

Original Article

Inhibition of phosphoinositide 3-kinase delta attenuates experimental autoimmune encephalomyelitis in mice

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Received September 12, 2015; Accepted November 8, 2015; Epub November 15, 2015; Published November 30, 2015

Abstract: The phosphoinositide 3-kinase delta (PI3K δ) has been implicated in multiple signaling pathways involved in autoimmune diseases. We here aimed to test the hypothesis that selective inhibition of PI3K δ may promote anti-inflammatory effects by inhibiting Th1 and Th17 cells. We investigated the therapeutic efficacy of a selective PI3K δ inhibitor IC87114 in experimental autoimmune encephalomyelitis (EAE), a murine model of multiple sclerosis (MS). The efficacy was evaluated based on clinical scores, histopathology, serum cytokines and inflammatory infiltrations in the central nervous system (CNS). Treatment of EAE mice with IC87114 reduced the clinical symptoms, histopathology and cellular infiltration into the CNS. And treatment of EAE with IC87114 suppressed the Th1 and Th17 cell ratios. Consistently, the serum levels of IL-1 β , IL-6, IL-17 and INF- γ were markedly reduced by IC87114. Taken together, our studies demonstrate that inhibition of PI3K δ may serve as novel therapy to suppress neuroinflammation seen during EAE.

Keywords: Experimental autoimmune encephalomyelitis, multiple sclerosis, phosphoinositide 3-kinase delta, Th1, Th17

Introduction

Multiple sclerosis (MS) is a chronic, demyelinating disease of the central nervous system (CNS) and multiple lines of evidence indicate that it is an autoimmune disease [1, 2]. Following breakdown of immunological tolerance to CNS antigens, immune cells invade the CNS and lead to activation of inflammatory signaling, resulting in CNS demyelination caused by oligodendrocyte loss and cytotoxic effects on myelinated axons [1]. The resulting paralysis and other neurological symptoms in MS patients are difficult to manage and are a major health and socioeconomic burden in many countries [3]. Experimental autoimmune encephalomyelitis (EAE) is the best studied model of MS in the mice. In EAE, CNS-autoreactive CD4⁺ T cells are activated or introduced, then invade the CNS and the resulting inflammatory cascade resembles human MS in many aspects of clinical and cellular pathogenesis. Recent evidence has revealed that the T cell lineage most likely to be driving EAE pathogenesis are Th17 cells, a pro-

inflammatory helper T cell type characterized by expression of the cytokine IL-17 [4]. Th17 cells differentiate down a separate lineage commitment pathway from Th1 and Th2 cells. Numerous studies in mice have now shown that Th17 cells drive organ-specific autoimmune inflammation and IL-17 has been strongly implicated in human autoimmune disease [5, 6]. Therefore, understanding the mechanisms controlling the generation of Th17 cells is an important step to study MS.

B cells produce plenty of autoantibodies, and proinflammatory cytokines, which play major roles in a number of autoimmune diseases [7]. Phosphoinositide 3-kinase (PI3K) signaling pathways regulate many essential functions of B cells and are therefore a promising target for preventing aberrant B cell response [8]. PI3K δ inhibition has now been shown to reduce the incidence and severity of autoimmune arthritis and autoimmune diabetes in non-obese diabetic mice [9, 10]. In this study, we aimed to investigate whether selective inhibition of

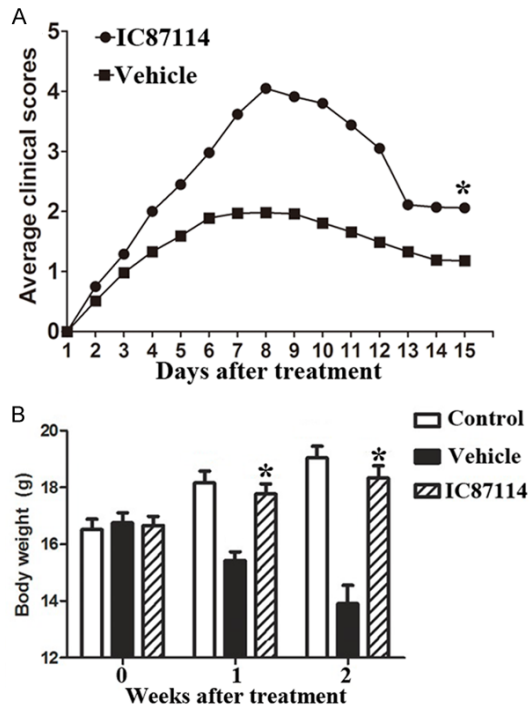


Figure 1. IC87114 attenuated EAE progression. A. IC87114 treatment significantly reduced mean clinical score of EAE mice. Inset denoted average clinical score of EAE mice. B. IC87114 treatment prevented against body weight loss of EAE mice. All data are presented as mean $\pm$ SD of the mean (n=10). *P<0.01.

