SUPPRESSION OF CD4+ T LYMPHOCYTE ACTIVATION *IN VITRO* AND EXPERIMENTAL ENCEPHALOMYELITIS *IN VIVO* BY THE PHOSPHATIDYL INOSITOL 3-KINASE INHIBITOR PIK-75

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Class IA phosphatidyl inositol-3 kinases (PI3-K) are important targets in cancer therapy and are essential to immune responses, particularly through costimulation by CD28 and ICOS. Thus, small PI3-K inhibitors are likely candidates to immune intervention. PIK-75 is an efficient inhibitor of the PI3-K p110α catalytic subunits that suppresses tumor growth, and its effects on immune and autoimmune responses should be studied. Here, we describe the effect of PIK-75 on different immune parameters *in vitro* and *in vivo*. PIK-75 at concentrations commonly used *in vitro* (≥0.1 μM) inhibited T and B cell activation by Concanavalin A and LPS, respectively, and survival of non-stimulated spleen cells. In naive CD4+ T lymphocytes, PIK-75 induced apoptosis of resting or activated cells that was prevented by caspase inhibitors. At low nanomolar concentrations (≤10 nM), PIK-75 inhibited naive CD4+ T cell proliferation, and IL-2 and IFN-γ production induced by anti-CD3 plus anti-CD28. In activated CD4+ T blasts costimulated by ICOS, PIK-75 (≤10 nM) inhibited IFN-γ, IL-17A, or IL-21 secretion. Furthermore, PIK-75 (20 mg/kg p.o.) suppressed clinical symptoms in ongoing experimental autoimmune encephalomyelitis (EAE) and inhibited MOG-specific responses *in vitro*. Thus, PIK-75 is an efficient suppressor of EAE, modulating lymphocyte function and survival.

Phosphatidyl inositol 3-kinases (PI3-K) are enzymes that phosphorylate the 3 position of the inositol ring of phosphoinositides present in cell membranes. Of these, class I PI3-K are characterized by phosphorylation of phosphatidylinositol 4,5-biphosphate to generate phosphatidylinositol 3,4,5-triphosphate (PIP_3). In turn, membrane PIP_3 recruits proteins, like Akt (PKB), initiating signaling cascades involved in the activation of cell metabolism, motility, survival, and proliferation (1, 2). Among class I PI3-K, the activation of class IA catalytic isoforms (p110α, p110β and p110δ) is typically dependent on tyrosine kinases, as class IA PI3-K form heterodimers with regulatory subunits (p85a, p55a, p50α, p85β, and p55γ) that bind Tyr-phosphorylated Y-x-x-M motifs through their SH2 domains. On the

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other hand, class IB catalytic subunits (p110γ) bind a different set of regulatory subunits (p101, p84/p87). Class IB p110γ, but also class IA p110β are activated by association to G-protein coupled receptors (GPCR) (3). Whereas the p110α and p110β isoforms have wide tissue expression, levels of p110δ and p110γ are particularly high in cells of hematopoietic origin. All class I PI3-K isoforms have oncogenic potential, but the catalytic p110α subunit is particularly important to tumor growth, mutations in the p110α gene enhancing its catalytic activity are particularly frequent in different types of tumor cells (4). In addition p110α activity is selectively activated in cancer-specific mutations of the p85α regulatory subunit (5). Of the p110δ and p110γ isoforms that are expressed at high levels in leukocytes (2), at least the p110δ isoform has shown potential as a therapeutic target for certain hematologic malignancies (6). Thus, class I PI3-K are important targets for novel anticancer therapeutics, and PI3-K inhibitors, particularly p110α- and p110δ-specific inhibitors, are actively investigated.

At the same time, data using mutant cells or mice as well as PI-3 kinase inhibitors have demonstrated a major role of class I PI3-K at different levels of the adaptive immune responses including lymphocyte differentiation, antigen activation, cytokine secretion and signaling, or cell motility. The high level of expression of the p110δ and p110γ isoforms in leukocytes suggests an important functional role for these subunits in immune responses, and indeed these subunits have a prime role in many aspects of T and B lymphocyte biology (7-10).

Interestingly, p110α catalytic subunits are expressed by T cells at levels similar to p110δ, whereas p110β levels are low in these cells (11). In addition, p110α catalytic subunits efficiently associate to regulatory moieties recruited by phosphorylated intracellular sequences of ICOS or CD28 (11). This raises the question as to the possible function of p110α isoforms in T lymphocyte function, as p110δ is needed for efficient early T-cell receptor signaling and cytokine secretion, yet p110α could have a similar, an opposite or no role, depending on the phenomenon considered (11). On the other hand, ICOS preferentially binds p110α (11), and can be determinant in accumulation of Treg promoting immune evasion in melanoma (12). In addition, some functions in immune cells might be mainly driven by p110α, even in the presence of high levels of other class IA catalytic subunits (13). The reason(s) for these preferences are not clear, but they have to be taken into account when establishing a therapeutic anti-tumor- or immunotherapeutic regime, so that the effect of PI3-K p110α inhibitors on immune responses needs to be carefully determined.

We previously observed that the p110α PI3-K inhibitor PIK-75 significantly decreased secretion of cytokines like IL-4 by T helper cell lines or $CD4^+$ T cell blasts (11). PIK-75 also inhibits inflammatory mediators like TNF-α or IL-6 secreted by human monocytes or synovial cells; and oral administration of PIK-75 can attenuate the inflammatory symptoms in dextran sulfate-induced colitis, a mouse model for human inflammatory bowel disease (14). Hence, PIK-75 is a good candidate to immune intervention, and we sought to further study the modulation potential of PIK-75 in T-cell activation and autoimmunity. Here, we have analyzed the effect of PIK-75 on T and B lymphocyte proliferation, cytokine secretion, and viability *in vitro*. Furthermore, we show that PIK-75, orally administered in a therapeutic regime, inhibited clinical symptoms of experimental autoimmune encephalomyelitis (EAE). The possible basis for these effects of PIK-75 is discussed.

MATERIALS AND METHODS

Mice

Specific pathogen-free C57BL/6J mice, aged 8-16 weeks were used. They were bred in the animal care facility of the Centro de Investigaciones Biológicas from stock purchased from Charles River. All experimental procedures were performed according to established institutional and national guidelines.

Antibodies and other reagents

Rat anti-mouse CD3 YCD3-1, rat anti-CD11b M1/70, and Armenian hamster anti-mouse/human ICOS (CD278) C398.4A were purified from hybridoma supernatants; Syrian hamster anti-mouse CD28 37.51 was purified as above or purchased from BD Biosciences. Lipopolysaccharide (LPS, Bacto lipopolysaccharide W *E. coli* 055:B5) was from Difco; recombinant mouse IL-7 was from Preprotech. Inhibitors of PI3-Kα (PIK-75, PIK-103), PI3-Kβ (TGX-221), PI3-Kγ (PI3-Kγ Inhibitor II), the Akt inhibitor Triciribine, and the IKK inhibitor Wedelolactone were from Calbiochem. The p110α inhibitor A66 was from Selleckchem. The PI3-

Kδ inhibitor IC87114 was from Symansis; the PI3-K inhibitor LY294002, the caspase inhibitor Z-VAD-FMK, and the DNA-PK inhibitor NU7026 were from Sigma. The characteristics of some PI-3K inhibitors used are summarized in Table I.

