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Research Article

Immunology

The establishment of T cell–mediated inflammation requires the migration of primed T lymphocytes from the blood stream and their retention in antigenic sites. While naive T lymphocyte recirculation in the lymph and blood is constitutively regulated and occurs in the absence of inflammation, the recruitment of primed T cells to nonlymphoid tissue and their retention at the site are enhanced by various inflammatory signals, including TCR engagement by antigen-displaying endothelium and resident antigen-presenting cells. In this study, we investigated whether signals downstream of TCR ligation mediated by the phosphoinositide-3-kinase (PI3K) subunit p110 $\delta$  contributed to the regulation of these events. T lymphocytes from mice expressing catalytically inactive p110 $\delta$  displayed normal constitutive trafficking and migratory responses to nonspecific stimuli. However, these cells lost susceptibility to TCR-induced migration and failed to localize efficiently to antigenic tissue. Importantly, we showed that antigen-induced T cell trafficking and subsequent inflammation was abrogated by selective pharmacological inhibition of PI3K p110 $\delta$  activity. These observations suggest that pharmacological targeting of p110 $\delta$  activity is a viable strategy for the therapy of T cell–mediated pathology.

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# T cell receptor–induced phosphoinositide-3-kinase p110 $\delta$ activity is required for T cell localization to antigenic tissue in mice

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**The establishment of T cell–mediated inflammation requires the migration of primed T lymphocytes from the blood stream and their retention in antigenic sites. While naive T lymphocyte recirculation in the lymph and blood is constitutively regulated and occurs in the absence of inflammation, the recruitment of primed T cells to nonlymphoid tissue and their retention at the site are enhanced by various inflammatory signals, including TCR engagement by antigen-displaying endothelium and resident antigen-presenting cells. In this study, we investigated whether signals downstream of TCR ligation mediated by the phosphoinositide-3-kinase (PI3K) subunit p110 $\delta$  contributed to the regulation of these events. T lymphocytes from mice expressing catalytically inactive p110 $\delta$  displayed normal constitutive trafficking and migratory responses to nonspecific stimuli. However, these cells lost susceptibility to TCR-induced migration and failed to localize efficiently to antigenic tissue. Importantly, we showed that antigen-induced T cell trafficking and subsequent inflammation was abrogated by selective pharmacological inhibition of PI3K p110 $\delta$  activity. These observations suggest that pharmacological targeting of p110 $\delta$  activity is a viable strategy for the therapy of T cell–mediated pathology.**

## Introduction

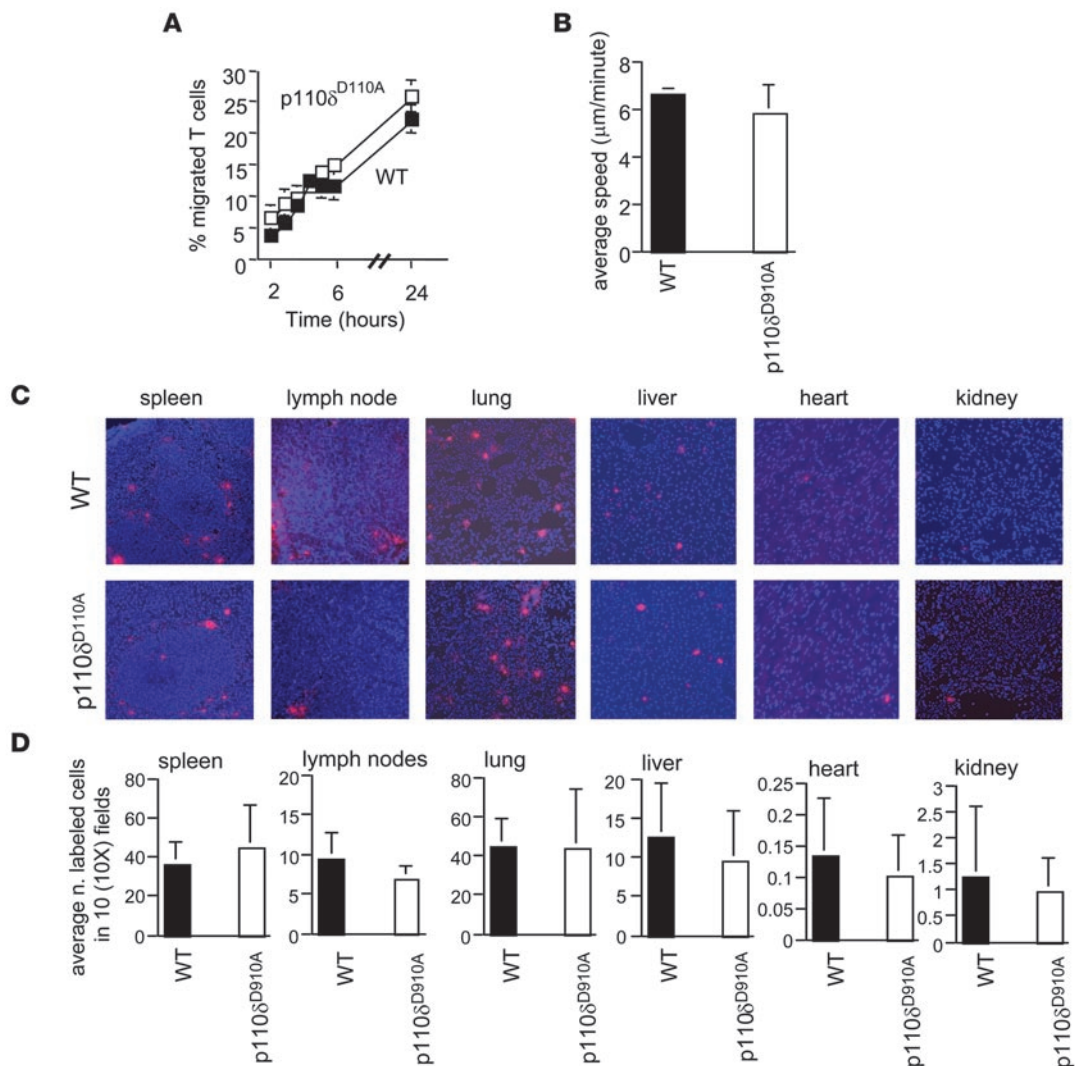
Coordinated migratory events guiding immune cell compartmentalization are essential to the development of effective and targeted immunity. Specific adhesive interactions and the responsiveness to chemotactic factors regulate cell-cell interactions leading to T cell activation, the provision of T cell help to B cells, and T cell access to target tissues. Naive T cells are programmed to recirculate predominantly in secondary lymphoid tissue. By contrast, primed T lymphocytes selectively localize in nonlymphoid antigenic sites upon detection of antigen (1). Antigen-presenting DCs help program primed T cells to establish specific molecular interactions that allow their access to various organs, such as the skin and the gut (1). Although these mechanisms provide a level of specificity by restricting the area to which primed T cells can recirculate, the extent of the vascular route that these cells must take to reach their target and the size of the anatomical site that they must patrol suggest that other mechanisms must be in place for T cells to locate and enter target tissues. We and others have shown that antigen recognition on the endothelium and parenchymal antigen-presenting cells contributes to the recruitment and retention of antigen-specific T cells into target tissue where antigen can be found. For example, we have reported that cognate recognition of MHC-peptides expressed by human ECs, under conditions that do not lead to T cell division or anergy (2), can enhance transendothelial migration of human T cells (3). Moreover, using a mouse model, we showed that TCR engagement by the endothelium leads to T

