LYMPHOID NEOPLASIA

Ibrutinib is an irreversible molecular inhibitor of ITK driving a Th1-selective pressure in T lymphocytes

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Key Points

- Ibrutinib is the first clinically viable irreversible ITK inhibitor.
- Ibrutinib inhibits the formation of Th2 but not Th1 immunity.

Given its critical role in T-cell signaling, interleukin-2–inducible kinase (ITK) is an appealing therapeutic target that can contribute to the pathogenesis of certain infectious, autoimmune, and neoplastic diseases. Ablation of ITK subverts Th2 immunity, thereby potentiating Th1-based immune responses. While small-molecule ITK inhibitors have been identified, none have demonstrated clinical utility. Ibrutinib is a confirmed irreversible inhibitor of Bruton tyrosine kinase (BTK) with outstanding clinical activity and tolerability in B-cell malignancies. Significant homology between BTK and ITK alongside in silico docking studies support ibrutinib as an immunomodulatory inhibitor of both ITK and BTK. Our comprehensive molecular and phenotypic analysis confirms ITK as an irreversible T-cell target of ibrutinib. Using ibrutinib clinical trial samples along with well-characterized neoplastic (chronic lymphocytic leukemia), parasitic infection (*Leishmania major*), and infectious disease (*Listeria monocytogenes*) models, we establish ibrutinib as a clinically relevant and physiologically potent ITK inhibitor with broad therapeutic utility. This trial was registered at www.clinicaltrials.gov as #NCT01105247 and #NCT01217749. (*Blood*. 2013;122(15):2539-2549)

Introduction

The interplay between antigen-presenting cells and T lymphocytes forms an indispensable component of adaptive immunity, yet certain neoplastic, autoimmune, parasitic, and infectious diseases subvert adaptive immunity by specifically misdirecting helper T-cell polarity.[1,2] A common mechanism of immune subversion is the aberrant recruitment of a Th2-dominant response that promotes B-cell antibody production and interferes with direct effector cell cytotoxicity. In contrast, a Th1-dominant response evokes cytotoxic effects with the production of interferon γ (IFN-γ) and interleukin 2 (IL-2), which contribute to effector-cell–based immune surveillance. Clearance of certain intracellular bacterial pathogens such as *Listeria* and parasites such as *Leishmania*, as well as tumor immune surveillance, hinge upon the capacity to elicit robust Th1 and CD8 T-cell responses.

IL-2–inducible kinase (ITK) is a T-cell–dominant member of the TEC-kinase family that drives proximal T-cell receptor (TCR) signaling.[3] Upon TCR ligation in Th1 and CD8 T cells, ITK and redundant resting lymphocyte kinase (RLK or TXK) activate phospholipase Cγ (PLCγ), launching a signaling cascade that includes the nuclear factor of activated T cells (NFAT), nuclear factor κB, and mitogen-activated protein kinase pathways, resulting in cellular activation, cytokine release, and rapid proliferation.[4] In cancer, ITK is a critical signaling motif important to acute lymphoblastic T-cell leukemia and Sézary syndrome/cutaneous T-cell lymphoma due to aberrant activation and heightened expression.[5] In healthy Th1-polarized and CD8 effector cells, ITK plays a supportive yet dispensable role to RLK. However, the epigenetic evolution of Th2 cells conserves a singular dominant role for ITK, pinning it as the Achilles' heel of Th2 T cells.[6-9]

Clinically applicable ITK inhibitors are sought by the medical community given their potential to inhibit a number of Th2-dominant autoimmune, inflammatory, and infectious diseases ranging from cancer immunosuppression and atopic dermatitis to inflammatory bowel disease and even HIV/AIDS.[10,11] Moreover, a viable ITK inhibitor would be a promising therapeutic advancement for many T-cell malignancies that are currently difficult to manage.[12,13] Although multiple chemical analogs have been reported,

Submitted June 10, 2013; accepted July 18, 2013. Prepublished online as *Blood* First Edition paper, July 25, 2013; DOI 10.1182/blood-2013-06-507947.

N.M., A.J.J., and J.C.B. contributed equally to this study.

Presented in part at the 54th annual meeting of the American Society of Hematology, Atlanta, GA, December 9, 2012.

