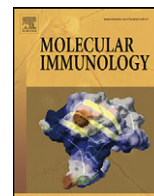




Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm



Role of phosphoinositide 3-kinase p110 δ in TLR4- and TLR9-mediated B cell cytokine production and differentiation

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ARTICLE INFO

Article history:

Received 30 December 2008

Received in revised form 12 March 2009

Accepted 14 March 2009

Available online xxx

Keywords:

B lymphocyte

Phosphoinositide 3-kinase

Toll-like receptor

TLR4

TLR9

LPS

CpG

Cytokines

Immunoglobulins

Plasma cells

ABSTRACT

Phosphoinositide 3-kinase (PI3K) enzymes play key roles in signaling via antigen receptors and cytokine receptors and isoform-selective PI3K inhibitors are being evaluated as targets for treatment of allergic and inflammatory diseases. The specific roles of PI3K isoforms in TLR-mediated activation of lymphocytes have not been defined. In this study we assess the role of p110 δ PI3K in TLR4, TLR9, or TLR4 + TLR9-mediated B cell responses. Utilizing both p110 δ -mutant mice and p110 δ -specific inhibitor IC87114, we find that signaling via p110 δ is required for optimal B cell proliferation, but is not required for TLR-mediated B cell differentiation into plasma cells or Ig isotype switch. However PI3K blockade led to increased frequencies of IgG1 and IgE expressing cells, and partially reversed ability of CpG to inhibit IgG1 and IgE. Examination of B cell cytokine production revealed that p110 δ blockade markedly reduced IL-6 and IL-10 production. In contrast, p110 δ signaling was clearly not required for IL-12 production, with p110 δ -mutant B cells in fact showing enhanced IL-12 p70 production. TLR4- and TLR9-ligands act in synergy to drive IL-6 and IL-10 production, but not IL-12, and this additive effect is independent of p110 δ signaling. Together, these results indicate that PI3K δ functions in influencing the type of B cell cytokine production and differentiation response induced by TLR-ligands.

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1. Introduction

Toll-like receptors (TLRs) detect pathogen associated molecular patterns (PAMPs) such as lipopolysaccharides (LPS) and unmethylated deoxycytidyl-deoxyguanosine (CpG)-containing microbial DNAs and play an essential role in the induction of both innate and adaptive immune responses (Pasare and Medzhitov, 2005b). Although TLRs can directly induce innate immunity providing immediate protective responses against pathogens, the mechanisms of TLR-mediated control of adaptive immunity are not fully understood.

Different immune cells express distinct subsets of the TLRs, which enables the immune system to tailor its responses against different pathogen classes (Iwasaki and Medzhitov, 2004). TLR4 and TLR9 are two major TLRs expressed in B cells that mediate potent and distinct biological effects (Peng, 2005). Naive mouse B cells proliferate and differentiate in response to LPS or CpG (Coutinho

et al., 1974; Krieg et al., 1995). LPS can directly activate mouse B cells in vitro, inducing proliferation, class switch recombination and plasma cell differentiation (Li et al., 1997). Unmethylated DNA from prokaryotic organisms and simple eukaryotic organisms, is known to act as adjuvant for mammalian B cell responses (Krieg et al., 1995). Synthetic oligodeoxynucleotides (ODNs) bearing the unmethylated CpG motifs (Krieg, 2002) mimic the immunostimulatory activity of bacterial DNA and are recognized by the TLR9. The use of synthetic CpG ODN, avoids ambiguity caused when bacterial DNA preparations are contaminated with other bacterial products such as LPS.

The essential requirement of TLR signaling in humoral immune response remains controversial. In MyD88-knockout mice, it has been demonstrated that activation of TLRs is required for maximal T cell-dependent B cell responses to immunization with LPS-containing antigens (Pasare and Medzhitov, 2005a). However, Gavin et al. (2006) showed that in mice deficient in the critical signaling components for TLR (MyD88^{-/-}/Trif^{Lps2/Lps2}), several typical adjuvants could elicit robust T cell-dependent B cell responses, indicating that TLR signals are not essential for induction of B cell activation under some conditions.

Most of our current understanding of TLR intracellular signaling mechanisms comes from studies in myeloid cells. Recent evidence

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indicates that phosphoinositide 3-kinases (PI3Ks) may be involved in modulating TLR signaling in these cell types (Ishii et al., 2002; Ojaniemi et al., 2003). PI3Ks are a family of signal-transducing enzymes known to be critical for adaptive immune responses (Fruman and Cantley, 2002; Okkenhaug and Vanhaesebroeck, 2003). Activation of PI3K by extracellular stimuli results in the phosphorylation of phosphoinositides on the third position of the inositol ring, leading to the transient accumulation of phospholipids in cell membranes (Fruman et al., 1998). These phospholipids serve as second messengers and/or signaling molecules to control many cellular events, such as cell differentiation, survival, cytoskeletal organization, vesicular trafficking, and phagocytosis in different cell types including those that are involved in innate and adaptive immunity (Fruman and Cantley, 2002; Okkenhaug and Vanhaesebroeck, 2003). PI3Ks are classified into several classes on the basis of their structural characteristics and substrate specificities. Class 1 PI3Ks comprise 4 distinct isoforms of p110 catalytic subunits, designated p110 α , p110 β , p110 γ , or p110 δ (Wymann and Pirola, 1998). PI3K γ and δ are preferentially expressed in cells of hemopoietic origin, whereas PI3K α and β are ubiquitously expressed. The p110 α and β knockout mice are embryonically lethal, whereas knockout mice for p110 γ and δ are viable and fertile (reviewed in Rommel et al., 2007). PI3K δ null or catalytically inactive mutant mice show defects in B cell functions such as BCR-signaling, basal immunoglobulin production and T-dependent Ab responses (Clayton et al., 2002; Okkenhaug et al., 2002).

