HemaSphere

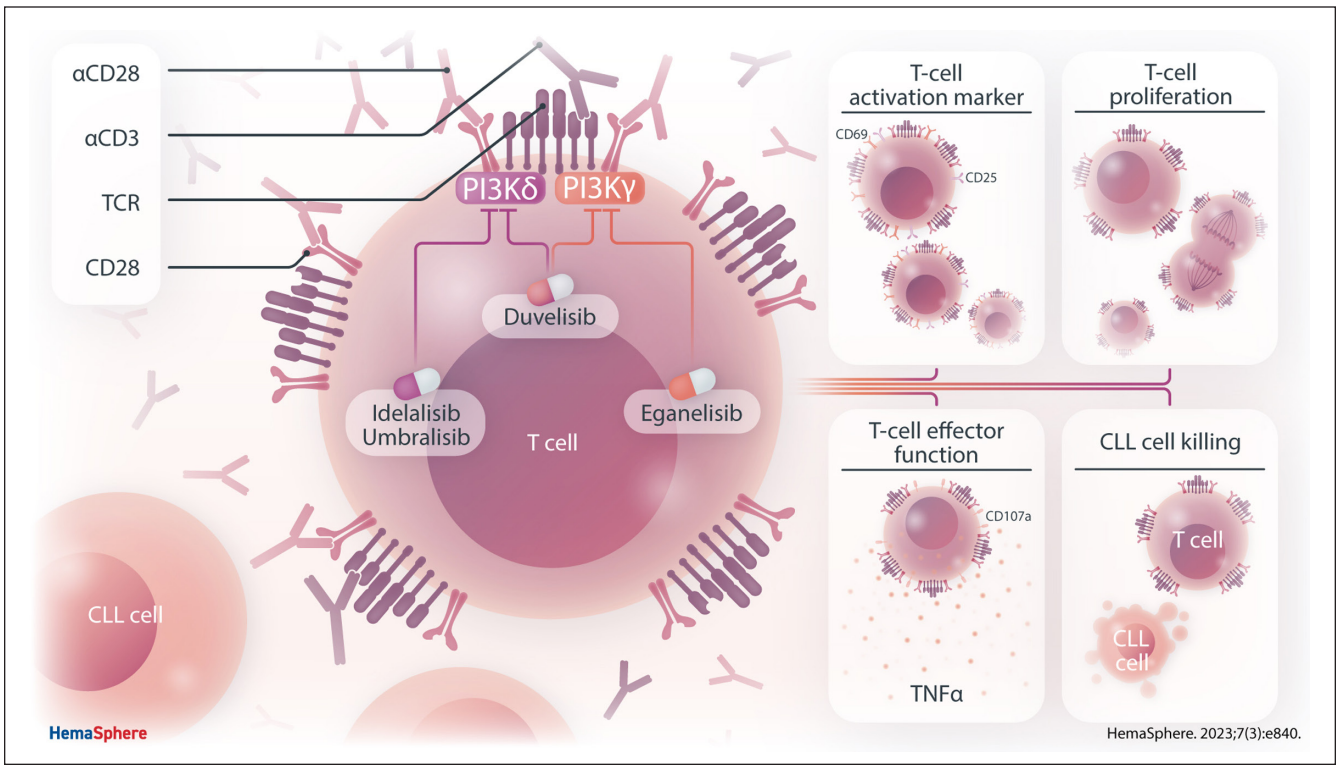
EHA EUROPEAN HEMATOLOGY ASSOCIATION

Article
Open Access

Simultaneous Inhibition of PI3Kgamma and PI3Kdelta Deteriorates T-cell Function With Implications for Chronic Lymphocytic Leukemia

Sebastian Faehling[1,2], Mariana Coelho[1,3], Alessia Floerchinger[1,3], Christof Schneider[4], Stephan Stilgenbauer[4], Peter Lichter[1], Martina Seiffert[1,*], Philipp M. Roessner[1,*]

GRAPHICAL ABSTRACT



HemaSphere

EHA EUROPEAN HEMATOLOGY ASSOCIATION

Article
Open Access

Simultaneous Inhibition of PI3Kgamma and PI3Kdelta Deteriorates T-cell Function With Implications for Chronic Lymphocytic Leukemia

Sebastian Faehling[1,2], Mariana Coelho[1,3], Alessia Floerchinger[1,3], Christof Schneider[4], Stephan Stilgenbauer[4], Peter Lichter[1], Martina Seiffert[1,*], Philipp M. Roessner[1,*]

Correspondence: Martina Seiffert (m.seiffert@dkfz.de) and Philipp M. Roessner (p.roessner@dkfz.de).

ABSTRACT

Chronic lymphocytic leukemia (CLL) is a common and incurable B-cell malignancy. Recent therapeutic approaches that target the B-cell receptor signaling pathway include inhibition of phosphatidylinositol-3-kinase (PI3K). The PI3K isoform delta is constitutively active in CLL, making it an attractive therapeutic target. However, the expression of PI3K isoforms is not exclusive to leukemic cells, as other immune cells in the tumor microenvironment also rely on PI3K activity. Subsequently, therapeutic inhibition of PI3K causes immune-related adverse events (irAEs). Here, we analyzed the impact of the clinically approved PI3Kδ inhibitors idelalisib and umbralisib, the PI3Kγ inhibitor eganelisib, and the dual-γ and -δ inhibitor duvelisib on the functional capacity of T cells. All investigated inhibitors reduced T-cell activation and proliferation *in vitro*, which is in line with PI3K being a crucial signaling component of the T-cell receptor signaling. Further, dual inhibition of PI3Kγ and PI3Kδ showed strong additive effects suggesting a role also for PI3Kγ in T cells. Extrapolation of this data to a clinical setting could provide an explanation for the observed irAEs in CLL patients undergoing treatment with PI3K inhibitors. Consequently, this highlights the need for a close monitoring of patients treated with PI3K inhibitors, and particularly duvelisib, due to their potentially increased risk of T-cell deficiencies and associated infections.

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is the most prevalent form of leukemia in adults in the western hemisphere.[1] It is characterized by the accumulation of mature B cells in lymphatic organs and peripheral blood, expressing CD5, CD20, CD19, and other B-cell antigens like CD23 and CD79b.[2] Although early and indolent courses of the disease are mainly surveilled using a "watch-and-wait" strategy, chemo-immunotherapy or targeted therapy-based regimens are common on progress or the appearance of CLL-related symptoms such as anemia or thrombocytopenia.[2]

To date there is no cure for this disease, but promising approaches combining targeted therapies are under investigation.[3] Among these novel treatment options, the phosphatidylinositol-3-kinase (PI3K) delta has served as a pivotal drug target, as this enzyme has been shown to be constitutively active in CLL.[4,5] Coherently, clinical inhibition of PI3Kδ using idelalisib or umbralisib, but also simultaneous inhibition of PI3Kγ and PI3Kδ by duvelisib has improved outcome for CLL patients tremendously.[1,6–8]

The PI3K enzyme family is grouped into 3 classes (I, II, and III) based on their subunits.[9–11] In mammals, PI3K class I contains 4 isoforms, according to their catalytic p110α, p110β, p110γ, or p110δ subunit. Although the p110α and p110β isoforms are expressed in all cell types, the expression of p110δ is largely restricted to leukocytes, and genetically inactivated PI3Kδ results in dysfunctional B, T, and NK cells in mice.[9,12,13] In contrast, genetic modification of p110γ causes defective myeloid cell function in mice.[9,14]

Two important drawbacks should be considered when treating patients with PI3K inhibitors: first, idelalisib, the pioneering PI3Kδ inhibitor, has been shown to inhibit other PI3K isoforms, which was improved by the development of umbralisib, a more recent PI3Kδ inhibitor.[10] Second, PI3Kδ is not exclusively expressed by CLL cells, but by all leukocytes, which could explain the immune-related adverse events (irAEs) that have been observed with these inhibitors: namely, transaminitis, arthritis, pneumonitis, colitis, T-cell infiltration into inflamed tissues, and opportunistic infections.[9,15–22]

These observations fueled investigations on the impact of PI3Kδ inhibitors on T cells, which are altered in their composition and function in CLL, as recently reviewed by us.[23] In brief, the disease is characterized by both an expansion of regulatory T-cell subsets (Tregs) as well as disease-controlling cytotoxic T cells.[23] There is strong evidence that PI3Kδ is involved in the

[1]Molecular Genetics, German Cancer Research Center (DKFZ), Heidelberg, Germany
[2]Medical Faculty, University of Heidelberg, Germany
[3]Faculty of Biosciences, University of Heidelberg, Germany
[4]Division of CLL, Department of Internal Medicine III, Ulm University, Germany
*MS and PMR have contributed equally to this work.
Supplemental digital content is available for this article.

