行政院國家科學委員會補助專題研究計畫成果報告

純化, cDNA Cloning, 生物特性分析 TEL-JAK2 致癌基因的磷酸化受腌質蛋白以說明血癌的生成機轉 (1/2 & 2/2)

Purification, cDNA cloning, and characterization of the phosphorylated substrate protein of TEL-JAK2 oncogene to elucidate the pathogenesis of leukemia (1/2 & 2/2)

計畫類別: 個別型計畫

計畫編號:

NSC 90-2314-B-002-181-(2/2)

執行期間: 89 年 8 月 1 日至 91 年 7 月 31 日

計畫主持人: 周獻堂

國立台灣大學醫學院小兒科

中華民國 91 年 10 月 28 日

中文摘要

JAK 在血液細胞的訊息傳遞路徑中扮演著重要的角色，並暗示在血液癌症的致病機轉扮有角色。最近 TEL-JAK2 致癌基因在 3 個血癌病人中被發現。其基因合併處分別發生在 TEL 的 337，337 和 155 胺基酸及 JAK2 的 811，505 和 711 胺基酸。TEL-JAK2 的致癌生成機轉，主要來自於其 kinase domain 上的酪氨酸基被持續磷酸化。因為 Y^{966} 是鼠 JAK2 kinase domain 內重要自體磷酸化的位置之一，與此位置結合的蛋白質可能是 TEL-JAK2 的下游受脢質蛋白，並且對血癌的生成機轉的訊息傳遞路徑的了解是非常重要的。

我們初始用 affinity isolation 來分析有那些訊息蛋白與此位置結合。驚人地，有許多訊息傳遞蛋白與含有磷酸化 $JAK2Y^{966}$ 的胜結合。這些蛋白是 PLC-r1，PLC-r2，PI3-kinase 的 P85 α，P85 β 和 P110 δ subunit，SHC 和 Stat5a/b。因為 TEL-JAK2 主導 PI3-kinase 傳遞路徑的持續活性以及 P110 δ subunit 的功能仍不清楚，我們就用基因剔除鼠模式來研究此基因。P110 δ 基因剔除鼠能存活，在 pro B 細胞分化階段受阻併成熟 B 細胞數目減少及 B1 細胞缺乏。IgM-receptor 引起的 Ca^{2+} flux 以及對 B 細胞 Mitogens 的增殖反應減弱，免疫球蛋白減少。對 T 細胞 independent 抗原的反應大大減低，並且對 T 細胞 dependent 抗原的反應完全消失。脾臟對抗原刺激形成 germinal center 的反應被瓦解。這些結果顯示 P110 δ 基因在 B 細胞的分化及功能的訊息傳遞路徑上扮演著無法取代的角色。

Abstract

The JAK (Janus protein tyrosine kinase) pathway has emerged as a major signal transduction mechanism in hematopoietic cells and suggests a potential role in the pathogenesis of hematological malignancies. The TEL-Jak2 fusion oncogene has recently been found in 3 leukemia patients. The fusion occurs within codon 337, 337, and 155 of TEL and codon 811, 505, and 711 of JAK2 respectively. The constitutive tyrosine phosphorylation of the tyrosine kinase domain of TEL-Jak2 plays the critical role for the leukemogenesis. Because one of the major sites of autophosphorylation within murine JAK2 tyrosine kinase domain is Y^{966}, then the proteins bound to this site may play the role of the down-stream substrate of the TEL-Jak2 and are very important for the understanding the signal transduction pathway for the leukemogenesis.

We initially determined the signaling proteins which would bind to this site by affinity isolation. Strikingly a number of proteins known to be involved in signal transduction bound a tyrosine-phosphorylated peptide derived from the amino acid sequence surrounding Jak2 Y^{966}. Among these proteins were PLC-γ1, PLC-γ2, the PI 3-kinase adapter subunits p85α and p85β, the PI 3-kinase catalytic subunit p110δ, SHC and Stat5a/b. Because the TEL-JAK2 mediates constitutive activation of the PI3-kinase signaling pathway and the function of PI 3-kinase catalytic subunit p110δ is still unclear, we derived enzyme-deficient mice to assess the role of this gene. The mice are viable, but have decreased numbers of mature B cells, a block in pro B cell differentiation, and a B1 B cell deficiency. IgM-receptor-induced Ca^{2+} flux and proliferation in response to B cell mitogens are both attenuated. Immunoglobulin levels are decreased substantially. The ability to respond to T cell independent antigens is markedly reduced, and the ability to respond to T cell-dependent antigens is completely abrogated. Germinal center formation in the spleen in response to antigen stimulation is disrupted. These results define a non-redundant signaling pathway(s) utilizing the δ isoform of p110 PI3-kinase for the development and function of B cells.

Background and Purposes

The JAK signal pathway is the main and the most important pathway among the hematopoietic system for signaling almost all cytokines' messages into the nucleus. The JAK kinase family (JAK1, 2, 3 and Tyk2) is cytoplasmic kinases that bind to cytokine receptors that have no kinase domain. Once cytokines bind to specific receptors, they will induce receptors' dimerization. The associated JAK kinases will respond by phosphorylating each other and also tyrosine residues on the receptor cytoplasmic domains, creating phosphotyrosine docking sites that allow SH2-containing signal proteins, including PI3-kinase or other down-stream substrate to bind.

The importance of the JAK pathway in cytokine signaling suggests a potential role in the leukemogenesis of hematological malignancies. The most direct evidence for involvement of JAK family in leukemogenesis is the chromosomal translocations from leukemic cells involving the JAK2 kinase gene on 9p24 and the TEL gene on 12p13(TEL-Jak2 oncogene) [1].

The TEL is a member of the ETS family of transcription factors. It contains a 60-amino acid oligomerization domain(PNT) at its N-terminus. The TEL-JAK2 oncogene was characterized to fuse the C-portion (catalytic kinase domain) of JAK2 to the N-portion (oligomerization domain) of TEL. The oligomerization by the PNT domain of TEL leads to constitutive activation of the JAK2 tyrosine kinase domains (JH1) activity (Fig.1.), which is critical and necessary for transformation of hematopoietic cells. We have done:

Clone TEL-JAK2 oncogene into retroviral vector:

The JAK kinase family share structural domains designated as JH segments 1-7. The critical domain which is involved in the TEL-JAK2 oncogene is the JH1, the kinase domain located at the C-terminus. The sequence of the human JAK2 was 90% identical to that of murine JAK2 at the nucleotide level and 96% identical at the protein level over the JH1 domain. We prepared a chimeric cDNA construct containing the sequence encoding for the human TEL PNT domain (nucleotide 1-1032) fused to the murine JAK2 kinase domain (JH1, nucleotide 2530-3492) by RT-PCR strategy (Fig.1) and cloned into PMSCV retrovirus vector with GFP selective marker.

Produce retrovirus and transfer into murine hematopoietic cell line (Ba/F3):

We prepared the retroviral stocks and used this harvested supernatant which contained the cDNAs encoding TEL-JAK2 retrovirus to transfect the murine Ba/F3 hematopoietic cell line, which is strictly dependent on IL-3 for survival and proliferation.

Check the protein expression of TEL-JAK2 by GFP marker(Fig. 2):

After 48-72 hours of transfection in the presence of IL-3, the infected Ba/F3 cells were sorted by checking the selective marker GFP in the vector by flow cytometry.

Check the TEL-JAK2 fusion protein activity by:

In Vitro: Immunoprecipitation and Western blotting:TEL-JAK2 oncogene results in the constitutive activation of JAK2 's tyrosine kinase activity (Fig. 3):

To check the TEL-induced oligomerization properties and protein kinase activity of TEL-JAK2, the Ba/F3/TEL-JAK2 cells were analyzed with in vivo-translated protein. We have checked the autophosphorylation of TEL-JAK2 which is mediated by the TEL-induced oligomerization by immunoblotting with a phosphotyrosine-specific antibody (anti-PTyr). The Ba/F3/TEL-JAK2 cell lysate was firstly specifically immunoprecipitated by antibodies directed against the C-terminal domain of JAK2 (anti-JAK2), then immunoblotted by anti-Ptyr and anti-JAK2 respectively. A higher level of tyrosine phosphorylation occurred with hTEL-mJAK2 protein than that with endogenous JAK2 protein. The expression and strong tyrosine-phosphorylation of the hTEL-mJAK2 fusion protein shows hTEL-mJAK2 fusion protein

is expressed and constitutively tyrosine-phosphorylated in Ba/F3/TEL-JAK2 cells.

In Vivo: To assay the transformation ability of TEL-JAK2 protein in cells: The constitutive activation of JAK2's tyrosine kinase activity transforms the IL-3-dependent Ba/F3 cells into growth factor(cytokine)-independent proliferation (Fig. 4):

We deprived the Ba/F3/TEL-JAK2 cells and parent Ba/F3 cells of IL-3 and monitored the cell proliferation by daily cell counting. The growth curve was recorded. In contrast to control cells (Ba/F3, no IL-3), which died with decreased cell number in the absence of IL-3, the similar proliferation curves were traced between the Ba/F3/TEL-JAK2 cells in the absence of IL-3 and Ba/F3 cells in its presence. According to the above data, the TEL-JAK2 has the property of constitutive activation of its protein tyrosine kinase activity. And this constitutively activated tyrosine kinase activity conferred cytokine-independent proliferation property to the IL-3-dependent Ba/F3 hematopoietic cell line.

Jak2 is activated in the context of a number of cytokine receptors. As a consequence is tyrosine phosphorylated at 10 predominant sites Among these sites, murine Jak2-Y^{966} was identified as a potentially important site based on mutational analysis(T. Matsuda and J.N. Ihle, unpublished data). The TEL-Jak2 fusion oncogene found in 3 leukemia patients with fusion occured within codon 337, 337, and 155 of TEL and codon 811, 505, and 711 of JAK2 respectively(all contain Y^{966})[1]. The constitutive tyrosine phosphorylation of the tyrosine kinase domain of TEL-Jak2 plays the critical role for the leukemogenesis. According to the above preliminary data (autophosphorylation of TEL-JAK2 in Fig.3) and because one of the major sites of autophosphorylation within murine JAK2 tyrosine kinase domain is Y^{966} then the proteins bound to this site may play the role of the down-stream substrate of the TEL-Jak2 and are very important for the understanding the signal transduction pathway for the leukemogenesis.

Results and Discussion

Therefore, we initially determined the signaling proteins which would bind to this site by peptide pulldown assay. The DA3 cells lysates were incubated with 20 ul Sepharose beads to which had been conjugated by glutaraldehyde either with TSQICKGMEYLGTKR or TSQICKGMEpYLGTKR. The bound proteins were eluted with Laemmli sample buffer, resolved by SDS-PAGE, and visualized by western blot analysis(Fig. 5). Strikingly a number of proteins involved in signal transduction bound a tyrosine-phosphorylated peptide derived from the amino acid sequence surrounding Jak2 Y^{966}. Among these proteins were PLC-γ1, PLC-γ2, the PI 3-kinase adapter subunits p85α and p85β, the PI 3-kinase catalytic subunit p110δ, SHC and Stat5a/b[2]. Because the TEL-JAK2 mediates constitutive activation of the PI3-kinase signaling pathway[3,4] and the function of PI 3-kinase catalytic subunit p110δ is still unclear, we derived enzyme-deficient mice to assess the role of this gene[5]. The mice are viable, but have decreased numbers of mature B cells, a block in pro B cell differentiation, and a B1 B cell deficiency. IgM-receptor-induced Ca^{2+} flux and proliferation in response to B cell mitogens are both attenuated. Immunoglobulin levels are decreased substantially. The ability to respond to T cell independent antigens is markedly reduced, and the ability to respond to T cell-dependent antigens is completely abrogated. Germinal center formation in the spleen in response to antigen stimulation is disrupted. These results define a non-redundant signaling pathway(s) utilizing the δ isoform of p110 PI3-kinase for the development and function of B cells (請詳見附件已發表文獻).

Self-Estimation

We have completed almost all of the expected goals by using different strategy. The result would be published in the journal of Molecular and Cellular Biology[2,5]. This study is very important to eluciate the function of the catalytic subunit p110δ of PI 3-kinase and the possible mechanism of leukemogenesis by the TEL-JAK2 oncogene.

Reference

1. Lacronique V, Boureux A, Valle VD, Poirel H, Quang CT, Mauchauffe M, Berthou C, Lessard M, Berger R, Ghysdael J, Bernard OA: A TEL-JAK2 fusion protein with constitutive kinase activity in human leukemia. Science 278:1309, 1997

2. Carpino N, Kobayashi R, Zang H, Takahashi Y, Jou ST, Feng J, Nakajima H, Ihle JN: Identification, cDNA cloning, and targeted deletion of p70, a novel, ubiquitously expressed SH3 domain-containing protein. Mol Cell Biol. 2002 Nov;22(21):7491-500.

3. Nguyen MH, Ho JM, Beattie BK, Barber DL: TEL-JAK2 mediates constitutive activation of the phosphatidylinositol 3'-kinase/protein kinase B signaling pathway. J Biol Chem. 2001 Aug 31;276(35):32704-13.

4. Santos SC, Lacronique V, Bouchaert I, Monni R, Bernard O, Gisselbrecht S, Gouilleux F: Constitutively active STAT5 variants induce growth and survival of hematopoietic cells through a PI 3-kinase/Akt dependent pathway. Oncogene. 2001 Apr 19;20(17):2080-90.

5. Jou ST, Carpino N, Takahashi Y, Piekorz R, Chao JR, Carpino N, Wang D, Ihle JN: An Essential, Non-redundant Role for the Phosphoinositide 3- Kinase p110δ in Signaling by the B Cell Receptor Complex. Mol Cell Biol. 2002 in press.