The PI3K pathway has been shown to be triggered by various TLR agonists and can function as a positive or negative regulator of TLR responses depending on the immune cell type and the TLR ligand used (reviewed in Fukao and Koyasu, 2003). The activation of PI3Ks by many microbial stimuli such as LPS, CpG DNA or CpG ODN plays an important role in regulating immune cell defensive mechanisms, including cytokine production, phagocytosis, and apoptosis (Fukao and Koyasu, 2003). Several studies have shown that global PI3K inhibitors such as wortmannin and LY294002 interfere with TLR-mediated cellular and molecular responses (Ishii et al., 2002; Ojaniemi et al., 2003; Park et al., 1997; Weinstein et al., 2000). However, few studies have investigated the isoform of PI3Ks responsible, and nothing is known about the specific roles of PI3Ks in TLR-induced B cell functions. In the present study, we use both p110 δ -mutant mice and p110 δ -specific inhibitor IC87114 to demonstrate that PI3K p110 δ signaling plays an important role in TLR4 agonist LPS- and TLR9 agonist CpG-mediated B cell functional responses.

2. Material and methods

2.1. Mice

p110 δ ^{D910A/D910A} mice have been described previously (Okkenhaug et al., 2002) and were backcrossed to C57BL6 for 9 generations. Age (6–12 wk) and sex matched wild-type (WT) mice were purchased either from Charles River Laboratories or bred locally. All animals were housed at the Central Animal Care Facility (University of Manitoba) in compliance with the guidelines established by the Canadian Council on Animal Care and were given standard rodent chow and water ad libitum.

2.2. TLR agonists and inhibitors

Endotoxin-free class B stimulatory CpG ODN-1826, 5'-TCCAT-GACGTTCTGACGTT-3' and control non-stimulatory, non-CpG ODN-1982, 5'-TCCAGGACTTCTCAGTT-3' were from InvivoGen. Hereafter, CpG ODN-1826 will be referred to as CpG and ODN-1982 will be referred to as ODN. Lipopolysaccharide was purchased from Sigma and ultrapure *Escherichia coli* LPS was

from InvivoGen. These TLR agonists were used at concentrations indicated in the text. Titration experiments using a range of concentrations of both CpG and control ODNs (5 μ M, 1 μ M, 0.1 μ M, 0.05 μ M, 0.025 μ M, 0.0125 μ M, 0.00625 μ M) and LPS (1000 ng/ml, 500 ng/ml, 100 ng/ml, 10 ng/ml) indicated that low dose (100 ng/ml) LPS was an appropriate concentration to investigate possible synergy between TLR4 and TLR9. The p110 δ selective inhibitor IC87114 (Sadhu et al., 2003) was provided by Dr. Kamal Puri (Calistoga Biopharma).

2.3. Splenic B cell isolation

After cervical dislocation, spleens were removed and homogenized to obtain a single cell suspension. B cells were purified by negative selection using the CD43 MicroBeads and MACS columns (Miltenyi Biotec) following the manufacturer's instructions. The purity of enriched B cells was evaluated by FACS analysis using CD19 staining (PE-anti-CD19, BD Biosciences), and was found to be >97% pure.

2.4. Cell culture and proliferation assay

Single cell suspensions (2 $\times$ 10⁵ cells/well) of purified B cells, from wild-type or p110 δ ^{D910A} mice, were cultured in complete medium (RPMI 1640, supplemented with 10% FCS, 2 $\times$ 10⁻⁵ M 2-ME) in 96-well flat-bottom plates at a final volume of a 200 μ l. Cells were stimulated with varying concentrations of LPS and/or CpG or control ODN, with or without p110 δ -specific small molecule inhibitor IC87114 (Sadhu et al., 2003), as indicated in the figure legends. On days 1, 2 and 3 of culture, the cells were pulsed with 1 μ Ci of [³H] thymidine (Amersham) for the last 12 h. Pulsed B cells were harvested on a filter paper using an automated plate harvester (Tomtec) and dried. Incorporation of radioactive thymidine was then determined using a liquid scintillation counter (Wallac).

2.5. ELISA assays

Supernatants of WT or p110 δ ^{D910A} B cell cultures were collected after 24 h, 48 h or 72 h of incubation and cytokines were assayed by ELISA. Ninety-six-well flat-bottom microtitre ELISA plates (MaxiSorp; Nunc) were coated overnight at 4 °C with purified rat anti-mouse IL-6, IL-10, or IL-12p70 (BD Biosciences) and serially-diluted respective cytokines were detected with biotinylated rat anti-mouse IL-6, IL-10, IL-12 (BD Biosciences), using recombinant cytokines: rIL-6, rIL-10, and rIL-12 (BD Biosciences) as standards.

2.6. Flow cytometry

Purified B cells were cultured in complete medium for 3 days with 100 ng/ml LPS or 0.1 μ M CpG ODN or 2 μ g/ml anti-CD40 (BD Bioscience) together with 20 ng/ml IL-4 (R&D Systems). IC87114 inhibitor was added at the indicated concentrations at the beginning of the culture period. To analyze the expression of plasmablast marker CD138, cells were surface-stained with PE-labeled anti-CD138 (BD Biosciences). For staining intracellular immunoglobulins, cells were pre-incubated with Fc blocking antibody (mAb 2.4G2), followed by fixation, permeabilization and staining with FITC-labeled anti-IgG1 and PE-labeled anti-IgE (BD Biosciences), essentially as described (Frasca et al., 2004). Stained cells were analyzed using a FACSCalibur flow cytometer and data plotted using CellQuest software.

2.7. Real-time PCR

RNA was extracted from purified splenic B cells using Trizol reagent. cDNA was synthesized from 1 μ g of RNA using SuperScript

II Reverse Transcriptase (Invitrogen). Quantitative RT-PCR was performed using SyberGreen amplification mix on the LightCycler System (Roche). BLIMP-1 forward, 5'-GGAGGATCTGACCCGAATCA; BLIMP-1 reverse, 5'-CTCCACCATGGAGGTCACATC (44). β -Actin was used as relative expression control to normalize sample variation.

2.8. Statistical analysis

Student's paired *t*-test was used to determine the statistical significance of the data. Statistical analysis was performed on GraphPad Prism Software. Significance was evaluated at a *p* value: **p* < 0.05, ***p* < 0.01, ****p* < 0.0001.

