PI3Kδ Is Essential for Tumor Clearance Mediated by Cytotoxic T Lymphocytes

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Abstract

Background: PI3Kδ is a lipid kinase of the phosphoinositide 3-kinase class 1A family and involved in early signaling events of leukocytes regulating proliferation, differentiation and survival. Currently, several inhibitors of PI3Kδ are under investigation for the treatment of hematopoietic malignancies. In contrast to the beneficial effect of inhibiting PI3Kδ in tumor cells, several studies reported the requirement of PI3Kδ for the function of immune cells, such as natural killer and T helper cells. Cytotoxic T lymphocytes (CTLs) are essential for tumor surveillance. The scope of this study is to clarify the potential impact of PI3Kδ inhibition on the function of CTLs with emphasis on tumor surveillance.

Principal Findings: PI3Kδ-deficient mice develop significantly bigger tumors when challenged with MC38 colon adenocarcinoma cells. This defect is accounted for by the fact that PI3Kδ controls the secretory perforin-granzyme pathway as well as the death-receptor pathway of CTL-mediated cytotoxicity, leading to severely diminished cytotoxicity against target cells in vitro and in vivo in the absence of PI3Kδ expression. PI3Kδ-deficient CTLs express low mRNA levels of important components of the cytotoxic machinery, e.g. prf1, grzmA, grzmB, fasl and trail. Accordingly, PI3Kδ-deficient tumor-infiltrating CTLs display a phenotype reminiscent of naive T cells (CD69lowCD62Lhigh). In addition, electrophysiological capacitance measurements confirmed a fundamental degranulation defect of PI3Kδ−/− CTLs.

Conclusion: Our results demonstrate that CTL-mediated tumor surveillance is severely impaired in the absence of PI3Kδ and that impaired immunosurveillance may limit the effectiveness of PI3Kδ inhibitors in long-term treatment.

Introduction

The common catalytic function of phosphoinositide 3-kinases (PI3Ks) is the phosphorylation of the D3-position of phosphatidylinositol. The PI3K family consists of three classes based on their primary structure, regulation, and in vitro liquid substrate specificity. Class I PI3Ks catalyze the phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP2) and thereby generate phosphatidylinositol 3,4,5-triphosphate (PIP3). PIP3 is selectively recognized by some pleckstrin homology domains and thus provides a membrane docking site for many different proteins, e.g. the serine-threonine-kinase AKT and its upstream activator the phosphoinositide-dependent kinase-1 (PDK1), the guanine nucleotide exchange factors for ARF6 ARNO (ARF nucleotide-site opener), the general receptor of phosphoinositiode-1 (GRP1), and non-receptor tyrosine kinases of the BTK and TEC-family. Accordingly, class I PI3Ks impinge on many cellular signaling cascades, which affect cell growth and survival, trafficking of vesicles and dynamics of the actin cytoskeleton. As a consequence, the PI3K/AKT/mTOR pathway has been shown to play an important role in apoptosis and cancer [1].

Class I PI3Ks are heterodimeric molecules comprising a catalytic and a regulatory subunit. There are four catalytic isoforms of class I PI3Ks (class IA p110α, p110β, p110δ and class IB p110γ). The isoforms p110α and p110β are ubiquitously expressed, whereas p110δ and p110γ are predominantly expressed in the hematopoietic system [2,3]. Currently, tools to study PI3K
signaling range from genetically modified mouse strains either lacking individual class I PI3K isoforms or harboring point mutations giving rise to catalytically inactive proteins, to PI3K isoform-specific small-molecule inhibitors [4].

T lymphocytes are of particular interest because they express all four catalytic isoforms. The enzymes can therefore be envisaged to have both, redundant and unique functions. In fact, T cells develop normally in mice with engineered deletions or kinase-dead (KD) versions of PI3Kδ [5,6], but in PI3Kδ-deficient mice T cells show partial defects in β-selection [7]. In contrast, mice deficient in both, PI3Kδ and PI3Kγ, suffer from a profound block at the pre-T cell receptor (pre-TCR) selection step of thymus development. In these mice the numbers of splenic CD4+ and CD8+ T cells are significantly reduced and the majority of peripheral CD4+ T cells display a memory phenotype [8,9]. Using small-molecule inhibitors, Ji et al [9] demonstrated that in mature T cells PI3Kδ, but not PI3Kγ, controls Th1 and Th2 cytokine secretion.

PI3Kδ is a key component of the signaling machinery downstream of the TCR and CD28 [10] and it is the most relevant isoform responsible for PI3K activation at the immunological synapse upon TCR activation [10,11]. Hence, PI3Kδ-deficient CD4+ T helper (Th) cells display defects in antigen-presenting cell-mediated stimulation and clonal expansion in vitro and in vivo [12]. PI3Kδ-KD CD4+ T cells proliferate moderately slower in response to anti-CD3 (aCD3) stimulation, but this defect is rescued by antibody-dependent co-stimulation of CD20 [10]. Upon physiological stimuli PI3Kδ-KD Th2 cells show reduced differentiation along the Th1 and Th2 lineages [12]. As a consequence of reduced Th2 responses, PI3Kδ-deficient mice are protected from experimentally-induced airway inflammation [13]. Additionally, a study by Haylock-Jacobs et al [14] showed that PI3Kδ is a key player in the pathophysiology of experimental autoimmune encephalomyelitis (EAE), a Th17-driven model of multiple sclerosis. Furthermore, loss of PI3Kδ was also shown to compromise the function of regulatory T cells [15], another CD4+ T cell lineage.

While the role of PI3Kδ in CD4+ T cells is understood in considerable detail, it is not clear whether the enzyme is important for the function of CD8+ cytotoxic T lymphocytes (CTLs). Thus, the aim of the present study was to determine the consequences of PI3Kδ-deficiency on CTL functions in vitro and in vivo. Our observations clearly show that PI3Kδ is indispensable at several stages of CTL biology. PI3Kδ-deficiency impedes the activation of CTLs and gives rise to inactive and quiescent CTLs, whose composition of the lytic machinery required for degranulation and target cell lysis is altered and functionally impaired. This defect severely curtails CTL-mediated antigen-specific cytotoxicity and impairs tumor surveillance. PI3Kδ-deficient mice develop significantly bigger solid tumors after inoculation with MC38 colon carcinoma cells. These results evoke safety concerns and challenge the use of PI3Kδ inhibitors in cancer treatment. Impaired CTL-mediated immunosurveillance might limit the effectiveness of PI3Kδ inhibitors by countering intended treatment effects on tumor target cells. However, we suggest that PI3Kδ inhibitors might be of therapeutic relevance in areas where suppression of CD8+ T cells is useful, e.g. in transplantation medicine or in the treatment of autoimmune diseases and chronic obstructive pulmonary disease (COPD).

