p110γ and p110δ isoforms of phosphoinositide 3-kinase differentially regulate natural killer cell migration in health and disease

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The mechanisms that regulate NK cell trafficking are unclear. Phosphoinositide-3 kinases (PI3K) control cell motility and the p110γ and p110δ isoforms are mostly expressed in leukocytes, where they transduce signals downstream of G protein coupled receptors (GPCR) or tyrosine kinase receptors, respectively. Here, we set out to determine the relative contribution of p110γ and p110δ to NK cell migration in mice. Using a combination of single-cell imaging analysis of transgenic cells reporting on PI3K activity in real time and small molecule inhibitors of p110γ and p110δ, we show here that the tyrosine-kinase coupled p110δ is linked to GPCR signaling and, depending on the GPCR, may even be preferentially activated over p110γ. Using gene-targeted mice, we showed that both isoforms were essential for NK cell chemotaxis to CXCL12 and to CCL3 and, in vivo, for normal NK cell migration during pregnancy and to the inflamed peritoneum. By contrast, only p110δ was indispensable for chemotaxis to S1P and CXCL10 and for NK cell distribution throughout lymphoid and nonlymphoid tissues and for extravasation to tumors. These results implicate p110δ downstream of GPCRs in NK cells and highlight its nonredundant role as a key regulator of NK cell trafficking in health and disease.

NK cells develop primarily in the bone marrow, thymus and lymph nodes and are distributed in lymphoid tissues, and in many organs throughout the body, including uterus, liver, lungs, intestine and peritoneum. Resident and recruited NK cells play important roles in reproduction and in immunological surveillance (1–3), and therefore migration is an important aspect of NK cell biology. Lymphocyte migration is organized by selectins, chemokines and integrins (4) and although some of the factors regulating NK cell trafficking are emerging, the picture is incomplete (1, 5). For example, L-selectin is indispensable for NK cell migration to lymph nodes (6), and some chemokines and chemokine receptors have been identified that orchestrate NK cell egression from the bone marrow (7), migration to the decidua (2), sites of inflammation (3), activated lymph nodes (8), and tumors (9–10). Recently, the GPCR sphingosine 1-phosphate receptor 5 (SIP5) has been identified as a key regulator of NK cell distribution (11). These studies indicate that the mechanisms that regulate NK cell trafficking are distinct from those that regulate the trafficking of other lymphocytes and therefore, in view of the promise of pharmacological inhibitors of leukocyte trafficking to treat disease (12), it is important to know how target molecules regulate trafficking of all leukocyte subsets. Phosphoinositide 3-kinases (PI3Ks) generate lipid second messengers that control disparate aspects of cell biology, including growth, survival, metabolism and motility (13) and are prominent pharmacological targets (14, 15). Class I PI3Ks are the most thoroughly studied in mammalian cells and are composed of 2 subclasses, which include 4 catalytic isoforms, subdivided in class IA (p110α, -β, and -δ) and class IB (p110γ). P110α and p110β are ubiquitously expressed, whereas p110γ and p110δ are mainly expressed in leukocytes (16). Small molecule inhibitors of PI3K isoforms have entered clinical trials to treat cancer and inflammation (14) and some of the expected therapeutic effects are through inhibition of leukocyte migration (12). PI3K-dependent leukocyte migration is controlled by p110γ through association to GPCRs, including chemokine receptors (17, 18), whereas p110α and p110δ regulate various aspects of cell migration in response to tyrosine kinase-linked receptors (19–23). These data fit with a model according to which class IA PI3K p110α, -β, and -δ isoforms are generally activated downstream of receptors signaling through tyrosine kinases, whereas the class IB PI3K p110γ isoform transduces signals downstream of GPCRs (18). However, p110β does signal downstream of GPCRs (24) and, in few cases, p110δ has been shown to regulate GPCR-dependent chemotaxis (22, 25). Thus, accumulating evidence suggests that the model may have to be reassessed. Moreover, >1 isoform may be necessary to provide sufficient signals for full activation of the PI3K pathway downstream of a given receptor. Indeed, p110γ and p110δ synergize during development of thymocytes (26, 27), differentiation, cytotoxicity and receptor-mediated IFN-γ production in NK cells (28, 29) and ROS production in neutrophils (30).

