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Abstract

Class IA phosphoinositide 3-kinases (PI3K) are essential to function of normal and tumor cells, and modulate immune responses. T lymphocytes express high levels of p110α and p110δ class IA PI3K. Whereas the function of PI3K p110δ in immune and autoimmune reactions is well established, the role of p110α is less well understood. Here, a novel dual p110α/δ inhibitor (ETP-46321) and highly specific p110α (A66) or p110δ (IC87114) inhibitors have been compared concerning T cell activation *in vitro*, as well as the effect on responses to protein antigen and collagen-induced arthritis *in vivo*. *In vitro* activation of naive $CD4^+$ T lymphocytes by anti-CD3 and anti-CD28 was inhibited more effectively by the p110δ inhibitor than by the p110α inhibitor as measured by cytokine secretion (IL-2, IL-10, and IFN-γ), T-bet expression and NFAT activation. In activated $CD4^+$ T cells re-stimulated through CD3 and ICOS, IC87114 inhibited Akt and Erk activation, and the secretion of IL-2, IL-4, IL-17A, and IFN-γ better than A66. The p110α/δ inhibitor ETP-46321, or p110α plus p110δ inhibitors also inhibited IL-21 secretion by differentiated $CD4^+$ T follicular (Tfh) or IL-17-producing (Th17) helper cells. *In vivo*, therapeutic administration of ETP-46321 significantly inhibited responses to protein antigen as well as collagen-induced arthritis, as measured by antigen-specific antibody responses, secretion of IL-10, IL-17A or IFN-γ, or clinical symptoms.

Hence, p110α as well as p110δ Class IA PI3Ks are important to immune regulation; inhibition of both subunits may be an effective therapeutic approach in inflammatory autoimmune diseases like rheumatoid arthritis.

Keywords

T lymphocytes

CD28

ICOS

Phosphatidyl inositol-3 kinase

PI3K inhibitors

Rheumatoid arthritis

Abbreviations

CIA, Collagen Induced Arthritis; ConA, Concanavalin A; EAE, Experimental autoimmune encephalomyelitis; KLH, keyhole limpet hemocyanin; PI3K, Phosphoinositide 3-kinase; RA, Rheumatoid Arthritis; SLE, Systemic lupus erythematosus.

Chemical Compounds

A-66

ETP-46321

IC-87114

1. Introduction

Class I phosphoinositide 3-kinases (PI3K) phosphorylate the 3 position of 4,5-biphosphate phosphoinositides located in cell membranes. This produces phosphatidylinositide 3,4,5-triphosphate (PIP_3) allowing the binding of proteins possessing pleckstrin homology (PH) domains. The recruited proteins are then activated to start metabolic cascades essential to different aspects of cell growth, proliferation, survival, differentiation, and migration [1-3]. Consequently, they are also essential to the development of normal and pathologic immune responses, including organ-specific or systemic autoimmune diseases like rheumatoid arthritis and systemic lupus erythematosus [4-7].

Class I PI3K are heterodimers formed by regulatory and catalytic subunits; recruitment of regulatory subunits serve to bring the catalytic subunits close to the membranes were both PI3K activators and substrates are located. The nature of regulatory subunits further differentiates class I PI3K into class IA and class IB: Class IA catalytic isoforms (p110α, p110β and p110δ) form heterodimers with the regulatory subunits p85α, p55α, p50α, p85β, and p55γ; activation is characteristically dependent on regulatory subunit recruitment to Tyr-phosphorylated Y-x-x-M sequence motifs. Class IB catalytic subunits (p110γ) bind regulatory subunits p101 and p84/p87; they are activated upon binding of their regulatory subunits to G-protein coupled receptors (GPCR).

Class I PI3K subunits have oncogenic potential; and particularly the p110α catalytic isoform is frequently mutated in different cancer cells [8]. Hence, PI3K inhibitors have been actively pursued as anti-cancer drugs. However, their impact on the immune system should be also carefully determined, in first place, because the importance of PI3K in the development of immune reactions confers an immunotherapeutic potential on PI3K inhibitors; secondly, because the overall impact of these inhibitors in tumor immunity needs to be determined [9].

Whereas the expression of p110α and p110β catalytic subunits is wide, the p110δ and p110γ polypeptides are mainly expressed in hematopoietic cells including T and B lymphocytes. Indeed, genetic and pharmacological data show that both the class IA p110δ and the class IB p110γ subunits are essential to adequate development, activation and differentiation of B and T lymphocytes, as well as interesting targets for therapy in immunopathology or cancer immunotherapy [5, 7, 10-12]. When T and B lymphocytes are considered, class IA p110δ catalytic subunits have specific functions in signaling

through the antigen receptor, amplification of signals by B cell or T cell costimulatory molecules (i.e., CD19, CD28, ICOS (CD278, also called H4 [13]), through TNF family molecules, or cytokine receptors.

Among Class IA PI3Ks, T lymphocytes express high levels of p110α and p110δ catalytic subunits and comparatively low levels of p110β [14]. Intriguingly, p110α binds better than p110δ to PI3K regulatory subunits, and consequently it is more efficiently recruited to costimulatory molecules like CD28 and ICOS [14], suggesting that p110α might play a relevant role in T lymphocyte function.

However, the data on the role of p110α in lymphocyte function are scarce, in part because of the embryonic lethality of p110α-deficient mice, in part because of the relative non-specificity of some p110α inhibitors [15, 16]. Still, specific silencing [14] or the use of highly specific inhibitors of p110α like A66 [17] show a clear, if minor, role of p110α on B cell and T cell activation. Here, the effect of ETP-46321, a dual inhibitor of the p110α and p110δ PI3K isoforms [18, 19] has been compared to the effect of highly specific p110δ- or p110α-specific inhibitors (IC87114 and A66, respectively). We show that ETP-46321 shares the characteristics of both inhibitors, and is a strong inhibitor of lymphocyte proliferation and the secretion of cytokines essential to antibody or inflammatory responses.

Rheumatoid arthritis (RA) is a systemic, inflammatory, autoimmune disease characterized by uncontrolled inflammation of the joints and the presence of autoantibodies directed against multiple autoantigens. Its precise etiology is unknown, yet T lymphocytes intervene in the pathogenic process, as shown by the presence of T cells in the inflammatory infiltrate of affected joints and the strong association of the disease with molecules involved in T cell activation [20]. Furthermore, blockade of CD28-dependent T-cell costimulation by abatacept (CTLA-4Ig) has provided a successful therapy for RA [21, 22].

Its relatively high incidence among the general population (approximately 1% worldwide) makes RA an important target for drug discovery. The animal model of collagen-induced arthritis (CIA) reproduces many features of human RA and is widely used to assess the effects of potential novel therapies [23].

The fact that p110δ PI3K contributes significantly to antigen activation of antibody producing B lymphocytes, but both the p110α and p110δ PI3K isoforms participate in

B lymphocyte development in the bone marrow and in B cell survival in the periphery [24] adds interest to the study of dual p110α and p110δ PI3K inhibitors in RA.
Our data indicate that therapeutic administration of ETP-46321 can be successfully used *in vivo* to inhibit secretion of antigen-specific antibodies and effector cytokines in response to protein antigen as well as in mice undergoing collagen-induced arthritis. Thus, PI3K inhibitors like ETP-46321 can be candidate drugs to treat abnormal adaptive immune responses, including autoimmune diseases where $CD4^+$ T lymphocytes and antibody responses have a prime role.

2. ***Materials and Methods***

2.1. Mice

C57BL/6 mice aged 8-16 weeks were used throughout this study. They were bred in the animal care facility of the Centro de Investigaciones Biológicas under specific pathogen-free conditions. For the collagen-induced arthritis experiments, female DBA/1OlaHsd mice were purchased from Harlan Laboratories (Horst, The Netherlands). They were housed in the animal facility of the Centro Nacional de Microbiología, Instituto de Salud Carlos III, in seal-safe cages under air flow supply. All the experimental procedures were performed according to established institutional and national guidelines.

2.2. Inhibitors

The PI3Kα/δ inhibitor ETP-46321 was synthesized by the Experimental Therapeutics Programme, Spanish National Cancer Research Centre (CNIO), as described [18, 19]. PI3Kα inhibitor A66 was from Selleck Chemicals (Houston, Texas); PI3Kδ inhibitor IC87114 was from Symansis Pty. (Timaru, New Zealand); LY 294002 was from Sigma-Aldrich (Saint Louis, Missouri). Some characteristics of these inhibitors are summarized in Table 1.

2.3. T lymphocyte isolation and activation

To obtain naive $CD4^+$ T lymphocytes ($CD4^+CD62L^+$ T cells), spleens were passed through a 70 μm mesh. After centrifugation, red blood cells were lysed and the cells washed in culture medium (Click's medium supplemented with 10% heat inactivated FCS). Then, naive $CD4^+$ T cells were isolated using Miltenyi $CD4^+CD62L^+$ T cell isolation kit II for mouse cells (Ref. 130-093-227, Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturers' instructions. The isolated cells were routinely >97% $CD4^+$, >95% $CD62L^+$. Cells (10^6) were cultured in 1 ml culture medium in 24-well culture plates (Costar) pre-coated with anti-CD3 antibody (YCD3-1 [30], 5 μg ml^{-1}). Where indicated, anti-CD28 (H57, 2.5 μg ml^{-1}, eBioscience, San Diego, California), DMSO or inhibitors dissolved in DMSO (1 μl per culture) were added. At 24h, the cultures were resuspended, centrifuged and the supernatants assayed for cytokine content.

