The PI3K p110delta isoform inhibitor idelalisib selectively inhibits human regulatory T cell function

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Running title: PI3Kδ signaling blockade in human Tregs

Abstract

In chronic lymphocytic leukemia (CLL) signaling through several pro-survival B-cell surface receptors activate the phosphatidyl-inositol-3-kinase (PI3K) signaling pathway. Idelalisib is a highly selective PI3K (PI3Kδ) isoform-specific inhibitor effective in relapsed/refractory CLL and follicular lymphoma (FL). 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, T-cell receptor signaling, phenotype, and suppressive function. Our results show that human Tregs are highly susceptible to PI3Kδ inactivation using idelalisib compared to $CD4^+$ and $CD8^+$ effector T cells (Teffs) as evident from effects on anti-CD3/CD28/CD2-induced proliferation (order of susceptibility (IC_{50}): Treg (0.5 μM) > $CD4^+$ Teff (2.0 μM) > $CD8^+$ Teff (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 to $CD4^+$ and $CD8^+$ Teffs. This Treg-selectivity could explain why idelalisib produces adverse autoimmune effects by breaking Treg-mediated tolerance. However, balancing effects on Treg sensitivity versus $CD8^+$ Teff insensitivity to idelalisib could still potentially be exploited to enhance inherent anti-tumor immune responses in patients.

Introduction

$CD4^+$ $FOXP3^+$ regulatory T cells (Tregs) are pivotal for the maintenance of self-tolerance and immune homeostasis (1, 2). Tregs suppress effector T cells (Teffs) and many other immune cell types and prevent autoimmune diseases, allergy and promote tolerance during pregnancy and after transplantations (1). On the other hand, the inappropriate number and function of Tregs in tumor microenvironments impairs anti-tumor immunity provided by tumor-infiltrating T lymphocytes (TILs) in many cancer types (3-6). Therefore, reducing the Treg numbers or their function may be beneficial to enhance the efficacy of anti-tumor therapies including immunotherapies (7). Despite the evidence of the immunosuppressive function of Tregs in the cancer microenvironment, the progress in targeting of Tregs in cancer has been slow (8, 9). The current lack of clinically useful reagents targeting Tregs and the overlapping intracellular signaling pathways from the T cell receptor (TCR) of human Tregs and Teffs pose a major hurdle in the selective modulation of Treg function (7, 9-11).

The phosphatidylinositol 3-kinase (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 and a 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 sub-divided 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 p87 regulatory subunits (13). The PI3Kα and PI3Kβ are ubiquitously expressed whereas, PI3Kγ and PI3Kδ expression are 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 activated through the TCR, CD28 and several cytokine receptors including interleukin (IL)-2 receptor (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 (FL), and small lymphocytic lymphoma (SLL) with an impressive clinical outcome (31). PI3Kδ-specific inhibitors block signaling from B cell pro-survival 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 hyper activated tumor cells is limited (32). Yet, the impressive efficacy of PI3K isoform inhibition can be explained by the extrinsic cues from TILs (32). However, it is not clear whether this is due to enhanced T-cell-mediated anti-tumor immune responses. Recent studies in mice indicated that targeting PI3Kδ through genetic inactivation or small molecule inhibitors in Tregs enhanced $CD8^+$ T cell mediated anti-tumor immune responses (22). 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 breaking tolerance by inhibiting human Tregs warrants further in-depth investigation and prompted us to evaluate the PI3Kδ isoform-specific regulation of Tregs and Teffs functions in humans in more detail.

In this study, using *ex vivo* isolated Tregs and Teffs from healthy donors and CLL patients, we demonstrate the selective effect of idelalisib on Treg proliferation, signaling, and suppressive function, and show that Tregs are significantly more sensitive to idelalisib than $CD8^+$ T cells.

Methods

Cell purification

Three matched pairs of blood samples were collected from CLL patients at baseline and at 90 days or more after starting idelalisib therapy (Table 1) with approval from the Regional Committee for Medical and Health Research Ethics of South-Eastern Norway and upon informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-paque (Axis-Shield Poc AS, Norway) buoyant density gradient centrifugation and cryopreserved in fetal bovine serum (FBS) with 10% dimethylsulfoxide (DMSO) until analyzed. Buffy coats from healthy human 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 (STEM CELL™ 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 Biotech, Germany) using FACS Aria (BD Biosciences, USA). Treg-depleted $CD3^+$ T cells from the same donor were used as Teffs. The isolated cells were maintained in RPMI 1640 with GlutaMax, non-essential amino acids and supplemented with 10% FCS, 1% penicillin-streptomycin (Gibco™ USA) at 37°C with 5% CO_2.

