

# Inactivation of PI3K $\gamma$ and PI3K $\delta$ distorts T-cell development and causes multiple organ inflammation

Hong Ji,<sup>1</sup> Felix Rintelen,<sup>1</sup> Caroline Waltzinger,<sup>1</sup> Dominique Bertschy Meier,<sup>1</sup> Antonio Bilancio,<sup>2</sup> Wayne Pearce,<sup>2</sup> Emilio Hirsch,<sup>3</sup> Matthias P. Wymann,<sup>4</sup> Thomas Rückle,<sup>1</sup> Montserrat Camps,<sup>1</sup> Bart Vanhaesebroeck,<sup>2</sup> Klaus Okkenhaug,<sup>5</sup> and Christian Rommel<sup>1</sup>

<sup>1</sup>Merck Serono S. A., Geneva Research Center, Geneva, Switzerland; <sup>2</sup>Ludwig Institute for Cancer Research, London, United Kingdom; <sup>3</sup>Department of Genetics, Biology and Biochemistry, University of Torino, Torino, Italy; <sup>4</sup>Institute of Biochemistry & Genetics, Department of Biomedicine, University of Basel, Basel, Switzerland; and <sup>5</sup>Laboratory of Lymphocyte Signaling and Development, The Babraham Institute, Cambridge, United Kingdom

**Mice lacking both the p110 $\gamma$  and p110 $\delta$  isoforms display severe impairment of thymocyte development. Here, we show that this phenotype is recapitulated in p110 $\gamma^{-/-}$ /p110 $\delta^{D910A/D910A}$  (p110 $\gamma^{KO\delta^{D910A}}$ ) mice where the p110 $\delta$  isoform has been inactivated by a point mutation. Moreover, we have examined the pathological**

**consequences of the p110 $\delta$  deficiency, which include profound T-cell lymphopenia, T-cell and eosinophil infiltration of mucosal organs, elevated IgE levels, and a skewing toward Th2 immune responses. Using small-molecule selective inhibitors, we demonstrated that in mature T cells, p110 $\delta$ , but not p110 $\gamma$ , controls**

**Th1 and Th2 cytokine secretion. Thus, the pathology in the p110 $\gamma\delta$ -deficient mice is likely to be secondary to a developmental block in the thymus that leads to lymphopenia-associated inflammatory responses. (Blood. 2007;110:2940-2947)**

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## Introduction

Class I phosphoinositide-3-kinases (PI3Ks) comprise a family of lipid kinases that phosphorylate the 3-OH of phosphatidylinositol(4,5)P<sub>2</sub> to generate PtdIns(3,4,5)P<sub>3</sub> that mediates many important functions such as proliferation, differentiation, survival, and migration.<sup>1,2</sup> The class I PI3K family consists of IA and IB subclasses. The class IA PI3K includes the p110 $\alpha$ , p110 $\beta$ , and p110 $\delta$  catalytic subunits, each of which is associated with a p85 regulatory subunit and is regulated by tyrosine kinase signaling.<sup>3,4</sup> The class IB PI3K includes only one catalytic subunit p110 $\gamma$ , which is associated with a p101 or p84/p87<sup>PIKAP</sup> regulatory subunit and is regulated by G-protein-coupled receptor signaling.<sup>5,6</sup> Unlike p110 $\alpha$  and p110 $\beta$ , which are ubiquitously expressed, the p110 $\gamma$  and p110 $\delta$  isoforms are predominantly expressed in the hematopoietic system,<sup>7</sup> suggesting a more specific and important role of PI3K $\gamma$  and PI3K $\delta$  in leukocyte development and function.

Mice with deletion or kinase inactivation of p110 $\gamma$  show impaired neutrophil and macrophage migration, mast cell degranulation, and a mild defect in thymocyte development.<sup>8-13</sup> Mice with deletion or kinase inactivation of p110 $\delta$  show impaired neutrophil chemotaxis, mast cell degranulation upon IgE receptor cross-linking, and compromised B-cell development and activation.<sup>14-18</sup> T-cell development is apparently unaffected, however, T-cell proliferation, Th1 and Th2 differentiation, and CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T-cell activity are compromised in p110 $\delta$ -deficient mice.<sup>17,19,20</sup> The multiple roles of p110 $\gamma$  and p110 $\delta$  in various immune cells have encouraged the development of small-molecule inhibitors of p110 $\gamma$  or p110 $\delta$ , alone or in combination, in treating immune-mediated inflammatory diseases.<sup>14,21-24</sup>

Immature T cells developing in the thymus pass through a number of selection check points. During the  $\beta$ -selection step, T cells that express a cell surface TCR $\beta$  chain in conjunction with a pre-T $\alpha$  chain receive a signal to develop into CD4<sup>+</sup>CD8<sup>+</sup> T cells. This selection depends on functional TCR signaling and is blocked in mice lacking Lck, ZAP-70, LAT, or SLP-76.<sup>25-29</sup> Recently, 2 groups have reported the importance of p110 $\gamma$  and p110 $\delta$  cooperativity at this selection step as well. T cells from p110 $\gamma\delta$  double-knockout (p110 $\gamma\delta^{-/-}$ ) mice are substantially blocked at the  $\beta$ -selection step, resulting in a dramatic reduction in CD4<sup>+</sup>CD8<sup>+</sup> T cells in the thymus.<sup>30,31</sup> Impaired signaling by the pre-TCR in p110 $\gamma\delta^{-/-}$  mice was correlated with reduced phosphorylation of ribosomal protein S6 and increased expression of Bim, suggesting defects in cell growth and survival at this critical selection stage.<sup>30</sup> Consequently, peripheral T-cell numbers are dramatically reduced in blood, spleen, and lymph nodes of p110 $\gamma\delta^{-/-}$  mice. In addition, most peripheral CD4<sup>+</sup> and CD8<sup>+</sup> T cells express high levels of CD44<sup>high</sup>, but low levels of CD62L, CD25, and CD69.<sup>30</sup> This pattern of expression is typical for lymphopenic mice.<sup>32</sup>

Under condition of lymphopenia-induced homeostatic expansion, T-cell clones that bear high ability to respond to homeostatic factors, such as self-peptide/MHC, tend to dominate the repertoire.<sup>33</sup> Therefore, lymphopenia is frequently linked to, although not always causes, autoimmunity.<sup>34</sup> A secondary factor is necessary to induce autoimmune diseases, including dysregulated regulatory T-cell function or the presence of local tissue inflammation.

