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CUTTING EDGE

Cutting Edge: Differential Roles for Phosphoinositide 3-Kinases, p110γ and p110δ, in Lymphocyte Chemotaxis and Homing[1]

Karin Reif,[2]* *Klaus Okkenhaug,*[†] *Takehiko Sasaki,*[‡] *Joseph M. Penninger,*[§] *Bart Vanhaesebroeck,*[¶] *and Jason G. Cyster*[3]*

***Despite the established role for PI3Ks in cell migration, the PI3Ks involved in lymphocyte chemotaxis are poorly defined. In this study, we report that p110γ-deficient T cells, but not B cells, show reduced chemotactic responses to the lymphoid chemokines, CCL19, CCL21, and CXCL12. As B cell and T cell chemotactic responses were both sensitive to the general PI3K inhibitors, wortmannin (WMN) and LY294002, we explored whether B cell responses were affected in mice lacking p110δ, a major PI3K isoform in lymphocytes. B cells deficient in p110δ showed diminished chemotactic responses, especially to CXCL13. Adoptive transfer experiments with WMN-treated wild-type B cells and with p110δ-deficient B cells revealed diminished homing to Peyer's patches and splenic white pulp cords. WMN selectively inhibited CXCR5-dependent B cell homing to Peyer's patches. These observations establish that p110γ and p110δ function in lymphocyte chemotaxis, and show differential roles for PI3K family members in B and T cell migration. The Journal of Immunology,* 2004, 173: 2236–2240.**

The process of immune surveillance depends on chemokine-directed migration of lymphocytes into and within lymphoid organs. Chemokines important for these events include: CXCL13 (BLC), a ligand for CXCR5, that guides B cells to lymphoid follicles; CCL19 (ELC) and CCL21 (SLC, 6Ckine), ligands for CCR7, that guide cells to lymphoid organ T zones; and CXCL12 (SDF1), the ligand for CXCR4, that has a broad spectrum of functions, including roles in B cell homing (1, 2).

Efforts to dissect the mechanisms of eukaryotic cell chemotaxis toward attractants acting via G protein-coupled receptors, principally performed in neutrophils and in the slime mold *Dictyostelium discoideum*, have established an important role for PI3Ks (3, 4). PI3Ks function to relay the chemoattractant gradient to the inside of the cell, generating high concentrations of phosphatidylinositol 3,4,5-trisphosphate at the leading edge (3, 4). Consistent with this, neutrophils and macrophages from mice lacking p110γ, a class IB PI3K that couples to heterotrimeric G proteins, are poorly responsive to chemoattractants (reviewed in Refs. 5, 6). By contrast, lymphocyte migration was not reported to be affected in these animals. However, studies with pharmacological inhibitors of PI3K, wortmannin (WMN)[4] and LY294002, and with dominant-negative forms of PI3K have provided evidence that PI3K contributes to lymphocyte chemotactic responses (2).

In this study, we examined the PI3K requirement for naive lymphocyte responses to lymphoid chemokines. We show that p110γ functions in T cell chemotaxis and that a class IA PI3K, p110δ, is required for maximal B cell chemotaxis to CXCL13 and for B cell homing to Peyer's patches (PPs) and to the splenic white pulp.

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Materials and Methods

Mice and bone marrow chimeras

CXCR5$^{-/-}$ mice and Ig^{HEL}-transgenic mice of the MD4 line were on a C57BL/6 (B6) background (7). Igh^{a}Thy1^{a}Gpi1^a B6 mice (termed Igh^a) were from The Jackson Laboratory (Bar Harbor, ME). B6 or B6-CD45.1 (Ly5.2) mice were from Charles River Laboratories (Wilmington, MA) or a University of California colony (San Francisco, CA). The mice deficient in p110γ (8) and p110δ (9) were three or six generations backcrossed to B6, respectively. To provide p110γ$^{-/-}$ or p110δ$^{D910A/D910A}$ cells for homing and migration assays, lethally irradiated B6-Ly5.2 mice were reconstituted as described (10) with p110γ$^{-/-}$, p110δ$^{D910A/D910A}$, or littermate control (+/+ or +/−) bone marrow.