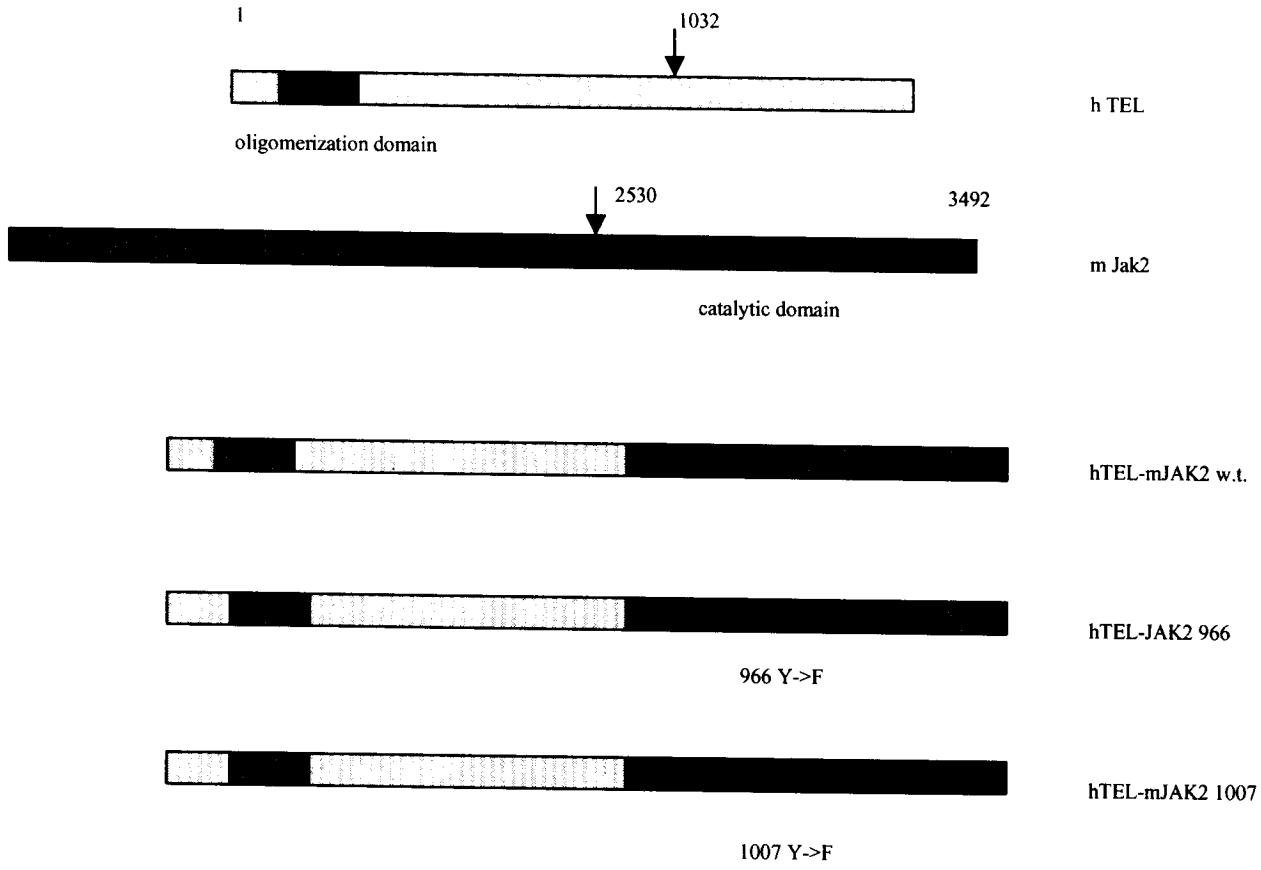


Fig. 1. hTEL-mJAK2 constructs. Schematic structure of human(h) TEL and murine(m) JAK2 proteins and the hTEL-mJAK2 fusion gene variants.The nucleotide positions are numbered according to the cDNA The arrows indicate the points where the chimeric TEL-JAK2 protein are fused.The TEL-JAK2 cDNA was constructed to encode the N-terminal residues of hTEL and the C-terminal residues of mJAK2 by RT-PCR strategy of the indicated regions of TEL and JAK2 cDNAs and subcloned into retrovirus vector. The hTEL-mJAK2 w.t. construct was used in this study to identify the constitutively phosphorylated substrate of this fusion oncoprotein. The other two (hTEL-JAK2 966 and hTEL-mJAK2 1007) are constructed by the site-directed mutagenesis of hTEL-mJAK2. They are being used to check the structure and functional regulation of the kinase domain of JAK2 for another study. The 966 and 1007 are the numbers of amino acid sequence. The Y indicates the tyrosine residule and the F indicates the phenylalanine residule.

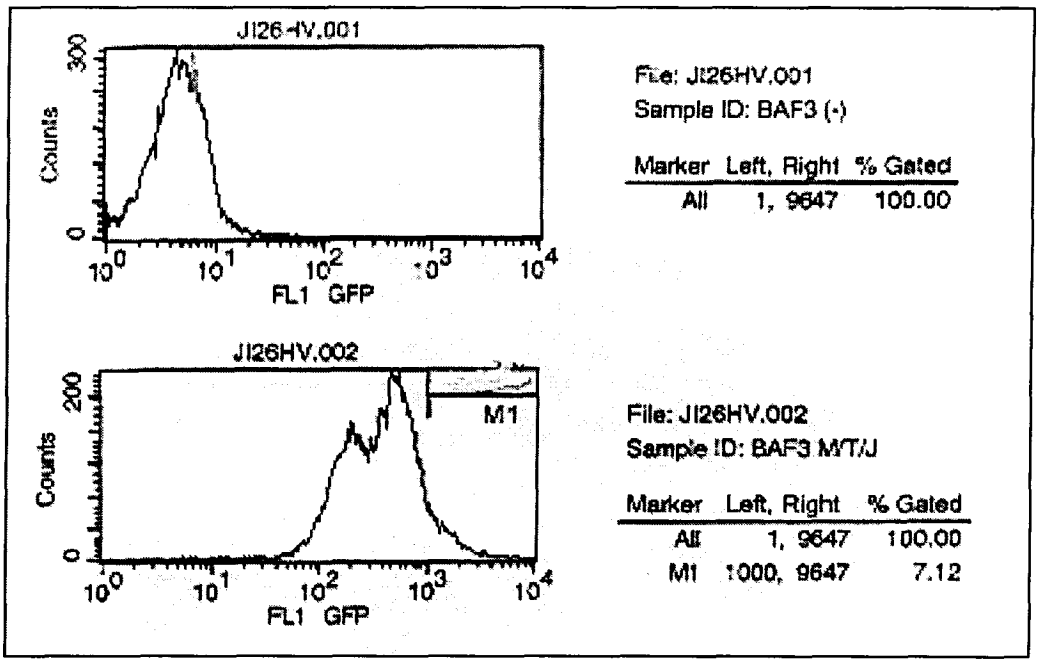


Fig.2. The Ba/F3/TEL-JAK2 cells were sorted by checking the GFP selective marker in the virus vector by flow cytometry. The JI26HV.001 shows the parent Ba/F3cells as the negative control. The M1 marker in JI26HV.002 denotes the top 7% GFP positive Ba/F3/TEL-JAK2 cells which are sorted for this study.

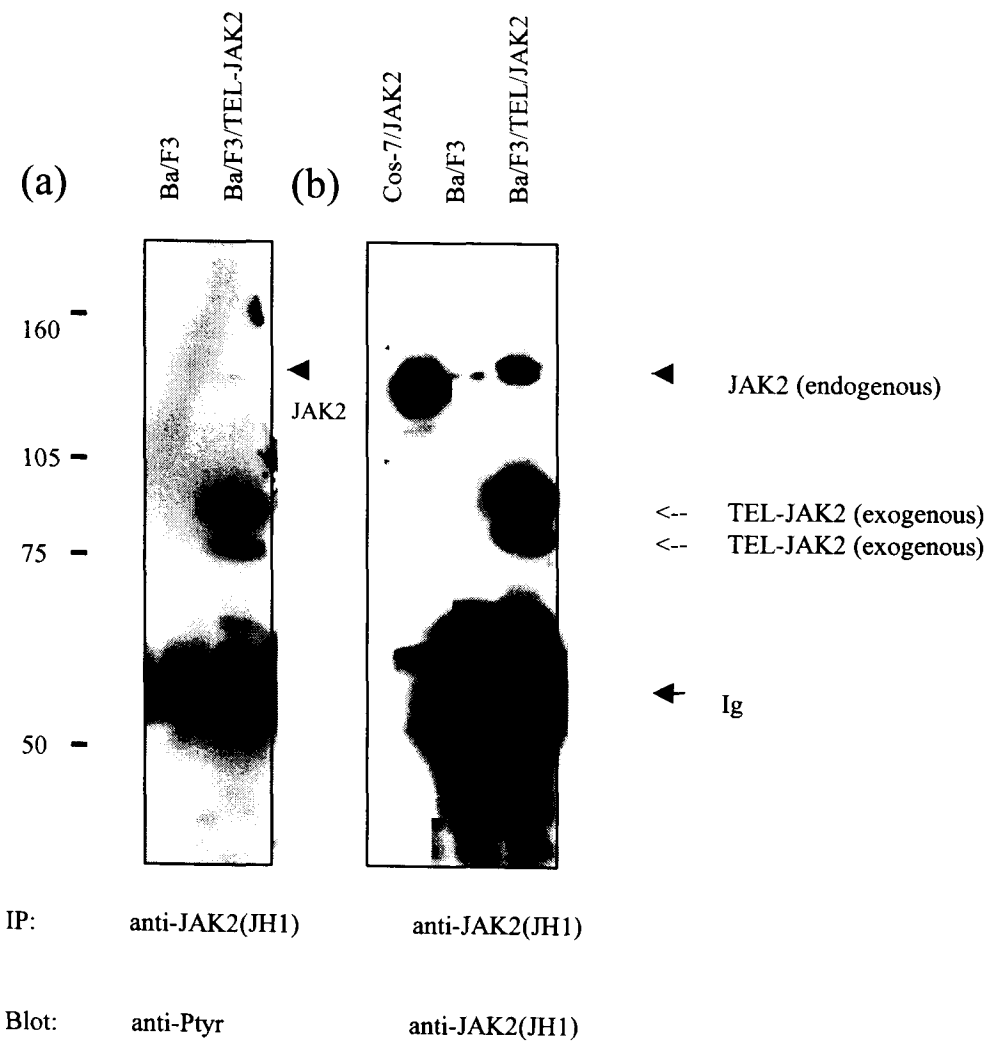


Fig.3a,b. Characterization of the expression of hTEL-mJAK2 fusion protein in Ba/F3/TEL-JAK2 cells.
Total cell lysates were prepared by using lysis buffer containing 1% Triton X-100. Lysates (1mg protein)
were immunoprecipitated with a rabbit anti-JAK2(JH1) antibody, resolved by 7.5% SDS-PAGE, transferred to PVDF membrane
and probed with an anti-Ptyr antibody in (a). The membrane was then stripped and reprobed with the same anti-JAK2 antibody initially used for IP in (b).
Cos-7 cells transfected by wild type mJAK2 were used as positive control. The hTEL-mJAK2 fusion proteins appear as doublets due to two start codon sites in the TEL[12].
A higher level of tyrosine phosphorylation occurred with hTEL-mJAK2 protein(double arrow) than that with endogenous JAK2 protein(arrow head).
The strong phosphorylation of hTEL-mJAK2 reflected its intrinsic tyrosine kinase activity. Ig indicates immunoglobulin.

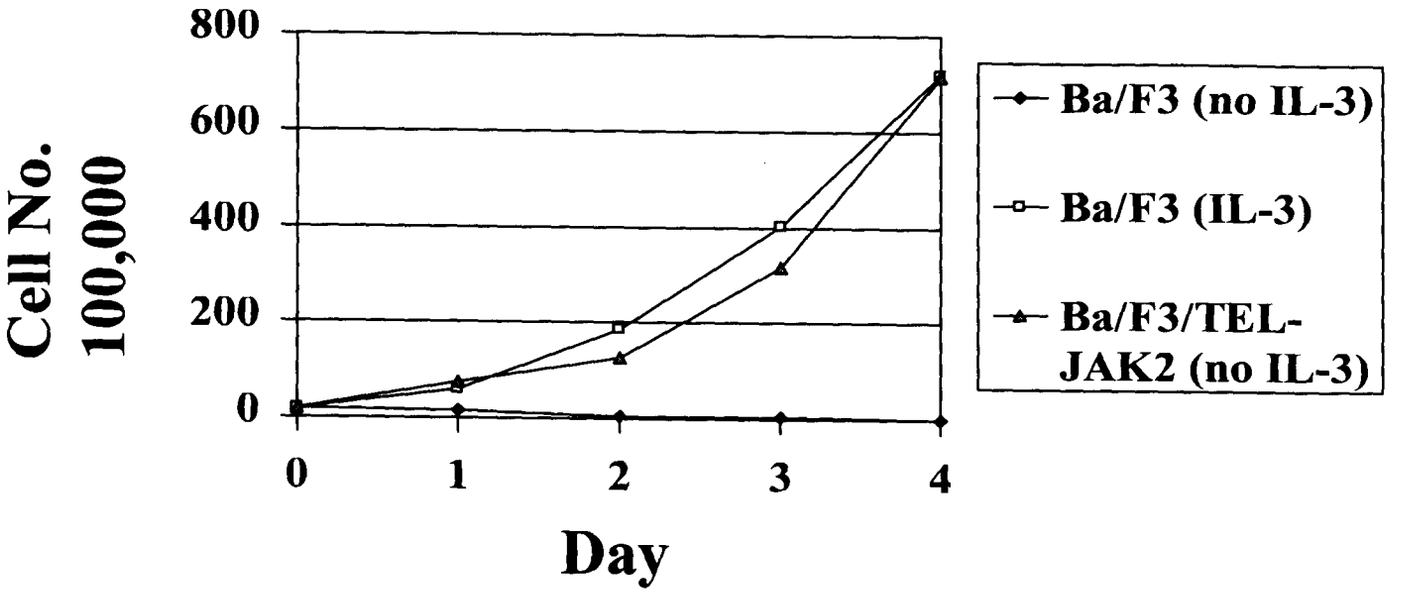


Fig. 4.TEL-JAK2 transforms the Ba/F3 cells to growth factor IL-3 independent growth. Ba/F3 cells were infected with retroviral expression vector encoding hTEL-mJAK2 w.t.. After 48 hours, the transfected cells were sorted by GFP marker and cultivated until the cells reached logarithmic growth in the absence of IL-3. The cells were counted daily.