HemaSphere (2023) 7:3(e840).
http://dx.doi.org/10.1097/HS9.0000000000000840.
Received: August 9, 2022 / Accepted: January 3, 2023

activation of T cells, resulting in their proliferation, differentiation, and cytokine production, processes that are impeded upon defective PI3Kδ signaling. More specifically, dysfunctional PI3Kδ resulted in reduced cytokine production of T helper cell subsets, reduced inhibitory function of Tregs, as well as diminished effector function of CD8+ T cells.[9,24,25] Of note, costimulation via CD28 or inducible T-cell co-stimulator (ICOS) has been shown to overcome the negative effect of PI3Kδ inhibition.[9,11]

PI3Kγ is a well-established signal transducer in myeloid cells but its function in T cells is less thoroughly investigated.[9] PI3Kγ has been shown to be involved in the T-cell receptor (TCR)-induced activation of T cells, as PI3Kγ-deficiency resulted in reduced proliferation and cytokine production upon αCD3 stimulation *in vitro*, which could be rescued by costimulation using αCD28 antibodies.[26,27]

As comprehensive analyses of the effect of clinically approved PI3K inhibitors on both CD4+ and CD8+ T-cell subsets are largely missing, we aimed at comparing effects of idelalisib, umbralisib, duvelisib, and the highly selective PI3Kγ inhibitor eganelisib. Thereby, we intended to elucidate the contribution of PI3Kγ and PI3Kδ to the function of T-cell subsets from healthy and leukemic individuals.

METHODS

Human blood samples

Patient samples were obtained after approval of study protocols by local ethics committees from the Department of Internal Medicine III of the University Clinic Ulm according to the declaration of Helsinki, and after obtaining informed consent of patients, as previously described.[28,29] Patients met standard diagnosis criteria for CLL, respectively. Patient characteristics such as age, mutational state, and Binet stage are provided in Suppl. Table S1. Buffy coats of healthy donors were obtained from the Institut für Klinische Transfusionsmedizin und Zelltherapie (IKTZ) Heidelberg gemeinnützige GmbH (Heidelberg, Germany).

Murine splenocytes

All animal experiments were carried out according to institutional and governmental guidelines approved by the local authorities (Regierungspräsidium Karlsruhe, permit numbers: G39/19 and DKFZ337).

PI3K inhibitors

PI3K inhibitors (duvelisib, idelalisib, umbralisib, eganelisib [all Sellekchem, Munich, Germany]) were reconstituted at a concentration of 20 mM (duvelisib, idelalisib, umbralisib) or 40 mM (eganelisib) in dimethyl sulfoxide (DMSO, Genaxxon Bioscience, Ulm, Germany) and all used at a final concentration of 1 µM in complete medium according to literature.[30,31]

Data acquisition and analysis

Fluorescently labeled single cell suspensions (for antibodies list, see Suppl. Table S2) were analyzed using FACSDiva software with a BD LSRFortessa flow cytometer (BD Biosciences, Heidelberg, Germany). Analysis of data was performed using FlowJo X software (version 10-07, FlowJo, Ashland, OR, USA).

Statistics

Sample size was determined based on expected variance of readout. No samples or animals were excluded from the analyses except when T-cell stimulation failed. For statistical analysis, GraphPad Prism software (version 8, GraphPad Software, La Jolla, CA, USA) or R studio (version 1.4.1717) was used. If data passed Shapiro-Wilk normality test, parametric statistical testing was applied. To compare data to DMSO control, fold change data was log2-transformed and 1 sample *T*-test or Wilcoxon test with Benjamini-Hochberg correction for multiple testing was applied. Comparison of 2 groups was performed using paired *T*-test or nonparametric Wilcoxon test. Comparisons of more than 2 paired groups were performed using repeated measures 1-way analysis of variance test applying Sidak's or Tukey's test for multiple comparisons. Nonparametric testing was performed using Friedman's test followed by Dunn's test for multiple comparisons. *P* values of <0.05 were considered statistically significant. Experiments were at least performed in 2 technical replicates. All graphs show individual data points as well as mean ± 95% confidence intervals .

RESULTS

PI3K inhibition by umbralisib or idelalisib has a similar negative impact on T-cell activity

Novel PI3K inhibitors specifically targeting p110δ are entering clinical trials, and a higher specificity of these compounds for PI3Kδ has been observed in comparison to idelalisib.[10] Recently, effects of umbralisib, a dual PI3Kδ and casein kinase-1ε (CK1ε) inhibitor, on T cells have been compared with idelalisib as well as duvelisib. The authors of this study concluded that umbralisib showed a unique immunomodulatory effect on T cells, but preserved number and immunosuppressive function of Tregs.[31] To comprehensively assess the effect of different PI3K inhibitors, namely idelalisib, umbralisib, eganelisib, and duvelisib, T cells of healthy donor-derived peripheral blood mononuclear cells (PBMCs) or splenocytes of mice were stimulated *in vitro* with soluble αCD3 or a combination of αCD3 and αCD28 antibodies in the presence of PI3K inhibitors or DMSO as control. Dose titrations revealed assay- and cell type-dependent IC_{50} concentrations for the 4 tested inhibitors, which were in general above 1 µM (Suppl. Tables S3, S4), a commonly used concentration in published *in vitro* studies.[30,31]

Investigation of the affected signaling pathways after stimulation with αCD3 or a combination of αCD3 and αCD28 revealed that all drugs negatively regulate the activation of the AKT/MTOR pathway (Suppl. Figure S1A–D), which is in line with previous reports.[9,31,32] In contrast, no consistent alterations of NF-κB signaling or between different inhibitors were noted (Suppl. Figure S1A–F).

By comparing the *in vitro* effects of umbralisib and idelalisib on murine CD4+ and CD8+ T cells, we observed no or only minor differences in the expression of the activation markers CD69 and CD25 (Figure 1A, Suppl. Figure S2A, B). However, TCR activation measured as Nr4a1-GFP reporter gene expression after antibody-mediated stimulation of umbralisib-treated T cells was higher, whereas cytokine production, measured as tumor necrosis factor α (TNFα) production, was comparable to idelalisib treatment (Suppl. Figure S2C, D). Ultimately, a higher proliferation rate of umbralisib-treated CD8+ T cells was noted, whereas no difference in the proliferation of CD4+ T cells was observed in comparison to idelalisib (Figure 1B).

In contrast, human CD4+ T cells, but not CD8+ T cells, retained higher CD69 expression and proliferation upon umbralisib treatment in comparison to idelalisib-treated cells (Figure 1C, D), while no difference in CD25 expression was noted (Suppl. Figure S2E).

To exclude an indirect effect of the inhibitors on T cells by blocking of PI3K in myeloid cells, T cells were sorted from murine splenocytes or human PBMCs and stimulated with αCD3- and αCD28-coated beads. Particularly the CD4+ subset retained a higher Nr4a1-GFP expression, higher CD25 expression and proliferation rate upon umbralisib compared with idelalisib treatment, while CD69 expression was not different (Figure 1E, F; Suppl. Figure S2F–I), suggesting that idelalisib affects the function of murine CD4+ T cells more than that of CD8+ T cells. However, there was no differential effect of umbralisib or idelalisib on isolated human T cells (Figure 1G),

