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The PI3K p110δ Isoform Inhibitor Idelalisib Preferentially Inhibits Human Regulatory T Cell Function

Stalin Chellappa,∗†,‡,§ Kushi Kushkhar,∗†,§ Ludvig A. Munthi,∗,‡ Geir E. Tjønnfjord,∗,‖ Einar M. Aandahl,∗†,‡,§ Klaus Okkenhaug,∗∗ and Kjetil Taskén,∗†,‡,§

In chronic lymphocytic leukemia (CLL), signaling through several prosurvival B cell surface receptors activates the PI3K signaling pathway. Idelalisib is a highly selective PI3K (PI3Kδ) isoform-specific inhibitor effective in relapsed/refractory CLL and follicular lymphoma. However, severe autoimmune adverse effects in association with the use of idelalisib in the treatment of CLL, particularly as a first-line therapy, gave indications that idelalisib may preferentially target the suppressive function of regulatory T cells (Tregs). On this background, we examined the effect of idelalisib on the function of human Tregs ex vivo with respect to proliferation, TCR signaling, phenotype, and suppressive function. Our results show that human Tregs are highly susceptible to PI3Kδ inactivation using idelalisib compared with CD4+ and CD8+ effector T cells (Teffs) as evident from effects on anti-CD3/CD28/CD2–induced proliferation (order of susceptibility [IC50]: Treg [5 μM] > CD4+ T eff [2.0 μM] > CD8+ T eff [6.5 μM]) and acting at the level of AKT and NF-κB phosphorylation. Moreover, idelalisib treatment of Tregs altered their phenotype and reduced their suppressive function against CD4+ and CD8+ Teffs. Phenotyping Tregs from CLL patients treated with idelalisib supported our in vitro findings. Collectively, our data show that human Tregs are more dependent on PI3Kδ-mediated signaling compared with CD4+ and CD8+ Teffs. This Treg-preferential effect could explain why idelalisib produces adverse autoimmune effects by breaking Treg-mediated tolerance. However, balancing effects on Treg sensitivity versus CD8+ T eff insensitivity to idelalisib could still potentially be exploited to enhance inherent antitumor immune responses in patients. The Journal of Immunology, 2019, 202: 000–000.

The PI3K pathway plays a central role in the growth, metabolism, survival, and motility in multiple cell types (12). PI3K isoforms are classified into three classes (class I, class II, and class III) based on structure and lipid substrate preferences (13). The class I PI3Ks are heterodimer kinases that consist of a regulatory subunit, and the catalytic subunit and control various aspects of immune cell functions (13, 14). The catalytic subunit has four isoforms: PI3K p110α, PI3K p110β, PI3K p110γ, and PI3K p110δ (referred to as PI3Kα–δ) and are further subdivided into the class IA group (PI3Kα, PI3Kβ, and PI3Kδ), which associates with the p85 type of regulatory subunit, and the class IB group (PI3Kγ), which binds to p101 and p85 regulatory subunits (13). The PI3Kα and PI3Kβ are ubiquitously expressed, whereas PI3Kγ and PI3Kδ expression is mostly restricted to immune cells, and their expression levels vary based on cell type and activation status/microenvironment (15). The PI3Kδ isoform has been shown to be the dominant isoform that is critical for controlling Teff functions and is

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S.C. designed experiments, performed experiments, collected data, analyzed the data, and wrote the manuscript; K.K. designed experiments, performed experiments, collected data, analyzed the data, and edited the manuscript; K.T., E.M.A., and K.O., designed experiments, analyzed the data, and edited the manuscript; G.E.T. and L.A.M. recruited patients, organized patient samples, interpreted data, and edited the manuscript. All authors reviewed the manuscript and approved the final version.

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activated through the TCR, CD28, and several cytokine receptors including IL-2R (13, 16–20). The inactivation of PI3Kδ-mediated signaling in mice leads to reduced numbers of peripheral Tregs and impaired suppressive function (21–23). In contrast, hyperactive PI3K signaling in Tregs can lead to destabilization of FOXP3 or lineage plasticity in mice and humans (24–29). Altogether, these findings indicate that a minimal level of PI3K signaling is necessary for Treg function and that subtle difference in activation can potentially antagonize or promote Tregs development and suppressive function (30).