Here, we show that p110γ and p110δ were necessary for NK cell migration to inflamed tissues and to the uterus during early pregnancy in vivo and for chemotaxis to CXCL12 and CCL3 in vitro. By contrast, p110δ was selectively required for NK cell distribution in steady-state and for extravasation to transplanted lymphomas in vivo and for chemotaxis to S1P and CXCL10 in vitro. Activation of the PI3K pathway downstream of CXCR4 in response to CXCL12 depended not only on p110γ, but also on p110δ, directly implicating p110δ in the signaling downstream of a GPCR. These results identify differential roles for p110γ and p110δ in NK cell trafficking and highlight p110δ as a specific, nonredundant regulator of NK cell recirculation and migration to lymphoma.

Results

Differential Requirement for p110γ and p110δ in NK Cell Chemotaxis.

The general PI3K inhibitor LY294002 reduced NK cell migration to the homeostatic chemokine CXCL12/SDF-1α, to S1P and to the inflammatory chemokines CCL3/MIP-1α and CXCL10/IP10, implicating PI3K downstream of the receptors for these...
parallel, we tested the effect of p110<sup>α</sup> NK cells from pharmacological inactivation of p110<sup>α</sup> contrast, migration to S1P was reduced by 50–60% by genetic or p110<sup>α</sup> kinase-dead p110<sup>α</sup> BChemotaxis of WT and individual mice or, for S1P, from 2 independent experiments including 7/H11006/CXCL10. The general PI3K inhibitor LY294002 did not cause a inactivation of either p110<sup>α</sup> the 2 PI3K isoforms. Thus, migration to CCL3 was reduced by inflammatory chemokines was also differentially controlled by noticed increased migration of p110<sup>α</sup> 5796/h20841Chemokine Receptor Expression on PI3K-Mutant NK Cells. Chemokine receptors CXCR4, CCR5 and CXCR3 are expressed on subsets of NK cells and regulate their trafficking to CXCL12, CCL3 and CXCL10 (7). Although CXCR4 expression was slightly reduced on p110<sup>α</sup>/H9254/CXCL12, S1P, CCL3 and CXCL10 (Fig. 1B). However, LY294002 did not completely abrogate NK cell chemotaxis, indicating that PI3K-independent pathways also contribute to NK cell migration. These results suggest that p110<sup>δ</sup> is selectively required for chemotaxis to S1P and CXCL10, whereas both p110<sup>γ</sup> and p110<sup>δ</sup> regulate chemotaxis to CXCL12 and CCL3.

GPCR-Induced PIP<sub>3</sub> Production in NK Cells Requires p110<sup>δ</sup>. GPCRs are thought to signal through p110<sup>γ</sup> or p110<sup>δ</sup> (24), therefore it was surprising to find that NK cells require p110<sup>δ</sup> to mediate PI3K-dependent chemotaxis to CXCL12, S1P, CCL3 and CXCL10. Although p110<sup>δ</sup> can regulate chemotaxis in various cell types, this occurs mostly downstream of tyrosine kinase-linked receptors. There is no direct evidence for p110<sup>δ</sup>-dependent PI3K activation downstream of GPCRs in lymphocytes, although this has been reported for neutrophils (25, 30). Indeed, using an imaging approach (31), we have recently shown that PI3K activation in response to CXCL12 in T cells is p110<sup>δ</sup>-dependent (32). We show here that p110<sup>δ</sup>-dependent NK cell chemotaxis to S1P and CXCL10 was pertussis toxin-sensitive (Fig. 2), linking p110<sup>δ</sup> to G protein signaling. P110<sup>γ</sup> and p110<sup>δ</sup>-dependent chemotaxis to CXCL12, as expected, was also PTx-sensitive. Moreover, CXCL12-induced accumulation of PI<sub>3</sub> at the plasma membrane was significantly reduced upon inactivation of p110<sup>δ</sup> (P = 0.001) and, as expected, p110<sup>γ</sup> (P = 0.002) (Fig. 3 A and B and Movies S1–S3). Similar data were obtained with mutant cells (p110<sup>δ</sup>, P = 0.01 and p110<sup>γ</sup>, P = 0.0001) (Fig. S4 A and C). S1P and CXCL10 elicited a weaker response than CXCL12, however, CXCL10-induced PI<sub>3</sub> accumulation did show a tendency to reduction upon inactivation of p110<sup>δ</sup>, but not p110<sup>γ</sup> (Fig. 3C and Fig. S4B). Inhibition of p110<sup>δ</sup> had no effect on chemotaxis to CXCL10 (data not shown). These results show that, in a cell that possess the whole complement of class I PI3Ks, the tyrosine-kinase coupled p110<sup>δ</sup> is preferentially linked to GPCR signaling over p110<sup>γ</sup>. P110<sup>δ</sup> activation downstream of GPCRs could occur indirectly through SRC kinases, which can be activated by Gai.

