Kδ in mediating signals required for CD8<sup>+</sup> T cells to support the proliferation of other responding cells.

PI3Kδ activity extrinsic to the responding CD8<sup>+</sup> T cells also contributes to their optimal expansion

The ability of coinfected WT OT1 T cells to provide help for the p110δ<sup>D910A</sup> T cells raised the question of whether other host cells also support Ag-specific CD8<sup>+</sup> T cell expansion in a PI3Kδ-dependent manner. To address this question, we injected WT OT1 T cells into either WT or p110δ<sup>D910A</sup> hosts (Fig. 6C). When the WT OT1 cells were infected with Lm-OVA 8 d previously. However, the expression of Cxcr3 did not require PI3Kδ activity within T cells because Cxcr3 expression on WT and p110δ<sup>D910A</sup> OT1 T cells transferred separately (Supplemental Fig. 2D) or together (Supplemental Fig. 2E) into WT hosts was similar. In contrast, when WT OT1 T cells were transferred into p110δ<sup>D910A</sup> hosts, Cxcr3 expression was reduced (Supplemental Fig. 2F). These results further demonstrate the lack of paracrine support for the differentiation of effector CD8<sup>+</sup> T cells in p110δ<sup>D910A</sup> mice.

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A previous study showed that CD8<sup>+</sup> T cells are themselves an important source of IL-2 production required for their optimal response to L. monocytogenes infections (50). The percentage of p110δ<sup>D910A</sup> OT1 T cells that produced IL-2 was similar to that seen with WT OT1 cells, regardless of whether the WT and p110δ<sup>D910A</sup> OT1 T cells were injected into separate or into the same WT host (Fig. 7A, 7B). To our surprise, a similar proportion of p110δ<sup>D910A</sup> OT1 T cells also produced IFN-γ and GzmB (Fig. 7C–F). The in vitro differentiation assays suggested that IFN-γ production by p110δ<sup>D910A</sup> T cells might be delayed rather than completely impaired. We therefore monitored IFN-γ production by Tet<sup>+</sup>CD8<sup>+</sup> T cells 5 d postinfection, which is the earliest a distinct population of OVA-specific T cells could be readily identified. Lm-OVA–infected YETI mice revealed that a similar proportion of WT and p110δ<sup>D910A</sup> Tet<sup>+</sup>CD8<sup>+</sup> T cells produced IFN-γ also at this earlier time point (Fig. 7G). We also monitored the rate of cell division by measuring the uptake of the nucleotide analog EdU. Surprisingly, we found similar or slightly increased proportions of Tet<sup>+</sup>CD8<sup>+</sup> p110δ<sup>D910A</sup> T cells that incorporated EdU (Fig. 7H) (even though the numbers of Tet<sup>+</sup>CD8<sup>+</sup> p110δ<sup>D910A</sup> T cells recovered were
Therefore, the reduced number of Tet⁺CD8⁺ p110<sup>D910A</sup> T cells does not likely reflect an inability to undergo cell division following antigenic stimulation in vivo. As an alternative explanation for the reduced magnitude of p110<sup>D910A</sup> Tet⁺CD8⁺ T cell expansion, we also considered that the loss of PI3K might increase the proportion of cells that undergo apoptosis. To test this, we measured the incorporation of the fluorescently labeled peptide FAM-VAD-FMK, which binds specifically to activated caspases by WT and p110<sup>D910A</sup> Tet⁺CD8⁺ cells isolated from mice infected 5 d previously (Fig 7I). However, a lower proportion of p110<sup>D910A</sup> Tet⁺CD8⁺ T cells than WT T cells showed signs of undergoing apoptosis. Hence, the requirement for PI3K by CD8⁺ T cells activated in vitro to produce IFN-γ and GzmB is not shared with p110<sup>D910A</sup> CD8⁺ T cells that respond to <i>L. monocytogenes</i> infection in vivo.