Lymphocyte activation

Spleen cells (10^6) were cultured in 0.2 ml of culture medium (Click's medium supplemented with 10% heat inactivated FCS) in round-bottom 96-well culture plates in the presence or absence of stimuli [LPS, 20 μg/ml, anti-CD3, 5 μg/ml, or Concanavalin A (ConA), 3 μg/ml] for the times specified in the results. Spleen $CD4^+$ T lymphocytes or naive ($CD4^+CD62L^+$) T cells were isolated with the Miltenyi isolation kit Miltenyi $CD4^+$ T cell isolation kit, mouse (130-090-860) or Miltenyi $CD4^+$ $CD62L^+$ T cell isolation kit II, mouse (130-093-227) according to the manufacturer's instructions. Purity of the preparations was routinely ≥95% of the relevant population. The purified cells were cultured (10^6/ml in culture medium) in 24-well plates (Costar) with plate-bound anti-CD3 plus soluble anti-CD28 antibodies or IL-7, as indicated in the Results section. Live cells were determined by trypan blue exclusion and counted in a hemocytometer; alternatively a colorimetric assay using MTT was used, as previously described (11).

Blast generation and activation

$CD4^+$ T cell blasts were obtained as described previously (15). $CD4^+$ T cells isolated from spleen (10^6/ml) were activated with Concanavalin A (ConA, 3 μg/ml) plus mitomycin-C-treated, T-cell depleted spleen cells at $5x10^5$/ml and IL-2. After 48 h, blasts were isolated in a discontinuous Percoll gradient, washed, adjusted to $2x10^5$/ml, and expanded in IL-2-containing culture medium for further 48-72 h. The blasts ($1x10^6$/ml) were activated in the presence or absence of inhibitors with plate bound anti-CD3 (10 μg/ml) plus anti-ICOS or control antibodies (20 μg/ml) for 16-24 h.

Apoptosis

Spleen cells were incubated for the times indicated in round 96-well plates (0.2 ml culture medium/well containing 10^6 cells) in the presence or absence of 5 μg/ml anti-CD3 or 20 μg/ml LPS. Isolated $CD4^+$ T lymphocytes were cultured in 24-well plates (1 ml medium, 10^6 cells/well). Where indicated, naive $CD4^+$ T lymphocytes were cultured in wells; these were coated or not with anti-CD3 antibody (20 μg/ml) plus anti-CD28 (2.5 μg/ml) or IL-7 (1 ng/ml). Viable cells were counted in a hemocytometer by trypan blue exclusion. Apoptosis was determined by flow cytometry in cells stained with AnnexinV-FITC and propidium iodide using the human Annexin V-FITC kit (eBioscience) according to the manufacturer's instructions.

Cytokine detection by ELISA

Cytokines in culture supernatants were determined by capture ELISA using Ready-Set-Go!® capture ELISA kits (eBiosciences) for mouse IL2, IL-4, IL-10, IL-17, or IL-21. Capture ELISA for mouse IFN-γ was performed as described in (16) using anti-IFN-γ R4-6A2 for capture, (BD Biosciences) and biotinylated XMG1.2 for detection.

EAE induction and measure

EAE was induced in 8-12 wk old C57BL/6 female mice as described in detail previously (16). Mice were injected s.c. in one flank with an emulsion of 300 μg rat MOG peptide (MOG_{35-55}) in saline in CFA containing 500 μg of heat-killed *Mycobacterium tuberculosis*. Pertussis toxin (0.5 μg in PBS i.p.) was injected the same day as MOG and 48 h later. The mice were boosted with MOG peptide in CFA in the other flank after one week. Clinical signs of EAE were graded according to the scale: 0, no clinical signs; 1, loss of tail tone; 2, wobbly gait; 3, hind limb paralysis; 4, hind and fore limb paralysis; 5, death; 0.5 gradations were given to intermediate scores. Disease scores were averaged daily for each group. The Disease Index was calculated by adding all the daily average disease scores; the result was divided by the average day of disease onset of each group and multiplied by 100. Other disease parameters were defined as described (16), as follows: The Day of Disease Onset was calculated by averaging the first day of clinical signs for each mouse in one experimental group. Maximum Disease Score was determined by averaging the highest score achieved by each individual mouse. PIK-75 was suspended in 0.5% carboxymethyl cellulose, 20% sucrose in water and administered p.o. (20 μg/kg) on days 9, 10, 12, 13, 14, and 15 after the first MOG injection. MOG-induced activation of cells from the draining lymph nodes was performed in round-bottom 96 wells in 0.2 ml at $2x10^6$ cells/well in the presence of MOG peptide (100 mg/ml). After 96 h of culture, the cells were counted and cytokines in the supernatant determined by ELISA.

Statistical analysis

Statistical significance was determined by the Student's *t*-test, or by the Mann-Whitney U test, as specified in the results. Differences with a value of $p < 0.05$ were considered as statistically significant.

RESULTS

Effect of PIK-75 on T and B lymphocyte activation and viability in vitro

Our previous data on activation of $CD4^+$ Th2 cell lines or $CD4^+$ T lymphocyte blasts for short times

Table I. *In vitro IC50 values μM) of PI3-K inhibitors for PI3-K catalytic isoforms.*

Drug	p110α	p110β	p110δ	p110γ	mTORC1/C2	DNA-PK
PIK-75 (α)	0.006	1.080	0.7	0.090	0.900/9.000	0.002
A66 (α)	0.070	20.0	18.05	18.81	>5.0	>5.0
TGX-221 (β)	5.000	0.007	0.100	3.500		
IC87114 (δ)	>100.0	1.820	0.070	1.240	>100.0	>100.0
LY294002 (All)	0.700	0.306	1.330	7.260	8.910	1.000

Data compiled from references (19, 28, 34) and (18, 21, 35).

showed a clear inhibition of IL-4 secretion by the p110α inhibitors PIK-75 or PIK-103 at micromolar concentrations (11). Then, we extended our analysis to fresh primary $CD4^+$ T lymphocytes using PIK-75 at micromolar or submicromolar concentrations previously used in other types of cells (14, 17-19). In early experiments, we found that, indeed, 1 μM PIK-75 strongly inhibited proliferation of cytokine (IL-2, IL-4, IFN-γ) secretion by $CD4^+$ T lymphocytes activated for three days with anti-CD3 plus anti-CD28 antibodies (data not shown). We also observed that, in these cultures, inhibition by PIK-75 was accompanied by early death of a fraction of the cultured cells. By contrast, in these cultures the p110δ inhibitor IC87114 at concentrations commonly used *in vitro* had weaker effects than PIK-75 in terms of cell proliferation and IL-2, IL-4, and IFN-γ secretion. We then set up experiments to determine the effect of PIK-75 on lymphoid cell viability using different concentrations of the inhibitor, times of culture, or stimuli.

As shown in Fig. 1, activation of spleen cells with anti-CD3, a polyclonal T lymphocyte activator, or the B lymphocyte activator LPS was strongly inhibited by PIK-75 at concentrations ≥0.1 μM, as determined by the number of live cells at 92 h of culture. A similar effect was observed in cultures of unstimulated cells, or using the same concentrations of another p110α inhibitor, PIK-103 (data not shown). As a comparison, the number of live cells was partially reduced in the presence of p110δ inhibitor IC87114 at 5 μM, a drug that is well known to inhibit T and B lymphocyte activation, but the effects were weaker than those displayed by PIK-75 (Fig. 1a).