**Fig. 2.** PI3K-mediated NK cell chemotaxis is pertussis toxin sensitive. Chemotaxis of WT splenic NK cells to the indicated concentrations of CXCL12, S1P, or CXCL10 in the presence of pertussis toxin (PTx, 100 ng/mL) or its vehicle control DMSO. Data are means ± SD of 2 independent experiments including 4 individual mice.
proteins in some circumstances (30). However, the Src kinase inhibitor PP2 did not reduce p110δ-dependent chemotaxis to CXCL10 (data not shown). Therefore, the molecular nature of the link between p110δ and GPCRs remains to be defined.

**NK Cell Distribution to Spleen, Lymph Nodes, and Liver Requires p110δ.** The percentages of splenic NK cells in WT, p110γ−/−, and p110δ/p110δ mice were comparable (Fig. 4A). By contrast, absolute numbers of NK cells were significantly reduced in spleens (P = 0.01) and lymph nodes (P = 0.002) of p110δ/p110δ mice and yet normal in bone marrow, peritoneal cavity, liver, lungs and blood of p110δ/p110δ mice, and in all tested tissues of p110γ−/− mice (Fig. 4B). The reduction in NK cell numbers in selected tissues of p110δ/p110δ mice may reflect a role for p110δ in NK cell distribution to these tissues. To directly test this possibility, we measured trafficking of mutant cells in competitive migration experiments in vivo. Spleen NK cells are not programmed to home to the spleen of host mice upon transfer and instead redistribute to all lymphoid and nonlymphoid tissues (1). At 24 h after transfer, the ratio between p110γ−/− and WT NK cells was similar to the initial 1:1 ratio, suggesting that p110γ−/− NK cells do not have a competitive disadvantage in trafficking (Fig. 4C). By contrast, p110δ/p110δ mice were significantly underrepresented in spleen (P = 0.004) and lymph nodes (P = 0.02), reflecting the reduced steady state numbers of NK cells in these tissues. Also liver (P = 0.035) and lungs contained less p110δ/p110δ cells (although the reduction in lung NK cells was not statistically significant). Bone marrow, peritoneal cavity and blood contained a 1:1 ratio of p110δ/p110δ and WT cells, suggesting that the trafficking defect of p110δ/p110δ NK cells is not generalized (Fig. 4C). The reduction of p110δ/p110δ NK cells was still detectable 48 h later, although it was less marked than at 24 h (Fig. S5). We conclude that p110δ controls NK cell distribution to spleen, lymph nodes and liver, excluding a role for p110γ.