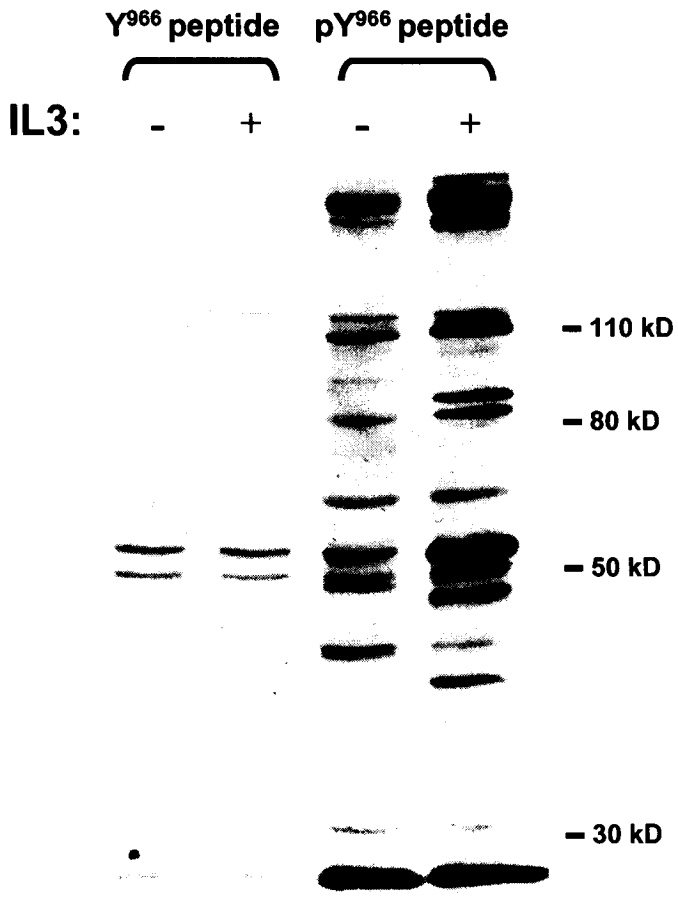


Figure 5. Peptide pulldown assay with Jak2-derived peptides.
Cells were starved of growth factor overnight, and either left untreated (-) or treated with IL3 for 5 min (+). Lysates were prepared and incubated with Sepharose beads to which had been conjugated non-phosphorylated Y^{966} peptide (TSQICKGMEYLGTKR) or tyrosine-phos-phorylated pY^{966} peptide (TSQICKGMEpYLGTKR). Bound proteins were separated by SDS-PAGE and tyrosine phosphorylated proteins were analyzed by Western Blot analysis with anti-phosphotyrosine antibodies.

MOLECULAR AND CELLULAR BIOLOGY

A publication of the American Society for Microbiology

Editor
ROGER J. DAVIS
Howard Hughes Medical Institute
Program in Molecular Medicine
University of Massachusetts Medical School
373 Plantation Street
Worcester, MA 01605-2377
Phone: (508) 856-8070
Fax: (508) 856-3210
E-mail: mebtavis@umassmed.edu

Journals Department
American Society for Microbiology
Journals Department
1752 N St., N.W.
Washington, DC 20036-2904
Phone: (202) 737-3600

September 12, 2002

Dr. James Ihle
Department of Biochemistry
St. Jude Children's Research Hospital
Memphis, TN 38105

RE: MCB 1034-02

I am pleased to inform you that your manuscript entitled "An Essential, Non-redundant Role for the Phosphoinositide 3- Kianse ..." has been accepted for publication in *Molecular and Cellular Biology*. I have forwarded the manuscript to the printer and you should shortly receive a formal letter of acceptance from the Editorial Office.

Sincerely,

Roger J. Davis, PhD, FRS
Editor

AMERICAN SOCIETY FOR MICROBIOLOGY
Journals Department

1752 N STREET, N.W.
WASHINGTON, DC 20036-2904
TEL: (202) 737-3600
FAX: (202) 942-9355

22 July 2002
Received: 07/19/02
Article: MCB 1034-02

Dr. James N. Ihle
Howard Hughes Medical Institute
Department of Biochemistry
St. Jude Children's Research
Hospital
Memphis, TN 38105

Title: An Essential, Non-Redundant Role for the Phoshoinositide 3- Kinase p110δ in Signaling by the B Cell Receptor Complex

The above manuscript has been received. It is being referred to Dr. Roger J. Davis, Howard Hughes Medical Institute, Program in Molecular Medicine, Univ. of Massachusetts Med. School, 373 Plantation St., Worcester, MA 01605-2377 (phone 508-856-8070, fax 508-856-3210, e-mail MCB_RDavis@umassmed.edu), who will notify you as to its acceptability or suggestions for improvement. In any communications relating to this paper, please refer to the article number shown above.

In submitting this manuscript to *Molecular and Cellular Biology,* the author(s) guarantees that a manuscript with substantially the same content has not been submitted or published elsewhere.

By publishing in the journal, the authors agree that any DNAs, viruses, microbial strains, cell lines, antibodies, and similar materials newly described in the article are available from a national collection or will be made available in a timely fashion, at reasonable cost, and in limited quantities to members of the scientific community for noncommercial purposes.

If "in press" references cited in the submitted manuscript are important for it to be judged, send three copies of each to Dr. Davis immediately. Failure to do so may delay the review.

If your manuscript is accepted for publication, a condition of acceptance is that you assign copyright to the American Society for Microbiology. A copyright transfer agreement is sent with each letter of acceptance.

If the manuscript is accepted for publication in a 2002 issue, page charges (subject to change without notice) will be assessed at $61 per page for the first nine pages and $76 for each page in excess of nine for a corresponding author who is an ASM member or $70 per page for the first nine pages and $87 for each page in excess of nine for a nonmember corresponding author. To obtain the member rate, a member corresponding author must include his 2002 member number on the reprint/page charge form that will be included with the proofs. To obtain the member rate, a nonmember corresponding author should complete the enclosed ASM membership application and submit it and payment of the membership fee to ASM. If the research was not supported, you may send a request for a waiver of page charges to the Director, Journals. This request must indicate how the work was financed and should be accompanied by copies of the title page and the Acknowledgments section of the paper.

If your manuscript is accepted for publication, you will be required to provide a current e-mail address in the correspondent footnote since proofs will be handled electronically.

Thank you for submitting your manuscript for consideration.

Rebecca L. Zwadyk
Production Editor
Molecular and
Cellular Biology

Enclosure

cc: All authors

An Essential, Non-redundant Role for the Phosphoinositide 3- Kinase p110δ in Signaling by the B Cell Receptor Complex

Shiann-Tarng Jou[2,3], Nick Carpino[2], Yutaka Takahashi[2], Roland Piekorz[1,2], Jyh-Rong Chao[2], Neena Carpino[2], Demin Wang[2,5] and James N. Ihle[1,2,4,6]

[1]Howard Hughes Medical Institute

[2]Department of Biochemistry

St. Jude Children's Research Hospital

Memphis, TN 38105

[4]Department of Biochemistry

University of Tennessee Health Science Center

Memphis, TN 38063

Present addresses:

[3] Department of Pediatrics, National Taiwan University Hospital, 7 Chung Shan S. Road, Taipei, 100 Taiwan,

[5] Blood Research Institute and Department of Microbiology and Molecular Genetics, Medical College of Wisconsin, 8728 Watertown Plank Rd. Milwaukee, WI 53226.

[6]Corresponding author

e-mail: james.ihle@stjude.org

Running title: p110δ is critical for BCR signaling

Keywords: B cell receptor, phosphatidylinositol 3-kinase, p110δ

Summary

Many receptor and non-receptor tyrosine kinases activate phosphatidylinositol 3-kinases. To assess the role of the δ isoform of the p110 catalytic subunit of PI3-kinases, we derived enzyme-deficient mice. The mice are viable, but have decreased numbers of mature B cells, a block in pro B cell differentiation, and a B1 B cell deficiency. IgM-receptor-induced Ca^{2+} flux and proliferation in response to B cell mitogens are both attenuated. Immunoglobulin levels are decreased substantially. The ability to respond to T cell independent antigens is markedly reduced, and the ability to respond to T cell-dependent antigens is completely abrogated. Germinal center formation in the spleen in response to antigen stimulation is disrupted. These results define a non-redundant signaling pathway(s) utilizing the δ isoform of p110 PI3-kinase for the development and function of B cells.

Introduction

Lymphocyte development and function are regulated through the coordinated action of receptors of the cytokine receptor superfamily and the B or T cell antigen specific receptors (BCR/TCR). Engagement of either receptor complex initiates tyrosine phosphorylation of a variety of intracellular substrates, including receptor chains, resulting in the initiation of cellular responses. Members of the cytokine receptor superfamily utilize JAK family cytoplasmic kinases (14) while the BCR/TCR complexes utilize members of the Src, Tec and Zap70/Syk families of tyrosine kinases. In BCR signal transduction, the Tec family kinase Btk plays a critical role as evidenced by the loss of a proliferative response to BCR engagement in Btk deficient B cells (15,16). Among the substrates typically phosphorylated and recruited to hematopoietic receptor complexes are the regulatory subunits for phosphoinositide 3-kinases (PI3K) (9,10,31). In mammals there are three genes that encode adapter proteins for PI3K catalytic subunits including p85α, p85β and p55γ. The adapter proteins facilitate association of the catalytic subunits with the receptor complex and are proposed to regulate enzyme activity. The disruption of the p85α gene, in a manner that deletes the p85 isoform as well as two splice variants of p55 and p50, results in defective BCR signaling comparable to that seen with Btk deficiency (11) (27). This strongly suggests that PI3K activity is critical in the BCR signal transduction.

Three of the known PI3Ks are regulated through their interaction with regulatory subunits including PI3Kα, PI3Kβ and PI3Kδ (31). The fourth PI3K, PI3Kγ, functions in the context of heterotrimeric G-protein coupled receptors and is essential for leukocyte function (12). The critical, non-redundant role that PI3Kα plays in cellular responses has been demonstrated through the derivation of mice lacking the gene. This deficiency results in an embryonic lethality at E9.5-E10.5 due to a severe proliferative defect in many tissues (2). Similarly, deletion of the PI3Kβ gene alone results in a very early embryonic lethality (1,2).

In contrast to PI3Kα and PI3Kβ, PI3Kδ is primarily expressed in hematopoietic cells (33). To identify proteins that are recruited to cytokine receptor complexes we have used affinity columns containing phosphopeptides corresponding to the major sites of tyrosine phosphorylation of JAK2. Two complexes which bound to a phosphopeptide affinity column derived from the amino acid sequence surrounding Jak2 Y^{966} were p85α/p110δ and p85β/p110δ. In order to determine the potential role of PI3Kδ in signaling we derived a strain of mice in which the gene was disrupted in a manner to yield a protein null mutant. As demonstrated here, a deficiency in PI3Kδ results in a very specific loss of function of the B cell receptor complex, while signaling through the cytokine receptor complexes is unaffected.

Materials and Methods

Construction of p110δ Targeting Vector

Approximately 9 kb of the p110d gene was isolated from a 129/J mouse genomic library in pBeloBacII (Incyte Genomics, St. Louis, MO) and subcloned into pBluescript. A neomycin resistance cassette was inserted into the StuI fragment encompassing exons 2 through 6. A diphtheria toxin A (DTA) cassette mediating negative selection was inserted in the 5'- end of the p110δ-neo construct.

Culture of ES Cells and Generation of p110δ-deficient Mice

E14 (129/Ola mouse strain) ES cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 15% fetal calf serum (FCS), 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 55 mM beta-mercaptoethanol, 10 ng/ml gentamicin, and 1,000 U/ml LIF (all from GIBCO-BRL, Rockville, MD). Irradiated SNLH9 fibroblasts were used as feeders. Twenty-five micrograms of SalI-linearized p110δ targeting construct was electroporated into the ES cells using a gene pulser (Bio-Rad, Hercules, CA) set at 0.23kV, 500 μF. Selection 24 hours after electroporation was in 350 ug/ml G418 (GIBCO-BRL). Drug-resistant ES clones were picked and expanded 7-10 days after electroporation. Four correctly targeted and karyotypically normal ES clones were injected into C57Bl/6 blastocysts as described (van Deursen, 1993), of which two gave germline transmission. The mice were maintained under specific pathogen-free conditions according to institutional guidelines. Genotyping of mice was performed by Southern blot analysis or by PCR.

Flow Cytometry

Single-cell suspensions were depleted of red blood cells using a hypotonic lysis buffer (pH 7.3) containing 150 mM NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM Na_2EDTA as described (20). Monoclonal antibodies conjugated to either FITC, PE, or CyChrome and specific to

murine B220/CD45R, IgM, IgD, CD5, CD43, Thy1.2, IL-2R (CD25), CD44, CD4, CD8, Mac-1 (CD11b), and Gr-1 (all from Pharmingen) were used to label $1x10^6$ cells for 30 min on ice. Fc-receptors were blocked with anti-FcγIII/II R Ab (Pharmingen). Cells were washed twice and analyzed on a FACSCalibur with CellQuest software (Becton Dickinson).

Immunoprecipitation, SDS-PAGE, and Western Blot Analysis

Immunoprecipitation, SDS-PAGE, and western blot analysis was performed as described(37). The polyclonal anti-p110δ antisera was generated by immunizing rabbits with a (KLH)-conjugated peptide derived from residues 1028-1043 of murine p110δ. Anti-phospholipase Cγ2 (PLCγ2) was from Santa Cruz Biotechologies, anti-Bruton's tyrosine kinase (BTK) was from Pharmingen, and anti-phosphotyrosine 4G10 (pTyr) from Upstate Biotechnology.

Immunizations

To measure the thymus-dependent immune response, wild type and mutant mice (five per group) were immunized IP with 100 μg of TNP-KLH (Biosearch Technologies) in a 1:1 emulsion with complete Freund's adjuvant (Sigma), followed by a second immunization 15 days later with 100 μg of TNP-KLH in a 1:1 emulsion with incomplete Freund's adjuvant (Sigma). Sera was collected 21 days after the first immunization and analyzed for TNP-specific Abs by ELISA. To measure the TI immune response, $p110\delta^{-/-}$ mice and wild-type littermates (five per group) were immunized IP with 50 μg TNP-LPS (TI-I antigen) (Sigma) or 25 μg TNP-Ficoll (TI-II antigen) (Biosearch Technologies) in phosphate-buffered saline. Sera collected 12 days after immunization were analyzed for TNP-specific Abs by ELISA.

ELISAs

Specific serum concentrations of IgM, IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, and IgA were measured by coating Nunc Maxisorp 96 well plates (Thomas Scientific, Swedesboro, NJ) with isotype-specific goat anti-mouse Abs (Southern Biotechnology Associates, Birmingham, AL).

Bound Ig from test sera was detected using alkaline phosphatase-conjugated goat anti-mouse isotype-specific Abs (Southern Biotechnology), followed by alkaline phosphatase substrate (p-nitrophenyl phosphate, Sigma). The absorbance of the reaction mixture at 405nm wavelength was quantitated using an ELISA plate reader (BIO-RAD Model 3550, Bio-Rad Laboratories). Each assay included the titration of affinity purified mouse IgM, IgG1, IgG2a, IgG2b, IgG3, and IgA Abs (Southern Biotechnology), which was used to construct a standard curve. The concentrations of serum Ig were calculated by comparing the optical density obtained to a standard curve of titrated Abs using linear regression analysis. Anti-TNP specific immunoglobulin isotype levels were measured by coating plates with TNP-bovine serum albumin (Biosearch Technologies).