3. Results

3.1. p110 δ inhibition results in impaired TLR-induced B cell proliferation

We characterized the contribution of p110 δ signaling to B cell proliferation induced by TLR4–ligand LPS and TLR9–ligand CpG. Splenic B cells isolated from wild-type and/or p110 δ PI3K-mutant mice were stimulated with LPS alone, CpG, control ODN or LPS + CpG or LPS + control ODN. Cell proliferation was measured by the uptake of [³H]-thymidine during the last 12 h of 48 h culture. LPS or CpG alone induced B cell proliferation, but no additive or synergistic proliferative effect was observed with combination of CpG plus suboptimal dose of LPS (Fig. 1A). The combination of control ODN plus LPS resulted in slightly diminished proliferation compared to LPS alone, suggesting some weak non-specific inhibitory effect of phosphothioate-modified ODNs, which is overcome when the ODN contains stimulatory CpG motifs. Consistent with previous findings (Okkenhaug et al., 2002), homozygous p110 δ enzymatic inactivation (p110 $\delta^{\text{D910A/D910A}}$) significantly impaired *in vitro* proliferation of LPS-stimulated B cells (Fig. 1B). Similarly, upon stimulation with CpG, p110 δ inactivated B lymphocytes showed significantly reduced cell proliferation (Fig. 1B). Similar results were observed at 24 h and 72 h (data not shown). To further validate the role of p110 δ signaling in TLR9-mediated B cell proliferation, we used IC87114, a small molecule inhibitor with selectivity for p110 δ (Sadhu et al., 2003). This pharmacologic intervention resulted in a similar reduction in proliferation of normal B cells in a dose-dependent manner (Fig. 1C). Together these findings demonstrate that PI3K p110 δ signaling is important for both TLR4- and TLR9-mediated B cell proliferation.

3.2. p110 δ activity is not required for TLR4-induced plasma cell differentiation

To determine the role of p110 δ signaling in TLR4-induced plasma cell differentiation, we evaluated the expression of CD138 (Syndecan-1) as a marker of B cell commitment to plasma cell differentiation. p110 δ^{D910A} B cells cultured with LPS + IL-4 showed substantially reduced frequencies of CD138⁺ plasma cells (Fig. 2C and D) suggesting that p110 δ signaling might be required for LPS-induced plasma cell differentiation. However, it is known that p110 δ^{D910A} spleens lack marginal zone B cells (Okkenhaug et al., 2002), which rapidly differentiate into plasma cells in response to LPS (Martin et al., 2001) and thus may contribute disproportionately to LPS-induced plasma cell differentiation in normal splenic B cell preparations. In contrast to results with p110 δ^{D910A} B cells, pharmacological inhibition of p110 δ signaling with IC87114 in WT B cells did not have a substantive effect on generation of CD138⁺ plasma cells. CpG + IL-4 treated cultures consistently result in very few CD138⁺ cells, and the frequency of CD138 positive cells was not altered by p110 δ blockade. Consistent with our measurements of CD138 expressing cells, we found that expression of BLIMP-1, a key transcription factor inducing plasma cell differentiation

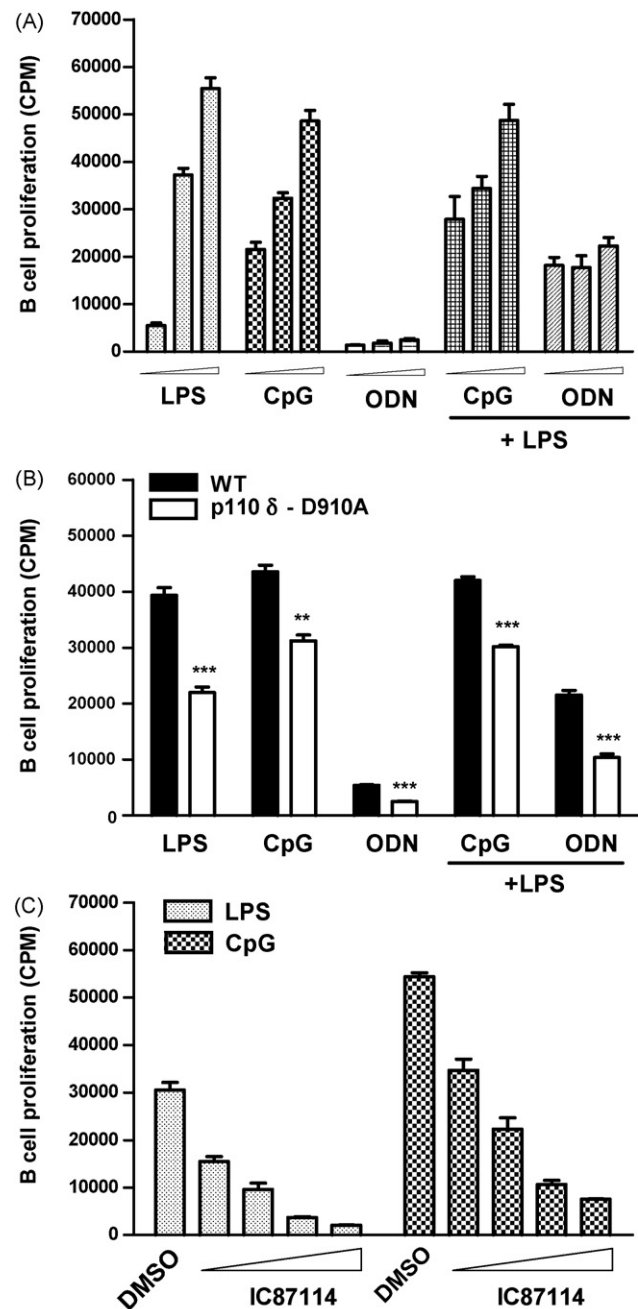


Fig. 1. B cell proliferation in response to LPS, CpG or LPS + CpG is partially dependent on p110 δ PI3K. (A) Purified splenic B cells were stimulated with LPS (10 ng/ml, 100 ng/ml, 1000 ng/ml), CpG or control ODNs (0.1 μ M, 0.05 μ M, 0.0025 μ M), or combination of LPS + CpG (using LPS at 100 ng/ml). [³H]-thymidine incorporation was measured during the last 12 h of the culture. Note that no evidence for additive or synergistic proliferation was observed. (B) Purified splenic WT or p110 δ^{D910A} B cells were stimulated with 100 ng/ml LPS or 0.1 μ M CpG or control ODN alone or in combination for 48 h and [³H]-thymidine incorporation was measured as in (A). (C) Purified splenic B cells were stimulated with 100 ng/ml LPS or 0.1 μ M CpG in the presence of DMSO (control) or various concentrations (2.5 μ M, 5.0 μ M, 10 μ M, 20 μ M) of p110 δ selective inhibitor IC87114 for 48 h and [³H]-thymidine incorporation was measured as in (A). Data are expressed as mean cpm $\pm$ SD. Results are representative of at least six independent experiments. ***p* < 0.01 as compared to the wild type, ****p* < 0.001 as compared to the wild type.