PI3K δ , using IC87114, attenuates CNS disease in EAE mice.

Materials and methods

Mice and EAE induction

Female C57BL/6 mice (6-8 weeks old) were purchased from the Shandong Provincial Animal Center. EAE induction was conducted as described previously [11, 12]. In brief, each mouse received subcutaneous injection of 100 μ l of complete Freund's adjuvant containing 300 μ g of MOG35-55 and 400 μ g of heat-inactivated *Mycobacterium tuberculosis* H37RA. Pertussis toxin (200 ng/mouse) was given intraperitoneally on the day of immunization and again two days later. The experimental study was approved by the Animal Institutional Review Board of our Hospital.

IC87114 administration

EAE was induced in naïve mice and 10 days later, these mice were completely randomized

into groups that received treatment with the selective PI3K δ inhibitor IC87114 (50 mg/kg/day, Santa Cruz) or the equivalent volume of vehicle control intraperitoneally as previously reported [10]. This treatment was continued every day throughout the remainder of the study. Clinical scores (0, no symptoms; 1, limp tail; 2, partial paralysis of hind limbs; 3, complete paralysis of hind limbs or partial hind and front limb paralysis; 4, tetraparalysis; 5, moribund; 6, death) were recorded on a daily basis. The mean score was calculated for each group every day.

H&E and luxol fast blue staining

Fifteen days post treatment, ten animals per group were euthanized by exposure to CO₂ and perfused with sterile PBS. Spinal cords were removed, post fixed in 10% formalin (Thermo Fisher Scientific, Waltham, MA, USA) and then embedded in paraffin. Sections were cut at 6 μ m thickness on a microtome and stained for H&E (Thermo Fisher) to reveal perivascular inflammatory infiltrates and luxol fast blue staining (Thermo Fisher) for myelin detection. Histological analysis was performed on a Nikon Eclipse E800 (Nikon, Melville, NY, USA) microscope, acquired with Slidebook version 5.0 software (Olympus, Center Valley, PA, USA) and analyzed using Fiji/Image J software. Quantification for each stain was performed on nine random sections per animal and five animals per group. All images were analyzed by investigators that were blinded to the status of the animal. The percent of inflammatory infiltrates was measured by the number of total cells.

Cytokine analysis and flow cytometry

EAE-induced mice were given vehicle, or IC87114 as indicated above. On day 15, blood was collected and serum was isolated for cytokine analysis. All ELISA kits used in cytokine analysis were purchased from Biolegend and used according to the manufacturer's instructions.

Spleens were excised prior to perfusion. Mice were then perfused with 10 mL heparinized PBS, and whole brain tissue was isolated. Tissues were homogenized separately into a single-cell suspension and subjected to red blood cell lysis. Mononuclear cells from whole