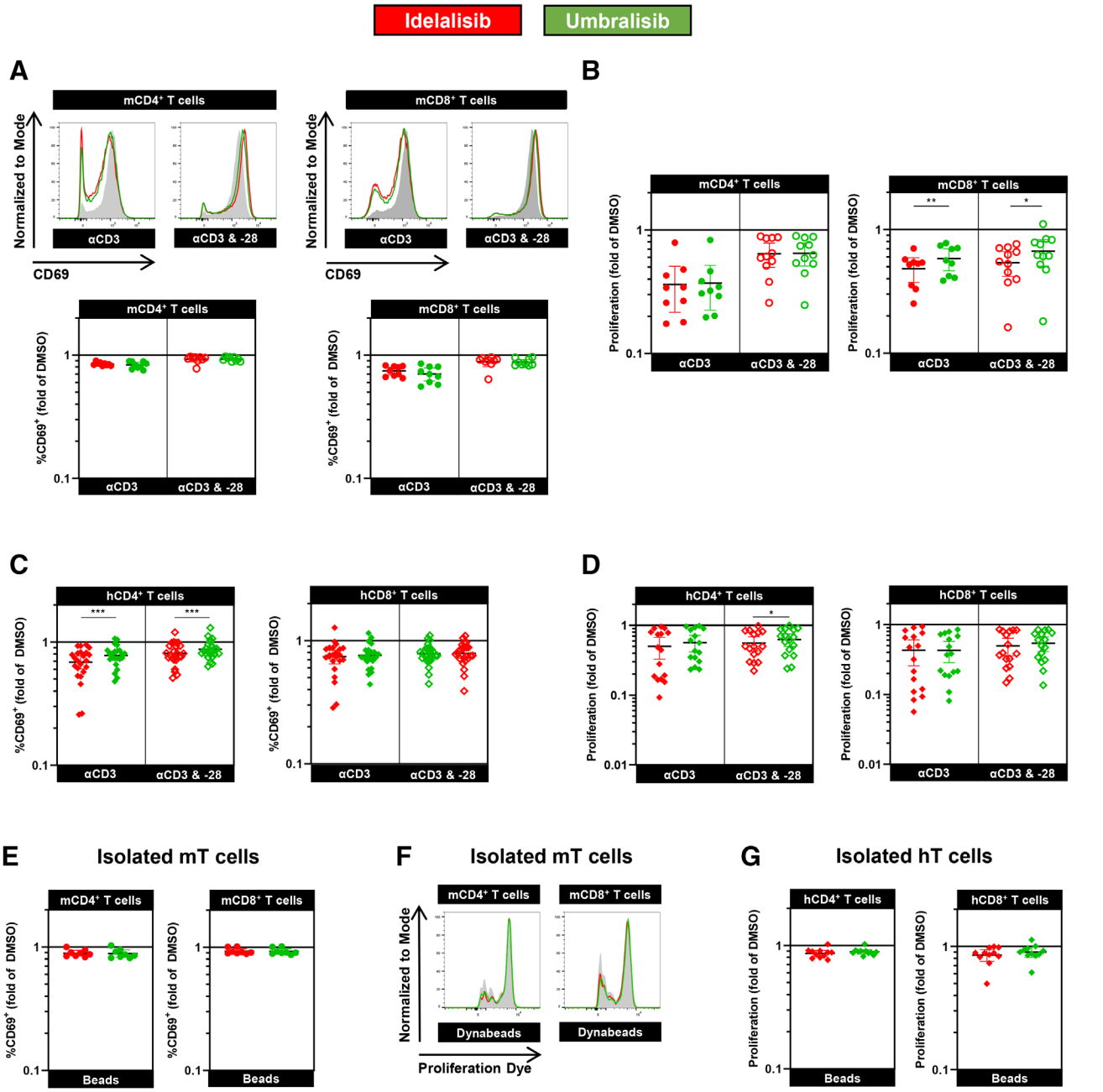


Figure 1. Dual PI3Kδ and CK1ε inhibitor umbralisib reduces T-cell function similar to idelalisib. (A and B) Murine splenocytes (m; circles) or (C and D) human PBMCs (h; rhombi) were stimulated with αCD3 (1 μg/mL) or a combination of αCD3 (1 μg/mL) and αCD28 (2 μg/mL) in the presence of DMSO control, idelalisib, or umbralisib (both 1 μM). (A) Representative histograms and frequency of CD69⁺ cells of CD4⁺ (left) and CD8⁺ T cells (right) after 6 h of stimulation. (B) Proportion of provliferated T cells after 72 h of stimulation with αCD3 (n = 9) or αCD3 and αCD28 (n = 11) normalized to DMSO control. (C) Frequency of CD69⁺ cells of human CD4⁺ (left) and CD8⁺ T cells (right) after 6 hrs of stimulation with αCD3 (n = 23) or αCD3 and αCD28 (n = 24) normalized to DMSO control. (D) Proportion of proliferated T cells after 72 h of stimulation normalized to DMSO control (n = 16). (E–G) T cells were isolated using magnetic bead-based negative enrichment and (E) frequency of murine CD69⁺ cells (n = 8) as well as (F) proliferated T cells were analyzed by flow cytometry. (G) Proliferation of human T cells relative to DMSO control (n = 11). All graphs show mean ± CI. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. CI = confidence interval; DMSO = dimethyl sulfoxide; PBMCs = peripheral blood mononuclear cells; PI3K = phosphatidylinositol-3-kinase.

allowing the general conclusion that dual PI3Kδ/CK1ε inhibition by umbralisib has a similar impact on T-cell activation as idelalisib.

Dual PI3Kγ and PI3Kδ inhibitor duvelisib strongly reduces T-cell activation

A beneficial effect of duvelisib, a dual PI3Kγ and PI3Kδ inhibitor, on the tumor microenvironment has been assumed, as PI3Kγ inhibition has been shown to reverse the tumor-supportive phenotype of myeloid cells without inducing cell death of healthy donor-derived T cells.[30,33,34] Apart from leukemia-supportive T cells, we recently showed that cytotoxic T cells exist in CLL that are able to control disease progression in mice.[23,29,35] These findings emphasize the need to analyze effects of duvelisib on CD4⁺ and CD8⁺ T-cell activity, expanding the already existing data on regulatory T cells.[31]