(Martins and Calame, 2008), was reduced only in LPS-stimulated p110 δ^{D910A} splenic B cells, not in wild-type B cells treated with IC87114 (Fig. 2E). Together these results suggest that LPS-induction of the plasma cell differentiation program is not strictly dependent on p110 δ PI3K signaling.

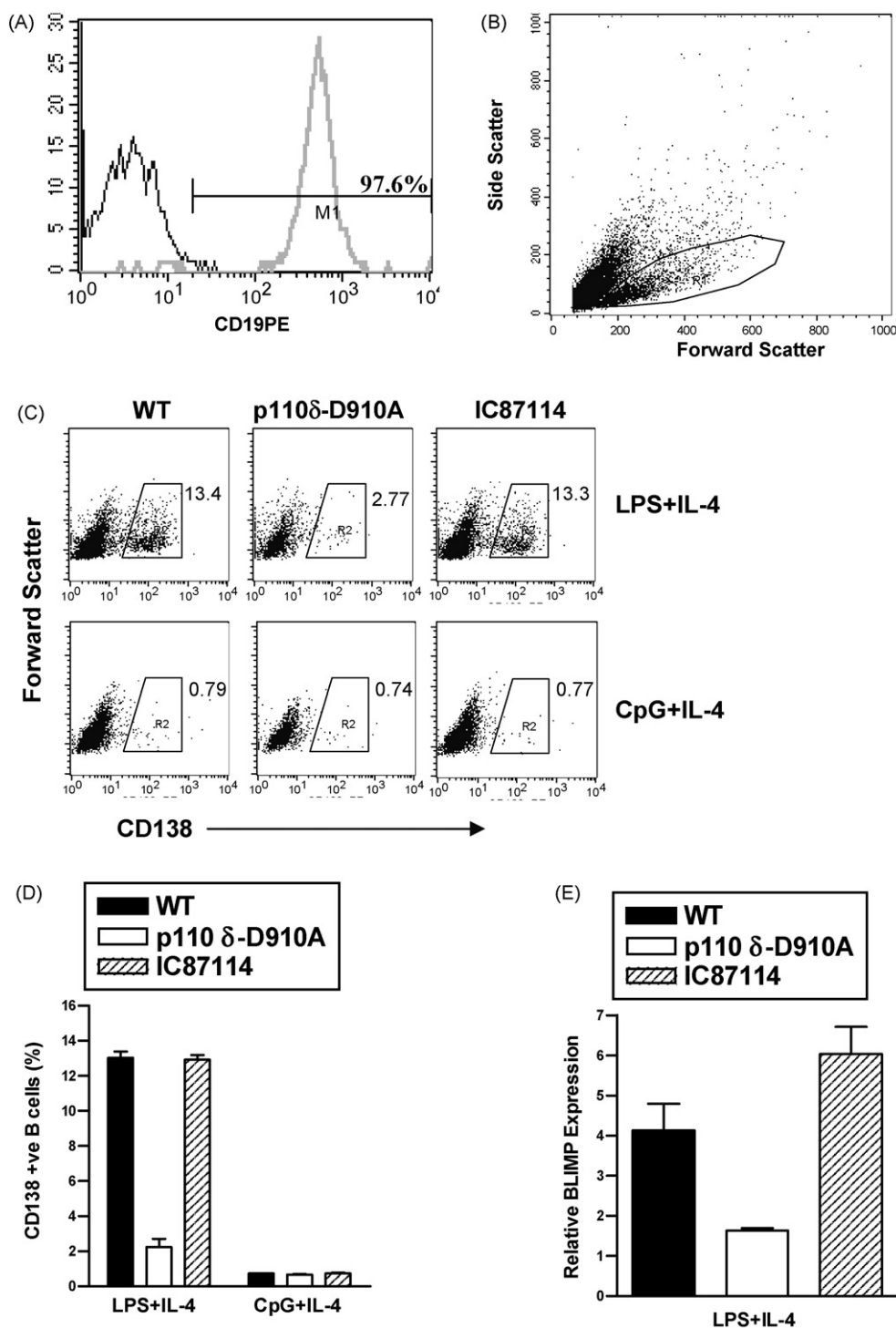


Fig. 2. p110 δ ^{D910A} B cells show reduced LPS-induced plasma cell differentiation, whereas inhibition of p110 δ in normal B cells does not reduce plasma cell frequencies. (A) Purity of splenic B cells isolated using CD43 microbeads, as determined by CD19 staining. (B) Forward and side scatter gate used for FACS analysis of cultured B cells. (C) Purified splenic WT or p110 δ ^{D910A/D910A} B cells or WT B cells treated with 10 μ M p110 δ selective inhibitor IC87114, were stimulated with LPS + IL-4 or CpG + IL-4. At day 3 cells were stained for the plasmablast marker CD138. (D) Average percent CD138⁺ B cells and SEM, and from three independent experiments. (E) At 24 h RNA was extracted and cDNA was subjected to quantitative RT-PCR, normalized BLIMP expression from 3 independent experiments. .