cell transendothelial migration and tissue infiltration in vivo (4). Similarly, islet-specific homing by insulin-specific H2-K<sup>d</sup>-restricted CD8<sup>+</sup> T cells was abrogated in mice lacking MHC class I expression or in mice displaying impaired insulin peptide presentation by local endothelium due to deficient insulin secretion, highlighting the ability of ECs to cross-present tissue antigens (5). Interestingly, the induction of TCR-mediated signals leading to either T cell division or anergy abrogates T cell migration, suggesting that qualitatively different signals may determine the outcome of TCR triggering on T cell migration. Although it is well established that TCR-derived signals can regulate T lymphocyte motility (6, 7) and immobilize migrating T cells (8), the molecular basis of these effects is still unclear. Class IA phosphoinositide 3-kinases (PI3Ks) are a family of p85/p110 heterodimeric lipid kinases that generate second messenger signals (e.g., PIP3) downstream of tyrosine kinases, thereby controlling various cell functions, including motility (9). The expression of the PI3K p110 $\delta$  subunit is mostly restricted to hematopoietic cells (9). The capacity of the TCR to induce PI3K signaling is dependent on the p110 $\delta$  subunit and has previously been shown to promote proliferation, differentiation, and regulation of T helper cells (10–12). In addition to antigen receptor signal transduction, PI3K activity has been associated to a variety of biological functions in both T cells and other leukocytes (13), including the regulation of their motility (14). In addition to signaling mediated by dedicated cytokines (DOCK2) (15), both class IB and class IA PI3K have been involved in lymphocyte responses to chemokines (14), although their relative contribution appears to be different in distinct lymphocyte subsets. Chemokine-induced migration of B cells in vitro mostly relies upon p110 $\delta$  activity, and it is not significantly affected by p110 $\gamma$  deficiency (16). In contrast, in vitro migration of p110 $\gamma$ -deficient T cells to CCL19, CXCL12, and CCL21 is mildly reduced, while p110 $\delta$ -mediated activity appears to be dispensable

**Nonstandard abbreviations used:** DOCK2, dedicator of cytokinesis 2; PI3K, phosphoinositide-3-kinase; rIL-2, recombinant IL-2.

**Conflict of interest:** Klaus Okkenhaug is a paid consultant of Piramed Inc., Slough, United Kingdom. The other authors have declared that no conflict of interest exists.

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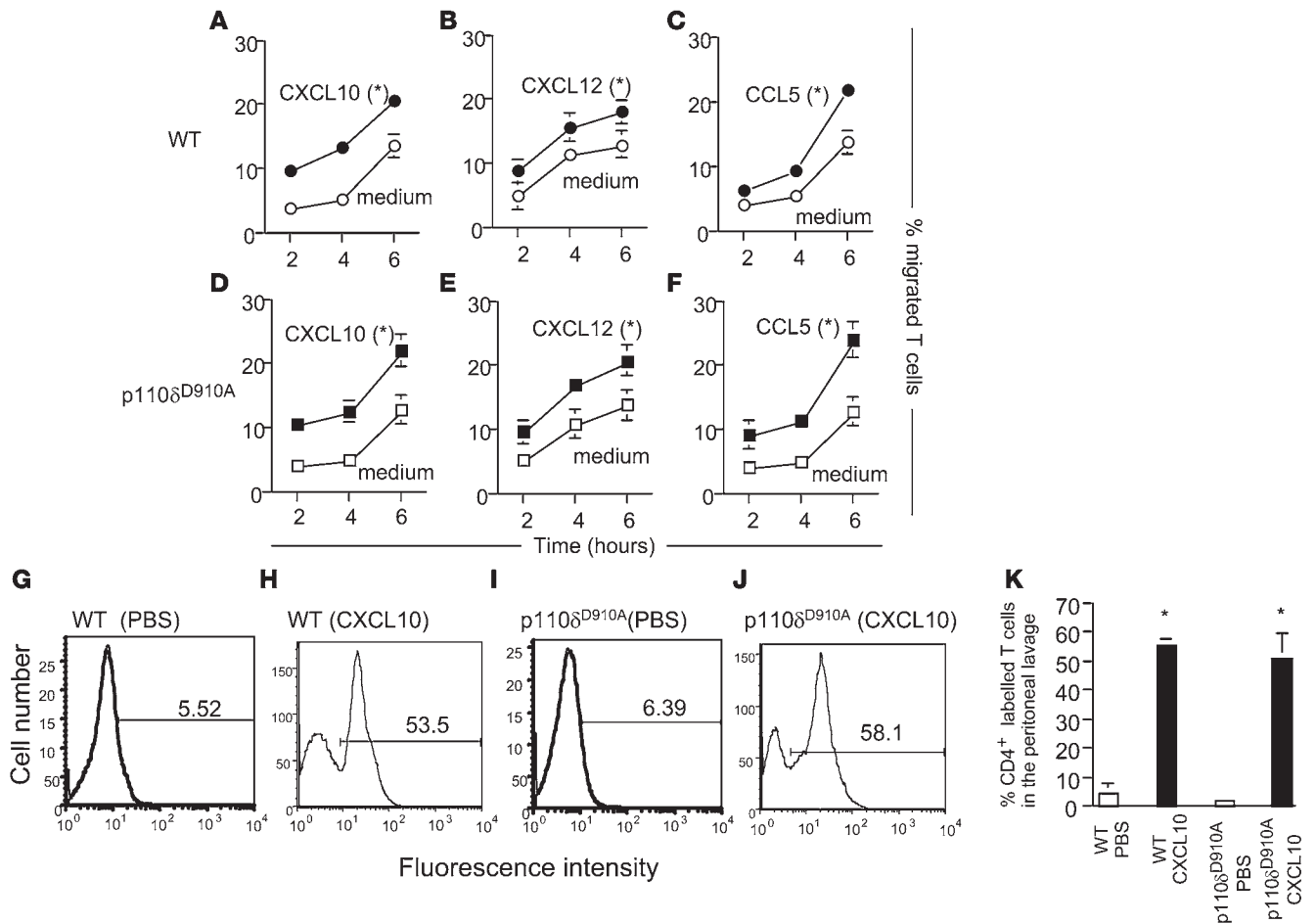
### Figure 1

PI3K P110 $\delta$  does not contribute to constitutive T cell migration. **(A)** HY-specific H2-A<sup>b</sup>-restricted WT and P110 $\delta$ <sup>D910A</sup> T cells ( $5 \times 10^5/\text{well}$ ) were seeded onto untreated syngeneic female-derived EC monolayers grown on 3- $\mu\text{m}$ -pore Transwells, and T cell migration was monitored as described in Methods. Results are expressed as a percentage of migrated T cells at the given time points and reported as the average of 3 experiments of identical design. Error bars are shown. **(B)** HY-specific H2-A<sup>b</sup>-restricted WT and P110 $\delta$ <sup>D910A</sup> T cells ( $5 \times 10^5/\text{well}$ ) were seeded onto ICAM-1-coated (2  $\mu\text{g}/\text{ml}$ ) 35-mm dishes, and their migration was observed for 25–30 minutes by time-lapse microscopy. A representative example of a series of 3 independent experiments with similar design is shown. Mean migration speed  $\pm$  SEM is shown. **(C and D)** PKH26-labeled HY-specific H2-A<sup>b</sup>-restricted CD4<sup>+</sup> WT and P110 $\delta$ <sup>D910A</sup> T cells were injected i.v. into syngeneic female mice. T cell localization in the indicated tissues was assessed 24 hours later by wide-field fluorescence microscopy. To minimize the effect of arbitrary choice of field, tissue infiltration was quantified by randomly selecting ten  $\times 10$ -magnified fields from tissue samples from at least 3 animals and assessing the number of fluorescent cells in each field. Each panel shows a representative tissue image. The mean T cell infiltration  $\pm$  SD observed in samples from at least 3 animals is shown in **D**.

(16) and CXCL12-induced PI3K activity is entirely dependent on p110 $\gamma$  and not p110 $\delta$  (12). Accordingly, PI3K $\gamma$ -deficient T but not B cells show a mild reduction in their homing efficiency to secondary lymphoid tissue (15) or on migration velocity within lymph nodes (17). T cells lacking both DOCK2 and PI3K $\gamma$  show a virtually abolished migration to chemokines *in vitro* and have a further reduced homing capacity toward secondary lymphoid organs (15). Recent data have indicated a role for the class IA regulatory subunit p85 in sustaining spontaneous motility of both T and B lymphocytes in the lymph node *in vivo* (18); however, Asperti-Boursin and col-

leagues reported that wortmannin, a potent PI3K inhibitor, had no effect on constitutive CCL19- and CCL21-dependent migration in lymph nodes (19). Thus, under some circumstances, PI3Ks may affect chemokine-dependent migration of primary mouse T cells; however, DOCK2 appears in most cases to be the main signal transducer employed by chemokine receptors.