In cultures of non-stimulated spleen cells, the number of live cells was markedly diminished by overnight culture in the presence of ≥0.1 μM PIK-75 (Fig. 1b). This was accompanied by enhanced apoptosis of lymphocytes, as determined for Annexin-V binding to cells gated for T (CD4) or B (CD19) lymphocyte markers. By contrast, the p110δ inhibitor IC87114 at 5 μM partially decreased the number of live cells, but had negligible effects on enhanced apoptosis.

Naïve $CD4^+$ T lymphocytes are sensitive to PIK-75 inhibition

We went on to analyze the effect of PIK-75 on the viability of isolated $CD4^+$ naive T cells, stimulated or not with anti-CD3 and costimuli (CD28) or cytokines promoting T-cell survival such as IL-7 (Fig. 2). Like in total spleen cell cultures, PIK-75 (0.1 μM) enhanced spontaneous apoptosis upon overnight culture of $CD4^+$ naive T lymphocytes; most of these cells were in the Annexin V^+PI^+ late apoptotic phase. Activation with anti-CD3 plus anti-CD28 or plus IL-7 prevented cell death in the absence of inhibitors; but not in the presence of PIK-75, as it induced apoptosis as strongly as in unstimulated cells. In the same conditions, the p110δ inhibitor IC87114 at 5 μM modestly enhanced apoptosis. Cell death of $CD4^+$ T cells induced by PIK-75 was strongly inhibited by the caspase inhibitor Z-VAD (Fig. 3). Cell viability was also reduced in cells from the Th2 cell line SR.D10 upon overnight culture in the presence of PIK-75 or other PI3-K p110α inhibitors, but not in the presence of inhibitors specific for other class I PI3-K, including p110γ or

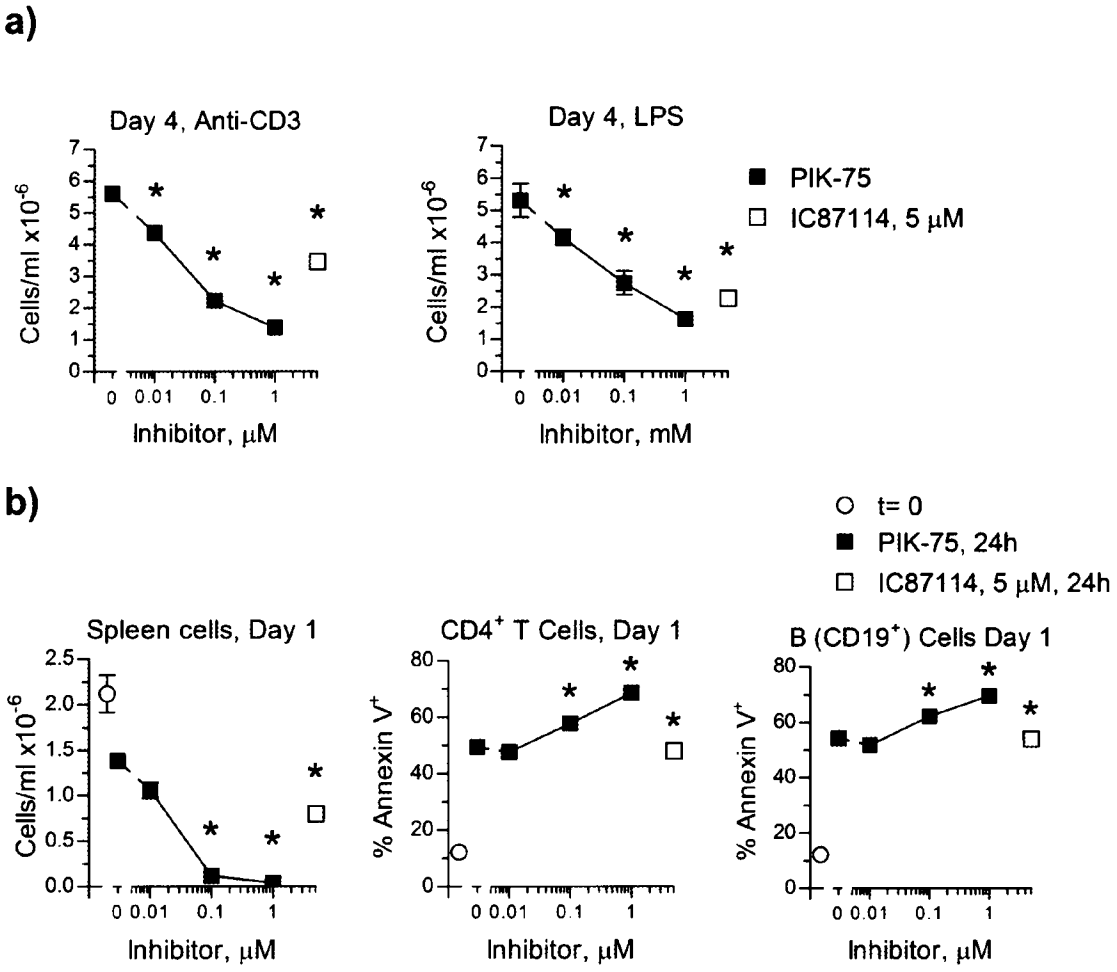

Fig. 1. *PIK-75 inhibits T and B cell activation and survival in spleen cell cultures.* ***a)*** *Spleen cells were cultured in the presence of T lymphocyte (anti-CD3 5 μg/ml, left) or B lymphocyte (LPS, 20 μg/ml, right) activating stimuli. PIK-75 (closed symbols) or the p110δ inhibitor IC87114 (5 μM, open symbols) were added, as indicated. After 4 days the cells were resuspended and counted. Mean ± s.e.m. of triplicate cultures. Asterisks indicate significant differences ($p<0.05$) with control cultures. Data from one representative experiment of three performed.* ***b)*** *Addition of PIK-75 (closed squares) or the p110δ inhibitor IC87114 (5 μM, open squares) decreases spleen cell survival and enhances spontaneous apoptosis at 24 h of culture. Open circles represent live cells or per cent apoptotic (Annexin-V⁺) at time 0. Mean ± s.e.m. of triplicate cultures. Asterisks indicate significant differences ($p<0.05$) with control cultures.*

p110β (Fig. 4a). Since PIK-75 efficiently inhibits the DNA-PK (Table I), the ability of DNA-PK inhibitors to enhance the effect of p110α-specific inhibitors was also determined. As shown in Fig. 4b, NU7026, a DNA-PK-specific inhibitor augmented the inhibition of T- and B-lymphocyte activation induced by A66, a p110α-specific inhibitor with no effect on DNA-PK.

When naive $CD4^+$ T lymphocytes were cultured for four days in the presence of anti-CD3 and anti-CD28, PIK-75 had an IC_{50} equal to, or lower than, 0.01 μM for either proliferation or secretion of IL-2 and IFN-γ (Fig. 5).