3.3. p110 δ activity regulates TLR9-induced isotype switch

We and others have recently reported that PI3K signaling represses isotype switch to IgG1 and IgE (Omori et al., 2006; Zhang et al., 2008). We thus determined whether signaling via p110 δ affects TLR9-induced isotype switch and/or the ability of CpG to inhibit switch to IgG1/E induced by IL-4. WT and p110 δ ^{D910A} B cells were stimulated with LPS + IL-4 or CpG + IL-4, and then IgG1 and IgE

positive cells were detected by flow cytometry (Fig. 3A). p110 δ ^{D910A} and IC87114-treated B cells showed a significantly enhanced isotype switch to both IgG1 and IgE. While TLR9 stimulation results in less switch to these Ig isotypes, blockade of p110 δ activity deregulated TLR9-induced switch to a similar extent as TLR4-induced switch (Fig. 3B). These results suggest that signaling via p110 δ actively inhibit isotype switch induced through multiple TLRs. To test whether p110 δ activity is required for CpG to actively inhibit

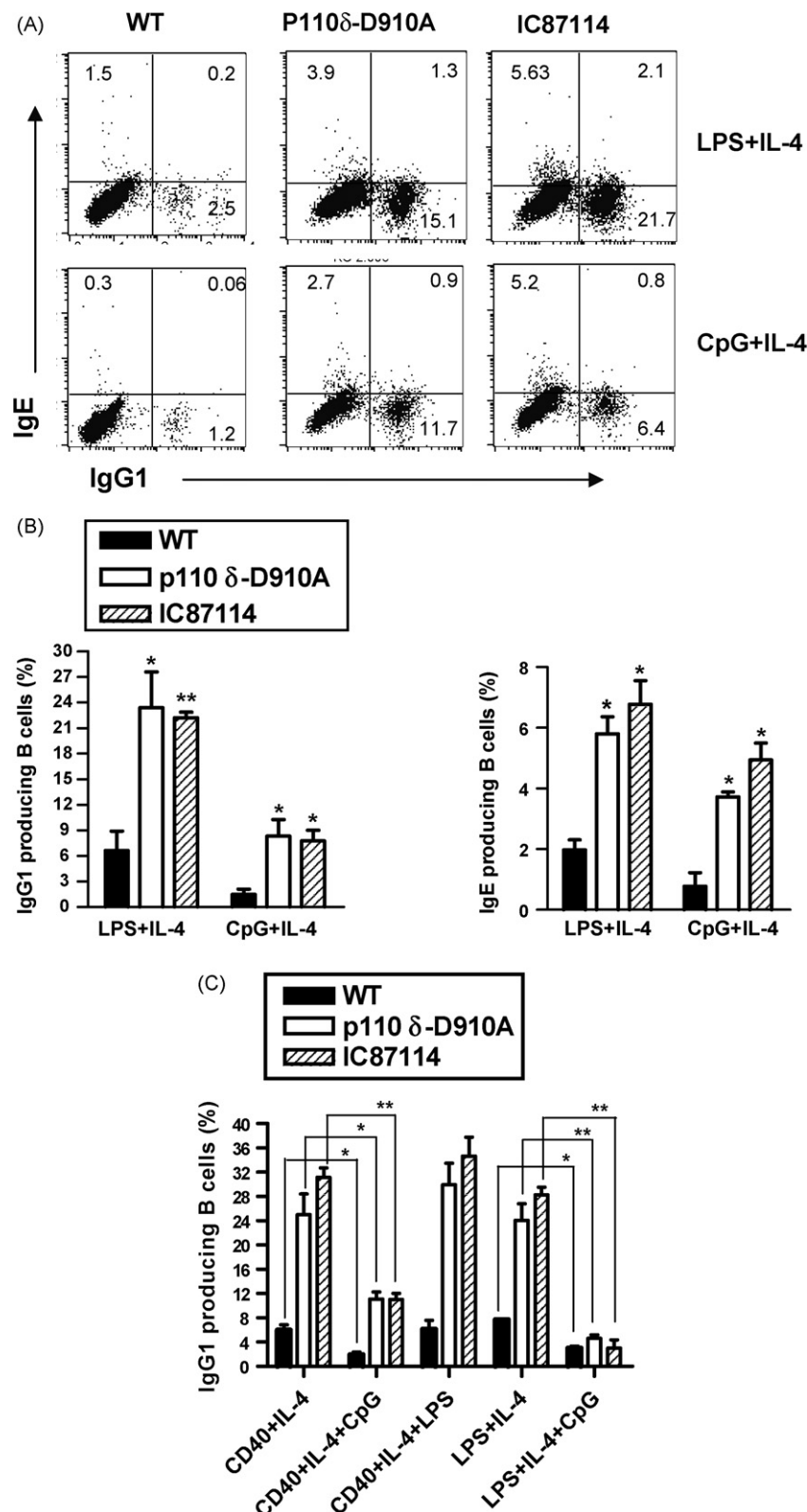


Fig. 3. p110 δ inhibition alters the pattern of TLR-induced Ig isotype switch. Purified splenic WT or p110 δ ^{D910A} B cells were cultured with the indicated combinations of stimulators, with or without 10 μ M p110 δ selective inhibitor IC87114. After 3 days of culture, cells were fixed and stained for intracellular Immunoglobulins. (A) IgG1 and IgE double staining. (B) Average percent IgG1⁺ and IgE⁺ cells and SEM from three independent experiments. (C) Average percent IgG1⁺ cells and SEM from three independent experiments. * p < 0.05, ** p < 0.01 as compared to wild type.

switch to IgG1 (Lin et al., 2004; Liu et al., 2003), we cultured WT or p110 δ ^{D910A} B cells with anti-CD40 + IL-4 to stimulate high levels of IgG1 switch, alone or together with either CpG or LPS. Consistent with previous studies, CpG, but not LPS, is able to inhibit

CD40 + IL-4 induced switch to IgG1 (Fig. 3C). Interestingly, high levels of IgG1 switch could be observed in the presence of CpG when p110 δ activity was blocked (Fig. 3C), indicating that the inhibitory effect is partially dependant on intact PI3K signaling.