Results
PI3Kδ Is Required to Induce T Cell Responses to Allogeneic Lymphocytes
As PI3K p110 isoforms might have redundant functions, we determined expression levels of PI3Kα, β, and γ in PI3Kδ−/−

 CTLs. Figure S1 illustrates no major alterations or compensations of other PI3K p110 isoforms in PI3Kδ−/− CTLs.

The mixed lymphocyte reaction (MLR) shall induce T cell activation and enhanced proliferation. We challenged carboxyfluorescein succinimidyl ester (CFSE)-labeled wild type and PI3Kδ−/− splenocytes (C57BL/6 background) in vitro with allogeneic lymphocytes from BALB/c mice (different MHC haplotype). Over a period of 84 hours the proliferation of CD8+ CFSE-labeled cells was assessed via flow cytometry according to the incremental reduction in CFSE-intensity due to cell division (Figure 1A). PI3Kδ−/− CTLs failed to react with enhanced proliferation upon challenge with allogeneic antigens, as their growth rates were comparable to unstimulated CD8+ T cells. Additionally, we performed pharmacological inhibition of PI3Kδ with CAL-101. This potent and selective inhibitor of PI3Kδ has already paved its way into human clinical trials [16], for review see [17,18]. The efficient inhibition of PI3Kδ by CAL-101 was confirmed by abrogated phosphorylation of the downstream target AKT. Further, we ruled out that in vitro treatment of CTLs with CAL-101 evoked toxic effects (Figure S2). Pharmacological inhibition of PI3Kδ resulted in a reduced proliferative response of CD8+ CFSE-labeled cells towards allogeneic lymphocytes, mimicking the phenotype of PI3Kδ−/− CTLs (Figure 1B). In contrast, wild type CTLs were rapidly activated and the cells proliferated significantly faster upon co-incubation with allogeneic splenocytes. These differences were not related to a general inability of PI3Kδ−/− CTLs to divide and grow, as proliferation in response to anti-CD3ε: Figure 1C) and interleukin-2 (IL-2, Figure 1D) was unaltered. Similarly, we failed to detect any alterations in the apoptotic behavior of PI3Kδ−/− CTLs after IL-2 withdrawal: apoptosis rates in wild type and PI3Kδ−/− CTLs were comparable, which was assessed via cell cycle staining with propidium iodide (data not shown). These experiments led us to the conclusion that PI3Kδ is dispensable for CTL proliferation per se, but required in the activation process of CD8+ CTLs when challenged with allogeneic lymphocytes.

Calcium (Ca2+) is released in response to TCR activation and is the final step to trigger CTL response. It was shown previously that Ca2+ influx into PI3Kδ−/− CD8+ T cells was impaired [19]. Accordingly, we examined, whether a defect in early TCR signaling or altered Ca2+-concentration in intracellular stores accounted for the missing response of PI3Kδ−/− CTLs to foreign antigens. Intracellular Ca2+-flux was monitored after preloading cells with the fluorescent indicator dye Indo-1. Importantly, PI3Kδ-deficiency did not interfere with Ca2+-influx triggered by TCR-crosslinking, indicating that early TCR signaling is intact in PI3Kδ−/− CD8+ T cells (Figure 1E). We also analyzed the size of the intracellular releasable storage pool in the endoplasmic reticulum by blocking the sarco/endoplasmic reticulum Ca2+-ATPase with thapsigargin. The ensuing increase in intracellular Ca2+ was virtually identical in PI3Kδ−/− and wild type CTLs (Figure 1F). These experiments ruled out that altered Ca2+-mobilization accounted for any functional failure of PI3Kδ-deficient CTLs.

PI3Kδ is Needed to Arm CTLs
The major task of CTLs is the eradication of cells containing non-self-antigens, i.e. cancer cells or virally infected cells. For their cytotoxic action CTLs rely on lytic granules filled with proteolytic enzymes such as granzymes and perforin. We evaluated the expression of these components in aCD3-activated CTLs and found significantly reduced mRNA levels for grzmA, grzmB and prf1 in PI3Kδ−/− CTLs (Figure 2A). This indicates that PI3Kδ is required to arm CTLs for efficient lysis. Moreover, CTLs are
Figure 1. Diminished reaction of PI3Kδ−/− CTLs to allogeneic mixed lymphocytes, but unaltered proliferation and Ca²⁺-response. A. WT and PI3Kδ−/− splenocytes were CFSE-labeled and cultured in the absence and presence of allogeneic (BALB/c), mitomycin C-treated splenocytes. At the indicated time points, cells were harvested and proliferation of responding CTLs was assessed by flow cytometry. Percentages of proliferating CFSE⁺CD8⁺ T cells with and without the stimulus of mixed lymphocytes are illustrated. Proliferating CD8⁺ T cells were discriminated from undivided T cells by the reduced levels of CFSE in daughter cells. B. WT splenocytes were CFSE-labeled and cultivated in analogy to A. Pharmacological inhibition of PI3Kδ was achieved by treatment with indicated concentrations of CAL-101 during the experimental procedure. DMSO-treatment served as negative control. C. Proliferation of WT and PI3Kδ−/− CTLs in response to aCD3 treatment was assessed in a CFSE proliferation assay. D. Proliferation of WT and PI3Kδ−/− aCD3-activated T cells was assessed under standard T cell medium conditions (in the presence of IL-2) and after deprivation from IL-2 by performing an [³H]-thymidine incorporation assay over 48 hours (with IL-2: WT: 12097±491 cpm; versus PI3Kδ−/−: 12413±501 cpm; without IL-2: WT: 1392±381 cpm; versus PI3Kδ−/−: 1140±160 cpm, n=6, values represent mean±SD). E., F. WT and PI3Kδ−/−
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splenocytes were stained with 1 μM Indo-1 AM. Ca²⁺ flux in response to αCD3ε followed by crosslinking with streptavidin (E) or thapsigargin (F) was measured in CD8+ T cells using flow cytometry. Treatment with ionomycin served as positive control. Three independent experiments were carried out and one representative experiment is shown, respectively.

doi:10.1371/journal.pone.0040852.g001

PI3Kδ-deficiency Impairs Antigen-specific Response in vitro and cytotoxicity of CTLs in vivo