The PI3Kδ-specific small molecule inhibitor idelalisib (previously CAL-101, GS-1101) was the first PI3K inhibitor approved for mono- or combination therapy for different human B cell malignancies, such as relapsed/refractory chronic lymphocytic leukemia (CLL), follicular lymphoma, and small lymphocytic lymphoma, with an impressive clinical outcome (31). PI3Kδ-specific inhibitors block signaling from B cell prosurvival receptors that converge on the PI3K signaling pathway (31). However, the mechanism of action of PI3K inhibition appears to extend beyond the direct regulation of tumor cell proliferation, as the capacity of the inhibitor to induce cell death in PI3K hyperactivated tumor cells is limited (32). Yet, the suppressive efficacy of PI3K isoform inhibition can be explained by the extrinsic cues from tumor-infiltrating T lymphocytes (32). However, it is not clear whether this is due to enhanced T cell–mediated antitumor immune responses. Recent studies in mice indicated that targeting PI3Kδ through genetic inactivation or small molecule inhibitors in Tregs enhanced CD8+ T cell–mediated antitumor immune responses (27). However, evaluation of the use of PI3Kδ-specific inhibitors to treat CLL in a primary rather than relapsed/refractory setting demonstrated that patients developed frequent immune-mediated adverse effects of the therapy such as hepatotoxicity, enterocolitis, skin rash, and pneumonitis (33–35). The authors observed reduced Treg numbers in idelalisib-treated patients and suggested that the autoimmunity could be due to the effects of idelalisib on Tregs (33). The opportunities as well as dangers of the use of PI3Kδ-specific inhibitors to treat CLL in a primary rather than relapsed/refractory setting demonstrated that patients developed frequent immune-mediated adverse effects of the therapy such as hepatotoxicity, enterocolitis, skin rash, and pneumonitis (33–35).

**Materials and Methods**

**Cell purification**

Three matched pairs of blood samples were collected from CLL patients at baseline and at 90 d or more after starting idelalisib therapy (Table I) with approval from the Regional Committee for Medical and Health Research Ethics of South-Eastern Norway and upon informed consent. PBMCs were isolated by ficoll-paque (Axis-Shield PoC AS, Norway) buoyant density gradient centrifugation and cryopreserved in PBS with 10% DMSO until analyzed. Buffy coats from healthy donors were obtained from Oslo University Hospital Blood Centre (Oslo, Norway) with the Ethics Committee approval and donor consent. CD3+ T cells were enriched using RosetteSep Human T Cell Enrichment Cocktail (STEMCELL Technologies, Canada). Tregs (CD4+CD25+CD127+ T cells) were FACs purified (unless otherwise specified) from enriched CD3+CD25+ T cells (CD25 Microbeads II Kit; Miltenyi Biotec, Germany) using FACs Aria (BD Biosciences). Treg-depleted CD3+ T cells from the same donor were used as Tefs. The isolated cells were maintained in RPMI 1640 with Glutamax and nonessential amino acids and supplemented with 10% FCS, 1% penicillin-streptomycin (Life Technologies) at 37°C with 5% CO2.

**T cell proliferation and suppression assays**

For proliferation assays, ex vivo–isolated Tregs and Teffs were labeled with 5 μM/mL of CFSE (Sigma-Aldrich). Briefly, 1.0 × 10^6 cells were plated in 96-well round-bottom plates and anti-CD2/CD3/CD28–coated beads (T Cell Activation/Expansion Kit; Miltenyi Biotec) at a ratio of 1:2 (bead to cells), vehicle (DMSO), and indicated concentrations of idelalisib (catalog no. S2226; Selleckchem) were added to a final volume of 200 μL. Cells were stimulated for 4 d without IL-2 or for 7 d with IL-2 (10 ng/mL). For Treg suppression, Tregs preactivated for 0–24 h (unless otherwise specified) were incubated at a 1:2 ratio with CFSE-labeled Teff cells and stimulated for 4 d with anti-CD2/CD3/CD28–coated beads at a 1:5 ratio (beads to cells). Idelalisib was added either during preactivation of Tregs or Teff and/or to the cocultures. The inhibition of Treg proliferation was calculated as suppression (%) = ([% proliferating Teffs alone − % proliferating Teffs after treatment with Treg]/% proliferating Teffs alone) × 100 as previously described (36). All flow cytometry data were acquired using the BD LSRLightsett instrument (BD Biosciences) and analyzed using FlowJo version 10 (Tree Star) unless otherwise specified.