Because lymphopenia and compensatory homeostatic expansion tend to create autoimmunity,<sup>34</sup> we sought to investigate the pathological consequences of lymphopenia caused by combined p110 $\gamma$  and p110 $\delta$  deficiency. In addition, we sought to determine

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whether p110 $\gamma$  and p110 $\delta$  act cooperatively to regulate mature T-cell responses.

## Materials and methods

### Animals

p110 $\gamma$  KO and p110 $\delta^{D910A/D910A}$  mice have been described previously.<sup>8,17</sup> p110 $\gamma$  KO and p110 $\delta^{D910A/D910A}$  mice were backcrossed with C57Bl/6 for 7 or 6 generations, respectively, and intercrossed to generate homozygous single- or double-mutant mice. Severe-combined immunodeficient (SCID) mice were purchased from Charles River Laboratories (L'Arbresle, France). Unless stated, we used 6- to 10-week-old mice in all experiments. Breeding and maintenance of animals were according to the regulations of the Swiss Veterinary Authority (Office Vétérinaire Cantonal).

### Histology and immunofluorescent staining

Organs were removed and 4- $\mu$ m sections were prepared from paraffin- or cryostat-embedded blocks and were stained with hematoxylin & eosin or May-Grünwald-Giemsa (Electron Microscopy Sciences, Hatfield, PA) or PE-conjugated anti-CD4 or anti-CD8 (BD Pharmingen, San Diego, CA) antibodies as indicated. Sections were examined with a Zeiss Axioplan microscope and photographs taken with a Zeiss Axiocam camera (Carl Zeiss SA, Zurich, Switzerland) and Nikon ACT-1 software (version 2.12).

### Fluorescence-activated cell sorting (FACS) analysis

With the exception of anti-GITR and anti-FoxP3 (eBioscience, San Diego, CA), all other antibodies were purchased from BD Pharmingen: anti-CD4, anti-CD8, anti-CD25, anti-CD44, anti-CD3, anti-CD28, anti-B220, anti-CD45. Cells were analyzed by the FACSCalibur and CellQuest software (BD Bioscience, San Jose, CA).

### ELISA for immunoglobulin isotype detection

Serum concentrations of IgG<sub>1</sub>, IgG<sub>2a</sub>, IgG<sub>2b</sub>, IgG<sub>3</sub>, IgM, and IgA were determined by mouse immunoglobulin isotyping enzyme-linked immunosorbent assay (ELISA) kit, and serum IgE concentrations were measured with IgE-specific ELISA (BD Pharmingen).

### T-cell proliferation

T cells negatively purified with anti-MHC-class II magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) were incubated in 96-well plates precoated with 10  $\mu$ g/mL antimouse CD3 $\epsilon$  mAb (BD Pharmingen) with or without 10  $\mu$ g/mL antimouse CD28 (BD Pharmingen) for 48 hours. For measuring T-cell proliferation, plates were pulsed with [<sup>3</sup>H]-thymidine at 1  $\mu$ Ci (0.037 MBq)/well for the last 6 hours. Cytometric bead array for mouse Th1/Th2 cytokines (BD Pharmingen) was used to detect cytokines from the supernatant.

### T regulatory cell (Treg) suppression assay

T cells negatively purified with anti-MHC-class II magnetic beads (Miltenyi Biotec) were further sorted by FACSARIA (BD Bioscience) to obtain CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> cells. CD4<sup>+</sup>CD25<sup>-</sup> cells ( $5 \times 10^4$ ) and/or CD4<sup>+</sup>CD25<sup>+</sup> cells ( $5 \times 10^4$ ) were incubated in 96-well plates with  $5 \times 10^4$  T-cell-depleted and mitomycin-treated splenocytes and 1  $\mu$ g/mL CD3 $\epsilon$  mAb (BD Pharmingen) for 72 hours for measuring proliferation and cytokine release. For measuring T-cell proliferation, plates were pulsed with [<sup>3</sup>H]-thymidine at 1  $\mu$ Ci (0.037 MBq)/well for the last 6 hours. Cytometric bead array for mouse Th1/Th2 cytokines (BD Pharmingen) was used to detect cytokines from the supernatant.

### Mixed leukocyte reaction

T cells were purified with Pan-T-cell isolation kit (Miltenyi Biotec). T cells ( $1 \times 10^5$ ) were cultured with  $2.5 \times 10^5$ ,  $5 \times 10^5$ , or  $10 \times 10^5$  mitomycin

C-treated T-depleted cells from spleen as antigen-presenting cells (APCs) for 48 hours. Plates were pulsed with [<sup>3</sup>H]-thymidine for the last 6 hours.

### Cell isolation from organs

Mice were perfused with PBS and salivary glands and stomach were removed. Stomach contents were washed out with PBS and organs were incubated in cell culture medium RPMI-1640 with collagenase IV (8 mg/mL) (Worthington, Lakewood, NJ) and 1% DNase for 1 hour at 37°C. Organs were then passed through a 70- $\mu$ m nylon mesh to obtain a single-cell suspension for further analysis.

### PI3K $\gamma$ and PI3K $\delta$ inhibitors

IC87114 at 3  $\mu$ M and/or AS604850 at 10  $\mu$ M were preincubated with purified T cells for 20 minutes.<sup>15,21</sup>