***In Vitro* B and T Cell Proliferative Responses**

For B cell proliferation assays, FACS-purified B cells (1x 10^5/well; >95% pure) were cultured in triplicate in 96-well U-bottom plates. Purified goat anti-mouse IgM (μ chain) (Jackson ImmunoResearch), anti-CD40 mAb (PharMingen), LPS (Sigma), or PMA and ionomycin (Sigma) were added as mitogens at the indicated concentrations. Cells were cultured for 48 hours, after which they were labeled with 1μCi/well ^{3}H-thymidine for 18 hours and harvested with a TOMTECH Harvester 96 MachII to measure incorporated radioactivity. T cell proliferation assays were conducted similarly. Percentages of Thy1.2$^+$ cells within splenocyte suspensions were identified by FACs analysis, and splenocytes were cultured in triplicate U-bottom 96-well plates such that a constant number of T cells (1.5 x 10^5/well) was used per assay. Anti-CD3 mAb (145-2C11, Pharmingen), PHA, ConA, and PMA + ionomycin (Sigma) were added at the indicated concentrations.

Ca^{+2} measurement and phosphotyrosine blots

Splenocytes were suspended in DMEM + 10% FCS, loaded with Indo-1 (10μM, Molecular Probes, Eugene, OR) for 30 min at room temperature, and stained with either PE-

anti-B220 or PE-anti-CD4 and FITC-anti-CD8 for an additional 20 min at 4°. Cells were stimulated with anti-mouse IgM (Jackson ImmunoResearch) for B cells or rat anti-mouse CD3 Ab (145-2C11) followed by anti-hamster IgG Ab (Jackson ImmunoResearch) for T cells. Ionomycin (Calbiochem) was used as a positive control. To induce tyrosine phosphorylation, splenic B cells were purified by complement lysis with anti-Thy1.2 Ab (AT83) and rabbit and guinea pig complement (Cedarlane Laboratory). Purified B cells (90%) were stimulated with anti-IgM (20ug/ml) for 3 min, and then lysed as indicated above.

Immunohistochemistry

For detection of a germinal center formation response, p110$\delta^{-/-}$ mice and their wild-type littermates were immunized intraperitoneally with TNP-KLH (Biosearch Technologies) as described above, or 150μl of a 10% suspension of sheep red blood cells (Colorado Serum, Denver, CO). Spleens were snap frozen in liquid nitrogen and sectioned. Frozen sections 8 nm thick were fixed with acetone for 15 min at -20 and rehydrated with PBS. Sections were double-stained with biotinylated B220 (1:200) and FITC-Thy1.2 (1:50), FITC-B220 (1:200) (all from PharMingen) and biotinylated PNA (10μg/ml, peanut agglutinin, Vector, Burlingame, CA) in PBS with 3% BSA for 2 hrs at room temperature. Sections were washed and subsequently counterstained with streptavidin-TRITC (1:1000) (Southern Biotechnology) for 1 hr at room temperature. After washing in PBS, the sections were mounted in Fluoromount G (Southern Biotechnology). All slides were evaluated and photographed by a Leica TCS confocal laser scanning microscope.

Bone Marrow Colony Assays

Cells were prepared from bone marrow in α-MEM medium (Life Technologies) containing 2% fetal bovine serum (StemCell Technologies) and counted in the presence of 3% acetic acid to lyse RBCs. Cells were cultured in 0.9% MethoCult M3230 (StemCell Technologies) with recombinant cytokines at the following concentrations: BFU-E, 3 U/ml rhEpo (Amgen)

+ 10 ng/ml rmIL-3 (R&D); CFU-Mix, 10 ng/ml rmIL-3 (R&D); CFU-Eos, 20 ng/ml rmIL-5 (R&D Systems); CFU-Meg, 50 ng/ml rhTpo (Genzyme); CFU-G, 10 ng/ml rhG-CSF (Amgen); CFU-GM, 10 ng/ml rmGM-CSF (R&D); and CFU-M 10 ng/ml rmCSF-1 (R&D). Pre-B cell assays were conducted with MethoCult M3630 using 10 ng/ml rhIL-7 (StemCell Technologies). Assays were plated in 35 mm culture dishes in duplicate and scored as previously described (29).

Results

Generation of p110δ mice

The p110δ gene was disrupted in murine E14 ES cells by the replacement of a 2.6kb fragment containing exons two through six with a *neo* gene expression cassette (Figure 1). The design of the targeting construct relied on the finding that deletion of >150 amino acids from the N-terminal region of p110δ abrogated kinase activity (33). Four correctly targeted ES clones were injected into C57Bl/6 blastocysts, two of which were transmitted to the germline. Both mouse lines had comparable phenotypes. Southern blot analysis confirmed the presence of the targeted locus (see Figure 1B). Heterozygous mice were bred to obtain mice homozygous for the targeted allele. Homozygous offspring were viable, were obtained with the expected Mendelian ratio and lacked p110δ protein as assessed by IP/Western analysis of splenic extracts (Figure 1C). The absence of p110δ had no consequence on the levels of p110α or p110β in splenic extracts (data not shown).

Lack of an effect of p110δ-deficiency on hematopoietic tissues

In contrast to the widely distributed p110α and p110β proteins, p110δ is primarily expressed in circulating neutrophils, monocytes, and lymphocytes as well as in lymphoid tissues including spleen, lymph nodes and thymus (33). Therefore, we examined a number of hematopoietic parameters in the mutant mice. There were no significant differences between wild type and mutant mice in the number of red cells (Table 1), hemoglobin levels, or hematocrits (data not shown). Similarly, there were no significant effects of p110δ deficiency on the numbers and morphology of peripheral blood leukocytes, including neutrophils, eosinophils, basophils, monocytes and lymphocytes (Table 1). The recovery of bone marrow cells was normal, and in colony assays of bone marrow progenitors, there were no significant alterations in the frequency and morphology of colonies in response to a variety of cytokines (Table 1).

Defective B cell deveplopment in p110δ$^{-/-}$ mice

Analysis of B cell populations indicated that the deficiency of p110δ had a significant consequence on the numbers and the various subpopulations of differentiating B cells. Although the total numbers of bone marrow cells were comparable to wild type mice (54.1 ± 5.3 verses 54.3 ± 2.3, n=8), there was an approximately 25% decrease in the percentage of bone marrow B lineage cells (21.1 ± 5.2 verses 15.6 ± 5.8). Among the bone marrow B cell subpopulations, the percentages of pro-B cells ($CD43^+B220^+$) were similar between wild type and p110δ-deficient bone marrow (Figure 2A). However the more mature B cell subpopulation of $B220^+CD43^-$ cells was reduced (Figure 2). Of the more mature populations there were significant reductions in both the percentage of $B220^+IgM^+$ cells and the more mature $B220^{++}IgM^{low}$ cells.

Gross macroscopic examination of lymphoid organs revealed a consistent reduction in the size of spleens and lymph nodes isolated from p110δ-deficient animals as compared to wild type littermate controls. In preparations of splenocytes, the percentages of total $B220^+$ lymphocytes was reduced from 42.3%±5.0% to 32.1%±11.4% (Table1, see also Figure 2B). As shown in Figure 2D, there was a marked reduction in the $IgM^{low}IgD^{high}$ population and in the most mature $B220^+IgM^-$ population. Similar to the spleen populations, the percentage of $B220^+$ lymphocytes in lymph nodes was reduced from 31.6%±4.5% to 19.0%±3.2% (n=6) (Table 1).

Both strains had comparable proportions of $CD4^+$ and $CD8^+$ peripheral T cells in their spleen and lymph node T cell compartments (Table 1) and the T cell subpopulations in the thymus were normal (Figure 2F) . Lastly, there was a complete absence of $CD5^+$ B1 cells in peritoneal lymphocytes (Figure 2C). Collectively, the phenotypic changes in the B cell subpopulations are similar to those observed in mice in which the B cell receptor signaling

pathway is disrupted by deficiencies in Btk (15,16), BLNK (22), PKC β (17), PLCγ2 (36), 85α (27), Vav-1/Vav-2 (11,28) and CD19 (7,24).

Decreased Serum Immunoglobulin and Defective Humoral Response in p110δ$^{-/-}$ Mice

The reduction in the number of peripheral B cells in p110δ knockout mice suggested that B cell function might be disrupted in the absence of p110δ. To further assess this, serum immunoglobulin levels were measured by ELISA. As illustrated in Figure 3, unimmunized p110δ-deficient mice had lower levels of serum immunoglobulin of all isotypes examined, with IgM and IgG_1 levels being the most dramatically lowered. The requirement for p110δ in the humoral response was examined by immunizing mice with T-independent and T-dependent antigens. Administration of TNP-LPS (a T-independent antigen) resulted in hapten-specific IgM and IgG_1 responses in the p110δ-deficient mice that were 30-50% lower than wild-type mice (Figure 4A). In contrast, the levels of hapten-specific IgG_{2a}, IgG_{2b}, and IgG_3 between the two strains were equivalent. P110δ-deficient mice, immunized with the type II antigen, TNP-ficoll, had significantly decreased production of hapten-specific IgM, IgG_1, IgG_{2b} and IgG_3, whereas IgG_{2a} responses were low in all mice (Figure 4B). Lastly p110δ$^{-/-}$ mice, immunized with the T-cell dependent antigen, TNP-KLH, failed to mount any detectable response (Figure 4C).

The lack of a response to TNP-KLH suggested that the deficiency of p110δ might also be associated with a defect in germinal center formation. As illustrated (Figure 5) immunocytochemical staining of spleen cryosections demonstrated that whereas wild type mice developed germinal centers, detected by peanut agglutinin (PNA) staining (arrows in Figure 5), mutant mice failed to generate germinal centers. To further confirm that p110δ is required for the process of germinal center formation, wild type and mutant mice were immunized with sheep red blood cells. Ten days later, at the peak of germinal center

formation, spleens were analyzed for germinal centers by PNA staining. In all p110δ$^{-/-}$ examined, there was a complete absence of germinal center formation (data not shown). In contrast to the effect of p110δ deficiency on peripheral B cells, we detected no difference in the T cell populations (Thy 1 positive cells) surrounding the splenic follicles between wild type and mutant mice (data not shown).

Impairment of *in vitro* B cell proliferation with p110δ deficiency

To establish whether the observed *in vivo* defects in p110δ deficient B cells were due to an intrinsic defect in signal transduction, *in vitro* B cell proliferation was examined. The abilities of splenocytes to respond to various concentrations of known activators are shown in Figure 6. As members of the PI3K family have been demonstrated to lie in the B cell receptor signaling pathway upstream of PLCγ2, we compared the mitogenic response of p110δ-deficient B cells with B cells isolated from PLCγ2$^{-/-}$ mice (36). As illustrated, the proliferation of p110δ deficient B cells in response to anti-IgM was markedly reduced. However, this reduction was not as severe as that associated with a deficiency of PLCγ2. There was also a decrease in the proliferative response of p110δ deficient B cells relative to wild type B cells after stimulation with anti-CD40, similar to the reductions observed with PLCγ2 deficient B cells. In addition, the response of p110δ$^{-/-}$ B cells to LPS was reduced relative to wild-type, while the response to phorbol ester and ionomycin was not significantly affected (Figure 6). Curiously, the response of p110δ$^{-/-}$ B cells to CD40 + IL4 was unaffected (data not shown). The response of splenic T lymphocytes was also examined (Figure 6B). The proliferative response to stimulation through the T cell receptor complex with anti-CD3 was not significantly affected. There was some reduction in the response to ConA and a more striking reduction in the response of lymphocytes to PHA. It should be noted however that the response to PHA is only approximately one tenth that seen in response to anti-CD3. Lastly the response of T cells to PMA and ionomycin was not

altered (Figure 6), as was the response to stimulation with anti-CD3 + anti-CD28, with or without addition of exogenous IL2 (data not shown).

The induction of an intracellular calcium flux is an early signal-transduction event that regulates many downstream cellular responses and is a sensitive marker for activation of PLCγ2 and its upstream activators (36). Therefore, we analyzed the ability of anti-IgM to induce an increase in intracellular Ca^{2+} in p110δ$^{-/-}$ B cells. In contrast to the absence of detectable Ca^{2+} mobilization in PLCγ2$^{-/-}$ B cells, p110δ$^{-/-}$ B cells induced an increase in intracellular Ca^{2+} that was approximately 25% that observed in wild type cells (Figure 7A). Lastly, mobilization of intracellular Ca^{2+} upon anti-CD3 stimulation was unimpaired in p110δ$^{-/-}$ T cells (Figure 7A).

A deficiency in p110δ could potentially affect the recruitment and activation of components of the B cell receptor complex that utilize pleckstrin homology (PH) domains to be localized in the complex, including Btk and PLCγ2. We therefore assessed the recruitment of these proteins into the complex by examining their extent of tyrosine phosphorylation following receptor engagement. As illustrated (Figure 7B), both Btk and PLCγ2 were inducibly tyrosine phosphorylated in p110δ deficient cells at levels comparable to that of wild type cells.

Discussion

The results demonstrate the essential, non-redundant role that p110δ plays in BCR signal transduction. The remarkable specificity of function was unexpected since p110δ is expressed in a variety of hematopoietic lineages. Indeed our interest in p110δ arose from studies of cytokine signaling in myeloid lineage cells. In particular, the cytoplasmic protein tyrosine kinase Jak2 is associated with many cytokine receptors (13) and with receptor engagement is activated resulting in phosphorylation of at least 10 major autophosphorylation sites (unpublished data). When one of these sites, Y^{966}, was used to develop phosphopeptide affinity columns, it was found to bind a number of signal transducing proteins including a complex of p85α/p85β with p110δ. Importantly, in the myeloid cell line used, complexes of p85α/p85β with p110α or p110β were not detected although both are expressed in these cells. The basis for this specificity is not known but could reside in the characteristics of p85α/p85β when bound to the Y^{966} phosphopeptide. In spite of the ability of Y^{966} is uniquely recruit the p85α/p110δ or p85β/p110δ complex, the absence of p110δ has no detectable affect on the responses of a number of cytokines that utilize Jak2, including IL-3 and Epo. The possibility exists that the recruitment of PI3K activity to these receptor complexes is not critical for their physiological function. Consistent with this, mutant mice that contain a truncated Epo receptor that lacks receptor domains required for optimal activation PI3K activity (39) have normal erythropoiesis. Alternatively, in the absence of p110δ, p110α or p110β may replace the requirement for p110δ. It should be noted however that there was no obvious evidence for altered Epo signaling in mice that are deficient in p85α thus a different regulatory subunit would also be involved.