**Phosphoflow cytometry signaling analysis**

Ex vivo–isolated CD3+ T cells (1 × 10^6 cells per well in 96-well plates) were incubated with indicated concentrations of idelalisib for 30 min at 37°C and stimulated for defined time course with biotinylated anti-CD2 (5 μg/mL), anti-CD28 (5 μg/mL), anti-CD3 (1 μg/mL), and avidin (25 μg/mL). The cells were fixed with BD Phos Flow Fix Buffer I (BD Biosciences) and barcoded three-dimensionally with Pacific Blue, Pacific Orange, and DyLight 594 (Life Technologies, Canada) as described elsewhere (36, 37). The barcoded cells were pooled together and permabilized with human FOXP3 buffer followed by BD Phos Flow Perm Buffer III (BD Biosciences) and stained for respective surface and intracellular lineage markers and phosphorylation site-specific Abs. The flow cytometry data were acquired with a BD LSRFortessa (BD Biosciences). The level of phosphorylation was presented as the arcsinh ratio of medians as previously described (38) and was calculated by normalizing to respective unstimulated cell control using Cytobank (https://cytobank.org).

**Cytokine quantification**

The culture supernatants were stored at –80°C until analyzed. IL-10, IL-2, TNF-α, and INF-γ were quantified using Magnetic Luminex Performance Assay multiplex kit (R&D Systems) and Bio-Plex Manager software version 6.1 according to manufacturer’s instructions (Bio-Rad).

**Flow cytometry reagents**

Abs used for phenotyping, anti-CD3 PerCP-Cy5.5 (UCHT1), anti-CD4 allophycocyanin-H7 (RPA-4), anti-CD8 PE-Cy7 (RPA-T8), anti-CD25 BV421 (M-A251), anti-CD25 PE (M-A251), anti-CD127 Alexa Fluor 647 (HIL-7R-M21), anti-Foxp3 Axl488 (259Cd/PE0, anti-ICOS PE (DX29), anti-CD38 allophycocyanin (HIT2), anti-Ki-67 BV421 (Ki-67), and human FOXP3 buffer were from BD Biosciences. Anti-LAP PE (TW4-6H10), anti–programmed cell death-1 (PD-1)–PE-Cy7 (EH12.2H7), anti-CD39 PE-Cy7 (A1), and anti-CTLA-4 allophycocyanin (L3D10) were from BioLegend. Phosphorylation site-specific Abs anti-CD3 (pY412, K25-407.69) Alexa Fluor 647, anti-SLP76 (pY128) (J141-668.36.58) Alexa Fluor 647, anti–NF-KB p50 (p5259) (K10-985.12.50) Alexa Fluor 647, and IgG1 isotype control (MOPC-21) Alexa Fluor 647 were purchased from BD Biosciences. Anti-AKT (pS473) (D9E) Alexa Fluor 647, anti-AKT (pT308) (C31E5E) Alexa Fluor 647, anti–NF-KB p536 (93H1) Alexa Fluor 647, anti-Erk1/2 (pT202/204) (E10) Alexa Fluor 647, and anti-S6 ribosomal protein (pS235/236) (D57.2.2E) Alexa Fluor 647 were purchased from Cell Signaling Technologies. CFSE was from Sigma-Aldrich. Avidin was from Life Technologies. Anti-CD28 (Biotin) (CD28.2) and anti-CD2 (Biotin) (RPA-2.10) were from eBiosciences. Anti-CD3 (Biotin) (OKT3) was from (Diatec AS, Norway).

**Statistics**

The p values were calculated using a two-tailed Student t test or one-way ANOVA with Bonferroni correction. Error bar represents mean ± SEM. p ≤ 0.05 was considered statistically significant. The statistical values were generated using GraphPad Prism version 7.