To obtain IL-2-expanded $CD4^+$ T cell blasts, $CD4^+$ T cells were isolated from spleen cell suspensions after red cell lysis using the Miltenyi $CD4^+$ T cell isolation kit for mouse (Ref. 130-090-860, Miltenyi Biotec) according to the manufacturers' instructions. The isolated cells were routinely >95% $CD4^+$ and were activated for 48h (10^6 ml^{-1}) with Concanavalin A (ConA, Sigma-Aldrich, 3 μg ml^{-1}) with mitomycin-C (Sigma-Aldrich) treated T-cell depleted spleen cells (APC, $0.5x10^6$ ml^{-1}) plus IL-2 (PeproTech, Rocky Hill, New Jersey), isolated in Percoll gradient, and expanded for two days in IL-2-containing medium (4-days, ConA-induced, IL-2 expanded $CD4^+$ T cell blasts) as described previously [31]. The resulting blasts were washed and activated for further 24h ($0.5\text{-}1x10^6$ ml^{-1}) in fresh medium with plate bound anti-CD3 (YCD3-1, 10 μg ml^{-1}) and anti-ICOS (Anti-H4 C398.4A [13, 32], 20 μg ml^{-1}) or control antibodies. Th17 and Tfh cells were generated from naive $CD4^+$ T cells activated with ConA for 4 days under conditions favoring the differentiation of Th17 cells (anti-IL-4 antibody 11B11, 5 μg ml^{-1}; anti-IFN-γ antibody XMG1, 10 μg ml^{-1}; IL-6 (PeproTech) 20 ng ml^{-1}, IL-23 (PeproTech) 20 ng ml^{-1}, TGF-β (R&D Systems Europe, Abingdon, UK) 5 ng ml^{-1}), or Tfh cells (anti-IL-4 antibody 11B11, 5 μg ml^{-1}; anti-IFN-γ antibody XMG1, 10 μg ml^{-1}; IL-6 20 ng ml^{-1}). The cells were then re-activated for 24h as described above for IL-2 blasts. In the experiments described in **Figures 3c and 4c**, 10^6 Th17 or Tfh cells were mixed with an equal number of antibody-coated latex beads (Anti-CD3, 10 μg ml^{-1} plus anti-ICOS or control antibodies at 20 μg ml^{-1}) [31]. After overnight culture in 1 ml of complete culture medium in round-bottom 5 ml polystyrene tubes, the tubes were centrifuged and the supernatant taken for cytokine analysis.
The secreted cytokines were determined with Ready-Set-Go!® capture ELISA kits (eBioscience) specific for mouse IL-2, IL-10, IL-17A, IL-21, IFN-γ, or TNF-α, or as described in [31] (mouse IL-4). All antibodies used were obtained and purified in our laboratory unless stated otherwise.

2.4. Nuclear extracts, cell lysates and immunoblot

To obtain nuclear extracts, naive $CD4^+$ T lymphocytes ($2\text{-}3x10^6$ ml^{-1} in 24-well culture plates) were activated overnight with anti-CD3 and anti-CD28 antibodies. The cells were resuspended, and nuclear extracts were obtained as described in detail in [33]. The extracts were separated by SDS-PAGE, and immunoblotted with rabbit antibodies to NFATc1 (sc-13033, Santa Cruz Biotechnology, Dallas, Texas) as previously described

in [14]. As a load control, part of the same gel was cut and silver stained, as described in [34].

To detect phosphorylated proteins in cell lysates, four day $CD4^+$ Tfh cells were washed with PBS and activated for 20 min in serum-free medium at $40x10^6$ cells ml^{-1} by mixing with an equal number of latex beads previously coated with antibodies (Anti-CD3, 10 μg ml^{-1} plus anti-ICOS or control antibodies at 20 μg ml^{-1}), as described [31]. PI3K inhibitors were added to cells 10 min before activation. The reaction was stopped by adding excess ice-cold PBS, 500 μM EDTA, 200 μM $NaVO_4$. After centrifugation, the cells were lysed for 15 min on ice at $2x10^7$ cells ml^{-1} in Triton X-100 lysis buffer (1% Triton X-100, 50 mM Tris/HCl, 150 mM NaCl, pH 7.6 containing 1mM $MgCl_2$, 1 mM EGTA, 10 μg ml^{-1} aprotinin, 10 μg ml^{-1} leupeptin, 1 mM PMSF, and 1 mM $NaVO_4$). Cell lysates were centrifuged, and post-nuclear lysates were mixed v:v with 2x reducing SDS Laemmli sample buffer. Separation of proteins by SDS-PAGE, and immunoblot with anti-phosphoSer^{473}Akt (Cell Signaling Technology, Danvers, Massachusetts, #4060), anti-phosphoThr^{308}Akt (Cell Signaling Technology, #13038) or anti-phospho-ERK (Cell Signaling Technology) were performed as described previously [14]. Load controls were performed using polyclonal rabbit anti-Akt or anti-Erk antibodies [31].

2.5. T-bet expression

T-bet expression was determined by flow cytometry in naive $CD4^+$ T lymphocytes before or after overnight activation in 24-well plates with plate-bound anti-CD3 plus anti-CD28 antibodies, as described above. Cells were resuspended, washed, fixed and permeabilized with the Transcription Factor Staining Buffer Set (Affimetrix/eBioscience). Then, the cells were stained with anti-human T-bet-PE (eBioscience 12-5825) or control isotype antibody according to the protocol for intracellular staining of transcription factors. Cells were then analyzed in a FC-500 flow cytometer (Beckman Coulter, Brea, California).

2.6. Immunization with KLH protein

100 μg of keyhole limpet hemocyanin (KLH) in Freund's complete adjuvant (both from Sigma-Aldrich) were injected s.c. in two sites in the base of the tail. ETP-46321 suspended in 0.5% carboxymethyl cellulose, 20% sucrose in water, or vehicle, was administered by oral gavage (20 mg kg^{-1}) on days 3 to 6 after KLH injection. On day 7,

mice were bled to obtain the serum, sacrificed, and the spleen and the draining inguinal lymph nodes were extracted. Cell suspensions were obtained and used to determine total cell counts and lymphocyte subpopulations. In addition, lymph node cells from individual mice ($2x10^6$cells per culture) were re-activated *in vitro* in 1 ml of culture medium with or without KLH (100 μg ml^{-1}) in round-bottom 5 ml polystyrene tubes. After three days, culture supernatants were taken, and live cells assessed with an MTT assay, as described [35]. Cytokine content in the supernatants was determined by ELISA as describe above. Serum anti-KLH specific antibodies of the IgM, IgG1, IgG2b, and IgG3 subclasses were determined by ELISA in 96-well ELISA plates (Costar Life Sciences, Tewksbury, Massachusetts) coated with KLH (20 μg ml^{-1} in borate saline) and horseradish peroxidase-coupled goat anti-mouse Ig class-specific antibodies (Southern Biotech, Birmingham, Alabama). Serum titer was calculated as the reciprocal of the dilution giving 50% of maximal optical density.

2.7. Collagen-induced arthritis: Induction, assessment, and treatment

DBA/1OlaHsd mice were immunized intradermally under anesthesia at the base of the tail with an emulsion of chicken type II collagen in Freund's complete adjuvant (200 μg per mouse) essentially as described previously in [36]. Clinical symptoms of arthritis were assessed daily by scoring each limb of the mice according to the following criteria: 0 normal, 1 slight swelling and erythema, 2 pronounced edematous swelling, and 3 joint rigidity. The degree of joint swelling for each paw (scored from 0 to 3) was assessed and expressed as the cumulative arthritis severity score for 4 paws, with a maximum possible clinical score of 12 per mouse. Arthritis onset (the first day in which clinical signs of arthritis were observed) appeared at 3-5 weeks after immunization. Graphs were used to represent the change in clinical score in relation to the day of onset.
Mice showing an illness score equal to or higher than 1 in one paw were randomly assigned to the experimental groups. Mice were treated daily during 11 days with ETP-46321 suspended in 10% Dimethylpirrolydone (DMP, Sigma-Aldrich) pH-3/90% PEG300 (Sigma-Aldrich), at a dose of 20 mg/kg body weight per mouse. Oral administration of the same vehicle was used as a control. The day after the last administration of ETP-46321, the mice were bled to obtain sera and sacrificed to obtain inguinal lymph nodes.

2.8. Analysis of type II collagen–specific responses.

Single cell suspensions of draining inguinal lymph nodes from individual mice were prepared and cultured ($2x10^5$/200 μl) in the presence or absence of 50 μg/ml chicken type II collagen. After 48 hours, 100 μl of the supernatant was collected to measure cytokine production by ELISA. 24 hours later, proliferation in the remaining cell cultures was evaluated by WST-1 Cell Proliferation Assay Kit from Roche Applied Science (Penzberg, Germany) as indicated by manufacturer´s instructions. IFN-γ and IL-17A were quantified with mouse Ready-Set-Go!® capture ELISA kits (eBioscience). Collagen-specific antibodies in the serum were measured by ELISA, as described for anti-KLH antibodies, using 96-well plates coated with 2 μg/ml of chicken type II collagen in PBS.

2.9. Statistical analysis

The statistical significance of differences was calculated with the Student's *t* test, or the Mann Whitney test, using the Excel (Microsoft Corporation, Redmond, Washington) or GraphPad Prism 4 (GraphPad Software, Inc, La Jolla, California) applications. Differences with P values ≤0.05 were considered significant.

3. Results

3.1 Effect of ETP-46321 on the activation of naive CD4$^+$ T lymphocytes

The effect of the dual p110α and p110δ PI3K inhibitor ETP-46321 on the activation of naive $CD4^+$ T lymphocytes by anti-CD3 plus anti-CD28 was assessed and compared to the effect of inhibitors specific for the p110α (A66) and p110δ (IC87114) subunits (**Figure 1a-d**). TCR/CD3 activation of these cells is strongly dependent on costimulation by CD28, a molecule that recruits and activates class IA PI3K. In these assays, ETP-46321 significantly inhibited IL-2, IL-10 and IFN-γ secretion after 24h of culture (**Figure 1a-c**) or cell proliferation at 72h (data not shown). Of note, IFN-γ secretion was ten-fold more sensitive to inhibition than IL-2, and more than fifty fold than IL-10 secretion (**Figure 1a-c**, summarized in Table 2). Differences among cytokines concerning their sensitivity to inhibitors were also found using the p110δ inhibitor IC87114, yet IL-10 secretion was not significantly inhibited. The p110α

inhibitor A-66 had a lower but significant effect in this assay (**Figure 1a-c**), fitting with our recent data showing efficient association of the p110α subunit to CD28 [14]. The high sensitivity of IFN-γ to all PI3-K inhibitors correlated with a strong inhibition of T-bet induction, the main transcription factor for IFN-γ secretion (**Figure 1d**).
Costimulatory molecules like CD28 and ICOS mediate part of their costimulatory effects through a PI3-kinase-, Itk-, phospholipase Cγ1-, and Ca^{2+} flux-dependent activation of NFATc1 resulting in its nuclear translocation [37]. Consequently, the effect of ETP-46321 on the presence of NFATc1 in nuclear extracts was compared to that of p110α- and p110δ-specific inhibitors. The activation of NFATc1 was slightly inhibited by the p110α-specific inhibitor A66, and strongly by the p110δ-specific inhibitor IC-87114. Yet, the dual inhibitor ETP-46321 was the most effective at blocking NFATc1 activation (**Figure 1e**).