## Results

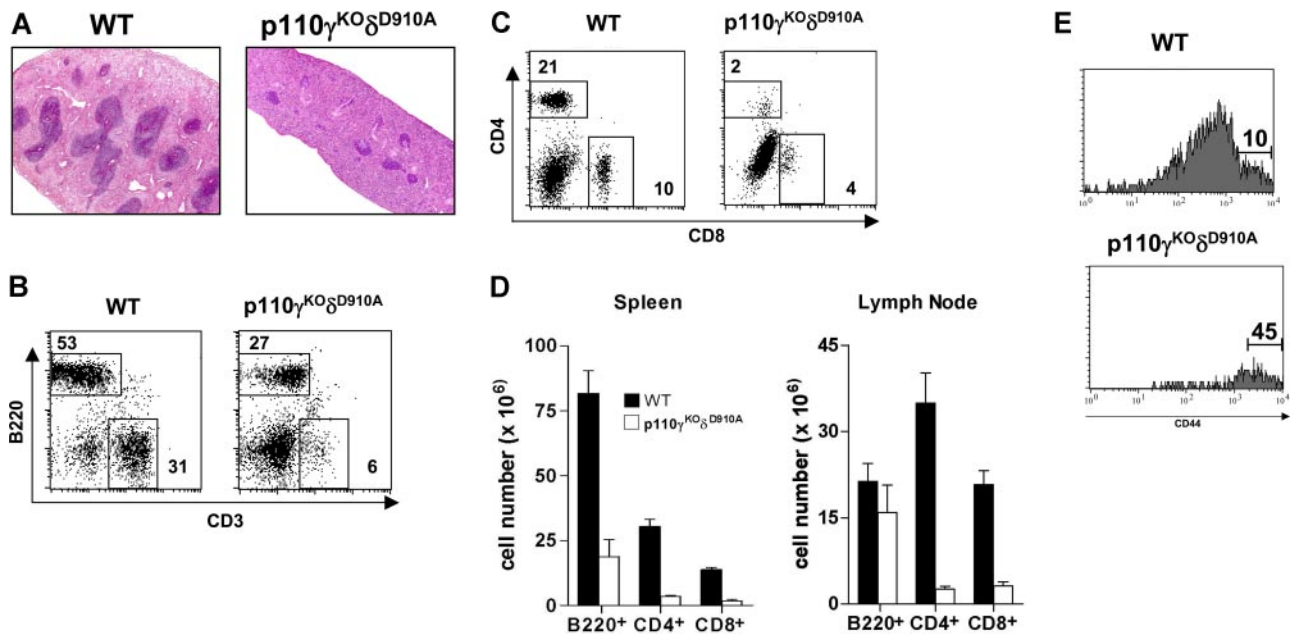
### Severe lymphopenia in p110 $\gamma^{KO\delta^{D910A}}$ mice

We crossed p110 $\gamma^{-/-}$  mice and p110 $\delta^{D910A/D910A}$  mice to generate p110 $\gamma^{-/-}$ p110 $\delta^{D910A/D910A}$  double-mutant mice (hereafter referred to as p110 $\gamma^{KO\delta^{D910A}}$  mice).<sup>8,17</sup> Similar to mice lacking both p110 $\gamma$  and p110 $\delta$ ,<sup>30,31</sup> p110 $\gamma^{KO\delta^{D910A}}$  mice displayed defective thymocyte development, demonstrated as thymus atrophy, reduced thymocyte numbers, loss of cortex-medulla organization, reduced cellularity especially of CD4<sup>+</sup>8<sup>+</sup> double-positive (DP) cells, and CD4<sup>+</sup>8<sup>-</sup> and CD4<sup>+</sup>8<sup>+</sup> single-positive (SP) cells, increased CD4<sup>+</sup>8<sup>-</sup> double-negative 3 (DN3)/DN4 ratio, and high level of CD25 expression on DN3 cells (Figure S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). Thus, our results are in line with previous reports that PI3K $\gamma$  and PI3K $\delta$  cooperativity is critical for thymocyte development.

As a consequence of the blocked thymocyte development, p110 $\gamma^{KO\delta^{D910A}}$  mice displayed spleen atrophy (Figure 1). Very few lymphoid follicles were observed in the white pulp of the p110 $\gamma^{KO\delta^{D910A}}$  mice (Figure 1A). Both B220<sup>+</sup> and CD3<sup>+</sup> cell (including CD4<sup>+</sup> and CD8<sup>+</sup> cell) numbers were dramatically reduced (Figure 1B-D), and most CD4<sup>+</sup> and CD8<sup>+</sup> T cells expressed high levels of CD44 (Figure 1E and data not shown). Whereas the reduction in B-cell numbers was comparable with that observed in p110 $\delta^{D910A/D910A}$  mice, the dramatically reduced proportion of T cells and the high expression of CD44 was observed only in p110 $\gamma^{KO\delta^{D910A}}$  mice.

### Markedly reduced immunoglobulin (Ig) production and relatively increased type 2 Ig isotype level in p110 $\gamma^{KO\delta^{D910A}}$ mice

We tested serum levels of different immunoglobulin isotypes of mice at ages between 2 weeks to 12 weeks and compared them with their littermates' levels. Compared with WT mice, p110 $\gamma^{KO\delta^{D910A}}$  mice produced less IgG2a, IgG2b, IgM, and IgA; however, IgG1 levels were normal, which resulted in increased IgG1/IgG2a ratio (Figure 2A,B). Unexpectedly, the amount of IgE was 10 to 20 times higher in the serum of p110 $\gamma^{KO\delta^{D910A}}$  mice (Figure 2C). These data indicate that type 2 immunity-associated Ig isotypes are preferentially formed in p110 $\gamma^{KO\delta^{D910A}}$  mice despite a severe defect in B-cell development and overall Ig production.



**Figure 1. Lymphopenia in  $p110\gamma^{KO\delta D910A}$  mice.** (A) H&E staining of spleen ( $4\times/0.12$  objective lens). (B-E) Flow cytometry analysis. (B) B220 and CD3 profile of spleen. (C) CD4 and CD8 profile of spleen. (D) Numbers of B220<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> cells from spleen and lymph nodes. (E) CD44 profile gated on CD4<sup>+</sup> cells from spleen. Data are from 6 mice in each group ( $\pm$  SEM). Numbers inside panels B and C indicate percentage of gated cells in total splenocytes; numbers in panel E, percentage of gated cells in CD4<sup>+</sup> splenocytes.

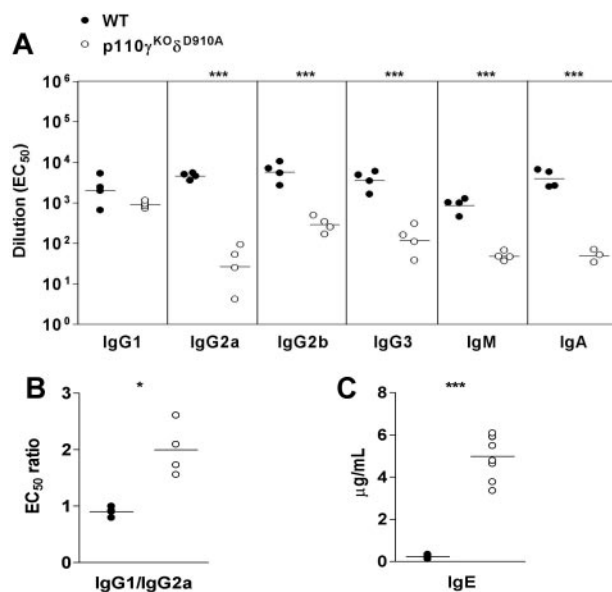
### Increased peripheral T-cell proliferation and Th2 cytokine production in $p110\gamma^{KO\delta D910A}$ mice