**Results**

Inhibition of PI3Kδ preferentially inhibits the TCR-induced proliferation of human Tregs compared with other T eff subsets

PI3K is an essential component of the TCR/CD28 signaling cascade that is responsible for T cell proliferation and survival (16–20). Therefore, we sought to evaluate the effects of inhibiting this pathway using the clinically approved PI3Kδ isoform–specific
inhibitor idelalisib on the proliferation of purified Tregs and Teffs. The Treg purification strategy and FOXP3 expression levels are shown in Supplemental Fig. 1A–C. The purified cells were labeled with CFSE and stimulated through their TCR and costimulation receptors CD2 and CD28 using anti-CD3-α, anti-CD2-β, and anti-CD28-coated beads for 96 h in culture medium with different concentrations of idelalisib, whereas inhibitor-untreated and DMSO (vehicle)-treated conditions served as assay controls. Based on CFSE dilution as an indicator of proliferation, we found that Tregs were highly susceptible to inhibition of proliferation at different concentrations of idelalisib compared with both the CD4+ Teff and CD8+ Teff subsets (Fig. 1A, 1B). The inhibitor-treated conditions were normalized to the untreated condition, and concentration-response curves were generated to compare the differential effects of idelalisib in different T cell subsets. As shown in Fig. 1C, the in vitro IC50 (concentration of idelalisib at which 50% of the proliferation was inhibited) values clearly indicated that Treg proliferation can be inhibited with a lower concentration of idelalisib (IC50 = 0.5 μM) compared with CD4+ Teffs (IC50 = 2.0 μM) and CD8+ Teffs (IC50 = 6.5 μM). In fact, the sensitivity of Treg and CD8+ Teff differed by one order of magnitude. To exclude the possibility of toxicity of idelalisib as a cause of the differential effects between the T cell subsets, we evaluated the viability of the cells after 96 h of idelalisib treatment. Dead cells were discriminated from viable cells using amine-reactive membrane-impermeable dye followed by flow cytometry analysis. Our results showed that the Treg viability was stable at the different concentrations of idelalisib tested, whereas the viability of the Teff populations was decreased with increasing concentrations of idelalisib (Fig. 1D). To exclude the possibility that the Treg susceptibility to inhibition is because of their low proliferative capacity, we added IL-2 to the cultures and stimulated the cells for 7 d. Addition of IL-2 increased the proliferation capacity of Tregs. However, although the Tregs remained sensitive to inhibition by idelalisib (IC50 = 2.1 μM), CD4+ Teffs and CD8+ Teffs became virtually insensitive to idelalisib (IC50 of 45.2 and 100.5 μM, respectively), maintaining the difference between Tregs and Teffs (Supplemental Fig. 2A, 2B). In addition, analysis of the in vivo proliferation of Tregs, CD4+ T cells, and CD8+ T cells from CLL patients at baseline and during idelalisib treatment by Ki-67 expression showed that the fraction of Ki-67+ Tregs tended to be reduced after initiation of idelalisib treatment (5.24% versus 2.4% at baseline and during idelalisib treatment), whereas smaller or no effect was observed for Teffs (Fig. 1E, 1F). Furthermore, the change in proliferation in vivo upon initiation of idelalisib treatment (8-Ki-67 cells) suggests that Tregs are significantly more sensitive than CD4+Teffs and CD8+Teffs (p < 0.05, Fig. 1G). This further supported our in vitro observations. Together, our results demonstrated that proliferation of Tregs is dependent on TCR signaling through PI3Kδ and thus susceptible to inhibition of PI3Kδ, whereas CD4+ Teffs and CD8+ Teffs can bypass this inhibition.