The contribution of PI3Ks to pathological effector T cell migration has recently been explored. In a model of lupus-like disease induced in p65PI3K-transgenic mice, in which class IA PI3K activity is elevated, genetic deletion of the class IB isoform p110 $\gamma$  did not



**Figure 2**

PI3K p110δ is not required for chemokine-induced T cell migration. (A and B) HY-specific H2-A<sup>b</sup>-restricted WT and P110δ<sup>D910A</sup> CD4<sup>+</sup> T cells (5 × 10<sup>5</sup>/well) were exposed to CXCL10 (300 ng/ml; filled symbols, A and D), CXCL12 (50 ng/ml; filled symbols, B and E), or CCL5 (100 ng/ml; filled symbols, C and F) through 5-μm-pore Transwells. In the experiments analyzing migratory responses to CXCL12, T cells were stimulated with plastic-bound anti-CD3 (1 μg/ml) and anti-CD28 (5 μg/ml) for 72 hours to induce CXCR4 expression. Spontaneous migration in chemotaxis medium (RPMI 0.5% FCS) alone was also measured (open symbols). The number of migrated T cells was monitored at the indicated time points. Results are expressed as the percentage of input T lymphocytes that had migrated through the filters at any given time point and represent the mean of at least 3 independent experiments ± SEM. \*P < 0.05 at all time points except 2 hours (B, C, and E) and 24 hours. (G–J) C57BL/6 female mice received an i.p. injection of CXCL10 (1,200 ng). One hour later, PKH26-labeled HY-specific H2-A<sup>b</sup>-restricted WT and P110δ<sup>D910A</sup> T cells (5 × 10<sup>5</sup>/well) T cells were injected i.v. After 6 hours, localization of PKH26-labeled T cells in the peritoneal cavity was assessed by flow cytometry. The panels (representative of 1 experiment) show the number of PKH26-labeled T cells in the CD4-gated T cell population. The mean T cell numbers ± SEM observed in samples from at least 3 animals are summarized in K. \*P < 0.02.

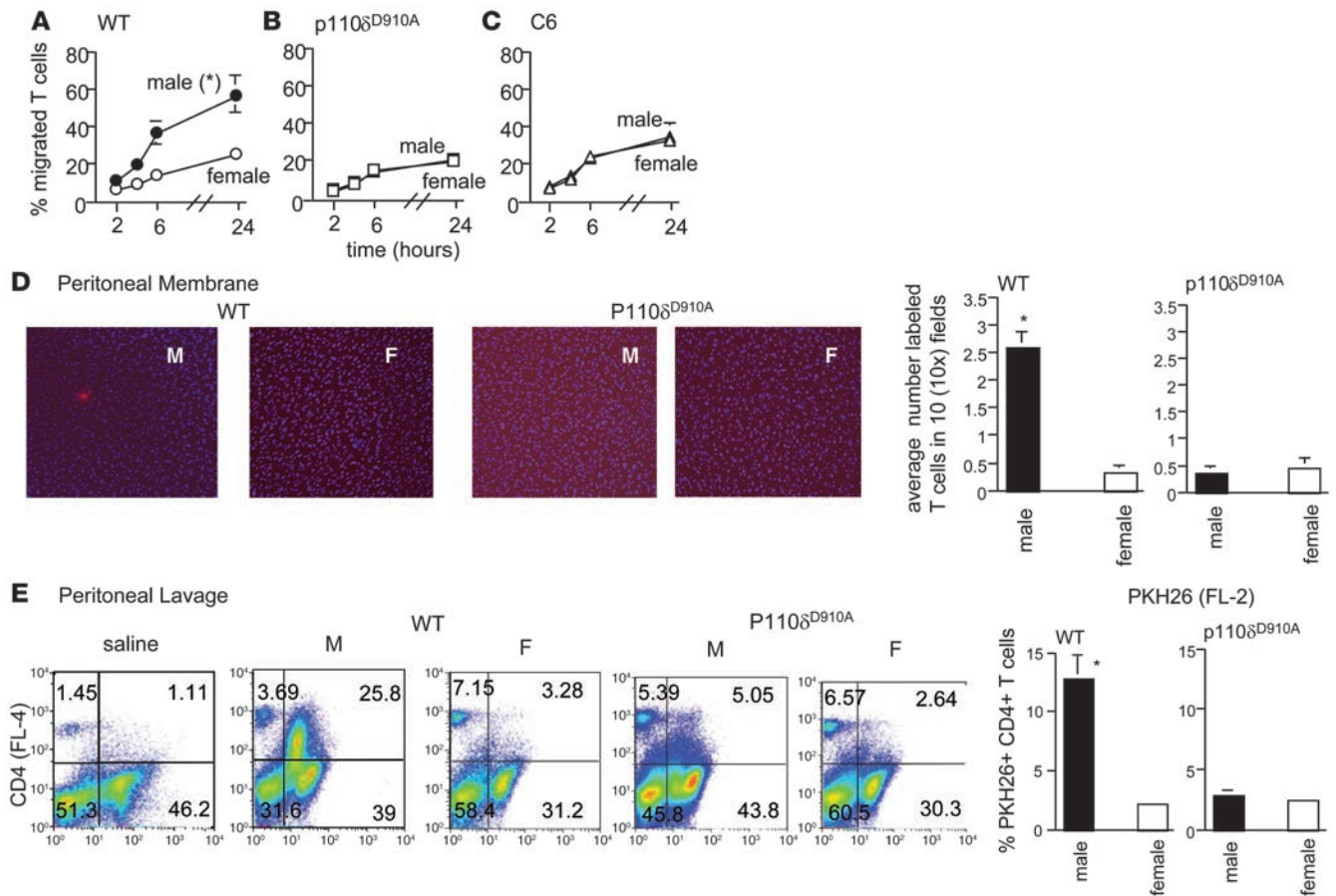
prevent tissue invasion by pathogenic CD4<sup>+</sup> T cells, although it ameliorated disease by reducing their survival (20). However, whether tissue infiltration was a direct result of PI3K-dependent trafficking or a more general loss of self tolerance in this model is not clear.

In this study we have investigated the contribution of TCR- and CD28-induced PI3K p110δ-mediated signals to the regulation of memory T cell trafficking to target tissue. Our observations suggest that PI3K p110δ is an essential mediator of primed T cell migration to antigenic sites and highlight its potential as a pharmacologic target for the control of T cell-mediated inflammation.

**Results**

*PI3K p110δ activity is not required for antigen-independent memory T cell transendothelial migration, chemotaxis, and constitutive trafficking.* Before investigating the effect of PI3K-mediated signals on TCR-driven

T cell trafficking, the ability p110δ<sup>D910A</sup> T cells to undergo antigen-independent migration in steady state and in response to inflammatory stimuli was analyzed. Memory HY-specific H2-A<sup>b</sup>-restricted CD4<sup>+</sup> T cells were generated by immunization of female C57BL/6 WT and p110δ<sup>D910A</sup> mice with syngeneic WT male splenocytes followed by in vitro expansion. The 2 T cell populations displayed similar phenotypes, as assessed 7 days after restimulation with male splenocytes and recombinant IL-2 (rIL-2) (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI33267DS1). Following further resting in medium alone and subsequent antibody stimulation with anti-CD3 and anti-CD28, HY-specific WT and p110δ<sup>D910A</sup> CD4<sup>+</sup> T cells equally upregulated surface expression of CD25, CD69, and the chemokine receptor CXCR4 (Supplemental Figure 2, A–D). However, fewer p110δ<sup>D910A</sup> CD4<sup>+</sup> T cells were induced to produce IFN-γ