The online version of this article contains a data supplement.

There is an Inside *Blood* commentary on this article in this issue.

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no ITK inhibitors have successfully transitioned into clinical trials.[14]

Ibrutinib is an irreversible inhibitor of Bruton tyrosine kinase (BTK) that blocks downstream B-cell receptor activation.[15,16] Numerous in vitro and in vivo studies confirm the specific activity of ibrutinib against BTK-restricted targets.[17,18] Ibrutinib has demonstrated clinical activity in phase 1 and 2 clinical trials, with durable remissions against a variety of B-cell malignancies including mantle cell lymphoma, follicular lymphoma, and chronic lymphocytic leukemia (CLL).[19-22] Intriguingly, ITK shares significant sequence and functional homology with BTK and both contain an ibrutinib inhibition motif consisting of an SH3 autophosphorylatable tyrosine (Tyr) and a covalent binding cysteine (Cys) residue within the hinge region connecting the C and N lobes of the active site.[23] ITK had previously been discounted as a relevant target of ibrutinib given a lack of sufficient in vitro evidence; not long after, however, Herman et al noted effects on T-cell cytokine production, reigniting the inquiry.[16,18]

The striking homology between BTK and ITK combined with intriguing in silico docking led to the hypothesis that ibrutinib is the first clinically viable ITK inhibitor. This was explored using healthy human T cells and human and murine CLL as a model system of dysregulated Th2-biased immunosuppression. In CLL, an increasingly defective immune synapse enables malignant B cells to evade immune detection by inducing T-cell anergy as well as improper Th2 polarization.[24,25] In addition to being incapable of responding to environmental pathogens, these improperly polarized T cells contribute both cytokine and direct signaling support to malignant B cells.[26,27] The end result of this immunosuppression is a high incidence of severe infections, which is the leading cause of patient mortality.[28,29]

Our molecular analysis confirms that ibrutinib irreversibly binds ITK and inhibits activation of Th2 cells after TCR stimulation. This inhibition is specific to Th2-polarized CD4 T cells, because RLK remains functional, thus providing a compensatory platform for activation of Th1 and CD8 T cells. These data demonstrate that CD4 T-cell populations isolated from CLL patients are skewed toward a Th1 profile after exposure to ibrutinib. Findings were validated using mouse models of leukemia, cutaneous leishmaniasis, and *Listeria monocytogenes* infection. Ibrutinib's immunomodulatory activity and ITK inhibition in humans were confirmed using irreversible ITK binding, cytokine, and T-cell signaling analysis from CLL patients treated with ibrutinib in 2 separate clinical trials. Together, these results confirm that ibrutinib is the first potent and irreversible inhibitor of ITK to achieve clinical viability, potentially repurposing the drug for a multitude of novel therapeutic applications.

Methods

Subject populations

Sera and peripheral blood mononuclear cells (PBMCs) were obtained from normal donors or patients with CLL in accordance with the Declaration of Helsinki. All subjects gave written informed consent for their blood products to be used for research under an institutional review board–approved protocol. Blood was collected at The Ohio State University Wexner Medical Center (Columbus, OH). For additional information, see supplemental Methods.

Cell culture, drug treatments, and T-cell polarization

Primary T cells were isolated using RosetteSep or EasySep T-cell enrichment kits (STEMCELL Technologies, Vancouver, BC, Canada). Cells were pretreated for 30 minutes with ibrutinib, washed 2 times, then stimulated with plate-bound anti-CD3 and soluble anti-CD28 (eBiosciences, San Diego, CA). For additional details, see supplemental Methods.

Calcium flux analysis

Jurkat cells were stained with Fluo4-AM (Invitrogen), washed twice, and resuspended in phenol-red free RPMI. Fluo4 fluorescence was measured using a plate reader at 535 nm. For additional information, see supplemental Methods.

Flow cytometry and cytokine bead array

Flow-cytometric analysis was performed using fluorochrome-labeled antibodies using conventional methods. For specific antibodies and experimental design, see supplemental Methods.