**FIGURE 3.** Intact formation of memory T cells and long-term memory responses in p110<sup>D910A</sup> mice. Tet⁺CD8⁺ T cells in blood, spleen, lymph nodes, and bone marrow 8 d postinfection with Lm-OVA are shown. (A) Representative FACS profiles showing CD127 and KLRG1 expression on WT or p110<sup>D910A</sup> Tet⁺CD8⁺ T cells isolated from the indicated organs 8 d after primary infection with Lm-OVA. (B and C) Graphical representation of CD127<sup>high</sup>KLRG1<sup>low</sup> MPECs (B) and CD127<sup>low</sup>KLRG1<sup>high</sup> SLECs (C) 8 d post infection. n = 6, representative experiment of three. *<i>p</i> < 0.05 by unpaired t test. (D and E) WT or p110<sup>D910A</sup> mice were infected with Lm-OVA on days 0 and 54 and the number of Tet⁺CD8⁺ T cells in the blood was enumerated by flow cytometry. (D) Mice were 9 wk old at the start of experiment. (E) Mice were 70 wk at the start of the experiment. (F) WT or p110<sup>D910A</sup> mice were infected with Lm-OVA on days 0 and 359 and the number of Tet⁺CD8⁺ T cells in the blood was enumerated at regular intervals by flow cytometry. n = 5-6/group. Statistical analyses: two-way ANOVA, days 0–20, <i>p</i> = 0.041; days 363–366, not significantly different. Results are representative of two independent experiments.
Moreover, the CD8+ T cells that are detected several days after infection proliferate normally and do not show increased susceptibility to apoptosis. These data suggest that the signaling lesion that causes impaired expansion of p110\textsuperscript{D910A} CD8+ T cells may manifest itself relatively early during the immune response and provide evidence that redundant pathways cooperate with PI3K\textsubscript{D} to promote cytokine expression and cytolytic function in response to infection in vivo.

**Discussion**

In this study, we have shown that PI3K\textsubscript{D} controls the magnitude of both primary and secondary CD8+ T cell responses in response to infection with the intracellular bacterial pathogen *L. monocytogenes*. However, inhibition of PI3K\textsubscript{D} does not prevent the differentiation of naive CD8+ T cells into competent long-lived CD8+ Tmem, nor for Teff to produce IFN-\gamma and GzmB in response to infection in vivo. The ability of CD8+ T cells to generate a full-magnitude primary response to *L. monocytogenes* infection depends in part on PI3K\textsubscript{D} activity within responding T cell populations.

Recent work from Hodgkin and colleagues (51) has demonstrated that CD8+ T cells integrate signals from the Ag receptor, costimulatory receptors, and cytokine receptors in a linear manner to determine their “division destiny,” which represents the total number of divisions each cell will undergo before contraction of the immune response. We propose that transduction of antigenic signals is weaker in p110\textsuperscript{D910A} T cells, and this has long-term consequences in terms of the magnitude of clonal expansion by limiting the number of CD8+ T cells that will reach their full proliferative capacity, even though a snapshot of T cells responding to the Ag (several days after infection) revealed unperturbed proliferation. Possible mechanisms that might explain such reduced proliferative capacity after initial activation may include fewer cells that respond to *L. monocytogenes*, delayed or sustained first divisions, or selective loss of daughter cells after the first division. We are currently analyzing the division of transferred T cells during the first 24–48 h postinfection to evaluate these possibilities. It is also possible that aberrant expression of homing receptors such as CD62L and Cxcr3 prevents p110\textsuperscript{D910A} T cells from fully engaging with the relevant APCs during the early stages of their response to infection. However, the reduced number of Tet+CD8+ Teff did not appear to be caused by altered anatomical redistribution, as we did not find any organ with increased numbers of endogenous Tet+CD8+ T cells in p110\textsuperscript{D910A} mice relative to WT mice infected with *L. monocytogenes*.

When we only injected 100 OT1 T cells, we observed comparable expansion of T cells from WT and p110\textsuperscript{D910A} donors. Similarly, we also demonstrated in OT1 cotransfer experiments that Ag-specific WT CD8+ T cells can provide help to other responding CD8+ T cells such that the p110\textsuperscript{D910A} OT1 T cells expand normally under conditions of cotransfer. Presumably, in both of these cases, there will be an excess of endogenous and/or cotransferred WT OVA-specific T cells also responding to Lm-OVA. Our data and previously published data suggest an endogenous pool of SIINFEKL-specific CD8+ T cells equivalent to 500

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**FIGURE 4.** Enhanced bacterial clearance by p110\textsuperscript{D910A} mice. (A) WT or p110\textsuperscript{D910A} mice were infected with Lm-OVA CFUs recovered from the spleen enumerated at indicated times after infection; \(n = 3–4\)/group, bars show SEM. Statistical analyses: \(p = 0.0002\) by two-way ANOVA. (B and C) Number of CFUs recovered from the spleen (B) or liver (C) 72 h postinfection from a separate experiment from (A) after injection of \(4 \times 10^7\) CFU Lm-OVA. Similar results were obtained from four similar experiments.