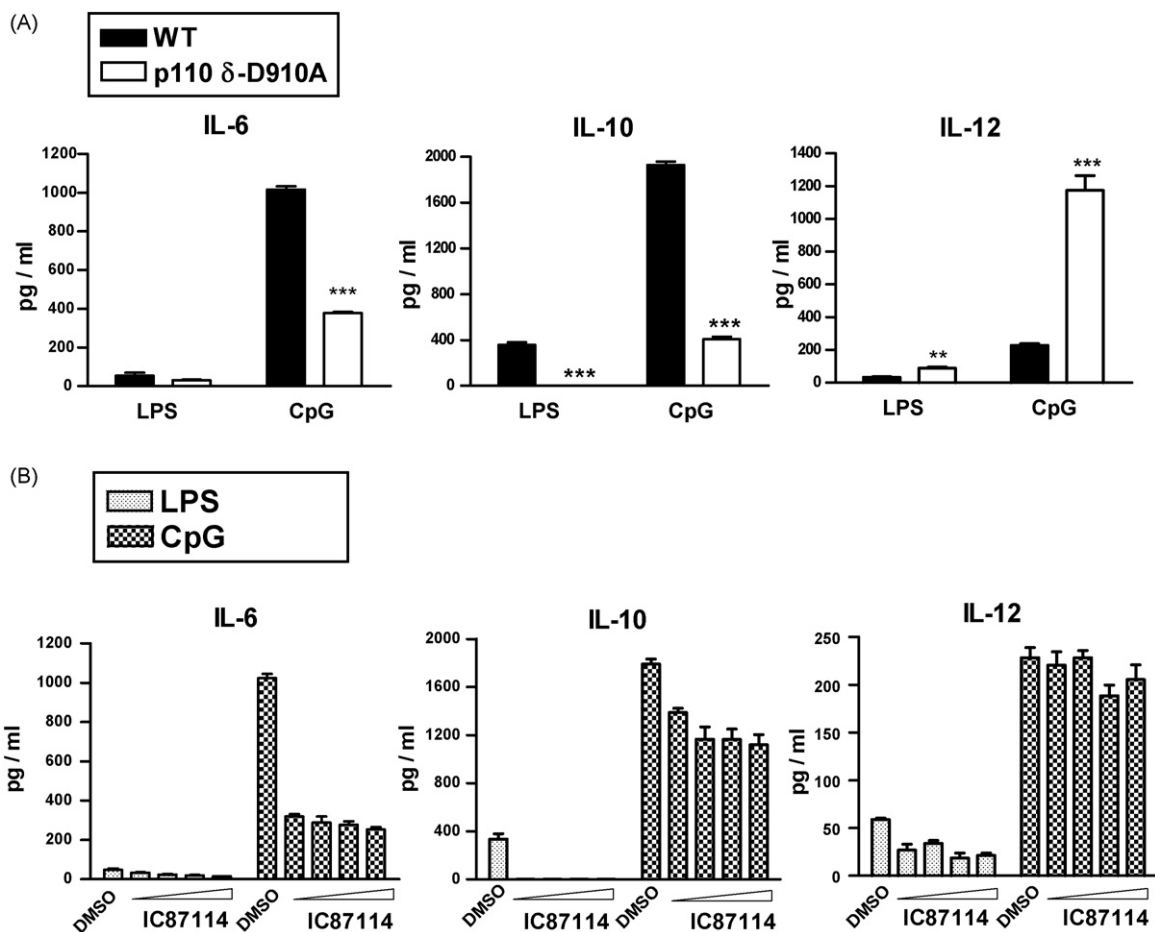


Fig. 4. p110 δ inhibition reduces LPS- or CpG-induced B cell IL-6 and IL-10 production, but not IL-12 production. (A) Purified splenic WT or p110 δ ^{D910A} B cells were cultured with 100 ng/ml LPS or 0.1 μ M CpG. (B) WT B cells were cultured with LPS or CpG as in (A) in the presence of DMSO (control) or increasing concentrations (2.5 μ M, 5.0 μ M, 10 μ M, 20 μ M) of p110 δ selective inhibitor IC87114. IL-6, IL-10, and IL-12 were measured in 48 h culture supernatants using ELISA. Data are presented as mean $\pm$ SD of three replicates of one representative experiment of six. ** p < 0.01, *** p < 0.001 as compared to the wild type.

Similar results were observed for CpG inhibition of IgE switch (data not shown).

3.4. p110 δ differentially regulates TLR-mediated B cell cytokine production

We and others have reported that PI3K signaling influences cytokine production patterns in T cells and dendritic cells (Fukao et al., 2002; Nashed et al., 2007). We investigated whether signaling via p110 δ is important for specific TLR-induced cytokine secretion in B cells. As shown in Fig. 4A, p110 δ ^{D910A} B lymphocytes show significantly reduced LPS- and CpG-mediated IL-6 and IL-10 production (Fig. 4A). IL-6 and IL-10 production by wild-type B cells was also reduced upon activation with LPS and CpG in the presence of IC87114 (Fig. 4B). In contrast, p110 δ inhibition did not inhibit production of IL-12 p70. In fact, p110 δ ^{D910A} B cells showed significantly enhanced LPS- and CpG-induced IL-12 production (Fig. 4A), despite the reduced proliferation in these cultures. No significant production of IL-4 or IFN γ was detectable under these conditions, and control ODN treatment did not lead to any detectable cytokine production (data not shown). These results indicate a selective requirement for p110 δ signaling in TLR-induced B cell cytokine production.

3.5. LPS and CpG act in synergy for IL-6 and IL-10 induction

Pathogens express several TLR–ligands that may engage different TLRs in distinct cellular locations. Recent evidence indicates

a remarkable synergy between cell surface TLR (such as TLR4) and intracellular TLR (such as TLR9) in activation of dendritic cell cytokine production (Napolitani et al., 2005). We sought to determine whether TLR4 and TLR9 act in synergy in the induction of B cell-associated cytokines and what role, if any, p110 δ signaling plays in this regard. Simultaneous addition of LPS- and CpG-induced significantly higher IL-6 and IL-10 levels, greater than the additive levels induced by individual agonists (Fig. 5). Although p110 δ ^{D910A} B cells produced lower amounts of IL-6 and IL-10, a similar pattern of synergistic induction of these cytokines was also observed in LPS+CpG stimulated cultures, ruling out any specific requirement for PI3K–p110 δ in the synergistic induction of IL-6 and IL-10. Unlike previous reports of DC cytokine production, there was no additive or synergistic effect of LPS and CpG stimulation on IL-12 induction either in WT or p110 δ ^{D910A} B cells. However, p110 δ ^{D910A} B cells exhibit a striking increase in TLR9-induced IL-12 production under all conditions.

