Leukemia
https://doi.org/10.1038/s41375-018-0318-3

ARTICLE

Chronic lymphocytic leukemia

PI3Kδ inhibition modulates regulatory and effector T-cell differentiation and function in chronic lymphocytic leukemia

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Received: 7 May 2018 / Revised: 27 September 2018 / Accepted: 29 October 2018

Abstract

Targeting B-cell receptor signaling using the PI3Kδ inhibitor idelalisib is a highly effective treatment option for relapsed/refractory chronic lymphocytic leukemia (CLL) patients. In addition to its direct impact on tumor cells, PI3Kδ inhibition can modulate the activity of regulatory T-cells (Tregs) resulting in enhanced anti-tumoral immune functions which may contribute to the success of PI3Kδ inhibitors in cancer therapy. The role of Tregs in CLL and their modulation by PI3Kδ inhibitors was so far poorly understood. Using the Eμ-TCL1 adoptive transfer model of CLL, we show that disease development induces the accumulation of activated and highly immunosuppressive Tregs. Depletion of $CD25^+$ Tregs using anti-CD25 antibodies resulted in enhanced $CD8^+$ T-cell activation, effector differentiation, and functional capacity. We further show that pharmacological inhibition of PI3Kδ effectively controlled disease and significantly decreased both $CD25^+$ and $CD25^-$ Treg numbers, proliferation and activation status in CLL-bearing mice. Nonetheless, this PI3Kδ-mediated decrease in Tregs did not translate into better $CD8^+$ T-cell function, as PI3Kδ inhibition concomitantly abrogated T-cell receptor signaling in $CD8^+$ T-cells leading to decreased activation, effector cell differentiation and proliferation. Collectively, these data highlight the strong immunomodulatory effects of PI3Kδ inhibitors in CLL and are of relevance for a rational design of idelalisib-based combination therapies in CLL.

Introduction

Chronic lymphocytic leukemia (CLL) is one of the most common adult leukemias in western countries that results from accumulation of mature $CD5^+$ B-cells in peripheral blood (PB), lymph nodes (LNs), and bone marrow (BM) [1]. CLL cells are characterized by apoptosis resistance and strong dependency on support from their microenvironment. Combination of alkylating agents and purine analogs with αCD20 antibodies (e.g., Rituximab) was considered as the gold standard for CLL therapy [2, 3]. Yet, large body of evidence over the last ten years emphasized the central role of B-cell receptor (BCR) signaling as a key survival pathway that is hyperactive in CLL cells, especially in secondary lymphoid organs [4, 5]. This led to a paradigm shift in CLL treatment and fueled the development of small molecule inhibitors that target key kinases in BCR pathway, such as Spleen tyrosine kinase (SYK), Bruton's tyrosine kinase (BTK), and phosphatidylinositol 3 kinase delta (PI3Kδ) [6–8].

Class I PI3Ks are a group of heterodimeric molecules comprised of a regulatory and catalytic subunit. There are four catalytic isoforms of class I PI3Ks (class IA p110α, p110β, p110δ, and class IB p110γ). While p110α and p110β isoenzymes are ubiquitously expressed, the p110γ and p110δ isoenzymes are predominantly expressed in hematopoietic cell types [9]. In B-cells, activation of the BCR induces PI3Kδ phosphorylation and activation of

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Supplementary information The online version of this article (https://doi.org/10.1038/s41375-018-0318-3) contains supplementary material, which is available to authorized users.

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Published online: 20 December 2018

AKT resulting in enhanced cell survival and proliferation [10]. Specific inhibitors of PI3Kδ (e.g., idelalisib and umbralisib) or PI3Kγ/δ dual inhibitors (e.g., duvelisib) have shown promising results in improving progression-free and overall survival of refractory CLL patients [7, 11, 12]. PI3Kδ inhibition in CLL induces apoptosis and suppresses AKT phosphorylation [13, 14]. In addition to blocking BCR signaling, inhibition of PI3Kδ is believed to interrupt CXCR4 and CXCR5 signaling and subsequently CLL cell homing, causing redistribution of these cells into circulation, removal from the lymph node microenvironment's pro-survival signals and sensitization to apoptosis [14]. Besides its direct impact on tumor cells, a large part of the activity of PI3Kδ inhibitors is attributed to their strong immunomodulatory effects on several cell types in the microenvironment in CLL [15]. One of the most plausible cell targets for this immunomodulatory activity are T-cells, where PI3Kδ represents a key component downstream of T-cell receptor (TCR) and CD28 signaling [16]. Thus, genetic or pharmacological inhibition of PI3Kδ have a broad spectrum of effects on different T-cell functions including, but not restricted to, helper T-cell polarization and regulatory T-cell (Treg) function [9].

Tregs are a subpopulation of $CD4^+$ helper T-cells with potent immunosuppressive activity that are characterized by the expression of the transcription factor Forkhead box protein 3 (FoxP3), and play a pivotal role in maintaining immune homeostasis, and preventing autoimmune and chronic inflammatory diseases [17]. Enrichment of Tregs is well-documented in many cancers as a result of their increased recruitment or in situ expansion in the local microenvironment due to antigenic exposure or in response to cytokine signals or metabolic changes [18]. In turn, Tregs can sabotage anti-tumoral immunity by fostering an immunosuppressive niche that is hostile for the infiltrating immune cells. Additionally, Tregs can directly induce cytotoxicity and limit the expansion and functional capacities of anti-tumoral cytotoxic T-cells or natural killer (NK) cells. Thus, Tregs have emerged as an attractive target for cancer immunotherapy in various tumor entities [19].

Considering the crucial role of PI3Kδ in Treg differentiation, it has been suggested that PI3Kδ inhibitors, such as idelalisib, can abrogate the immunosuppressive activity of Tregs resulting in enhanced anti-tumoral activity [9, 20]. Although increased Treg numbers have been reported in CLL [21–25], the role and functional relevance of these cells, as well as their potential modulation by PI3Kδ inhibition remain largely unstudied in this disease. Due to the functional relevance of PI3Kδ in several lymphocyte subsets aside from Tregs, the overall impact of PI3Kδ inhibition on anti-tumoral immune responses is likely to be very contextual [9], and thereby can be hardly predicted using in vitro models. Thus, the current study utilized the Eμ-TCL1 (TCL1) mouse model of CLL [26] to study in vivo effects of Tregs, their modulation by PI3Kδ inhibition and its result on anti-tumoral immune functions in CLL.

Materials and methods

Mouse models

Eμ-TCL1 (TCL1) mice on C57BL/6 background were kindly provided by Dr. Carlo Croce (Ohio State University) [26]. C57BL/6 wild-type (WT) mice were purchased from Charles River Laboratories (Sulzfeld, Germany), and $Nr4a1^{GFP}$ mice [27] were kindly provided by Prof. Markus Feuerer (DKFZ, Heidelberg).

Adoptive transfer of TCL1 tumors was performed as previously described [28, 29]. Briefly, $1–2 \times 10^7$ leukemic TCL1 splenocytes ($CD5^+$ $CD19^+$ content of purified cells was typically above 90%) were transplanted by intravenous (i.v) injection into 2–3-months-old C57BL/6 WT females. All animal experiments were carried out according to governmental and institutional guidelines and approved by the local authorities (Regierungspräsidium Karlsruhe, permit numbers: G-36/14, G-123/14, G-16/15, and G-53/15).