T cell proliferation and suppression assays

For proliferation assays, *ex vivo* isolated Tregs and Teffs were labeled with 5 µM/ml of carboxyfluorescein succinimidyl ester (CFSE) (Sigma Aldrich, USA). Briefly, 1.0 x 10^5 cells were plated in 96-well round-bottom plates, and α-CD2/CD3/CD28 coated beads (T cell activation/Expansion Kit, Miltenyi Biotech, Germany) at a ratio of 1:2 (bead to cells), vehicle (DMSO) and indicated concentrations of idelalisib (Selleckchem, CAT. No. S2226) were added to a final volume of 200 µl. Cells were stimulated for 4 days without IL-2 or for 7 days

with IL-2 (10 ng/ml). For Treg suppression, Tregs pre-activated for 0 – 24 h (unless otherwise specified) were incubated at a 1:2 ratio with CFSE-labeled Teff cells and stimulated for 4 days with α-CD2/CD3/CD28 coated beads at a 1:5 ratio (beads to cells). Idelalisib was added either during pre-activation of Tregs or Teff and/or to the co-cultures. The inhibition of Teff 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 LSR Fortessa™ instrument (BD Biosciences, USA) and analyzed using FlowJo™ version 10 (TreeStar Inc. USA) unless otherwise specified.

Phosphoflow cytometry signaling analysis

Ex vivo isolated $CD3^+$ T cells (1×10^6 cells/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 α-CD2 (5 µg/ml), α-CD28 (5 µg/ml), α-CD3 (1 µg/ml) and Avidin (25 µg/ml). The cells were fixed with BD Phosflow Fix Buffer I (BD biosciences, USA) and barcoded three-dimensionally with pacific blue, pacific orange and dylight 594 (Life Technologies, Canada) as described earlier (36, 37). The barcoded cells were pooled together and permeabilized with Human FOXP3 buffer followed by BD Phosflow Perm Buffer III (BD biosciences, USA) and stained for respective surface and intracellular lineage markers and phosphorylation site-specific antibodies. The flow cytometry data was acquired with a BD LSR Fortessa™ (BD Biosciences, USA). 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 INFγ were quantified using Magnetic Luminex® Performance Assay multiplex kit (R&D systems, USA) and Bio-plex manager software version 6.1 according to manufacturer's instructions (Bio-Rad, USA).

Flow cytometry reagents

Antibodies used for phenotyping, α-CD3 PerCP-Cy5.5 (UCHT1), α-CD4 APCH7 (RPA-T4), α-CD8 PE-Cy7 (RPA-T8), α-CD25 BV421 (M-A251), α-CD25 PE (M-A251),α-CD127 Alexa Fluor 647 (HIL-7R-M21), α-Foxp3 Ax488 (259D/C7), α-ICOS PE (DX29), α-CD38 APC (HIT2), α-Ki-67 BV421 (Ki-67), and Human FOXP3 buffer were from BD Biosciences, USA. α-LAP PE (TW4-6H10), α-PD-1 PE-Cy7 (EH12.2H7), α-CD39 PE-Cy7 (A1), α-CTLA-4 APC (L3D10), were from BioLegend, USA. Phosphorylation site-specific antibodies α-CD3ζ (pY142) (K25-407.69) Alexa Fluor 647, α-SLP76 (pY128) (J141-668.36.58) Alexa Fluor 647, α-NFκB (pS529) (K10-895.12.50) Alexa Fluor 647, and IgG1κ isotype control (MOPC-21) Alexa Fluor 647 were purchased from BD Biosciences, USA. α-AKT (pS473) (D9E) Alexa Fluor 647, α-AKT (pT308) (C31E5E) Alexa Fluor 647, α-NF-κB (pS536) (93H1) Alexa Fluor 647, α-Erk1/2 (pT202/Y204) (E10) Alexa Fluor 647, and α-S6 Ribosomal Protein (pS235/236) (D57.2.2E) Alexa Fluor 647 were purchased from Cell Signaling Technologies, USA. CFSE was from Sigma Aldrich. Avidin was from Life Technologies, Canada. α-CD28 (Biotin) (CD28.2) and α-CD2 (Biotin) (RPA-2.10) was from eBiosciences, USA. α-CD3 (Biotin) (OKT3) was from Diatec AS, Norway.