4. Discussion

Phosphoinositide 3-kinases are key enzymes in immune cell activation through several types of receptors (Okkenhaug and Vanhaesebroeck, 2003), and various isoforms are currently being targeted for treatment of inflammatory diseases (Knight et al., 2006; Rommel et al., 2007). Thus, the identification of specific immunomodulatory activities of PI3K isoforms is critical. In this study we have identified roles for the p110 δ isoform of PI3K in

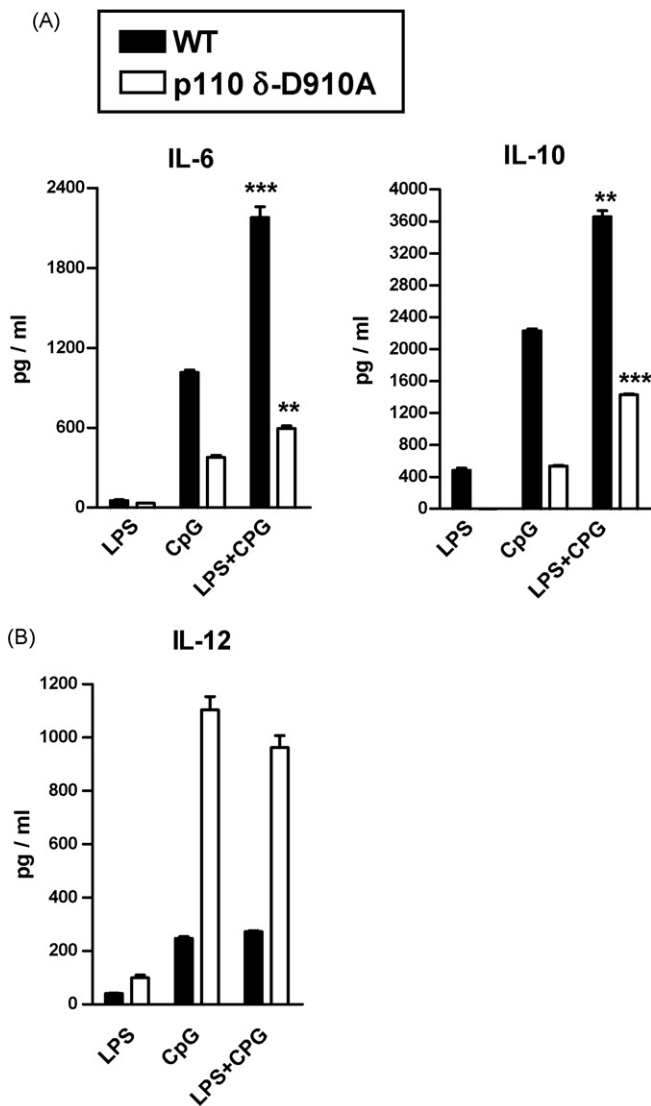


Fig. 5. Additive stimulation of IL-6 and IL-10 production by combination of LPS and CpG occurs irrespective of p110 δ inhibition. Purified splenic WT or p110 δ ^{D910A} B cells were cultured with 100 ng/ml LPS, 0.1 μ M CpG or both in combination. IL-6, IL-10, and IL-12 were measured in 48 h culture supernatants by ELISA. Note that LPS + CpG stimulation leads to increased IL-6 and IL-10 production exceeding the total amount produced with either stimuli alone and this effect is independent of p110 δ signaling. Data are presented as mean $\pm$ SD of three replicates of one representative experiment of four. * $p < 0.01$, *** $p < 0.001$ as compared to LPS or CpG alone.

TLR4- and TLR9-mediated B cell proliferation, cytokine production and isotype switching. We investigated the role of p110 δ signaling in TLR activation of B cells using both genetic and pharmacological inactivation, to avoid the caveats inherent in either genetic or pharmacological inhibition alone. We assessed affects on activation via two different classes of TLR (cell surface TLR4 and endosomal TLR9), and also in the context of synergistic TLR stimulation. These studies provide evidence for selective immunomodulatory roles of p110 δ , influencing B cell production of IL-10 versus IL-12, and isotype switch.

Most studies analysed the role of PI3K signaling in TLR-mediated responses in different cell types by single TLR agonist. PI3K has been shown to be activated in macrophages and dendritic cells by TLR agonists, such as LPS, poly (I:C), and CpG (Aksoy et al., 2005; Diaz-Guerra et al., 1999; Saegusa et al., 2007). In addition, various genetic models have supported a role for PI3K in proliferation and/or survival of splenic B cells, following stimulation

with TLRs and other mitogenic stimuli (reviewed in Fruman, 2004). Our data confirm that p110 δ is required for optimal B cell proliferation to both TLR4- and TLR9-ligands, as well as combined stimulation with both. Numerous studies have indicated that different TLR agonists can profoundly alter immune responses to one another (Dobrovolskaia et al., 2003; Kalis et al., 2005; Lehner et al., 2001). We did not observe evidence of synergy between TLR4- and 9-ligands in terms of the B cell proliferative response, however IL-6 and IL-10 responses were increased with dual stimulation to levels greater than the added effects of the individual ligands. It is possible that a potential synergistic proliferative effect of LPS and CpG may be masked by significantly greater amounts of IL-10 produced under these conditions. IL-10 has been shown to act as “fine-tuner” of microbial product-induced B-cell activation by inhibiting CpG-induced B-cell proliferation (Bernasconi et al., 2003).

TLR4 and TLR9 are representative of different classes of TLR, with different signaling mechanisms. TLR4 is expressed on the cell surface and initiates intracellular signaling by TIRAP-MyD88 and TRIF-TRAM pathway on LPS stimulation. By contrast, TLR9 has an intracellular location and is recruited to endosomal/lysosomal compartments on stimulation with CpG and activates the MyD88 pathway without TIRAP. There is evidence for direct PI3K activation by both LPS and CpG, with one report suggesting that MyD88 binds directly to the p85 regulatory subunit of PI3K (Gelman et al., 2006). Some models hypothesize that endosomal TLRs such as TLR9 may be more dependant on PI3K signaling (Takeshita et al., 2004); however, we find little evidence for any preferential requirement of p110 δ PI3K in TLR9-mediated B cell activation. On the contrary, we find that p110 δ signaling may function to restrain CpG-induced IL-12 production.