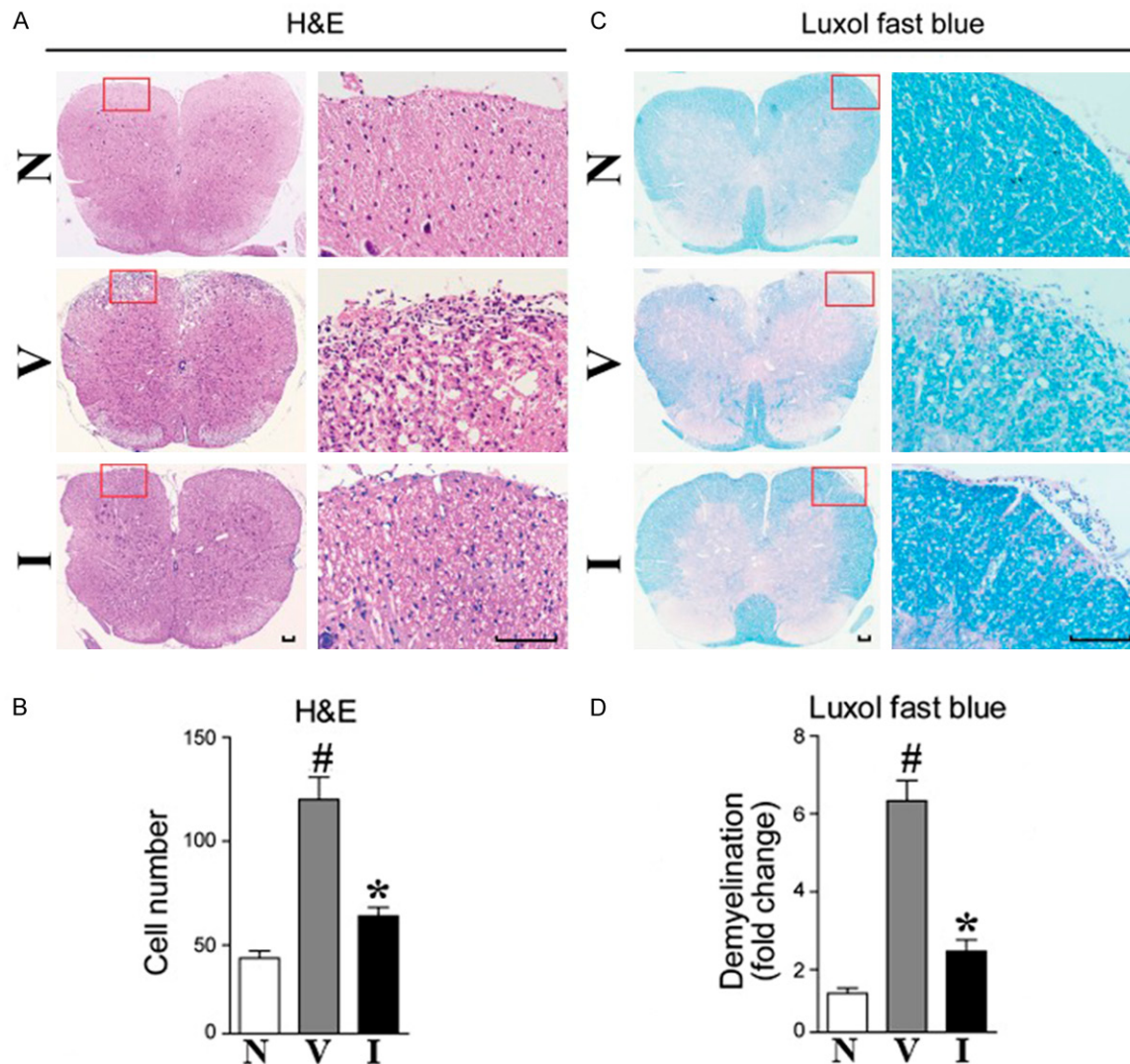


Figure 2. IC87114 alleviated CNS infiltrates and demyelination in EAE mice. (A) H&E staining and (B) Luxol fast blue staining of paraffin sections of spinal cords isolated from naive (N), vehicle (V), or IC87114 (I)-treated EAE mice. Scale bar, 100 μ m. (C and D) Quantification of CNS infiltrates and demyelination in (A and B). n=10, data are Mean $\pm$ SD. #P<0.01, compared with naive mice; *P<0.01, compared with vehicle-treated group.

brain homogenates were isolated using 33% Percoll. Cells were counted and stained with fluorescently tagged mAbs. The following mAbs were purchased from Biolegend (San Diego, CA, USA): Fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD4 mAb, Phycoerythrin (PE) conjugated anti-mouse CD25, PE-conjugated anti-mouse IL-17 and PE-conjugated anti-mouse IFN- γ .