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[4] Abbreviations used in this paper: WMN, wortmannin; LN, lymph node; PP, Peyer's patch; HEL, hen egg lysozyme; HEV, high endothelial venule; CMTMR, 5- (and 6-)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine.

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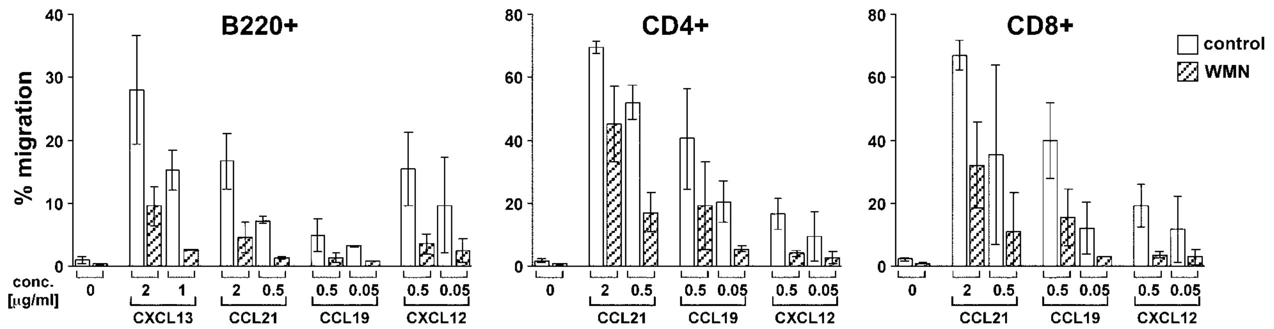

FIGURE 1. Effect of WMN treatment on B and T cell chemotactic responses to lymphoid chemokines. The panels show percentage of input cells that migrated in response to the indicated concentration of chemokine in the presence (WMN, ▨) or absence (control, □) of 100 nM WMN. Bars show average (± SD) for two to six experiments where each assay was performed in duplicate.

Adoptive transfer assays

Donor splenocytes were prepared at 10^6/ml (10). For WMN experiments followed by histological examinations, B6 or Ig^{HEL}-transgenic splenocytes were labeled for 10 min at 37°C with 1.4 μM or 0.14 μM CFSE (Molecular Probes, Eugene, OR), cotreated with either 200 nM WMN (Sigma-Aldrich, St. Louis, MO) or carrier, and washed three times in RPMI 1640 plus 10% FBS. Carrier- and WMN-treated cells were transferred alone (3×10^7) or after mixing (4×10^7), and injected i.v. into Igh^a B6 recipients. Spleens were harvested and analyzed as described (10). For WMN treatment experiments followed by flow cytometry, $CXCR5^{-/-}$ or wild-type splenocytes were treated for 30 min at 37°C with 200 nM WMN or carrier, and labeled at the same time with either 1 μM CFSE or 10 μM CellTracker Orange 5- (and 6-)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine (CMTMR) (Molecular Probes), washed, and mixed at a 1:1 ratio, and 3×10^7 cells were transferred. Splenocytes from $p110\delta^{D910A/D910A}$, $p110\gamma^{-/-}$, or littermate control chimeras labeled with 1.4 μM CFSE and from Ig^{HEL}-transgenic mice labeled with 0.14 μM CFSE were mixed at a 3:1 ratio and 4×10^7 total cells were transferred as above. The absolute frequency of p110 knockout or wild-type B cells to cotransferred control B cells in any given tissue was determined by dividing the absolute number of recovered cells by the absolute number of cotransferred Ig^{HEL} control cells. Blood sample numbers are based on a total volume of 2 ml of blood per mouse. The frequency was corrected for differences in the input ratio of p110 knockout or wild-type cells and Ig^{HEL} control cells, and transfer data were plotted as the number of cells of each type recovered in each recipient per 10^6 transferred cells as described (11). The absolute number of WMN-treated vs carrier-treated $CXCR5^{-/-}$ or $CXCR5^{+/+}$ B cells were determined as for the p110 transfer experiments, except that numbers were not normalized to a cotransferred control cell population because carrier- or WMN-treated cells were cotransferred into the same recipient mouse. All statistical analyses were conducted using a two-tailed, two sample, unequal variance (unpaired) Student's *t* test.