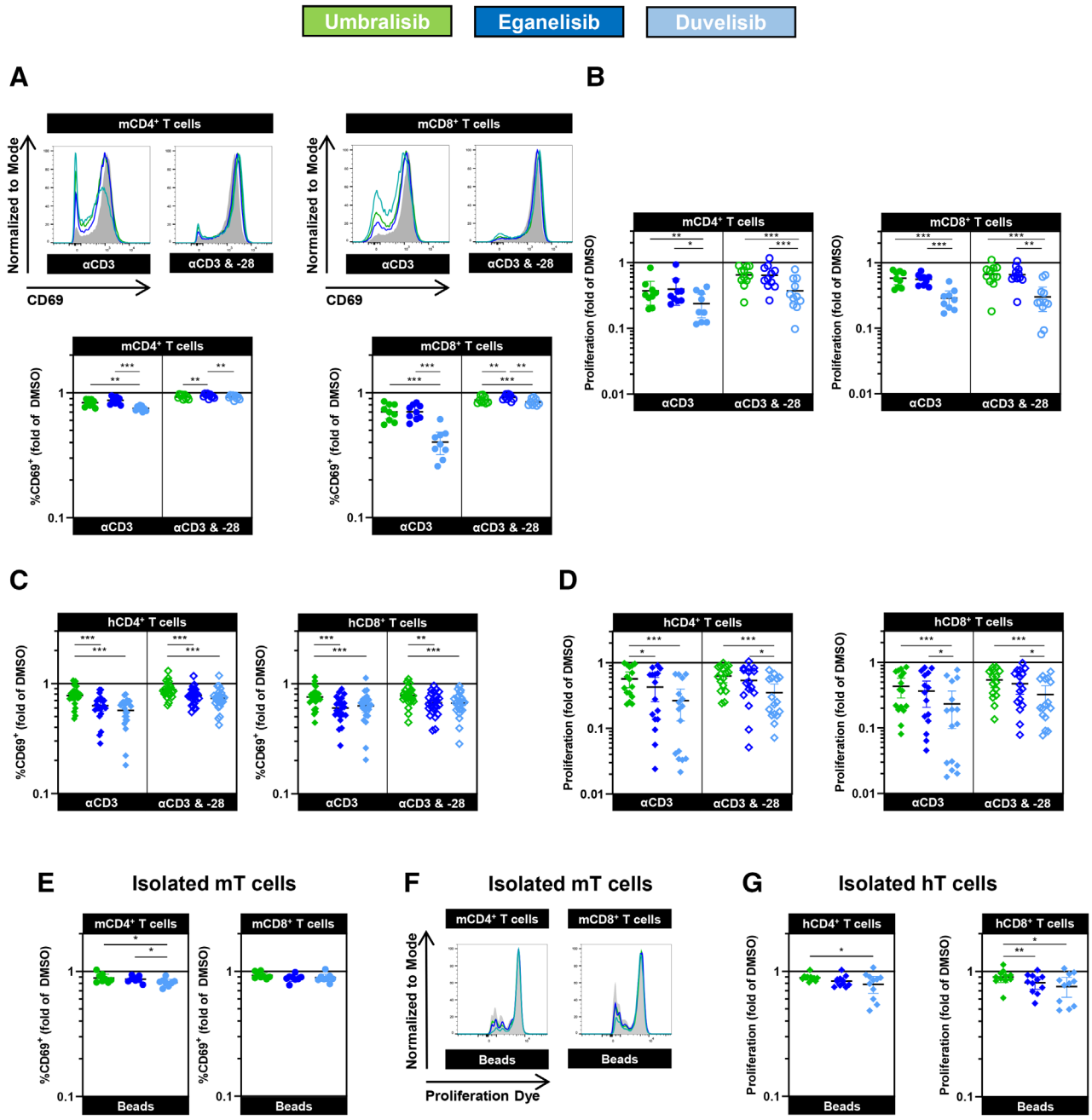


Figure 2. The dual PI3Kγ and PI3Kδ inhibitor duvelisib reduces T-cell activation more potently than single isoform inhibitors. (A and B) Murine splenocytes (m; circles) or (C and D) human PBMCs (h; rhombi) were stimulated with αCD3 (1 µg/mL) or a combination of αCD3 (1 µg/mL) and αCD28 (2 µg/mL) in the presence of DMSO control, umbralisib, eganelisib, or duvelisib (all 1 µM). (A) Representative histograms and frequency of CD69⁺ cells of CD4⁺ (left) and CD8⁺ T cells (right) after 6 h of stimulation with αCD3 (n = 9) or αCD3 and αCD28 (n = 11). (B) Proportion of proliferated T cells after 72 h of stimulation with αCD3 (n = 23) or αCD3 and αCD28 (n = 24) normalized to DMSO control. (C) Frequency of CD69⁺ cells of human CD4⁺ (left) and CD8⁺ T cells (right) after 6 h of stimulation normalized to DMSO control. (D) Proportion of proliferated T cells after 72 h of stimulation normalized to DMSO control (n = 16). (E–G) T cells were isolated using magnetic bead-based negative enrichment and (E) frequency of murine CD69⁺ cells (n = 8) as well as (F) proliferated T cells were analyzed by flow cytometry. (G) Proliferation of human T cells relative to DMSO control (n = 11). All graphs show mean ± CI. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. CI = confidence interval; DMSO = dimethyl sulfoxide; PBMCs = peripheral blood mononuclear cells; PI3K = phosphatidylinositol-3-kinase.

Intriguingly, duvelisib reduced the expression of activation markers CD69 and CD25 (Figure 2A; Suppl. Figure S3A, B), TCR activity (Suppl. Figure S3C) and secretion of TNFα (Suppl. Figure S3D) of murine CD4⁺ as well as CD8⁺ T-cell subsets. These effects were more potent in comparison to umbralisib and the PI3Kγ-specific inhibitor eganelisib, and could not be completely overcome by co-stimulatory αCD28 antibodies. Along this line, the proliferation rate of CD4⁺ and CD8⁺ T-cell subsets was most strongly reduced in the presence of duvelisib (Figure 2B).

Likewise, duvelisib was the most potent inhibitor for human T-cell subsets, as shown by reduced CD69 and CD25 expression (Figure 2C; Suppl. Figure S3E) as well as lower proliferation rates (Figure 2D) of CD4⁺ and CD8⁺ T cells regardless

of costimulation with αCD28. Intriguingly, single PI3Kγ inhibition using eganelisib decreased T-cell activity more strongly than umbralisib (Figure 2C, D; Suppl. Figure S3E), which is in contrast to the results observed with murine cells.

To exclude that duvelisib impacts on T cells via PI3Kγ-expressing myeloid cells which are present in the *in vitro* assays of splenocytes or PBMCs, isolated T cells were investigated. Since a similar trend of reduced activation and proliferation of murine (Figure 2E, F; Suppl. Figure S3F–I) and human CD4+ and CD8+ T cells (Figure 2G) was seen with sorted and unsorted cells, a direct impact of PI3Kγ inhibition on T-cell activity can be suggested. In summary, these results uncover an unexpected role of PI3Kγ in activated human T cells *in vitro*, which likely explains the observed stronger effects of the dual inhibitor duvelisib compared with umbralisib on the activity of murine and human T cells.

Duvelisib impairs the activity and leukemic cell death of antigen-specific T cells

To assess the effect of PI3K inhibitors on the activity of antigen-specific T cells and their induction of leukemia cell death, a cytotoxic T-cell assay was performed. In brief, antigen-specific murine CD8+ T cells of P14-transgenic mice were treated for 1 hour with PI3K inhibitors and subsequently, cocultured with a cognate antigen-primed TCL1 leukemia cell line.[36] In line with results described above, idelalisib-treated T cells showed a slightly lower activity in comparison to umbralisib-treated cells, as evidenced by a lower proportion of degranulation measured as CD107a+ cells, and a lower expression of TNFα (Figure 3A, B; Suppl. Figure S4A, B). Despite the slightly lower T-cell activity, no differences in leukemia cell death were observed between these 2 drugs (Figure 3C, D; Suppl. Figure S4C).

In contrast, the dual inhibitor duvelisib reduced the activity of antibody-activated Tcells more severely. This was supported by duvelisib showing a remarkable reduction of CD107a- and TNFα-expressing T cells in comparison to umbralisib- and eganelisib-treated T cells (Figure 3A, B; Suppl. Figure S4A, B). Of note, even though eganelisib did not reduce the percentage of CD107a or TNFα-expressing T cells, it reduced TNFα-expression per T cell, similarly as idelalisib (Suppl. Figure S4B). Importantly, reduced activity of duvelisib-treated T cells also resulted in a reduced leukemia cell death in comparison to umbralisib and eganelisib (Figure 3D). These data underline the detrimental effects of duvelisib on T-cell activity and antigen-specific tumor cell killing.

PI3Kγ and PI3Kδ additively control T-cell activity

As our data showed that PI3Kγ inhibition reduced T-cell activity, we further investigated whether PI3Kγ and PI3Kδ additively influence T-cell activity. Thus, murine splenocytes as well as human PBMCs were treated simultaneously with the PI3Kγ inhibitor eganelisib and one of the PI3Kδ inhibitors idelalisib or umbralisib. Expression of the early activation marker CD69 as well as TCR activation were more strongly reduced in murine T cells upon simultaneous PI3Kγ and PI3Kδ inhibition compared with single treatments (Figure 4A, B). Similar effects were seen when targeting both PI3K isoforms in human T cells (Figure 4C; Suppl. Figure S5A). The effect of a combination of PI3Kγ- and PI3Kδ-specific inhibitors was comparable to the effect of the dual inhibitor duvelisib (Figure 4A–C). These results highlight an additive impact of PI3Kγ and PI3Kδ on T-cell activation, which is in line with the data obtained with the dual inhibitor duvelisib, thus demonstrating the need for PI3Kγ signaling in T cells for complete functionality.

Dual PI3Kγ and PI3Kδ inhibitor duvelisib decreases the activity of CLL patient-derived T cells

While above-mentioned findings highlight the role of PI3Kγ and PI3Kδ in leukemia-naive T cells, it is essential to translate these findings to CLL-experienced T cells, as the composition of T-cell subsets is substantially altered by this disease.[23] Therefore, we investigated the *in vitro* effect of PI3K inhibitors on the activity of T cells derived from splenocytes of the Eμ-TCL1 (TCL1) mouse model of CLL which mimics alterations of the T-cell compartment in CLL patients, as well as of T cells from treatment-naive CLL patients.[23,35] In contrast to T cells from healthy mice, PI3K inhibition of TCL1-derived T cells showed only a modest negative impact on the expression of the activation markers CD69 and CD25 in comparison to DMSO control, which was more pronounced in CD8+ T cells (Suppl. Figure S6A, B). Interestingly, applying co-stimulatory signals combining αCD3 and αCD28 antibodies showed that the effect of the inhibitors could be mostly overcome (Suppl. Figure S6A, B). The lack of strong inhibitory effects of the drugs in TCL1 T cells could be due to their prior *in vivo* activation in the CLL microenvironment, which results in high basal expression of activation markers leaving only a small window for further *in vitro* stimulation and therefore, inhibition of activation.[35] The minor impact of PI3K inhibition on TCL1-experienced T cells led to no consistent differences between the investigated inhibitors, suggesting neither idelalisib nor duvelisib as being more effective than any investigated inhibitor.