Treatment studies

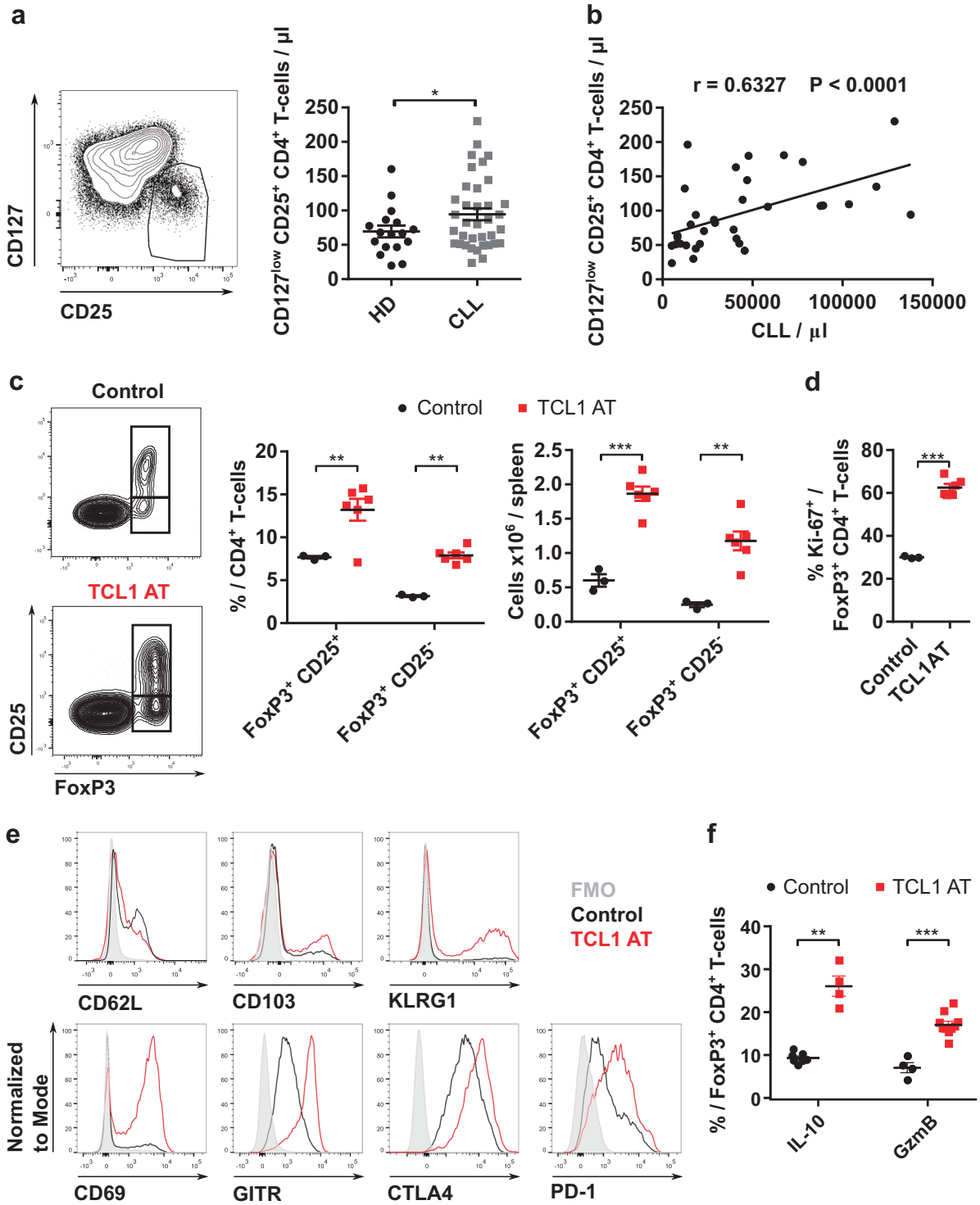
Two to three weeks after tumor cell transplantation, mice were allocated to different treatment arms according to the percentage of $CD5^+$ $CD19^+$ CLL cells out of $CD45^+$ cells in PB. For Treg depletion, mice were injected i.p. with 0.5 mg of αCD25 (clone: PC-61.5.3) or rat IgG1 isotype control antibody (clone: HRPN; both from BioXcell, West Lebanon, NH), followed by subsequent doses of 0.5 mg antibody every 3 days for another 2 weeks. The PI3Kδ-specific inhibitor PI-3065 (Selleckchem, Munich, Germany) was dissolved in 0.5% methylcellulose with 0.2% Tween 80 and administered by oral gavage once daily for two weeks at 75 mg/kg [20, 30].

Flow cytometry and functional testing

Detailed description of flow cytometric assays and gating strategies are described in Supplementary material.

Statistical analysis

Data were analyzed using Prism 5.04 GraphPad software. Data were analyzed using unpaired Student's *t*-test with Welch approximation to account for unequal variances, or one-way ANOVA followed by multiple comparison testing. When appropriate, paired Student's *t*-test was applied. Correlation between two parameters was calculated using



Spearman's rank correlation coefficient. Sample size was determined based on expected variance of read-out. No samples or animals were excluded from the analyzes. No randomization or blinding was used in animal studies. The statistical test used for each data set is indicated in the figure legends. Values of $p < 0.05$ were considered to be statistically significant. All graphs show means ± SEM.

Results

Expansion of regulatory T-cells in CLL patients and the TCL1 adoptive transfer model

To evaluate a potential role of regulatory T-cells (Tregs) in CLL, we performed phenotyping of the $CD4^+$ T-cell

◀ **Fig. 1** Expansion of activated and highly suppressive regulatory T-cells in the TCL1 adoptive transfer model of CLL. **a** Flow cytometry analysis of the absolute numbers of Tregs ($CD127^{low}$ $CD25^+$ $CD4^+$) in peripheral blood (PB) of CLL patients ($n = 36$) and matched healthy donor (HD) controls ($n = 17$). **b** Spearman's correlation of the absolute numbers of Tregs (y-axis) and tumor load (x-axis), defined as the absolute number of $CD3^-$ lymphocytes, in PB of CLL patients ($n = 36$). **c** Flow cytometry analysis of percentages (left panel) and absolute number (right panel) of $FoxP3^+$ $CD25^+$ and $FoxP3^+$ $CD25^-$ Tregs out of splenic $CD3^+$ $CD4^+$ T-cells from TCL1 adoptive transfer (TCL1 AT) mice ($n = 6$) and matched wild-type (WT) controls ($n = 3$). **d** Flow cytometry analysis of percentual Ki-67 expression in splenic $FoxP3^+$ $CD4^+$ Tregs from TCL1 AT mice ($n = 6$) and matched WT controls ($n = 3$). **e** Flow cytometry analysis of CD62L, CD103, KLRG1, CD69, GITR, CTLA4, and PD-1 expression on splenic $FoxP3^+$ $CD4^+$ Tregs from TCL1 AT mice ($n = 6$) and matched WT controls ($n = 3$). Representative histograms are shown. **f** Splenocytes from TCL1 AT mice and matched WT controls ($n = 4$–10) were stimulated ex vivo with PMA/ionomycin, and IL-10 and granzyme B (GzmB) expression in splenic $FoxP3^+$ $CD4^+$ Tregs were quantified by intracellular flow cytometry. Results are representative of at least 3 independent experiments. Graphs show means ± SEM. Statistical analysis was performed using unpaired Student's *t*-test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

compartment in CLL patients' peripheral blood. Analysis of $CD127^{low}$ $CD25^+$ $CD4^+$ Tregs showed a significant increase in their numbers (Fig. 1a, Suppl. Figure 1A shows gating strategy), yet with no significant change in their percentage out of total $CD4^+$ T-cells (Suppl. Figure 1B). Moreover, Treg numbers positively correlated with tumor load (Fig. 1b). In addition, we detected a higher number of Tregs in CLL patients having unfavorable Binet stage (Suppl. Figure 1C).