3.2. Effect of ETP-46321 on cytokine secretion by activated $CD4^+$ T cells

As a potential immunotherapeutic agent, it was also important to determine the effect of ETP-46321 or the p110α and p110δ inhibitors on the function of activated, effector $CD4^+$ T cells. The effect of ETP-46321 on cytokine secretion by activated T cells "in vitro" was firstly assessed using ConA-activated, IL-2 expanded $CD4^+$ T blasts. They were then reactivated with anti-CD3 antibody alone or, as these activated cells express the CD28-like, PI3K-binding costimulatory molecule ICOS, they were also activated by anti-CD3 plus anti-ICOS antibodies. ICOS costimulation enhanced secretion of some cytokines (IL-2, IL-4, IL-17A, IFN-γ), but not others (IL-10, **Figure 2**).
ETP-46321 efficiently inhibited all the cytokines analyzed in culture supernatants after 24 h of blast reactivation (IL-2, IL-4, IL-10, IL-17A, and IFN-γ), and particularly IL-2, IL-4, IL-17A, and IFN-γ (**Figure 2, Table 2**). The p110δ inhibitor IC87114 showed a similar profile, except for inhibition of IL-17A and IL-10, that was clearly less efficient (**Figure 2, Table 2**).
In this assay, the p110α inhibitor A66 needed micromolar concentrations to achieve significant inhibition of cytokine secretion, and was poor as an inhibitor of IL-10 (**Figure 2**). These data suggest that, in these cells, most cytokines depend more on p110δ than on p110α; on the other hand IL-10 secretion might be dependent on different PI3K isoforms.

3.3. Effect of p110α and p110δ inhibition on IL-21 secretion by Th17 and Tfh effector cells

IL-21 is essential to germinal centre formation and antibody production that is supported by Th17 cells and Tfh cells [38]. Since IL-21 is produced in an ICOS- and PI3K-dependent fashion [39, 40], its production was investigated in the $CD4^+$ blasts described above. However IL-21 levels were barely detectable in the IL-2-expanded, ConA-induced cells used, this could be due to an inhibitory effect of IL-2 [41]. Hence, to compare the effect of ETP-46321 and isoform-specific PI3K inhibitors on IL-21 secretion we used cells previously differentiated for four days into Th17 or Tfh cells (**Figures 3, 4, Table 2**). Both types of cells, and particularly Tfh cells, expressed high levels of ICOS in their surface. As shown in **Figure 3a**, differentiated Th17 cells produced much higher amounts of IL-17A than the IL-2 blasts cells depicted in **Figure 2d**, and IL-21 levels were clearly detectable (**Figure 3b**). IL-17A secretion was enhanced by ICOS costimulation, and was inhibited by all the PI3K inhibitors at concentrations two to five-fold lower than those observed in $CD4^+$ IL-2 blasts (compare **Figure 2d**, **Figure 3a**, see also Table 2). Yet, the pattern of IL-17A sensitivity to ETP-46321 or the p110α (A66) and p110δ (IC87114) inhibitors was similar to that previously observed in IL-2 blasts (**Figure 3a,** compare to **Figure 2d**).

IL-21 production was not enhanced by ICOS costimulation in differentiated Th17 cells (**Figure 3b**). ETP-46321 inhibited IL-21 secretion, although at micromolar rather than the nanomolar concentrations that inhibited IL-17A secretion. In contrast, IL-21 secretion was not significantly inhibited either by A66 or by IC87114 (**Figure 3b, Table 2**), suggesting that both p110α and p110δ contribute to IL-21 secretion in these differentiated cells. This was confirmed in experiments where Th17 cells were activated in the presence of the p110α- and p110δ-specific inhibitors A66 and IC87114 alone or combined; only the combination of both inhibitors significantly diminished IL-21 levels, as did the dual inhibitor ETP-46321 (**Figure 3c**). When the levels of IL-17A were analyzed, A66 and IC87114 showed an additive effect.

We also set up experiments in Tfh-differentiated cells that express high levels of ICOS to determine whether ICOS enhanced early signals like Akt and MAPK activation or cytokine secretion in these IL-21 producing cells, as well as the role of PI3K in these events. As shown in **Figure 4a**, Akt phosphorylation in Ser^{473} and Thr^{308} induced by CD3 re-activation of differentiated Tfh cells was efficiently enhanced by ICOS

costimulation, as did MAPK (Erk) activation. Inhibition of p110δ by IC87114 clearly inhibited Akt or Erk phosphorylation, yet the dual inhibitor ETP-46321 was an even more efficient inhibitor of these parameters. The p110α inhibitor A66 did not induce significant effects on Akt or Erk phosphorylation in these cells (**Fig. 4a**).
As in other activated cells expressing ICOS, in Tfh cells ICOS ligation significantly enhanced CD3-induced secretion of IL-17A, but not the secretion of IL-21. As observed in $CD4^+$ IL-2 blasts or Th17 cells, ETP-46321 significantly inhibited secretion of both IL-17A and IL-21 in Tfh (**Fig. 4b**). As in Th17 cells, in Tfh cells the p110α- and p110δ-specific inhibitors A66 and IC87114 significantly inhibited IL-17A, but not IL-21 secretion(**Fig. 4b,** and Table 2).

3.4. ETP-46321 inhibits ongoing immune responses to protein antigen "in vivo"
Oral treatment with ETP-46321 is effective *in vivo* as an antitumoral, and has well established pharmacokinetic properties, low "in vivo" clearance and good oral bioavailability [18, 19]. In view of its effects on lymphocyte activation "in vitro", experiments were set out to determine the ability of ETP46321 to inhibit immune responses "in vivo". Mice were immunized with the protein antigen KLH. After three days, the mice were orally administered ETP-46321 for four days at doses (20 mg kg^{-1}) that achieve micromolar concentrations in the serum [19], according to the schedule summarized in **Figure 5a**. The day after the last administration of ETP-46321, serum was collected and cells from the spleen and draining lymph nodes were obtained. The total number of cells in the spleen or lymph nodes was not significantly different in the ETP-46321-treated mice, nor was the percentage of different T and B lymphocyte subpopulations as determined by flow cytometry, including total B lymphocytes ($CD19^+$ cells) or germinal center ($CD19^+PNA^+$) B cells, total T lymphocytes ($CD3^+$ cells), $CD4^+$ and $CD8^+$ T cells, Treg cells ($CD3^+CD4^+Foxp3^+$), Tfh ($CD3^+CD4^+CXCR5^+ICOS^+$), or NK ($CD3^-CD16^+$) cells.
We then checked the levels of anti-KLH antibodies in the sera of the immunized mice; IgM antibodies were significantly enhanced, whereas the IgG3 antibodies (that are largely dependent on IFN-γ) were significantly diminished in ETP-46321-treated mice. Antibody titers of the IgG1 and IgG2b isotypes were not significantly modified (**Figure 5b**). To analyze the effect of ETP-46321 on antigen-specific proliferation and cytokine production, lymph node cells from individual mice were re-stimulated with KLH "in

vitro". Proliferation or IL-2 secretion by antigen-activated cells from ETP-46321-treated mice was not significantly modified, yet secretion of IL-4, IL-10, IL-17A, or IFN-γ was significantly lower in these mice (**Figure 5c**). This confirms the potential of ETP-46321 to control an ongoing immune response "in vivo".

3.5. ETP-46321 significantly inhibits collagen-induced arthritis

Since ETP-46321 inhibits T-dependent antibody responses as well as the production of cytokines that are involved in the development of arthritis and other autoimmune diseases, we checked the effect of oral administration of ETP-46321 in a therapeutic fashion in a model of collagen-induced arthritis. Daily administration of ETP-46321 (20 mg kg^{-1}) or vehicle started once clear clinical symptoms appeared; mice that did not develop arthritis were not included in the assay. As shown in **Figure 6**, administration of ETP-46321 for 11 days significantly inhibited the development of clinical symptoms in treated mice as compared to mice treated with vehicle; these mice continued to increase the severity of arthritis.

At the end of the treatment, mice were bled and checked for anti-collagen antibodies (**Figure 6b**). Total anti-collagen IgG antibody was lower in ETP-46321-treated mice than in vehicle-treated control mice, but the difference was not statistically significant. In contrast, anti-collagen antibody of the arthritis-relevant isotypes IgG1 and IgG2a was significantly lower in the mice treated with ETP-46321.

Cells from the draining lymph nodes were reactivated with collagen and checked for antigen-specific proliferation and cytokine secretion "ex vivo". No significant differences in the number of cells recovered from the lymph nodes, the proportion of the main functional T and B lymphocyte subsets, or the proliferation induced by collagen were observed in ETP-treated mice (**Figure 6c**, and data not shown). Interestingly, collagen-induced IFN-γ secretion was clearly and significantly lower in ETP-46321 treated mice (**Figure 6c**); IL-17A was also lower in ETP-46321-treated mice, but the difference was not significant. In contrast, proliferation or cytokine secretion in response to a polyclonal stimulus like anti-CD3 antibodies was not statistically significantly inhibited by ETP-463231 administration "in vivo" (data not shown).

4. Discussion

Class I PI3-kinases are essential to different steps in the development of normal immune responses, including antigen activation of T and B lymphocytes and their expansion and differentiation into effector cells. In T cells, class IA PI3-kinases participate in antigen signaling through the TCR/CD3 complex as well as in costimulation by the CD28 family molecules CD28 and ICOS. Class IA PI3K catalytic subunits (p110α, p110β, p110δ) are differentially expressed in distinct tissues. T lymphocytes and T cell lines have similar levels of the widely expressed p110α or the p110δ catalytic subunit preferentially expressed by cells of hematopoietic origin [14]. Abundant genetic and pharmacological data show a major role of the p110δ in the initiation and development of normal and pathological immune responses (reviewed in [3, 7, 10, 11]). Intriguingly, we have recently observed that p110α is the main catalytic isoform bound to the costimulatory molecules CD28 and ICOS through the recruitment of PI3K regulatory subunits [14]. This poses the question of the role of p110α in T cells.