Perhaps one of the most striking features is the similarity of the p110δ deficiency with deficiencies of a number of other genes on BCR signaling. Firstly, the similarities seen with deficiencies of p85α and p110δ would indicate that p110δ uniquely associates with p85α in the context of the BCR. This is again surprising since both p110α and p110β associate with p85α and have previously been shown to be present in B cells (33). Moreover, deficiency of p110δ does not detectably alter the levels of p110α or p110β in splenic lymphocytes (data not shown). The specificity may reside in the BCR complex and its ability to recruit specific p85α/p110 complexes. For example, CD19 is uniquely required for B cell responses to mitogens, generation of PIP_3, PLC activation and Ca^{2+} mobilization following BCR engagement and the deficiency of CD19 results in a very similar *in vivo* phenotype to that seen with a deficiency of p110δ (7,24). Given the lack of a significant effect of p110δ-deficiency on T cell receptor signaling pathways (see below), it can by hypothesized that the lack of T-dependant antibody responses in p110δ-/- mice is directly related to impaired CD19 signaling. Interestingly, the properties of CD19 deficient B cells has led to the proposal that tyrosine phosphorylation of CD19 on Y^{484} and Y^{515} is uniquely required for activation of PI3K activity which, in turn, is required for phosphoinositide hydrolysis and Ca^{2+} mobilization (3).

The deficiency in p110δ also has a very similar consequence to that of a deficiency in PLC-γ2 (36). It should be noted however that the deficiency in PLC-γ2 affects a number of receptors of the Fc family of receptors that are not detectably affected by the p110δ deficiency. Thus, only within the context of the BCR, is p110δ required for PLC-γ2 function. It is possible that in the context of other receptor complexes, which do not require CD19, the p85/p110 complexes may function redundantly or have different specificities. The results demonstrate that in the absence of p110δ, PLC-γ2 is recruited to the receptor complex based

on its induced tyrosine phosphorylation. However, functionally it is not fully activated as evidenced by the greatly reduced Ca^{2+} mobilization. It can be proposed that the engagement of the pleckstrin homology domain of PLC-γ2 may be required for its appropriate localization or activation. In this regard it should also be noted that a deficiency in the PIP-3 phosphatase, PTEN, results in B cell hyperactivity (6). In addition to potentially affecting PLC-γ2 activity in the complex, p110δ may also affect Btk function. As shown here, Btk is recruited to the complex and is inducibly tyrosine phosphorylated in the absence of p110δ. However, the naturally occurring Xid mutation in mice involves a single amino acid change in the pleckstrin homology domain (23,30) and creates a phenotype that is similar to that seen with Btk deficiency (15,16,32,34) and deficiencies in other components of the complex.

Based on the above observations it can be proposed that the p85α/p110δ is uniquely recruited to the BCR complex, most likely through a specificity involving CD19. Once recruited, PIP-3 generation is required to form binding sites for both Btk and PLC-γ2 that may stablize the complex or contribute to the overall activation of the complex. The essential function of the complex is the hydrolysis of membrane phosphoinositides and the generation of IP_3 and diacylglyerol (DAG). These mediators, in turn, are essential for Ca^{2+} mobilization and the activation of PKC β. Again, the critical role for PKC β is evident in the phenotype of PKC β deficient mice which have BCR defects identical to the deficiencies of the other components (18). The other possibility is that PI3K activity is required for the activation of Akt, the major isoform being Akt1. However, mice deficient in neither Akt1 or in Akt2 have been reported to have B cell defects comparable to those seen in PKC β deficient mice (4,5).

The other essential components of the BCR complex, based on the phenotypes of deficient mice, are the adapter protein Blnk (19,22), the PI3K binding protein BCAP (38) and members of the Vav family of GTP exchange factors. The adapter protein Blnk is

critical for the recruitment and activation of PLC-γ2 through direct interaction of the SH2 domains of PLC-γ2 with tyrosine phosphorylated Blnk and Blnk deficiency results in the elimination of the Ca^{2+} signal in BCR signaling. A deficiency in BCAP also creates a very similar phenotype and, biochemically, is also associated with loss of PLC-γ2 function but retention of the recruitment and tyrosine phosphorylation of PLC-γ2 in the BCR complex. Based on these observations it can be postulated that BCAP is required to recruit, activate and/or stablize the p85/p110δ complex in the context of the BCR signalsome. In the case of Vav, a deficiency of Vav-1 has a severe consequence to T cell development and a lesser consequence on B cell development (8,40). However, the absence of both Vav-1 and Vav-2 dramatically affects BCR signaling and results in a phenotype comparable to that seen in p110δ deficient mice (28). Importantly the absence of Vav-1/Vav-2 eliminates the Ca^{2+} flux induced by BCR engagement and the activation of Vav-1/Vav-2 has been proposed to require binding of PIP_3 by their PH domains. The precise nature by which Vav-1 and Vav-2 contribute to the BCR complex signaling is not known.

In contrast to the BCR complex, the deficiency in p110δ did not affect signaling through aggregation of the T cell receptor complex with anti-CD3 either in terms of a mitogenic response or in the induction of a Ca^{2+} flux. However, there was a reduction in the much weaker responses of T cells to PHA and ConA, the significance of which is not known. Although the absolute numbers of splenic and lymph node T cells are slightly reduced, it is likely that this reduction is related to the overall reduced size of the spleen and lymph nodes. In addition to there being no differences in peripheral blood lymphocytes numbers between wild-type or p110δ-deficient mice, no significant differences in thymic size or cellularity was observed. Importantly, many of the components of the BCR complex are distinct from those of the TCR complex although are often family members. For example, PLC-γ2 is not required for a Ca^{2+} flux following TCR aggregation although PLC-γ1 is likely essential.

Similarly, the TCR complex requires Rlk and Itk (25) and not Btk. As noted above, it has been suggested that Vav-1 is critical to TCR signaling while Vav-1 and Vav-2 redundantly are required for BCR signaling. In the TCR complex the adapter protein Blnk is replaced by the family member SLP-76. Further downstream the critical role of PKC β in B cell responses is replaced by PKC θ in T cells (26). Thus, although the ultimate function of the receptor complex might be comparable, the functional components of the complex are different and are likely to have evolved to function synergistically.

Hypogammaglobulenima in man is associated with mutations in the B cell receptor complex components Btk and BLNK. It might be anticipated that other components, identified through gene disruptions in mice, would contribute to those cases in which an etiology is not known. Recent studies (35), however, failed to identify causative mutations in PLCγ2 in 32 such cases. It should be noted however that the deficiency of PLCγ2 in mice affects many receptors of the immunoglobulin superfamily including the collagen receptor complex on platelets and therefore a spectrum of phenotypic changes would be expected in individuals with a deficiency in PLCγ2. In contrast, as described here, p110δ deficiency results in a very selective defect on B cells and consequently is a potential candidate for hypogammaglobulenima in humans.

Following submission of this manuscript, an article appeared in which the properties of a mouse strain were described in which homologous recombination was used to create a dominant negative form of p110γ (21). Similar to the properties of mice in which a null mutation exists, signaling through the BCR complex was affected although the degreee of loss of the calcium response was less. This may due to a role for p110δ in forming a stable BCR complex in addition to its enzymatic role, possibly through its interaction with BCAP. A significant difference existed however in the conclusions regarding T cell responses. As noted above we have not detected any consistent differences in the response of T cells to

TCR engagement in numerous experiments. In mice expressing the dominant negative form of p110δ the responses to anti-CD3 were reduced 30-50% while the responses to anti-CD3 + anti-CD28 were either not decreased or increased. It was concluded that the reduction in the T cell responses were responsible for the dramatic reduction in T-dependent antibody responses, a phenotype also seen in our null mutants. As noted above however, the absence of CD19 also results in loss of T-dependent responses and its role in the BCR complex might equally explain the loss of T-dependent antibody responses, independent of a functional role for p110δ in T cell responses. Lastly, we have not observed an inflammatory bowel disease in mice containing a null mutation similar to that described for mutant mice containing the dominant negative mutation. This difference could potentially be due differences in animal housing conditions coupled with the B cell defects. Alternatively, the dominant negative protein may create a phenotype in other lineages of cells.

Acknowledgements

The authors would like to acknowledge Ken Sato, Jean-Christophe Marine, Evan Parganas, Catriona McKay, Richard Moriggl, Mei-Hwei Chang and Kai-Hsin Lin for valuable discussions. We would also like to thank John Raucci for the injection of ES cells into blastocysts and the staff of the Animal Resources facility for help in animal maintenance. The FACS sorting and Ca^{2+} flux studies were performed by Richard Ashmun and Mahnaz Paktinat and general technical support was provided by Linda Synder and Kristen Rothammer. The ES cells were kindly provided by Jan van Deursen. S-T J was supported by the National Taiwan University Hospital and Taiwan National Science Council (NSC89-2314-B-002-399; NSC90-2314-B-002-181). This work is supported by the Cancer Center CORE grant CA21765, by the grant RO1 DK42932 to J. N. I., by grant PO1 HL53749, and by the American Lebanese Syrian Associated Charities (ALSAC).

Figure legends

Figure 1. Targeted disruption of murine p110δ gene.

(A). A targeting construct was developed that would replace the indicated exons with a neomycin resistance cassette. The locations of the 3' external probe and PCR primers (P1, P2, and P3) for genotyping are indicated. Restriction enzyme sites are as follows: Sa, SacI; K, KpnI; St, StuI. (B) Southern blot analysis of KpnI-digested genomic DNA from p110δ heterozygous mice probed with the indicated probe. The positions of the mutant 5.8 Kb and wild-type 4.3 Kb KpnI fragments are indicated. (C) Absence of p110δ protein expression in p110δ-/- mice. Total splenic lysate (2mg) was used for immunoprecipitation and western blot analysis using rabbit polyclonal anti-p110δ antibodies (see Experimental Procedures).

Figure 2. Flow cytometric analysis of B- and T-cell development in the primary and secondary lymphoid organs.

Total cells isolated from 3- to 4- month-old mice were stained with the indicated antibodies and analyzed by FACS. The numbers in each region indicate the percentage of the total cells plotted. The data shown are representative of a minimum of six pairs of mice examined. (A) The early and late stages of B cell maturation in the bone marrow (left and right, respectively) are displayed. Left panel, B220 vs. CD43 profiles of IgM^--gated cells; right panels, the B220 vs. IgM profiles of $CD43^-$-gated cells (Fraction D, E and F, Hardy et al., 1991). (B) Analysis of splenic B and T cells is displayed., profile of B220 vs. Thy1.2 gated lymphocytes. (C) Analysis of peritoneal B1a cells is displayed as the profile of $IgM^+/CD5^+$ cells within the lymphocyte gate. (D) Maturation stages of splenic B cells are displayed. Left panels, B220 vs. IgM profiles of gated lymphocytes; right panels, IgD vs. IgM profiles of gated lymphocytes. (E) Analysis of lymph node B cells, B220 vs. IgM

profile of gated lymphocytes. (F) Maturation of thymocytes is unimpaired. CD4 vs. CD8 profile of gated thymocytes.

Figure 3. Decreased serum immunoglobulin concentrations in p110$\delta^{-/-}$ mice.

Serum levels of immunoglobulin isotype levels in unimmunized wild type or p110δ-deficient mice were determined by ELISA. The values for each individual mouse tested are plotted on a logarithmic graph. Mean values are indicated by bold hashmarks. P values as determined by Student's t test are indicated.

Figure 4. Defective humoral response to thymus-independant antigens and lack of a humoral response to a thymus-dependent antigen in p110δ-deficient mice.

Wild-type (filled circle) or p110δ-deficient mice (open circle) were immunized either with (A) TNP-LPS, a TI-I antigen, or (B) TNP-Ficoll, a TI-II antigen, or (C) TNP-KLH, a T-dependant antigen. Serum TNP-specific immunoglobulin isotype response was measured by ELISA. The relative absorbance values at 405 nm wavelength for the indicated dilution are plotted for each individual animal. Mean values are indicated by bold hashmarks. Statistically significant differences ($p < 0.05$) are indicated by **.

Figure 5. Germinal center formation in TNP-KLH-stimulated 4-month old mice.

Germinal centers were stained with biotinylated PNA followed by streptavidin-TRITC[red]. B cells were stained with FITC-B220[green].

Figure 6. Impaired in vitro proliferative response of p110δ-deficient lymphocytes.

(A). Purified splenic B cells from 3- to 4-month old wild type (black bar), p110$\delta^{-/-}$ mice (hatched bars), and PLC$\gamma 2^{-/-}$ mice (open bars) were incubated in medium alone, or medium

containing indicated concentrations of various stimuli. Proliferative responses are expressed as mean counts of triplicates from a single [^{3}H]thymidine incorporation assay. Results are representative of four to six experiments. (B) Splenic T cells were incubated in medium alone, or medium containing indicated concentrations of various stimuli. Proliferative responses are expressed as mean counts of triplicates from a single [^{3}H]thymidine incorporation assay. Results are representative of four to six experiments.

Figure 7. Induction of Ca^{+2} mobilization and tyrosine phosphorylation in p110δ-deficient mice.

(A). Induction of Ca^{2+} was measured by flow cytometry following stimulation of Indo-1-labeled splenic B cells with anti-IgM or splenic T cells with anti-CD3 followed by anti-IgG. Arrow 1 indicates the time point of specific stimulation, and arrow 2 indicates the time point of ionomycin addition. The results are representative of four independent experiments. (B) Purified splenic B cells from wildtype and p110δ $^{-/-}$ mice were stimulated with anti-IgM (20ug/ml) for 3 min and analyzed for the induction of tyrosine phosphorylation of Btk and PLCγ2.

References

1. **Bi, L., I. Okabe, D. J. Bernard, and R. L. Nussbaum**. 2002. Early embryonic lethality in mice deficient in the p110beta catalytic subunit of PI 3-kinase. Mamm.Genome **13**:169-172.

2. **Bi, L., I. Okabe, D. J. Bernard, A. Wynshaw-Boris, and R. L. Nussbaum**. 1999. Proliferative defect and embryonic lethality in mice homozygous for a deletion in the p110alpha subunit of phosphoinositide 3-kinase. J.Biol.Chem. **274**:10963-10968.

3. **Buhl, A. M., C. M. Pleiman, R. C. Rickert, and J. C. Cambier**. 1997. Qualitative regulation of B cell antigen receptor signaling by CD19: selective requirement for PI3-kinase activation, inositol-1,4,5- trisphosphate production and Ca2+ mobilization. J.Exp.Med. **186**:1897-1910.