PI3Kδ blockade selectively inhibits the TCR signaling pathway in T cell subsets

PI3Kδ is the main signal transducer of PI3K signaling downstream of TCR in human T cells (13, 16). We evaluated the effect of PI3Kδ inhibition on phosphorylation of known proximal and distal signaling proteins associated with TCR signaling pathways using our well-established phosphoflow cytometry method (36, 37). Briefly, CD3+ T cells were stimulated using soluble biotinylated anti-CD3, anti-CD2, and anti-CD28 Abs in the absence or presence of various concentrations of idelalisib for indicated time periods. As the CD127 epitope is damaged by the fixation process for staining intracellular phospho-epitopes, Tregs were gated as CD4+CD25+FOXP3+ T cells, whereas Teffs were gated based on CD4 and CD8 expression. Our results showed that phosphorylation of protein kinase B, also known as AKT (a serine/threonine-specific protein kinase), the immediate downstream target of PI3Kδ, on amino acid residues T308 and S473 was inhibited in a concentration-dependent manner (Fig. 2A, 2B). AKT phosphorylation can also enhance the activation of NF-κB through its association with the caspase recruitment domain-containing membrane-associated guanylate kinase protein 1 (CARM1), which facilitates the formation of the CARD11-BACL10-MALT1 (CBM) complex, a step critical for NF-κB activation (39). Idelalisib-mediated reduction of AKT phosphorylation affected NF-κB activation in Tregs and to a lesser extent in Teff subsets (Fig. 2A, 2B). By contrast, downstream effects of PI3K signaling such as S6 ribosomal protein (rpS6) phosphorylation was not affected by PI3Kδ inhibition in human T cell subsets as reported earlier (16), suggesting a possible redundancy in signaling events that lead to rpS6 activation (40) (Supplemental Fig. 3). Furthermore, PI3K-independent signaling events such as phosphorylation of ERK (Erk) and of TCR-proximal proteins such as CD3ζ-chain and SH2 domain–containing leukocyte protein of 76 kDa (SLP76) were unaffected by idelalisib in both Treg and Teff subsets (Supplemental Fig. 3). These findings confirm that PI3Kδ is indeed the main PI3K signal transducer in human Tregs as previously shown for human T eff subsets (16). In addition, as for inhibition of proliferation in Fig. 1A and 1B, the sensitivity to inhibition of Treg signaling by idelalisib was effective at lower concentrations (0.15 μM) compared with that of the CD4+ Teff and CD8+ Teff subsets, which remained incompletely inhibited at 1.0 μM. Overall, these results substantiate previous findings from mice that PI3Kδ signaling is essential for Tregs, which may explain why Teff subsets are less sensitive to PI3Kδ inhibition (22, 41–43).

Blockade of PI3Kδ signaling alters Treg phenotype

Studies in mice suggest that PI3K/AKT signaling is essential to maintain a Treg identity (21–23, 44) and thereby suppresses anticancer immune responses (22, 45). In addition, both murine and human Tregs have been shown to require continuous TCR and CD28 costimulation receptor–mediated signaling to maintain their phenotype in the periphery (36, 46–48). Therefore, we analyzed the expression of essential Treg-related markers after 48 h of stimulation through TCR, CD2, and CD28 costimulation receptors in the presence of 0.5 μM idelalisib (Fig. 3A, 3B). The expression levels of FOXP3, CD25, ICOS, and PD-1 were significantly downregulated in idelalisib-treated Tregs (p < 0.05), and a tendency to decreased levels was observed also for CTLA-4, CD39, and latency-associated peptide (LAP) (Fig. 3A, 3B). Furthermore, we investigated the effects of idelalisib on Treg cells in CLL patients (Table I) (n = 1) compared with the baseline from the same patients. In line with the in vitro data from healthy donors, decrease in the expression levels of Treg-specific markers such as CD25, ICOS, CTLA4, PD-1, and CD39 were observed during idelalisib treatment (Fig. 3C, 3D). These changes were not statistically significant with the three patients we could examine prospectively inside the remit of this study, but the observed trend in two of the patients suggests that a prospective study with a larger cohort may be warranted. Together, these results suggest that blockade of PI3Kδ signaling in human Tregs affects the expression of Treg signature markers associated with their phenotype and suppressive activity.