Western blot

Brain cortices of mice were homogenized in CellLyticTM MT mammalian tissue lysis reagent supplemented with protease inhibitor cocktail

(Sigma). Afterwards, the homogenate was centrifuged at 15,000 rpm and 4°C for 10 min. Protein concentration of the supernatants was quantified by BCA method. Thirty microgram of each samples were loaded to a 12% SDS-PAGE gel. After electrophoresis, proteins were transferred to PVDF membranes (Millipore) by an electrophoretic transfer system (Bio-Rad). The membranes were blocked with 5% skim milk in PBST for 1 hr and then incubated with respective primary antibodies at 4°C overnight. After thoroughly washed by PBST, the membranes were further incubated with horseradish peroxidase conjugated secondary antibodies and

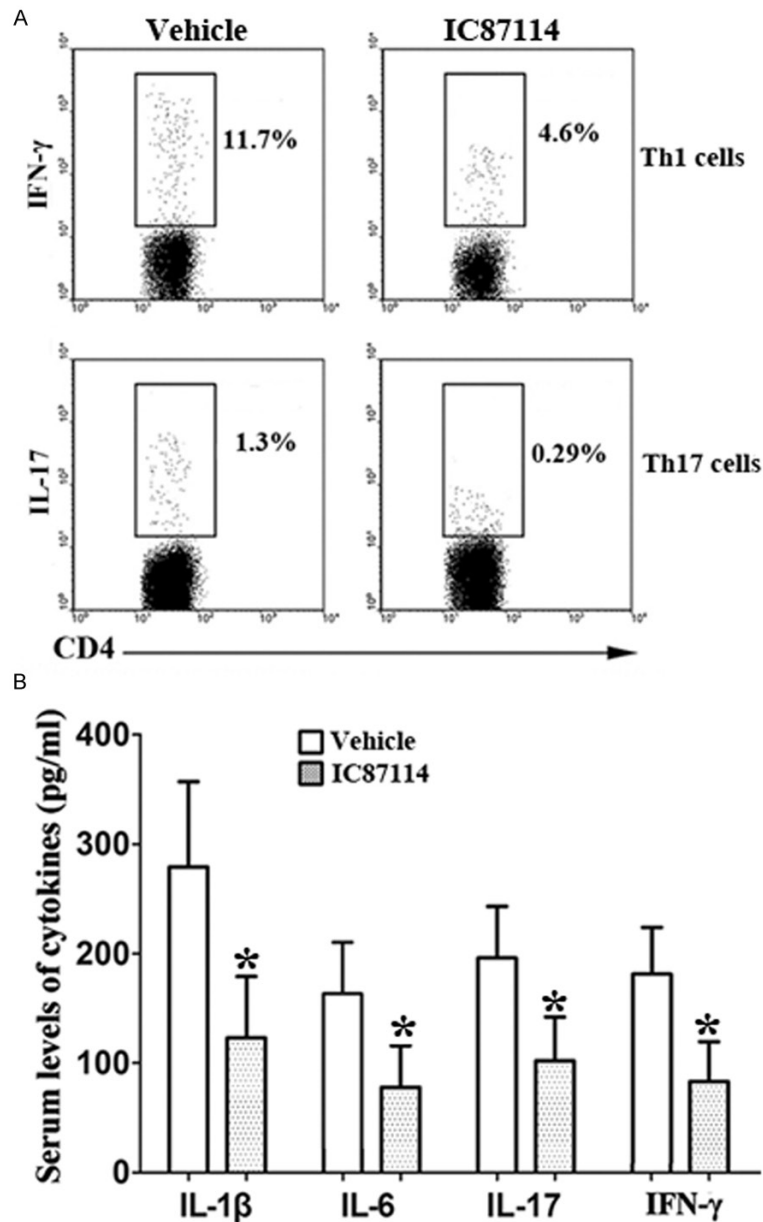


Figure 3. IC87114 treatment reduced the number of total Th1 and Th17 cells. A. Cells in the spinal cords were analyzed by flow cytometry. B. Levels of cytokines in the serum. Values are the mean $\pm$ SD from n=10 mice per group in each experiment). *P<0.01, compared with vehicle-treated group.

visualized with enhanced chemiluminescence reagents (Qiagen, China).

Statistics

Results are expressed as mean $\pm$ SD. Statistical analysis was performed using SPSS16.0. One Way ANOVA was used to compare data among groups and Student t test was used to compare data between groups. A P-value of ≤ 0.05 was

considered to be statistically significant.

Results

IC87114 treatment mitigated the clinical symptoms of EAE

First, we investigated the effects of IC87114 after immunization with MOG (post-treatment), on the clinical course of EAE. Clinical scores revealed that IC87114 treatment markedly delayed the onset of EAE and reduced the clinical symptoms compared to vehicle-treated EAE mice (**Figure 1A**).