Analysis of p110α subunit's role in immune responses has been hampered by the embryonic lethality of p110α deficiency, and by specificity problems of some p110α inhibitors. Available data on the role of p110α in T cell function are scarce, but show that p110α silencing or inhibition induces low or moderate inhibition of cell viability [14, 28]. Proliferation and secretion of cytokines including IL-2, IL-4, or IFN-γ are also inhibited by p110α-specific inhibitors [14, 17]. Early Akt phosphorylation was significantly inhibited by p110α silencing or inhibition, whereas p110α inhibitors had low effect on Erk activation, and p110α silencing enhanced Erk phosphorylation [14]. Here, we have extended this analysis to compare the effect of the p110α and p110δ inhibitors A66 and IC87114, or the dual inhibitor ETP-46321, on the activation of naive and activated CD4 T cells to secrete cytokines like IL-2 involved in T cell growth, or IL-10, IL-17A, IL-21, or IFN-γ, that are critically involved in the control of inflammation and antibody production (**Figures 1-4**, Table 2, and data not shown).

Our titration of the PI-3 kinase inhibitors allowed a better assessment of the role of specific catalytic isoforms in the activation of naive T lymphocytes. CD28 costimulation was necessary for effective activation of naive $CD4^+$ T cells to proliferate or secrete IL-2, IL-10, and IFN-γ, or to induce the T-bet transcription factor essential to IFN-γ production. The p110α inhibitor A66 inhibited these parameters, albeit not as

effectively as IC87114; ETP-46321 was more effective than either inhibitor, in agreement with data showing that joint inhibition of p110α and p110δ is more effective than p110δ inhibition alone [14, 17]. Our results also show that IFN-γ secretion is clearly more sensitive to inhibition than cell growth or IL-2 secretion.
Naive $CD4^+$ T lymphocyte activation and differentiation into effector cells is mediated by CD28/ICOS and PI3K activation of NFAT in a Tec-kinase dependent pathway [37, 42, 43]. As in other parameters, in NFAT activation we observed a low effect of p110α inhibition, a clear effect of p110δ inhibition, and an enhanced effect upon dual inhibition by ETP-46321.

All these data indicate that ETP-46321 is a very efficient inhibitor of the initial steps of $CD4^+$ T naive activation. To assess its suitability in immunotherapy, we went on to determine its effects on the effector phase of $CD4^+$ T cell responses, as well as its possible advantages over p110α- and p110δ-specific inhibitors. Particularly, we were interested in determining its effect on cytokine secretion by $CD4^+$ T activated cells, including the secretion of IL-17 and IL-21 that characterize differentiated Th17 and Tfh cells, or the role of ICOS costimulation. CD28 and ICOS, as well as p110δ, are required for the differentiation of Th17 cells [44, 45]. On the other hand, ICOS and p110δ PI3K are involved in IL-21 production by activated T cells and Tfh differentiation [39, 40, 46, 47]. Th1, Th17, and Tfh contribute to autoimmune arthritis and other inflammatory diseases [48-50].
In activated cells, including Th17 and Tfh cells, ICOS clearly costimulated secretion of most cytokines assayed (IL-2, IL-4, IL-17A, or IFN-γ), but not IL-10 or IL-21 secretion (**Figures 2-4**). The effect of the different PI3-K inhibitors tested was similar in cultures activated or not in the presence of ICOS ligands. At the concentrations used, A66 significantly inhibited all cytokines assayed except for IL-21, and was particularly effective on IL-2 and IFN-γ secretion, yet the p110δ inhibitor IC87114 was roughly ten-fold more effective. As in naive T cells, ETP-46321 was more effective than the isoform-specific inhibitors (**Figures 2-4**, summarized in Table 2). Interestingly, only the dual inhibitor ETP-46321 could significantly inhibit IL-21 secretion by Th17 or Tfh cells; furthermore, this effect was mimicked by combining the p110α and p110δ inhibitors, suggesting a non-redundant function of these PI3K subunits (**Figure 4**).

The fact that, unlike most cytokines, IL-21 secretion was not enhanced by ICOS costimulation, or significantly inhibited by the p110δ inhibitor IC87114 was surprising as, in agreement with previous data [39, 51], we observed that ICOS clearly enhanced early activation of Akt, Erk, or IL-17 secretion in the same cells, that were significantly inhibited by IC87114. Previous reports show that IL-21 production by activated cells is fostered by ICOS costimulation in a PI3K-dependent way, with a relevant role for p110δ [39, 40, 51, 52]. As IL-21 in these previous studies was analyzed in cells activated for two days, one possible explanation is that ICOS and p110δ might be important for production during the early phases of differentiation of IL-21 producing cells, but not for already differentiated effector cells such as those used in our assays. In this regard, ICOS is essential for Tfh differentiation [38, 46, 47] in its early steps [53, 54]. This might be due in part to enhanced IL-21 secretion during differentiation via enhanced NFATc activation by ICOS, as NFAT binding sites are present in the IL-21 promoter [55].

As in IL-21, only the dual inhibitor ETP-46321significantly inhibited IL-10 secretion by activated, $ICOS^{+}CD4^{+}$ T cells. IL-10 is a peculiar cytokine because i) it can be secreted by different lymphocyte and leukocyte populations; ii) its secretion is controlled by many different signaling pathways and transcription factors; and iii) the weight of different signals and transcription factors in IL-10 secretion greatly vary among distinct leukocyte populations and lymphocyte subpopulations (see [56], for a review). Erk and c-Maf are involved in IL-10 secretion in most T lymphocytes, with variable contribution of other factors including STAT3,4 or 6, Jun, and SMAD. The reason(s) for the poor inhibition of IL-10 secretion by PI3-K inhibitors, as well as the need for dual p110α and p110δ inhibition for partial suppression of IL-10 in these cells are unclear. Since p110δ inhibition alone is very efficient in blocking Erk activation in different mouse and human T cells ([14, 57], Figure 4), the effect of PI3-K inhibitors on pathways other than Erk needs to be further explored.

As ETP-46321 inhibited IL-17A, IL-21, or IFN-γ, that are key players in autoimmune diseases like rheumatoid arthritis, SLE, or EAE [50], we determined its therapeutic potential in suppressing an ongoing immune response “in vivo” against the protein antigen KLH or collagen-induced arthritis. In the T-cell-dependent anti-KLH response, administration of ETP-46321 began on day three after immunization with KLH; at this

time the differentiation of effector cells like Tfh is close to its maximum [46, 53, 54, 58]. In fact, we did not find significant differences in the number of Tfh cells in ETP-46321-treated or –untreated, KLH-immunized mice (data not shown). In contrast, we found significantly diminished antibody responses to KLH of the IgG3 isoform. The effect of ETP-46321 on IgG3 might be due to inhibition of IFN-γ production, as IgG3 production is dependent on IFN-γ, a cytokine that was clearly reduced in lymph node cells from ETP-46321-treated mice. Thus, ETP-46321 might also have an impact on IFN-γ- and IgG3-dependent autoimmune diseases like lupus nephritis [59, 60]. It should be noted that IFN-γ, ICOS, and PI3K play important roles in murine models of SLE or human lupus [50, 61-66]. IL-21 also plays a role in mouse models of lupus [62, 67], yet IL-21 levels in our cultures of cells from immunized mice were below the detection limits of the assay. Therapeutic treatments with ETP-46321 "in vivo" also inhibited IL-17A. This raised the possibility of using ETP-46321 in autoimmune diseases where IFN-γ, IL-17 and PI3K have a prime role in the pathogenesis, including EAE [45], psoriasis [68], or rheumatoid arthritis [69, 70]. Indeed, oral administration of the dual inhibitor ETP-46321 significantly inhibited the clinical symptoms of collagen induced arthritis, the titer of pathogenic anti-collagen antibodies in serum, or the production of IFN-γ by collagen-activated lymph node cells.

In summary, our data using specific inhibitors show that p110δ is functionally the main class IA PI3K in CD28-dependent proliferation and differentiation of $CD4^+$ T lymphocyte into cytokine-producing cells; similar effects were observed in already differentiated effector cells "in vitro". By contrast, p110α has a variable, non-redundant role with p110δ in T cell signaling and function, being more important to the function of activated $CD4^+$ T cells. Clear differences among cytokines were observed when considering the role of ICOS, as well as concerning their sensitivity to inhibition by p110α- or p110δ- specific PI3-kinase. The dual inhibitor ETP-46321 was clearly the most effective and produced a significant inhibition in most parameters considered, in agreement with the abundance of p110α and p110δ, but not p110β, in lymphocytes. Furthermore, administration of the p110α and p110δ PI3K inhibitor ETP-46321 to immunized mice significantly inhibited certain antibody and cytokine responses to a protein antigen, pointing to its potential as a therapeutic agent in autoimmune diseases, which was confirmed in a model of collagen-induced arthritis.

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Disclosures

The authors declare no financial conflict of interest.

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Figure Legends

Figure 1. Impact of PI3-kinase inhibitors on the activation of T CD4$^+$ naive lymphocytes.

Naive ($CD4^+CD62L^+$) T lymphocytes were activated with anti-CD3 antibodies in the presence (closed squares) or absence (open squares) of soluble anti-CD28, or left unstimulated (triangles). PI3K inhibitors specific for p110α and p110δ (ETP-46321), p110α (A66), or p110δ (IC87114) were added, as indicated. After 24 h, the content of **a)** IL-2, **b)** IL-10, and **c)** IFN-γ in culture supernatants was determined by ELISA. Data are the mean ± SEM of triplicate samples from one representative experiment of three performed; *, significant differences ($p < 0.05$, Student's t-test) with samples with vehicle. In **d)** and **e)** the cells were activated for 16-24 h with anti-CD3 and anti-CD28 antibodies plus inhibitors (5 μM), as shown. In **d)**, the presence of the T-bet transcription factor was determined by flow cytometry. In **e)**, nuclear extracts were separated by SDS-PAGE and the NFATc1 nuclear factor detected by immunoblot. To assess the protein load, the lower part of the same SDS-PAGE gel was cut silver stained; The relative O.D. in each lane is plotted in the right panel.