Effect of PIK-75 on secretion of cytokines by activated $CD4^+$ T cells

Both CD28 and the CD28-like costimulatory molecule ICOS associate class IA PI3-K, with a bias towards the p110α subunit (11). ICOS is

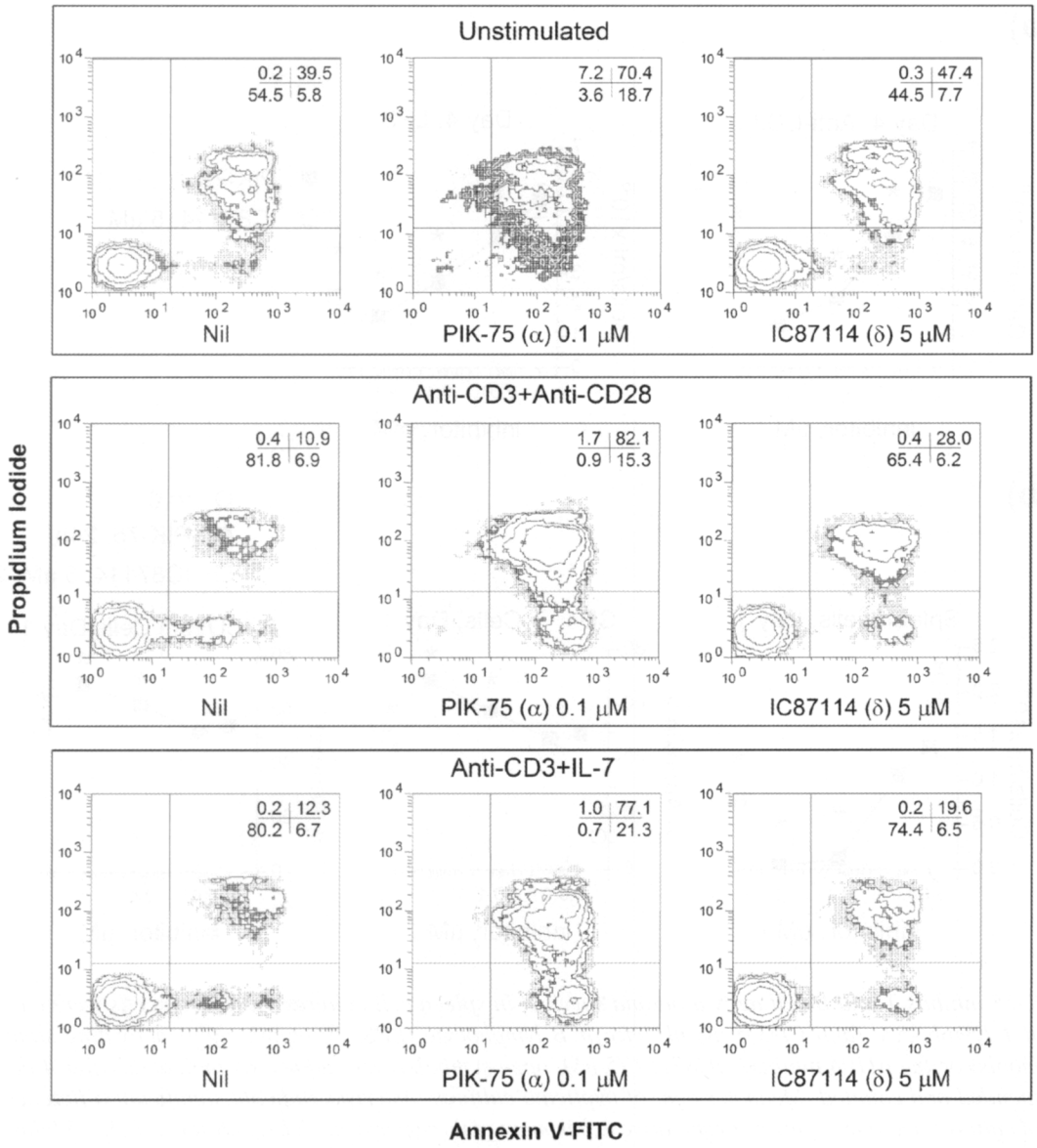

Fig. 2. *Effect of PIK-75 and IC87114 on the apoptosis of resting or activated naive ($CD4^+CD62L^+$) T lymphocytes. Unstimulated $CD4^+CD62L^+$ T cells (top panel) or cells activated by plate-bound anti-CD3 (20 μg/ml) plus anti-CD28 (2.5 μg/ml) (middle panel) or IL-7 (1 ng/ml) (bottom panel) were cultured for 24 h, then analyzed by flow cytometry for early (Annexin V^+ PI^-) and late (Annexin V^+ PI^+) apoptosis. Where indicated, PI3-K inhibitors PIK-75 (0.1 μM) or IC87114 (5 μM) were added. Vehicle (DMSO) was added to control cultures. Inset Fig.s indicate percentage of cells in each quadrant.*

expressed upon antigen activation of T cells, so that its costimulatory effect is particularly important to activated cells. Our previous data on ICOS costimulation in short-term activation of $ICOS^+$ Th2 cell lines and $CD4^+$ T blasts show that p110α inhibitors like PI-103 or PIK-75, at micromolar concentrations, inhibit early phosphorylation of Akt and IL-4 secretion, but not IL-10 secretion [(11), and unpublished data]. Here, we checked the effect of low concentrations of PIK-75 (≤0.01 μM) on the secretion of cytokines by $CD4^+$ T blasts activated overnight with TCR/CD3 plus anti-ICOS antibodies