To study the functional relevance of Tregs in CLL, we turned to the Eμ-TCL1 mouse model which recapitulates the major changes observed in human CLL microenvironment [28, 31]. We adoptively transferred splenocytes from leukemic TCL1 mice into syngeneic wild-type recipients (TCL1 AT) and analyzed the $CD4^+$ T-cell compartment after establishment of disease (Suppl. Figure 2A shows gating strategy). Adoptive transfer of TCL1 tumors induced a substantial increase in the percentage and absolute number of $FoxP3^+$ $CD25^+$ cells, representing the dominant natural Treg population, and $FoxP3^+$ $CD25^-$ Tregs, representing committed regulatory cells that regain CD25 expression upon homeostatic expansion [32], in comparison to untransplanted control mice (Fig. 1c). The accumulation of Tregs following TCL1 AT varied in different adoptive transfer experiments and is likely due to their increased proliferation rates, as evidenced by a higher percentage of $Ki\text{-}67^+$ cells, which was more pronounced for $CD25^-$ Tregs (Fig. 1d, Suppl. Figure 2B+C). Phenotypical analysis of Tregs in TCL1 AT mice showed that they have low CD62L expression, and express higher levels of differentiation and maturation markers, like CD103 and KLRG1, which were primarily expressed in the $CD25^+$ population (Fig. 1e, Suppl. Figure 2D). Moreover, they showed a higher expression of activation markers, like CD69 and GITR (Fig. 1e). We next evaluated whether the expanding Treg population in TCL1 AT mice exhibits a stronger suppressive capacity. Tregs exert their regulatory function via multiple effector pathways, including cytokine capture via IL-2 receptor, expression of cell surface inhibitory receptors, like CTLA4 and PD-1, secretion of suppressive cytokines, like IL-10, or direct cytotoxicity [17]. We did not observe a significant difference of IL-2Rα (CD25) levels on $CD25^+$ Tregs of TCL1 AT and control mice (Suppl. Figure 2E). But TCL1 AT Tregs expressed significantly higher levels of the inhibitory molecules CTLA4 and PD-1 compared to control Tregs (Fig. 1e). In addition, a higher percentage of TCL1 AT Tregs produced IL-10, and the cytotoxic molecule granzyme B (GzmB) (Fig. 1f, Suppl. Figure 2F) following PMA/ionomycin stimulation, compared to Tregs from control mice. Of note, the change in Treg percentage and phenotype after TCL1 AT was time- and tissue-dependent. Two weeks after TCL1 AT, corresponding to a tumor load in the blood of 5–10%, we detected an increase in Treg activation status and production of IL-10 (Suppl. Figure 2G-I). This increase was more pronounced 6 weeks after TCL1 AT (Suppl. Figure 2G-I). Moreover, percentages of Tregs in TCL1 AT mice were higher in spleen and BM compared to PB, with the latter containing Tregs with lower activation status (Suppl. Figure 2J-K), indicating that Treg activation and expansion happens primarily in lymphoid organs. Collectively, these data show that CLL development in TCL1 AT mice induces the accumulation of mature and activated Tregs with increased expression of suppressive molecules.

Treg depletion enhances $CD8^+$ T-cell effector differentiation, activation, and functional activity

Tregs have the ability to regulate the function of a broad spectrum of innate and adaptive immune cells. To evaluate the role of Tregs in CLL progression, we induced their depletion using αCD25 antibodies in mice three weeks after TCL1 AT. This resulted in a considerable decrease in percentage and absolute numbers of $FoxP3^+$ $CD25^+$ Tregs in spleen and LN, yet with no significant impact on the $FoxP3^+$ $CD25^-$ T-cell population (Suppl. Figure 3A-B). In these mice, we further tested whether the lack of Tregs impacts on the activity of anti-tumoral $CD8^+$ T-cells, as we have recently shown that CLL development in TCL1 mice induces the differentiation of an anti-tumoral, oligoclonal $CD8^+$ effector T-cell population that controls tumor development in an IFNγ-dependent manner [33]. Accordingly, we analyzed the $CD8^+$ T-cell composition by flow cytometry using CD44 and CD127, which divide $CD8^+$ T-cells into $CD127^{hi}$ $CD44^{low}$ naive, $CD127^{hi}$ $CD44^{hi}$ memory, and $CD127^{low}$ $CD44^{int\text{-}hi}$ effector subsets (Fig. 2a). In line with our published data, $CD127^{low}$ $CD44^{int\text{-}hi}$ effector $CD8^+$ T-cells in isotype