Figure 2. PI3-kinases p110α and p110δ modulate the activation of T CD4$^+$ blasts by TCR/CD3 stimulus and ICOS costimulus.

ConA-induced, IL-2 expanded $CD4^+$ T cell blasts were reactivated with anti-CD3 antibodies in the presence (closed squares) or absence (open squares) of anti-ICOS antibodies as a costimulus. Results from unstimulated cultures are shown as open triangles. PI3K inhibitors (p110α/p110δ, ETP-46321, p110α, A66; p110δ, IC87114) were added, as indicated. After overnight culture, supernatants were analyzed by ELISA for cytokine content, as follows: **a)** IL-2, **b)** IL-4, **c)** IL-10, **d)** IL-17A, and **e)** IFN-γ. Data are the mean ± SEM of triplicate samples from one representative experiment of three performed; *, significant differences ($p < 0.05$, Student's t-test) with anti-CD3 plus anti-ICOS activated samples with vehicle. Triangles show data from unstimulated cultures.

Figure 3. Effect of ICOS and PI3K inhibitors on cytokine secretion by Th17 cells.

$CD4^+$ T cells were cultured for four days under Th17-differentiation conditions. In **a)** and **b)**, cells were reactivated for 24h with anti-CD3 antibodies in the presence (closed squares) or absence (open squares) of anti-ICOS antibodies as a costimulus, or left unstimulated (open triangles). PI3K inhibitors (p110α/p110δ, ETP-46321; p110α, A66; p110δ, IC87114) were added, as indicated. After overnight culture, supernatants were analyzed by ELISA for **a)** IL-17A, and **b)** IL-21 content. **c)** Th17 cells were reactivated for 24h with anti-CD3 antibodies plus anti-ICOS antibodies in the presence of PI3K inhibitors (p110α/p110δ, ETP-46321; p110α, A66; p110δ, IC87114), as indicated. Black triangles indicate data from cultures receiving A66 plus IC87114 (p110α + p110δ). Data are the mean ± SEM of triplicate samples from one representative experiment of four performed; *, significant differences ($p < 0.05$, Student's t-test) with control cultures with vehicle.

Figure 4. Effect of ICOS and PI3-kinases on early signals, IL-17A, and IL-21 secretion by Tfh cells.

$CD4^+$ T cells were cultured for four days under Tfh-differentiation conditions. Then, **a)** Tfh cells were activated for 20 min with anti-CD3 antibodies in the presence or absence of anti-ICOS antibodies, and phosphorylation of Akt ($pSer^{473}$Akt (top); $pThr^{308}$Akt (middle)), or Erk (pErk, bottom) was determined in cell lysates by immunoblot. Anti-Akt or anti-Erk antibodies were used for loading controls, as shown in the figure. Where indicated, 1 μM PI3K inhibitors (p110α/p110δ, ETP-46321 (α/δ); p110α, A66 (α); p110δ, IC87114 (δ)) were added. The relative O.D. in each lane is plotted in the right panels. *, significant differences with samples in lane 3 ($p < 0.05$, Student's t-test).
b) Tfh cells were reactivated overnight with anti-CD3 plus anti-ICOS antibodies (closed symbols) or anti-CD3 antibody alone (open squares). PI3K inhibitors (p110α/δ, ETP-46321; p110α, A66; p110δ, IC87114) or vehicle were added as indicated in the figure. Culture supernatants were analyzed for IL-17A or IL-21 content by ELISA. Data are the mean ± SEM of triplicate samples from one representative experiment of four performed; *, significant differences with control cultures with vehicle ($p < 0.05$, Student's t-test).

Figure 5. Effect of ETP-46321 administration "in vivo" on KLH-specific antibody and cytokine responses.

a) Mice (n=5 per group) were immunized with KLH on day 0; ETP-46321 (20 mg kg^{-1}) was administered p.o. four days beginning the third day after immunization and sacrificed the next day, as indicated.
b) Serum was obtained on day 7 and individually analyzed for KLH-specific antibodies of the IgM, IgG1, IgG2b, and IgG3 subclasses.
c) Cells from the draining lymph nodes of the same mice (n=5 per group) were obtained on day 7 and reactivated with KLH for three days "in vitro". Then, cell growth was determined by a colorimetric assay using MTT (left panel), and IL-2, IL-4, IL-10, IL-17A, and IFN-γ in culture supernatants was determined by ELISA, as indicated. *, significant differences between samples from different mice ($p < 0.05$, Mann Whitney test). N.S.: Non significant.

Figure 6. Administration of the PI3K p110α/δ inhibitor ETP-46321 inhibits collagen-induced arthritis and collagen-specific immune responses.
a) Mice were immunized with chicken type II collagen; appearance of clinical symptoms of arthritis was assessed daily and scored in each limb, as follows: 0 normal, 1 slight swelling and erythema, 2 pronounced edematous swelling, 3 joint rigidity (maximum possible score of 12 per mouse). Upon appearance of clinical symptoms (clinical score ≥1) each mouse was randomly assigned to treatment with vehicle or ETP-46321 (20 mg kg^{-1}) p.o. for 11 days (Vehicle n=9; ETP-46321 n=10). Data represents the mean ±SE for each experimental group. *, significant differences between samples ($p < 0.05$).
b) Mice immunized with chicken type II collagen and treated with vehicle or ETP-46321 (20 mg kg^{-1}) p.o. 11 days, as described in **a)**. On day 12 after initiation of treatment, mice were bled and the serum from each mouse was individually analyzed for whole IgG collagen-II-specific antibodies, or the clinically relevant IgG1 and IgG2a subclasses (Vehicle n=7; ETP-46321 n=6).
c) Cells from the draining lymph nodes of each treated mice described in **b)** were individually activated "in vitro" with type II chicken collagen II (Vehicle n=7; ETP-46321 n=6). After 72 h of culture, supernatants were taken and antigen-specific proliferation determined by a colorimetric assay using WST-1. Secreted IL-17A and IFN-γ was determined by ELISA. *, significant differences between samples ($p < 0.05$); N.S.: Non significant.

Table 1. IC50 values (nM) of PI3K inhibitors for catalytic activity of different PI3K isoforms.

	p110α	p110β	p110δ	p110γ	mTORC1/C2
A66 (α)	32.0	20 000.0	18 050.0	18 810.0	
IC87114 (δ)	>100 000.0	1 820.0	70.0	1 240.0	
ETP-46321 (α,δ)	2.4	549.0	14.0	153.0	>5 000.0
LY294002 (Broad)	700.0	306.0	1 330.0	7 260.0	8 910.0

Data compiled from references [17-19, 25, 26] and [27-29].

Table 2. Cytokine secretion by $CD4^+$ T cells: Sensitivity to PI3-K inhibitors

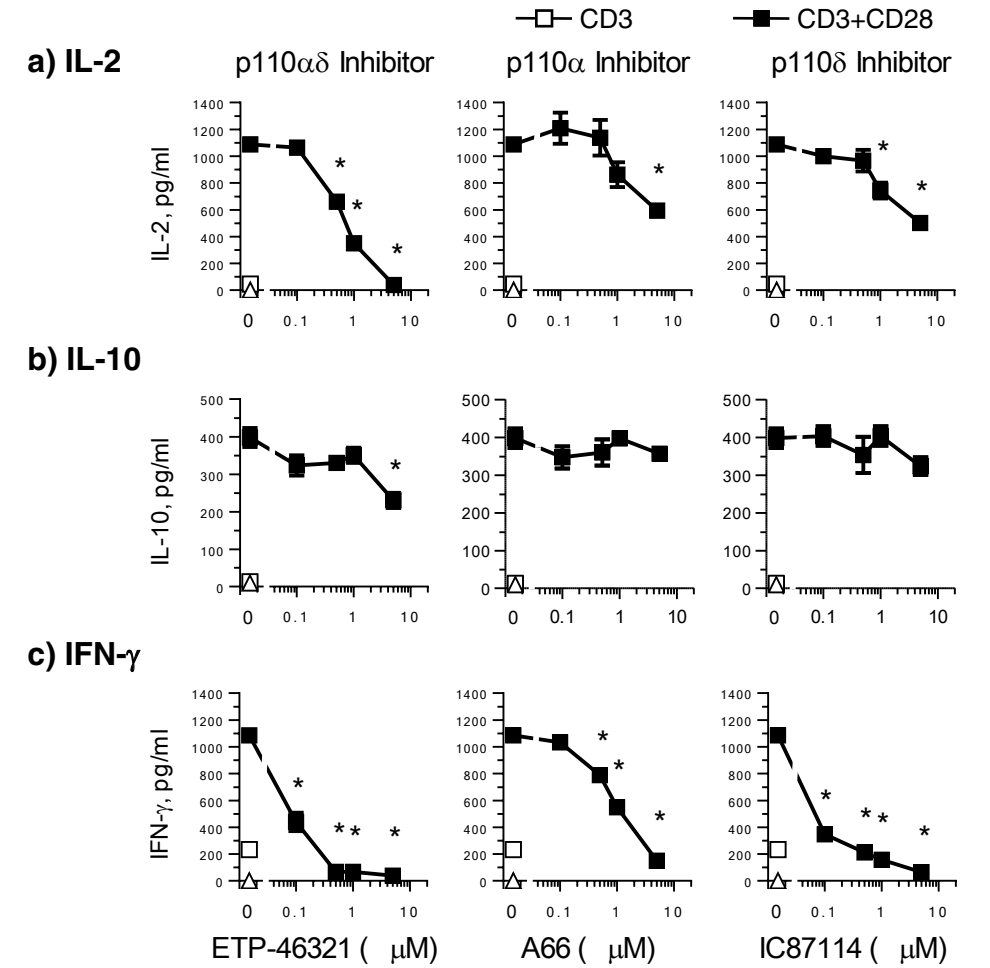
Inhibitor (Specificity)	Cells	IC50 (μM)					
		IL-2	IL-4	IL-10	IL-17A	IL-21	IFN-γ
ETP-46321	Naive $CD4^+$ T cells [a]	0.70		>5.00			0.07
(p110α/δ)	T $CD4^+$ IL-2 Blasts [b]	0.20	0.10	5.00	1.00	n.d.[c]	0.20
	Th17 Cells [b]				0.20	2.50	
	Tfh Cells [b]				0.20	0.90	
A66	Naive $CD4^+$ T cells [a]	5.00		>10.00			1.00
(p110α)	T $CD4^+$ IL-2 Blasts [b]	2.00	2.20	>10.00	5.00	n.d.[c]	3.20
	Th17 Cells [b]				2.50	>10.00	
	Tfh Cells [b]				3.50	>10.00	
IC87114	Naive $CD4^+$ T cells [a]	3.50		>10.00			0.06
(p110δ)	T $CD4^+$ IL-2 Blasts [b]	0.10	0.07	>10.00	5.00	n.d.[c]	0.10
	Th17 Cells [b]				1.00	>10.00	
	Tfh Cells [b]				0.80	>10.00	