Chemotaxis, flow cytometry, and immunohistochemistry

Chemotaxis assays were with splenocytes as described (10) using uncoated 5-μm transwell filters (Corning Costar, Acton, MA) and chemokines from R&D Systems (Minneapolis, MN). In some experiments, cells were pretreated for 10 min with the indicated concentrations of WMN, with 5 μM LY294002 (Calbiochem, San Diego, CA) or carrier before the assay. Flow cytometry for chemokine receptors and immunohistochemical analysis was as described (10, 12). Cell enumeration was performed by locating well-defined mucosal addressin cell adhesion molecule-1-stained (BD Pharmingen, San Diego, CA) white pulp cords from serial sections costained with either IgM^b and IgD^b or hen egg lysozyme (HEL) to detect transferred B6 B cells or Ig^{HEL}-transgenic (transgenic for IgM and IgD with Igh^a) B cells, respectively. Cotransferred B cells were enumerated within three serial white pulp cord sections per mouse.

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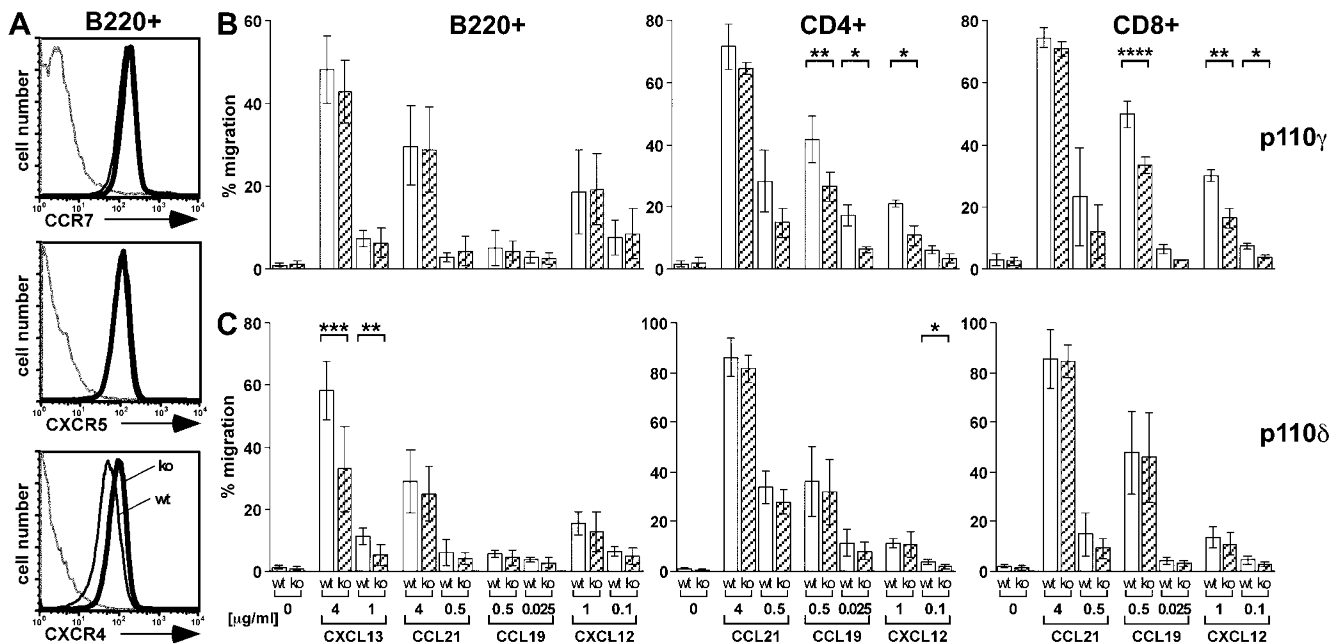