Opposite of what we observed in TCL1 T cells, the activity of T cells derived from treatment-naive patients with CLL was deteriorated by all investigated inhibitors both for αCD3 stimulation and the combination with αCD28 antibodies (Suppl. Figure S7A). When comparing idelalisib and the dual PI3Kδ/CK1ε inhibitor umbralisib, the latter preserved the function of CD4+ T cells better (Figure 5A), which is in line with the results of T cells from healthy donors (Figure 1C). Furthermore, inhibition of PI3Kγ by eganelisib or dual PI3Kγ and PI3Kδ inhibition by duvelisib strongly attenuated T-cell activation in comparison to PI3Kδ-specific inhibition by umbralisib (Figure 5B, C), which is again in line with the results obtained with healthy donor-derived T cells (Figure 2C).

DISCUSSION

PI3K is an interesting therapeutic target in CLL, as indicated by the approval of several drugs inhibiting this enzyme.[10] But as PI3K family members are also expressed in T cells, these inhibitors may have clinically relevant effects on their function. To decipher these effects, we performed *in vitro* treatment studies using healthy as well as CLL-derived T cells and analyzed their function.

We observed that all investigated PI3K inhibitors had an impact on the activation and proliferation of both CD4+ and CD8+ T cells, which confirms previous literature.[31,37] This effect seemed to be independent of antigen-presenting cells, as a comparable inhibition of T-cell proliferation was seen upon treatment of unsorted and isolated T cells. Interestingly, not only inhibition of PI3Kδ but also PI3Kγ reduced T-cell activation and proliferation, suggesting that both isoforms are crucial for T-cell function. Although the contribution of PI3Kδ to physiological T-cell function is broadly accepted, the role of PI3Kγ is less known.[9,10] Here, we show that eganelisib, a very specific but not exclusive inhibitor of PI3Kγ, reduced murine T-cell function similarly as the PI3Kδ inhibitor umbralisib, while in human T cells, an even more potent effect of eganelisib was observed. This is in line with data showing a reduced *in vitro* proliferation of T cells derived from PI3Kγ$^{-/-}$ compared with wild-type mice, which was dependent on TCR signaling and functional PI3K downstream of CD3.[27] Collectively, these data suggest that murine and human CD4+ as well as CD8+ T cells require both functional PI3Kδ and PI3Kγ for their activation and proliferation.

Since the first approval of the PI3Kδ inhibitor idelalisib, further drugs with improved specificity for PI3Kδ, including

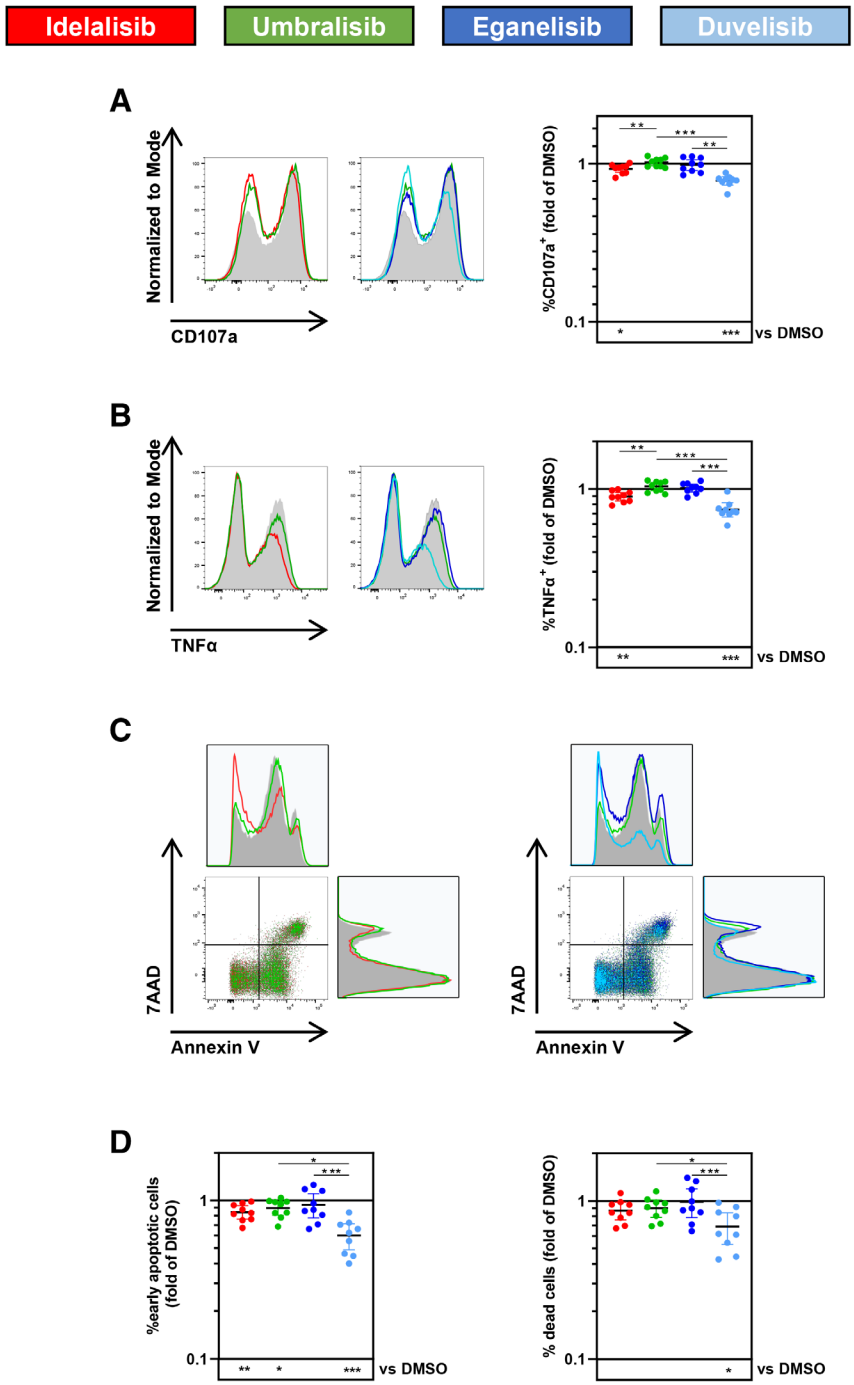


Figure 3. Duvelisib reduces the activity of antigen-specific T cells. Splenic P14 CD8+ T cells were incubated for 1 h with respective PI3K inhibitors and cocultured with cognate antigen-pulsed TCL1 leukemic cells (n = 9). (A and B) Degranulation and cytokine production and T cells after 6 h and (C and D) leukemia cell death after 24 h were analyzed. (A) Frequency of CD107a+ and (B) TNFα+ cells depicted as representative histograms and quantification. (C) Frequency of early apoptotic (Annexin V+ 7AAD−), and all dead cells (Annexin V+/− 7AAD+) depicted as representative graphs and (D) quantification relative to DMSO control. All graphs show mean ± CI. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. CI = confidence interval; DMSO = dimethyl sulfoxide; PI3K = phosphatidylinositol-3-kinase.

umbralisib which also targets CK1ε, have entered clinical trials.[10] Recently, it has been described that umbralisib treatment exhibits unique immunomodulatory effects on CLL T cells, but exerts less effects on reducing Treg activity and immunosuppressive function compared with idelalisib or duvelisib.[31] Our study showed no consistent differences in the activation and proliferation of CD4+ or CD8+ T cells *in vitro* by umbralisib in comparison to idelalisib. Even though we did not investigate the specific effect of umbralisib treatment on Tregs, secondary effects mediated by improved Treg function due to umbralisib treatment[31] are most likely not sufficient to overcome the direct and impeding effects of PI3K inhibition on CD4+ and CD8+ T cells.