So far we demonstrated that PI3Kδ–/– CTLs do not respond to foreign antigens with enhanced proliferation, that they express reduced levels of important components of the lytic machinery, and that they have a severe degranulation defect. Hence, all these data reveal an essential role of PI3Kδ for CTL-mediated cytotoxicity at different stages in the canonical killing pathway. To investigate the antigen-specific cytotoxicity of CTLs in vivo, wild type and PI3Kδ–/– mice were immunized subcutaneously with the SIINFEKL peptide in combination with an adjuvant (CpG). One week thereafter mice received syngeneic target cells containing CFSElow splenocytes, CFSEmid splenocytes pulsed with an irrelevant peptide and CFSEhigh splenocytes pulsed with SIINFEKL mixed in a 1:1:1 ratio intravenously. Following 18 hours, the draining inguinal lymph nodes and spleens of wild type and PI3Kδ–/– mice were analyzed by flow cytometry. Figure 4A depicts a representative histogram plot obtained from lymph nodes of immunized wild type and PI3Kδ–/– mice: whereas the splenocytes loaded with SIINFEKL peptide were drastically reduced in lymph nodes from wild type mice after immunization, this effect was observed to a significantly lower extent in PI3Kδ–/– mice (summarized in Figure 4B). These experiments document a reduced cytotoxic ability of CTLs in the absence of PI3Kδ. To further support this finding, we additionally challenged PI3Kδ–/– CTLs in an in vitro experimental set-up. We again immunized wild type and PI3Kδ–/– mice with SIINFEKL peptide plus adjuvant over a period of two weeks. Thereafter, splenocytes were prepared and co-cultivated with SIINFEKL-loaded splenocytes for five days. The thymoma cell lines EL4 and its corresponding ovalbumin (OVA)-expressing cell line EG7 were used to test the cytolytic capacity of the splenocytes. EL4 cells should not be recognized and were used as targets to determine unspecific background killing, which was comparable between wild type and PI3Kδ–/– CTLs (Figure 4C). In contrast, OVA-expressing EG7 cells were efficiently recognized and killed by primed wild type CTLs, whereas primed PI3Kδ–/– CTLs failed to induce any detectable antigen-specific cytotoxicity above background levels (Figure 4D). To confirm the impact of PI3Kδ
Figure 2. Reduced expression of cytotoxic components in PI3Kδ−/− CTLs. WT and PI3Kδ−/− splenocytes were activated for 3 days with aCD3ε and cultured in T cell medium. A. mRNA expression of grzmA (WT: 0.097 ± 0.036; versus PI3Kδ−/−: 0.014 ± 0.01, n = 6, p = 0.0003), grzmB (WT: 0.030 ± 0.012; versus PI3Kδ−/−: 0.005 ± 0.002, n = 6, p = 0.003), and prf1 (WT: 0.045 ± 0.012; versus PI3Kδ−/−: 0.005 ± 0.002, n = 6, p = 0.003). B. mRNA expression of trail (WT: 0.097 ± 0.036; versus PI3Kδ−/−: 0.014 ± 0.01, n = 6, p = 0.003) and fasl (WT: 0.030 ± 0.012; versus PI3Kδ−/−: 0.005 ± 0.002, n = 6, p = 0.003). C. IFN-γ expression in medium and +4h IL-12. D. IFN-γ production in medium and +4h IL-12.
on CTL-mediated antigen-specific cytotoxicity we isolated splenocytes from OT-I mice and co-cultivated them with SIINFEKL-loaded splenocytes as described above. The CTLs were treated with the PI3Kδ inhibitor CAL-101 2 hours prior and during the in vitro cytotoxicity assay. Again, OVA-expressing E67 cells were efficiently lysed by DMSO-treated OT-I CTLs, whereas CAL-101-treated OT-I cells showed significantly reduced cytotoxicity (Figure S3).

PI3Kδ is Required for CTL Activation and Tumor Surveillance

At this point we speculated that PI3Kδ inhibition – besides beneficial effects on tumor growth restriction – might entail adverse effects on the immune system. Thus, we wanted to challenge the concept of PI3Kδ inhibition in the treatment of malignancies and tested whether impaired CTL-mediated cytotoxicity observed in PI3Kδ−/− mice was irrelevant for tumor surveillance. We made use of the colon adenocarcinoma cell line MC38, which is recognized and lysed in a CTL-dependent manner [23]. Fifteen days after subcutaneous injection of 10⁶ cells into the flanks of wild type and PI3Kδ−/− mice, the animals were sacrificed and the tumors analyzed. As depicted and summarized in Figure 5A and 5B significantly larger tumors had developed in PI3Kδ−/− mice compared to wild type recipient mice. When we characterized the tumor-infiltrating lymphocytes, we found comparable numbers of CD3+CD8+ effector cells irrespective whether a tumor evolved in wild type or PI3Kδ−/− recipients (Figure 5C). Investigating resting CTLs from untreated healthy mice, we found comparable expression profiles of the activation markers CD45RB and CD69 on wild type and PI3Kδ−/− lymphocytes, whereas the expression of CD44 was reduced in PI3Kδ−/− lymphocytes (Figure 5D). Pharmacological inhibition of PI3Kδ in mature wild type CTLs over a time period of 3 days resulted in reduced expression of CD44 while no difference was observed in the expression of CD69 compared to DMSO-treated wild type controls (Figure 5E). On tumor-infiltrating CTLs the expression of CD44 and CD45RB was comparable (Figure 5E, F); no consistent and significant differences in their expression levels could be detected. In contrast, we found a significantly reduced expression of the activation marker CD69 on PI3Kδ−/− tumor-infiltrating CTLs. Thus, although PI3Kδ−/− CTLs migrated to the tumor, they were activated to a lower extent. Further proof for this concept was obtained by analyzing CD62L, which is down-regulated upon activation. CD62L was consistently higher in PI3Kδ−/− CTLs (compare Figure 5D depicting expression profiles of unchallenged, resting CTLs and Figure 5E, F showing expression levels on tumor-infiltrating CTLs). Moreover, prolonged pharmacological inhibition of PI3Kδ in spleenic wild type CTLs resulted in significantly increased expression of CD62L compared to DMSO-treated wild type controls (Figure 5E, F). Spleenic CD8+ T cells comprise different subsets, amongst which naive T cells are the most prevalent ones. We could not observe any toxic effect of CAL-101 on the entity of CD8+ T cells. Hence, we are convinced that the differences in CD44 and CD62L are not the result of different CD8+ subset susceptibilities to CAL-101.

In summary, these experiments clearly verified reduced CTL-mediated tumor surveillance in PI3Kδ−/− mice accompanied by decreased activation of PI3Kδ−/− CTLs (Figure 5D-F).

