PI3K p110 / regulates T cell cytokine production during primary and secondary immune responses in mice and humans

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Abstract

We have previously described critical and non-redundant roles for the PI3K p110 f during the activation and differentiation of na ve T cells and p110 f inhibitors are currently being developed for clinical use. However, to effectively treat established inflammatory or autoimmune diseases it is important to be able to inhibit previously activated or memory T cells. In this study, using the isoform-selective inhibitor IC87114, we show that sustained p110 f activity is required for IFN e production. Moreover, acute inhibition of p110 f inhibits cytokine production and reduces hypersensitivity responses in mice. Whether p110 f played a similar role in human T cells was unknown. Here we show that IC87114 potently blocked TCR-induced PI3K signaling by both na ve and effector/memory human T cells. Importantly, IC87114 reduced cytokine production by memory T cells from healthy and allergic donors and from inflammatory arthritis patients. These studies establish that previously activated memory T cells are at least as sensitive to p110 f inhibition as na ve T cells and show that mouse models accurately predict p110 f function in human T cells. There is therefore a strong rationale for p110 f inhibitors to be considered for therapeutic use in T cell-mediated autoimmune and inflammatory diseases.

Introduction

In many immune-mediated diseases, T cells with an activated or memory phenotype accumulate at the site of tissue destruction. Genetic susceptibility to autoimmunity is often linked to the MHC locus and other loci that affect T cell biology, thus implying pathological roles for T cells in autoimmunity[1,2]. Indeed there is mounting evidence that perturbation of

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antigen receptor signaling in T cells often contributes to autoimmune diseases[3]. Therapeutics affecting T cells such as glucocorticoids, methotrexate, cyclosporine (CS), CTLA4-Ig, and rapamycin are used effectively to treat or ameliorate immune-related disorders. However, these therapies can be associated with undesirable side-effects and/or unresponsiveness in some patients[4-7]. Hence, there is a real need for additional drugs that target T cells but do not compromise organ function or leave the patient unduly susceptible to infections.

The Class I PI3Ks phosphorylate phosphatidylinositol-(4,5)-phosphate to produce phosphatidylinositol-(3,4,5)-phosphate (PIP3). PIP3 acts as a second messenger by recruiting pleckstrin homology (PH) domain-containing proteins to the plasma membrane where they activate signaling pathways that promote proliferation, survival, differentiation, and chemotaxis[8]. Class I PI3Ks are sub-divided into two groups based on their structure: Class IA PI3Ks are heterodimers consisting of one regulatory subunit (p85 c,p85

p110 f in effector and memory T cells from mice and humans and monitored intracellular signaling events, proliferation, cytokine production and inflammatory responses.

We found that sustained p110 f activation is required for IFN e production and that IC potently blocked effector responses from effector/memory T cells in both species. p110 f inhibition also reduced contact-hypersensitivity (CHS) reactions in mice, and cytokine production by T cells from patients with allergy or reactive arthritis (ReA). We conclude that p110 f plays an important role in the activation of effector and memory T cells in humans and mice.

Materials and Methods

Mice

p110 f^{D910A}

Anti-CD3 and Concanavalin A (ConA) injections

Mice were dosed once orally with 30 mg/kg IC in 1% methylcellulose (MCL) or with 1% MCL vehicle control alone 30 minutes before they were injected intravenously with anti-CD3 (0.01 mg/kg) or ConA (15 mg/kg). 1.5 hours after injection, the mice were terminally anaesthetized and bled by heart puncture. Cytokine levels in the serum were measured using a FlowCytomix kit from Bender Medsystems.

CHS studies

100 nl of 7% TNCB in a 4:1 acetone/olive oil mix or acetone/olive oil alone (for unsensitized controls) was applied to the shaved abdomens of p110$\delta^{D910A/D910A}$ or C57BL/6J controls. Six days later, ear thickness was measured with a micrometer (Kroeplin). Mice were subsequently dosed orally with 30 mg/kg IC in 1% MCL or with 1% MCL vehicle control alone 1 h before and 5 h after elicitation of CHS. To elicit CHS, 1% TNCB in a 9:1 olive oil/acetone mix was applied to an ear. Ear thickness was measured again 24 h later. The change in ear thickness was measured as the size difference before and after re-challenge.