Ibrutinib probe assay

Protein lysates were labeled with a biotinylated derivative of ibrutinib and added to a Streptavidin-coated plate, washed 3 times, and incubated with mouse anti-ITK. After washing with SULFO-TAG–conjugated anti-mouse antibody (MSD, catalog #R32AC-5), lysates were read on an SI2400. For additional details, see supplemental Methods.

Immunoblot analysis

Experiments were conducted using conventional sodium dodecyl sulfate polyacrylamide gel electrophoresis methodology. For specific antibodies and densitometry, see supplemental Methods.

Confocal immunofluorescence microscopy

Cells were centrifugally concentrated on microscope slides and stained with monoclonal antibodies. Images were taken using a ×60 objective and ×4 digital zoom with Olympus Fluoview 1000 laser scanning confocal microscope at The Ohio State University Microscopy and Imaging Facility. See supplemental Methods for details.

Mouse models

All animal procedures were performed in accordance with Federal and Institutional Animal Care and Use Committee requirements. Detailed methods for mouse models are provided in supplemental Methods.

ELISA

An enzyme-linked immunoabsorbent assay (ELISA) assay was performed for each immunoglobulin G (IgG) subisotype using a clonotyping system (B6/C57J-AP-5300-04B; Southern Biotech, Birmingham, AL) according to the manufacturer's instructions. For additional details, see supplemental Methods.

Statistics

Unless otherwise noted, a 2-tailed Student *t* test was used for normal data at equal variance. Significance was considered for $P < .05$. For detailed statistics, see supplemental Methods.

Results

Ibrutinib is an irreversible inhibitor of ITK

As a Th2-critical TEC family kinase, ITK retains significant structural and functional homology to ibrutinib's known irreversible target BTK, including a Cys442 putative covalent binding moiety located within the hinge region of the active site and an autophosphorylatable Tyr180 in the SH3 domain (Figure 1A). In silico