To confirm the results of clinical score, we monitored the body weight of mice weekly. As shown in **Figure 1B**, mice showed persistent body weight loss with the progression of EAE. However, IC87114 treatment prevented against body weight loss of EAE mice, which indicated the therapeutic effect of this compound.

IC87114 attenuated neuro-inflammation and demyelination of EAE Mice

Spinal cords taken from vehicle-treated EAE mice displayed cellular infiltration, particularly within the myelinated regions, and the accumulation of these inflammatory cells was accompanied by immense tissue damage compared to naïve mice (**Figure**

2A). EAE mice treated with IC87114 showed dramatically reduced levels of infiltration and altered tissue morphology compared to vehicle-treated EAE mice (**Figure 2A, 2B**).

To further examine whether IC87114 treatment could lessen demyelination of CNS, luxol fast blue staining was conducted on spinal cord sections. As shown in **Figure 2B** and **2D**, compared with naïve mice, EAE mice displayed

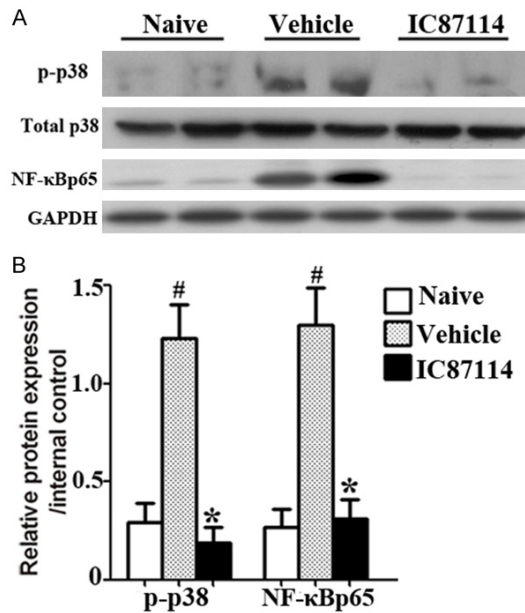


Figure 4. Akt and NF- κ B activation was inhibited in CNS of IC87114-treated mice with EAE. A. Representative Western blots of Akt and NF- κ B p65 in spinal cords. GAPDH was used as internal control. B. Quantification of the NF- κ Bp65 and p-Akt/GAPDH ratio. In the histograms, the data are the mean $\pm$ SEM for 10 mice per group. [#]P<0.01, compared with naive mice; ^{*}P<0.01, compared with vehicle-treated group.

severe demyelination (**Figure 2C**). In contrast, those mice treated with IC87114 exhibited significantly improved demyelination of EAE Mice by the enhanced luxol fast blue staining (**Figure 2C, 2D**).

Th1 and Th17 cells were reduced in CNS of IC87114-treated mice with EAE

To evaluate the effect of IC87114 on T cell responses, we examined the frequencies of Th1 and Th17 cell numbers in the spinal cords in vehicle- and IC87114-treated mice with EAE. The percentage of INF- γ -producing CD4⁺ Th1 cells was obviously decreased in the spinal cords of IC87114-treated mice with EAE (**Figure 3A**). Similarly, the number of IL-17-producing Th17 cells showed a marked reduction in the spinal cords of IC87114-treated mice with EAE (**Figure 3A**).

Furthermore, the related cytokine levels at the immediate sites of inflammation including the serum of IL-1 β , IL-6, IL-17 and INF- γ were significantly decreased in IC87114-treated mice with EAE compared with vehicle-treated mice, with a statistically significant difference (**Figure 3B**).

Akt and NF- κ B activation was inhibited in CNS of IC87114-treated mice with EAE

Akt and NF- κ B are downstream molecules of PI3K activation. We assessed the Akt and NF- κ B activation after treatment with IC87114 (**Figure 4**). Elevated Akt and NF- κ B activation was observed in EAE mice in comparison with naïve mice. In IC87114-treated mice, p-Akt and NF- κ Bp65 protein expression was significantly reduced compared with controls.

Discussion

In this study, we investigated the efficacy of the selective PI3K δ inhibitor IC87114 in mouse models for EAE. IC87114 reduced clinical symptoms of EAE and prevented brain and spinal cord inflammation. Interestingly, we found that IC87114 treatment suppressed the production of Th1, Th17 cells and cytokine production. These findings suggest that the PI3K δ is responsible for EAE progression and PI3K δ inhibitors are effective in EAE treatment.