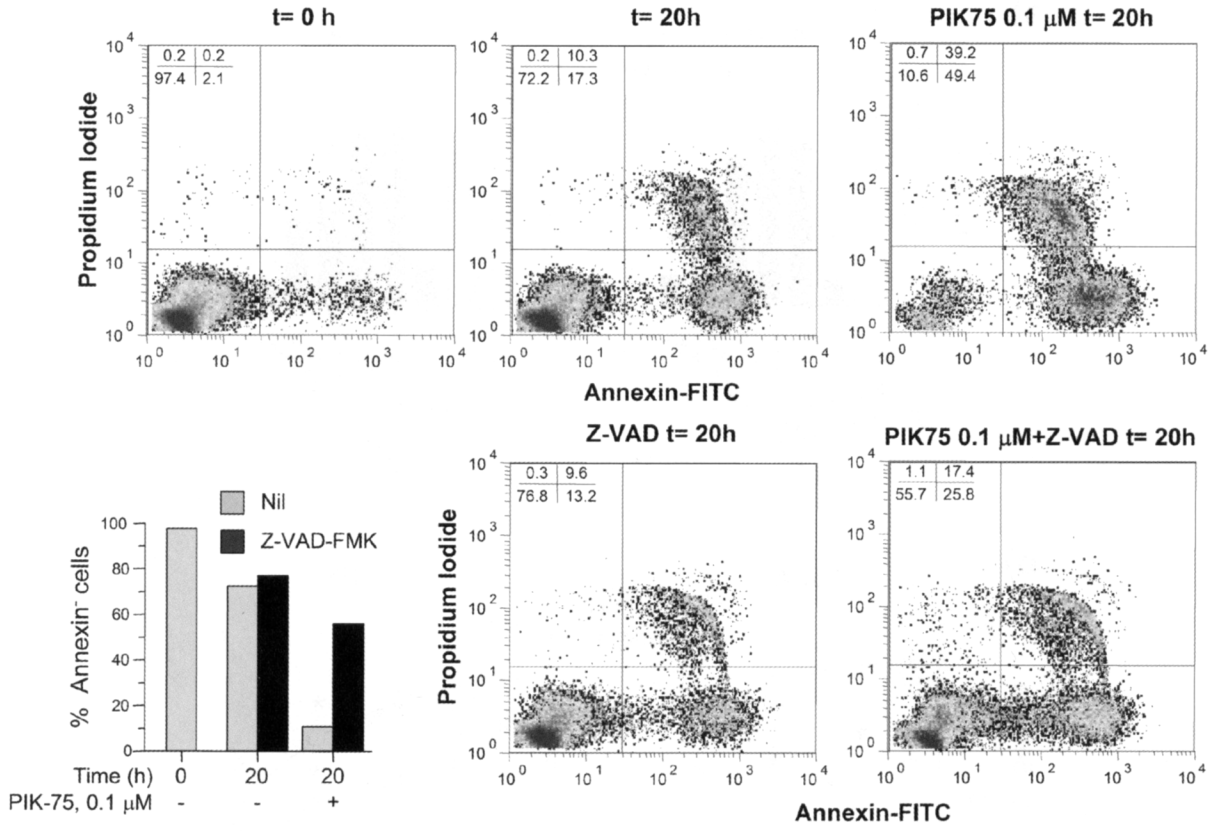

Fig. 3. *Apoptosis and death of CD4⁺ T lymphocytes induced by PIK-75 is blocked by the caspase inhibitor Z-VAD-FMK. Isolated naive CD4⁺ T lymphocytes (2x10⁶/ml) were cultured for 20 h in 24 well plates in the presence or absence of PIK-75 (0.1 μM) and Z-VAD-FMK (40 μM). Then, the cells were analyzed for cells in early (Annexin V⁺PI⁻) or late (Annexin V⁺PI⁺) apoptosis by flow cytometry, and compared to apoptosis before culture (t = 0h).*

(Fig. 6). Interestingly, in these conditions, PIK-75 had an IC_{50} lower than 0.01 μM in IL-17, IL-21, or IFN-γ secretion. All these cytokines are important for the development of efficient antibody responses and/or inflammatory reactions relevant to autoimmune diseases. On the other hand, its effect on the secretion of IL-2 and the anti-inflammatory cytokines IL-10 and IL-4 was negligible at the concentrations used (Fig. 6).

Effect of PIK-75 in experimental autoimmune encephalitis

The effects of PIK-75 on the proliferation and survival of naive or activated T cells, as well as its effect on secretion of inflammatory cytokines, suggested that it might be useful as a therapeutic agent in autoimmune diseases. In fact, previous data show that oral administration of PIK-75 significantly inhibits IL-6 secretion in monocytes and ameliorates dextran sulfate sodium (DSS)-induced colitis, an experimental model of inflammatory bowel disease (14). Thus, we sought to determine the effect of PIK-75 in a model of EAE that is dependent on CD4⁺ T cells. A therapeutic administration regime for PIK-75 was chosen starting on day 9 after the initial injection with the encephalitogenic MOG peptide, a time when most mice had begun to show clinical symptoms of EAE. Oral administration of PIK-75 had a significant effect on most clinical parameters considered, including the average score, the average maximal disease score, or the average disease index (Fig. 7b-d). The average day of disease onset was not significantly changed, as expected from the late beginning of PIK-75 administration (Fig. 7a). Since

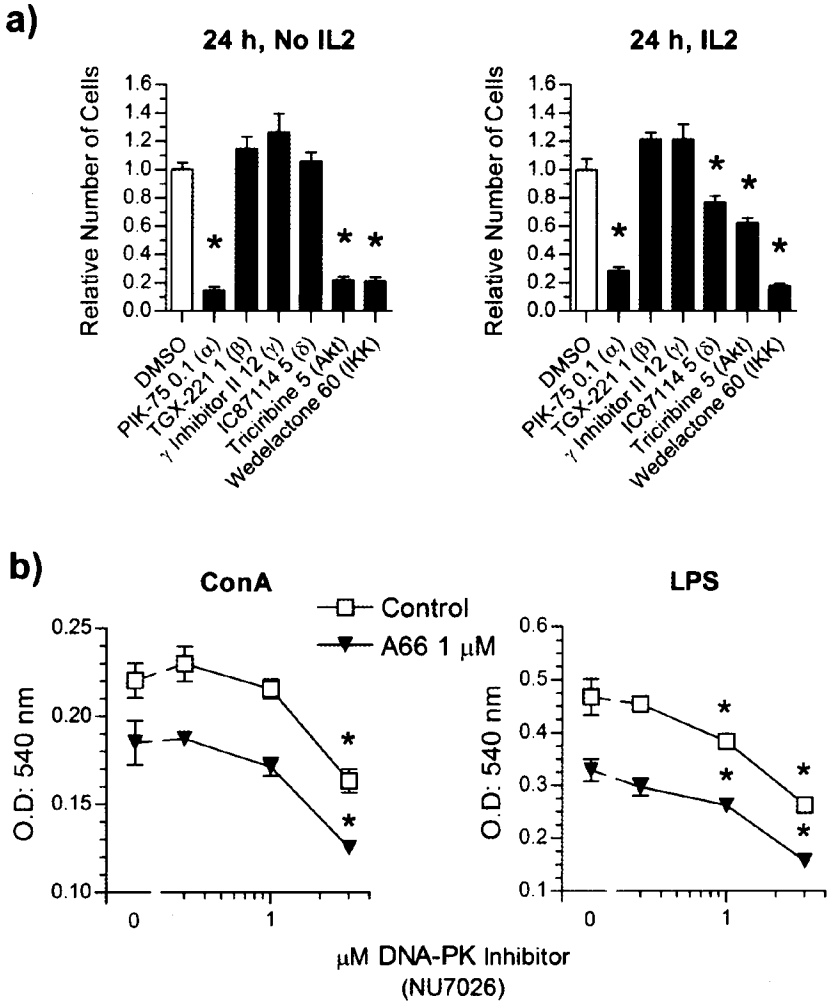

Fig. 4. *Effect of inhibitors of class I PI3-K, Akt, NF-kB and DNA-PK on survival of the D10 Th2 lymphocyte T cell line (a) or on spleen cell activation by ConA and LPS.* ***a)*** *Survival of the D10 Th2 lymphocyte T cell line is significantly inhibited by inhibitors of p110α, p110δ, Akt and NF-kB. D10 cells (10^6/ml) were cultured in 24-well culture plates for 24 h in the absence (left) or presence (right) of mouse IL-2 (10 U/ml). Inhibitors of PI3-K p110α (PIK-75, 0.1 μM), p110b (TGX-221, 1 μM), p110γ (p110γ inhibitor II, 12 μM), p110δ (IC87114, 5 μM), Protein kinase B (PKB, Akt, Triciribine, 5 μM), or IKKα,β- mediated activation of NK-kB (Wedelactone, 60 μM) in DMSO were added, as indicated. The number of live cells was normalized in each case to the number of viable cells in control cultures with DMSO only. Mean ± s.e.m. of triplicate cultures. *, $p<0.05$ with control cultures.* ***b)*** *Inhibition of DNA-PK fosters the effect of PI3-K p110α inhibitors on the activation of spleen cells by T (ConA) and B (LPS) stimuli. Spleen cells (10^6) were cultured in 0.2 μl in round-bottom 96 well culture plates in the presence of stimuli (ConA, 3 μg/ml, LPS, 20 mg/ml) for 72 h. The PI3-K p110α inhibitor (A66, 1 μM) and the DNA-PK inhibitor NU7026 in DMSO were added, as shown in the Fig. Control cultures received DMSO only. Asterisks indicate significant differences ($p<0.05$) with cultures without DNA-PK inhibitor.*