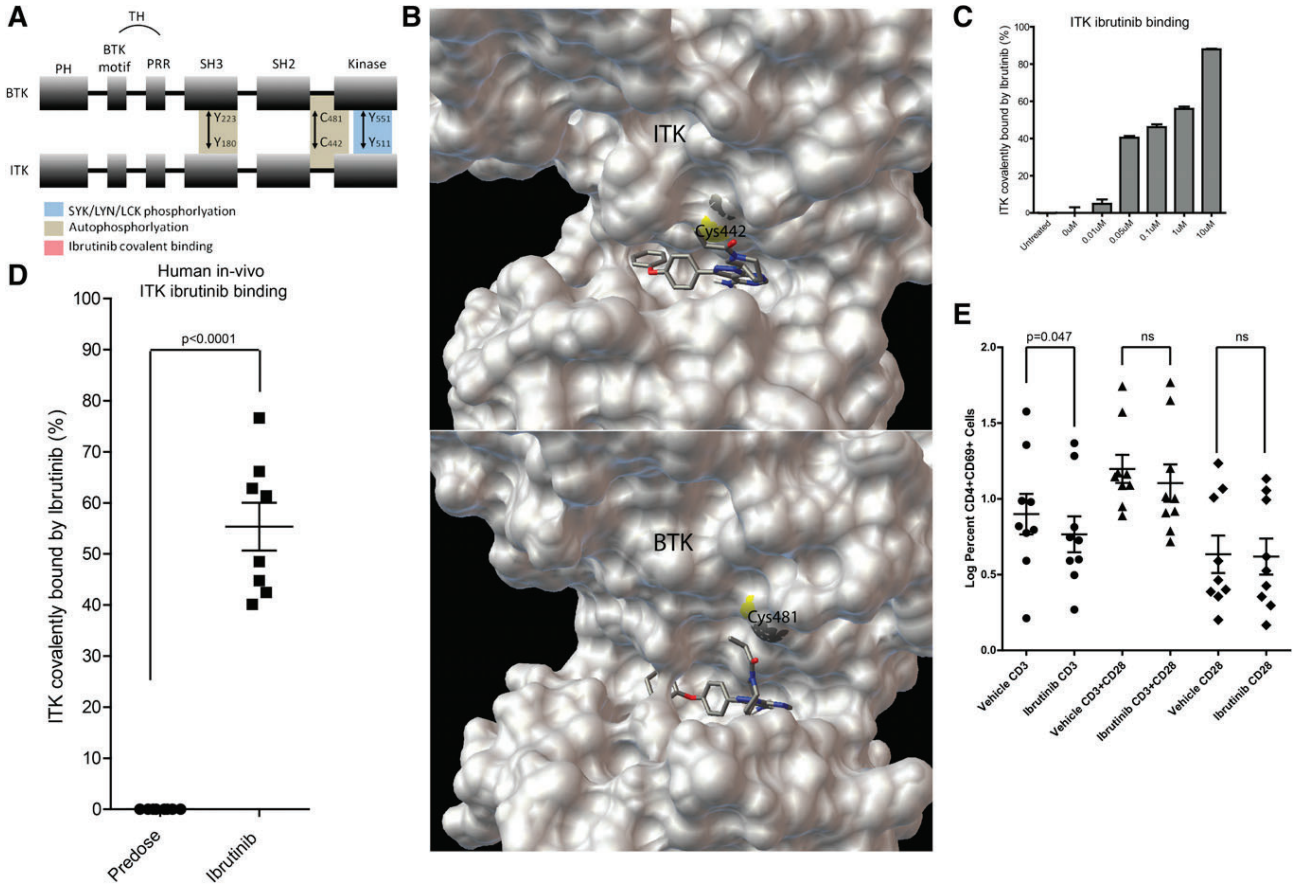

Figure 1. Ibrutinib is an irreversible molecular inhibitor of ITK, displaying BTK-independent antileukemic potential. (A) A graphical depiction of the sequence and domain homology between ITK and BTK. The relevant phosphorylation sites as well as ibrutinib irreversible covalent binding sites are labeled. (B) In silico representation of docked ibrutinib within the active site of crystallized ITK (top panel) (Protein Data Bank code 3QGW) or BTK (bottom panel) showing close approximation of Cys442 or Cys481 to reactive moiety of ibrutinib. Shape and chemical complementarity of ibrutinib are shown in surface representation. (C) A molecular probe assay was used to calculate the percent irreversible occupancy of total ITK in Jurkat whole-cell lysates irreversibly bound by ibrutinib. Error bars represent standard error of the mean (SEM). (D) A molecular probe assay was used to calculate the percent irreversible occupancy of ITK by ibrutinib in cryopreserved PBMCs obtained from patients immediately prior to (predose) and 8 days into (ibrutinib) daily oral ibrutinib therapy for CLL (n = 8). Error bars represent SEM. (E) Primary CD4 T cells isolated from healthy donors were pretreated with ibrutinib (1 μM) or vehicle and subjected to stimulation with anti-CD3, anti-CD28, or anti-CD3/anti-CD28 for 6 hours and analyzed via fluorescence-activated cell sorter for CD69 surface expression. Baseline (unstimulated) CD69 percentage was subtracted and data are represented in log percent $CD69^{+}CD4^{+}$ T cells. A 2-tailed paired Student *t* test was used for statistical analysis (nonsignificant [ns] = $P > .05$). Error bars represent SEM.

docking studies showed potential covalent binding of ITK at Cys442 and occupancy of the active site similar to that achieved when ibrutinib irreversibly binds BTK (Figure 1B). In vitro probe binding assays confirmed that ibrutinib was capable of irreversibly binding a significant percentage of endogenous ITK in the Jurkat T-cell leukemia cell line (Figure 1C).

To confirm that ibrutinib irreversibly binds ITK in vivo, we conducted an ITK probe assay on PBMC samples obtained from CLL patients in a phase 2 clinical trial of ibrutinib. Samples were tested immediately prior to receiving ibrutinib and after 8 days of daily oral administration (420 mg/day). The data revealed between 40% and 80% of ITK is covalently bound by ibrutinib, similar to that achieved in vitro (Figure 1D). Multimerization is an established ITK regulatory mechanism that sequesters inactive ITK within the cytoplasm, potentially blocking complete ibrutinib occupancy of ITK. To explore this, we disrupted the cytoplasmic architecture and observed near-complete occupancy of the ITK active site at drug concentrations nearing 0.3 μM (supplemental Figure 1).