Discussion

T cells express all four catalytic isoforms of PI3K. Robust networks are engineered to be resilient by built-in redundancies: because of redundant trajectories, removal of one nod does not affect the flow of signals through the network. In each instance, the product of PI3Kδ-dependent catalysis is PI(3,4,5)P3, a lipid second messenger that supposedly activates identical effectors. It was therefore surprising to observe that removal of PI3Kδ had such a profound effect on the function of CD8+ cytotoxic cells supported by the following key findings: (i) The components required for their eponymous action were only expressed at low levels in PI3Kδ-deficient CTLs (granryme A and B, perforin, FasL, TRAIL, and IFN-γ). (ii) Granule release and (iii) antigen-induced clonal expansion was impaired in PI3Kδ-deficient CTLs. (iv) Predictably, in the absence of PI3Kδ, CTLs failed to restrict MC38 tumor cell growth in vivo. Our findings suggest that the absence of PI3Kδ in CD8+ T cells phenocopies the deficiencies that have been observed in PDK1-deficient CTLs and CTLs treated with an inhibitor of AKT1 [24], suggesting that low expression levels of granymes, perforin and FasL might be due to disrupted AKT-dependent phosphorylation of Foxo3 (forkhead box O3). Similarly, CTLs treated with an AKT1-inhibitor or deficient in PDK1 also failed to mount a proliferative response upon antigen challenge. PI(3,4,5)P3, the second messenger produced by PI3Kδ, activates clearly more effectors than only AKT and PDK1. However, PI3Kδ-deficiency, AKT-inhibition and abrogation of PDK1 expression result all in similar phenotypic consequences, i.e. impaired expression of cytotoxic proteins. Hence we speculate that the pertinent signaling module that mediates extracellular input to transcriptional control is composed of PI3Kδ, PDK1, AKT and Foxo3. In this module, the requirement for PI3Kδ is absolute; the other PI3K-isoforms cannot compensate for its absence. Similarly, antigen-induced, TCR-dependent cell proliferation is contingent on the module comprising PI3Kδ, PDK1 and AKT. Our observations suggest that this is not necessarily the case for other stimuli such as IL-2, a conclusion that was reached by McIntrye et al [24] in a similar way.

CTLs can kill infected or malignant cells via degranulation. We demonstrate here for the first time that PI3Kδ is indispensable for degranulation of CTLs. Our observations extend comparable findings with mast cells [23] and Natural Killer (NK) cells [26]. Whereas the indispensable role of PI3Kδ in degranulation and cytokine secretion is beyond dispute, its relevance for NK cell cytotoxicity is seen controversially [26–31]. It is very likely that the deficiency in PI3Kδ impairs degranulation by effector mechanisms other than downstream signaling via AKT. The following arguments support this conjecture: (i) Capacitance measurements...
Figure 3. PI3Kδ is indispensable for CTL degranulation. A. Using the whole cell patch clamp technique, the cellular capacitance of αCD3-activated and in T cell medium cultivated WT and PI3Kδ−/− CTLs was determined. Membrane capacitances before (ctr) and after stimulation with PMA and ionomycin (PMA/iono) at the single cell level are summarized from two representative experiments (WT: ctr: 6.3 ± 1.2pF, PMA/iono: 9 ± 1.8pF, n = 10, p = 0.01; versus PI3Kδ−/−: ctr: 6.7 ± 1.7pF, PMA/iono: 6.6 ± 1.8, n = 9, p = 0.4; paired t-test; values represent mean ± SEM). B. Fold increase in membrane capacitance (fold increase after PMA/iono).
membrane capacitance due to stimulation with PMA/iono is calculated (WT: 1.46±0.13, n=10, p = 0.0064; versus PI3KΔ-/-: 0.94±0.06%, n=9, p = 0.3). Additionally, WT CTLs were pre-incubated with the PI3K isoform-specific inhibitor IC-87114 (1 μM, 4 μM, 1 hour), DMSO-treatment served as control (DMSO: 2.14±0.18, n=11, p = 0.0001; 1 μM IC-87114: 0.98±0.03, n=11, p = 0.49; 4 μM IC-87114: 0.98±0.01, n=9, p = 0.18; values representing mean±SEM. One sample t-test). C. In vitro cultivated WT and PI3KΔ-/- CTLs were challenged with PMA and ionomycin. The percentage of CD107a+ T cells was measured via flow cytometry before and after the stimulus (WT: ctr: 15.5±2.4%, PMA/iono: 41.2±1.3%, p<0.0001; versus PI3KΔ-/-: ctr: 24.6±2.7%, PMA/iono: 29.3±1.7%, p = 0.085; n = 8, paired t-test, values represent mean±SEM). D. Accordingly, the degranulation of WT CTLs upon pharmacological inhibition of PI3K with a specific inhibitor was tested. (C57BL/6; 8.7±1.7%; DMSO: 12.3±2.9%; p = 0.0001; n = 9, pre-incubated with 1 μM IC-87114; 0.8±2.1%, n = 9, p = 0.18; values representing mean±SEM. One-way ANOVA revealed p = 0.0001, Tukey’s Post-Hoc test was significant p = 0.001 for 1 μM and highly significant p = 0.001 for 5 μM compared to DMSO control). E. WT and PI3KΔ-/- CTLs were cultivated in T cell medium and their expression of CD107a was measured via flow cytometry under basal conditions and after electrostimulation or electrostimulation plus the degranulation inhibitor Concanaemycin A, respectively (WT: ctr: 11.5±1.2% CD107a+ T cells, electrostimulation: 41.3±3.3% CD107a+ T cells, p = 0.0001; versus PI3KΔ-/-: ctr: 11.3±1.3% CD107a+ T cells, electrostimulation: 12.0±0.8% CD107a+ T cells, p = 0.54, paired t-test, values represent mean±SEM). All experiments were performed at least two times independently and summarized in the depicted graphs.

The disclosure of the indispensable role of PI3KΔ for CTL function led us to envision potential limitations or safety concerns regarding the clinical applicability of PI3KΔ inhibitors in humans. Thus, we explored how PI3KΔ-deficiency affected CTL-mediated tumor surveillance in vivo, thereby challenging the therapeutic concept of PI3KΔ inhibition. PI3KΔ-/- CTLs infiltrated evolving tumors with unaltered frequency, but CD62L expression was severely reduced. Increase in CD62L expression is directly linked to T-lymphocyte activation [36]. Additionally, we observed a concomitant enhanced CD62L expression, which is indicative of reduced activation [37]. The failure of PI3KΔ-/- cells to down-regulate CD62L has also been observed by others, who documented the importance of CD62L as regulator of lymphocyte recirculation [38]. Whereas defective T cell recirculation may explain the increased susceptibility of PI3KΔ-/- mice towards Leishmania major second infection [39], it is unlikely that it accounts for the increased tumor growth we observed. We conclude that several deficiencies contribute to impaired immunosurveillance by PI3KΔ-deficient CTLs: (i) In vivo, PI3KΔ-/- CTLs acquire a phenotype reminiscent of naive cytotoxic T cells. In contrast to terminally differentiated effector CTLs (CD62Llow, full equipment with lytic machinery), naive cells (CD62Lhigh) are only partially equipped and do not function properly. Indeed, the cytolytic capacity of PI3KΔ-deficient T cells was found to be severely impaired both in vivo and in vitro. (ii) An additional factor leading to this naive-like phenotype is the inability of PI3KΔ-/- CD8+ T cells to react to foreign antigens via clonal proliferation. This was substantiated by the results obtained in the mixed lymphocyte reaction. (iii) Impaired secretion of IFN-γ by PI3KΔ-/- CTLs is likely to contribute to defective tumor surveillance [40], given the observation that PI3KΔ regulates IFN-γ production in several lymphocytic lineages [26,28] and is required for TCR-induced IFN-γ production [39,41].