4. **Chen, W. S., P. Z. Xu, K. Gottlob, M. L. Chen, K. Sokol, T. Shiyanova, I. Roninson, W. Weng, R. Suzuki, K. Tobe, T. Kadowaki, and N. Hay**. 2001. Growth retardation and increased apoptosis in mice with homozygous disruption of the Akt1 gene. Genes Dev. **15**:2203-2208.

5. **Cho, H., J. Mu, J. K. Kim, J. L. Thorvaldsen, Q. Chu, E. B. Crenshaw, III, K. H. Kaestner, M. S. Bartolomei, G. I. Shulman, and M. J. Birnbaum**. 2001. Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta). Science **292**:1728-1731.

6. **Di Cristofano, A., P. Kotsi, Y. F. Peng, C. Cordon-Cardo, K. B. Elkon, and P. P. Pandolfi**. 1999. Impaired Fas response and autoimmunity in Pten+/- mice. Science **285**:2122-2125.

7. **Engel, P., L. J. Zhou, D. C. Ord, S. Sato, B. Koller, and T. F. Tedder**. 1995. Abnormal B lymphocyte development, activation, and differentiation in mice that lack or overexpress the CD19 signal transduction molecule. Immunity. **3**:39-50.

8. **Fischer, K.-D., A. Zmuidzinas, S. Gardner, M. Barbacid, A. Bernstein, and C. Guidos**. 1995. Defective T-cell receptor signalling and positive selection of Vav-deficient $CD4^+CD8^+$ thymoctyes. Nature **374**:474-477.

9. **Fruman, D. A. and L. C. Cantley** . 2002. Phosphoinositide 3-kinase in immunological systems. Semin.Immunol. **14**:7-18.

10. **Fruman, D. A., R. E. Meyers, and L. C. Cantley**. 1998. Phosphoinositide kinases. Annu.Rev.Biochem. **67**:481-507.

11. **Fruman, D. A., S. B. Snapper, C. M. Yballe, L. Davidson, J. Y. Yu, F. W. Alt, and L. C. Cantley**. 1999. Impaired B cell development and proliferation in absence of phosphoinositide 3-kinase p85alpha. Science **283**:393-397.

12. **Hirsch, E., V. L. Katanaev, C. Garlanda, O. Azzolino, L. Pirola, L. Silengo, S. Sozzani, A. Mantovani, F. Altruda, and M. P. Wymann**. 2000. Central role for G protein-coupled phosphoinositide 3-kinase gamma in inflammation. Science **287**:1049-1053.

13. **Ihle, J. N.** 1994. The Janus kinase family and signaling through members of the cytokine receptor superfamily. Proc.Soc.Exp.Biol.Med. **206**:268-272.

14. **Ihle, J. N.** 1996. Janus kinases in cytokine signalling. Philos.Trans.R.Soc.Lond.B.Biol.Sci. **351**:159-166.

15. **Kerner, J. D., M. W. Appleby, R. N. Mohr, S. Chien, D. J. Rawlings, C. R. Maliszewski, O. N. Witte, and R. M. Perlmutter**. 1995. Impaired expansion of mouse B cell progenitors lacking Btk. Immunity **3**:301-312.

16. **Khan, W. N., F. W. Alt, R. M. Gerstein, B. A. Malynn, I. Larsson, G. Rathbun, L. Davidson, S. Muller, A. B. Kantor, and L. A. Herzenberg**. 1995. Defective B cell development and function in Btk-deficient mice. Immunity **3**:283-299.

17. **Leitges, M., C. Schmedt, R. Guinamard, J. Davoust, S. Schaal, S. Stabel, and A. Tarakhovsky**. 1996. Immunodeficiency in protein kinase cbeta-deficient mice. Science **273**:788-791.

18. **Leitges, M., C. Schmedt, R. Guinamard, J. Davoust, S. Schaal, S. Stabel, and A. Tarakhovsky**. 1996. Immunodeficiency in protein kinase cbeta-deficient mice. Science **273**:788-791.

19. **Minegishi, Y., J. Rohrer, E. Coustan-Smith, H. M. Lederman, R. Pappu, D. Campana, A. C. Chan, and M. E. Conley** . 1999. An essential role for BLNK in human B cell development. Science **286**:1954-1957.

20. **Moriggl, R., D. J. Topham, S. Teglund, V. Sexl, C. McKay, D. Wang, A. Hoffmeyer, J. van Deursen, M. Y. Sangster, K. D. Bunting, G. C. Grosveld, and J. N. Ihle**. 1999. Stat5 is required for IL-2 induced cell cycle progression of peripheral T cells. Immunity **10**:249-259.

21. **Okkenhaug, K., A. Bilancio, G. Farjot, H. Priddle, S. Sancho, E. Peskett, W. Pearce, S. E. Meek, A. Salpekar, M. D. Waterfield, A. J. Smith, and B. Vanhaesebroeck**. 2002. Impaired B and T cell antigen receptor signaling in p110delta PI 3- kinase mutant mice. Science **297**:1031-1034.

22. **Pappu, R., A. M. Cheng, B. Li, Q. Gong, C. Chiu, N. Griffin, M. White, B. P. Sleckman, and A. C. Chan**. 1999. Requirement for B cell linker protein (BLNK) in B cell development. Science **286**:1949-1954.

23. **Rawlings, D. J., D. C. Saffran, S. Tsukada, D. A. Largaespada, J. C. Grimaldi, L. Cohen, R. N. Mohr, J. F. Bazan, M. Howard, N. G. Copeland, N. A. Jenkins, and O. N. Witte**. 1993. Mutation of unique region of Bruton's tyrosine kinase in immunodeficient XID mice. Science **261**:358-361.

24. **Rickert, R. C., K. Rajewsky, and J. Roes**. 1995. Impairment of T-cell-dependent B-cell responses and B-1 cell development in CD19-deficient mice. Nature **376**:352-355.

25. **Schaeffer, E. M., J. Debnath, G. Yap, D. McVicar, X. C. Liao, D. R. Littman, A. Sher, H. E. Varmus, M. J. Lenardo, and P. L. Schwartzberg**. 1999. Requirement for Tec kinases Rlk and Itk in T cell receptor signaling and immunity. Science **284**:638-641.

26. **Sun, Z., C. W. Arendt, W. Ellmeier, E. M. Schaeffer, M. J. Sunshine, L. Gandhi, J. Annes, D. Petrzilka, A. Kupfer, P. L. Schwartzberg, and D. R. Littman**. 2000. PKC-theta is required for TCR-induced NF-kappaB activation in mature but not immature T lymphocytes. Nature **404**:402-407.

27. **Suzuki, H., Y. Terauchi, M. Fujiwara, S. Aizawa, Y. Yazaki, T. Kadowaki, and S. Koyasu**. 1999. Xid-like immunodeficiency in mice with disruption of the p85alpha subunit of phosphoinositide 3-kinase. Science **283**:390-392.

28. **Tedford, K., L. Nitschke, I. Girkontaite, A. Charlesworth, G. Chan, V. Sakk, M. Barbacid, and K. D. Fischer**. 2001. Compensation between Vav-1 and Vav-2 in B cell development and antigen receptor signaling. Nat.Immunol. **2**:548-555.

29. **Teglund, S., C. McKay, E. Schuetz, J. van Deursen, D. Stravopodis, D. Wang, M. Brown, S. Bodner, G. Grosveld, and J. N. Ihle**. 1998. Stat5a and Stat5b proteins have essential and non-essential, or redundant, roles in cytokine responses. Cell **93**:841-850.

30. **Thomas, J. D., P. Sideras, C. I. Smith, I. Vorechovsky, V. Chapman, and W. E. Paul**. 1993. Colocalization of X-linked agammaglobulinemia and X-linked immunodeficiency genes. Science **261**:355-358.

31. **Toker, A. and L. Cantley**. 1997. Signalling through the lipid products of phosphoinositide-3-OH kinase. Nature **387**:673-676.

32. **Tsukada, S., D. C. Saffran, D. J. Rawlings, O. Parolini, R. C. Allen, I. Klisak, H. Kubagawa, T. Mohandas, S. Quan, J. W. Belmont, M. D. Cooper, M. E. Conley, and O. N. Witte**. 1993. Deficient expression of a B-cell cytoplasmic tyrosine kinase in human X-linked agammaglobulinemia. Cell **72**:279-290.

33. **Vanhaesebroeck, B., M. J. Welham, K. Kotani, R. Stein, P. H. Warne, M. J. Zvelebil, K. Higashi, S. Volinia, J. Downward, and M. D. Waterfield**. 1997. P110delta, a novel phosphoinositide 3-kinase in leukocytes. Proc.Natl Acad.Sci.U.S.A. **94**:4330-4335.

34. **Vetrie, D., I. Vorechovsky, P. Sideras, J. Holland, A. Davies, F. Flinter, L. Hammarstrom, C. Kinnon, R. Levinsky, M. Bobrow, C. I. E. Smith, and D. R. Bentley**. 1993. The gene involved in X-linked agammaglobulinaemia is a member of the src family of protein-tyrosine kinases [published erratum appears in Nature 1993 Jul 22;364(6435):362] [see comments]. Nature **361**:226-233.

35. **Wang, D., E. C. Boylin, Y. Minegishi, R. Wen, C. I. Smith, J. N. Ihle, and M. E. Conley**. 2001. Variations in the human phospholipase Cgamma2 gene in patients with B- cell defects of unknown etiology. Immunogenetics **53**:550-556.

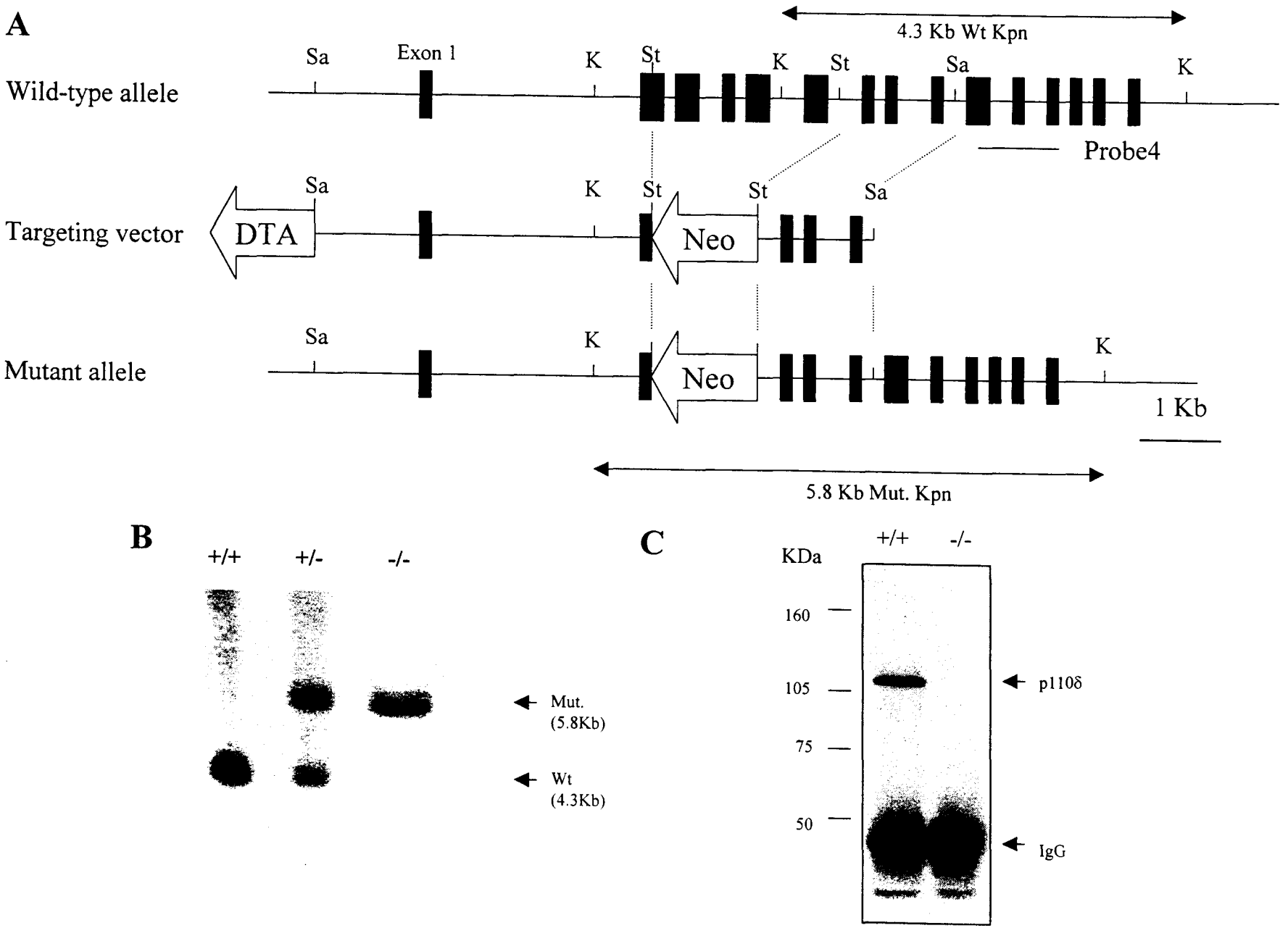
36. **Wang, D., J. Feng, R. Wen, J. C. Marine, M. Y. Sangster, E. Parganas, A. Hoffmeyer, C. W. Jackson, J. L. Cleveland, P. J. Murray, and J. N. Ihle**. 2000. Phospholipase Cgamma2 is essential in the functions of B cell and several Fc receptors. Immunity. **13**:25-35.

37. **Wang, D., D. Stravopodis, S. Teglund, J. Kitazawa, and J. N. Ihle**. 1996. Naturally occurring dominant negative variants of Stat5. Mol.Cell.Biol. **16**:6141-6148.

38. **Yamazaki, T., K. Takeda, K. Gotoh, H. Takeshima, S. Akira, and T. Kurosaki**. 2002. Essential immunoregulatory role for BCAP in B cell development and function. J.Exp.Med. **195**:535-545.