In their initial description of ibrutinib, Honigberg et al did not find inhibition of the T-cell activation marker CD69 after stimulation with anti-CD3 and anti-CD28.[18] Based upon this evidence, a T-cell–specific target had been ruled out. One reason for these divergent results could be the fact that CD28 costimulation alone can induce CD69 surface expression in a TCR- and ITK-independent fashion. To explore this possibility, we examined the CD69 activation marker in CD4 T-cells isolated from healthy donors and stimulated with anti-CD3, anti-CD28, or anti-CD3/anti-CD28 (Figure 1E).[30] Ibrutinib significantly attenuated anti-CD3–induced surface expression of CD69. However, we did not observe any significant alteration to CD69 in CD4 T cells stimulated via CD28 or via CD3/CD28, indicating that CD28 costimulation provides an additional non-inhibited pathway that elicits surface presentation of CD69.

Ibrutinib inhibits ITK signaling and molecular characteristics of TCR-induced activation in primary CD4 T cells and Jurkat cells

T-cell activation is predicated upon robust downstream nuclear factor κB, mitogen-activated protein kinase, and NFAT signaling; therefore, components of each pathway were examined to determine the T-cell–specific effects of ibrutinib. Ibrutinib treatment yielded a dose-dependent inhibition of ITK autophosphorylation at Y180

resulting in downstream inactivation of IkBα, JunB, and NFAT signaling in both primary CD4 and Jurkat T cells (Figure 2A-B; supplemental Figure 2). Notably, inhibition of both JunB and STAT6 was observed, both of which are critical components of the IL-4 pathway.[31,32] Although JAK3 inhibition could explain some of our in vitro data, our target validation studies demonstrate that ibrutinib does not directly influence this kinase in cell-based assays (supplemental Figure 3).

To confirm that TCR-induced activation events preceding ITK autophosphorylation were not altered, we examined the proximal pathway components using both primary CD4 and Jurkat T cells. Immunoblot data revealed that upstream phosphorylation of LCK, ZAP70, and LAT remain unchanged (Figure 2C; supplemental Figure 4). Furthermore, we used the PKC activator, phorbol 12-myristate 13-acetate, and the calcium ionophore, ionomycin, to confirm that distal elements of TCR activation, including NFAT activity and IkBα phosphorylation, were engaged regardless of ibrutinib treatment in Jurkat cells (Figure 2D).

To functionally confirm our molecular data set, we examined NFAT nuclear translocation via confocal microscopy. As expected, NFAT nuclear translocation was inhibited by ibrutinib (Figure 2E-F). Remnant populations of activated CD4 T cells were observed, indicating that not all CD4 T cells were inhibited. We also confirmed that TCR-induced proliferation as well as naïve, central, effector, and terminal memory subsets were unaffected by in vitro ibrutinib treatment (supplemental Figures 5 and 6).

We sought to confirm functional ITK inhibition in patients receiving oral ibrutinib. Because PLCγ1 is directly phosphorylated at Tyr783 by active ITK, we conducted pPLCγ1-Tyr783 phosphoflow analysis on CD3/CD28-stimulated CD4 T cells collected from CLL patients receiving ibrutinib as part of a phase 2 clinical trial. Results reveal a significant decrease in TCR-induced pPLCγ1 activation, confirming inhibition of CD4 T-cell ITK signaling in these patients (Figure 2G; supplemental Figure 7).

It has been demonstrated in mice that loss of ITK attenuates, yet does not ablate, intracellular calcium flux in response to TCR signaling.[33,34] Ibrutinib treatment of Jurkat cells yielded similar results (Figure 2H-I), demonstrating that ibrutinib-based ITK inhibition significantly reduces intracellular calcium flux in response to TCR stimulation.

Ibrutinib-induced ITK-C_{442}–irreversible inhibition provides a selective advantage to RLK-expressing Th1 and CD8 T cells

To confirm that the primary molecular target of ibrutinib in CD4 T cells was ITK, TCR-induced activation of NFAT, pSTAT6, pIkBα, and JunB was evaluated in primary CD4 T cells pretreated with ibrutinib or 1 of 2 alternative BTK inhibitors, AVL-292 and PCI-45292, which do not target ITK (50% inhibition/inhibitory concentration > 22.5 nM) (Figure 3A; supplemental Figures 8-13). Only ibrutinib (ITK 50% inhibition/inhibitory concentration = 2.2 nM) was capable of inhibiting TCR downstream molecular activation.