As inhibition of PI3Kγ in myeloid cells was shown to reverse the protumoral phenotype of these cells in solid tumors,[33,38] one might speculate that dual PI3Kγ and PI3Kδ inhibition would improve control of CLL. The PI3Kγ/δ inhibitor duvelisib has also

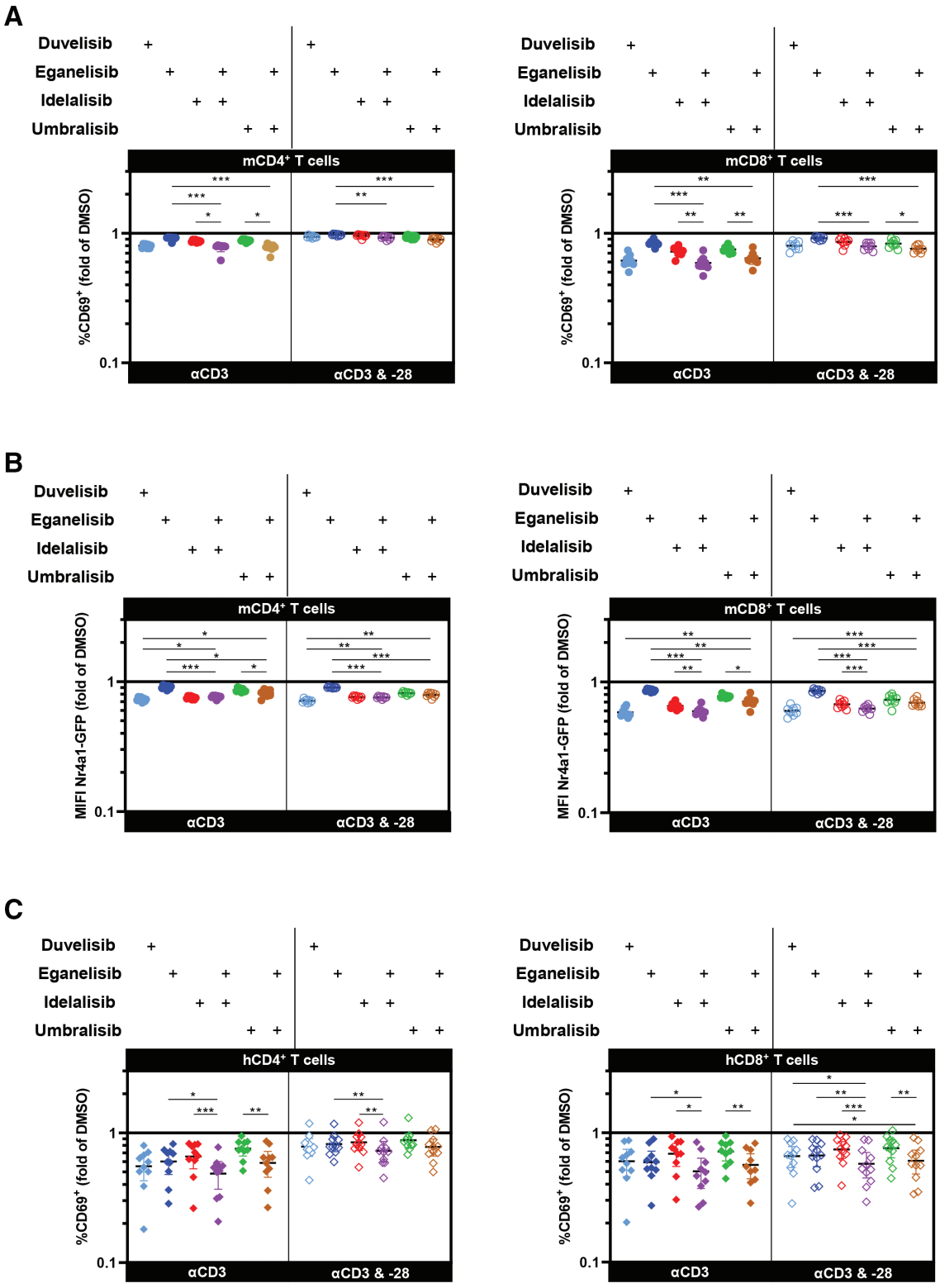


Figure 4. PI3Kγ and PI3Kδ additively control T-cell function. (A and B) Murine splenocytes (m; circles) or (C) human PBMCs (h; rhombi) were stimulated with αCD3 (1 μg/mL) or a combination of αCD3 (1 μg/mL) and αCD28 (2 μg/mL) in the presence of DMSO control, duvelisib, eganelisib, idelalisib, umbralisib, or a combination of those (all 1 μM). (A) Frequency of CD69+ cells (n = 8) and (B) MFI of Nr4a1-GFP (n = 8) expression of CD4+ (left) and CD8+ T cells (right) normalized to DMSO control. (C) Proportion of CD69+ cells out of human CD4+ (left; n = 10) and CD8+ T cells (right; n = 11) normalized to DMSO control. All graphs show mean ± CI. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. CI = confidence interval; DMSO = dimethyl sulfoxide; MFI = median fluorescence intensity; PBMCs = peripheral blood mononuclear cells; PI3K = phosphatidylinositol-3-kinase.

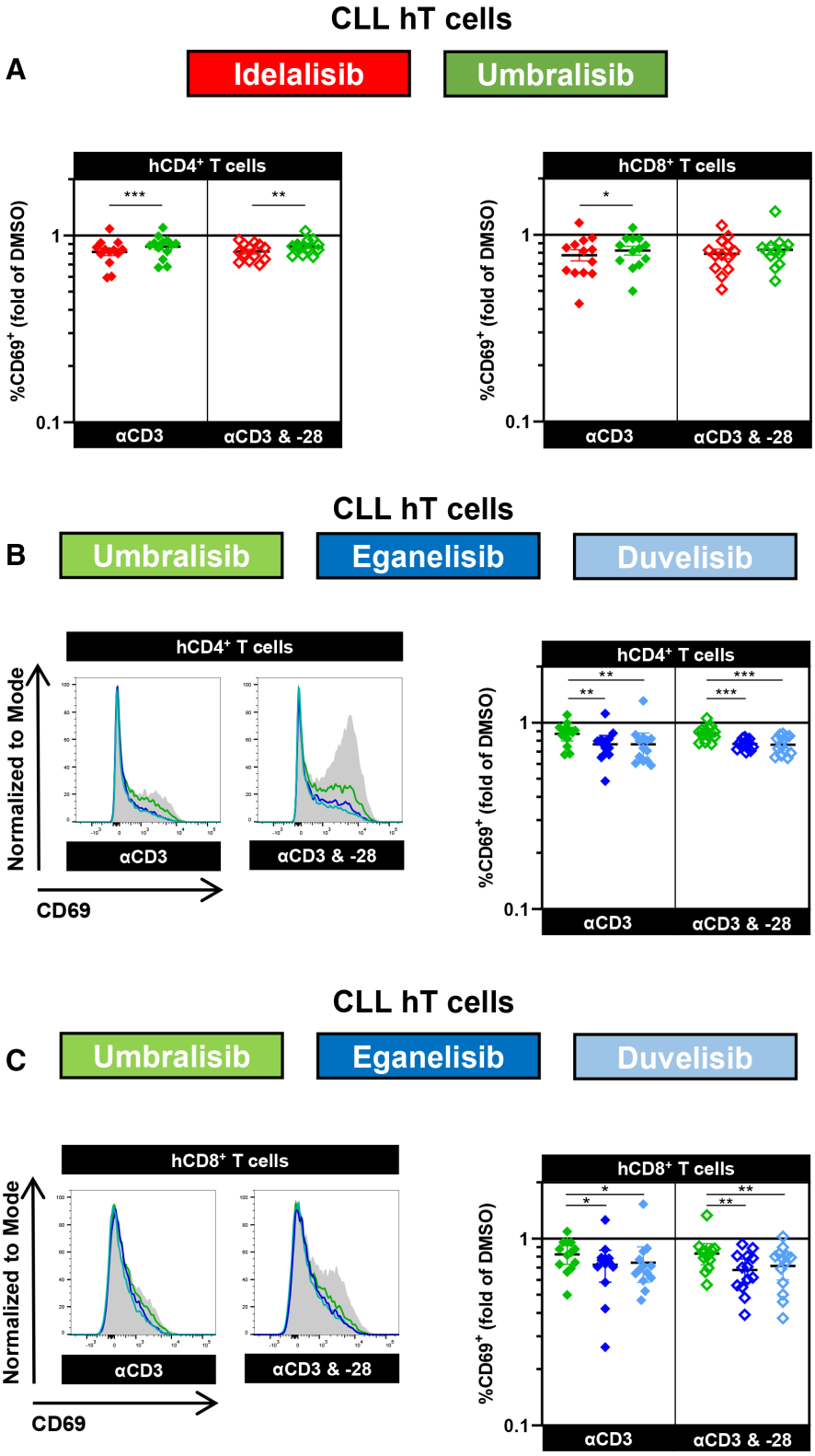


Figure 5. Duvelisib, a dual PI3Kγ and PI3Kδ inhibitor potently reduces activity of CLL patient-derived T cells. PBMCs of CLL patients were stimulated with αCD3 (1 µg/mL) or a combination of αCD3 (1 µg/mL) and αCD28 (2 µg/mL) in the presence of DMSO control, or the indicated PI3K inhibitors (all 1 µM). (A) Frequency of CD69+ cells out of CD4+ (left) and CD8+ T cells (right) after 6 h of stimulation comparing idelalisib and umbralisib-treated cells normalized to DMSO control (n = 13). (B and C) Representative histograms as well as frequency of CD69+ cells out of (B) CD4+ (n = 13) and (C) CD8+ T cells (n = 13) comparing umbralisib-, eganelisib-, and duvelisib-treated cells normalized to DMSO control. All graphs show mean ± CI. *$P < 0.05$; **$P < 0.01$. CI = confidence interval; CLL = chronic lymphocytic leukemia; DMSO = dimethyl sulfoxide; PBMCs = peripheral blood mononuclear cells; PI3K = phosphatidylinositol-3-kinase.