**Figure 3**

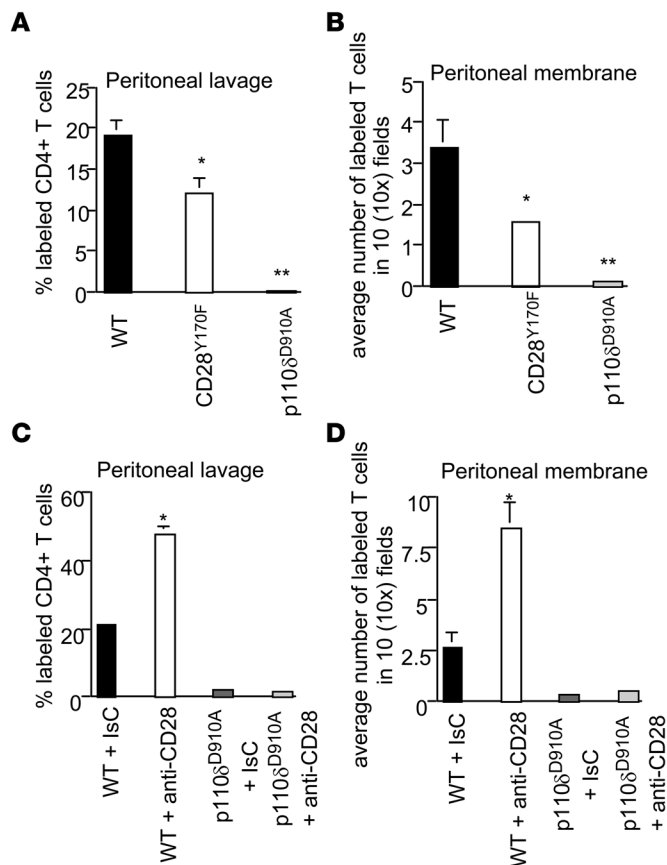
PI3K P110 $\delta$  is instrumental to antigen-induced T cell recruitment. (A–C) HY-specific H2-A<sup>b</sup>-restricted WT and p110 $\delta$ <sup>D910A</sup> CD4<sup>+</sup> T cells or HY-specific H2-K<sup>k</sup>-restricted CD8<sup>+</sup> C6 T cells were seeded onto IFN- $\gamma$ -treated female- and male-derived syngeneic EC monolayers grown on 3- $\mu$ m-pore Transwells. T cell migration was assessed as described in Figure 1. Error bars for 3 averaged experiments are shown. \**P* < 0.05 versus migration through female ECs at all time points except 2 hours. (D and E) Male and female C57BL/6 mice were injected i.p. with 600 U IFN- $\gamma$  or PBS. Two days later, mice received an i.v. injection of PKH26-labeled HY-specific CD4<sup>+</sup> T cells (10<sup>7</sup>/mouse). The presence of labeled cells in the peritoneal membrane (D) and cavity (E) was assessed 24 hours later by wide-field fluorescence microscopy and flow cytometry, respectively. A cluster of 3 PKH26-labeled cells is visible in the membrane of male recipient of WT HY-specific T cells. Due to the presence of an autofluorescent population of non-T cells often detected in FL-2 (also in control mice that received IFN- $\gamma$  but no T cells; far-left dot plot, “saline”), cells were double stained with an APC-conjugated anti-CD4 antibody following harvesting, and the percentage of PKH26 (FL-2)-labeled T cells gated in the CD4<sup>+</sup> T cell population is shown in the dot plots and the graph representing cumulative data from at least 3 animals. The mean  $\pm$  SEM observed in samples from at least 3 animals is shown on the right. \**P* < 0.04 versus female mice.

(Supplemental Figure 2E), consistent with previous results (21). HY-specific WT and p110 $\delta$ <sup>D910A</sup> CD4<sup>+</sup> T cells migrated equally through resting (Figure 1A) and TNF- $\alpha$ -treated (data not shown) syngeneic, female-derived EC monolayers and displayed similar migration speed over plastic-bound recombinant ICAM-1 (Figure 1B) in vitro. Thus, p110 $\delta$  does not appear to regulate basal motility in absence of cognate antigen.

To investigate whether constitutive memory T cell trafficking was affected by lack of p110 $\delta$  activity in physiological settings, WT female mice were injected with either PKH26-labeled WT or p110 $\delta$ <sup>D910A</sup> T cells (10<sup>7</sup>/mouse), and their localization in several tissues was analyzed 16–24 hours later by wide-field microscopy. Both WT and p110 $\delta$ <sup>D910A</sup> T cells recirculated normally and could be detected in the spleen, lung, liver, and kidney of recipient mice in similar numbers (Figure 1, C and D). Notably, WT and p110 $\delta$ <sup>D910A</sup> T cells displayed similar patterns of distribution within the various

organs and localized in the liver, kidney, and heart in a scattered pattern, while they clustered in restricted areas in lymph nodes. These observations suggest that p110 $\delta$  signaling is not involved in constitutive memory T cell trafficking. The inefficient localization of both WT and p110 $\delta$ <sup>D910A</sup> T cells to the lymph nodes as compared to the spleen was likely due to lack of expression of CD62L and CCR7 (Supplemental Figure 1; ref. 22).

As PI3K activity is associated with chemokine-induced T cell migration (14), we also compared the recruitment of WT and p110 $\delta$ <sup>D910A</sup> T cells in response to chemokines. WT and p110 $\delta$ <sup>D910A</sup> T cell migration was equally enhanced by the chemokines CXCL10, CXCL12, and CCL5 in vitro (Figure 2, A–F). As both WT and p110 $\delta$ <sup>D910A</sup> T cells were found to express CXCR4 only following antibody-mediated stimulation with plastic-bound anti-CD3 (1  $\mu$ g/ml) and anti-CD28 (5  $\mu$ g/ml) (Supplemental Figure 2), in the experiments assessing migration to CXCL12, T cells were used fol-



**Figure 4**

PI3K p110δ activation by TCR but not CD28 is required for antigen-dependent T cell recruitment. **(A and B)** H2-A<sup>b</sup>-restricted HY-specific WT (black bars), CD28<sup>Y170F</sup> (white bars), and p110δ<sup>D910A</sup> (light gray bars) CD4<sup>+</sup> T cells were injected i.v. into male mice (10<sup>7</sup>/mouse) that had received an i.p. injection of IFN-γ (600 U) 48 hours earlier. The following day, mice were sacrificed, and the presence of fluorescently labeled cells in the peritoneal cavity **(A)** and membrane **(B)** was assessed by flow cytometry and wide-field fluorescence microscopy, respectively. To facilitate visualization by flow cytometry, cells were double stained with an APC-conjugated anti-CD4 antibody following harvesting. The mean T cell numbers ± SEM observed in samples from at least 3 animals are shown. **A:** \**P* < 0.04, CD28<sup>Y170F</sup> versus WT T cells; \*\**P* < 0.001, p110δ<sup>D910A</sup> versus WT T cells; *P* < 0.005, p110δ<sup>D910A</sup> versus CD28<sup>Y170F</sup> T cells. **B:** \**P* < 0.03, CD28<sup>Y170F</sup> versus WT T cells; \*\**P* < 0.002, p110δ<sup>D910A</sup> versus WT T cells; *P* < 0.007, p110δ<sup>D910A</sup> versus CD28<sup>Y170F</sup> T cells. **(C and D)** HY-specific WT or p110δ<sup>D910A</sup> CD4<sup>+</sup> T cells that had either undergone antibody-mediated CD28 ligation (30 minutes at 37°C, PKH26-labeled) or had been pretreated with an antibody isotype control (IsC) (CFSE-labeled) were injected i.v. (10<sup>7</sup>/mouse) into male mice that had received an i.p. injection of IFN-γ 48 hours earlier. The presence of fluorescently labeled cells in the peritoneal cavity **(C)** and membrane **(D)** was assessed 24 hours later as described for **A** and **B**. The mean T cell number ± SEM observed in samples from at least 3 animals is shown. **C:** \**P* < 0.02 versus WT + IsC; **D:** \**P* < 0.02 versus WT + IsC.