The therapeutic concept of PI3KΔ inhibition in the treatment of hematopoietic malignancies is based on the knowledge that the PI3KΔ isoform is important in sustaining growth of leukemic cells [4,16,26,42]. Accordingly, the specific PI3KΔ inhibitor CAL-101 has already entered clinical trials for the treatment of hematopoietic malignancies [17,18,43,44]. Similarly, inhibitors for AKT are under development [45]. However, AKT is a questionable target. Due to its wide expression pattern, its inhibition is predicted to affect many cellular functions. Isoform-specific inhibitors are not yet available and their applicability is considered doubtful [46]. At the current stage, specific PI3KΔ-inhibitors can be assumed to be superior to AKT-inhibitors. We believe that these compounds will have fewer side effects (due to restricted expression pattern of PI3KΔ) and be more efficacious.

Nevertheless, PI3KΔ-specific inhibition has unintended consequences on the functional activity of CTLs at all levels: activation, antigen-induced clonal expansion, degranulation and cytotoxicity. Therefore, unintended side effects such as impaired CTL-mediated tumor surveillance might counteract or even reverse intended treatment effects of PI3KΔ-inhibitors. As a consequence, in the clinical development of PI3KΔ-inhibitors, rigorous monitoring of tumor development should be implemented. Additionally, contraindications should be carefully defined, especially with respect to pre-existing immunodeficiency and/or tumor development in a patients history. However, while impaired CTL-function may be a drawback in cancer therapy, it is evident that the suppression of CTLs would be of profound benefit in the treatment of autoimmune diseases and other CD8+ T cell-associated diseases, such as COPD (chronic obstructive pulmonary disease) [47] and even more, in transplantation medicine. This concept is supported by a recent study of Ying et al [48], who investigated the therapeutic potential of pharmacological PI3KΔ inactivation in murine models of heart and skin transplantations.

Materials and Methods

Ethics Statement

All experiments were conducted in accordance with protocols approved by the Animal Welfare Committee of the Medical University of Vienna (66.009/0155-I/3b/2011) and the Austrian Federal Ministry of Science and Research.

Mice and Cell Lines

Wild type (WT) C57BL/6 and BALB/c mice were purchased from Charles River Laboratories. PI3KΔ-/- animals [5] were backcrossed to C57BL/6 background. OT-1 mice were a kind gift from A.M. Dohnal (St. Anna Children’s Cancer Research Institute).
Institute, Vienna, Austria). All animals were housed under pathogen-free conditions according to recommendations of the European Laboratory Animal Science Associations. All experiments were done with gender- and age-matched 8- to 12-week old animals. The murine colon adenocarcinoma cell line MC38 (kindly provided by V. Sexl, University of Veterinary Medicine, Vienna, Austria [23]), the murine thymoma cell line EL4 and OVA-expressing EG7 cell line (a kind gift from D. Stoiber-Sakaguchi, Ludwig Boltzmann Institute for Cancer Research, Vienna, Austria [49]) were propagated in Dulbeccos Modified Eagle Medium (DMEM, PAA) high glucose supplemented with 10% heat-inactivated fetal calf serum (FCS, PAA), 50 μM 2-mercaptoethanol (Sigma), 100 U/ml penicillin and 100 μg/ml streptomycin (PAA).

Figure 4. PI3Kδ-deficiency leads to severely impaired CTL cytotoxicity in vivo and in vitro. A., B. WT and PI3Kδ−/− animals were immunized with SIINFEKL and CpG. Seven days later mice received CFSE-labeled targets and peptide-specific CTL activity in draining inguinal lymph nodes was analyzed by flow cytometry. A. A representative FACS histogram for each genotype is depicted. B. SIINFEKL-specific target cell killing was calculated from three independent experiments as described in Materials and Methods (WT: 80±6% antigen-specific lysis; versus PI3Kδ−/−: 52±10% antigen-specific lysis, n=12; values represent mean±SD, unpaired t-test, p<0.0001). No specific killing was observed in control mice (data not shown). C., D. WT and PI3Kδ−/− animals were immunized with SIINFEKL peptide and CpG and boosted 7 days later. To generate effector cells, splenocytes were isolated at day 14 and co-cultured for 5 days with irradiated SIINFEKL-pulsed splenocytes. To determine peptide-reactive CTL cytotoxicity in vitro, CFSE-labeled EL4 (C) and OVA-expressing EG7 (D) target cells were co-cultured with effectors in ratios of 30:1, 15:1, 5:1 and 1:1. Specific in vitro target cell killing was quantified by flow cytometry (EG7, E:T=30:1: WT: 63% specific lysis; versus PI3Kδ−/−: 15% specific lysis). One representative experiment out of three is shown.

doi:10.1371/journal.pone.0040852.g004

Pharmacological Inhibition of PI3Kδ

The selective PI3Kδ inhibitor CAL-101 was purchased from Selleck Chemicals, IC-87114 was provided by ICOS (Bothell, WA), and 2-mercaptoethanol (Sigma).
Figure 5. Impaired activation of PI3Kδ−/− CTLs by tumor cells. A. 1×10^6 MC38 colon adenocarcinoma cells were injected subcutaneously into the flanks of WT and PI3Kδ−/− animals. Fifteen days later tumor weights were analyzed. Depicted is the summary of 3 independent experiments (WT: 0.19±0.19 g; versus PI3Kδ−/−: 1.16±0.33 g, n = 12, values represent mean±SD). B. Representative pictures of MC38 solid tumors grown in WT.
and P13Kδ−/− animals. C. After removal, tumors were minced and digested with collagenase D and DNase I. Tumor-infiltrating CD3+CD8+ CTLs were quantified using flow cytometry (WT: 1.9±0.5%; versus P13Kδ−/−: 1.8±0.7%, n = 8, values represent mean±SD). D, E. Representative histograms showing expression levels of CD44, CD45RB, CD62L and CD69 on splenic CD3+CD8+ T cells from untreated WT and P13Kδ−/− mice (D) and on MC38 tumor-infiltrating CD3+CD8+ CTLs of WT and P13Kδ−/− mice (E). F. Summary of the expression levels of CD44 (WT: median = 10040, IQR = 16670-24220; versus P13Kδ−/−: median = 15053, IQR = 12750-26440, n = 8), CD45RB (WT: median = 3222, IQR = 2907-4142; versus P13Kδ−/−: median = 3945, IQR = 2579-5833, n = 8), CD62L (WT: median = 194, IQR = 146-219; versus P13Kδ−/−: median = 228, IQR = 187-333, n = 8, Mann-Whitney test: p = 0.0572) and CD69 (WT: median = 2673, IQR = 1825-7606; versus P13Kδ−/−: median = 1428, IQR = 1334-1698, n = 8, Mann-Whitney test: p = 0.0037) on tumor-infiltrating WT and P13Kδ−/− CD3+CD8+ CTLs.

doi:10.1371/journal.pone.0040852.g005

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WA). Inhibitors were used at the indicated concentrations. DMSO (ROTH) treatment served as negative control.