Statistics

P values were calculated using a 2 tailed, student's *t*-test or 1-way ANOVA with Bonferroni correction. Error bar represents mean ± SEM. $P \leq 0.05$ 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 to other Teff 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 **Supplementary figure 1 (A, B and C).** The purified cells were labeled with CFSE and stimulated through their TCR and co-stimulation receptors CD2 and CD28 using anti-CD3, anti-CD2 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 to both the $CD4^+$ Teff and $CD8^+$ Teff subsets (**Figure 1A and B**). 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 **Figure 1C**, the *in vitro* IC_{50} (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 (IC_{50} =0.5 µM) compared to $CD4^+$ Teffs (IC_{50} =2.0 µM) and $CD8^+$ Teffs (IC_{50} =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 (**Figure 1D**). To exclude the possibility that the Treg susceptibility to inhibition is due to their low proliferative capacity, we added IL-2 to the cultures and stimulated the cells for 7 days. Addition of IL-2 increased the proliferation capacity of Tregs. However, while the Tregs remained sensitive to inhibition by idelalisib (IC_{50} =2.1 µM), $CD4^+$ Teffs and $CD8^+$ Teffs became virtually insensitive to idelalisib (IC_{50} of 45.2 µM and 100.5 µM, respectively) maintaining the difference between Tregs and Teffs (**Supplementary figure 2A** and **B**). 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% vs 2.4% at baseline and during idelalisib treatment) whereas smaller or no effect was observed for Teffs (**Supplementary figure 3 A and B**). Furthermore, the change in proliferation *in vivo* upon initiation of idelalisib treatment (delta-Ki-67^+ cells) suggest that Tregs are significantly more sensitive than $CD4^+$Teffs and $CD8^+$Teffs ($p<0.05$, **Supplementary figure 3C**). 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 antibodies 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 T303 and S473 was inhibited in a concentration-dependent manner (**Figure 2, A and B**). AKT phosphorylation can also enhance the activation of nuclear factor-κB (NF-κB) through its association with the caspase recruitment domain-containing membrane-associated guanylate kinase protein 1 (CARMA1) which facilitates the formation of the CARD11-BCL10-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 (**Figure 2, A and B**). 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) (**Supplementary figure 4**). Furthermore, PI3K independent signaling events such as phosphorylation of extracellular signal-regulated kinase (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 (**Supplementary figure 4**). These findings confirm that PI3Kδ is indeed the main PI3K signal transducer in human Tregs as previously shown for human Teff subsets (16). In addition, as for inhibition of proliferation in **Figure 1 A and B**, the sensitivity to inhibition of Treg signaling by idelalisib was effective at lower concentrations (0.15 µM) compared to 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 anti-cancer immune responses (22, 45). In addition, both murine and human Tregs have been shown to require continuous TCR and CD28 co-stimulation 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 co-stimulation receptors in the presence of 0.5 µM idelalisib (**Figure 3 A and B**). The expression levels of FOXP3, CD25, Inhibitor of co-stimulation (ICOS), and programmed cell death-1 (PD-1) were significantly down-regulated in idelalisib-treated Tregs ($p<0.05$), and a tendency to decreased levels was observed also for cytotoxic T lymphocyte antigen-4 (CTLA-4), CD39, and latency-associated peptide (LAP) (**Figure 3 A and B**). Furthermore, we investigated the effects of idelalisib on Treg cells in CLL patients (n=3) compared to 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 (**Figure 3C and D**). These changes were not statistically significant with the three patients we could examine prospectively inside the permit 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 pre-treated with idelalisib during their activation and differentiation process using anti-CD3, anti-CD2 and anti-CD28 coated beads for 24 h. Tregs pre-treated with idelalisib were further co-cultured with CFSE-labelled $CD3^+CD25^-$ T cells for 96 h (**Figure 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 (**Figure 4 A and B**). In addition, the Treg-associated suppressive cytokine IL-10 was found to be significantly reduced in the co-cultures (**Figure 4C**). We also validated the reduced Treg suppressive capacity by quantifying the secreted cytokines in the co-culture supernatants after 24 h. The results confirmed a significant increase in secretion of IL-2 and tumor necrosis factor alpha (TNF-α) but not interferon gamma (INFγ) from $CD3^+$ T cells ($p<0.05$) corroborating the reduced suppressive capacity of Tregs (**Figure 4 D**). Next, in a series of experiments, we assessed the effects of idelalisib on pre-activated and differentiated Tregs and Teffs. First, we tested the Tregs that were not exposed to idelalisib during their pre-activation process but were treated along with $CD3^+$ T cells directly in the co-culture for 96 h. This significantly increased the suppressive capacity of Tregs against $CD4^+$ Teffs and $CD8^+$ Teffs (**Supplementary figure 5 A**) and came in addition to the inhibitory effect of idelalisib on Teffs (**Supplementary figure 5 C).** Secondly, this effect was mitigated when Tregs were inhibited with idelalisib both during their pre-activation process as well as in the co-cultures (**Supplementary figure 5 B**). In the third set of experiments, the $CD3^+$ T cells were pre-activated 48 h prior to co-culture 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 co-culture (**Supplementary figure 5 D**). Taken together these results