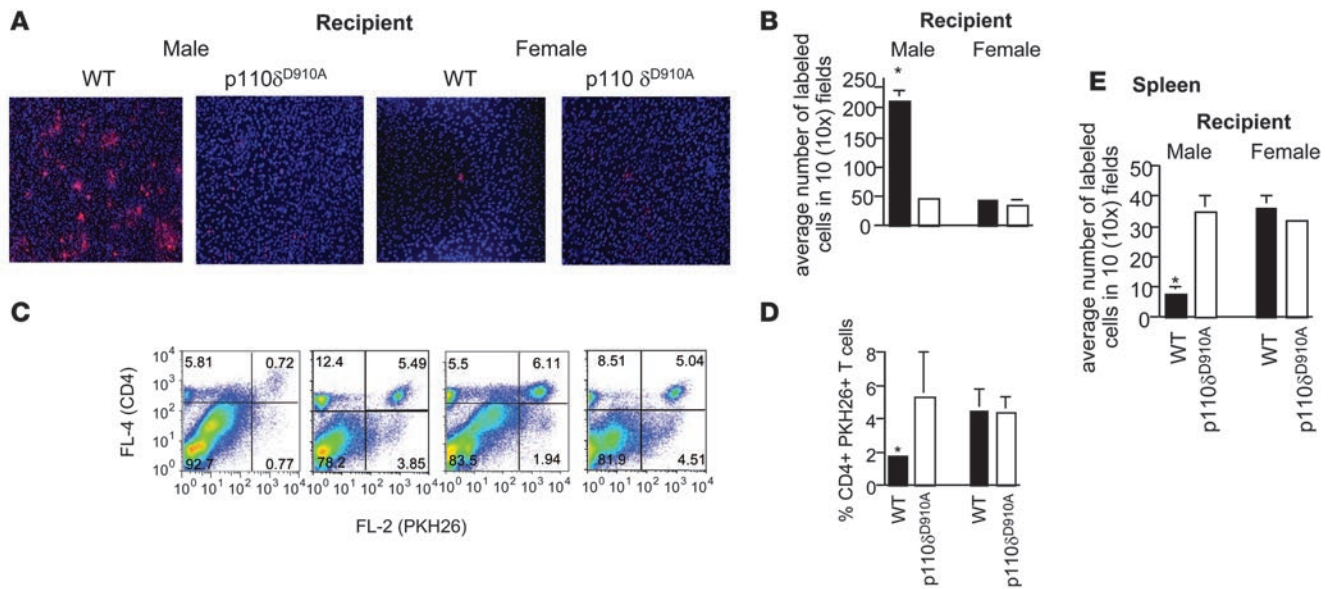
lowing exposure to these antibodies for 72 hours in vitro. In these experiments, the increase in migration in response to CXCL12 was quite low, possibly due to the low expression of CXCR4, but p110δ and WT T cells responded similarly.

As chemokine-induced T cell adhesion and subsequent transendothelial migration is regulated by shear flow (23), the potential contribution of PI3K p110δ activity to T cell migratory response to chemokines in vivo was investigated. HY-specific PKH26-labeled CD4<sup>+</sup> WT or p110δ<sup>D910A</sup> T cells (10<sup>7</sup>/mouse) were injected i.v. into syngeneic female mice that had received an i.p. injection of CXCL10 (1,200 ng/mouse) 1 hour earlier. CXCL10 was used in these experiments because of the high level of expression of its counterreceptor CXCR3 by both WT and p110δ<sup>D910A</sup> T cells (Supplemental Figure 1) and because CXCL10 elicited strong chemotactic responses in vitro. Control mice received an equal volume of saline solution i.p. T cell migration to the peritoneal cavity was assessed 6 hours later by flow cytometric analysis of the peritoneal lavage. As shown in Figure 2, G–K, WT and p110δ<sup>D910A</sup> CD4<sup>+</sup> T cells localized to the peritoneal cavity in response to chemokine challenge with similar efficiency. In line with previous observations (15, 16), these data suggest that PI3K p110δ activity is not required for constitutive or chemokine-induced T cell migration and that the machinery required to leave the circulation and cross the endothelial barrier in response to a chemokine gradient is intact in p110δ<sup>D910A</sup> T cells.

*PI3K p110δ activity is essential for antigen-dependent T cell recruitment.* We and others have previously reported that antigen displayed by the endothelium contributes to the recruitment of specific T cells into the antigenic tissue. As PI3K activity is regulated by TCR signaling (9) and has the potential to regulate cytoskeletal

rearrangements (24), we sought to investigate whether its activity contributes to antigen-dependent T cell recruitment. To explore this, the effect on migration after cognate recognition of syngeneic WT male- or female-derived endothelial cells by HY-specific WT and p110δ<sup>D910A</sup> T cells on their migration was first compared using an in vitro transendothelial migration assay that we have previously described (25). T cells were purified on a Ficoll gradient and seeded onto IFN-γ-treated EC monolayers (derived from female or male WT mice) grown on a Transwell insert, and the number of transmigrated T cells recovered in the bottom well was monitored hourly. As shown in Figure 3, A–C, WT HY-specific T cells displayed a much greater rate of migration through MHC class II H2-A<sup>b</sup>-expressing male-derived ECs (Figure 3A). By contrast, migration of p110δ<sup>D910A</sup> T cells through male-derived EC monolayers was not enhanced when compared with the migration through female-derived ECs (Figure 3B). The male and female EC monolayers mediated comparable levels of migration of third-party control MHC class I-restricted CD8<sup>+</sup> T cells (HY-specific K<sup>k</sup>-restricted C6 T cells; Figure 3C).

To assess the physiological relevance of the dependence of p110δ on antigen-mediated transendothelial migration, we compared the recruitment of HY-specific CD4<sup>+</sup> WT and p110δ<sup>D910A</sup> T cells from the circulation into the peritoneum of male and female WT mice. In this model, HY-specific T cells localize in the peritoneal membrane and cavity of male but not female mice following induction of local MHC molecule upregulation (and subsequently antigen presentation) by i.p. injection of IFN-γ (4). PKH26-labeled WT or p110δ<sup>D910A</sup> H2-A<sup>b</sup>-restricted CD4<sup>+</sup> T cells (10<sup>7</sup>/mouse) were injected i.v. into male or female WT C57BL/6 mice that had received an



**Figure 5**

PI3K p110δ is required for tissue infiltration by antigen-specific T cells. Female and male C57BL/6 mice were treated i.p. with 600 U IFN-γ. After 48 hours, 3 × 10<sup>6</sup> PKH26-labeled WT or p110δ<sup>D910A</sup> HY-specific H2-A<sup>b</sup>-restricted CD4<sup>+</sup> T cells (10<sup>7</sup>/mouse) were injected i.p. The presence of labeled T cells in the peritoneal membrane and lavage was analyzed after 24 hours by wide-field fluorescence microscopy (A and B) and flow cytometry (C and D), respectively. In addition, the presence of labeled cells in the spleen was quantified by wide-field fluorescence microscopy (E). Original magnification, ×10. Tissue infiltration was quantified by randomly selecting ten ×10-magnified fields and assessing the number of fluorescent cells in each field. The mean values ± SD observed in samples from at least 3 animals are summarized in B and E (infiltration of the peritoneal membrane and spleen, respectively) and D (cells retrieved in the peritoneal lavage). B and D: \*P < 0.004 versus p110δ<sup>D910A</sup>; E: \*P < 0.02 versus p110δ<sup>D910A</sup>.