It is well established that T cells in lymphopenic mice undergo excessive expansion and acquire effector/memory-like phenotype.<sup>35</sup> Indeed, in contrast to the single isoform-deficient mice that retained normal responses,<sup>8,17</sup>  $p110\gamma^{KO\delta D910A}$  T cells proliferated rigorously to both allogeneic (H-2<sup>s</sup>) and self-MHC (H-2<sup>b</sup>) in mixed leukocyte reaction (MLR) assay (Figure 3A,B). In addition,  $p110\gamma^{KO\delta D910A}$  T cells exhibited increased proliferation as well as

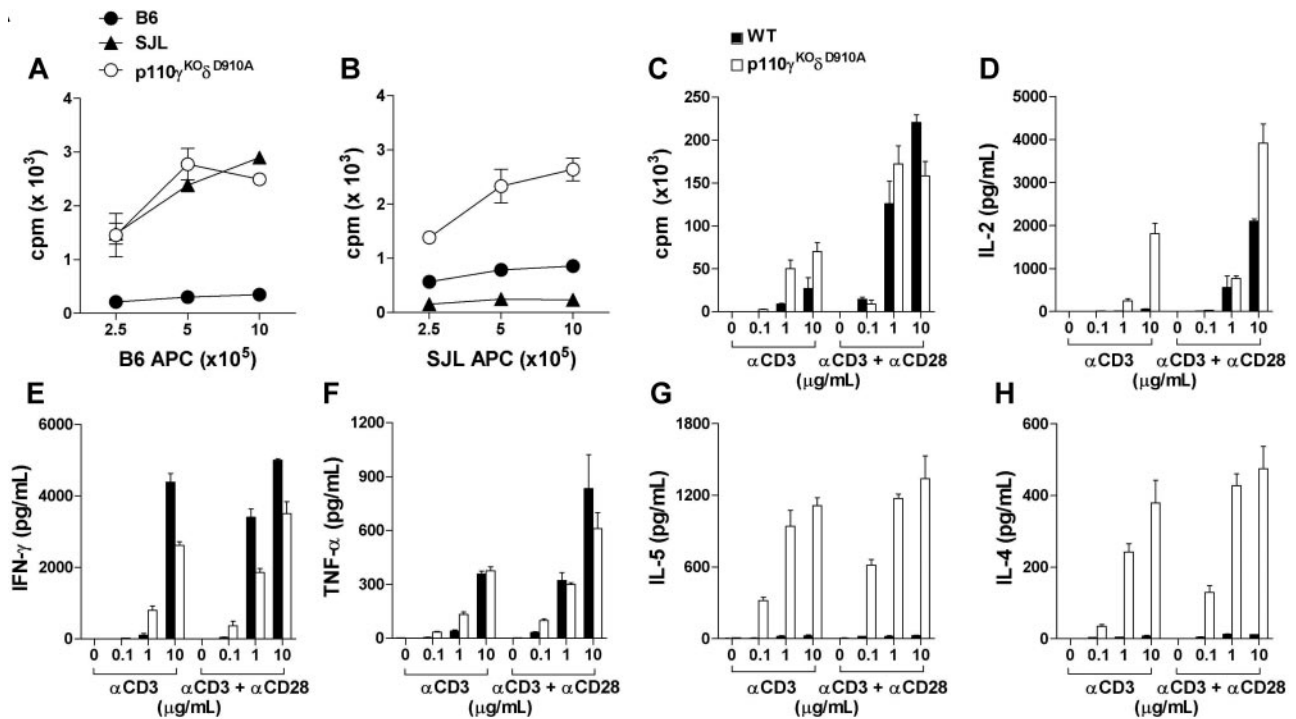
IL-2 release upon  $\alpha$ -CD3 stimulation, but were comparable with WT T cells in response to  $\alpha$ -CD3/ $\alpha$ -CD28 activation (Figure 3C,D).  $p110\gamma^{KO\delta D910A}$  T cells produced marked increase of Th2 cytokines IL-5 and IL-4 and reduced Th1 cytokine IFN- $\gamma$  and a comparable amount of TNF- $\alpha$  to levels secreted by WT T cells (Figure 3E-H). Therefore, the substantially augmented T-cell response in lymphopenic  $p110\gamma^{KO\delta D910A}$  mice was skewed toward Th2-associated cytokines.

### Partially impaired suppressing function of CD4<sup>+</sup>CD25<sup>+</sup> cells in $p110\gamma^{KO\delta D910A}$ mice

CD4<sup>+</sup>CD25<sup>+</sup> regulatory cells (Tregs) play important roles in maintaining T-cell homeostasis and modulating immune responses.<sup>36-38</sup> Foxp3 is a master control gene for the development and function of natural CD4<sup>+</sup>CD25<sup>+</sup> Treg cells, and its expression serves as a specific and stable marker of Tregs.<sup>39</sup> In contrast, glucocorticoid-induced TNFR-related protein (GITR), expressed on the surface of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells, negatively regulates their immunosuppressive function.<sup>40,41</sup> In  $p110\delta^{D910A/D910}$  mice, there are increased Tregs found in the thymus, whereas the numbers of Tregs are reduced in the peripheral lymphoid organs.<sup>20</sup> Similarly, the percentage of CD4<sup>+</sup>Foxp3<sup>+</sup> cells in  $p110\gamma^{KO\delta D910A}$  mice was 2 to 3 times higher than WT in thymus, however, in the lymph nodes the proportions of Tregs were increased, while they were comparable in the spleen (Figure 4A). The relative expression levels of Foxp3 and GITR were reduced in thymus but relatively normal in spleen and lymph nodes of  $p110\gamma^{KO\delta D910A}$  mice (Figure 4B,C).  $p110\delta^{D910A}$  Tregs show impaired suppression in in vitro assays.<sup>20</sup> We measured the suppression function of the CD4<sup>+</sup>CD25<sup>+</sup> cells isolated from WT and  $p110\gamma^{KO\delta D910A}$  mice. Purified CD4<sup>+</sup>CD25<sup>+</sup> and/or CD4<sup>+</sup>CD25<sup>-</sup> cells were cultured in vitro in the presence of WT APCs and  $\alpha$ -CD3. WT and  $p110\gamma^{KO\delta D910A}$  CD4<sup>+</sup>CD25<sup>-</sup> T cells proliferated to a similar extent, but in both cases the Tregs were refractory to proliferation (Figure 4D first and second bar groups). When cocultured with WT CD4<sup>+</sup>CD25<sup>-</sup> cells, WT



**Figure 2. Altered immunoglobulin production in  $p110\gamma^{KO\delta D910A}$  mice.** (A) Ig isotype level. (B) IgG1/IgG2a ratio and (C) IgE level in serum taken from WT and  $p110\gamma^{KO\delta D910A}$  mice at 9 to 10 weeks of age. Each dot represents one individual mouse (representative of at least 3 experiments). Horizontal lines indicate mean value of each group.