PI3Kδ inhibition primarily affects the differentiation and suppressive function of human Tregs

First, we evaluated the suppressive capacity of Tregs that were pretreated with idelalisib during their activation and differentiation...
FIGURE 1. PI3Kδ inhibition strongly affects the TCR-induced proliferation of human Tregs compared with other Teff subsets. Representative CFSE dilution flow cytometric histograms (A) and compiled data (B) show the proliferative capacity of purified CD4+CD25+CD127− Tregs and Treg-depleted CD3+CD25− Teffs further gated on CD4+ Teff and CD8+ Teff after 96 h in absence or presence of indicated concentrations of idelalisib. (C) Concentration-response curves showing IC50 for inhibition of proliferation of Tregs, CD4+ Teffs, and CD8+ Teff subsets (data points were normalized to 100%). (D) Compiled bar graphs show the percentage of dead cells measured using fixable viability dye in the 96-h proliferating cultures of Tregs and total CD3+ Teff cells. (E) A representative flow cytometry dot plot and (F) compiled frequencies of Ki-67 expression in Tregs, CD4+ Teff, and CD8+ Teff cells at baseline and during idelalisib therapy in CLL patients. (G) Changes in the (Figure legend continues)
process using anti-CD3–, anti-CD2–, and anti-CD28–coated beads for 24 h. Tregs pretreated with idelalisib were further cocultured with CFSE-labeled CD3+CD25+ T cells for 96 h (Fig. 4A). The suppressive capacity of Tregs to inhibit CD3+ T cell proliferation (further gated on CD4+ Teffs and CD8+ Teffs) was reduced significantly with all concentrations of idelalisib tested (Fig. 4A, 4B). In addition, the Treg-associated suppressive cytokine IL-10 was found to be significantly reduced in the cocultures (Fig. 4C). We also validated the reduced Treg-suppressive capacity by quantifying the secreted cytokines in the coculture supernatants after 24 h. The results confirmed a significant increase in secretion of IL-2 and TNF-α but not IFN-γ from CD3+ T cells (p < 0.05), corroborating the reduced suppressive capacity of Tregs (Fig. 4D). Next, in a series of experiments, we assessed the effects of idelalisib on preactivated and differentiated Tregs and Teffs. First, we tested the Tregs that were not exposed to idelalisib during their fraction of T cells that proliferate in vivo on and off idelalisib (δ-Ki67+ cells) in Tregs, CD4+, and CD8+ Teff subsets. Statistics; error bar represents mean ± SEM, horizontal bar represents the median, each dot represents one patient, *p ≤ 0.05, ****p ≤ 0.0001. Healthy donors (n = 3) and CLL patients (n = 3).

ns, not significant.
preactivation process but were treated along with CD3+ T cells directly in the coculture for 96 h. This significantly increased the suppressive capacity of Tregs against CD4+ Teffs and CD8+ Teffs (Supplemental Fig. 4A) and came in addition to the inhibitory effect of idelalisib on Teffs (Supplemental Fig. 4C). Secondly, this effect was mitigated when Tregs were inhibited with idelalisib (Supplemental Fig. 4A) and came in addition to the inhibitory effect of idelalisib on Teffs (Supplemental Fig. 4C). Secondly, this effect was mitigated when Tregs were inhibited with idelalisib

Table I. The clinical characteristics of included patients with CLL

<table>
<thead>
<tr>
<th>UPN</th>
<th>Gender</th>
<th>Age (y)</th>
<th>Time from Diagnosis to Procurement (mo)</th>
<th>Clinical Stage</th>
<th>Treatment prior to Procurement</th>
<th>IGHV-Gene Usage</th>
<th>Homology with Germline (%)</th>
<th>Chromosomal Aberrations</th>
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</thead>
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<tr>
<td>CLL103</td>
<td>M</td>
<td>61</td>
<td>166</td>
<td>Binet stage C</td>
<td>FCR in 2012</td>
<td>HV3-48</td>
<td>99.4</td>
<td>Del (13q14)</td>
</tr>
<tr>
<td>CLL198</td>
<td>M</td>
<td>69</td>
<td>123</td>
<td>Binet stage C</td>
<td>FCR in 2008</td>
<td>HV5-A3</td>
<td>100</td>
<td>No del (17p13) or TP53 mutation del (11q22)</td>
</tr>
<tr>
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<td>6</td>
<td>Binet stage C</td>
<td>None</td>
<td>HV3-9</td>
<td>98.8</td>
<td>Del (13q14), del (17p13), and TP53 mutation exon 7 c.734G &gt; A</td>
</tr>
</tbody>
</table>

Del, deletion; FCR, fludarabine, cyclophosphamide, rituximab; M, male; UPN, unique patient number.