Ibrutinib presumably requires a cysteine residue within the hinge region to form an irreversible covalent bond and inhibit a kinase target. In ITK, that cysteine moiety resides at amino acid 442. Therefore, as molecular confirmation that ITK is the sole irreversible target in CD4 T cells, a stably transduced Jurkat line was generated with ITK-C_{442}A, a version of ITK that lacks the putative covalent binding site for ibrutinib (supplemental Figure 14). The ITK-C_{442}A Jurkat line maintained NFAT activation to drug concentrations exceeding 8 μM, whereas the parental line and Jurkat cells transfected with wild-type ITK were inhibited at 2 to 4 μM (Figure 3B). These data were confirmed by intracellular calcium flux showing that the ibrutinib-resistant ITK-C_{442}A Jurkat line significantly and completely restored calcium response (Figure 3C; supplemental Figure 15).

A key reason why ITK inhibitors retain clinical interest is the potential to selectively inhibit Th2 T cells, which do not contain the compensatory RLK kinase. To elucidate the differential inhibition of Th2-polarized T cells in relation to Th1, naïve CD4 T cells were polarized in vitro to obtain enriched cultures of IFN-γ–producing Th1 cells and IL-4–producing Th2 cells (Figure 3D). In contrast to Th1, Th2 cultures were sensitive to pharmacologically relevant levels of ibrutinib as demonstrated by reduced IL-4 production (Figure 3E). Additionally, ibrutinib inhibited NFAT and IkBα activation in Th2 T cells, whereas Th1-polarized CD4 and CD8 T cells were resistant (Figure 3F).

In Th1 CD4 and CD8 T cells, RLK plays a redundant role to ITK; however, neither Th2 polarized CD4 T cells nor Jurkat cells express RLK.[35] To test the hypothesis that RLK expression protects Th1 T cells from ibrutinib inhibition, Jurkat cells stably transduced to express RLK were tested (supplemental Figure 16). TCR downstream activation of NFAT was protected in the Jurkat-RLK cell line, whereas both the parental and empty vector stable transfectants were susceptible to ibrutinib inhibition (Figure 3G). Confirmatory intracellular calcium release experiments demonstrate a significant restoration of calcium flux in Jurkat cells stably expressing RLK (Figure 3H; supplemental Figure 17). This result demonstrates that RLK can compensate for ibrutinib-inhibited ITK, thereby providing an alternate activation platform for specific RLK-expressing T-cell subsets.

Ibrutinib selectively limits Th2 activation, thereby initiating a Th1-selective pressure in a mixed population of CD4 T cells in vitro, in vivo, and in human CLL patients receiving ibrutinib

To evaluate the effects of ibrutinib on the Th1/Th2 polarization of a CD4 T-cell population over time, CD4 T cells isolated from healthy donors were cultured for 3 days following ibrutinib pretreatment. Outgrowth of IFN-γ–positive T cells was confirmed by intracellular staining analysis (Figure 4A). This outgrowth correlated with a decrease in the Th2-dominant transcription factor JunB and an increase in the Th1-specific transcription factor T-bet (Figure 4B).

To confirm the functional relevance of these results in the setting of CLL, intracellular staining was performed for IFN-γ and IL-4 in ibrutinib-treated, TCR-stimulated CD4 T-cell cultures purified from CLL patients not previously treated with ibrutinib. Following stimulation, a significant decrease was identified in the IL-4–producing Th2 population of CD4 T cells, whereas IFN-γ–producing Th1 cells were largely unaffected (Figure 4C). These results very closely matched data obtained on primary CD4 T cells from healthy donors (supplemental Figure 18). These data confirm that a significant divergence of the 2 populations can be achieved in a purified T-cell culture at ibrutinib doses ranging from 0.1 to 1 μM. This dose range is consistent with serum concentrations observed in vivo during pharmacokinetic studies of ibrutinib in both mouse and human trials.[18,36]