been shown to overcome the protective effect of stromal cells for CLL cells and their migration and adhesion in co-cultures.[30,39] A direct cytotoxic effect of duvelisib has neither been observed for healthy donor-derived T cells nor for CLL patient-derived T cells.[30,31] Nevertheless, chemotaxis of T cells has been repeatedly found to be reduced by duvelisib *in vitro* and *in vivo*.[34,40] Here, we show that duvelisib strongly affects T cells *in vitro*, as it dampens the activation, proliferation, and antigen-specific cytotoxicity of T cells of healthy donors and CLL patients. Intriguingly, this effect is not mediated by bystander myeloid cells, as similar effects were observed in monocultures of purified T cells. In functional assays, impaired *in vitro* cytotoxicity and cytokine production by duvelisib could be observed, while by combining specific inhibitors of PI3Kγ and PI3Kδ, an additive inhibition of T cells was seen. These findings support our conclusion that dual PI3Kγ and PI3Kδ inhibitors will have detrimental effects on T-cell function.

Using a stimulation cocktail that combines αCD3- and αCD28-coated beads with interleukin-2 (IL-2), which is a considerably stronger stimulus as the regimens used in this manuscript, a reduced proliferation of $CD4^+$ but not $CD8^+$ T cells of CLL patients has been shown after duvelisib treatment *in vitro*.[40] Of note, IL-2 signaling has distinct functions in $CD4^+$ and $CD8^+$ T-cell subsets,[41] and IL-2-induced expansion of cytotoxic $CD8^+$ T cells was found to be independent of functional PI3Kδ, which might be one possible explanation for the divergent results.[42] Similarly, treatment of CLL patients for 1 week with duvelisib resulted in the relative loss of $CD4^+$ T cells and an accumulation of $CD8^+$ T cells in blood.[22] Interestingly, genetically engineered PI3Kγ- and PI3Kδ-deficient mice showed severely impaired thymic development, resulting in reduced $CD4^+$ and $CD8^+$ T-cell counts in spleen as well as lymph nodes,[43] which is in line with our conclusion that both PI3Kγ and PI3Kδ are important for T-cell function.

In summary, our study clearly shows that the PI3Kγ and PI3Kδ inhibitor duvelisib and dual inhibition of PI3Kγ and PI3Kδ are potent inhibitors of T-cell activation and proliferation *in vitro*, and that umbralisib does not preserve T-cell activity in comparison to idelalisib, which contrasts with published literature.[31]

Given our observations *in vitro*, one might speculate that they can serve as an explanation for the observed irAEs in CLL patients treated with PI3K inhibitors. T cells are a very heterogeneous group of cells that harbor distinct, often counteracting functions, highlighting the complexity of their systemic activity.[23] Immunosuppressive Tregs are enriched in CLL, and strong evidence exists that PI3Kδ inhibition reduces the number, activity, and function of Tregs in this disease.[9,24,25,31] The effect of PI3K inhibition on $CD8^+$ T cells in the same context is more controversial, as both a beneficial effect on their function due to the alleviation of inhibitory activity of Tregs, and a negative impact due to direct inhibition of $CD8^+$ T-cell function have to be considered.[9,10,24,25] Although an enhanced anti-tumor control of $CD8^+$ T cells was observed in many cancer entities due to a reduced Treg function caused by inhibition of PI3K,[9,10,44] there are still no such results in the context of CLL. Immunophenotyping of blood T cells from duvelisib-treated CLL patients by mass cytometry recently revealed that the onset of duvelisib-associated irAEs correlated with a decrease in naive $CD4^+$ and $CD8^+$ T-cell subsets, and with the accumulation of activated $CD8^+$ as well as Th17 T cells.[22] Alternatively, intermittent (3 days on, 2 days off drug) dosing of PI3Kδ inhibitor treatment in murine models did not impact on tumor control, but remarkably reduced the generation of irAE-causing T cells within the tumor microenvironment in comparison to continuous drug dosing,[45] which could also provide an alternative treatment schedule for reintroduction of PI3K inhibition in CLL. Here, we provide evidence for healthy donor- as well as CLL patient-derived $CD8^+$ T cells that pharmacological inhibition of PI3Kδ, PI3Kγ as well as dual PI3Kγ and PI3Kδ inhibition *in vitro* limits the activation and proliferation of this cell type. Follow-up studies in mouse models as well as relevant analysis of $CD4^+$ and $CD8^+$ T-cell populations in CLL patients under treatment with PI3K inhibitors would be ultimately needed to prove a connection between PI3K inhibition and irAEs.

AUTHOR CONTRIBUTIONS

SF performed experiments, analyzed, and interpreted data, prepared figures, and wrote the manuscript. MC and AF performed experiments, analyzed, and interpreted data and wrote the manuscript. CS and SS provided clinical samples and information and reviewed the paper. PL critically advised the study and reviewed the paper. MS supervised the study, interpreted data, provided budget, and wrote the manuscript. PMR designed and supervised the study, performed experiments, analyzed, and interpreted data, prepared figures, and wrote the manuscript.

DISCLOSURES

CS received speaker's bureau from AbbVie and AstraZeneca. MS received research funding from Bayer AG. All the other authors have no conflicts of interest to disclose.

SOURCES OF FUNDING

SF was supported by the German Cancer Aid grant number 70113937. MC and AF have been supported by Helmholtz International Graduate School stipends awarded by the German Cancer Research Center. This project was funded by Deutsche Krebshilfe to MS. PMR has been funded by a fellowship of the DKFZ Clinician Scientist Program, supported by the Dieter Morszeck Foundation. SS and CS were supported by DFG SFB1074 subproject B1.

REFERENCES

1. Yosifov DY, Wolf C, Stilgenbauer S, et al. From biology to therapy: the CLL success story. *HemaSphere*. 2019;3:e175.
2. Hallek M, Cheson BD, Catovsky D, et al. iwCLL guidelines for diagnosis, indications for treatment, response assessment, and supportive management of CLL. *Blood*. 2018;131:2745–2760.
3. Jain N, Keating M, Thompson P, et al. Ibrutinib and venetoclax for first-line treatment of CLL. *N Engl J Med*. 2019;380:2095–2103.
4. Ringshausen I, Schneller F, Bogner C, et al. Constitutively activated phosphatidylinositol-3 kinase (PI-3K) is involved in the defect of apoptosis in B-CLL: association with protein kinase Cdelta. *Blood*. 2002;100:3741–3748.
5. Herman SE, Gordon AL, Wagner AJ, et al. Phosphatidylinositol 3-kinase-delta inhibitor CAL-101 shows promising preclinical activity in chronic lymphocytic leukemia by antagonizing intrinsic and extrinsic cellular survival signals. *Blood*. 2010;116:2078–2088.
6. Furman RR, Sharman JP, Coutre SE, et al. Idelalisib and rituximab in relapsed chronic lymphocytic leukemia. *N Engl J Med*. 2014;370:997–1007.
7. Flinn IW, Hillmen P, Montillo M, et al. The phase 3 DUO trial: duvelisib vs ofatumumab in relapsed and refractory CLL/SLL. *Blood*. 2018;132:2446–2455.
8. Sharman JP, Coutre SE, Furman RR, et al. Final results of a randomized, phase III study of rituximab with or without idelalisib followed by open-label idelalisib in patients with relapsed chronic lymphocytic leukemia. *J Clin Oncol*. 2019;37:1391–1402.
9. Aydin E, Faehling S, Saleh M, et al. Phosphoinositide 3-kinase signaling in the tumor microenvironment: what do we need to consider when treating chronic lymphocytic leukemia with PI3K inhibitors? Review. *Front Immunol*. 2020;11:595818.
10. Vanhaesebroeck B, Perry MWD, Brown JR, et al. PI3K inhibitors are finally coming of age. *Nat Rev Drug Discovery*. 2021;20:741–769.
11. Okkenhaug K, Vanhaesebroeck B. PI3K in lymphocyte development, differentiation and activation. *Nat Rev Immunol*. 2003;3:317–330.
12. Okkenhaug K, Bilancio A, Farjot G, et al. Impaired B and T cell antigen receptor signaling in p110delta PI 3-kinase mutant mice. *Science*. 2002;297:1031–1034.
13. Guo H, Samarakoon A, Vanhaesebroeck B, et al. The p110 delta of PI3K plays a critical role in NK cell terminal maturation and cytokine/chemokine generation. *J Exp Med*. 2008;205:2419–2435.