To measure a secondary CHS response, mice were sensitized and elicited as above but without drug treatment during the primary elicitation. Four weeks after the initial sensitization, mice were drug treated and re-elicited as above, and the change in ear thickness calculated after 24 h.

Human cell purification

PBMCs from healthy humans were isolated using a Ficoll gradient. In some experiments, cells were negatively selected according to manufacturer's instructions using RosetteSep Human T cell Enrichment Cocktail (for total T cells), or EasySep Human Na ve CD4+ T cell or Memory CD4+ T cell Enrichment kits (for na ve or effector/memory T cells respectively) (Stem Cell Technologies). Total CD4$^+$ T cells were negatively selected from atopic donors using Human CD4$^+$ T Cell Isolation Kit II (Miltenyi), and autologous APCs isolated from the positively selected fraction. Purified cells were >95% pure. Mononuclear cells were also isolated from the synovial fluid of patients fulfilling ESSG criteria for spondyloarthritis, and had clinical reactive arthritis (ReA)[42]

Human T cell biochemistry

For Western blot analysis, total (CD3$^+$) T cells were pre-treated with DMSO alone, LY or IC and subsequently stimulated by first adding 0.3 ng/mL anti-CD3 (Clone OKT3) and 1 ng/mL anti-CD28 (Clone CD28.2; Immunotech). After 2 min, these Abs were cross-linked for 5 min with 10 ng/mL goat anti-mouse IgG, F(ab)$_2$ (Jackson ImmunoResearch). Lysates were probed with polyclonal rabbit anti-phospho-Ser^{473}-Akt, -Thr^{308}-Akt, -Thr^{202}/Tyr^{204}-Erk1/2, total Akt and total Erk1/2 (all Cell Signaling Technologies).

For fluorescent cell bar-coding experiments, total T cells were pre-treated with DMSO alone, LY or IC and subsequently stimulated by first adding 0.3 ng/mL biotinylated anti-CD3 and 5 ng/mL biotinylated anti-CD28. After two min, cells were cross-linked with 20 ng/mL avidin (Zymed) at 37C, and aliquots were withdrawn at the indicated time points. Cells were fixed for 10min at 37 C with Fix Buffer I (BD Biosciences), washed, and then fluorescently bar-coded as described previously[43]. Briefly, aliquots were stained for 20 min at room temp in different dilutions of Alexa Fluor 488 and Pacific Blue succinimidyl ester (Invitrogen), providing each sample with a unique fluorescent signature. Cells were washed and then pooled for further staining. Cells were permeabilized in Perm Buffer III (BD Biosciences), washed and stained with phosphospecific Abs (Cell Signal Technology: Alexa 647-conjugated pSer^{473}-Akt clone D9E, pThr^{202}/Tyr^{204}-Erk1/2 clone E10, or pSer$^{235/236}$-S6

clone D57.2.2E; or unconjugated pSer473 SGK3 d followed by Alexa-647 goat anti-rabbit IgG (Invitrogen)) and lineage markers (CD3 clone SK7, CD4 clone SK3, CD45RO clone UCHL1; all BD Biosciences) for 30 min at room temp. T cell subsets were defined as follows: Na ve CD4+ ($CD3^+CD4^+CD45RO^-$), effector/memory $CD4^+$ ($CD3^+CD4^+CD45RO^+$), na ve $CD8^+$ ($CD3^+CD4^-CD45RO^-$), and effector/memory $CD8^+$ ($CD3^+CD4^-CD45RO^+$). Cells were processed on a FACS Canto II flow cytometer (BD Biosciences). Data were analyzed using the Stanford University Cytobank program (http://cytobank.stanford.edu/). Arcsinh medians with a co-factor of 150 were used for the statistical calculation of fold change in Alexa-647 between individual samples using the following equation:

$$\ln\left(x/150+\left((x/150)^2+1\right)^{1/2}\right)-\ln\left(\text{control}/150+\left((\text{control}/150)^2+1\right)^{1/2}\right).$$

Results were visualized either as heatmaps or as overlaid histograms.