Generation of Splenocytes and Expansion of aCD3-activated T Cells in vitro

Spleens from WT and P13Kδ−/− animals were collected and forced through a 70 µm cell strainer. The resulting single cell suspension was treated with red blood cell lysis buffer. T cells were activated in vitro by stimulation with aCD3ε antibody (clone 145-2C11; 0.5 µg/µl) and supplemented with 10% FCS, 50 µM 2-mercaptoethanol, 1 mM sodium pyruvate (Gibco), non-essential amino acids (PAA), 100 U/ml penicillin, 100 µg/ml streptomycin and 100 U/ml rhIL-2 (Proleukin®, Novartis).

Mixed Lymphocyte Reaction (MLR)

BALB/c splenocytes were growth arrested by treatment with mitomycin C (50 µg/ml, 20 min, Sigma). C57BL/6 WT and P13Kδ−/− splenocytes were labeled with 2.5 µM carboxyfluorescin succinimidyl ester (CFSE). 1×10⁵ CFSE+ responding splenocytes (C57BL/6) were co-cultured in a ratio of 1:1 and 1:4 with stimulating splenocytes (BALB/c). Pharmacological inhibition of PI3Kδ was achieved by treatment of C57BL/6 WT splenocytes with 0.1 µM, 0.5 µM and 1 µM CAL-101 during the entire experimental procedure. After 24 h, 36 h, 48 h, 60 h, 72 h and 84 h co-cultures and controls were harvested and stained with aCD8-APC to determine CD8+ specific T cell proliferation by flow cytometry. Dead cells were excluded from analysis via gating in the FSC/SSC dot plot, % proliferating CD8+ cells define the percentage of cytotoxic T cells showing reduced CFSE intensity compared to the initial CFSE staining intensity.

CFSE Proliferation Assay

Splenocytes were labeled with 2.5 µM CFSE and cultured in T cell medium supplemented with 0.5 µg/ml aCD3ε antibody (clone 145-2C11) at a concentration of 5×10⁵ cells/ml. After 12 h, 48 h, 62 h and 86 h splenocytes were harvested and stained with aCD8-APC antibody to determine CD8+ specific T cell proliferation characterized by the incremental loss of CFSE intensity in a flow cytometer. Dead cells were excluded from analysis via gating in the FSC/SSC dot plot.

[3H]-thymidine Incorporation Proliferation Assay

aCD3-stimulated T cells were plated at a density of 5×10⁵/96 round-bottom well in triplicates in the presence of 0.1 µCi/well [3H]-thymidine (PerkinElmer) for 48 h. Incorporated radioactivity was analyzed by adding Rotiszint® eco plus (ROTH) and measured in a liquid scintillation analyzer (Tri-Carb 1900 CA, PerkinElmer, Waltham, MA).

Ca²⁺ Measurement

1×10⁶ splenocytes were stained with 1 µM Indo-1 AM (Invitrogen) in 100 µl cell loading buffer (Hanks Buffered Salt Solution (HBSS, Invitrogen)) containing 1 mM Ca²⁺, 1 mM Mg²⁺ and 1% (v/v) FCS for 30 min at 37°C and 5% CO₂. After washing twice, cells were again incubated for 30 min at 37°C in cell loading buffer. Indo-1 AM loaded cells were stained for CD8 (clone 53-6.7). After staining, cells were resuspended in cell loading buffer. Cells were gated for CD8 expression and analyzed at 37°C on a LSRII flow cytometer (BD Bioscience, Heidelberg, DE). Ca²⁺ flux was either induced by using 1 µM thapsigargin (Sigma) or by stimulation with a biotinylated aCD3 antibody (10 µg/ml) followed by crosslinking with streptavidin (Sigma) (10 µg/ml). 3.3 mM iomonomycin (Sigma) was used to induce full-scale deflection of internal Ca²⁺. The median fluorescence intensity of the Indo-1 AM violet versus Ca²⁺ expression and analyzed at 37°C. Measurement of intracellular Ca²⁺ concentration were started under superfusion (DAD-8-VC superfusion system).

Quantitative Real-time PCR

1×10⁶ in vitro expanded aCD3-activated splenocytes were analyzed under basal conditions and after stimulation with 5 ng/ml IL-12 (R&D Systems) for 4 h. Total RNA was purified using Trifast reagent (Peqlab) followed by alcohol precipitation. 1 µg of RNA was reversely transcribed using the iSCRIPT cDNA synthesis kit (Biorad). qRT-PCR was performed on an Eppendorf Mastercycler® realplex (Hamburg, DE) with SsoFast® EvaGreen® Supermix (Biorad). Glycerinealdehyd-3-phosphat-dehydrogenase (Gapdh) was used for normalization. Following primers were used: gapdh forward: 5′-TGTGTCGTCGTCGATCTGA-3′ and gapdh reverse: 5′-CCTGCCCTCAACACCCTTCCTTG-3′; ifng forward: 5′-AAGTTGGCATGATGTTGAGG-3′ and ifng reverse: 5′-GAATTGACATCCTTTTGGGCTG-3′; granzyme A (grznA) forward: 5′-TTCATTTGGGGCTCACTC-3′ and grznA reverse: 5′-GCGATCTGTTTCTGTTTGCT-3′; granzyme B (grznB) forward: 5′-CCCACTGATAGTTGGCCGGG-3′ and grznB reverse: 5′-GGCTGACAGCATATGTTTCC-3′; perforin 1 (pf1) forward: 5′-GATGTTGAACCTTGGCCAAGA-3′ and pf1 reverse: 5′-CCTGCTTACACCTTTCCTGGA-3′. Primers for P13Kδ p110α (pik3ca), p110β (pik3cb), p110γ (pik3cg) and p110δ (pik3dd) are described in [50].

IFN-γ ELISA

1×10⁶ splenocytes were cultured in 96 round bottom wells with or without concanavalin A (ConA, Sigma) (10 µg/ml) for 48 h. IFN-γ release was measured using Quantakine Mouse IFN-γ ELISA according to the manufacturer’s protocol (R&D Systems).