To validate these findings in human patients, serial serum cytokine levels were investigated in relapsed or refractory CLL patients receiving ibrutinib as part of a phase 1 study. The data demonstrated a decrease in serum Th2-type cytokines, including IL-10, IL-4, and

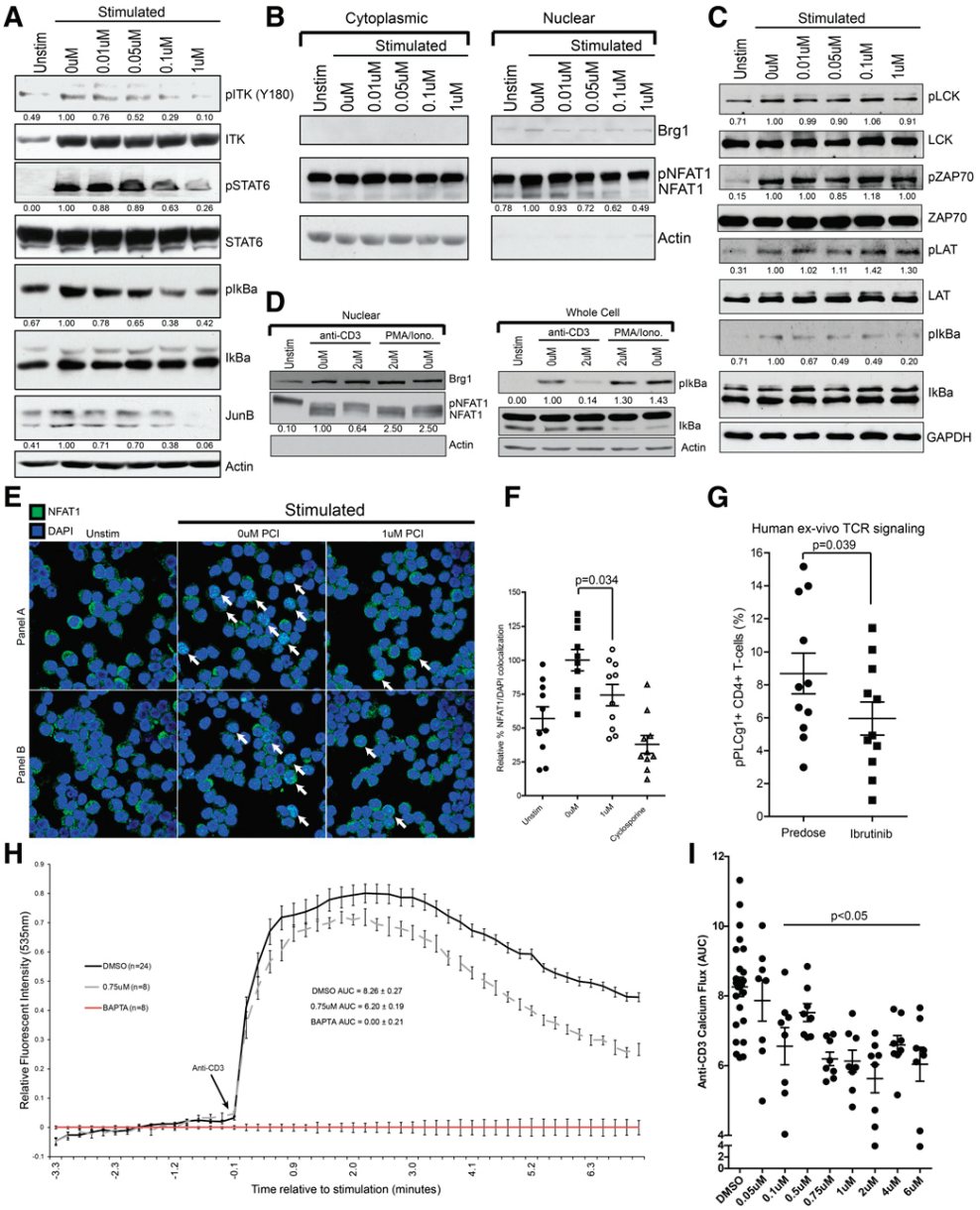

Figure 2. In T cells, ibrutinib specifically targets ITK, inhibiting TCR-induced cellular signaling and activation. (A) Immunoblot analysis of freshly isolated ibrutinib pretreated primary CD4+ cells from a healthy donor, anti-CD3/anti-CD28 stimulated (or unstimulated), whole-cell lysates. Blot probed for pITK-Y_{180}, total ITK, pSTAT6-Y_{641}, total STAT6, pIkBα-$S_{32/36}$, total IkBα, JunB, and actin. Densitometry analysis normalized to dimethylsulfoxide (DMSO)-treated (0 μM) sample. (B) Immunoblot analysis of freshly isolated ibrutinib pretreated primary $CD4^+$ cells from a healthy donor, anti-CD3/anti-CD28–stimulated (or unstimulated), cytoplasmic, and nuclear lysates. Blots probed for NFAT (and activated hyperdephosphorylated NFAT), Brg1, and actin. Densitometry analyses are normalized to the DMSO-treated (0 μM) sample. (C) Immunoblot analysis of freshly isolated ibrutinib-pretreated primary $CD4^+$ cells from a healthy donor, anti-CD3/anti-CD28–stimulated (or unstimulated), whole-cell lysates. Blots were probed for pZAP70-Y_{319}, total ZAP70, pLAT-Y_{191}, total LAT, pLCK-Y_{505}, total LCK, pIkBα-$S_{32/36}$, total IkBα, and actin. Densitometry analyses are normalized to the DMSO-treated (0 μM) sample. (D) Nuclear or whole-cell lysate immunoblot analysis of Jurkat cells pretreated with ibrutinib and stimulated with either anti-CD3/anti-CD28 or phorbol 12-myristate 13-acetate/ionomycin for 45 minutes. Blots were probed with Brg1, NFAT1, and actin (nuclear lysates) or pIkBα-$S_{32/36}$, total IkBα, and actin (cellular lysates). (E) Immunofluorescent microscopy of ibrutinib-pretreated, freshly isolated, primary $CD4^+$ cells from healthy donors (panels A and B) were stimulated for 45 minutes