**NK Cell Migration to the Uterus During Pregnancy Depends on p110γ and p110δ.** We then set out to assess the relative contribution of p110γ and p110δ in models of NK cell trafficking in pregnancy, inflammation and cancer. NK cells accumulate in the uterine mucosa during early pregnancy in human and mice. Although the mechanisms underlying this evolutionarily conserved process are unclear, one possibility is selective recruitment of circulating NK cells (2, 33). The mutant:WT ratio of recovered NK cells was measured 48 h later, in competitive migration experiments upon transfer into WT mice coinciding with midgestation, when the peak of uterine NK cells is attained (33). In the uterus of nonpregnant mice the frequency of donor cells was too low to accurately measure it, suggesting that the trafficking to the uterus of nonpregnant animals is minimal. By contrast, sizable populations of donor NK cells were found in the uterus of pregnant host mice. In these mice, both p110γ−/− and p110δ/p110δ NK cells were underrepresented in the uterine mucosa (Fig. 4D). These results show that both p110γ and p110δ are required to respond to pregnancy-specific chemotactic cues.

**NK Cell Recruitment to the Inflamed Peritoneum Depends on p110γ and p110δ.** In a model of LPS-induced acute peritonitis, the number of NK cells increased 29-fold in WT mice, suggesting a strong recruitment. This was profoundly decreased in both p110γ−/− and p110δ/p110δ mice, in which only an ~2-fold increase was detected (Fig. 5A), thus extravasation of NK cells to the site of acute inflammation requires both isoforms. The decreased recruitment was due to cell autonomous defects in both p110γ−/− and p110δ/p110δ NK cells, because similar data were obtained in competitive migration experiments upon transfer into WT mice (Fig. 5B). Moreover, migration in this model was largely dependent on GPCRs, because pertussis toxin-treated WT cells showed a strong competitive disadvantage (Fig. 5B).

**NK Cell Extravasation to Tumors Requires p110δ.** We have recently shown that p110δ/p110δ mice show effective NK cell cytotoxicity...
in vitro, yet do not reject lymphoma cells (34) in a model of tumor immunity in vivo that depends on NK cells and their recruitment (35, 36). We therefore hypothesized that NK cell extravasation to tumors is p110δ-dependent. To test this hypothesis, we administered RMA-S lymphoma cells i.p. into 3 groups of lymphocyte-deficient Rag2−/−Il2rg−/− mice, one of which was reconstituted with WT or PI3K-mutant spleen cells i.v., so that the adoptively transferred NK cells had to extravasate to the peritoneum. The second group of Rag2−/−Il2rg−/− mice was reconstituted with WT or p110δ0910A spleen cells i.p., so that the adoptively transferred NK cells could come into direct contact with tumor cells. The third group of Rag2−/−Il2rg−/− mice was not reconstituted with any spleenocytes. The latter group did not reject tumors (Fig. 6A), whereas mice reconstituted with WT, p110γ−/− or p110δ0910A NK cells in situ readily rejected RMA-S cells (Fig. 6B) and so did mice reconstituted intravenously with WT or p110γ−/− NK cells (Fig. 6C). By contrast, mice reconstituted intravenously with p110δ0910A NK cells failed to reject tumor cells (Fig. 6C). These results suggest that p110δ is selectively required to control NK cell extravasation to the site of tumor growth. Moreover, these results show that p110δ can reject RMA-S cells in vivo, confirming that the lack of p110δ function does not intrinsically hamper NK cell cytotoxicity. To directly quantify NK cell migration to tumors, a 1:1 mixture of PI3K-mutant and WT spleenocytes was injected intravenously into Rag2−/−Il2rg−/− mice that were given RMA-S i.p. The p110δ0910A:WT NK cell ratio recovered in the peritoneal lavage 48 h after adoptive transfer was smaller than the initial 1:1 ratio, whereas the p110γ−/−:WT NK cell ratio was similar to the initial 1:1 ratio, showing that p110δ is selectively required for NK cell extravasation to tumors (Fig. 6D). In line with these data, whereas WT NK cells migrated strongly in a dose-dependent manner to the peritoneal lavages obtained from tumor-bearing mice, p110δ0910A NK cells showed 50–60% reduced migration (Fig. 6E). Based on published evidence (37) we hypothesized that p110δ is required for CXCR3-dependent tumor clearance. Purified CXCR3+ and CXCR3− NK cell subsets from WT, p110γ−/− and p110δ0910A mice were tested for tumor clearance as in Fig. 6C. Both subsets cleared tumor cells, although WT and p110γ−/− CXCR3+ cells were more potent (~4.5- and 3.1-fold more, respectively), confirming that CXCR3 is required for tumor clearance. By contrast, the differences between CXCR3+ and CXCR3− p110δ0910A NK cells was negligible (1.3-fold), indicating p110δ is required for CXCR3-dependent migration in vivo (Fig. 6F).