Human T cell activation

$CD3^+$ human primary T cells were activated with T Cell Expander Dynabeads (Invitrogen) in a 1:1 bead to cell ratio. Supernatants for IL2 and IFN e measurements were collected after 16 h. $CD45RA^+$ or $CD45RO^+$ $CD4^+$ T cells were stimulated with 3.75 ng/mL plate-bound anti-CD3 (Clone UCHT1; BD Bioscience) for 48 h before measuring proliferation and cytokine production. Total PBMCs were stimulated with 1 ng/mL tetanus toxin (Calbiochem) for seven days before measuring proliferation and cytokine production. $CD4^+$ T cells from PBMCs of atopic donors were mixed 1:1 with autologous APCs and stimulated with 25 ng/mL house dust mite antigen (Greer Laboratories) for six days before measuring cytokine production. Mononuclear cells from the synovial fluid of ReA patients presenting in clinic were stimulated with plate-bound anti-CD3 for 72 h before measuring proliferation and cytokine production. Cytokine concentrations from culture supernatants were assayed by ELISAs from R&D Systems or MesoScale Discovery.

Statistics

In mouse experiments, the effects of genetic or pharmacological inactivation of p110 f were compared to uninhibited wild type (WT) controls in mice using Student's *t* tests, except for the CHS studies where the one way ANOVA with a Bonferroni post-test was used. Responses of human cells treated with IC were compared to DMSO controls using one-sided repeated measures ANOVA on log-transformed data. Responses of cells treated with LY or CS were compared to DMSO controls using Student's *t* test on log-transformed data. Probabilities were calculated using GraphPad Prism or SPSS, and *p* values are annotated on figures with the following symbols: *0.01 p 0.05 **0.001 p 0.01 ***p 0.001. % inhibition of human cells was calculated as: 100* (1-((inhibited sample ±unstimulated sample)/ (uninhibited sample ±unstimulated sample)), and median values are shown below the graphs with negative numbers indicating an enhanced response.

Results

Naïve and effector/memory mouse $CD4^+$ T cells are sensitive to p110 / inhibition

We have previously shown that p110 f is required for PIP3 accumulation for at least 30 min after TCR stimulation in $CD4^+$T cells[17]. However, the kinetics of PI3K activation after this time point is unknown. $CD8^+$T cells only required PI3K activation for the first nine hours after activation to induce a full proliferative program[44]. This suggested that prolonged activation of PI3Ks (including p110 f) may not be required in T cells.

more swollen after the second elicitation than the first (Figure 3G and data not shown), indicating that a memory-type response was being measured. Similar to the primary response (Figure 3F), a ~50% reduction in swelling was observed in IC-treated WT mice as well as in p110 ^{D910A} mice with or without IC treatment (Figure 3G). We conclude that p110 f participates in primary and secondary responses *in vivo*.

p110 / is the main transducer of PI3K signals in TCR-stimulated human T cells

We have shown that TCR-induced phosphorylation of Akt in mouse T cells is highly sensitive to p110 f inhibition[12,17,19]. Because Akt phosphorylation only occurs if PIP3 is produced, these results demonstrated that p110 f is the main PI3K isoform activated by the TCR. To determine if human T cells were also dependent on p110 f to activate the PI3K pathway, primary T cells from healthy donors were stimulated for five minutes with anti-CD3 and anti-CD28 in the presence of DMSO alone, IC or LY, and phosphorylation of Akt and Erk were subsequently analyzed by immunoblotting (Figure 4A). Both LY and IC blocked Akt phosphorylation in a dose-dependent manner. IC inhibited Akt phosphorylation as potently as LY, suggesting that p110 f transmits PI3K signals from the TCR and CD28 in human T cells and that other isoforms cannot substantially compensate. Erk phosphorylation was only partially blocked by 10 nM of either inhibitor, consistent with a non-essential but accessory role for PI3K in regulating Erk in T cells under some stimulatory conditions[12,19].