PIK-75 had a strong effect on lymphocyte viability *in vitro*, the mice were sacrificed on day 36 and analyzed for the number of cells and lymphoid cell populations in the draining lymph nodes, or in the spleen. At this time, twenty days after the end of PIK-75 administration, we observed no significant differences in the number of cells in these lymphoid organs between the group of mice treated with

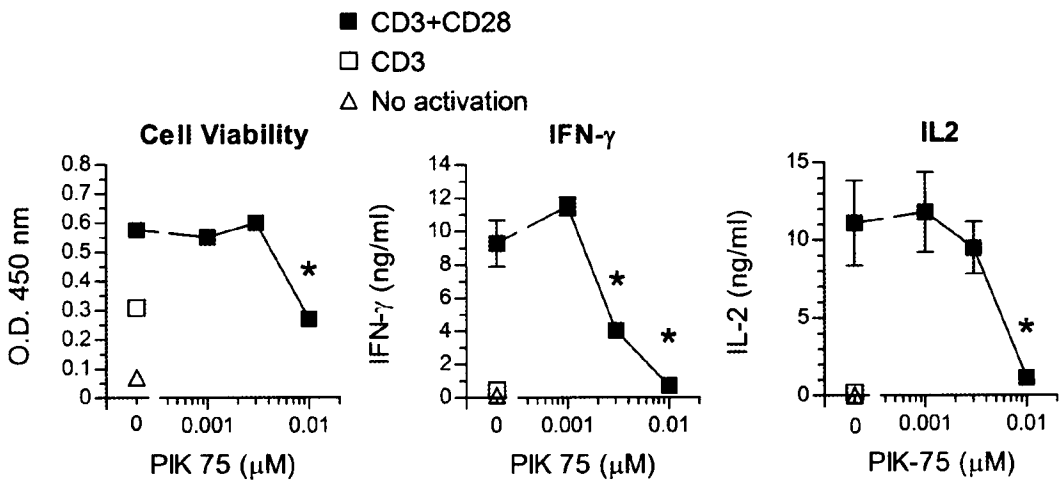

Fig. 5. *Effect of low concentrations of PIK-75 on naive ($CD4^+CD62L^+$) T lymphocyte activation. Purified naive $CD4^+CD62L^+$ T cells were activated by plate-bound anti-CD3 (10 μg/ml) alone (open squares), or in the presence of anti-CD28 (2.5 μg/ml, closed symbols). After 96 h, cell viability was assessed using a colorimetric assay (left); cytokine content in the supernantant (IFN-γ, IL-2) was also determined. PIK-75 or diluent (DMSO) were added, as indicated. Open triangles: unstimulated cultures. Mean ± s.e.m. of triplicate cultures. *: $p<0.05$ with control cultures. Data from one representative experiment of four performed.*

PIK-75 and the control group. Furthermore, the percentage of different lymphocyte subpopulations was also similar in both experimental groups. These included T cell populations ($CD4^+$, $CD8^+$, γδ, NKT, $CD4^+Foxp3^+$ Treg cells), B lymphocytes ($CD19^+$), or NK cells ($NK1.1^+$) (data not shown). When re-activated *in vitro* with antigen (MOG peptide), the lymph node cells from the PIK-75-treated mice still showed reduced proliferation (Fig. 7e). The analysis of cytokines involved in the pathogenesis of encephalomyelitis showed that IL-17A was reduced in the PIK-75-treated group; however, the differences were not statistically significant at this late time after PIK-75 administration. IFN-γ secretion was similar in both groups (Fig. 7e).

DISCUSSION

PIK-75 was developed as a potent PI3-K p110α inhibitor that suppressed growth of tumor cells *in vitro* or in human xenograft models *in vivo* (17). Deregulation of the PI-3-kinase activity is a common trait in many human cancers; this occurs by mutation, augmented expression, or amplification of activity by mutation of specific phosphatases. Among PI3-K, the catalytic p110α subunit has a wide tissue distribution, and gain-of-function mutations in the p110α gene that enhance its catalytic activity are common in several types of tumor cells. Even cancer-specific mutations in the p85α regulatory subunit of class I PI3K function through specifically enhanced activity of the bound p110α subunits (4, 5). Thus, it makes sense to investigate p110α inhibitors like PIK-75 in the treatment of solid tumors. Other inhibitors of p110α like PI-103 and PIK-90 inhibit leukemias through inhibition of CXCR4 signaling [a key receptor for Chronic Lymphocytic Leukemia (CLL) cell migration and adhesion to marrow stromal cells] and are potent inducers of cell apoptosis (20). In another study, isoform-specific inhibitors of p110α, p110β, or p110δ, but not p110γ, induced CLL apoptosis, suggesting a direct role of these isoforms in maintaining CLL cell viability (21), with p110α inhibition having the highest incidence in CLL cell survival (21). In the same study, inhibitors of p110α (AS702630) or p110δ (IC87114) at 10 μM reduced cell survival of human B lymphocytes, but not T lymphocytes.

Exploring the effect of PI3-K p110α specific inhibitors on cell viability and function is important