**Discussion**

The functional versatility of NK cells is the result of subset heterogeneity and of appropriate localization, but the mechanisms regulating NK cell trafficking are unclear and a better understanding will help appreciate how these innate lymphocytes deliver functions to the appropriate anatomical site, ultimately offering opportunities for selective pharmacological manipulation. A picture is emerging suggesting that regulation of NK cell trafficking is distinct from that of other lymphocytes. For example, egress of B and T lymphocytes from lymphoid organs in response to S1P is regulated by S1P1 (38), whereas in NK cells is regulated by S1P3 (11). Most available evidence suggests that p110γ is the dominant PI3K subunit in leukocyte trafficking, but our results indicate that p110δ is a key regulator of NK cell migration in health and disease, because it was dispensable for migration during pregnancy, inflammation, steady-state recirculation and extravasation to tumors, whereas the requirement for p110γ was restricted to pregnancy and inflammation only.

Leukocyte migration is a multistep process involving selectin-mediated rolling, chemokine-induced activation, stable adhesion via integrins and transmigration (4). PI3Ks can regulate leukocyte motility by modulating all 3 steps (39–42). We detected normal levels of α4β7 (CD11a), α4β2 (LFA-1) and L-selectin in p110γ−/− and p110δ0910A NK cells (data not shown). Although the results of our preliminary experiments do not exclude that either p110γ or p110δ can regulate integrin activation and signaling, or expression of selectins, here we show that p110γ and p110δ do regulate NK cell responses to chemokines. Both isoforms were required for chemotaxis to CXCL12 and CCL3, whereas only p110δ was dispensable for chemotaxis to S1P and CXCL10.

Differential roles for PI3Ks subunits have been reported before in lymphocyte migration. Thus, p110γ regulates T cell migration to CCL19, CCL21 and CXCL12 (23) and to the site of vaccinia virus infection (43), whereas p110δ regulates T cell localization to antigenic tissues (12) and B-cell chemotaxis to CXCL13 (13). P110γ is recruited to phosphotyrosine-containing complexes downstream of IMLP in neutrophils (30), but evidence that GPCRs activates p110δ in lymphocytes was lacking. Similarly to neutrophils, p110γ and p110δ are both equally required for PIP3 production in response to CXCR4 in NK cells, whereas in T cells the response to the same GPCR is exclusively p110δ-dependent (32). The way p110δ can be activated downstream of GPCRs is not clear. Src kinases have been implicated...
downstream of Grα- and Grβ1-related GPCRs in neutrophils (30). However, the failure of PP2 to block the chemotactic response excludes Src kinases as the critical link in the context of NK cell chemotaxis.