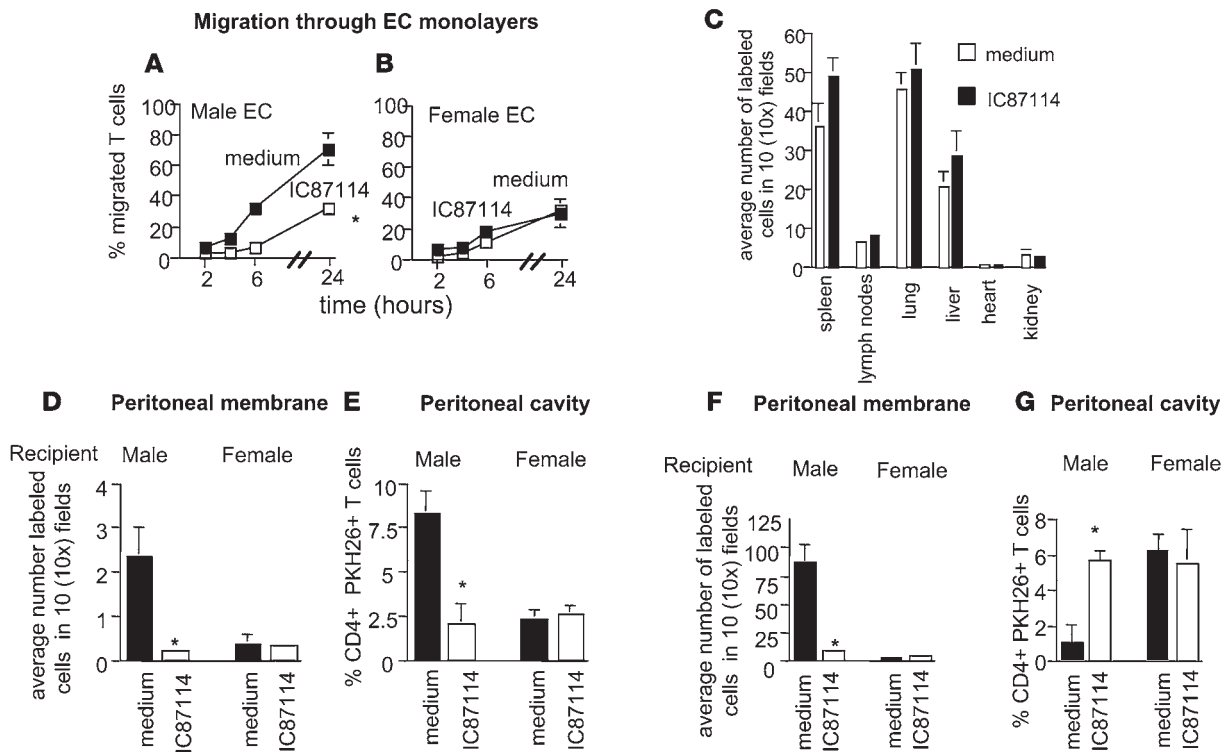
i.p. injection of IFN-γ (600 U) 48 hours earlier. Significant amounts of WT T cells were detected in the peritoneal membrane (Figure 3D) and cavity (Figure 3E) of male mice. By contrast, HY-specific p110δ<sup>D910A</sup> T cells failed to localize to the peritoneal membrane and were not found in the peritoneal lavage of IFN-γ-treated male mice. As expected, neither WT nor p110δ<sup>D910A</sup> T cells migrated the peritoneal membrane or cavity of IFN-γ-treated female mice. Similar observations were made when TCR-dependent recruitment of HY-specific CD8<sup>+</sup> T cells was analyzed, suggesting that PI3K p110δ activity is required for antigen-driven localization of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Supplemental Figure 3).

*TCR-induced PI3K recruitment selectively mediates antigen-dependent T cell localization.* We have previously shown that the PI3K binding motif in CD28 is necessary for the efficient localization of CD8<sup>+</sup> memory T cells to nonlymphoid tissue (22). However, this is also required for Grb2 and Gads binding, and hence a requirement of PI3K had been implied but not proven. Constitutive low expression of CD80 molecules by murine endothelium provided an opportunity to discriminate the relative contribution of TCR- and CD28-induced PI3K activity in antigen-dependent T cell localization. To test the role of PI3K specifically in the context of CD28 signaling, we used HY-specific H2-A<sup>b</sup>-restricted CD4<sup>+</sup> CD28<sup>Y170F</sup> T cells (which carry a mutation in the cytoplasmic tail of CD28 that abrogates PI3K recruitment without leading to defects in clonal expansion; ref. 26) and compared their responses to those by p110δ<sup>D910A</sup> T cells, where PI3K signaling is uncoupled from both TCR and CD28 signaling.

First, localization of HY-specific WT, p110δ<sup>D910A</sup>, and CD28<sup>Y170F</sup> CD4<sup>+</sup> T cells to the peritoneal membrane and cavity of male mice was compared by the antigen-dependent peritoneal recruitment

model described above. PKH26-labeled HY-specific CD4<sup>+</sup> T cells derived from WT, CD28<sup>Y170F</sup>, or p110δ<sup>D910A</sup> T cells (10<sup>7</sup>/mouse) were injected i.v. into male C57BL/6 mice that had received an i.p. injection of IFN-γ (600 U) 72 hours earlier. Infiltration of the peritoneal membrane by labeled T cells and enrichment of HY-specific T cells in the peritoneal lavage were assessed 24 hours later as described for the experiments in Figure 3, D and E. WT T cells were readily identified from the peritoneal membrane and in the peritoneal lavage, demonstrating efficient recruitment from the circulation. The recruitment of HY-specific CD28<sup>Y170F</sup> CD4<sup>+</sup> T cells to the peritoneal cavity of male mice was moderately impaired, whereas the recruitment of p110δ<sup>D910A</sup> T cells was virtually undetectable (Figure 4, A and B). In line with our previous findings, these observations suggest that CD28- and TCR-mediated PI3K activation can independently regulate T cell migration.

We have previously shown that anti-CD28 can stimulate trafficking of T cells into nonlymphoid tissues and that this was dependent on the tyrosine within a YNM motif in the cytoplasmic domain of CD28 (13). As this tyrosine can bind the SH2 domains of Grb2 and Gads in addition to the SH2 domains of p85, we sought to independently determine whether PI3K p110δ is required for CD28-dependent trafficking. To this end, HY-specific CD4<sup>+</sup> WT or p110δ<sup>D910A</sup> T cells (10<sup>7</sup>/mouse) that had either undergone CD28 ligation (PKH26-labeled) or had been pretreated with an isotype control antibody (CFSE-labeled) were coinjected i.v. into male C57BL/6 mice that had received an i.p. injection of IFN-γ (600 U) 72 hours earlier, as previously described (13). Tissue infiltration by labeled T cells and enrichment of HY-specific T cells in the peritoneal lavage were assessed 24 hours later as described



**Figure 6** Pharmacologic inhibition of PI3K p110 $\delta$  prevents antigen-dependent T cell localization to antigenic sites. HY-specific H2-A<sup>b</sup>-restricted CD4<sup>+</sup> T cells were treated with 5  $\mu$ M IC87114 or with medium containing the vehicle (1% DMSO) for 30 minutes at room temperature and subsequently washed before use. (A and B) Untreated (filled symbols) and treated (open symbols) T cells ( $5 \times 10^5$ /well) were seeded onto syngeneic female- or male-derived IFN- $\gamma$ -treated EC monolayers. T cell migration was monitored as described in Figure 1. The percentage of migrated T cells at the given time points is reported as the average of 3 experiments of identical design. \* $P < 0.05$ , except at 2 and 4 hours. (C) PKH26-labeled HY-specific untreated (black bars) or IC87114-treated (white bars) T cells were injected i.v. into syngeneic female mice. Infiltration of the indicated tissues was assessed 24 hours later as described in Figure 1, B and C. The mean T cell infiltration  $\pm$  SD observed in samples from at least 3 animals is shown. (D–G) Female and male C57BL/6 mice were treated i.p. with 600 U IFN- $\gamma$ . After 48 hours,  $10^7$  labeled untreated (black bars) or IC87114-treated (white bars) HY-specific T cells were injected i.v. ( $10^7$  T cells/mouse, D and E) or i.p. ( $3 \times 10^6$  T cells/mouse, F and G). The presence of labeled T cells in the peritoneal membrane and cavity was analyzed after 24 hours as described in the legends to Figures 3 and 5, respectively. The mean values  $\pm$  SD observed in samples from at least 3 animals are shown. \* $P < 0.02$  (D), \* $P < 0.05$  (E), \* $P < 0.02$  (F), \* $P < 0.03$  (G) versus untreated T cells.