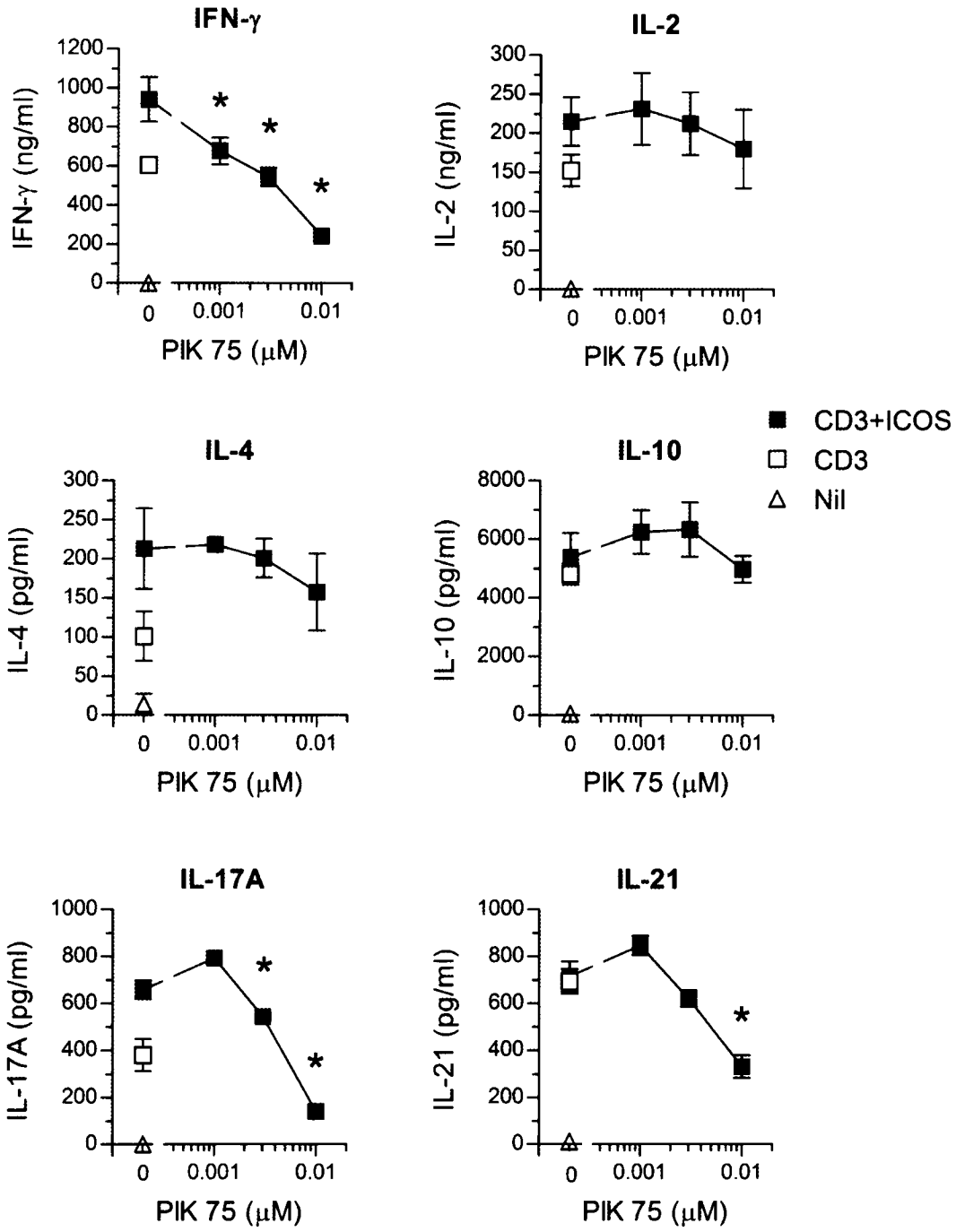

Fig. 6. *PIK-75 differentially inhibits cytokine secretion by ICOS-costimulated CD4⁺ T cell blasts at nanomolar concentrations. Concanavalin A activated, IL-2 expanded CD4⁺ T cells were re-activated for 24 h with plate-bound anti-CD3 (10 μg/ml) plus anti-ICOS (20 μg/ml, closed symbols) or a control antibody (open squares). Then, supernatants were taken and the cytokines IFN-γ, IL-2, IL-4, IL-10, IL-17A, or IL-21 were determined. Open triangles: unstimulated cultures. Mean ± s.e.m. of triplicate cultures. *: $p<0.05$ with control cultures lacking the inhibitor. Data from one experiment of two performed with similar results.*

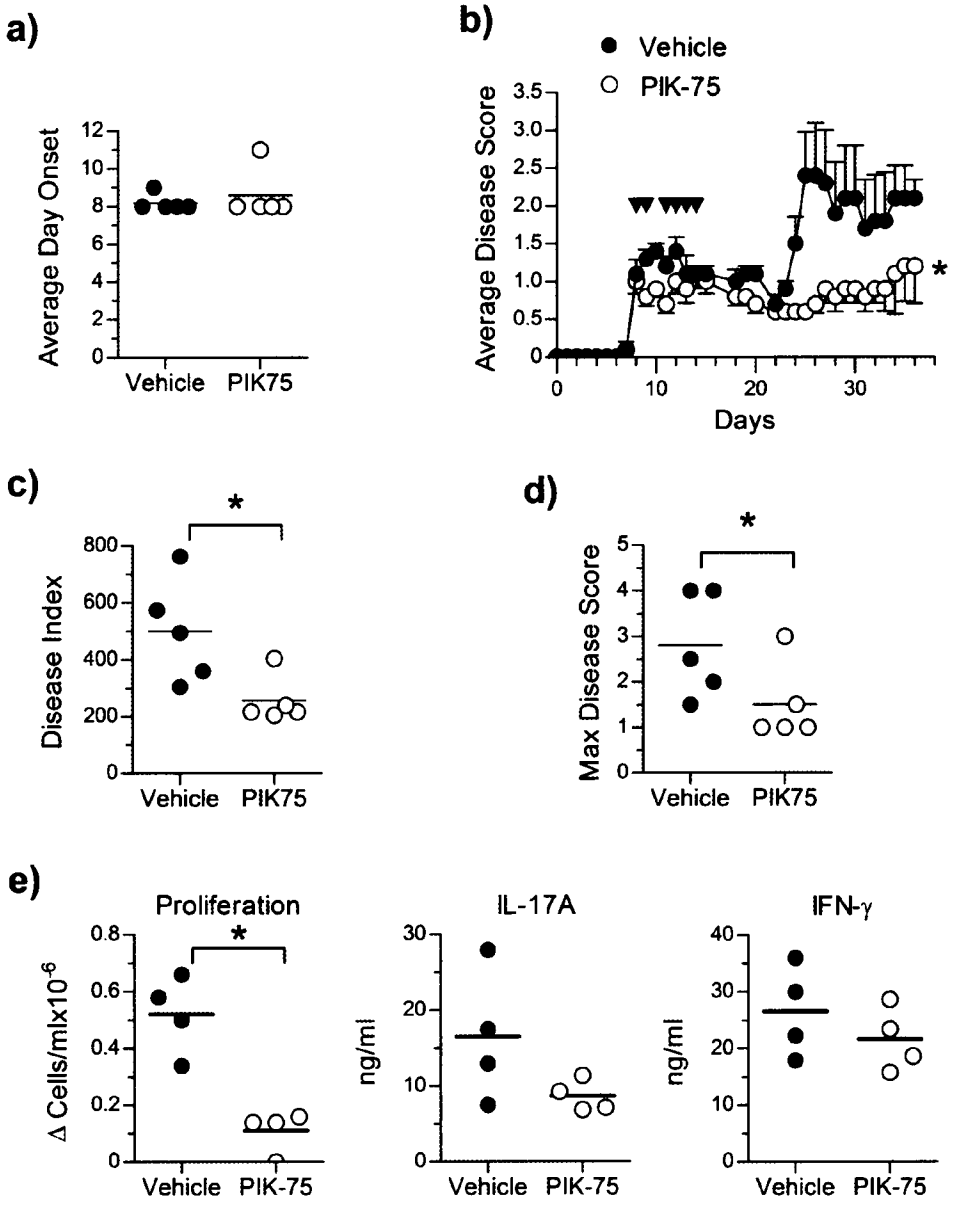

Fig. 7. *Administration of PIK-75 alleviates clinical symptoms of MOG-induced EAE in vivo and inhibits MOG-specific responses in vitro. PIK-75 (20 μg/kg) was administered orally to mice (n = 5/group) on days 8, 9, 11, 12, 13, and 14 after the first MOG immunization, as indicated by inverted triangles in (b). Clinical symptoms recorded were (a) Average day of disease onset; (b) Average disease score; (c) Disease index; (d) Maximal disease score. e) On day 36 after the first MOG immunization, cells from the draining axillary and inguinal lymph node cells from control or PIK-75-treated mice (n =4) were re-stimulated for 96 h in vitro with or without MOG (100 mg/ml); the number of live cells in the cultures (left panel), and IL-17A and IFN-γ in culture supernatants (center and right panels) were determined. Means are indicated by horizontal bars (a, c-e). (b) Mean ± s.e.m. of individual mice. *: $p<0.05$ with mice treated with vehicle, determined by the Mann-Whitney U test (b) or with the Student's t test (c, d, e).*