FIGURE 2. Chemokine receptor expression and chemotactic responses of B and T lymphocytes deficient in the p110γ or p110δ catalytic subunit of PI3K. *A*, Flow cytometric analysis of chemokine receptor levels on $B220^+$ B cells deficient in p110δ (ko, thick black line) and wild-type cells (wt, thin black line). Staining control is indicated in a thin gray line. *B* and *C*, Chemotaxis of B lymphocytes ($B220^+$), $CD4^+$ or $CD8^+$ T cells deficient (ko, ▨) from p110γ (*B*) or p110δ (*C*) deficient mice compared with cells from littermate controls (wt, □). The percentage of the input cell population that responded to the chemokine indicated is shown. Bars indicate average (± SD) for a minimum of three to six mice per group. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; ****, $p < 0.0005$ in Student's *t* test.

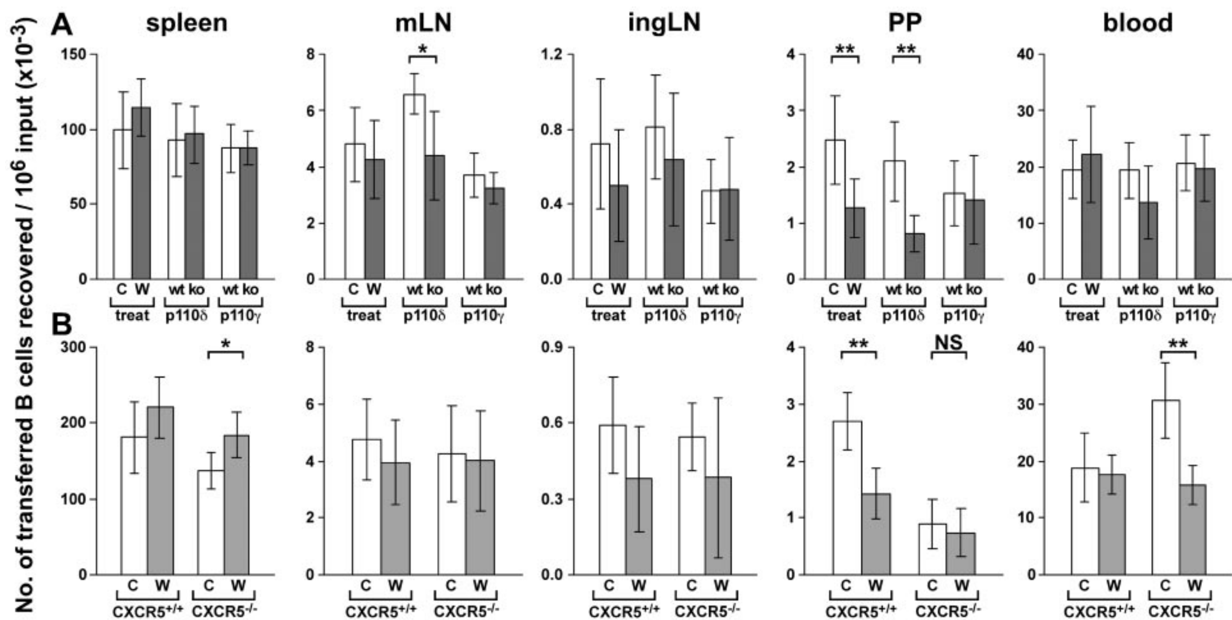

FIGURE 3. Defective homing of p110δ-deficient or WMN-treated B cells to PPs. *A*, CFSE-labeled (1.4 μM) Igh[b] splenocytes from p110$\delta^{D910A/D910A}$ (p110$\delta^{-/-}$), p110$\delta^{+/+}$, p110$\gamma^{-/-}$, or p110$\gamma^{+/-}$ chimeras were cotransferred with CFSE-labeled (0.14 μM) Ig[HEL]-transgenic B cells into Igh[a] congenic mice; or CFSE- or CMTMR-labeled splenocytes treated in vitro before transfer with WMN (W) were cotransferred respectively with CMTMR- or CFSE-labeled carrier-treated (C) splenocytes into recipient mice. After 1.5 h, blood and lymphoid tissues were examined for the frequency of transferred cells as described in *Materials and Methods*. Spleen numbers correspond to half of the spleen. *B*, Transfer experiments were performed as in *A* for the WMN- and carrier-treated group, except that CXCR5$^{-/-}$ and CXCR5$^{+/+}$ donor cells were used. Absolute numbers of recovered WMN- (W) or carrier-treated (C) B cells in blood and secondary lymphoid tissues are shown. The average (± SD) for 5–11 recipients per group is shown. The data are representative of two to four experiments using chimeras made from at least two different knockout and wild-type bone marrows. mLN, mesenteric LN; ingLN, inginual LN. *, $p < 0.005$; **, $p < 0.0005$ in Student's *t* test.

Results and Discussion

PI3K dependence of lymphocyte chemotaxis to lymphoid chemokines

To examine the PI3K dependence of naive lymphocyte responses to lymphoid chemokines, spleen cells were incubated with 100 nM WMN and subjected to transwell chemotaxis assays. B cell responses to each of the lymphoid chemokines were inhibited between 65 and 85% by WMN treatment, with the inhibitory effect being most prominent at the lower chemokine doses (Fig. 1). By contrast, T cell responses were more resistant to the inhibitor, especially the responses to the