TLR9 is known to have unique properties of skewing immune responses towards production of Type 1 cytokines and antibody isotypes and inhibiting Type 2-associated responses (Ehlers et al., 2006). Previously, such effects upon B cells were presumed to reflect the indirect effect of skewing towards and/or preferential survival of Th1 cells, but recent studies are indicative of direct effect on B cells where CpG, but not LPS, upregulates T-bet expression in murine B cells and inhibits IgG1 and IgE production (Lin et al., 2004; Liu et al., 2003; Peng et al., 2002). The mechanism of CpG-induced inhibition of IgG1 and IgE is not clear, but appears to be independent of T-bet, B-cell lymphoma protein 6 (Bcl-6) or inhibitor of DNA binding protein 2 (Id2) (Kusunoki et al., 2005; Peng et al., 2003). PI3K signaling has been shown to inhibit isotype switch to IgG1 and IgE (Omori et al., 2006; Zhang et al., 2008), and the present results indicate that CpG is unable to fully inhibit switch to these isotypes when p110 δ activity is blocked. These results suggest that TLR9-induced activation of p110 δ may be part of the mechanism for its suppression of IgG1 and IgE. On the other hand, CpG treatment did partly reduce the frequency of IgG1 and IgE-switched cells under conditions of p110 δ blockade, indicating that additional p110 δ -independent inhibitory mechanisms exist.

Terminal differentiation of B cells to immunoglobulin-secreting plasma cells is a crucial step in the immune response, and de-regulation of plasma cell differentiation is thought to contribute to autoimmune disease. The impaired antibody production observed in mice deficient in PI3K signaling (Okkenhaug and Vanhaesebroeck, 2003) could be interpreted as a B cell-intrinsic failure of plasma cell differentiation. Indeed one recent study has suggested that PI3K signaling is important for plasmablast generation, primarily based on enhanced plasma cell generation by PTEN-deficient B cells (Omori et al., 2006). Our results indicate that signaling through the major PI3K isoform in B cells is not intrinsically required for TLR4-induced plasma cell generation. As previously reported, p110 δ -D910A mice lack marginal zone B cells (Okkenhaug et al., 2002), which leads to reduced numbers of LPS-induced plasma cells in a short-term in vitro assay, due to the

disproportionately strong responses of these cells to LPS. However, p110 δ -D910A B cells can still generate appreciable levels of BLIMP1⁺, CD138⁺ cells, and treatment of WT B cells with p110 δ inhibitor had no effect on BLIMP-1 induction or frequency of CD138⁺ cells. We conclude that it is unlikely that p110 δ has a critical B cell-intrinsic role in induction of the plasmablast differentiation program.

In addition to proliferation, isotype switch and plasma cell differentiation, TLR stimulation promotes a variety of B cell regulatory functions, including antigen presentation and the production of cytokines (Peng, 2005). Evidence is mounting that these B cell regulatory functions have critical importance in autoimmunity and infectious disease (Lund, 2008). Our data here indicate that simultaneous B cell stimulation with LPS and CpG leads to greater than additive IL-6 and IL-10 production. This is in marked contrast to studies in murine DC where these two TLR-ligands did not induce any additive or synergistic effects in terms of IL-6 and IL-10 production (Napolitani et al., 2005). This is likely due to cell-type specific differences between DC and B cells. In contrast, several groups (Napolitani et al., 2005; Theiner et al., 2008), report a synergistic IL-12 production on combined TLR4 and TLR9 stimulation in DCs. However, we observe no additive or synergistic effect in terms of IL-12 production by B cells. One possible explanation of this difference might come from the fact that IL-10 and IL-12 have been shown to have an inverse relationship (Kuhn et al., 1993; Rennick et al., 1997). Since combined stimulation of B cells with LPS and CpG resulted in additive induction of IL-10, and IL-10 negatively regulates IL-12, perhaps the potential synergistic IL-12 production is obscured.

We find that both TLR4- and TLR9-mediated IL-6 and IL-10 productions are partially dependent on p110 δ signaling. Recent studies suggest the existence of “regulatory” B cell populations, phenotypically similar to B1 and marginal zone B cells, that produce a large amount of IL-10 (Barr et al., 2007; Lund, 2008). Thus, one possible interpretation of our results is that p110 δ activity is required for the optimal development and/or activity of IL-10-producing regulatory B cells. Our data indicate a striking increase in TLR9-induced IL-12 production in p110 δ -D910A B cells. While treatment of wild-type B cells with p110 δ inhibitor did not show this striking increase, there is still more IL-12 production on a per-cell basis, taking into account reduced proliferation. The opposite effect of p110 δ inhibition on IL-12 production might occur through a direct inhibitory effect of p110 δ activity in IL-12 induction or indirectly through reduction in IL-10 levels.

The identification of the immunomodulatory activities for isoform-specific PI3K inhibitors will be important in identifying clinical applications for these compounds. Our results regarding p110 δ in B cells are consistent with studies on p85 regulatory subunit deficiency, which show that PI3K activity is an endogenous suppressor of IL-12 production triggered by Toll-like receptor (TLR) signaling in dendritic cells and limits excessive Th1 polarization (Fukao et al., 2002). The inhibitory function of PI3K in TLR4 and 9 stimulated IL-12 production in macrophages, DCs and now B cells suggests that PI3K may negatively regulate TLR-induced Type 1 inflammatory responses in multiple cell types and systemically. Indeed we found previously that p110 δ -D910A mice generate enhanced systemic Type 1 cytokine production in an ovalbumin immunization model (Nashed et al., 2007). Together these results collectively suggest that targeting p110 δ PI3K may lead to unexpected enhancements in innate immune responses involving IL-12 and other type I cytokines.

Conflict of interest

The authors have no financial conflict of interest.

Acknowledgements

The authors gratefully acknowledge Drs. Klaus Okkenhaug and Bart Vanhaesebroeke for providing p110 δ ^{D910A} mice and Dr. Kamal Puri for providing IC87114 inhibitor. We thank Ting-ting Zhang, Liu Dong, Sen Hou, Dr. Baher Nashed and Dr. Monther AlAlwan for technical assistance. N.D. was supported by a fellowship from the Canadian Institutes of Health Research National Training Program in Allergy and Asthma. A.M. is supported by a Canada Research Chair. This project was supported by a grant from the Canadian Institutes for Health Research.

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