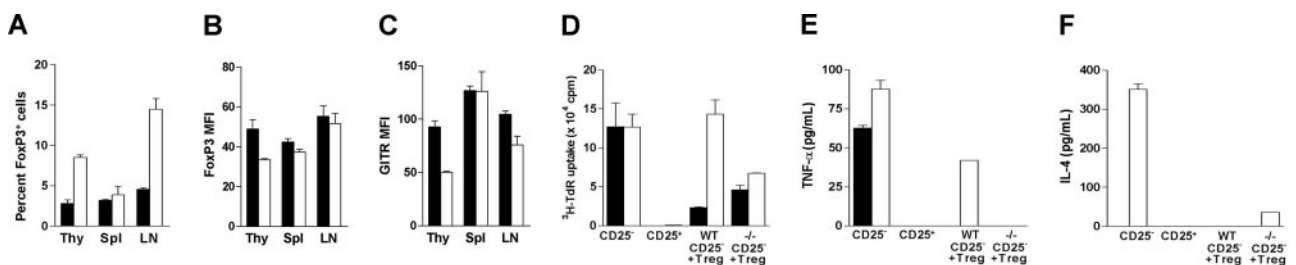
**Figure 3. Altered peripheral T-cell function in *p110 $\gamma$ <sup>KO $\delta$</sup> D910A* mice.** (A–B) Purified T cells were cultured with purified and mitomycin C–treated T-cell–depleted splenocytes from WT (B6) or SJL mice for 48 hours. <sup>3</sup>H-thymidine (TdR) was added at 42 hours. (C–H) Purified T cells from lymph nodes of WT or *p110 $\gamma$ <sup>KO $\delta$</sup> D910A* mice were cultured with plate-coated  $\alpha$ -CD3 (at indicated concentrations,  $\mu$ g/mL) with or without  $\alpha$ -CD28 (10  $\mu$ g/mL) for 48 hours. Proliferation was measured as in panel A and cytokines from supernatant were measured by cytometric bead arrays. Data represent at least 2 experiments with triplicates for each sample ( $\pm$  SEM).

CD4<sup>+</sup>CD25<sup>+</sup> Tregs markedly suppressed the proliferation and cytokine production, but *p110 $\gamma$ <sup>KO $\delta$</sup> D910A* CD4<sup>+</sup>CD25<sup>+</sup> Tregs failed to do so (Figure 4D–F third bar group). However, WT and *p110 $\gamma$ <sup>KO $\delta$</sup> D910A* CD4<sup>+</sup>CD25<sup>+</sup> Tregs suppressed the proliferation and cytokine production of *p110 $\gamma$ <sup>KO $\delta$</sup> D910A* CD4<sup>+</sup>CD25<sup>-</sup> cells to a similar extent (Figure 4D–F fourth bar group). Taken together, *p110 $\gamma$ <sup>KO $\delta$</sup> D910A* mice harbored higher proportion of CD4<sup>+</sup>CD25<sup>+</sup> Tregs with reduced Foxp3 and GITR expression that still efficiently suppressed self but not WT CD4<sup>+</sup>CD25<sup>-</sup> cell activity.

**Eosinophil- and T-cell–dominated inflammation in multiple organs but undetectable circulating autoantibodies in *p110 $\gamma$ <sup>KO $\delta$</sup> D910A* mice**

By histologic examination, we found that all *p110 $\gamma$ <sup>KO $\delta$</sup> D910A* mice (n = 12) demonstrated gastritis and sialadenitis with approximately 80% to 90% of eosinophil and 10% of neutrophil infiltrates in submucosa of stomach and interstitial tissue of salivary glands

(Figure 5A). Eosinophilic inflammation was also observed in other organs of different individuals to various extents (Table 1; Figure 5B). Predominant amount of CCR3<sup>+</sup> cells (data not shown), and to a lesser extent, CD4<sup>+</sup> and CD8<sup>+</sup> T cells, was also detected in the lesions (Figure 5C). Accordingly, higher percentage of CD45<sup>high</sup> CD4<sup>+</sup> or CD45<sup>high</sup> CD8<sup>+</sup> cells was recovered from stomach and salivary glands (Figure 5D). Other organs, such as pancreas, kidney, lung, liver, and heart were normal (data not shown). No sign of inflammation in any of the organs examined was observed in the WT control mice (n = 12). No parasite was found in the tissues in both strains. Augmented levels of proinflammatory cytokines such as TNF- $\alpha$ , IFN- $\gamma$ , IL-6, and MCP-1 were also detected in *p110 $\gamma$ <sup>KO $\delta$</sup> D910A* serum (Figure S2). To examine if there are circulating tissue-specific autoantibodies, we probed stomach and salivary gland sections from SCID.B6 mice with serum from *p110 $\gamma$ <sup>KO $\delta$</sup> D910A*, WT, and lupus-like MRL.lpr/lpr mice (as positive control) and revealed the Ab isotypes with secondary  $\alpha$ -IgG and



**Figure 4. Partially impaired CD4<sup>+</sup>CD25<sup>+</sup> Treg function in *p110 $\gamma$ <sup>KO $\delta$</sup> D910A* mice.** (A–C) Cells from thymus, spleen, and lymph nodes of WT and *p110 $\gamma$ <sup>KO $\delta$</sup> D910A* mice were stained with fluorescence-conjugated antibodies against surface marker CD4, et al. (A). Percentage of Foxp3<sup>+</sup> cells gated from CD4<sup>+</sup>CD8<sup>-</sup> cells in thymus or CD4<sup>+</sup> cells in spleen and lymph nodes. (B,C) Mean fluorescent intensity of Foxp3 and GITR on CD4<sup>+</sup>CD8<sup>-</sup>CD25<sup>+</sup> cells in thymus or CD4<sup>+</sup>CD25<sup>+</sup> cells in spleen and lymph nodes. (D) Purified 5 × 10<sup>4</sup> CD4<sup>+</sup>CD25<sup>+</sup> and/or CD4<sup>+</sup>CD25<sup>-</sup> cells from WT or *p110 $\gamma$ <sup>KO $\delta$</sup> D910A* (–/–) mice were incubated with T-cell–depleted and mitomycin-treated splenocytes and 1  $\mu$ g/mL  $\alpha$ -CD3 in 96-well plate for 72 hours for measuring proliferation. (E,F) TNF- $\alpha$  and IL-4 produced by T cells shown in panel D. Data represent at least 2 experiments with triplicates for each sample ( $\pm$  SEM). ■ represents WT; □, *p110 $\gamma$ <sup>KO $\delta$</sup> D910A*.