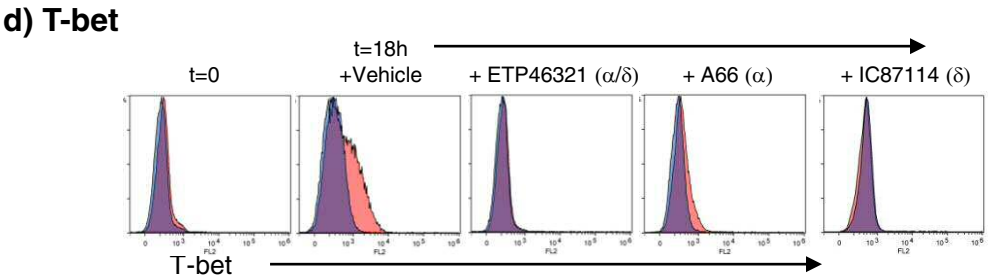
[a] Activated 24h by anti-CD3 plus anti-CD28 antibodies.

[b] Activated 24h by anti-CD3 plus anti-ICOS antibodies.

[c] n.d.: Not detected in these cells

Figure 1.ppt





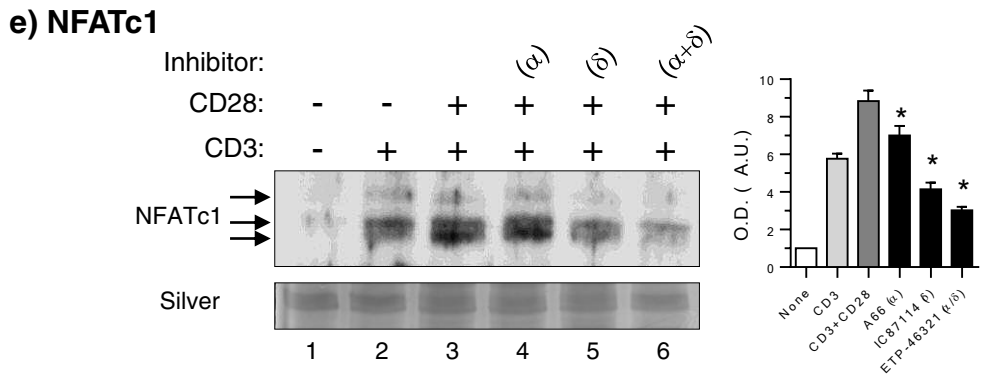


Figure 01e-f. Activation of T CD4+ naive lymphocytes

Figure 2.ppt

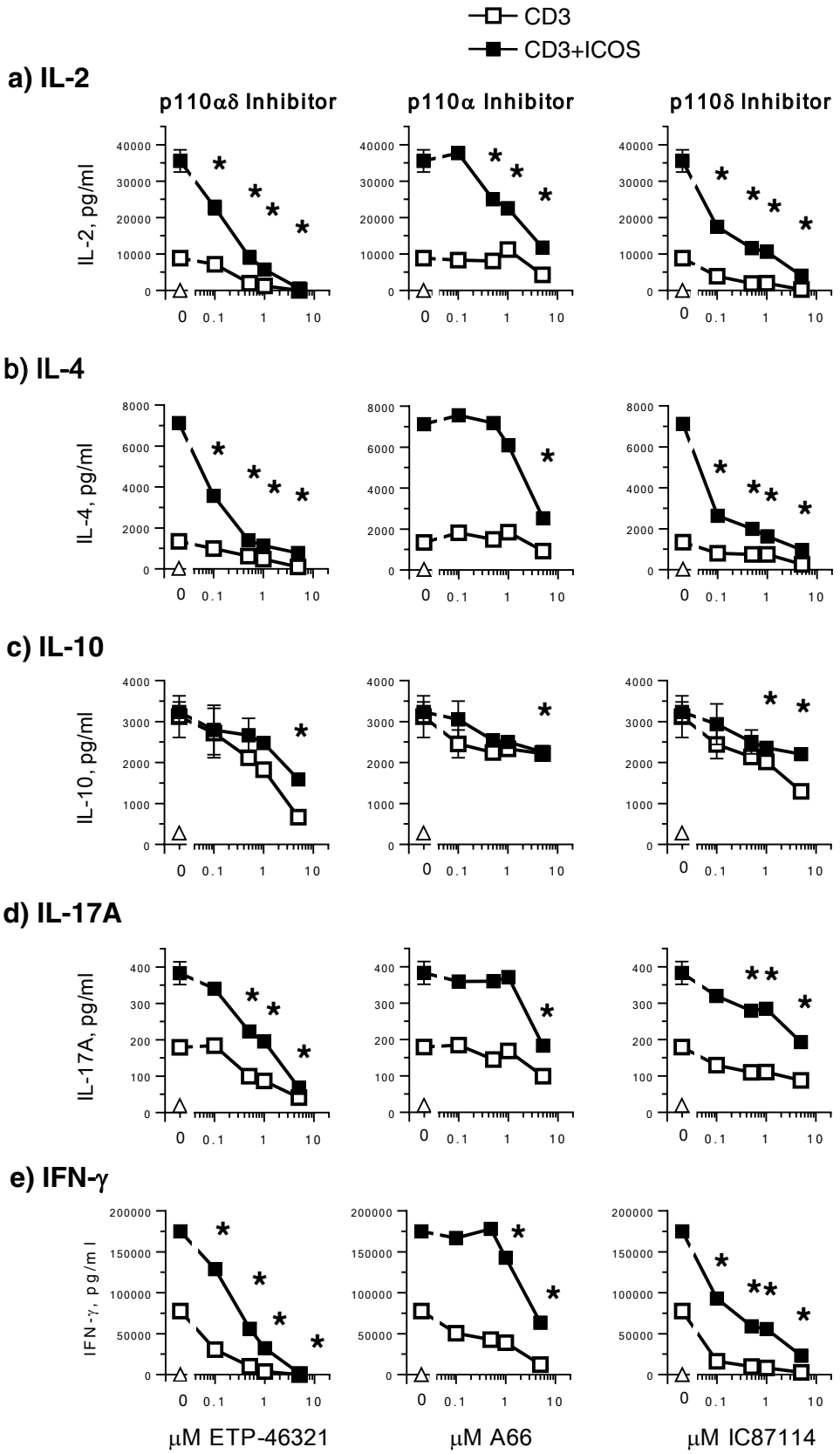


Figure 2. Activation of T CD4+ blasts

Figure 3.ppt

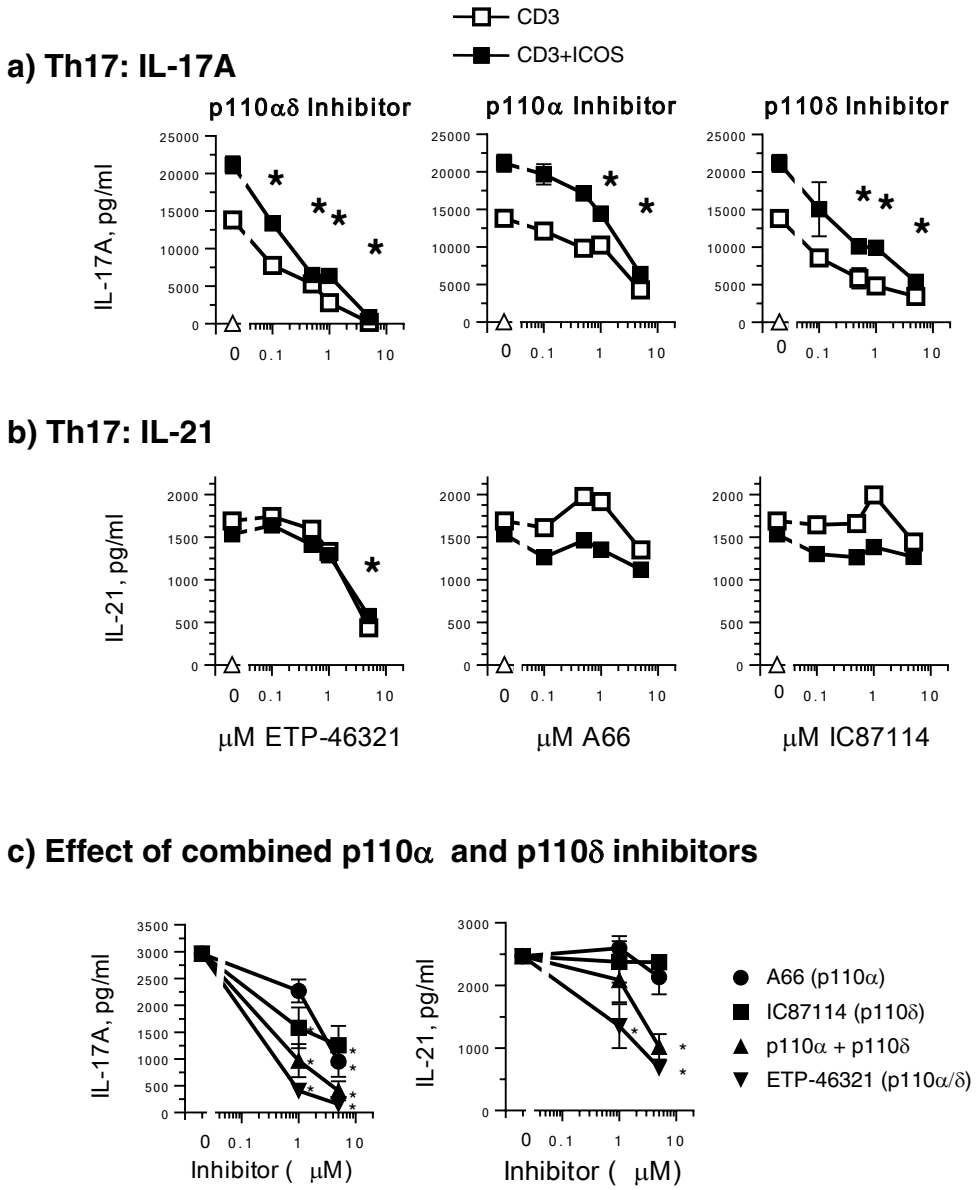


Figure 03. Effect of ICOS and PI3-K inhibitors on CD3 activation of cytokine secretion by Th17 cells.