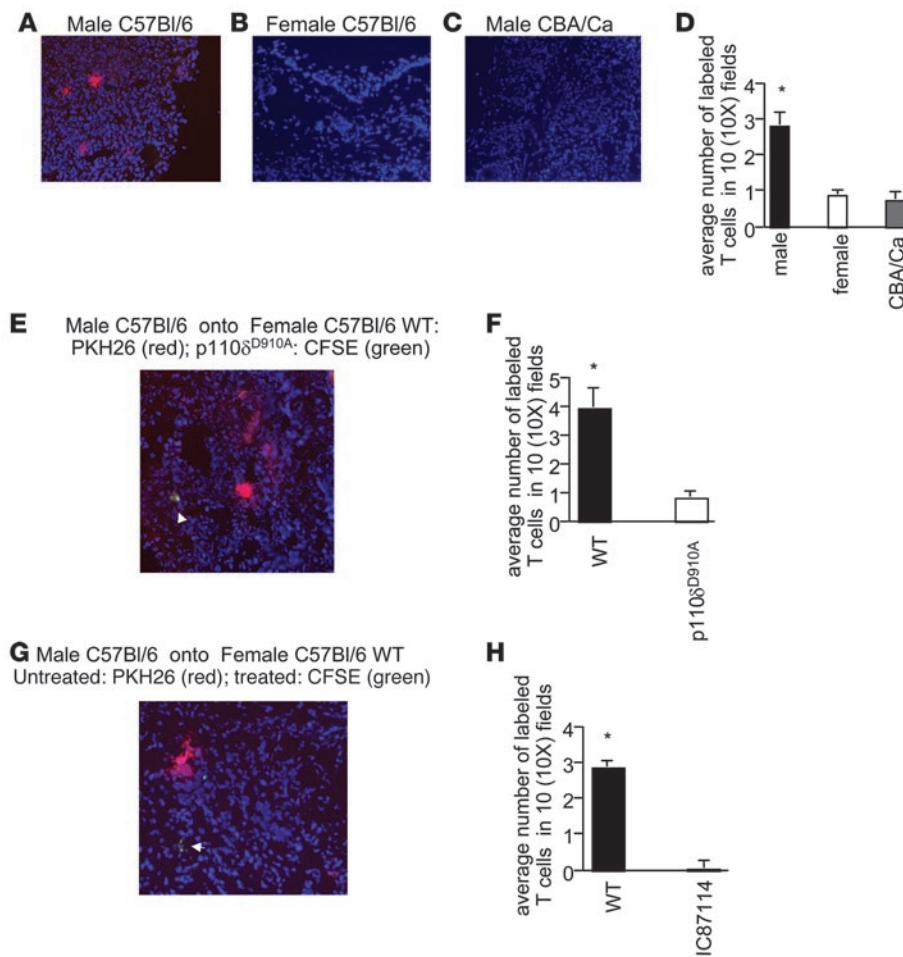
above and in Methods. CD28 ligation significantly enhanced the recruitment of WT T cells, which were detected in the peritoneal membrane and lavage (Figure 4, C and D). By contrast, peritoneal recruitment of HY-specific p110 $\delta^{D910A}$  T cells was not reconstituted by CD28 triggering. Isotype control antibody-treated and untreated T cells displayed similar localization (data not shown). These results show that CD28 through its YNM motif signals via p110 $\delta$  to promote tissue infiltration.

*PI3K p110 $\delta$  activity is instrumental in retention of antigen-specific T cells into antigenic tissue.* Once T cells have reached the antigenic sites, they are retained in the tissue by mechanisms involving antigen presentation by resident antigen-presenting cells (27). To investigate the contribution of TCR-induced PI3K p110 $\delta$  activity on this effect, we used an established model of antigen-dependent tissue infiltration (22, 25). In this model, tissue infiltration is strictly dependent on recognition of HY antigen within the peritoneal tissue. As in the model described above, male or female C57BL/6 mice were injected with 600 U IFN- $\gamma$ . Two days later, PKH26-labeled, HY-specific WT or p110 $\delta^{D910A}$  T cells ( $3 \times 10^6$  cells/mouse) were injected directly into the peritoneum. The fol-

lowing day mice were sacrificed, and T cells that had infiltrated the peritoneal membrane or remained in the peritoneal lavage were detected by wide-field fluorescence microscopy and flow cytometry, respectively. HY-specific WT T cells efficiently localized into the peritoneal membrane of male recipients (Figure 5, A and B) and were depleted from the peritoneal cavity (Figure 5, C and D), whereas only a few p110 $\delta^{D910A}$  T cells could be detected in the peritoneal membrane (Figure 5, A and B) and were instead readily recovered from the peritoneal lavage of male recipients (Figure 5, C and D).

As expected, HY-specific WT and p110 $\delta^{D910A}$  T cells were not detected in the peritoneal membrane but were readily recovered from the peritoneal cavity of female recipients. In female recipient mice, the few T cells that had entered the peritoneum also entered the general circulation as evidenced by their recovery from the spleens (Figure 5E). By contrast, in male recipients, few WT T cells could be recovered from the spleens, reflecting their retention in the peritoneal wall. In contrast, p110 $\delta^{D910A}$  T cells were readily detected in the spleens of male recipient mice as well. We conclude that p110 $\delta$  controls both the recruitment and retention





### Figure 7

Lack of PI3K p110 $\delta$  activity prevents T cell localization into HY-mismatched skin grafts. C57BL/6 female mice received 3 skin grafts: one from a syngeneic male mouse (A), one from a syngeneic female mouse (B), and the third from a CBA/Ca male mouse (C). Four days following grafting, HY-specific A<sup>b</sup>-restricted WT (PKH26-labeled) CD4<sup>+</sup> T cells (10<sup>7</sup>/mouse), were injected i.v. Graft infiltration by labeled T cells was assessed 24 hours later by wide-field fluorescence microscopy, as described in the legend to Figure 1, C and D. Original magnification,  $\times 10$ . The mean values  $\pm$  SD observed in samples from at least 3 animals are summarized in D. \* $P < 0.03$  versus female and CBA/Ca graft infiltration. Parallel experiments were run in which C57BL/6 female mice engrafted with C57BL/6 male-derived skin 4 days earlier were co-injected with either WT (PKH26-labeled, red) or p110 $\delta^{\text{D910A}}$  (CFSE-labeled, green) HY-specific CD4<sup>+</sup> T cells (E and F) or with untreated (PKH26-labeled, red) or IC87114-treated (CFSE-labeled, green) HY-specific CD4<sup>+</sup> T cells (G and H). Grafts were removed 24 hours later, and tissue infiltration was quantified by randomly selecting ten  $\times 10$ -magnified fields from tissue samples from at least 3 animals and assessing the number of fluorescent cells in each field. The few CFSE-labeled cells are indicated by an arrowhead. Nuclei are stained by DAPI (blue). A–C show representative  $\times 10$ -magnified tissue images, while the images shown in E and G were taken at a  $\times 20$  magnification. The mean T cell infiltration  $\pm$  SD observed in samples from at least 3 animals is shown; \* $P < 0.03$  (F); \* $P < 0.01$  (H).

of T cells in nonlymphoid tissues without affecting passive entry into the general circulation. These findings raise the possibility that p110 $\delta$ -mediated signals may be required for the establishment of antigen-dependent stable interactions with conventional antigen-presenting cells.

*Pharmacological inhibition of PI3K p110 $\delta$  abrogates antigen-dependent T cell recruitment and retention into antigenic tissue.* Having established its role in mediating TCR-dependent T cell recruitment, we sought to investigate the effectiveness of pharmacological inhibition of p110 $\delta$

activity on this effect. The IC87114 compound has been reported to selectively inhibit p110 $\delta$  at doses up to 5–10  $\mu\text{M}$  both in vitro and in vivo (28). In all the experiments described below, control T cells were treated with PBS containing vehicle (1% DMSO).

First we carried out studies to establish whether IC87114 might affect the viability of ECs or their ability to mediate migration of T cells. IC87114-treated ECs did not display increased cell death or permeability following exposure to up to 5  $\mu\text{M}$  of the inhibitor for 30 minutes to 24 hours (data not shown). In addition, compared with untreated endothelium, female-derived, IFN- $\gamma$ -treated ECs were equally capable of mediating migration of HY-specific T cells following exposure of ECs to 5  $\mu\text{M}$  IC87114, suggesting that EC function was not adversely affected by inhibition of PI3K p110 $\delta$  activity (data not shown). Nonetheless, in order to exclude effects that IC87114 might have on other cell types, T cells were treated with the inhibitor and subsequently washed prior to use. In addition, exposure of T cells to the inhibitor (5  $\mu\text{M}$ ) for up to 48 hours did not affect their viability (data not shown).