efficacious T cell attractant, CCL21, where the maximal inhibitory effect was 35–65% for CD4 cells (Fig. 1). Increasing the WMN concentration to 250 nM led to only a slight increase in inhibition, and there was no further increase when 500 or 1000 nM concentrations were used (data not shown). Similar findings were obtained using a second PI3K inhibitor, LY294002 (data not shown). As expected from its covalent mechanism of action, WMN inhibitory effects were still evident at ~75% of the initial level 6 h after the inhibitor had been removed from the cultures (data not shown). These findings extend previous studies showing that lymphocyte responses to CCL5 (RANTES) and CXCL12 can be reduced or inhibited by WMN treatment (reviewed in Ref. 2), and they indicate that different lymphoid chemokine receptors have differing dependence on PI3K for mediating chemotactic responses. The basis for the incomplete block in migration is unclear, but may indicate the involvement of PI3K-independent pathways of chemotaxis in lymphocytes (1, 2, 4).

Role of p110γ in T cell migration and p110δ in B cell migration

Although previous studies of p110γ-deficient mice had not indicated a role for this PI3K isoform in lymphocyte migration, our findings above led us to test whether p110γ-deficient lymphocytes showed partial defects in chemotactic responsiveness. Flow cytometric analysis revealed that chemokine receptor levels on p110γ-deficient B and T cells were equivalent to wild-type controls (data not shown). However, the migration of p110γ-deficient CD4 and CD8 T cells to CCL19 and CXCL12, and to low doses of CCL21, was reduced by 30–65% (Fig. 2*B*). By contrast, B cell migration to chemokines was not significantly affected by p110γ deficiency (Fig. 2*B*).

As B cell chemotactic responses were equally or more sensitive than T cell responses to the effects of WMN (Fig. 1), we explored the possibility that p110δ, a major PI3K isoform in lymphocytes (5, 6), was involved in B cell chemotaxis. PI3K p110δ-deficient B cells had normal amounts of CCR7 and CXCR5 but exhibited slightly elevated CXCR4 levels (Fig. 2*A*). Elevated CXCR4 levels were also seen in B cells following WMN treatment (data not shown). CCR7 and CXCR4 levels on p110δ-deficient T cells were equivalent to the wild-type controls. Strikingly, p110δ-deficient B cells showed a defect in B cell chemotaxis to all concentrations of CXCL13 (Fig. 2*C*). Responses to the CCR7 and CXCR4 ligands were less affected, but slight reductions in migration were detected at the lower CCL19 and CXCL12 concentrations (Fig. 2*C*). T cell responses were unaffected by p110δ deficiency, with the exception that responses to low doses of CXCL12 were reduced (Fig. 2*C*).