suggest that PI3Kδ signaling activated through TCR and CD2, CD28 co-stimulation play 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 anti-tumor immunity (49). Especially, the presence of $CD8^+$ cytotoxic T-lymphocytes (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 anti-tumor immune response (50). However, therapeutic strategies to selectively modulate Treg number and functions in cancer have been challenging, mainly due to 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 antibodies 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 idelalisib to treat CLL patients have suggested that the observed enhanced T-cell mediated immunity may be due to reduced Treg

numbers in idelalisib-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 idelalisib 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 cytokine IL-10 that further enhanced CTLs mediated tumor elimination (22). This suggested that the PI3Kδ isoform regulates the suppressive activity of Tregs without compromising the anti-tumor function of CTLs. These findings are consistent with our results showing that idelalisib treatment of *ex vivo* isolated cells from healthy blood donors preferentially blocked Treg proliferation at a lower dose compared to $CD4^+$ Teffs and $CD8^+$ Teffs. This is also in line with a report by Abu-Eid et al, where purified Tregs were inhibited by a different PI3Kδ inhibitor (IC-87114) compared to $CD4^+$ Teffs (52), 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. Examining the expression of Ki-67 in circulating Tregs, $CD4^+$ Teffs and $CD8^+$ Teffs from CLL patients on idelalisib therapy confirmed our findings in healthy donor T cells.

Our phosphoflow cytometry based signaling analysis revealed that idelalisib 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 to $CD8^+$ Teffs. This confirms the notion that human Tregs are more dependent on PI3Kδ signaling activated through TCR and co-stimulation receptors such as CD2 and CD28 compared to the Teff subsets. Although PI3Kδ signaling has been shown to regulate the cytotoxic function of $CD8^+$ Teffs (16, 42, 53, 54), it appears that once the $CD8^+$ Teffs are differentiated into a memory cell type, they are less dependent on PI3Kδ signaling (41-43, 53). 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 idelalisib treatment.

We found that inhibition of PI3Kδ signaling blocked the TCR and co-stimulation 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 idelalisib mainly affects the Treg activation and differentiation process. The cytokine profiles from Treg and Teff co-cultures confirmed that idelalisib 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, idelalisib 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 co-culture, 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, 55). However, there is no clinical report yet that substantiates these findings in human malignancies. Interestingly, human clinical trials testing idelalisib in different B-cell malignancies such as CLL, FL, and SLL show remarkable results (31). Particularly, the first evidence of reduced circulating Treg numbers was reported in CLL patients treated with idelalisib (33). There is a possibility that the enhanced tumor clearance in these patients could be due to the generation of improved anti-tumor immune responses. Our results addressing the inhibitory mechanisms of idelalisib are consistent with these clinical findings.