$\alpha$ -IgM fluorescence-conjugated Abs. Both IgG and IgM autoantibodies were detected from MRL.lpr/lpr (1:50 dilution) but not WT (1:50 dilution) or  $p110\gamma^{KO\delta^{D910A}}$  serum (even up to 1:5 dilution when background staining could be detected from WT serum) (Figure S3). Similarly, no signal was detected with  $\alpha$ -IgE FITC-conjugated Ab (data not shown). Thus, eosinophils and T cells but not autoantibodies likely mediated multiple organ inflammation in the mucosal tissues of  $p110\gamma^{KO\delta^{D910A}}$  mice.

#### Pharmacological inhibition of PI3K $\gamma$ and PI3K $\delta$ does not promote Th2 cell switching in vitro

To assess the effect of PI3K $\gamma$  and PI3K $\delta$  isoforms on mature peripheral T cells, we treated naive T cells from WT lymph nodes with PI3K $\gamma$  and PI3K $\delta$  selective inhibitors (AS-604850 and IC87114, respectively).<sup>15,21</sup> In line with previously reported phenotype of  $p110\delta^{D910A}$  T cells,<sup>17</sup> PI3K $\delta$  inhibitor IC87114 at concentration of 3  $\mu$ M abrogated T-cell proliferation and IL-2 production in response to  $\alpha$ -CD3 but not to  $\alpha$ -CD3/ $\alpha$ -CD28 stimulation (Figure 6A,B). In addition, IC87114 annulled Th1 cytokine IFN- $\gamma$  and TNF- $\alpha$  as well as Th2 cytokine IL-5 production (note, the IL-5 level in cells treated with vehicle was minimal and IL-4 was not detectable under the current experimental condition) despite normal proliferation and IL-2 secretion upon  $\alpha$ -CD3/ $\alpha$ -CD28 activation (Figure 6A-E). In contrast, the PI3K $\gamma$  inhibitor AS-604850 did not exert any effect on any of the parameters examined, and when given together with IC87114, the inhibition effect was not further strengthened, indicating a negligible role of PI3K $\gamma$  in mature T-cell activation and cytokine secretion under these circumstances (Figure 6A-E). Neither IC87114 nor AS-604850 affected  $p110\gamma^{KO\delta^{D910A}}$  T-cell response (data not shown), indicating that the inhibitors at the applied concentrations were specific. Thus, while  $p110\gamma$  and  $p110\delta$  act synergistically during  $\beta$ -selection in the thymus, prolif-

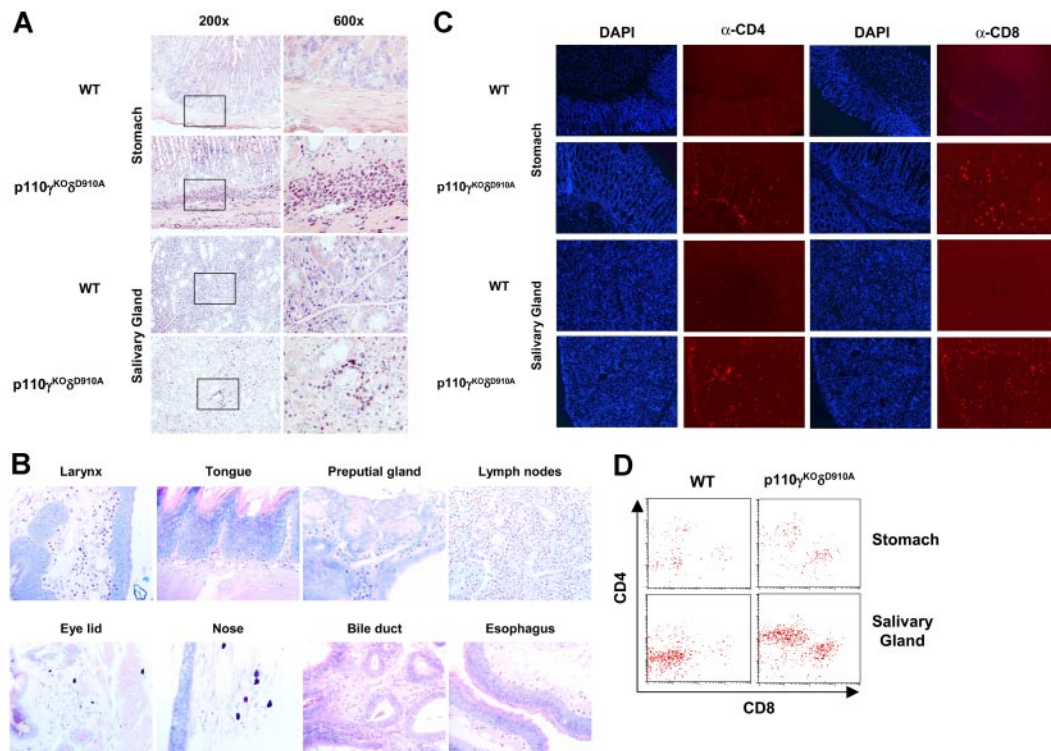
eration and cytokine responses by mature T cells are primarily  $p110\delta$  dependent, with minimal input from  $p110\gamma$ .

## Discussion

We demonstrated that  $p110\gamma^{KO\delta^{D910A}}$  mice, which suffered from severe blockade of thymocyte development and lymphopenia, displayed homeostatic peripheral expansion of Th2 cells and type 2 inflammation in multiple organs. In contrast, blocking of the PI3K $\gamma$  and PI3K $\delta$  isoforms with selective inhibitors did not promote Th2 cytokine production in vitro.

In  $p110\gamma^{KO\delta^{D910A}}$  mice, the thymocyte output was severely compromised as evidenced by an approximately 90% reduction of CD4 SP and 60% reduction of CD8 SP thymocytes (Figure S1). This significant block in T-cell development most likely led to homeostatic expansion of the few T-cell clones that developed. Despite the prolific expansion of peripheral T cells, normal T-cell numbers were not restored, that is, approximately 10% of CD4<sup>+</sup> and 15% of CD8<sup>+</sup> cells were found in spleen and lymph nodes of  $p110\gamma^{KO\delta^{D910A}}$  mice compared with WT controls (Figure 1). As in other lymphopenic hosts,  $p110\gamma^{KO\delta^{D910A}}$  T cells responded rigorously to allogeneic as well as self-peptide/MHC complex (Figure 3).