14. Hirsch E, Katanaev VL, Garlanda C, et al. Central role for G protein-coupled phosphoinositide 3-kinase gamma in inflammation. *Science*. 2000;287:1049–1053.
15. Weidner AS, Panarelli NC, Geyer JT, et al. Idelalisib-associated colitis: histologic findings in 14 patients. *Am J Surg Pathol*. 2015;39:1661–1667.
16. Coutre SE, Barrientos JC, Brown JR, et al. Management of adverse events associated with idelalisib treatment: expert panel opinion. *Leuk Lymphoma*. 2015;56:2779–2786.
17. O'Brien SM, Lamanna N, Kipps TJ, et al. A phase 2 study of idelalisib plus rituximab in treatment-naive older patients with chronic lymphocytic leukemia. *Blood*. 2015;126:2686–2694.
18. Pleyer C, Wiestner A, Sun C. Immunological changes with kinase inhibitor therapy for chronic lymphocytic leukemia. *Leuk Lymphoma*. 2018;59:2792–2800.
19. Zelenetz AD, Barrientos JC, Brown JR, et al. Idelalisib or placebo in combination with bendamustine and rituximab in patients with relapsed or refractory chronic lymphocytic leukaemia: interim results from a phase 3, randomised, double-blind, placebo-controlled trial. *Lancet Oncol*. 2017;18:297–311.
20. Lampson BL, Kasar SN, Matos TR, et al. Idelalisib given front-line for treatment of chronic lymphocytic leukemia causes frequent immune-mediated hepatotoxicity. *Blood*. 2016;128:195–203.
21. Kienle DL, Stilgenbauer S. Approved and emerging PI3K inhibitors for the treatment of chronic lymphocytic leukemia and non-Hodgkin lymphoma. *Expert Opin Pharmacother*. 2020;21:917–929.
22. Gadi D, Griffith A, Tyekucheva S, et al. A T cell inflammatory phenotype is associated with autoimmune toxicity of the PI3K inhibitor duvelisib in chronic lymphocytic leukemia. *Leukemia*. 2021;36:723–732.
23. Roessner PM, Seiffert M. T-cells in chronic lymphocytic leukemia: guardians or drivers of disease? *Leukemia*. 2020;34:2012–2024.
24. Hanna BS, Roessner PM, Scheffold A, et al. PI3Kdelta inhibition modulates regulatory and effector T-cell differentiation and function in chronic lymphocytic leukemia. *Leukemia*. 2019;33:1427–1438.
25. Rohrbacher L, Brauchle B, Ogrinc Wagner A, et al. The PI3K partial differential-selective inhibitor idelalisib induces T- and NK-cell dysfunction independently of B-cell malignancy-associated immunosuppression. *Front Immunol*. 2021;12:608625.
26. Sasaki T, Irie-Sasaki J, Jones RG, et al. Function of PI3Kgamma in thymocyte development, T cell activation, and neutrophil migration. *Science*. 2000;287:1040–1046.
27. Alcazar I, Marques M, Kumar A, et al. Phosphoinositide 3-kinase gamma participates in T cell receptor-induced T cell activation. *J Exp Med*. 2007;204:2977–2987.
28. Roessner PM, Hanna BS, Ozturk S, et al. TBET-expressing Th1 CD4(+) T cells accumulate in chronic lymphocytic leukaemia without affecting disease progression in Emicro-TCL1 mice. *Br J Haematol*. 2020;189:133–145.
29. Roessner PM, Llao Cid L, Lupar E, et al. EOMES and IL-10 regulate antitumor activity of T regulatory type 1 CD4(+) T cells in chronic lymphocytic leukemia. *Leukemia*. 2021;35:2311–2324.
30. Balakrishnan K, Peluso M, Fu M, et al. The phosphoinositide-3-kinase (PI3K)-delta and gamma inhibitor, IPI-145 (Duvelisib), overcomes signals from the PI3K/AKT/S6 pathway and promotes apoptosis in CLL. *Leukemia*. 2015;29:1811–1822.
31. Maharaj K, Powers JJ, Achille A, et al. The dual PI3Kdelta/CK1epsilon inhibitor umbralisib exhibits unique immunomodulatory effects on CLL T cells. *Blood Adv*. 2020;4:3072–3084.
32. Chellappa S, Kushekhar K, Munthe LA, et al. The PI3K p110delta isoform inhibitor idelalisib preferentially inhibits human regulatory T cell function. *J Immunol*. 2019;202:1397–1405.
33. Kaneda MM, Messer KS, Ralainirina N, et al. PI3Kgamma is a molecular switch that controls immune suppression. *Nature*. 2016;539:437–442.
34. Peluso M, Faia K, Winkler D, et al. Duvelisib (IPI-145) inhibits malignant B-cell proliferation and disrupts signaling from the tumor microenvironment through mechanisms that are dependent on PI3K-δ and PI3K-γ. *Blood*. 2014;124:328–328.
35. Hanna BS, Roessner PM, Yazdanparast H, et al. Control of chronic lymphocytic leukemia development by clonally-expanded CD8(+) T-cells that undergo functional exhaustion in secondary lymphoid tissues. *Leukemia*. 2019;33:625–637.
36. Chakraborty S, Martines C, Porro F, et al. B-cell receptor signaling and genetic lesions in TP53 and CDKN2A/CDKN2B cooperate in richter transformation. *Blood*. 2021;138:1053–1066.
37. Martinelli S, Maffei R, Fiorcari S, et al. Idelalisib impairs T-cell-mediated immunity in chronic lymphocytic leukemia. *Haematologica*. 2018;103:e598–e601.
38. Kaneda MM, Cappello P, Nguyen AV, et al. Macrophage PI3Kgamma drives pancreatic ductal adenocarcinoma progression. *Cancer Discov*. 2016;6:870–885.
39. Ali AY, Guan Q, Wu X, et al. Expression and function of phosphoinositide 3-kinase delta in mesenchymal stromal cells from normal and leukaemic bone marrow. *Br J Haematol*. 2019;185:883–887.
40. Chen SS, Ham S, Rai KR, et al. Dual inhibition of PI3K-delta and gamma by duvelisib (IPI-145) impairs CLL B- and T-cell migration, survival and proliferation in a murine xenograft model using primary chronic lymphocytic leukemia cells. *Blood*. 2015;126:1753–1753.
41. Ross SH, Cantrell DA. Signaling and function of interleukin-2 in T lymphocytes. *Annu Rev Immunol*. 2018;36:411–433.
42. Macintyre AN, Finlay D, Preston G, et al. Protein kinase B controls transcriptional programs that direct cytotoxic T cell fate but is dispensable for T cell metabolism. *Immunity*. 2011;34:224–236.
43. Ji H, Rintelen F, Waltzinger C, et al. Inactivation of PI3Kgamma and PI3Kdelta distorts T-cell development and causes multiple organ inflammation. *Blood*. 2007;110:2940–2947.
44. Ali K, Soond DR, Pineiro R, et al. Inactivation of PI(3)K p110delta breaks regulatory T-cell-mediated immune tolerance to cancer. *Nature*. 2014;510:407–411.
45. Eschweiler S, Ramirez-Suastegui C, Li Y, et al. Intermittent PI3Kdelta inhibition sustains anti-tumour immunity and curbs irAEs. *Nature*. 2022;605:741–746.