Some signaling differences have been observed in na ve, activated and memory T cells, and between CD4 $^{+}$ and CD8 $^{+}$ T cells[38,45,46]. Hence, it was plausible that IC may affect T cell subsets differently. To compare the potency of IC on different T cell subsets, purified primary T cells were treated with DMSO alone, IC, or LY before stimulation with anti-CD3 and anti-CD28, and PI3K targets were analyzed by phospho-flow cytometry at various time points (Figure 4 B-F). By analyzing populations separately on the basis of surface phenotype, the effects of inhibitors could be assessed in na ve CD4 $^{+}$ (CD3 $^{+}$CD4 $^{+}$CD45RO $^{-}$), effector/memory CD4 $^{+}$ (CD3 $^{+}$CD4 $^{+}$CD45RO $^{+}$), na ve CD8 $^{+}$ (CD3 $^{+}$CD4 $^{-}$CD45RO $^{-}$), and effector/memory CD8 $^{+}$ T cells (CD3 $^{+}$CD4 $^{-}$CD45RO $^{+}$). Raw data were then converted into a heat map comparing the change in fluorescence to an unstimulated control. Histograms for samples treated with DMSO alone (Figure 4 B-E) are shown to demonstrate the levels of phosphorylation represented by each color in the heat map (Figure 4F). Each subset reacted similarly to IC, with phosphorylation of Akt, S6 and GSK3 d reduced at even the lowest concentration of IC while Erk phosphorylation was only diminished at 10 nM IC (Figure 4F). These data demonstrate that in each T cell subset, CD3 and CD28 activate p110 f and that prior activation status does not influence sensitivity to IC. Moreover, these data reveal redundancy in signals leading to GSK3 and S6 phosphorylation as these phosphorylation events were less sensitive to PI3K inhibition than was Akt phosphorylation. Indeed, Erk and Rsk are known to contribute to these signaling events independently of PI3K[47,48].

Because IFN e is primarily secreted by previously activated T cells, we next sub-fractionated CD4 $^{+}$ T cells isolated from healthy donors into na ve CD45RA $^{+}$ and effector/memory CD45RO $^{+}$ populations, and stimulated for 48 h with anti-CD3 in the presence DMSO alone, LY or IC. Up-regulation of the activation marker CD69 was undiminished in all but the highest concentration of PI3K inhibitors which correlated with the level of Erk inhibition (Figure 5A, B). However, IC concentrations between 1-10 nM diminished proliferation of both CD45RA $^{+}$ and CD45RO $^{+}$ CD4 $^{+}$ T cells to a similar extent as LY (Figure 5C, D). IFN e and IL17 production was undetectable in CD45RA $^{+}$ cells from most donors, but LY and IC profoundly blocked production of IFN e and IL17 by CD45RO $^{+}$ cells (Figure 5F, H). Individual donors varied in sensitivity to IC at the lowest concentration used (0.1 nM). To indicate the most common level of inhibition observed, % inhibition was calculated for each donor as described in Materials and Methods and the median value shown beneath the

graph. These data suggest that human effector/memory T cells are at least as sensitive to p110 f inhibition as na ve T cells.

Because CD28 costimulation has been shown, under some circumstances, to be insensitive to p110 f inhibition[12], we also stimulated human T cells with anti-CD3 and anti-CD28. Consistent with previous results in mouse[12], we found that anti-CD28 stimulated robust IL2 production in absence of p110 f activity. However, IFN e production was inhibited by IC in a dose dependent manner. The ability of LY, but not IC to inhibit IL2 production may reflect a role for mTOR in blocking IL2 production as LY, but not IC, targets the catalytic activity of this kinase[41].

$CD45RO^+$ cells include both recently activated effector and resting memory cells. As a second, more definitive approach to monitor the sensitivity of human memory T cells to p110 f inhibition, unfractionated PBMCs were pulsed with tetanus toxin in the presence of DMSO alone or IC. Tetanus toxin will only elicit a response from individuals who have previously been vaccinated against tetanus and the response is mediated by memory cells.

possible to tune the level of p110 f inhibition in the clinic to preferentially affect pathogenic T cells. We also demonstrated that signals transduced by p110 f 24 h or more after initial TCR stimulation can potently promote IFN e production, suggesting that p110 f inhibitors

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Figure 1. p110 **f** regulates cytokine production late after TCR stimulation

$CD8^+$ OT1 (left column) or $CD4^+$ OT2 (right column) T cells were stimulated with irradiated APCs+1 mM OVA peptide or left unstimulated. 10 mM LY or 5 mM IC was added either at the start of co-culture, seven or 24 h later. Vehicle control (DMSO) was added at the experiment start for uninhibited controls. After 48 h, proliferation (A, B), IL2, (C,D) and IFN e production (E, F) were measured. Data shown are mean SEM of triplicate readings and are representative of two independent experiments.