How do p110γ and p110δ regulate NK cell migration in vivo? PI3K-dependent cell survival may be relevant to the trafficking experiments performed over 2–3 days, however, it is unlikely to contribute to the outcomes, because neither p110γ nor p110δ are essential for NK cell survival (28, 29, 44). NK cells lacking either isoforms migrate less to CXCL12 and CCL3 in vitro, thus reduced chemotaxis to these homeostatic and inflammatory stimuli may explain the reduced trafficking to the gravid uterus and to the inflamed peritoneum. Chemotaxis to S1P and CXCL10 is instead exclusively p110δ-dependent. Thus, the abnormal redistribution of p110δ-mutant NK cells to lymphoid and nonlymphoid tissues may partially be due to defective S1P5 signaling, although the phenotype of the S1P5−/− mice and the p110δ mutant mice do not overlap completely. CXCR3, the receptor for CXCL10, is directly implicated in NK cell recruitment to the tumor in the peritoneum (35). Because TNF-α is not directly toxic to RMA-S cells, it may be required to produce other chemotactic factors that recruit NK cells to the peritoneum. Production of TNF-α, IFN-γ and GM-CSF is impaired in p110δ−/− NK cells (28) and, although we have not extensively analyzed cytokine secretion in p110δ−/− NK cells, we have evidence that they produce less IFN-γ (data not shown). Because CXCL10 production is induced by IFN-γ, it is possible that reduced CXCL10 production in p110δ−/− mice may account for the reduced NK cell recruitment to the lymphoma. Nevertheless, we showed that the defective recruitment is NK cell-autonomous and possibly confined to the CXCR3+ compartment, implicating p110δ downstream of CXCR3 in vivo.

Although p110δ emerges here as an essential signaling molecule for NK cell trafficking to lymphomas, its role in cytoxicity is redundant (28, 29, 36). In contrast to this notion, Zebedin et al. (45) have recently shown evidence of impaired NK cell cytoxicity in p110δ−/− mice on a mixed (129xC57BL/6) background. We think the discrepancy with the results published by us and others, using mice on C57BL/6 (29, 36) or C57BL/10 background (28), could be due to the genetic background itself. Indeed, our observation confirms that cytoxicity is impaired in p110δ−/− 129xC57BL/6 mice (Fig. S9), supporting the hypothesis that the 129 genome contributes modifier genes that impact on NK cell cytoxicity in the absence of p110δ. Guo et al. (46) have recently confirmed our data by showing p110δ-dependent tumor clearance, however, they did not find a migration defect. The conditions used by Guo et al. (46) are very different from ours in terms of kinetics and number of tumor cells administered, making a direct comparison difficult.

In conclusion, our results show that p110γ and p110δ differentially regulate NK cell migration in health and disease and highlight p110δ as one key catalytic PI3K isoform in NK cell functions. Because isoform-specific PI3K inhibitors are in clinical trials to treat several conditions including cancer and inflammation, an understanding of their action on NK cell functions is a prerequisite for an effective and safe use of the small molecule inhibitors. For example, p110δ inhibitors may be effective to treat Acute Myeloid Leukemia (47, 48), a malignancy that is targeted by allogeneic donor NK cells upon bone marrow transplantation (49). It will be important to know whether interfering with p110δ functions may alter the net effect of donor NK cells on host tumor cells, which is the result of a balance between p110δ-dependent NK cell migration and p110δ-independent NK cell cytoxicity.

Methods

Chemotaxis Assays. Fresh splenocytes or bone marrow cells enriched for NK cells by magnetic beads (NK cell isolation kit, Miltenyi Biotec) were resuspended in RPMI-1640 0.1% BSA without fatty acids (Calbiochem) and incubated 30 min at 37 °C. Cells were either pretreated for 20 min at 37 °C with the pan-PI3K inhibitor LY294002 (10 μM) or 30 min at 37 °C with the p110γ-specific inhibitor AS252424 (1 μM), the p110δ-specific inhibitor IC87114 (1 μM), the p110β-specific inhibitor TGX-221 (10 nM), pertussis toxin from Bordetella Pertussis (100 ng/mL), the Src kinase inhibitor PP2 (1 μM) or its inactive form PP3 (1 μM) before the assay or directly loaded (10^5 cells in 100 μL) in the top chamber of a 6.5-mm diameter 5-μm pore polycarbonate transwell insert (Corning Costar). The transwell chambers were then placed in wells containing BSA medium with CXCL10, CCL3 (Peprotech), CXCL12 (R&D Systems), S1P (Sigma), or different dilutions of peritoneal lavages isolated from WT tumor-bearing mice and incubated for 3 h at 37 °C. Cells migrating from the top to the bottom chamber were collected and NK1.1+ CD3− cell numbers evaluated by flow cytometry.