The ability of p110δ to function downstream of chemokine receptors in lymphocyte chemotactic responses is consistent with the finding that dominant-negative p85 reduces chemotaxis to CXCL12 (13, 14). Recently, a small molecule inhibitor of p110δ was identified and shown to reduce neutrophil chemotaxis to fMLP in under agarose assays, with ICAM1 as an

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adhesive substrate (15). The mechanism of p110δ coupling to chemokine receptors is unclear, but because tyrosine kinases can be activated by chemoattractant receptors, the conventional pathway of class IA PI3K activation may be involved (4). Consistent with this scenario, microinjection of p110δ neutralizing Abs inhibited tyrosine-kinase receptor-mediated migratory responses of a macrophage cell line to CSF-1 (16).

Requirement of p110δ for B cell homing

To explore the role of PI3K in B cell homing in vivo, we transferred wild-type B cells that had been treated in vitro with WMN, or cells from p110γ- or p110δ-deficient mice, into wild-type recipients for 1.5 h. These experiments revealed reduced homing to PP of both WMN-treated and p110δ-deficient B cells, but normal homing of p110γ-deficient B cells (Fig. 3*A*). p110δ deficiency also caused a reduction in homing to mesenteric lymph node (LN), although this effect was weaker than the effect in PP and it was not evident with WMN-treated cells. Entry into peripheral LN and to the spleen was not reduced by WMN treatment or by p110δ or p110γ deficiency (Fig. 3*A*). Analysis of lymphoid organs in p110δ-deficient mice revealed that B cell numbers were severely diminished in PP ($1.55 \pm 1.2 \times 10^6$, $n = 4$, in wild-type vs $0.07 \pm 0.01 \times 10^6$, $n = 5$, in p110δ-deficient) whereas numbers in LNs and spleen were not significantly reduced. This finding is consistent with p110δ playing a nonredundant role in B cell accumulation in PP.

As p110δ deficiency strongly affected CXCL13 responses in vitro, and other studies have shown that entry to PP but not peripheral LN depends partially on CXCR5 (11), we tested whether the PI3K requirement for B cell entry to PP was due to PI3K acting downstream of CXCR5. For these experiments, cells from wild-type or CXCR5-deficient mice were treated with WMN or with the carrier, and then transferred to wild-type recipients. Analysis 1.5 h later revealed that homing of CXCR5-deficient B cells to PP was reduced ~65% (Fig. 3*B*) as expected (11). However, WMN treatment did not cause a further reduction in the PP homing of the CXCR5-deficient cells, in contrast to its inhibitory effect on the homing of wild-type cells (Fig. 3*B*). Therefore, we conclude that p110δ has a selective role in B cell entry to PP via CXCL13$^+$ high endothelial venules (HEV). Although WMN treatment was not found to affect lymphocyte attachment to PP HEV in a previous study (17), it was not indicated whether T or B lymphocytes were being analyzed and it seems possible that the HEV under investigation were the larger CXCL13-negative vessels present in the T zone. Alternatively, the role of p110δ may be restricted to a CXCL13-dependent step downstream of attachment but required for B cell accumulation within the organ.

When the distribution of WMN-treated B cells within the spleen was examined 1.5 h after transfer, a strong reduction was observed in B cell entry to splenic white pulp cords (Fig. 4, *A* (*upper panels*) and *B*). Reduced white pulp entry was also observed for p110δ-deficient cells, whereas p110γ deficiency had no effect on entry (Fig. 4*B*). B cell entry into the splenic white pulp cords depends on CXCR5, and to a lesser degree on CCR7 (18) and on the integrins $\alpha_L\beta_2$ and $\alpha_4\beta_1$ (12). Therefore, the PI3K requirement during B cell entry to white pulp cords may reflect the role of p110δ in CXCR5-mediated chemotaxis or possibly a role in CXCR5-mediated integrin activation.