**FIGURE 5.** CD8 T cell–intrinsic role for p110\textsubscript{D} is dose-dependent. The indicated number of purified WT OT1 T cells (CD45.1+) or p110\textsuperscript{D910A} OT1 T cells (CD45.2+) were transferred into WT recipient mice (CD45.1+CD45.2+), which were infected 1 d later with Lm-OVA and the number of OT1 T cells and endogenous Tet+CD8+ CD8 T cells in the blood was determined by flow cytometry. The left panel shows number of transferred OT1 T cells recovered from blood samples (filled symbols indicate WT OT1; empty symbols indicate p110\textsuperscript{D910A} OT1). The right panel shows endogenous Tet+CD8+ T cells of WT mice receiving WT OT1 cells (filled symbols) or p110\textsuperscript{D910A} OT1 T cells (empty symbols); \(n = 4–8\)/group. Results are representative of two to four independent experiments.
FIGURE 6. T cell–intrinsic and –extrinsic roles for p110δ during primary and secondary responses. (A) Five hundred WT (CD45.1) or p110δ<sup>D901A</sup> (CD45.2) OT1 T cells were transferred into WT hosts (CD45.1 or CD45.2) on day −1. Mice were then infected with Lm-OVA on days 0 and 40. Graphs show the number of OT1 T cells enumerated in the blood following primary and secondary infection by flow cytometry; n = 7–8/group. Filled symbols indicate WT OT1; empty symbols indicate p110δ<sup>D901A</sup> OT1 T cells. Statistical analyses: two-way ANOVA, days 6–10, p = 0.0415; days 45–25, p = 0.0409. Results are representative of three independent experiments. (B) Two hundred fifty WT (CD45.1) and 250 p110δ<sup>D901A</sup> (CD45.2) OT1 T cells were cotransferred into WT hosts (CD45.1/CD45.2) and infected with Lm-OVA the next day and 62 d later. The number of WT and p110δ<sup>D901A</sup> OT1 T cells in the blood is shown. Filled symbols indicate WT OT1 T cells; empty symbols indicate p110δ<sup>D901A</sup> OT1 T cells; n = 8/group. Statistical analyses: two-way ANOVA, days 2–43 and days 66–69 genotypes, not significantly different. Results are representative of three independent experiments. (C) Five hundred WT OT1 T cells (CD45.1) were transferred to either WT (CD45.1/CD45/2) or p110δ<sup>D901A</sup> (CD45.2) mice and infected the following day and then again on day 60 with Lm-OVA. The number of WT OT1 T cells in blood is shown. Filled symbols indicate OT1 cells in WT hosts; empty symbols indicate OT1 cells in p110δ<sup>D901A</sup> hosts; n = 8/group. Statistical analyses: day 1–12, p = 0.0061; days 62–94, not statistically different (two-way ANOVA). (D–F) Number of donor OT1 cells recovered from indicated organs at the peak of the secondary or tertiary responses in repeat experiments, corresponding to (A)–(C). (D) Mice were infected on days 0, 43, and 111 and the number of OT1 T cells in the blood, spleen lymph nodes, and bone marrow was enumerated by flow cytometry at peak tertiary response (day 5 following third infection); n = 7–8/group. Results are representative of two independent experiments. (E) Mice were infected on days 0 and 43 and the number of OT1 T cells in the blood, spleen, lymph node, and bone marrow was enumerated by flow cytometry at peak secondary response (day 5 following second infection); n = 7–8/group. Results are representative of two independent experiments. (F) Mice were infected on days 0 and 52 and the number of WT OT1 T cells in the blood, spleen, lymph nodes, and bone marrow was enumerated by flow cytometry at peak secondary response (day 5 after second infection); n = 8/group. Results are representative of three independent experiments. *p < 0.05 by Student unpaired t test.
transferred OT1 cells. Hence endogenous SIINFEKL-specific CD8+ T cells would exceed transferred p110\textsuperscript{D910A} OT1 cells by a ratio of 5:1 when 100 cells were injected. Similar calculations estimate the ratio of WT to p110\textsuperscript{D910A} to be 3:1 in the cotransfer experiments (500 WT endogenous Tet+CD8+ cells and 250 cotransferred WT OT1 cells against 250 p110\textsuperscript{D910A} OT1 cells). It seems, therefore, that the WT Ag-specific CD8+ T cells need to outnumber the p110\textsuperscript{D910A} T cells by a factor of \(\sim 3:1\) or more to provide sufficient signals to support normal expansion of the p110\textsuperscript{D910A} OT1 T cells. We also consider the alternative hypothesis that p110\textsuperscript{D910A} OT1 cells may impede the expansion of cotransferred WT OT1 cells. However, when larger numbers of p110\textsuperscript{D910A} T cells were injected into WT hosts, they did not appear to be more potent than WT OT1 cells at suppressing endogenous responses (which would be expected if p110\textsuperscript{D910A} T cells suppressed the expansion of WT T cells). Hence, we favor the hypothesis that CD8+ T cells responding to the same Ag can support one another to promote optimal proliferation and that their ability to provide such help depends on PI3K\textsuperscript{δ}.