Microscopy. For live cell imaging, purified NK cells were plated on glass coverslips coated with poly-L-lysine (Sigma-Aldrich). Cells were then stimulated with 10 ng/mL of CXCL10 and imaged in RPMI supplemented with 0.5% FCS, using an objective heater. For p110γ and p110δ specific inhibition, NK cells were preincubated with isoform-specific drugs (IC87114 at 1 μM or AS252424 at 1 μM) at 37 °C for 15 min before being imaged. Images were taken every 15 s with an Olympus FV1000 system consisting of an Olympus IX81 microscope fitted with a Plan-SuperApochromat x60/1.4 NA oil objective. Images were analyzed using Velocity software (Improvision, Ltd.). The Akt/IKK-GFP translocation to the membrane was quantified by drawing regions surrounding the plasma membrane outside and inside the cells. When the plasma membrane could not be clearly identified a mask, created from the plasma membrane of a WT cells responding to CXCL12 or CXCL10, was used.

Competitive Migration Assays in Vivo. WT, p110γ−/− or p110δ−/− spleen cells were labeled for 10 min at 37 °C with 5 μM or 0.5 μM 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) Molecular Probes) and washed twice with RPMI medium 1640 5% FBS. A total of 10^5 CFSE-labeled spleen cells mixed at a 1:1 ratio (CFSE low WT cells and CFSE high mutant spleen cells) were injected intravenously into WT naive mice. Twenty-four and 48 h after injection, ratios between mutant and WT NK cells were measured in different organs by flow cytometry. In the pregnancy model, WT females were timed and received 1:1 mixed spleen cells intravenously at 7.5 day of pregnancy (identification of copulation plug defined as gestation day 0.5). Forty-eight hours after injection, ratios between mutant and WT cells were measured in the decidua by flow cytometry as in ref. 50. Cells from multiple implants of 1–10 individual pregnant mice at the same stage of gestation were pooled together for each experiment. In the tumor model, WT mice were administered live tumor cells (2×10^5 RMA and 2×10^5 RMA-S cells) i.p. and 1:1 mixed splenocytes intravenously. Forty-eight hours after injection, ratios between mutant and WT NK cells were measured in the spleen and the peritoneum by flow cytometry.

Migration to Inflamed Peritoneum. LPS (10 μg in 500 μL of PBS) was injected i.p. into WT, p110γ−/− or p110δ−/− mice or in WT mice that received a 1:1 ratio of CFSE-labeled WT, WT treated with pertussis toxin, p110γ−/− or p110δ−/− splenocytes as described above for the competitive migration assays. Twenty-four hours later, the number of NK1.1+ CD3− cells was evaluated by flow cytometry in the peritoneum.

Tumor Clearance upon Adoptive Transfer. Live tumor cells (2×10^5 RMA and 2×10^5 RMA-S) labeled with 5 μM 5,6-carboxyfluorescein diacetate, 5,6-carboxytetramethylrhodamine (CMTMR) were injected i.p. in a volume of 0.5 mL of PBS into Rag2−/−Il2rg−/−. These mice were then injected i.p. or intravenously with 10^5 WT, p110γ−/− or p110δ−/− splenocytes or intravenously with 10^5 CXCR3+ or CXCR3− purified NK cells from each genotype. Forty-eight hours later, peritoneal cells were recovered and analyzed by flow cytometry for CMTMR detection and expression of MHC class I by which RMA and RMA-S can be distinguished.
ACKNOWLEDGMENTS. We thank the Babraham Small Animal Facility, Joao-Pedro Pereira for advice (University of California S.F.); Takehiko Sakata (Akita University) for the AktPH-GFP mice; Emilio Hirsch (University of Turin) for the p110α−/− mice; Suhasini Kulkarni, Martin Turner, and the members of the Colucci group for discussions; Dan Hampshire for proof reading; and Phil Hawkins for critically reading the manuscript and for insightful discussions. This work was supported by Cancer Research U.K. (F.C.), the Babraham Institute (K.O. and F.C.), and the Swiss National Science Foundation (to AMF).