PI3K δ also plays an important role in TCR-induced T-cell activation [13] and PI3K δ inhibition blocks TCR-induced cytokine production by both naïve and memory human T cells in vitro [14]. In particular, PI3K δ signaling is important for IL-17 production by mouse and human T cells [14]. Th17 cells have been implicated in a number of autoimmune diseases including EAE [15]. Enhanced phosphoinositide 3-kinase δ activity is a frequent event in systemic lupus erythematosus that confers resistance to activation-induced T cell death [16]. These data show that PI3K δ over-activation is implicated in autoimmune diseases.

As a selective PI3K δ inhibitor, IC87114 has been shown to suppress the severity of autoimmune arthritis in mice [17], and inhibit the progression of mouse autoimmune diabetes [18]. The ability of PI3K δ inhibitors to reduce the severity of these autoimmune diseases results not only from their actions on B cells, but also from inhibitory effects on other immune cells that contribute to autoimmune disease [19]. IC87114 inhibits the trafficking of neutrophils into inflamed tissues [20] and prevents the activation of mast cells [21]. In the present study, we show that treatment of EAE mice with IC87114 reduced the clinical symptoms, histopathology and cellular infiltration into the CNS.

Notably, AKT and NF- κ B were demonstrated to be critical in the context of systemic autoimmunity. Inhibition of PI3K signaling and Akt pathways have been proposed as therapeutic targets in autoimmune diseases [22, 23]. In addition, PI3K/Akt was implicated in the Th17 cell differentiation [24]. Our data in the present study indicated that Akt and NF- κ B activation was reduced by PI3K inhibitor, suggesting that IC87114 appears to be as a particularly attractive target via inhibiting the AKT/NF- κ B axis. Consistently, treatment of EAE with IC87114 suppressed the Th1 and Th17 cell ratios. And, the serum levels of IL-1 β , IL-6, IL-17 and INF- γ were markedly reduced by IC87114.

In summary, these present data support that PI3K δ inhibition could have an therapeutic potential for EAE. Further studies in other strains of EAE animal models and humans will be needed to explore the effects and mechanism of IC87114, in order to provide its clinical application for human MS.

Disclosure of conflict of interest

None.

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References

- [1] Ramagopalan SV, Dobson R, Meier UC and Giovannoni G. Multiple sclerosis: risk factors, prodromes, and potential causal pathways. *Lancet Neurol* 2010; 9: 727-739.
- [2] Ishiguro A, Akiyama T, Adachi H, Inoue J and Nakamura Y. Therapeutic potential of anti-interleukin-17A aptamer: suppression of interleukin-17A signaling and attenuation of autoimmunity in two mouse models. *Arthritis Rheum* 2011; 63: 455-466.
- [3] Reese JP, John A, Wienemann G, Wellek A, Sommer N, Tackenberg B, Balzer-Geldsetzer M and Dodel R. Economic burden in a German cohort of patients with multiple sclerosis. *Eur Neurol* 2011; 66: 311-321.
- [4] Wang X, Ma C, Wu J and Zhu J. Roles of T helper 17 cells and interleukin-17 in neuroautoimmune diseases with emphasis on multiple sclerosis and Guillain-Barré syndrome as well as their animal models. *J Neurosci Res* 2013; 91:871-881.
- [5] Robinson KM, Manni ML, Biswas PS and Alcorn JF. Clinical consequences of targeting IL-17 and TH17 in autoimmune and allergic disorders. *Curr Allergy Asthma Rep* 2103; 13: 587-595.
- [6] Jadidi-Niaragh F and Mirshafiey A. Regulatory T-cell as orchestra leader in immunosuppression process of multiple sclerosis. *Immunopharmacol Immunotoxicol* 2011; 33: 545-567.
- [7] Yanaba K, Bouaziz JD, Matsushita T, Magro CM, St Clair EW and Tedder TF. B-lymphocyte contributions to human autoimmune disease. *Immunol Rev* 2008; 223: 284-299.
- [8] Puri KD and Gold MR. Selective inhibitors of phosphoinositide 3-kinase delta: modulators of B-cell function with potential for treating autoimmune inflammatory diseases and B-cell malignancies. *Front Immunol* 2012; 3: 256.
- [9] Randis TM, Puri KD, Zhou H and Diacovo TG. Role of PI3Kdelta and PI3Kgamma in inflammatory arthritis and tissue localization of neutrophils. *Eur J Immunol* 2008; 38: 1215-1224.
- [10] Durand CA, Richer MJ, Brenker K, Graves M, Shanina I, Choi K, Horwitz MS, Puri KD and Gold MR. Selective pharmacological inhibition of phosphoinositide 3-kinase p110delta opposes the progression of autoimmune diabetes in non-obese diabetic (NOD) mice. *Autoimmunity* 2013; 46: 62-73.
- [11] Wu X, Pan W, He Y, Hsueh H and Kastin AJ. Cerebral interleukin-15 shows upregulation and beneficial effects in experimental autoimmune encephalomyelitis. *J Neuroimmunol* 2010; 223: 65-72.
- [12] Peiris M, Monteith GR, Roberts-Thomson SJ and Cabot PJ. A model of experimental autoimmune encephalomyelitis (EAE) in C57BL/6 mice for the characterisation of intervention therapies. *J Neurosci Meth* 2007; 163: 245-254.
- [13] Okkenhaug K, Patton DT, Bilancio A, Garçon F, Rowan WC and Vanhaesebroeck B. The p110delta isoform of phosphoinositide 3-kinase controls clonal expansion and differentiation of Th cells. *J Immunol* 2006; 177: 5122-5128.
- [14] Soond DR, Bjørge E, Moltu K, Dale VQ, Patton DT, Torgersen KM, Galleway F, Twomey B, Clark J, Gaston JS, Taskén K, Bunyard P and Okkenhaug K. PI3K p110delta regulates T-cell cytokine production during primary and secondary immune responses in mice and humans. *Blood* 2010; 115: 2203-2213.
- [15] Ambrosi A, Espinosa A and Wahren-Herlenius M. IL-17: a new actor in IFN-driven systemic autoimmune diseases. *Eur J Immunol* 2012; 42: 2274-2284.
- [16] Suárez-Fueyo A, Barber DF, Martínez-Ara J, Zea-Mendoza AC and Carrera AC. Enhanced