Capacitance Measurements

Capacitance measurements were performed in the whole-cell mode of the patch-clamp technique using the time domain method of Lindau and Neher [51]. As described for NK cells [26], as soon as stable whole-cell conditions were established, recordings of aCD3-stimulated and in vitro expanded T cells were started under superfusion (DAD-8-VC superfusion system).
with control solution (140 mM NaCl, 2.5 mM KCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 10 mM HEPES NaOH, pH 7.4) with a sample rate of 330 kHz and continued under superfusion with Ca$^{2+}$-ionophore (1 μM ionomycin dissolved in DMSO) and 500 nM phorbol myristate acetate (PMA, Sigma). IC-67114 was used at 1 and 4 μM concentrations added to standard medium 1 hour prior to the analysis; DMSO treatment served as control. Cell capacitance was calculated by integrating the area under the capacitive current transiently elicited by a 20 ms voltage step from −120 to −80 mV. This area was divided by the applied change in voltage (40 mV). Capacitance-measurements were performed at room temperature (22 ± 1.5 °C) using an Axoclamp 200B patch clamp amplifier (Axon Instruments, Foster City, CA). Pipettes were pulled from aluminum silicate glass (AF150-100-10, Science Products, Hofheim, Germany) with a P-97 horizontal puller (Sutter Instruments, Novato, CA), heat-polished on a microforge (MF-830, Narishige, Japan), and had resistances between 1 and 2 MΩ when filled with the recording pipette solution (105 mM CsF, 10 mM NaCl, 10 mM 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin). 5 × 10$^5$ splenocytes of immunized or control mice were cocultured with 2 × 10$^5$ irradiated, SIINFEKL-pulsed splenocytes in 1 ml T cell medium for 5 days.

### Generation of Peptide-reactive T Cells
Mice were immunized by subcutaneous injection of the OVA agonist peptide SIINFEKL (0.1 mg/mouse) in combination with the adjuvant CpG-ODN 1668. After seven days, mice were boosted by a second injection. Splenocytes were obtained at day 14 and suspensions of splenocytes were obtained from control mice and immunized mice as described above. In parallel, splenocytes from WT mice were prepared, irradiated (30 Gy – gamma ray) and pulsed with SIINFEKL (10 μg/ml in RPMI-1640 medium containing L-glutamine and supplemented with 10% FCS, 50 μM 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin). 5 × 10$^5$ splenocytes of immunized or control mice were cocultured with 2 × 10$^5$ irradiated, SIINFEKL-pulsed splenocytes in 1 ml T cell medium for 5 days.

### In vitro Cytotoxicity Assay
Splenocytes of control mice and splenocyte suspensions containing peptide-reactive T cells were cocultured with 5 × 10$^4$ CFSE-stained (2.5 μM) EL4 or EG7 target cells at effector-to-target (E:T) ratios of 30:1, 15:1, 5:1 and 1:1 in triplicates in 96-well plates. In parallel, tumor cells were incubated in the absence of splenocytes to assess the extent of spontaneously occurring apoptosis. After 18 h, 5 × 10$^4$ PKH26-stained control cells were added to each well as internal control and cytotoxicity was quantified via flow cytometry. 10$^4$ labeled cells (either CFSE+ or PKH26+) were counted and the CFSE+ target cells were calculated as percentage of labeled tumor cells. Dead target cells were discriminated from living cells in a control staining with propidium iodide (Sigma) and further distinguished by determination of forward and sideward scattering. The percentage of specific lysis was calculated as [1– (%CFSE$^{high}$/%CFSE$^{low}$)] × 100.

### Chronic Low-frequency Electrical Stimulation (CLFES)
WT and P3Kδ−/− aCD3-activated CTLs were seeded at a cellular density of 3 × 10$^5$ cells/ml T cell medium. Electrical stimulation was performed using the C-Pace EP from IonOptix Corporation (Milton, USA). Stimulation pulses of 5 V amplitude and 5 ms duration were delivered via two carbon electrodes to 3.5cm dishes at a frequency of 1 Hz. Chronic stimulation protocols and data acquisition were performed with pclamp 6.0 software (Axon Instruments) through a 12-bit A-D/D-A interface (Digitida 1200; Axon Instruments).

### In vivo CTL Assay
In vivo cytotoxicity was measured according to Schellack et al [52]. In brief, mice were immunized by subcutaneous injection of 0.1mg/mouse SIINFEKL (Bachem) in combination with the adjuvant CpG-ODN 1668 (Eurofins). Seven days later control mice and immunized mice received syngeneic splenocytes labeled with three different concentrations of the intracellular dye CFSE: 2.5, 0.25 and 0.025 μM. The CFSE$^{high}$ population was pulsed with the relevant SIINFEKL peptide (10 μg/ml), the CFSE$^{mid}$ population was pulsed with the irrelevant peptide m-TRP2181-188 (10 μg/ml, Bachem) and the CFSE$^{low}$ population remained untreated. The three CFSE$^+$ populations were mixed in a 1:1:1 ratio and 3 × 10$^5$ cells were injected via the tail veins of recipient WT and P3Kδ−/− mice. After 18 hours spleens and draining lymph nodes were removed. Single cell suspensions were analyzed by flow cytometry. Specific killing was calculated as [1– (% CFSE$^{high}$/% CFSE$^{low}$)] × 100.

### Antibodies and Flow Cytometric Analysis
The following antibodies were purchased from eBioscience: aCD3ε-APC, aCD8α-Alexa Fluor 488, aCD44-PE, aCD45RB-biotin, aCD62L-APC-eFluor 780, Streptavidin-PerCP-Cy5.5 and aCD16/CD32 as Fc receptor block. The antibodies aCD3ε-PerCP-Cy5.5, aCD8α-PE, aCD69-PE-Cy7 and aCD107a-FTTC were ordered from BD Biosciences. The following cell dyes were used according to the manufacturer’s protocol: Cell Trace CFSE Cell Proliferation Kit (Invitrogen) and PKH26L Red Fluorescent Cell Linker Kit (Sigma). Samples were analyzed using a FACSCanto™ II flow cytometer (BD Biosciences, Heidelberg, DE) and analyzed with FACSDiva software version 6.1.2 (BD Biosciences) or with FlowJo software version 7.6.1 (Tree Star).

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(Descriptive text about the role of PI3Kδ in CTL-mediated immunity.)
Cell Cycle Analysis

Cell cycle and apoptosis analysis were conducted according to Hoelbl et al. [53]. Dead cells were defined as cells with DNA contents lower than 2 n (sub-G0/G1).