$p110\gamma^{KO\delta^{D910A}}$  mice displayed strikingly skewed type 2 profile, including elevated IgG1/IgG2a ratio and significant amount of IgE in serum (Figure 2), increased secretion of IL-4 and IL-5 by peripheral T cells upon stimulation in vitro (Figure 3), and eosinophilic inflammation in multiple mucosal organs (Figure 5). Lymphopenia-induced T-cell expansion does not inevitably promote type 2 responses; on the contrary, it has been shown that Th1 T cells are preferably activated and dominantly proliferated during homeostatic expansion.<sup>42</sup> The molecular mechanism underlying



**Figure 5. Inflammation in multiple organs.** (A) Stomach and salivary gland from WT and  $p110\gamma^{KO\delta^{D910A}}$  ( $-/-$ ) mice stained with May-Grünwald-Giemsa. (B) Organs from  $p110\gamma^{KO\delta^{D910A}}$  ( $-/-$ ) mice stained with May-Grünwald-Giemsa (40 $\times$ /0.75 objective). (C) Stomach and salivary gland from WT and  $p110\gamma^{KO\delta^{D910A}}$  ( $-/-$ ) mice stained with DAPI and PE conjugated  $\alpha$ -CD4 or  $\alpha$ -CD8 Ab (100 $\times$ /1.30 oil objective). (D) Flow cytometry analysis of cells from stomach and salivary gland of WT and  $p110\gamma^{KO\delta^{D910A}}$  mice (n = 4).

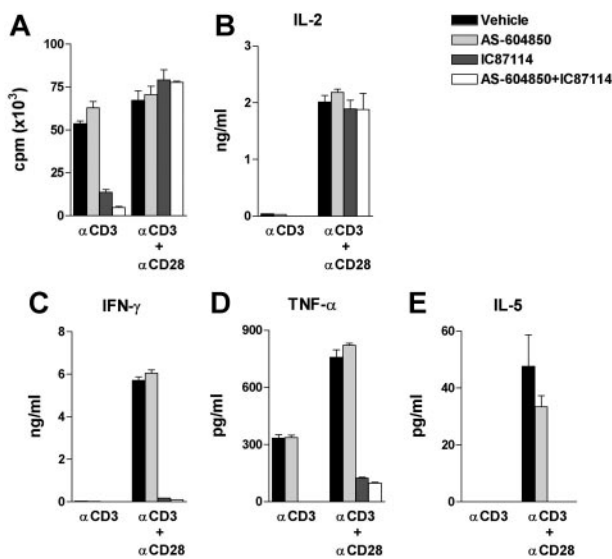
**Table 1. Hematology and histology of p110 $\gamma$ <sup>KO $\delta$ D910A</sup> mice**

Phenotype/parameter	Frequency	Individual mouse			
		F1	F2	M1	M2
<b>Hematology</b>					
Neutrophilia	4/4	+++	+++	++	+++
Eosinophilia	4/4	+++	++	+++	+++
Lymphocytopenia	4/4	+++	+++	+++	+++
Thrombocytosis	4/4	+	+	+	+
Erythrocytosis	2/4	+	-	-	+
Erythrocytopenia	1/4	-	-	+	-
<b>Histology</b>					
Lymphoid organs					
Thymus, diffuse lymphoid atrophy	4/4	+++	++	+++	++
Spleen, diffuse lymphoid depletion	4/4	+++	+++	+++	+++
Spleen, extramedullary hematopoiesis	4/4	+	+++	+	++
Bone marrow, granulocytic hyperplasia	4/4	+	+	+	+
Small intestines, Peyer patches	2/4	Absent	Remnants	Remnants	Absent
Large intestines, Peyer patches	4/4	Remnants	Remnants	Remnants	Remnants
Inflammation					
Gastritis, acute and diffuse	4/4	++	++	++	++
Sialoadenitis submandibular, acute & focally extensive	3/4	+	-	+++	+++
Sialoadenitis, parotid, acute & focally extensive	2/4	-	+++	-	+++
Enteritis, acute & diffuse	1/4	-	-	-/+	-
Laryngitis, acute	1/4	-	+	-	-
Glossitis, acute	2/4	-	+	-	-/+
Blepharitis, acute	1/4	-	-	-/+	-
Rhinitis	1/4	-	-	-/+	-
Cholecystitis	1/4	-	-	-	-/+
Esophagitis	1/4	-	-	-	-/+
Lymphadenitis (submandibular), acute	1/4	-	-	-	+++
Epididymitis, chronic & active	1/4	-	-	-	+++
Posthitis, acute	1/4	-	-	-	+

+++ indicates marked; ++, moderate; +, mild; -, normal; and +/-, minimal.

PI3K activity and Th cell differentiation is not fully understood. Akt/PKB, a downstream target of PI3K, has been shown to promote both Th1 and Th2 differentiation.<sup>43,44</sup> In line with this,

mice lacking p110 $\delta$  activity display reduced Th1 and Th2-skewed phenotype in vitro and in vivo.<sup>19</sup> On the other hand, Itk and Pdk1, also PI3K-dependent kinases, are required for Th2 differentiation.<sup>45,46</sup> Ca<sup>2+</sup> influx and NFAT activity upon activation of TCR have been shown to control Th cell differentiation.<sup>47</sup> NFATc2-deficient mice display modestly increased Th2 cytokines and prolonged maintenance of IL-4 transcription,<sup>48</sup> and NFATc2 and NFATc3 double-knockout mice produce extremely high amounts of IL-4, IL-5, and other Th2 cytokines as well as elevated serum IgG1 and IgE, leading to severe allergic inflammation.<sup>49</sup> TCR-induced Ca<sup>2+</sup> influx is dramatically reduced in p110 $\delta$ <sup>-/-</sup> CD4<sup>+</sup> T cells, while only mildly affected in p110 $\delta$ <sup>-/-</sup> CD4<sup>+</sup> T cells.<sup>31</sup> It is possible that the severity of impairment of TCR activation-mediated cytoplasmic Ca<sup>2+</sup> mobilization affects activation of different components of NFAT activity, thereby influencing Th cell skewing. The reduced Ca<sup>2+</sup> signaling in the absence of PI3K $\gamma$  and PI3K $\delta$  might reduce or diminish NFAT component activation, which in concert with the lymphoproliferative response, may lead to the profound Th2 skewing phenotype. In support of this hypothesis, mice harboring a LAT point mutant incapable of recruiting PLC- $\gamma$ 1 show largely impaired calcium flux and fail to activate NFATc1 and NFATc2 upon TCR cross-linking.<sup>50</sup> Consequently, these mice develop tissue eosinophilia in lung, liver, and kidney due to massive Th2 development.<sup>50,51</sup> The type 2 immune responses in p110 $\gamma$ <sup>KO $\delta$ D910A</sup> mice may have been further exacerbated by the reported capacity of p110 $\delta$  to suppress class-switching recombination.<sup>52</sup> The exaggerated type 2 cytokine and IgE and IgG1 resulted in overreacted immune response to otherwise innocuous bacterial flora in mucosa and organs directly connected