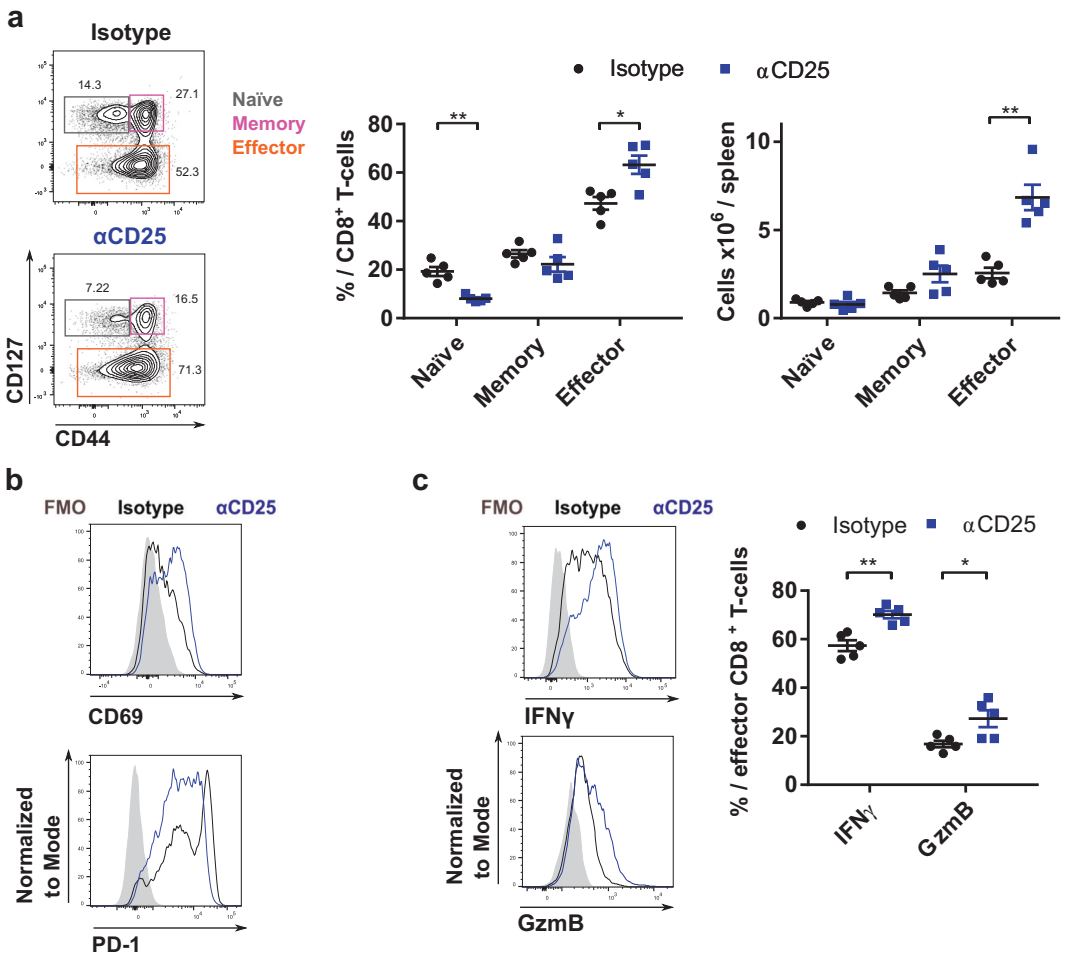


Fig. 2 Treg depletion enhances CD8$^+$ T-cell effector differentiation, activation and functional activity. C57BL/6 mice were transplanted with splenocytes from leukemic TCL1 mice and after 3 weeks assigned to treatment with αCD25 antibody or the corresponding isotype control according to percentage of CLL cells in blood. Mice were sacrificed after 2 weeks of treatment. **a** Flow cytometry analysis of splenic CD3$^+$ CD8$^+$ T-cells from isotype- or αCD25-treated mice ($n = 5$). Cell subsets were defined as naive (CD127^{hi} CD44^{low}), memory (CD127^{hi} CD44^{hi}), and effector (CD127^{low} CD44$^{int\text{-}hi}$) cells. Right panel shows absolute numbers of splenic CD8$^+$ T-cell subsets from isotype- or αCD25-treated mice ($n = 5$). **b** Flow cytometry analysis of CD69 and PD-1 expression on splenic effector CD8$^+$ T-cells from isotype- or αCD25-treated mice ($n = 5$). **c** Cell cytotoxicity of CD8$^+$ T-cell subsets was assessed by IFNγ and GzmB expression in effector CD8$^+$ T-cells from isotype- or αCD25-treated mice ($n = 5$) upon ex vivo PMA/ionomycin stimulation. Results are representative of 2 independent experiments. Graphs show means ± SEM. Statistical analysis was performed using unpaired Student's *t*-test. $^*p < 0.05$, $^{**}p < 0.01$

antibody-treated mice represented the major CD8$^+$ T-cell subset. Of interest, Treg depletion by αCD25 resulted in a significant increase in the percentage and absolute number of this effector population (Fig. 2a). In addition, these effector cells exhibited a higher activation state, as evident by higher CD69 expression (Fig. 2b, Suppl. Figure 3C). Moreover, the expression of the inhibitory receptor PD-1 on the effector population was lower in Treg-depleted animals (Fig. 2b, Suppl. Figure 3D). We then tested whether these changes translate into enhanced T-cell function. Therefore, we evaluated the cytokine production and cytotoxic abilities of T-cells ex vivo following PMA/ionomycin stimulation. Our results indeed show that Treg depletion resulted in a higher percentage of IFNγ-producing effector CD8$^+$ T-cells (Fig. 2c). Moreover, the cytotoxic capacity of these cells, as measured by GzmB production, was significantly enhanced after Treg depletion (Fig. 2c). Nonetheless, αCD25 treatment had no significant impact on disease load in spleen, BM and LN, or spleen size (Suppl. Figure 3E-F), indicating that partial Treg depletion by CD25-specific antibodies in leukemic mice is not sufficient to effectively control progression of disease.

Pharmacological PI3Kδ inhibition effectively controls CLL progression and decreases Tregs in the TCL1 AT model

As Treg depletion resulted in enhanced CD8$^+$ T-cell function, we investigated potential therapeutic interventions that

can target Tregs in CLL. PI3Kδ plays a crucial role in Treg differentiation and function [9]. Therefore, we tested the impact of PI3Kδ inhibition on Tregs and anti-tumoral immune function in the TCL1 AT model using PI-3065, a highly specific PI3Kδ inhibitor [20]. We adoptively transferred malignant splenocytes from TCL1 mice into syngeneic WT recipients and subsequently assigned the mice according to tumor load in blood to treatment with vehicle or PI-3065 (75 mg/kg once daily), as previously described [20]. PI-3065 treatment in CLL-bearing mice for two weeks resulted in a substantial decrease in tumor development in PB, spleen and liver (Fig. 3a, Suppl. Figure 4A). Moreover, CLL cell proliferation rates, as measured by Ki-67 staining, were considerably lower in spleen and BM of PI-3065 treated mice (Suppl. Figure 4B), confirming the efficient cytotoxic activity of the drug for CLL cells.

We then evaluated the effect of PI-3065 treatment on Treg differentiation and function. We observed a considerable decrease in percentage of FoxP3$^+$ CD25$^+$ and FoxP3$^+$ CD25$^-$ Tregs in PI-3065 treated mice (Fig. 3b). Treg proliferation, as measured by Ki-67 staining, was significantly lower in PI-3065 treated animals (Fig. 3c). This was associated with decreased expression of Treg maturation and activation markers, like KLRG1, CD103, CD69, and GITR (Fig. 3c). Moreover, Treg production of GzmB, but not IL-10, upon ex vivo stimulation was significantly lower after treatment (Fig. 3d). In summary, these data indicate that PI3Kδ inhibition in the TCL1 AT model results in decreased Treg numbers and immunosuppressive activity.

Pharmacological PI3Kδ inhibition abrogates the expansion of effector CD8$^+$ T-cells and impairs their function in the TCL1 AT model

Treg inhibition by PI3Kδ ablation has been suggested to unleash CD8$^+$ T-cells and increase their activation and effector differentiation resulting in greater anti-tumoral immune responses [20]. Therefore, we investigated whether the observed changes in Tregs following PI-3065 treatment translate into better CD8$^+$ T-cell function in TCL1 AT mice. We first analyzed the effect of the drug on CD8$^+$ effector T-cell numbers. As expected, CLL development induced a sizable expansion of CD127^{low} CD44$^{int\text{-}hi}$ effector CD8$^+$ T-cells in vehicle-treated mice (Fig. 4a). In contrast to our initial hypothesis, PI-3065 treatment resulted in a dramatic drop in the percentages and numbers of CD8$^+$ effector T-cells (Fig. 4a, Suppl. Figure 5A). The decrease in effector CD8$^+$ T-cell percentages was more pronounced than that of Tregs as evident by the lower ratio of CD8$^+$ effector cells/Tregs in mice treated with PI-3065 (Fig. 4b). In line with that, CD8$^+$ T-cell proliferation, as measured by Ki-67, was significantly lower in PI-3065 treated mice (Fig. 4c). In addition, PI3Kδ inhibition resulted in a decreased expression of activation markers, like CD137 and CD69, on CD8$^+$ T-cells (Fig. 4d). The drop in activation markers was also observed when focusing the analysis on CD8$^+$ effector T-cells (Suppl. Figure 5B). On the other hand, no significant difference could be detected in the expression of exhaustion markers PD-1, Lag3, and CD244 on the surface of CD8$^+$ effector T-cells (Suppl. Figure 5C).