Whole Cell Extracts and Western Blot Analysis

Cells were lysed in 50 mM Tris/HCl pH 8.0, 10% (v/v) glycerol, 25 mM EDTA, 150 mM NaCl (all from ROTH), 2 mM DTT, 0.5% NP40 (Igepal CA-630), 25 mM NaF, 1 mM sodium vanadate, 0.5 mM PMSE, SIGMA/AES Protease Inhibitor (all from Sigma Aldrich Austria), and cell debris removed by centrifugation and re-suspended in 50 μl 2 x Laemml sample buffer. Proteins were separated with SDS-PAGE and blotted onto nitrocellulose membranes (Hybond, GE Healthcare Austria). PageRuler® Prestained Protein Ladder (Fermentas ThermoScientific Austria) was used as molecular weight standard. Membranes were a kind gift from J. Werzowa (Department of Clinical Pharmacology, Medical University of Vienna, Vienna, Austria). The peroxidase-conjugated secondary rabbit antibody was from GE Healthcare (Austria).

Statistical Analysis

Student’s t-test, Mann-Whitney U test, paired t-test, One-sample t-test and One-Way ANOVA were performed using GraphPad Prism® version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. Statistical analysis is indicated for each experiment specifically (*... p<0.05; **... p<0.01; ***... p<0.001).

Supporting Information

Figure S1 Expression of class I PI3K catalytic isoforms in PI3Kδ-/- CTLs. WT and PI3Kδ-/- splenocytes were activated for 3 days with aCD3ε and cultured in T cell medium in order to obtain highly purified CTLs. mRNA expression of PI3Kz (pik3cz): WT: 0.0034±0.0006; versus PI3Kδ-/-: 0.0033±0.0005, n=5, p=0.89), PI3Kβ (pik3cb): WT: 33e-5±5.7e-5; versus PI3Kδ-/-: 37e-5±7.3e-5, n=5, p=0.66), PI3Kγ (pik3cg): WT: 0.015±0.0017; versus PI3Kδ-/-: 0.011±0.0008, n=5, p=0.08) and PI3Kδ (pik3d): WT: 44e-5±11e-5; versus PI3Kδ-/-: not detected (n.d.), n=5) was quantified via qRT-PCR and normalized to the house-keeping gene gapdh. Values represent means±SEM, unpaired t-test. (TIF)

Figure S2 Pharmacological properties of the selective PI3Kδ inhibitor CAL-101. A. aCD3-activated C57BL/6 WT CTLs were cultivated in T cell medium supplied with varying concentrations of CAL-101, DMSO, or left untreated (w/o), respectively. Cell cycle analysis revealed that short-term (3 h, 6 h) treatment with CAL-101 did not affect CTL cell viability significantly (3 h: w/o: 41.2±2.9%; dms: 42.4±3.6%; 0.5 μM: 41.0±7.0%; 0.5 μM: 40.5±4.2%; 1 μM: 40.5±4.6%; 5 μM: 41.3±2.4%; 6 h: w/o: 40.4±0.9%; dms: 38.3±2.6%; 0.5 μM: 37.1±6.0%; 0.5 μM: 36.8±1.6%; 1 μM: 37.6±3.3%; 5 μM: 33.9±1.1%). B. Cell cycle profiles were comparable between CTLs without treatment (w/o): G0/G1: 76.9±1.7%, S: 15.5±1.3%, G2: 7.3±0.4%); DMSO (G0/G1: 76.6±1.1%, S: 14.6±1.1%, G2: 6.6±0.2%); 0.1 μM CAL-101 (G0/G1: 78.8±0.6%, S: 14.2±0.8%, G2: 6.6±0.2%); 0.5 μM CAL-101 (G0/G1: 77.6±0.5%, S: 15.1±0.5%, G2: 7.1±0.0%); 1 μM CAL-101 (G0/G1: 78.4±0.8%, S: 14.5±0.7%, G2: 6.8±0.1%) and 5 μM CAL-101 (G0/G1: 78.9±0.6%, S: 14.1±0.2%, G2: 6.8±0.4%). n=2. Values represent means±SEM. One-Way ANOVA did not reveal any statistically significant differences. C. Western Blot analysis of cell lysates derived from aCD3-activated WT CTLs treated for 2 hours with indicated concentrations of CAL-101 or DMSO, respectively. Two different WT CTL preparations are depicted. P-AKT signals disappeared already upon PI3Kδ inhibition with 0.1 μM CAL-101. NFKB p65 served as loading control. (TIF)

Figure S3 In vitro cytotoxicity of WT OT-1 CTLs upon pharmacological inhibition of PI3Kδ. To generate effector cells, spleenocytes from an OT-1 mouse were isolated and co-cultured for 5 days with irradiated SIINFEKL-pulsed splenocytes. To determine peptide-reactive CTL cytotoxicity in vitro, CFSE-labeled OVA-expressing EG7 target cells were co-cultured with effectors in ratios of 15:1, 5:1 and 1:1. Specific in vitro target cell killing was quantified by flow cytometry (E:T=15:1: WT: 22% specific lysis; versus PI3Kδ-/-/- : 9% specific lysis). (TIF)

Figure S4 CTL surface expression of CD44, CD62L and CD69 upon pharmacological inhibition of PI3Kδ. In vitro assay on primary mature WT CTLs treated for three days with 0.5 μM or 1 μM CAL-101 or DMSO, respectively. Every 24 hours the expression levels of the activation and maturation markers CD44, CD62L and CD69 were measured on CD3+CD8+ CTLs via flow cytometry. Pharmacological inhibition of PI3Kδ gave rise to CTLs with reduced expression of CD44 and elevated levels of CD62L, while no differences were observed in the expression of CD69 compared to DMSO-treated WT controls. (Day 1: CD44: DMSO: 50.8±5.6, 0.5 μM: 32.6±2.6; 1 μM: 31.9±2.7; CD62L: DMSO: 13.1±0.8, 0.5 μM: 25.9±0.3, 1 μM: 27.5±1.1; CD69: DMSO: 10.9±0.3, 0.5 μM: 11.9±0.4, 1 μM: 12.1±0.3; n=4, values represent mean fluorescent intensities±SEM, One-Way ANOVA and Tukey’s Post-Hoc Test). (TIF)

Acknowledgments

We thank B. Strobl, K.H. Hilber, S. Sakaguchi and D. Stoiber-Sakaguchi for their help and support. We are especially grateful to S. Bolzer, G. Koppel as well as G. Schöppel and the mouse facility of the Institute of Pharmacology (MUV) for taking excellent care of the mice.

Author Contributions

Conceived and designed the experiments: EMP MPM FF VS EZB. Performed the experiments: EMP MPM OAS FF XK EZB. Analyzed the data: EMP MPM FF VS EZB. Contributed reagents/materials/analysis tools: HS RP MM VS. Wrote the paper: EMP MFP FF HS MF VS EZB.

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