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

Conflict-of-interest disclosure

K.O. is a consultant for Karus Therapeutics, Oxford, UK. K.O. has received consultancy or speaker fees from Merck, GSK, Gilead, and Incyte. KT has a research collaboration with GSK, has consulted for Pfizer and given expert testimony for Gilead. S.C., K.K., L.A.M, G.E.T, and E.M.A have no potential conflicts of interest.

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Foot Notes

Authorship Contribution

S.C. designed experiments, performed experiments, collected data, and wrote the manuscript; K.K. performed experiments, collected 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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Figure legends

Figure 1. PI3Kδ inhibition strongly affects the TCR induced proliferation of human Tregs compared to other Teff subsets. Representative CFSE dilution flow cytometric histograms (A) and compiled data (B) shows 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 IC_{50} for inhibition of proliferation of Tregs, $CD4^+$ Teffs and $CD8^+$ Teff subsets (data points were normalized to 100%). (D) Compiled bar graphs show the percent of dead cells measured using fixable viability dye in the 96 h proliferating cultures of Tregs and total $CD3^+$ Teff cells. Statistics; error bar represents mean ± SEM, ns=non-significant, **** $P \leq 0.0001$. n=3, healthy donors.

Figure 2. PI3Kδ blockade selectively inhibits the TCR signaling in Treg and Teff subsets. (A) A representative flow cytometry histogram overlays and (B) Phosphoflow cytometry analysis of TCR signaling proteins in Tregs, $CD4^+$ Teff and $CD8^+$ Teff subsets, gated from total $CD3^+$ T cells in absence or presence of indicated concentrations of idelalisib. Mean fluorescence intensity is represented as an arcsinh ratio of medians as described in the methods section. Statistics; error bar represents mean ± SEM, ns=non-significant, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, **** $P \leq 0.0001$. n=3, healthy donors.

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 non-stimulated and 48 h stimulated Tregs from healthy donors prior to co-culture 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 mean fluorescence intensity (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. ns=non-significant, *$P \leq 0.05$. Healthy donors (n=3) and CLL patients (n=3).

Figure 4. Compromised suppressive functions of idelalisib-treated Tregs (A) Representative overlaid flow cytometric histograms show the suppressive capacity of Tregs against $CD3^+$ T cells further gated on $CD4^+$ Teffs (blue) and $CD8^+$ Teffs (red). (B) The compiled data shows the percent suppression of Teff proliferation and (C) IL-10 levels in the supernatant of the co-cultures after 96 h (D) The levels of IL-2, TNF-α and INFγ in the supernatant of the co-cultures after 24 h; n=3 to 5 individual healthy donors. Percent suppression was calculated as [(% Teff cells alone - % Teff cells after treatment with Treg)/ % Teff cells alone] × 100. Statistics; error bar represents mean ± SEM, ns=non-significant, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

Fig. 1 Chellappa et. al.