Figure 2. p110

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Figure 3. IC reduces T cell cytokine production and contact hypersensitivity responses in vivo

WT B6 mice were given one oral dose of IC (30 mg/kg) 30 minute before being administered anti-CD3 or ConA intravenously. Cytokines concentrations in the serum 1.5 h post injection were measured. A: IL2, B: IL4, C: IL17, D: IFN e, E: TNF c. F-G: WT and p110 f^{D910A} mice were sensitized on the abdomen with TNCB and then re-challenged with TNCB on one ear 6 days (F; primary response) or 6 and 30 days (G; secondary response) later. Mice were dosed twice orally with 30 mg/kg IC or methylcellulose (MCL) alone on the day of the final re-challenge, and the change in ear thickness was measured 24 h later. The effect of p110 f genetic or pharmacological inactivation was compared to WT mice receiving MCL only, and *p* values were calculated using one way ANOVA with Bonferroni

post-test, *p* values are represented with the following symbols: *0.01 p 0.05 **0.001 p 0.01 ***p 0.001. Data is representative of 3 experiments for primary responses and 1 experiment for secondary responses.

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Figure 4. p110 **f** regulates TCR signaling by human T cells

(A) T cells from healthy human donors were pre-treated with DMSO alone, IC or LY and stimulated with anti-CD3 and anti-CD28 for 5 min. Lysates were immunoblotted to examine the levels of total and phosphorylated Akt and Erk. Data represents one of three donors. (B-F) T cells from healthy human donors were pre-treated with DMSO alone, IC or LY and stimulated with anti-CD3 and anti-CD28 for different time points. The pooled bar-coded aliquots were stained for surface markers and phospho-proteins and analyzed by flow cytometry. Populations were gated into na ve (N) and effector/memory (E/M) and CD4$^+$ CD8$^+$ T cells and changes in phosphorylation were assessed for each population. Histograms showing dynamics of phosphorylation of Akt (B), GSK3 d (C), Erk (D) and S6

(E) in DMSO treated samples are shown. (F) The data for DMSO and inhibitor-treated samples were converted into a heat map showing fold change compared to unstimulated control. The data presented is representative or three independent experiments with different donors

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population, and median values shown below the graph. I-F: T cells were stimulated for 16 h with anti-CD3 and anti-CD28 in the presence of DMSO alone, IC or LY, and IL2 (I) or IFN e (J) production was measured. Data shows mean SEM of triplicate readings and is representative of one of three donors.

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Figure 6. p110 **f** inhibition blocks recall responses

PBMCs from healthy donors were stimulated with tetanus toxin in the presence of DMSO alone or IC. After 7 days, proliferation (A), IFN e (B), TNF c (C), and IL17 (D) production were measured. Each dot represents an individual donor. To calculate *p* values, one-way repeated measures ANOVA was used to compare DMSO alone to IC. *p* values are represented with the following symbols:*0.01 p 0.05 **0.001 p 0.01 ***p 0.001. % inhibition was calculated for each donor to determine the range of sensitivity in the population, and median values are shown below the graph.

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Figure 7. p110 **f** inhibition blocks recall responses from disease samples

(A,B) PBMCs from atopic individuals were pulsed with house dust mite antigen in the presence of DMSO alone or IC. After 6 days, IL5 (A) and IL13 (B) were measured. (C-F) Mononuclear cells from synovial fluid of ReA patients were stimulated with anti-CD3 in the presence of DMSO alone, IC or CS. Proliferation (C), IFN e (D), IL17 (E) and TNF c (F) production were measured at 72 h. Each dot represents an individual donor. To calculate *p* values, Student's *t* test was used to compare DMSO alone to CS, and one-way repeated measures ANOVA was used to compare DMSO alone to IC. *p* values are represented with the following symbols: *0.01 p 0.05 **0.001 p 0.01 ***p 0.001. % inhibition was

calculated for each donor to determine the range of sensitivity in the population, and median values are shown below the graph.

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