We next analyzed the functional capacity of CD8$^+$ T-cells isolated from treated mice by intracellular flow cytometric staining following ex vivo mitogen PMA/ionomycin stimulation. Notably, we observed that IFNγ and GzmB production from CD8$^+$ T-cells were substantially reduced by PI-3065 (Fig. 5a). In addition, degranulation capacity of CD8$^+$ T-cells, as measured by CD107a presentation at the cell surface, significantly dropped in PI-3065-treated mice, demonstrating poor cytotoxic T-cell capacity in these mice (Fig. 5b). The decrease in T-cell effector function was primarily due to the inhibition of effector T-cell differentiation after PI-3065 treatment. Nevertheless, IFNγ and GzmB production and degranulation capacity were also decreased when focusing the analysis on the minor CD8$^+$ effector T-cell population that was still present after PI-3065 treatment (Fig. 5c, Suppl. Figure 5D). This suggests that PI3Kδ inhibition in the TCL1 AT mouse model of CLL impairs the differentiation and the functional capacity of CD8$^+$ effector T-cells.

PI3Kδ inhibition impairs T-cell receptor signaling, activation, and proliferation of CD8$^+$ T-cells

To investigate the underlying mechanism of PI-3065-induced effects in CD8$^+$ T-cells, we used the Nr4a1-GFP mouse model, which is reliably used to monitor T-cell receptor (TCR) activity [27]. Splenocytes of these mice were pretreated with PI-3065 or vehicle in vitro and then stimulated with αCD3 antibody for 3 h. This stimulation resulted in a substantial increase in Nr4a1 in DMSO-treated controls, while PI-3065 treatment decreased the Nr4a1-GFP signal in a dose-dependent manner (Fig. 6a), indicating a reduction in TCR activity. This was accompanied by a dose-dependent decrease in expression of the early activation marker CD69 (Fig. 6b). Moreover, the expression of differentiation markers, such as CD44, CD25, and CD137, was decreased after 24 h of αCD3 stimulation (Suppl. Figure 6A). Furthermore, PI-3065 pre-treatment resulted in a decline of CD8$^+$ T-cell proliferation rates, as measured by CFSE dilution after 48 h of αCD3 stimulation, which was pronounced at a concentration of 1 μM (Suppl. Figure 6B). To further analyze whether treatment of human CD8$^+$ T-cells with idelalisib, a clinically used PI3Kδ inhibitor, resulted in similar effects, we stimulated human PBMCs after pre-treatment with idelalisib with αCD3 antibody and

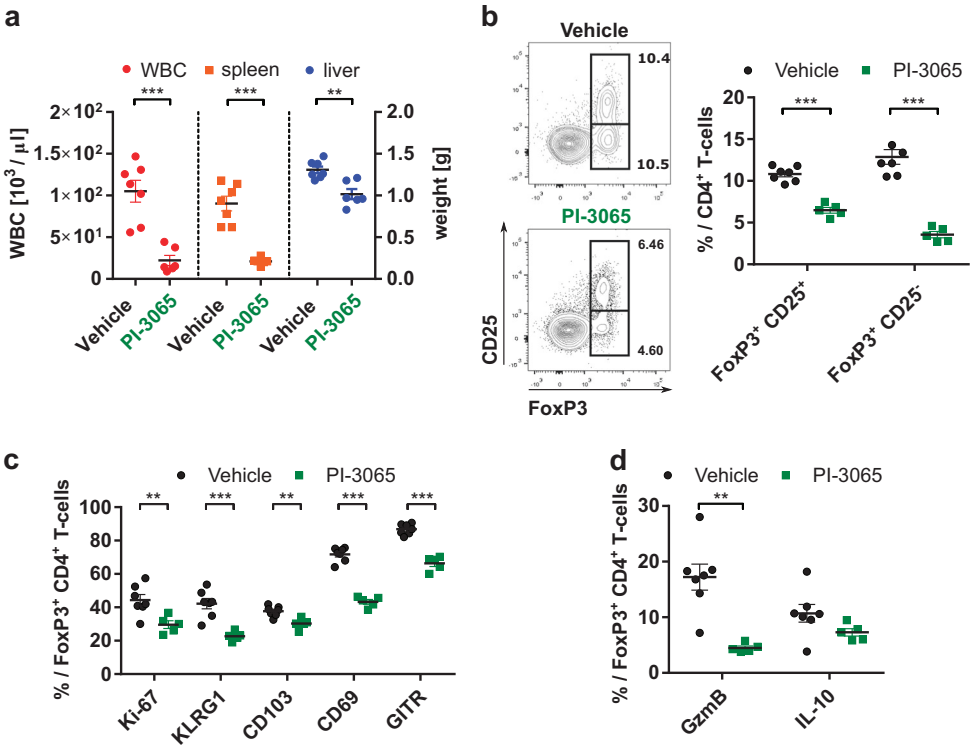


Fig. 3 Pharmacological PI3Kδ inhibition effectively controls CLL progression and decreases Tregs in the TCL1 AT model. C57BL/6 mice were transplanted with splenocytes from leukemic TCL1 mice, and after 2 weeks assigned to treatment with the PI3Kδ inhibitor PI-3065 (75 mg/kg once daily) or vehicle control according to percentage of CLL cells in blood. Mice were sacrificed after 2 weeks of treatment. **a** White blood cell count (WBC) in PB and spleen, and liver weight of vehicle- or PI-3065-treated mice ($n = 5$–7) are shown. **b** Analysis of percentages of FoxP3$^+$ CD25$^+$ and FoxP3$^+$ CD25$^-$ Tregs out of splenic CD3$^+$ CD4$^+$ T-cells from vehicle- or PI-3065-treated mice ($n = 5$–7) by flow cytometry. **c** Expression of Ki-67, KLRG1, CD103, CD69, and GITR on splenic FoxP3$^+$ CD4$^+$ Tregs from vehicle- or PI-3065-treated mice ($n = 5$–7), as measured by flow cytometry. **d** GzmB and IL-10 expression of FoxP3$^+$ CD4$^+$ Tregs from vehicle- or PI-3065-treated mice ($n = 5$–7) following ex vivo stimulation with PMA/ionomycin was analyzed by intracellular flow cytometry. Graphs show means ± SEM. Statistical analysis was performed using unpaired Student's *t*-test. $^{**}p < 0.01$, $^{***}p < 0.001$