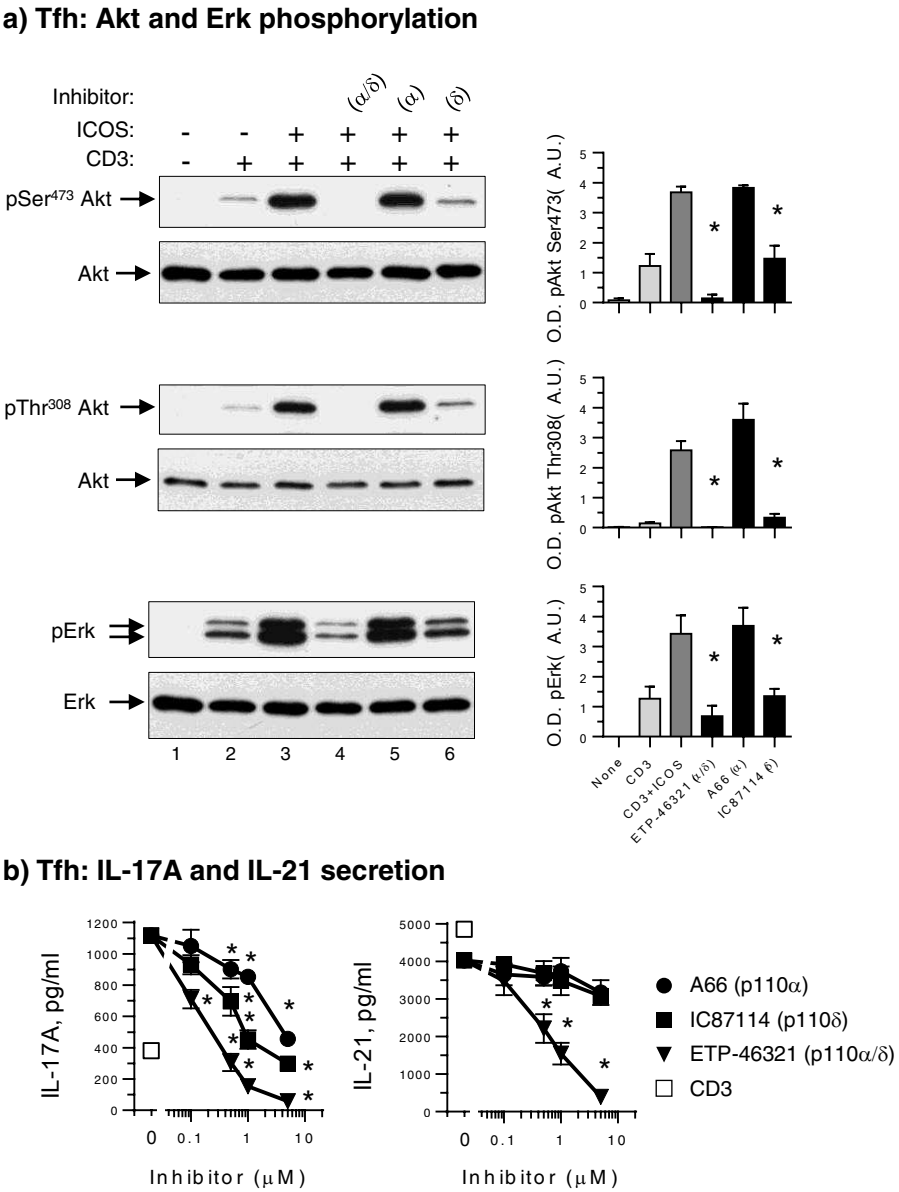


Figure 04. Effect of ICOS and ETP-46321 on CD3 activation of Tfh cells. a) Early Akt and Erk phosphorylation; b) IL-17A and IL-21 secretion.

Figure 5.ppt

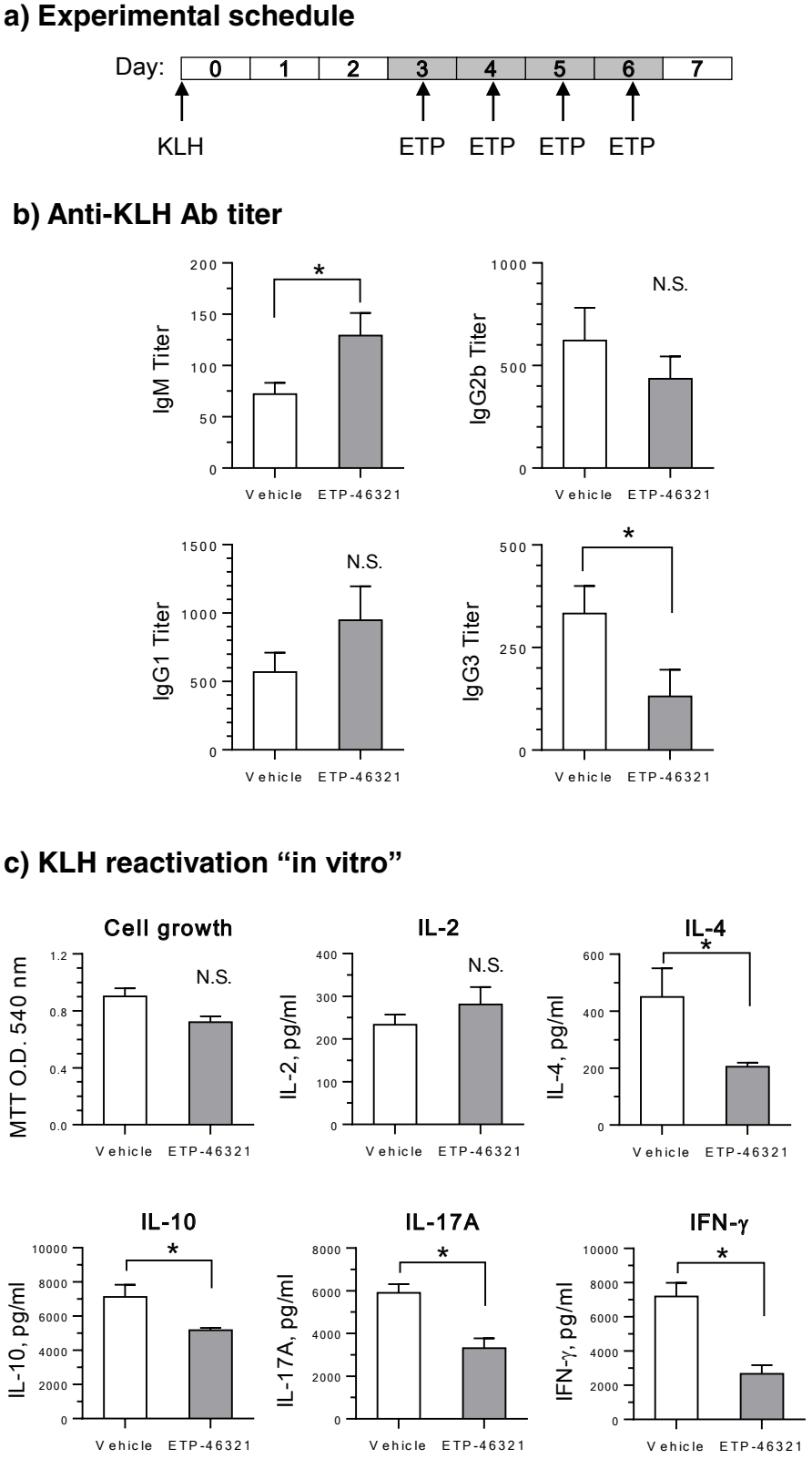


Figure 05. Effect of ETP-46321 administration "in vivo" on antibody and cytokine responses to KLH.