FIGURE 3. Blockade of PI3Kδ signaling alters the phenotypic characteristics of Tregs. (A) Representative flow cytometry histogram overlays and (B) compiled bar graphs with mean fluorescence intensity (MFI) show the expression level of Treg cell markers in nonstimulated and 48-h stimulated Tregs from healthy donors prior to coculture with Teffs either in absence or presence of an indicated concentration of idelalisib. (C) Representative flow cytometry histogram overlays and (D) compiled bar graphs with MFI show the expression level of indicated markers from circulating Tregs in CLL patients prior to therapy and during idelalisib therapy. Statistics; error bars represent mean ± SEM. *p ≤ 0.05. Healthy donors (n = 3) and CLL patients (n = 3). ns, not significant.
both during their preactivation process as well as in the cocultures (Supplemental Fig. 4B). In the third set of experiments, the CD3+ T cells were preactivated 48 h prior to coculture with 24-h pre-activated Tregs. Interestingly, the activated CD4+ Teffs and CD8+ Teffs completely resisted the activated Treg-mediated suppression irrespective of the increased concentrations of idelalisib in the coculture (Supplemental Fig. 4D). Taken together, these results suggest that PI3Kδ signaling, activated through both the TCR and the costimulation receptors CD2 and CD28, plays an important role in the activation and differentiation of human Tregs but not for the acute suppression mediated by already activated and differentiated Tregs.

**Discussion**

Several experimental and clinical studies have shown that T cells play a pivotal role in shaping antitumor immunity (49). Especially the presence of CD8+ CTLs is strongly associated with favorable outcome in several human malignancies (3). However, tumor-infiltrating Tregs can be detrimental and promote tumor progression by suppressing the tumor-specific CTL functions (6, 8), and tumor-infiltrating Tregs are associated with poor clinical outcome in some cancers (4, 5). Depleting Tregs in mouse models have been shown to be beneficial by enhancing the clearing of tumor cells by an improved antitumor immune response (50). However, therapeutic strategies to selectively modulate Treg number and functions in cancer have been challenging, mainly because of limitations in clinically useful reagents. In addition, the great overlap of human Treg and Teff phenotypes makes it difficult to selectively target Tregs based on conventional surface markers such as Abs against CD25 (9–11, 50). Currently, clinical inhibitors that could efficiently and selectively target Tregs without hampering Teff functions are necessary for tumor immunomodulation.
PI3K isoforms play an enigmatic role in regulating the immune functions of Tregs and Teff subsets (13, 14), and the fundamental difference in PI3K isoform signaling in Tregs and Teffs offers an attractive opportunity that could potentially be exploited in selectively targeting the Tregs in cancer (16–22, 24–30).

The emerging evidence from clinical trials using idealisib to treat CLL patients has suggested that the observed enhanced T cell–mediated immunity may be due to reduced Treg numbers in idealisib-treated patients. This could be one of the primary reasons for the impressive clinical response to PI3Kδ inhibition in CLL (33, 34). It should be noted that idealisib treatment of patients is also associated with adverse autoimmune effects that are clinically managed with steroids, which indicate a breach of immune tolerance (35). Against this backdrop, we evaluated the PI3Kδ isoform–specific regulation of Treg and Teff functions in humans in more detail.