analyzed CD8$^+$ T-cell activation and proliferation. In line with our results of murine T-cells, idelalisib treatment resulted in decreased activation marker expression and decreased proliferation of human T-cells (Fig. 6c, d). As our in vivo results indicated that CD8$^+$ effector T-cells are more sensitive to PI3Kδ inhibition (Fig. 4b), we tested the differential impact of the PI3Kδ inhibitor PI-3065 on TCR signaling, as measured by Nr4a1 induction, in CD8$^+$ effector T-cells and Tregs. In comparison to Tregs and all other T-cell subsets, CD8$^+$ effector T-cells from TCL1 AT mice showed the lowest response to αCD3 stimulation (Fig. 6e). Accordingly, Tregs exhibited higher levels of Nr4a1 than CD8$^+$ effector T-cells even at very high doses of PI-3065 (Fig. 6e), indicating that the very small window of response to TCR stimulation in CD8$^+$ effector T-cells renders them more sensitive to TCR manipulation by PI3Kδ inhibition. This low response to TCR stimulation and higher sensitivity to PI-3065 was specific to CD8$^+$ effector T-cells from leukemic TCL1 AT mice and not observed in WT tumor-naive mice where Tregs showed the lowest response to TCR stimulation and were therefore most sensitive to PI3Kδ inhibition (Fig. 6f). In sum, these data demonstrate the direct deleterious impact of PI3Kδ inhibition on CD8$^+$ effector T-cells in CLL.

Discussion

Among several BCR-targeting molecules, PI3Kδ inhibitors have proven high efficacy in relapsed/refractory CLL patients [7, 12]. It is believed that the clinical success of these drugs relies also on their ability to target crucial tumor-promoting components of the microenvironment [15]. For example, idelalisib has been shown to impair the release of pro-inflammatory cytokines by CD4$^+$ helper T-cells resulting in abrogating their tumor-supportive functions in CLL [13]. The presented data further demonstrates the impact of PI3kδ inhibition in CLL on two main T-cell populations; Tregs and CD8$^+$ T-cells.

Here, we show that CLL progression in the TCL1 AT mouse model induces the accumulation of activated Tregs, which is in line with previous reports about increased Treg

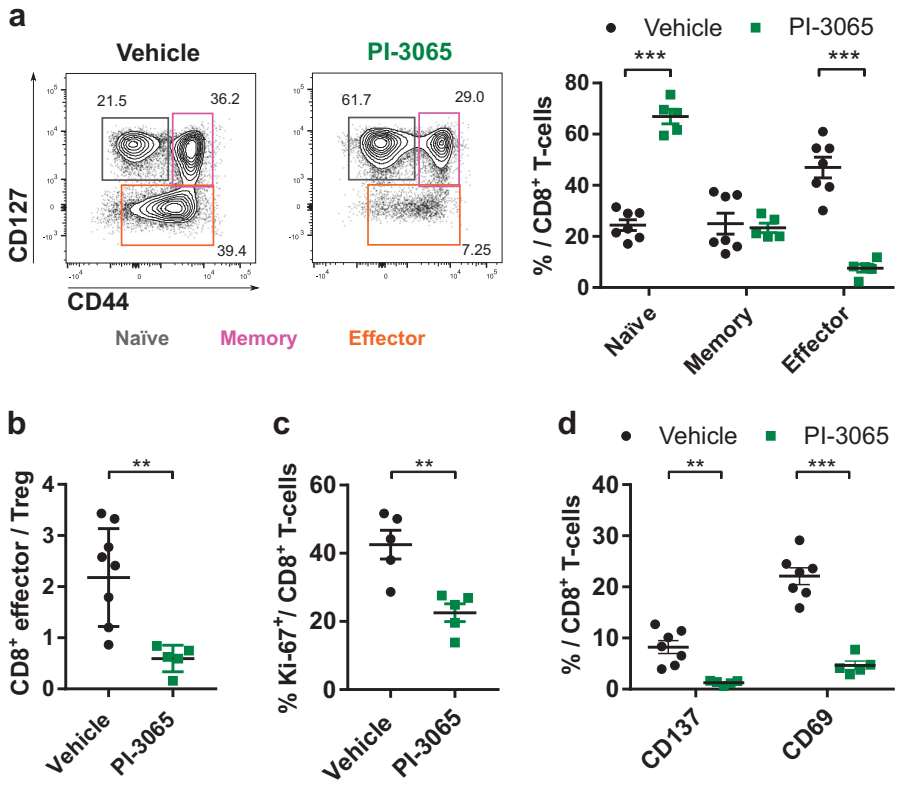


Fig. 4 Pharmacological PI3Kδ inhibition abrogates $CD8^+$ effector T-cell expansion in the TCL1 AT model. C57BL/6 mice were transplanted with splenocytes from leukemic TCL1 mice, and after 2 weeks assigned to treatment with the PI3Kδ inhibitor PI-3065 (75 mg/kg once daily) or vehicle control according to percentage of CLL cells in blood. Mice were sacrificed after 2 weeks of treatment. **a** Flow cytometry analysis of splenic $CD3^+$ $CD8^+$ T-cells from vehicle- or PI-3065-treated mice ($n = 5$–7). Cell subsets were defined as naive ($CD127^{hi}$ $CD44^{low}$), memory ($CD127^{hi}$ $CD44^{hi}$), and effector ($CD127^{low}$ $CD44^{int\text{-}hi}$) cells. **b** Ratio of $CD8^+$ effector T-cells and $FoxP3^+$ $CD4^+$ Tregs in spleens of vehicle- or PI-3065-treated mice ($n = 5$–7). **c** Flow cytometry analysis of Ki-67, **d** CD137 and CD69 expression on $CD8^+$ T-cells from vehicle- or PI-3065-treated mice ($n = 5$–7). Graphs show means ± SEM. Statistical analysis was performed using unpaired Student's *t*-test. $^{**}p < 0.01$, $^{***}p < 0.001$

numbers in end-stage CLL patients [21]. Several mechanisms of Treg induction in CLL have been proposed. CLL cells secrete the immunosuppressive cytokine IL-10, which is known to induce Treg differentiation through FoxP3 upregulation [34, 35]. Moreover, CLL-associated myeloid cells overexpress indoleamine 2,3-dioxygenase 1 (IDO-1) which induces Treg differentiation [36]. The presented data provides the first in vivo analysis of the interplay of Tregs and $CD8^+$ T-cells in CLL, their modulation by PI3Kδ inhibition, and its impact on disease progression. Various reports showed that $CD8^+$ T-cells from CLL patients feature decreased cytotoxicity and proliferation and show signs of exhaustion, a dysfunctional state of T-cells described primarily in chronic viral infections and characterized by overexpression of inhibitory molecules, like PD-1, and decreased effector functions [37, 38]. While these functional defects were believed to be directly induced by chronic antigenic stimulation by leukemic cells [39], our data further shows that Tregs exacerbate $CD8^+$ T-cell dysfunction and limit their expansion and cytotoxic ability. Nonetheless, the enhancement of $CD8^+$ T-cell differentiation and functional capacities attained by antibody-mediated depletion of $CD25^+$ Tregs was not sufficient to achieve better tumor control in the TCL1 AT model. This is likely due to the ability of CLL cells to hijack the expanding anti-tumoral immune cell populations in a process of adaptive immune evasion. Moreover, it remains possible that $CD25^-$ $FoxP3^+$ $CD4^+$ Tregs that are unaffected by αCD25 treatment can compensate for the loss of $CD25^+$ Tregs. Further studies are required to evaluate the efficacy of immunotherapies targeting Treg suppressive functions (e.g., CTLA4 blockade) in human and murine CLL [40].