**Figure 6. Pharmacological inhibition of PI3K $\gamma$  and PI3K $\delta$  of T-cell function.** Purified WT lymph node T cells were cultured with plate-coated  $\alpha$ -CD3 (at 10  $\mu$ g/mL) with or without  $\alpha$ -CD28 (10  $\mu$ g/mL) for 48 hours. AS-604850 (10  $\mu$ M) and/or IC87114 (3  $\mu$ M) were preincubated with cells 20 minutes before stimulation. Data represent 4 experiments with triplicates for each sample ( $\pm$  sem). (A) Proliferation was measured by <sup>3</sup>H-thymidine incorporation as in Figure 3A. (B-E) Cytokines from supernatant were measured by cytometric bead arrays.

to mucosa, hence further releasing proinflammatory cytokines and chemoattractants that recruited large amount of eosinophils (Figure 5). Further investigation using reconstitution models, such as transfer mutant T cells into immune-deficient host, should shed light on the underlying pathogenic mechanism in  $p110\gamma^{KO\delta D910A}$  mice.

In  $p110\gamma^{KO\delta D910A}$  mice, Treg function was partially impaired in assays with WT, but curiously not when  $p110\gamma^{KO\delta D910A}$  responder cells were included in the cultures (Figure 4D-F). On face value, this indicates that  $p110\gamma^{KO\delta D910A}$  T cells are more suppressible than WT T cells. Given the higher ratio of  $CD4^+CD25^+$  to  $CD4^+CD25^-$  in peripheral lymphoid organs of  $p110\gamma^{KO\delta D910A}$  mice, these results suggested that the development and homeostatic expansion of Tregs was less affected than non-Treg population, and that the T-cell inflammation in  $p110\gamma^{KO\delta D910A}$  may not necessarily be a consequence of diminished Treg function. A relative increase in the frequency of Tregs has also been observed in other murine models as well as human lymphopenic individuals, resulting from more efficient homeostatic expansion of peripheral Tregs, which appears to outcompete the nonregulatory  $CD4^+$  T-cell pool (review see Krupica et al<sup>34</sup>).

Although the nature of the inflammation in  $p110\gamma^{KO\delta D910A}$  mice is not yet known, the fact that significant amount of IgE has also been detected in  $p110\gamma\delta^{-/-}$  mice from another group (data not shown) suggests that it is not due to a specific SPF hygiene status in our laboratory. It is possible that increased responsiveness to local "normal" bacterial flora produces proinflammatory cytokines as the secondary factor that combined with severe lymphopenia in  $p110\gamma^{KO\delta D910A}$  mice to promote multiple mucosal organ inflammation, despite the absence of uncontrolled infection. This phenomenon, in some aspects, resembles human immunodeficiency virus (HIV)-associated immune reconstitution inflammatory syndromes (IRISs), which is the most well-defined autoimmune syndrome associated with lymphopenia in humans.<sup>53</sup> Although inflammation in IRIS patients often appears in tissues with previous infection, a T-cell-associated inflammatory response has been described.<sup>53</sup> Moreover, profound lymphocyte expansion in draining lymph nodes and elevated serum IL-6 levels are also observed in patients with IRIS.<sup>54</sup>

$p110\gamma^{KO\delta D910A}$  mice displayed similar defects in thymocyte development as well as similarly increased serum IgE level to that of  $p110\gamma\delta^{-/-}$  mice. Our data show that PI3K $\delta$  kinase activity, and not just protein per se, is required for cooperativity with PI3K $\gamma$  in thymocyte development, and likely, causing the pathological consequence in  $p110\gamma^{KO\delta D910A}$  mice. Thus, suppressing both PI3K $\gamma$  and PI3K $\delta$  kinase activity might block certain immune-mediated diseases, but on the other hand, might risk imposing the danger of developing type 2 eosinophilic inflammatory diseases. However, the fact that selectively blocking PI3K $\gamma$  and PI3K $\delta$

function with isoform-specific inhibitors reduced Th1 cytokine release without elevated Th2 response in vitro suggests that acute inhibition of  $p110\delta$  and  $p110\gamma$  is not sufficient to direct Th2 differentiation in mature T cells.

In summary, mice lacking PI3K $\gamma$  and PI3K $\delta$  function developed type 2 responses in immune system and corresponding eosinophilic inflammation in multiple mucosal organs. These symptoms were likely due to a defect in thymocyte development that results in severe lymphopenia-induced homeostatic lymphocyte expansion, and, probably, opportunistic infection enforced by lymphopenic condition. On the contrary, combined treatment of PI3K $\gamma$  and PI3K $\delta$  in adults, in whom the maintenance of the peripheral cell pool occurs primarily via thymic-independent pathway, the effect on thymocyte development will be negligible. On the other hand, the combined treatment will likely reduce Th1 without promoting Th2 response in vitro, although it remains to be determined under in vivo and pathologic conditions when Th1-Th2 balance is lost. Currently, the nature of the ligand(s) that operate PI3K $\gamma$ - and PI3K $\delta$ -dependent mechanism in T cells is unknown.

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## Authorship

Contribution: H.J. designed and performed the research, analyzed data, and wrote the paper; K.O. and C.R. participated in designing the research and writing the paper; B.V. contributed vital reagent and participated in designing the research; F.R. and D.B.M. performed the research and participated in analyzing the data; C.W. participated in designing and performing the research and analyzing the data; A.B. and W.P. participated in performing the research; and E.H., M.P.W., T.R., and M.C. contributed vital reagents.

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Correspondence: Hong Ji, Target Research, Merck Serono S.A., Geneva Research Center, 9 chemin des Mines, 1211 Geneva, Switzerland; e-mail: hong.ji@merckserono.net.

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