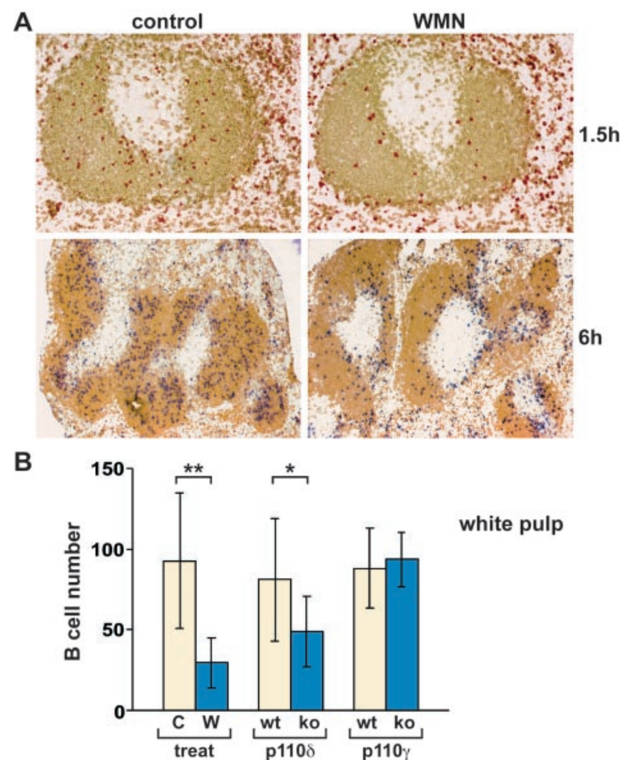

FIGURE 4. PI3K functions in B cell entry and localization in splenic white pulp cords. *A*, Effect of WMN pretreatment on B cell distribution in the spleen. *Upper panels*, Adjacent spleen sections of Igh^a B6 mice that had received a mixture of WMN-treated Ig^{HEL}-transgenic B cells (WMN) and carrier-treated Igh^b B cells (control) 1.5 h before. Transferred Ig^{HEL} B cells were detected by staining with HEL (red, *upper right panel*) and transferred Igh^b B cells were stained for IgM^b plus IgD^b (red, *upper left panel*). Original magnification, ×10. *Lower panels*, Distribution of Ig^{HEL}-transgenic B cells preincubated with carrier (control) or WMN at 6 h after transfer to B6 recipient mice. Spleen sections were stained with HEL (blue) to detect Ig^{HEL} transgenic B cells. B cell follicles (B220$^+$) are stained brown. Original magnification, ×5. *B*, Enumeration of transferred p110δ-, p110γ-deficient, or 200 nM WMN-treated B cell numbers in splenic white pulp cords compared with littermate control or carrier-treated B cells (C) 1.5 h after transfer. For WMN-treated cells, in half the transfers the WMN-treated cells were the Ig^{HEL}-transgenic cells, and in half they were the nontransgenic Igh^b cells. Transfers were normalized to total numbers of input cells as described in *Materials and Methods*. Bars show average (± SD) for two to four experiments with five to seven recipient mice per group. *, $p < 0.005$; **, $p < 0.00005$ in Student's *t* test.

We also examined whether PI3K function was important for B cell localization within the splenic white pulp by analyzing sections from mice 6 h after transfer of WMN-treated cells. By this time point, many WMN-treated B cells had entered the white pulp. However, these cells were largely excluded from lymphoid follicles and instead accumulated at the boundary of the B and T zones (Fig. 4*A*, *lower panels*). This might be explained by the greater sensitivity of CXCR5 to inhibition by WMN treatment compared with CCR7 (Fig. 1), because slight reductions in CXCR5 expression have been found to shift the balance of chemokine responsiveness sufficiently to promote B cell positioning in this location (7, 10).

In summary, we establish differential roles for p110γ and p110δ in T and B cell chemotaxis, demonstrating a particularly prominent role for p110δ in B cell responses to CXCL13. Differential PI3K usage by different chemokine receptors and different cell types is likely to be important in allowing cells to

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appropriately interpret complex chemokine environments, such as those that exist within lymphoid organs.

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