PI3Kδ is required for the homeostasis and function of Tregs [41]. Therefore, it was assumed that Treg impairment by PI3Kδ inhibition also contributes to treatment success by enhancing anti-tumoral activity. Mice lacking PI3Kδ specifically in Tregs mount more effective $CD8^+$ T-cell anti-tumoral responses and show better survival [20]. However, tumor control was weakened in mice lacking PI3Kδ systemically in comparison to Treg-specific knockout mice,

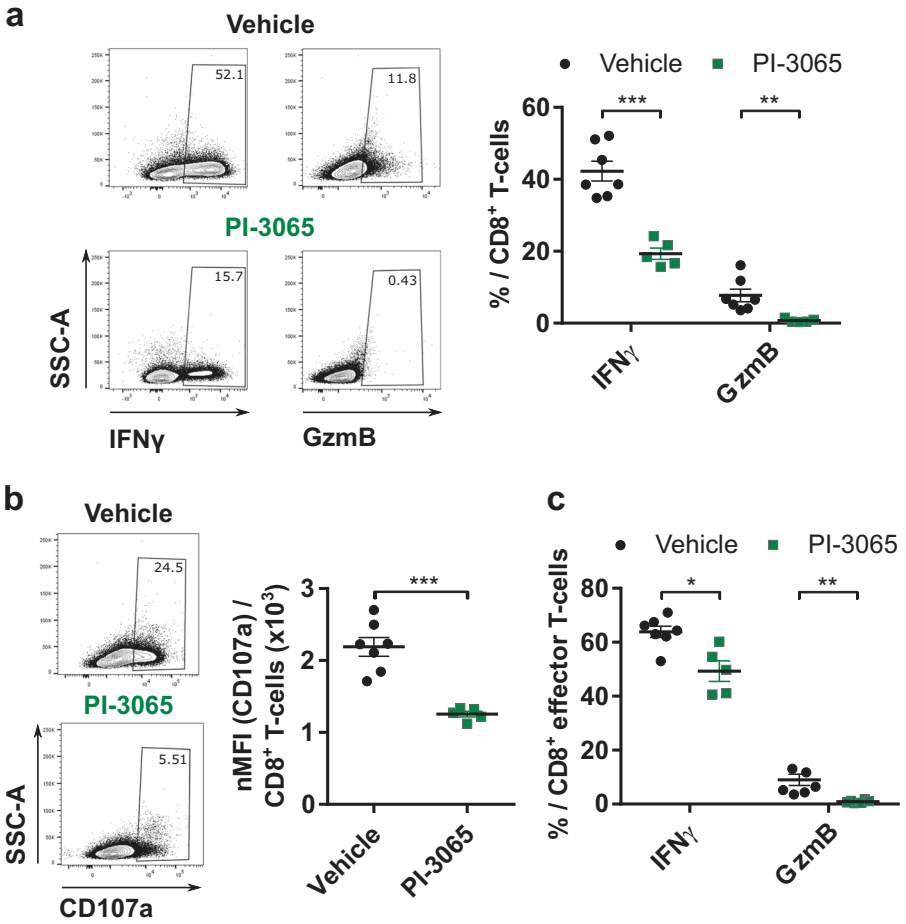


Fig. 5 Pharmacological PI3Kδ inhibition impairs CD8$^+$ T-cell function in the TCL1 AT model. C57BL/6 mice were transplanted with splenocytes from leukemic TCL1 mice, and after 2 weeks assigned to treatment with the PI3Kδ inhibitor PI-3065 (75 mg/kg once daily) or vehicle control according to percentage of CLL cells in blood. Mice were sacrificed after 2 weeks of treatment. **a** Cell cytotoxicity of CD8$^+$ T-cells was assessed by GzmB and IFNγ expression, and **b** degranulation capacity, as measured by CD107a expression on the cell surface after ex vivo PMA/ionomycin stimulation. **c** IFNγ and GzmB production was quantified in CD8$^+$ effector T-cells after stimulation. Graphs show means ± SEM. Statistical analysis was performed using unpaired Student's *t*-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; nMFI normalized median fluorescence intensity

signifying the deleterious effects of PI3Kδ loss in other anti-tumoral cell types [20]. Previous work has shown that PI3Kδ is required for various aspects of CD8$^+$ T-cell responses in vivo [9]. PI3Kδ-deficient CD8$^+$ T-cells fail to expand and generate effector T-cells in response to various infectious agents [42, 43]. Nonetheless, some functional aspects are maintained in PI3Kδ-deficient CD8$^+$ T-cells, such as generation of long-term memory responses [42]. Ali and colleagues suggested that Tregs are more dependent on PI3Kδ than CD8$^+$ T-cells [20]. The authors proposed that the beneficial impact of Treg impairment by PI3Kδ inhibition can supposedly outbalance the block in CD8$^+$ T-cell activation and differentiation, collectively resulting in a better net effect on anti-tumoral immunity [20]. This is in contrast to our observations in the TCL1 AT model, where CD8$^+$ effector T-cells showed a weaker TCR activity and therefore higher sensitivity to PI3Kδ inhibition compared to Tregs. Accordingly, it seems plausible that the outcome of PI3Kδ inhibition is contextual and depends on the relative abundance and contribution of Tregs and effector CD8$^+$ T-cells, which are likely to vary in different tumor microenvironments. Our data show that pharmacological inhibition of PI3Kδ in CLL results in deteriorated CD8$^+$ T-cell activation and differentiation, albeit of the treatment-induced decrease in Treg numbers and function. Similarly, PI3Kδ inhibition in mouse models of colon carcinoma and leukemia resulted in weakened immune surveillance by NK and CD8$^+$ T-cells [44–46].

Despite the very potent cytotoxic effects of PI3Kδ inhibitors in CLL, the broad immune effects of these drugs have several implications on their use in CLL. In addition to the well-documented autoimmune adverse effects in PI3Kδ inhibitor-treated patients [47], our data suggest that the block of CD8$^+$ T-cell differentiation by PI3Kδ inhibition can compromise the immune status of CLL patients. In line with that, Gracias and colleagues raised concerns about potential defects in microbial surveillance due to defective CD8$^+$ T-cell function in patients continuously receiving PI3Kδ inhibitors [43]. This is of high relevance in light of recent reports about increased infection rates, including cytomegalovirus (CMV) reactivation, with idelalisib when used as front line therapy in CLL [15, 48]. Considering the poor immune status in a large fraction of CLL patients, precautionary measures in individuals receiving idelalisib is recommended (e.g., antibiotic prophylaxis and CMV viremia monitoring) [15, 49]. Moreover, further studies are