In addition to the CD8+ T cells themselves, it is likely that PI3K\textsuperscript{δ} expressed by other cell types also contributes to the production of cytokines and/or costimulatory ligands that promote CD8+ T cell expansion. Thus, when WT OT1 cells were transferred into a p110\textsuperscript{D910A} host, the T cells showed impaired expansion and differentiation when compared with a WT host. We have previously shown that the deletion of Pten within activated CD4+ T cells leads to enhanced Tet+CD8+ T cell responses to Lm-OVA infection (52). CD4 T cells express a number of ligands and cytokines that help facilitate optimal CD8+ T cell responses, and PI3K\textsuperscript{δ}-deficient T cells produce less IL-2, IFN-\(\gamma\), IL-21, and CD40L (6, 53–55). It is also possible that PI3K\textsuperscript{δ}-deficient innate immune cells fail to support optimal CD8+ T cell expansion.

Our studies showed that whereas the Teff response was diminished in p110\textsuperscript{D910A} mice, the production and maintenance of Tmem were largely intact. Administration of a low dose of rapamycin has been shown to enhance the generation of memory T cells following infection (23). Whether the level of inhibition of mTOR in those studies was comparable to that observed when PI3K\textsuperscript{δ} is inactivated remains to be determined. It is nevertheless interesting that in addition to promoting Tmem, rapamycin is the only drug that has been proven to delay age-related decline in mice (56). We have previously shown that genetic alterations that prolong life by altering PI3K and/or mTOR signaling also preserve the balance of naive

**FIGURE 7.** T cell cytokine and granzyme expression is not dependent on p110\textsuperscript{D910A} in vivo. (A–F) Five hundred WT or p110\textsuperscript{D910A} OT1 T cells were transferred to WT hosts. Mice were infected the next day with Lm-OVA and killed on day 8 postinfection. Proportion of CD44\textsuperscript{high} transferred cells in the spleen producing IL-2 (A and B), IFN-\(\gamma\) (C and D), or GzmB (E and F) from mice in which the OT1 cells were transferred into different hosts (A, C, and E) or cotransferred into the same host (B, D, and F). Representative plots are shown to the right of the graphs; \(n=5–8\) group. No statistically significant differences were found by an unpaired \(t\) test. Results are representative of two independent experiments. (G) WT or p110\textsuperscript{D910A} mice on the YETI background were infected with Lm-OVA. Tet+CD8+ T cells harvested from the spleen were analyzed for YFP fluorescence 5 d later. (H) Incorporation of the EdU by Tet+CD8+ T cells 5 d postinfection. (I) Staining with the caspase-binding peptide FAM-VAD-FMK by Tet+CD8+ T cells isolated from spleens 5 d postinfection. **\(p<0.01\).
T cells to Tmem (which is higher in young mice than in old mice) (57, 58). In this context, it is pertinent to note that although p110\(^{D910A}\) mice mounted reduced magnitude CD8\(^+\) T cell responses, old p110\(^{D910A}\) mice did not show as strong evidence of age-related decline as displayed by the WT mice. Further studies are needed to establish whether PI3K\(\delta\) inhibition can delay ageing-related decline of the immune system.