- phosphoinositide 3-kinase δ activity is a frequent event in systemic lupus erythematosus that confers resistance to activation-induced T cell death. *J Immunol* 2011; 187: 2376-2385.
- [17] Randis TM, Puri KD, Zhou H and Diacovo TG. Role of PI3Kdelta and PI3Kgamma in inflammatory arthritis and tissue localization of neutrophils. *Eur J Immunol* 2008; 38: 1215-1224.
 - [18] Puri KD and Gold MR. Selective inhibitors of phosphoinositide 3-kinase delta. modulators of B-cell function with potential for treating autoimmune inflammatory diseases and B-cell malignancies. *Front Immunol* 2012; 3: 256.
 - [19] Fung-Leung WP. Phosphoinositide 3-kinase delta (PI3K δ) in leukocyte signaling and function. *Cell Signal* 2011; 23: 603-608.
 - [20] Puri KD, Doggett TA, Douangpanya J, Hou Y, Tino WT, Wilson T, Graf T, Clayton E, Turner M, Hayflick JS and Diacovo TG. Mechanisms and implications of phosphoinositide 3-kinase delta in promoting neutrophil trafficking into inflamed tissue. *Blood* 2004; 103: 3448-3456.
 - [21] Ali K, Camps M, Pearce WP, Ji H, Rückle T, Kuehn N, Pasquali C, Chabert C, Rommel C and Vanhaesebroeck B. Isoform-specific functions of phosphoinositide 3-kinases: p110 delta but not p110 gamma promotes optimal allergic responses in vivo. *J Immunol* 2008; 180: 2538-2544.
 - [22] Foster JG, Blunt MD, Carter E, and Ward SG. Inhibition of PI3K signaling spurs new therapeutic opportunities in inflammatory/autoimmune diseases and hematological malignancies. *Pharmacol Rev* 2012; 64: 1027-1054.
 - [23] O'Sullivan B, Thompson A and Thomas R. NF-kappa B as a therapeutic target in autoimmune disease. *Expert Opin Ther Targets* 2007; 11: 111-122.
 - [24] Nagai S, Kurebayashi Y and Koyasu S. Role of PI3K/Akt and mTOR complexes in Th17 cell differentiation. *Ann N Y Acad Sci* 2013; 1280: 30-34.