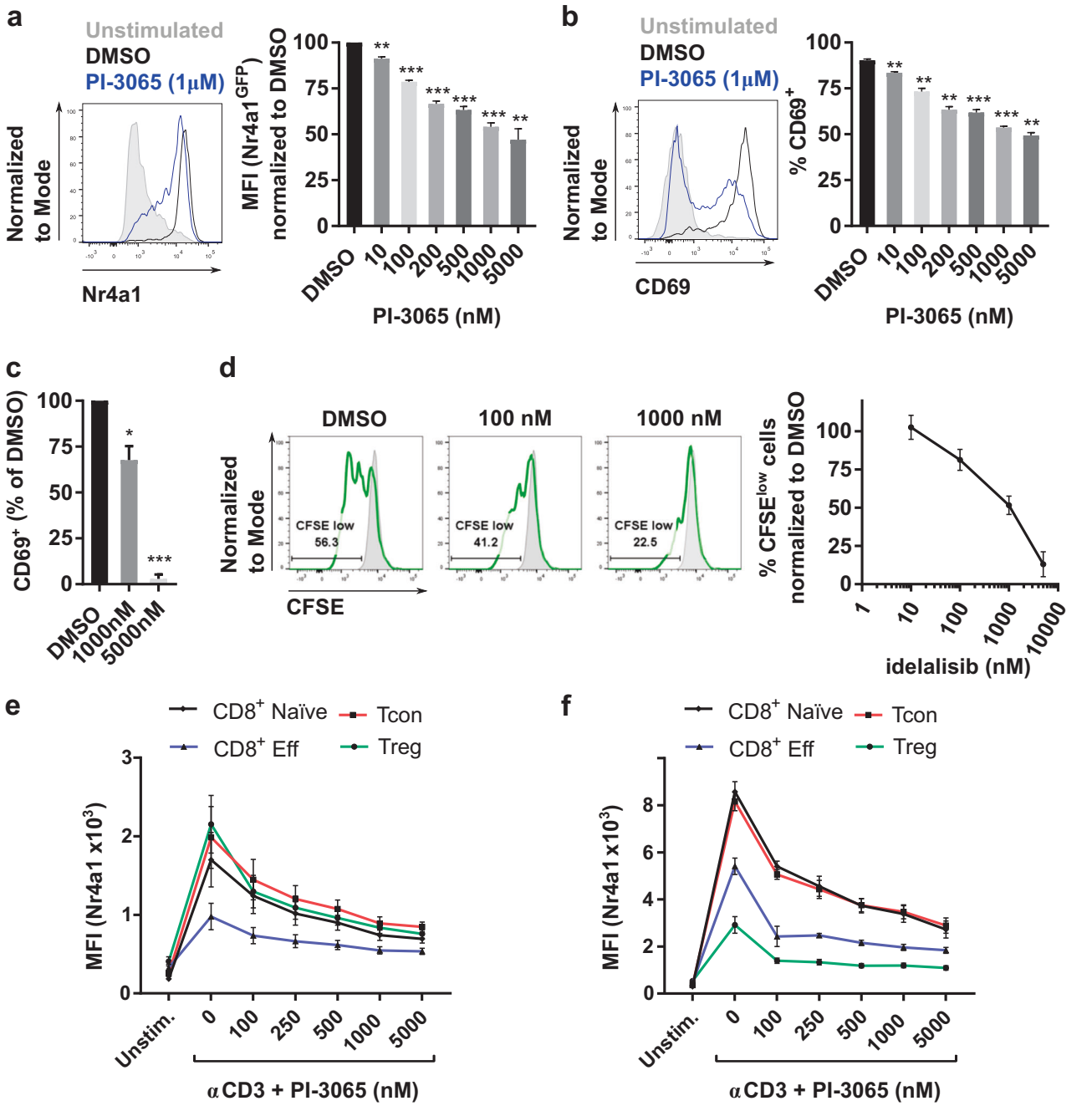


Fig. 6 PI3Kδ inhibition impairs T-cell receptor signaling, activation and proliferation of $CD8^+$ T-cells. **a**, **b** Splenocytes from $Nr4a1^{GFP}$ transgenic mice ($n = 4$) were pretreated with different concentrations of PI-3065 or DMSO for 30 min, and then stimulated with anti-CD3 antibody for 3 h. **a** Nr4a1-GFP, and **b** CD69 expression was analyzed by flow cytometry in viable 4′,6-Diamidino-2-Phenylindole (DAPI)-negative, single $CD8^+$ T-cells. **c**, **d** PBMCs from healthy donors ($n = 5$) were labeled with 5 μM CFSE, pretreated with different concentrations of idelalisib or DMSO for 30 min and then stimulated with anti-CD3 antibody. **c** CD69 expression after 24 h, and **d** proliferation as measured by CFSE dilution after 72 h were analyzed by flow cytometry in viable, single $CD8^+$ T-cells. **e**, **f** Splenocytes were pretreated with different concentrations of PI-3065 or DMSO for 30 min, and then stimulated with anti-CD3 antibody for 3 h. Intracellular Nr4a1 signal was analyzed by flow cytometry in $FoxP3^+$ $CD4^+$ Tregs, $FoxP3^-$ $CD4^+$ conventional T-cells (Tcons), naive, and effector $CD8^+$ T-cells from **e** TCL1 AT ($n = 8$ mice), and **f** untransplanted controls ($n = 5$ mice). Graphs show means ± SEM. Statistical analysis was performed using one-way ANOVA followed by multiple comparison testing. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$; MFI median fluorescence intensity

warranted to identify prognostic biomarkers that define patient subgroups with higher susceptibility to these adverse effects. The difference in the spectrum of these immune side effects between specific PI3Kδ inhibitors and PI3Kγ/δ dual inhibitors like duvelisib (IPI-145) remains to be studied [12]. Inhibition of PI3Kγ can decrease pro-tumoral myeloid

cells [50], but can further dampen T-cell function because of the essential role of PI3Kγ in T-cell migration and activation [51, 52]. Moreover, the strong immunomodulatory effects of PI3Kδ inhibition are likely to influence the design of combination therapies involving idelalisib or other PI3K inhibitors. Chemotherapeutic agents that reduce immune functions are likely to aggravate the side effects of PI3Kδ inhibition [53]. Moreover, combination of PI3K inhibitors with immunotherapeutic approaches should be thoroughly evaluated. Combination of idelalisib with immune checkpoint blockade is currently under investigation in CLL and other malignancies (NCT02332980, NCT03257722). In addition, several studies are ongoing to evaluate the efficacy of idelalisib in combination with the immunomodulatory drug lenalidomide (NCT01838434, NCT01644799). The success of these immunotherapeutic approaches, especially immune checkpoint blockers, relies on the expansion of tumor-reactive T-cells and on enhancing their cytotoxic activity [54]. Therefore, it needs to be evaluated whether these immunotherapies can circumvent the block of $CD8^+$ T-cell differentiation caused by PI3Kδ inhibition. Collectively, the presented study highlights the strong immunomodulatory effects of PI3Kδ inhibitors on Tregs and $CD8^+$ T-cells which can inform the design of PI3K inhibitor-based therapies in CLL.

Acknowledgements This study was supported by the German José Carreras Foundation (R14/23) to MS, the DFG (SFB1074 project B1) to StSt, AS, and BMCJ, the BMBF-Network "PRECiSe" (031L0076A) and the ERA-NET TRANSCAN-2 program JTC 2014–project FIRE-CLL to StSt, PL, and MS. PMR was supported by the German Cancer Aid grant number 112069.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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