The results presented in the present study have potential clinical implications. The PI3K\(\delta\) inhibitor idelisib is now an approved drug for the treatment of chronic lymphocytic leukemia (59, 60). So far, there is little evidence that PI3K\(\delta\) inhibitors increase the risk of infection in these patient populations. The results presented in the present study suggest that, at least in some contexts, PI3K\(\delta\) inhibition may in fact help limit bacterial infection. Furthermore, our findings raise the possibility that PI3K\(\delta\) inhibitors could be used to augment immune function in the context of certain acute and chronic viral and bacterial infections.

We have recently shown that p110\(^{D910A}\) mice are resistant to cancer and that this resistance is dependent on CD8\(^+\) T cells (33). Moreover, p110\(^{D910A}\) mice that rejected an initial tumor inoculation were highly resistant to a second challenge with a higher tumor dose, suggesting intact formation of CD8\(^+\) Tmem also in the context of cancer (33). However, the resistance to cancer was more striking in mice in which p110\(^{\delta}\) was only deleted in regulatory T cells, indicating that PI3K\(\delta\) inhibition did have some adverse effects on effector cells, possibly including CD8\(^+\) T cells (33). In this context, it is interesting that delayed inhibition of PI3K\(\delta\) (drug added 24 h after activation) inhibited CD4\(^+\) T cells, but less so CD8\(^+\) T cells (6). Moreover, the inhibition of PI3K\(\delta\) within differentiated CD8\(^+\) T cells has minimal effect on the ability of CTLs to kill cancer cells (33, 61). The present study may thus form the basis for designing strategies that will favor more robust CD8\(^+\) T cell responses while maintaining suppression of regulatory T cell function in the context of PI3K\(\delta\) inhibition. Indeed, tumor vaccines, which can include L monocytes, may be one such approach (62).

Activated PI3K\(\delta\) syndrome patients that harbor activating mutations in the gene that encodes p110\(^{\delta}\) (PIK3CD) suffer from recurrent bacterial and viral infections (63, 64). Interestingly, the patients have activated but exhausted CD8\(^+\) cells and lack CD8\(^+\) Tmem. In these patients, constitutive PI3K\(\delta\) signaling may thus have favored the differentiation of CD8\(^+\) T eff over Tmem, in direct contrast to the p110\(^{D910A}\) mice in which CD8\(^+\) Tmem persist, but CD8\(^+\) T eff are less abundant. It will be of interest to determine whether PI3K\(\delta\) inhibitors can restore normal CD8\(^+\) T cell function in activated PI3K\(\delta\) syndrome patients.

In summary, PI3K\(\delta\) inhibition attenuates immediate CD8\(^+\) T eff responses, but it is not detrimental to the generation of long-lived CD8\(^+\) Tmem responses. These observations should help inform therapeutic use of PI3K\(\delta\) inhibitors for diverse clinical indications.

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Disclosures
The authors have no financial conflicts of interest.

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Supplemental Figure 1. CD62L and CCR7 expression by Tet⁺CD8⁺ T cells in the blood at day 8 post infection

CD44 and CD62L expression by Tet⁺CD8⁺ T cells in the blood at day 8 post infection. Representative of two independent experiments.
Supplemental Figure 2. T cell response to non-replicating antigens and Cxcr3 expression

A. 500 OT1 cells were transferred into WT or p110δ^{D910A} hosts which were immunized with SIINFEKL peptide resuspended in Pam4CK (A) or Ova protein resuspended in LPS (B). The number of OT1 cells per ul blood is shown. A. OT1 cells detected 5 days after immunization. B. OT1 cells detected 6-8 days after immunization.

C. Cxcr3 expression on Tet^+CD8^+ T cells isolated from WT (black line) or p110δ^{D910A} (red line) mice that had been infected with *Lm*-ova 8 days previously.

D, E. Cxcr3 expression on WT (black) or p110δ^{D910A} (red) OT1 cells that had been transferred into different (D) or the same (E) WT hosts which were then infected with *Lm*-ova. The OT1 T cells were recovered 8 days later.

F. WT OT1 cells that had been transferred into WT (black) or p110δ^{D910A} (red) host and recovered 8 days after infection with *Lm*-ova.