with anti-CD3/anti-CD28 (or unstimulated), fixed, and stained for NFAT (green) and nuclei (4,6 diamidino-2-phenylindole [DAPI], blue). Activated cells are characterized by influx of NFAT into nuclear region (green overlay with blue = cyan) and are denoted by white arrows. (F) Percent relative NFAT1/DAPI colocalization derived from Pearson correlation analysis of 10 independent immunofluorescent microscopy fields (different donors than pictured in panel E and normalized to the average unstimulated value. Cyclosporin A (CSA) treatment was used as an additional negative control. Error bars represent SEM. (G) Phosphoflow analysis of pPLCγ1-Tyr_{783} in 1hr anti-CD3/anti-CD28–stimulated cryopreserved PBMCs obtained immediately predose or after 8 days of receiving ibrutinib therapy for CLL (n = 11). A minimum of 400 000 events were collected. Graph displays the overall percent of live $CD3^+CD4^+$pPLCγ1^-Tyr783^+ events in each sample. Error bars represent SEM. (H) Calcium flux analysis of ibrutinib (n = 8), vehicle (n = 24), or BAPTA-AM (n = 8) pretreated Jurkat cells after TCR stimulation by anti-CD3. Area under the curve (AUC) is presented for each dataset in the center. All data were normalized to baseline and BAPTA-treated fluorescent averages. Time points depicted on horizontal axis are relative to stimulation with anti-CD3. (I) AUC for calcium flux of various concentrations of ibrutinib. Each symbol indicates a single replicate experiment. Statistical analysis represented on graph is relative to DMSO treatment. PMA/Iono., phorbol 12-myristate 13-acetate and ionomycin; Unstim, unstimulated.

IL-13, from pretreatment to day 28 of ibrutinib therapy (Figure 4D). This was in sharp contrast to an increase in the Th1 cytokine IFN-γ. To rule out the potential contribution of B cells to the observed Th1 cytokines, we analyzed peripheral $CD19^+$ B-cell and CLL messenger RNA levels and found no such alteration in B-cell cytokine expression (supplemental Figure 19).

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