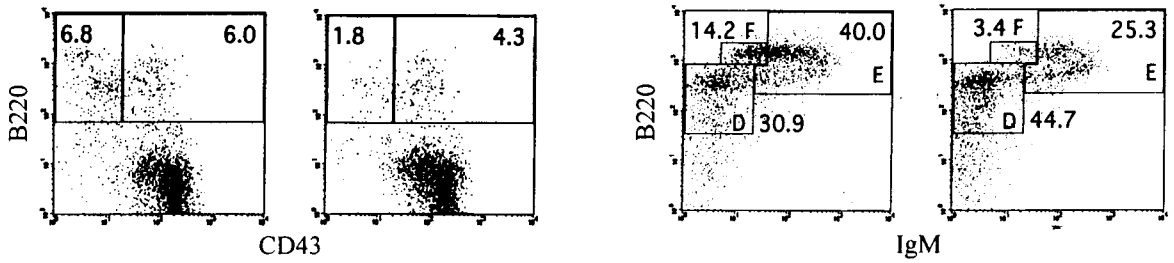
39. **Zang, H., K. Sato, H. Nakajima, C. McKay, P. A. Ney, and J. N. Ihle**. 2001. The distal region and receptor tyrosines of the Epo receptor are non- essential for in vivo erythropoiesis. EMBO J. **20**:3156-3166.

40. **Zhang, R., F. W. Alt, L. Davidson, S. H. Orkin, and W. Swat**. 1995. Defective signalling through the T- and B-cell antigen receptors in lymphoid cells lacking the vav proto-oncogene. Nature **374**:470-473.

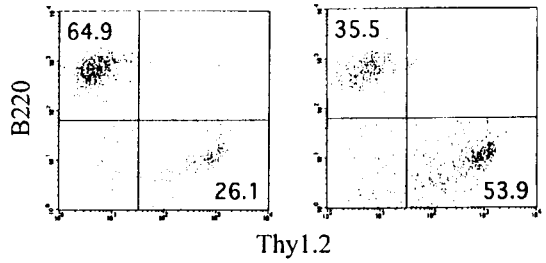

A
4.3 Kb Wt Kpn
Sa
Exon 1
K
St
K
St
Sa
K
Wild-type allele
Probe4
Sa
K
St
St
Sa
Targeting vector
DTA
Neo
Sa
K
K
Mutant allele
Neo
1 Kb
5.8 Kb Mut. Kpn
B
+/+
+/-
-/-
Mut.
(5.8Kb)
Wt
(4.3Kb)
C
+/+
-/-
KDa
160
105
75
50
p110δ
IgG