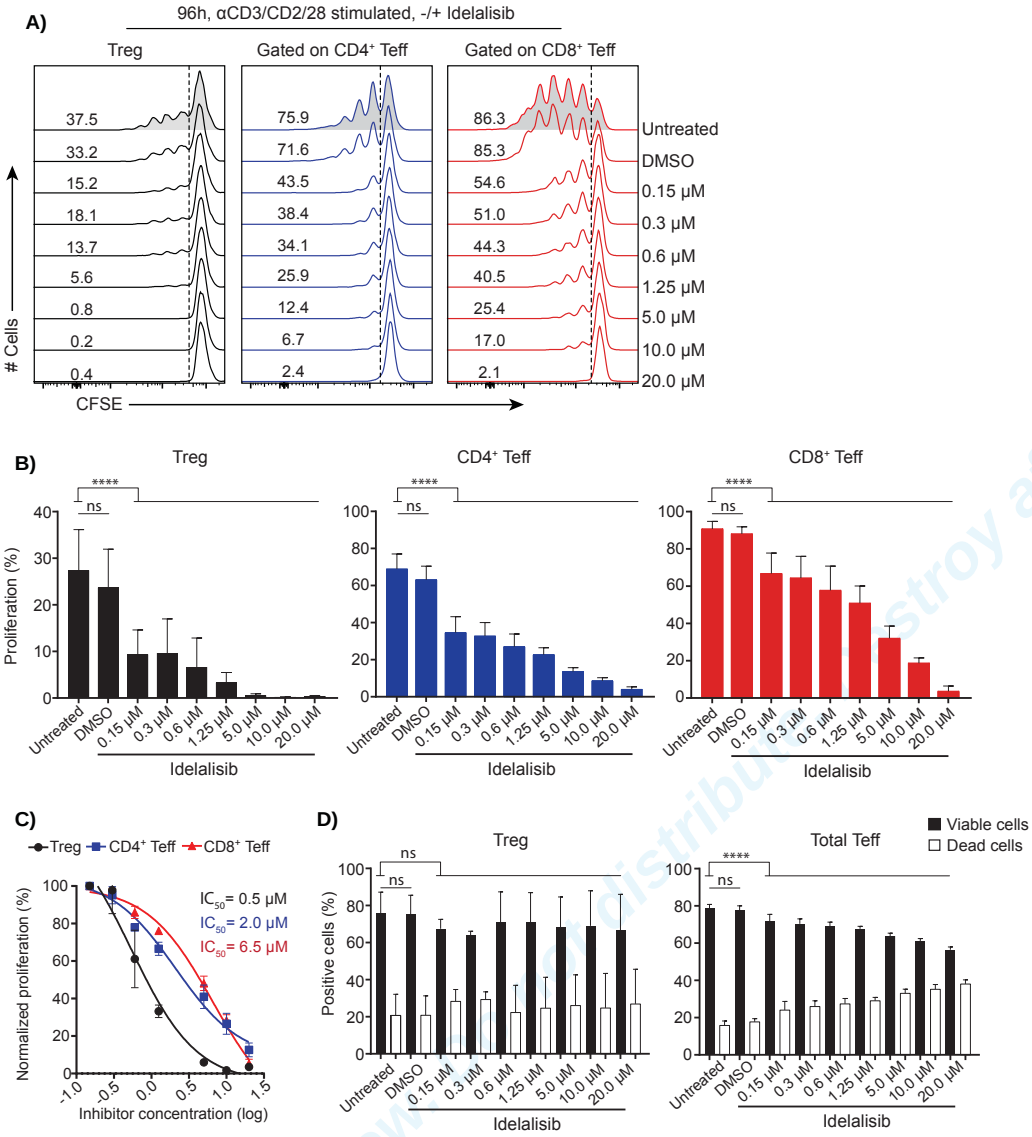
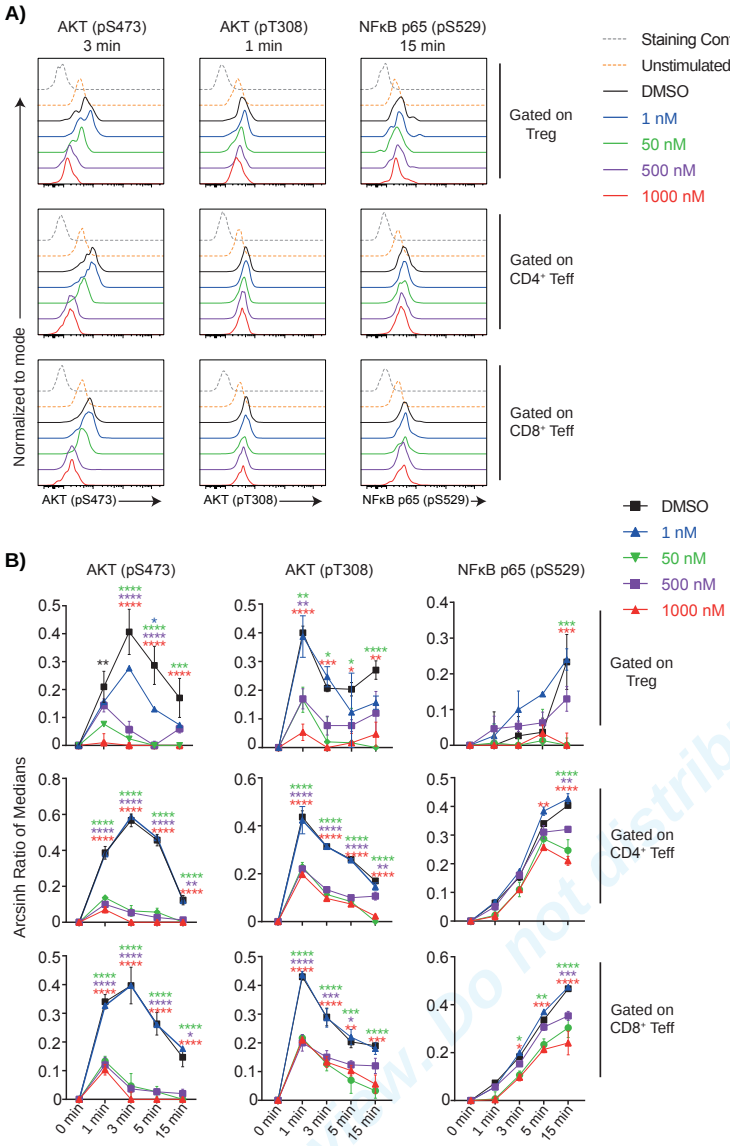