to the control of anti-tumor as well as to pathologic immune reactions. Some functions in innate or adaptive immunity are controlled by p110α, even in the presence of high levels of other class IA catalytic subunits (13, 24-26). In our system, survival of activated or resting spleen cells or isolated $CD4^+$ T cells was strongly reduced by PIK-75 at concentrations ≥0.1 μM, and moderately reduced by IC87114 (5 μM). The proliferation of naive $CD4^+$ T lymphocytes, as well as IL-2 or IFN-γ secretion were still reduced at very low concentrations (≤0.01 μM) of PIK-75. Particularly, the sensitivity of IFN-γ to inhibition by PIK-75 might be of interest concerning the effect of PI3-K inhibitors in tumor growth, as it could have different outcomes depending on the particular tumor. For instance, IFN-γ and TNF-α induce senescence in numerous mouse and human cancers, and this might be a general mechanism for arresting cancer progression (22). However, at least in chronic myeloid leukemia, IFN-γ secreted by T cells can support cancer growth by inducing proliferation of leukemia stem cells (23).

We have recently observed that in $CD4^+$ T cells, p110α was expressed at levels similar to those of p110δ (11). Intriguingly, p110α was preferentially recruited by phosphorylated sequences of the costimulatory molecules ICOS and CD28, because p110α binding to PI3-K regulatory subunits was stronger than other isoforms [(11), and Y.A., U.D., P.P., and J.M.R., unpublished results]. We observed that p110α as well as p110δ contributed to early phosphorylation of Akt or Erk, and to secretion of IL-4 by T-cell lines or blasts activated by TCR ligands, with or without ICOS costimuli (11). Recent data by So et al. show that inhibitors of p110α (A66, MLN1117) or p110β (TGX-221) have a minor but significant role in B cell activation, with p110δ inhibitor (IC87114) being the most effective (27). Here, we show that B cell activation by LPS was also clearly inhibited by the p110δ inhibitor IC87114 (Fig. 1). Yet, unlike the p110α inhibitors A66 or MLN1117, we observed that PIK-75 was strongly inhibitory to LPS B cell activation at low concentrations (≥0.1 μM). These concentrations also enhanced the apoptosis of unstimulated B cells (Fig. 1). The diverse effects of PIK-75 and other p110α inhibitors like A66 cannot be fully explained in terms of their different IC_{50}, and additional mechanisms could be involved (see below).

In T lymphocytes, 0.1-1 μM PIK-75 strongly inhibited TCR/CD3 activation of spleen cells. In isolated naive $CD4^+$ T cells, activated or not by anti-CD3 plus CD28 or IL-7, PIK-75 enhanced apoptosis (Figs. 1, 2, 3, and data not shown). At lower concentrations (0.01 μM) PIK-75 did not markedly inhibit lymphocyte viability, but still inhibited CD28 costimulation-dependent proliferation of naive $CD4^+$ T lymphocyte, or secretion of IL-2 and IFN-γ (Fig. 5). Interestingly, inhibition of IFN-γ as well as IL-17A or IL-21 was also observed in $CD4^+$ T blasts at these nanomolar doses of PIK-75 (Fig. 6). Data on the effect of PI3-K p110α inhibitors in T lymphocytes are scarce. As mentioned above, the p110α inhibitor AS702630 (10 μM) did not significantly reduce survival of normal human T lymphocytes (21). The p110α inhibitors A66 or MLN1117 or the p110δ inhibitor IC87114 (1-2 μM) did not significantly inhibit mitogen- or antigen-induced proliferation of human or mouse T cells unless they were combined (27). Indeed, we have also found that T-cell proliferation is more resistant than B-cell activation concerning their inhibition by IC87114 (Fig. 1); our own data using A66 and IC87114 at the same concentrations used by So et al. are in agreement with their results in terms of their effect on T-cell proliferation or IFN-γ, and show significant but low effects on cell viability and apoptosis at concentrations higher than 1 μM (Fig. 4, and Rojo J.M. et al., manuscript in preparation). In contrast, using PIK-75, we have observed strong effects on activated or resting T cells at ten-fold lower concentrations (≥0.1 μM), and clear effects on cytokine secretion or proliferation using concentrations as low as 0.01 μM PIK-75. These differences among inhibitors are not likely due to differences in their p110α IC_{50} [0.006 and 0.032 mM, respectively, for PIK-75 and A66 (19)], and seem specific for lymphocytes, as an IC_{50} ≥20 mM for PIK-75 toxicity in monocytes and synovia have been reported (14). Inhibition of other Class I PI3-K isoforms might be involved as the IC_{50} of PIK-75 is relatively low (0.080, 0.164 and 0.033 mM for p110β, p110δ and p110γ, respectively) (19). However, in T-cell lines or primary T cells we have failed to observe any effect of p110β or p110γ inhibitors on T-cell activation or survival

in vitro (Fig. 4) (11). A different possibility is that inhibition of DNA-dependent protein kinase (DNA-PK) might be involved in some PIK-75 effects, particularly those concerning cell viability. PIK-75 has an IC_{50} for DNA-PK similar to its IC_{50} for p110α (18) (Table I). The DNA-dependent protein kinase belongs to the class IV family of PI3-K, or PI3K-related protein kinases that also includes proteins like mTOR (28). Within this family, the DNA-PK, the ataxia telangiectasia mutated gene product (ATM) or the ataxia telangiectasia related (ATR) protein all interact with DNA and are involved in DNA damage responses by repairing double strand DNA breaks (DSB). Defects in DNA-PK enhance sensitivity to radiation damage and produce severe combined immunodeficiency (29); furthermore, DNA-PK directly induces phosphorylation and activation of Akt (30, 31). Inhibition of Akt strongly affects T-cell survival in our system (Fig. 4). Interestingly, recent data show that inhibition of DNA-PK synergizes with etoposide (an agent that promotes double-stranded DNA breaks), in inducing cell death of activated $CD4^+$ T lymphocytes (32). In agreement with these considerations, we observed that the specific inhibitor of DNA-PK NU726 cooperates with the A66 selective p110α inhibitor in suppressing T and B lymphocyte responses to ConA and LPS, respectively (Fig. 4b).

Taking into account previous data showing that administration of PIK-75 alleviates dextran sulfate-induced colitis (14), and in view of its effect on lymphocyte subpopulations and cytokines involved in EAE (33), we analyzed the effect of PIK-75 administration on MOG-induced EAE. Administration of PIK-75 during the effector phase of EAE inhibited the clinical symptoms of treated mice (Fig. 7). The PIK-75 treatment was well tolerated, and normal numbers and percentages of the main lymphocyte populations were observed 20 days after termination. Previous results have also shown that PIK-75 inhibits secretion of inflammatory cytokines in cells from patients with active rheumatoid arthritis (14). This suggested that PIK-75 might be useful as a therapeutic agent in arthritis. However, in our hands, administration of PIK-75 in an experimental model of proteoglycan-induced arthritis in mice did not result in significant improvement of clinical symptoms (data not shown)

In summary, whereas the mechanism(s) and targets of action of PIK-75 in lymphocytes, particularly dual targeting of PI3-K p110α and DNA-PK, deserve further analysis, our data supports its efficacy as a modulator of lymphocyte survival and effector function, and its therapeutic potential in autoimmune diseases.

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