Figure 6

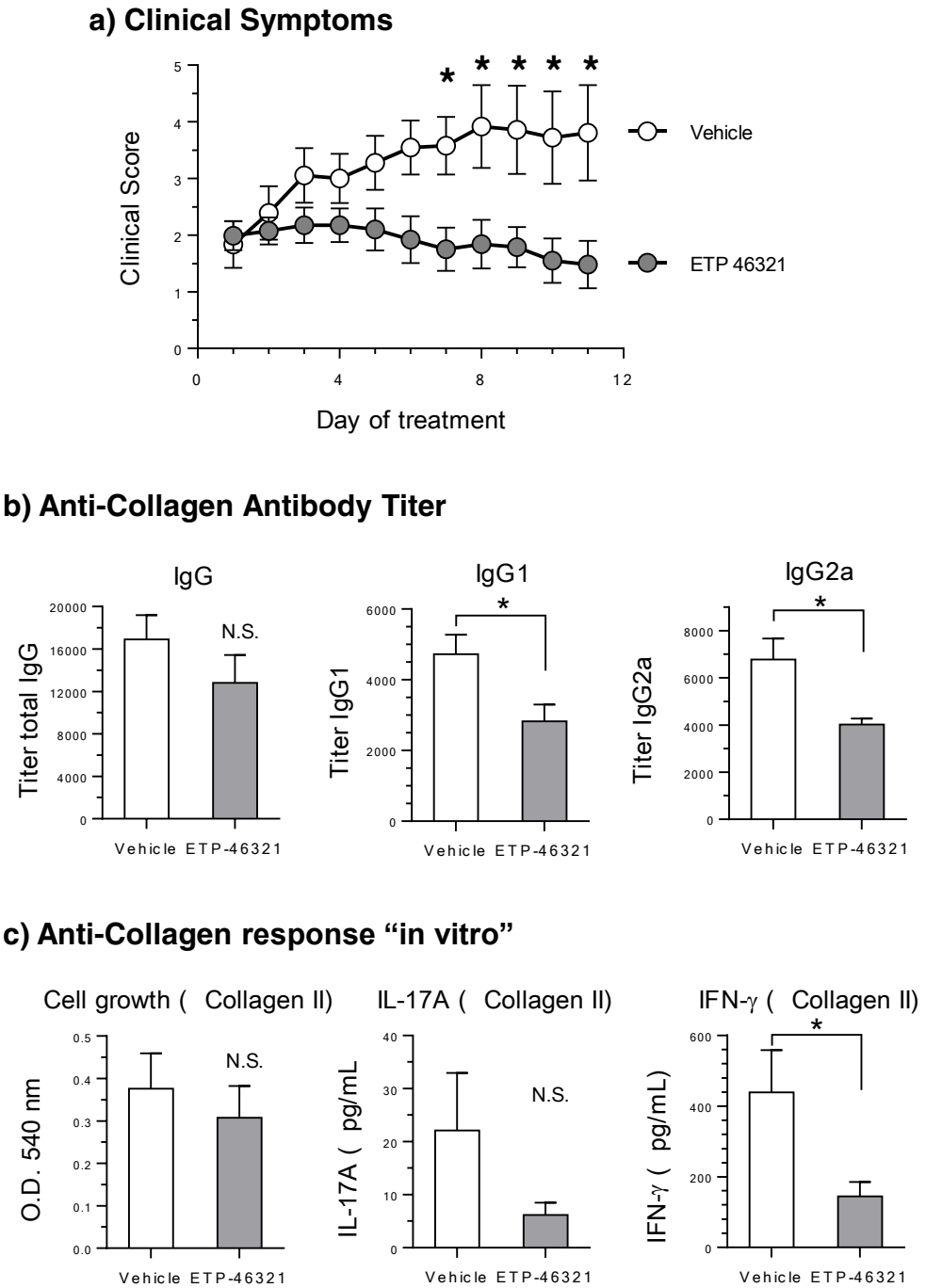


Figure 06. Effect of ETP-46321 administration "in vivo" on collagen-induced arthritis and anti-collagen response.

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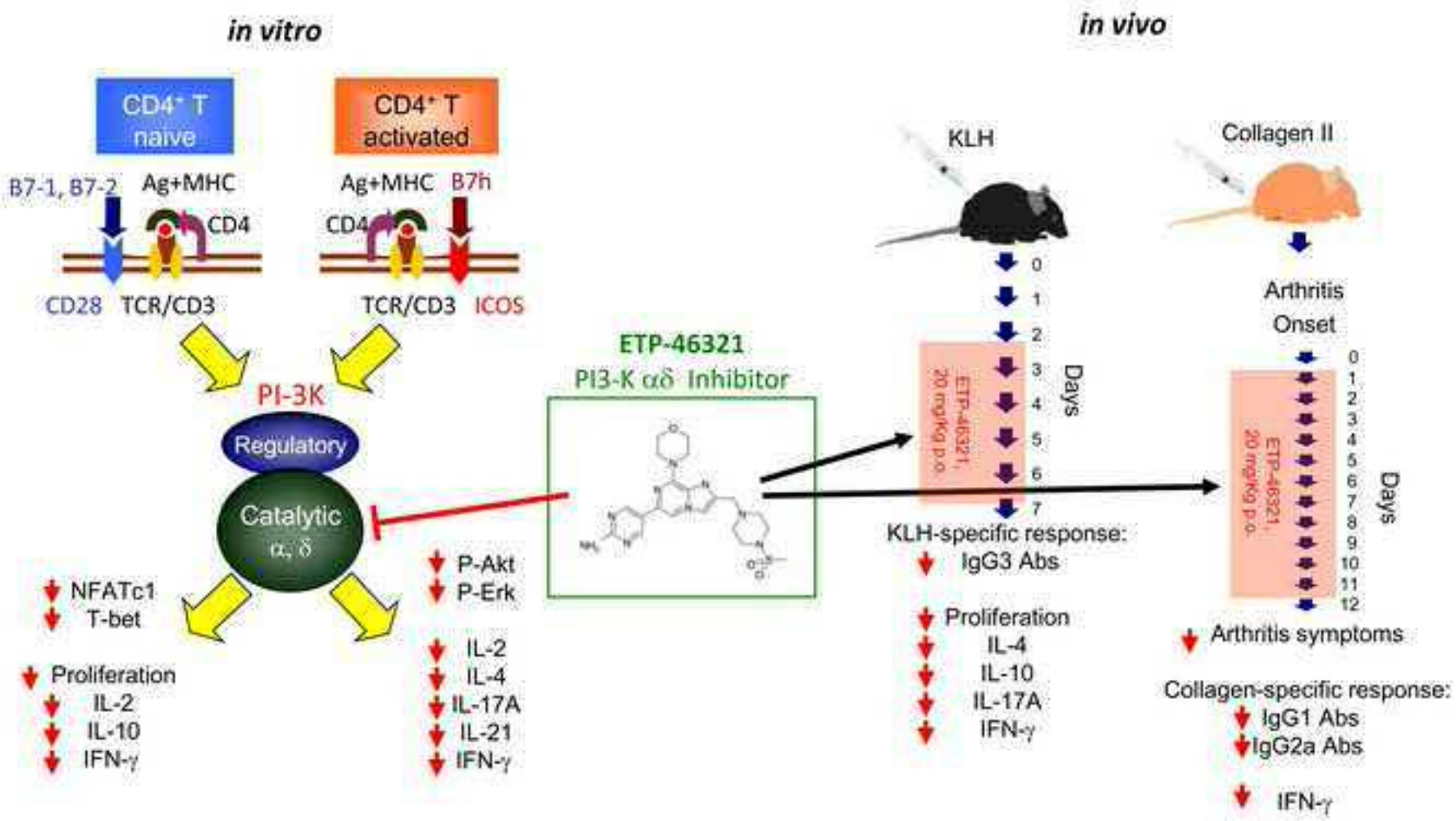

Graphical Abstract

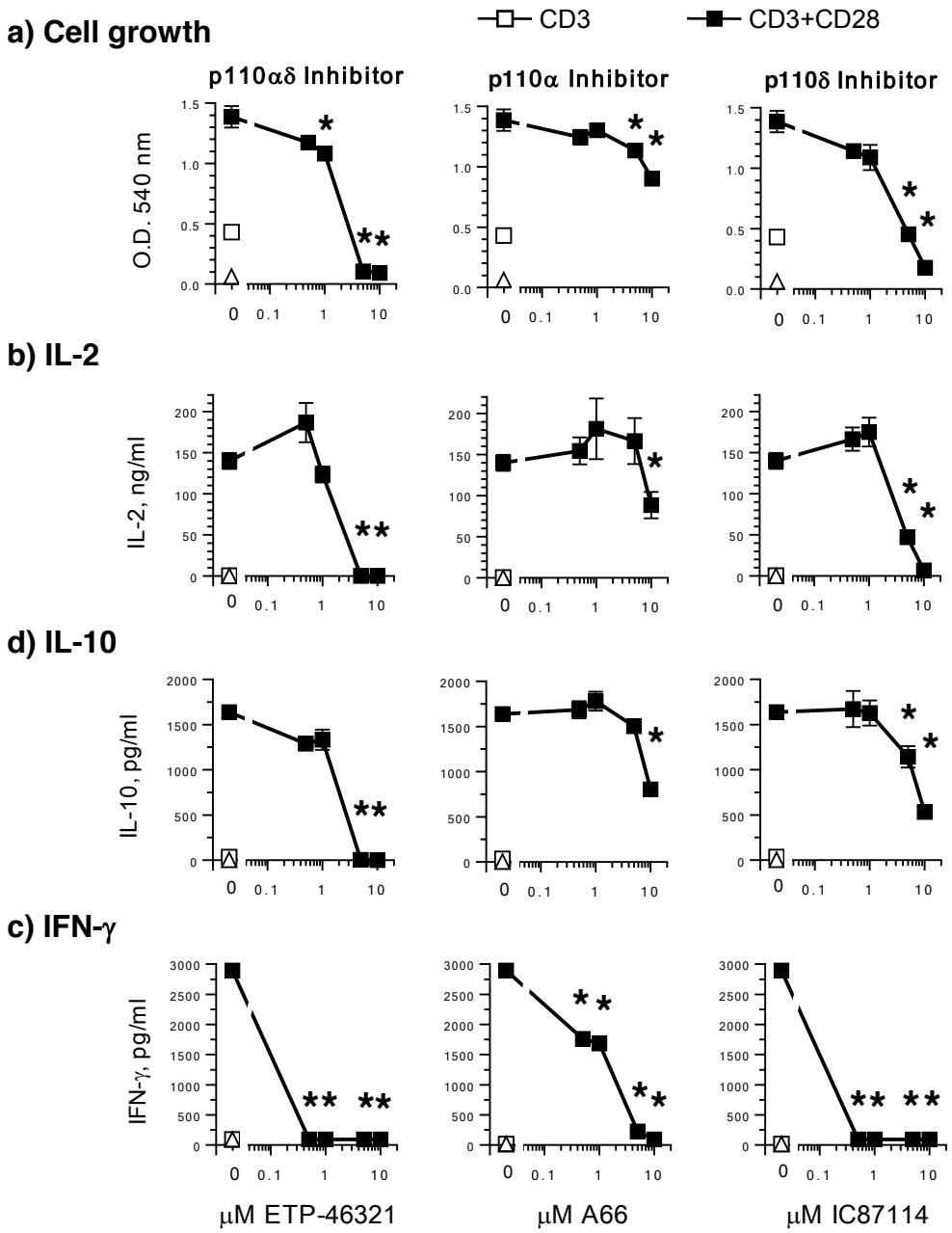

Figure 1S. Activation of T CD4$^+$ naive lymphocytes (72h).
Naïve CD4$^+$ T cells (CD4$^+$CD62L$^+$) cells (10^6) were cultured in 1 ml culture medium in 24-well culture plates (Costar) pre-coated with anti-CD3 antibody (YCD3-1 5 μg ml^{-1}). Where indicated, anti-CD28 (H57, eBiosciences, 2.5 μg ml^{-1}), DMSO or inhibitors dissolved in DMSO (1 μl per culture) were added. At 72h, the cultures were resuspended, centrifuged and the supernatants taken. a) The cells were suspended in fresh culture medium and assayed for live cells using MTT . The supernatants were checked for b) IL-2, c) IL-10, or d) IFN-γ content.