PI3K signaling downstream of TCR and CD28 stimulation plays an essential role in T cell activation, clonal expansion, cytokine secretion, motility, and survival (13, 51). In murine studies, Treg-specific inactivation of PI3Kδ reduced their suppressive function (21) and secretion of the cytokine IL-10 that further enhanced CTL-mediated tumor elimination (22). This suggested that the PI3Kδ isoform regulates the suppressive activity of Tregs without compromising the antitumor function of CTLs. These findings are consistent with our results showing that idealisib treatment of ex vivo–isolated cells from healthy blood donors preferentially blocked Treg proliferation at a lower dose compared with CD4+ Teffs and CD8+ Teffs. This is also in line with a report by Abu-Eid et al. (52) in which purified Tregs were inhibited by a different PI3Kδ inhibitor (IC-87114) compared with CD4+ Teffs, although contrary to that study, we confirm that PI3Kδ activation is required for AKT phosphorylation in all T cell subsets but that Teffs require higher drug concentrations for complete inhibition. Tregs have been shown to be less responsive to TCR stimulation compared with Teff subsets, and the presence of exogenous IL-2 does not completely rescue this phenotype in vitro (53). Moreover, studies in mice suggest that IL-2–dependent proliferation of Treg and Teff is PI3Kδ independent (17, 21). Therefore, it is possible that the preferential inhibition of Treg proliferation by idealisib might be associated with their hyporesponsiveness to TCR stimulation as compared with CD4+ and CD8+ Teffs. However, Tregs critically depend on continuous TCR stimulation for their proliferation and suppressive function in vivo (46, 47). Therefore, we further examined the expression of the cell proliferation marker Ki-67 in circulating Tregs, CD4+ Teffs, and CD8+ Teffs from CLL patients on idealisib therapy and found that idealisib treatment of patients reduced the expression of Ki-67 more in Tregs than Teffs, mirroring our in vitro results.

Our phosphoflow cytometry–based signaling analysis revealed that idealisib inhibited PI3Kδ–AKT signaling in Tregs, CD4+ Teffs, and CD8+ Teffs in a concentration-dependent manner. However, the downstream inhibitory effect on proliferation inhibition was more pronounced in Tregs compared with CD8+ Teffs. This confirms the notion that human Tregs are more dependent on PI3Kδ signaling activated through TCR and costimulation receptors such as CD2 and CD28 compared with the Teff subsets. Although PI3Kδ signaling has been shown to regulate the cytotoxic function of CD8+ Teffs (16, 42, 54, 55), it appears that once the CD8+ Teffs are differentiated into a memory cell type, they are less dependent on PI3Kδ signaling (41–43, 54). These cell-intrinsic differences in dependency on PI3Kδ signaling may be the molecular basis for the difference we observed between Treg and CD8+ Teff sensitivity to idealisib treatment.

We found that inhibition of PI3Kδ signaling blocked the TCR and costimulation receptor–induced expression of important Treg stability and activation markers including FOXP3, CD25, CTLA-4, ICOS, PD-1, and CD39. Furthermore, in a series of in vitro suppression assays, we found that idealisib mainly affects the Treg activation and differentiation process. The cytokine profiles from Treg and Teff cocultures confirmed that idealisib–treated Tregs during their activation process subsequently impaired the capacity to secrete IL-10 and further suppression of the secretion of IL-2 and TNF-α from Teff subsets. Interestingly, idealisib did not affect the suppressive capacity of Tregs that are already being activated and differentiated. However, in a similar experimental condition where the Teffs were activated and differentiated longer than the Tregs prior to coculture, the Teffs strongly resisted the Treg-mediated suppression. Together, we conclude that PI3Kδ signaling is required for human Treg activation and differentiation but not for acute suppression mediated by already activated Treg cells.

Data mainly from studies in mice suggest that in vivo decrease in Treg numbers and proliferation combined with enhanced CTL infiltration in cancer implants can be achieved using PI3Kδ inhibitors but not with PI3Kα- or PI3Kβ–specific inhibitors (21, 22, 52, 56). However, there is no clinical report yet that substantiates these findings in human malignancies. Interestingly, human clinical trials testing idealisib in different B cell malignancies such as CLL, follicular lymphoma, and small lymphocytic lymphoma show remarkable results (31). Particularly, the first evidence of reduced circulating Treg numbers was reported in CLL patients treated with idealisib (33). There is a possibility that the enhanced tumor clearance in these patients could be due to the generation of improved antitumor immune responses. Our results addressing the inhibitory mechanisms of idealisib are consistent with these clinical findings.

In conclusion, the differential role of PI3Kδ signaling in Tregs and Teff subsets may be used to preferentially downregulate the suppressive function of Tregs in human malignancies while leaving CTL functions intact. Further human preclinical and clinical studies are required to validate our findings in vivo.

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