+/+ -/- +/+ -/-

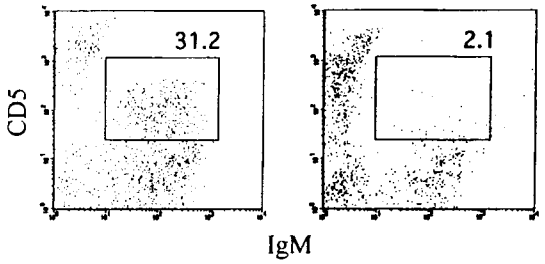
A. Bone Marrow



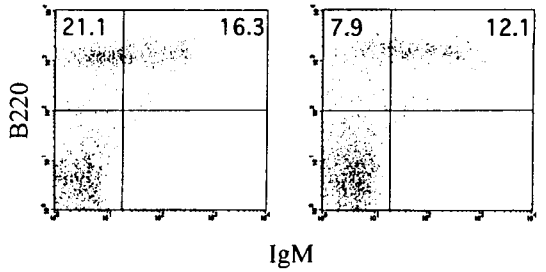
B. Spleen



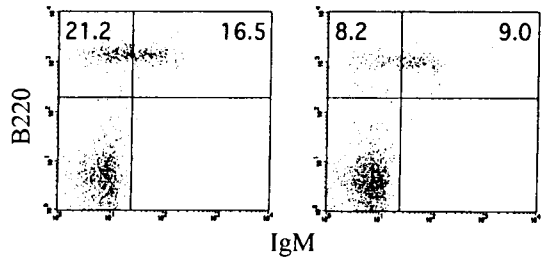
C. Peritoneum



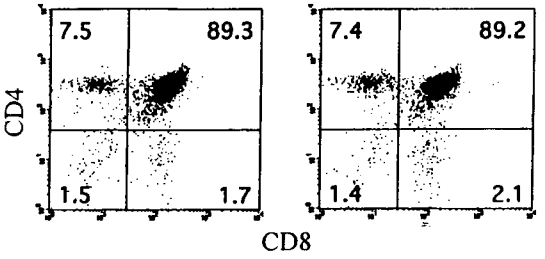
D. Spleen

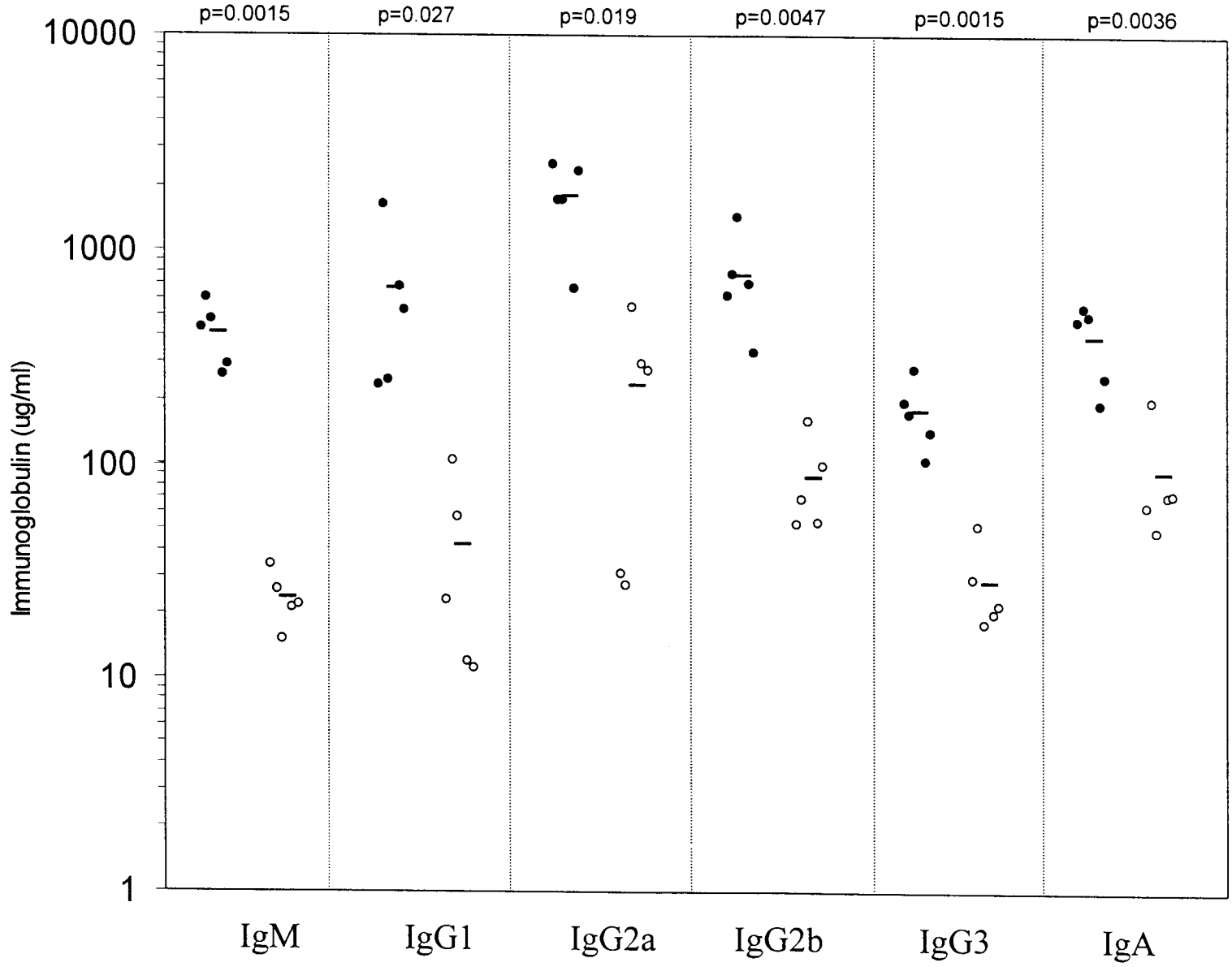


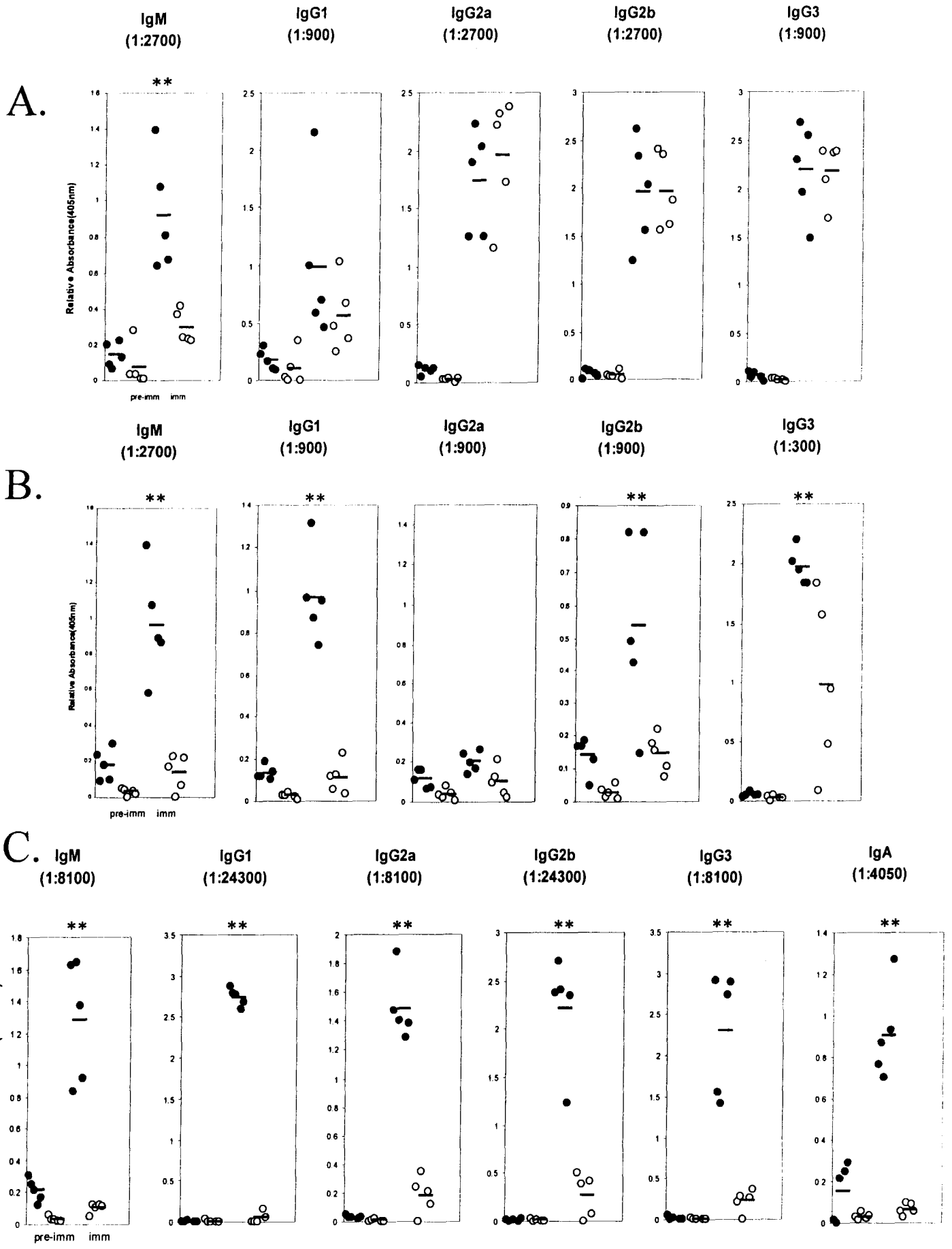
E. Lymph Node

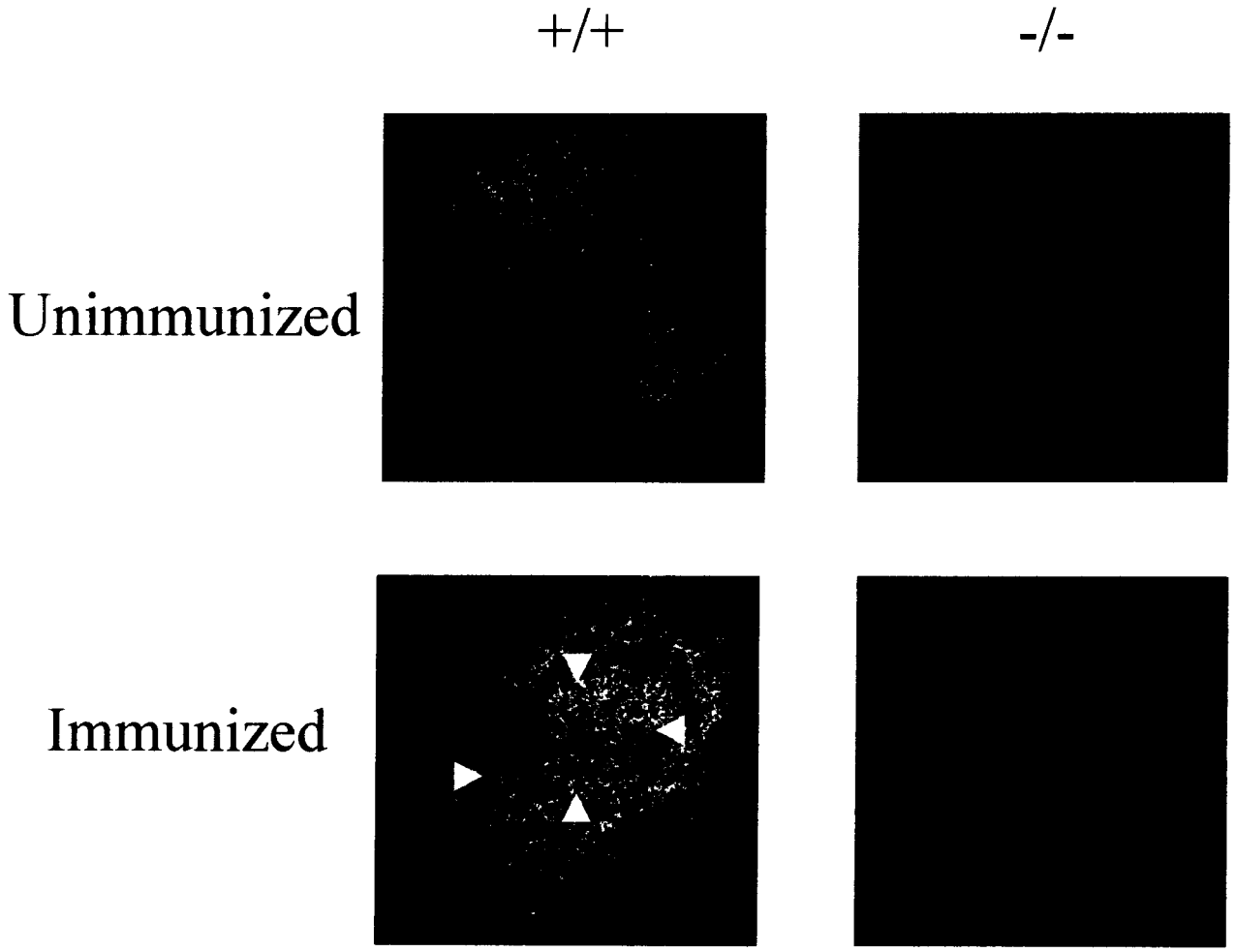


F. Thymus

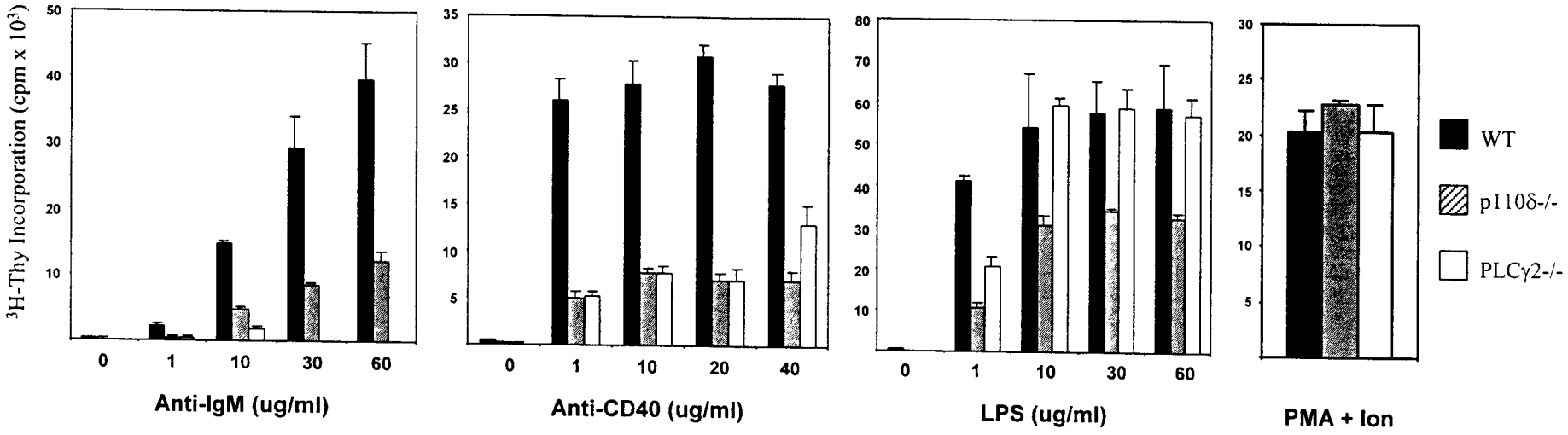



p=0.0015
p=0.027
p=0.019
p=0.0047
p=0.0015
p=0.0036
10000
1000
100
10
1
Immunoglobulin (ug/ml)
IgM
IgG1
IgG2a
IgG2b
IgG3
IgA

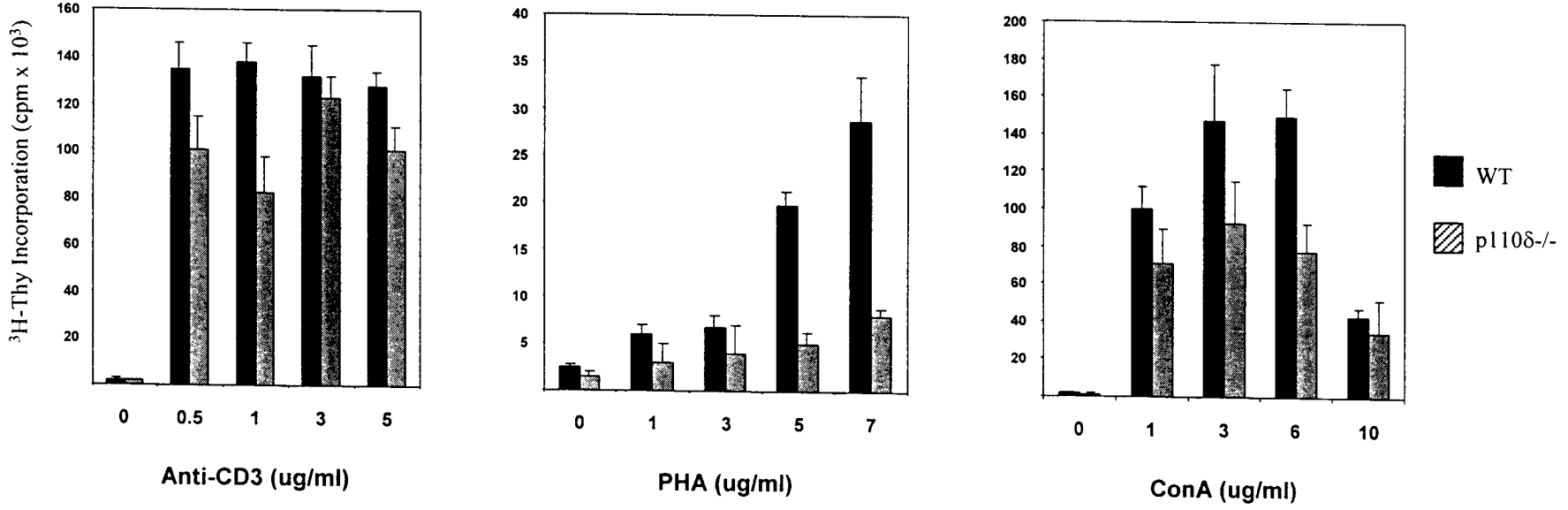

A.
IgM (1:2700)
IgG1 (1:900)
IgG2a (1:2700)
IgG2b (1:2700)
IgG3 (1:900)
**
Relative Absorbance(405nm)
pre-imm
imm
B.
IgM (1:2700)
IgG1 (1:900)
IgG2a (1:900)
IgG2b (1:900)
IgG3 (1:300)
**
Relative Absorbance(405nm)
pre-imm
imm
C.
IgM (1:8100)
IgG1 (1:24300)
IgG2a (1:8100)
IgG2b (1:24300)
IgG3 (1:8100)
IgA (1:4050)
**
Relative Absorbance (405nm)
pre-imm
imm

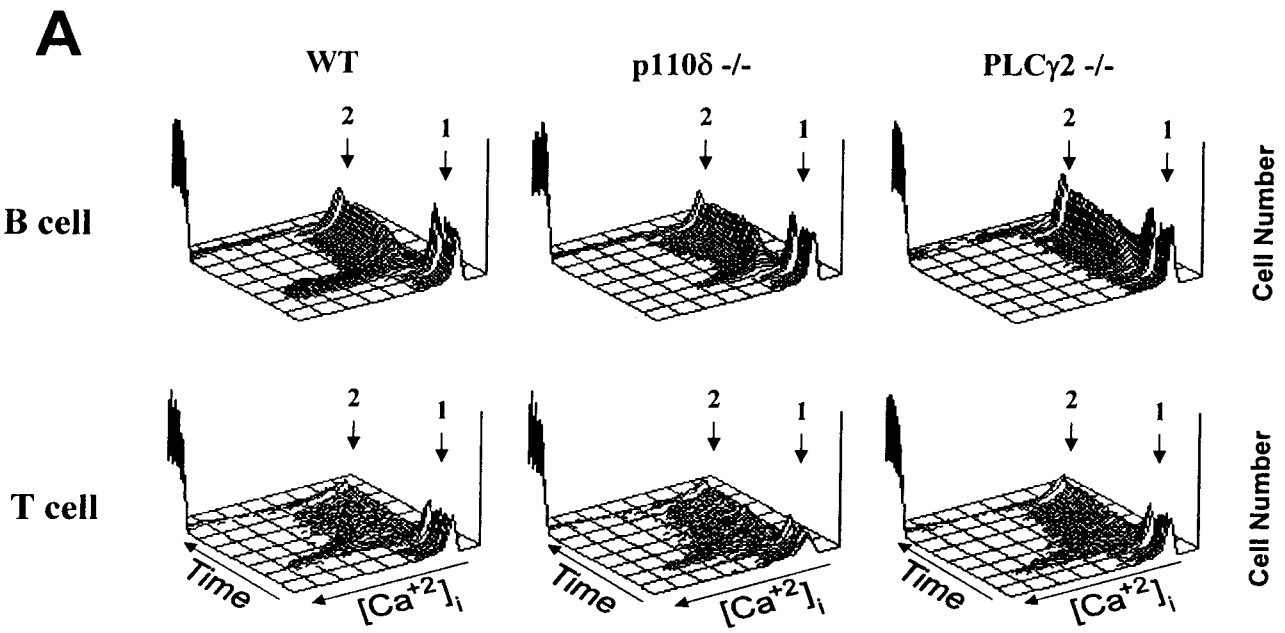

+/+
-/-
Unimmunized
Immunized

A


3H-Thy Incorporation (cpm x 10³)
Anti-IgM (ug/ml)
Anti-CD40 (ug/ml)
LPS (ug/ml)
PMA + Ion
WT
p110δ-/-
PLCγ2-/-

B


3H-Thy Incorporation (cpm x 10³)
Anti-CD3 (ug/ml)
PHA (ug/ml)
ConA (ug/ml)
WT
p110δ-/-


A
WT
p110δ -/-
PLCγ2 -/-
B cell
T cell
2
1
Cell Number
Time
[Ca+2]i

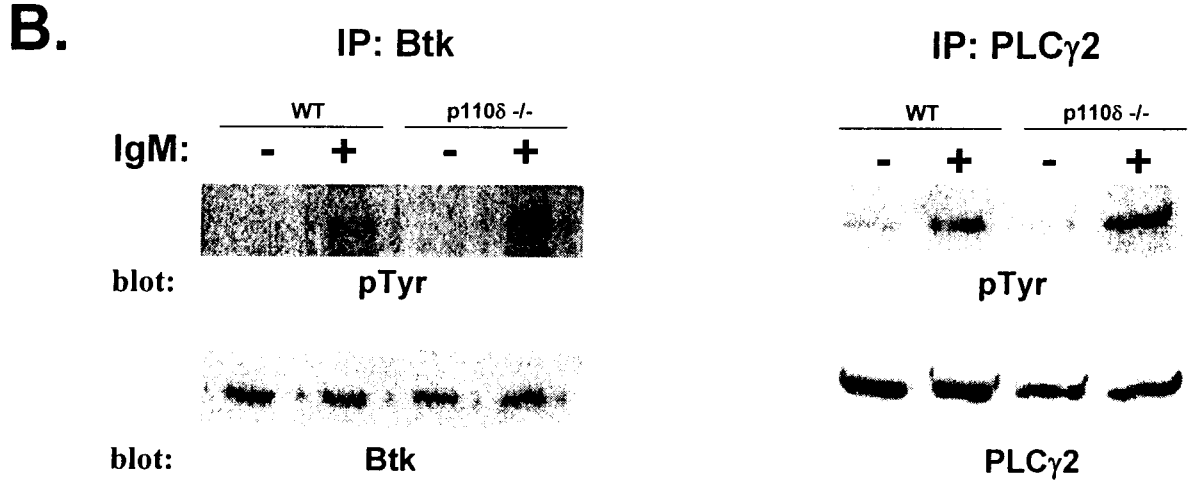

B.
IP: Btk
IP: PLCγ2
WT
p110δ -/-
IgM:
-
+
blot:
pTyr
Btk
PLCγ2

Table 1.

In Vitro Colony Formation by bone marrow hematopoietic progenitors from wildtype(+/+) and p110δ-deficient (–/–) mice in response to various cytokines

Cytokine Colony Type	Epo+IL-3 BFU-E	IL-3 CFU-Mix	IL-5 CFU-Eos	IL-7 Pre-B	Tpo CFU-Meg	G-CSF CFU-G	GM-CSF CFU-GM	CSF-1 CFU-M
(+/+)	3.3±0.8	24±10	12.2±2.5	52±11	2.6±0.6	74±15	347±30	351±63
(-/-)	2.6±0.7	20±11	7.3±1.6	47±13	2.7±0.7	72±8	361±21	406±39

Peripheral Blood Cell Numbers in Wild-Type and p110δ-Deficient Mice (n=11)

Parameter	Total WBC (/μl)	Neutrophil (%)	Lymphocyte(%)	Monocyte(%)	Eosinophils(%)	Basophils(%)	Erythrocytes(x10^6/μl)	Platelets(x10^3/μl)
(+/+)	9093±2849	23.7±12.6	71.8±12.6	3.7±1.2	0.6±0.3	0.2±0.2	8.38±0.78	1245.8±143.4
(-/-)	11206±2729	23.8±9.4	70.9±9.3	4.2±2.4	0.8±0.5	0.3±0.2	8.54±0.64	1589.2±562.2

Lymphocyte Populations in Wild-Type and p110δ-Deficient Mice (in Millions)

Spleen (n=8)

	Total Cells	Total B cells(%) (B220$^+$)	IgM$^+$ (%)	CD4$^+$ T (%)	CD8$^+$ T (%)
(+/+)	47.8±13.0	42.3±5.0	27.1±4.6	19.1±6.1	11.6±1.1
(–/–)	30.4±11.5	32.1±11.4	20.3±7.3	21.7±5.7	12.5±3.3

Peritoneum (n=8)

	Total Cells	B lineage (%) (IgM$^+$)	B1a (%) (CD5$^+$IgM$^+$)
(+/+)	3.56±0.85	31.7±6.2	14.0±3.6
(–/–)	1.11±0.28	2.4±0.6	0.4±0.1

Lymph nodes (n=6)

	Total Cells	Total B (%) (B220$^+$)	CD4$^+$ T (%)	CD8$^+$ T (%)
(+/+)	9.80±3.59	31.6±4.5	38.7±10.2	25.5±3.7
(–/–)	6.07±2.14	19.0±3.2	45.6± 4.4	27.7±0.8

The phenotype was determined by flow cytometry. The numbers and percentages of each group of cells were determined for each mouse, and the mean value and standard deviation were calculated.