Fig. 2 Chellappa et. al.



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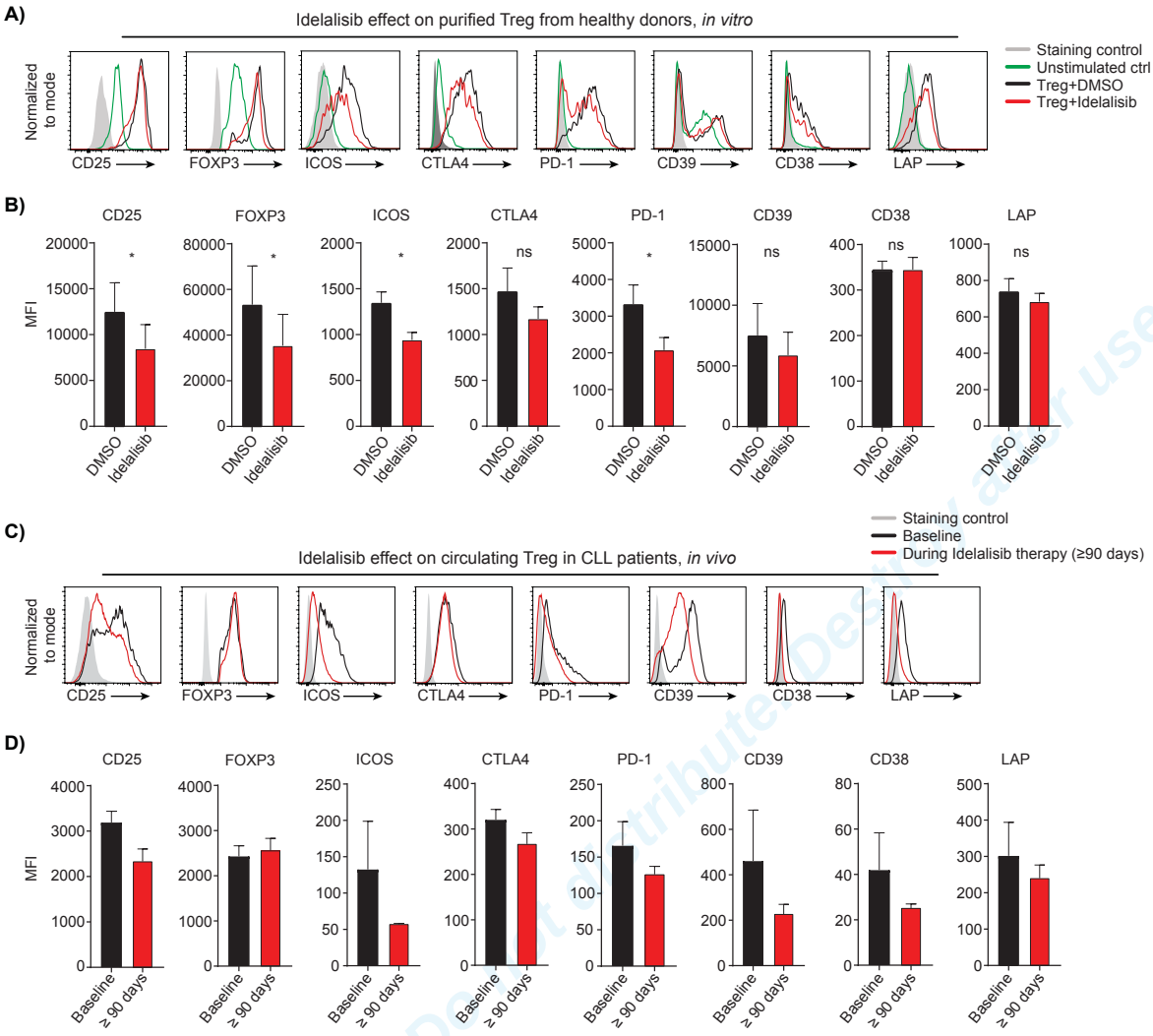

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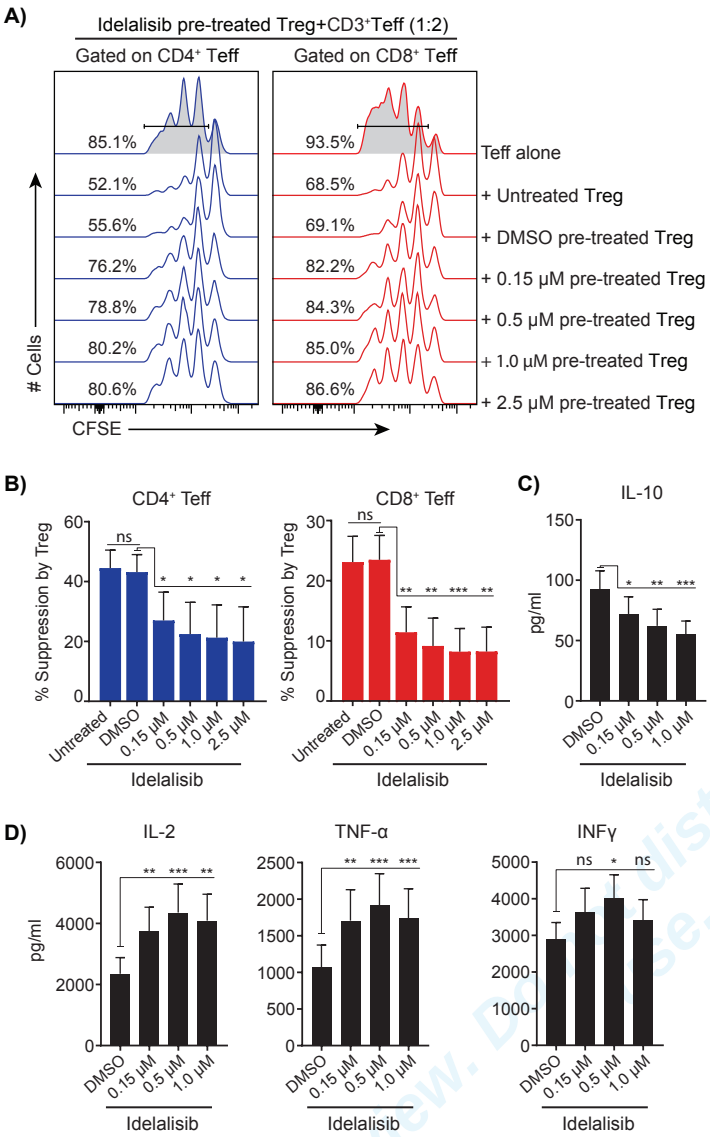

Table I. The clinical characteristics of included patients with CLL

UPN	Gender	Age (years)	Time from diagnosis to procurement (months)	Binet stage	Treatment prior to procurement	IGHV-gene usage	Homology with germ line	Chromosomal abberations
CLL103	M	61	166	C	FCR in 2012	HV3-48	99,4%	del(13q14) no del(17p13) or *TP53* mutation
CLL198	M	69	123	C	FCR in 2008	HV5-A3	100%	del(11q22) no del(17p13) or *TP53* mutation
CLL200	M	61	6	C	None	HV3-9	98,8%	del(13q14), del(17p13), and *TP53* mutation exon 7 c.734G>A

FCR=fludarabine, cyclophosphamide, rituximab