Migration of untreated and IC87114-treated HY-specific T cells through IFN- $\gamma$ -treated EC monolayers derived from C57BL/6 male or female mice and grown on a Transwell insert in vitro was compared. As shown in Figure 6, A and B, inhibition of PI3K p110 $\delta$  prevented antigen-induced enhancement of HY-specific T cell migration, while it did not affect T cell migration through female-derived ECs.

To assess the effect of pharmacological inhibition of PI3K p110 $\delta^{\text{D910A}}$  on constitutive T cell trafficking in vivo, we compared the recruitment of HY-specific untreated and IC87114-treated T cells from the circulation into various tissues as described in Figure 1. PKH26-labeled untreated or IC87114-treated (5  $\mu\text{M}$ , 1 hour at 37 $^{\circ}\text{C}$ ) H2-A<sup>b</sup>-restricted CD4<sup>+</sup> T cells (10<sup>7</sup>/mouse) were injected i.v. into C57BL/6 WT female mice. Similar numbers of T cells localized in lymphoid or different nonlymphoid tissues, suggesting that pharmacological inhibition of PI3K p110 $\delta$  does not affect constitutive memory T cell trafficking (Figure 6C).

Next, to assess the effect of pharmacological inhibition of PI3K p110 $\delta^{\text{D910A}}$  on antigen-dependent T cell localization to antigenic sites in vivo, we compared the recruitment of HY-specific untreated and IC87114-treated T cells from the circulation into the



peritoneum of male and female mice as described above. PKH26-labeled untreated or IC87114-treated (5  $\mu$ M, 1 hour at 37°C, CFSE-labeled) H2-A<sup>b</sup>-restricted CD4<sup>+</sup> T cells (10<sup>7</sup>/mouse), were injected i.v. into male or female C57BL/6 mice that had received an i.p. injection of IFN- $\gamma$  (600 U) 48 hours earlier to induce local HY antigen presentation in male recipients. As shown in Figure 6, D and E, significant numbers of untreated T cells were detected in the peritoneal membrane and cavity of male mice. By contrast, HY-specific IC87114-inhibited T cells failed to localize to the peritoneal membrane and lavage of male mice. As expected, neither untreated nor IC87114-treated T cells localized in the peritoneal membrane and lavage of IFN- $\gamma$ -treated female mice.

Finally, the effect of pharmacological inhibition of p110 $\delta$  activity on tissue infiltration by HY-specific CD4<sup>+</sup> T cells was investigated by experiments similar to those described in Figure 5. T cells were treated with IC87114 prior to i.p. injection into male and female C57BL/6 mice that had received an i.p. injection of IFN- $\gamma$  (600 U) 48 hours earlier. As shown in Figure 6, F and G, pharmacologic inactivation of PI3K p110 $\delta$  dramatically inhibited tissue infiltration by HY-specific T cells in male mice, which remained localized in the peritoneal cavity.

Taken together, these observations suggest that selective pharmacological inhibition of PI3K p110 $\delta$  activity is effective in preventing the recruitment of antigen-specific T cells and their localization into target tissue.

*Genetic and pharmacological inactivation of PI3K p110 $\delta$  activity prevents HY-specific T cell infiltration of HY-mismatched skin grafts.* Having established that p110 $\delta$ -mediated signals are required for antigen-dependent T cell recruitment into – and infiltration of – antigenic nonlymphoid tissue, we investigated the effect of PI3K p110 $\delta$  inactivation on the development of HY-specific T cell-mediated inflammation in HY-mismatched skin grafts.

First, we assessed the effect that antigen display exerts on T cell localization in this model. C57BL/6 female mice received 3 skin grafts: one from a syngeneic female mouse, one from a syngeneic male mouse (usually rejected between days 19 and 31 in WT female mice), and the third from a CBA/Ca male mouse (usually rejected by day 12). Four to 9 days following grafting, mice were injected i.v. with PKH26-labeled HY-specific CD4<sup>+</sup> T cells (10<sup>7</sup>/mouse), and T cell infiltration in the grafts was assessed the following day (after sacrifice). It has to be noted that CBA/Ca-derived grafts bear MHC molecules of the H2-K haplotype, while the HY-specific T cells used here are H2-A<sup>b</sup> restricted. As shown in Figure 7, A–D, HY-specific T cells selectively infiltrated antigenic (male) grafts, suggesting that antigen location plays a major role in determining primed T cell localization. Graft infiltration was assessed 24 and 48 hours after T cell transfer with similar results (data not shown).

Next, we investigated the effect of p110 $\delta$  inactivation on the infiltration of HY-mismatched skin grafts by HY-specific H2-A<sup>b</sup>-restricted CD4<sup>+</sup> T cells. C57BL/6 female mice that had received syngeneic male mouse skin graft were coinjected i.v. with PKH26-labeled, HY-specific WT CD4<sup>+</sup> T cells (10<sup>7</sup>/mouse) together with either CFSE-labeled HY-specific p110 $\delta$ <sup>D910A</sup> T cells (Figure 7, E and F) or CFSE-labeled IC87114-pretreated (5  $\mu$ M, 1 hour at 37°C; Figure 7, G and H) WT T cells. Genetic (Figure 7, E and F) or pharmacological (Figure 7, G and H) inhibition of PI3K p110 $\delta$  strongly inhibited male skin graft infiltration by HY-specific CD4<sup>+</sup> T cells (the very few green-labeled cells are indicated by arrows in the figure). No differences were observed in T cell infiltration of female-derived skin grafts, which was virtually absent (data not shown).

## Discussion

TCR-derived signals can determine the fate of T cells in many ways. Antigen stimulates T cells to divide and differentiate or, alternatively, to become anergic. TCR-derived signals are also instrumental for directing T cells to target tissue where antigen can be found. The outcome of TCR signaling is also influenced by other receptor systems. For example, the delivery of costimulatory signals determines whether T cells expand or become unresponsive, whereas TCR triggering in the presence of certain cytokines induces the development of different T helper cell subsets. Thus, extrinsic factors, such as the microenvironment in which antigen presentation takes place and the type of antigen-presenting cell, help influence the outcome of TCR signaling. Here we present evidence that in addition to regulating T cell proliferation and differentiation, PI3K p110 $\delta$  activity is required for TCR-dependent localization of T cells to tissues that play host to cognate antigens.

Although PI3Ks have been suggested to play a key role in chemotaxis of *Dictyostelium* and neutrophils, PI3Ks are not major regulators of chemotaxis in T cells (15–17). Where PI3K do play a role, it is the class IB PI3K p110 $\gamma$  that is required (~10% of the total response) (15). The main regulator of chemotaxis in T cells is DOCK2, which appears, to a large extent, to regulate migration independently of PI3Ks (15).

In line with previous observations (16), our data show that primed T cell lacking PI3K p110 $\delta$  display intact constitutive trafficking and chemotactic responses both in vitro and in vivo; however, they fail to localize to antigenic tissue, suggesting that PI3K p110 $\delta$  is required for T cell migration to antigenic sites independently of chemokine signaling. This effect does not appear to be subset restricted, as both CD4<sup>+</sup> and CD8<sup>+</sup> memory T cells display similar requirements for p110 $\delta$  activity to be recruited in an antigen-dependent manner. In this context, class I PI3K regulatory subunit p85 has been shown to contribute to spontaneous lymphocyte motility in the lymph node (18). As PI3Ks have been implicated in signaling pathways leading to integrin activation, it is possible that p85 activity may be required for efficient cell-cell interactions in the lymph nodes. Alternatively, as it has been suggested that PI3K requirement for lymphocyte migration may depend on their differentiation as well as the microenvironment in which T cell locomotion takes place (14), it is possible that the requirement for class I activity may differ between naive T cells in the LN and differentiated T cells migrating to nonlymphoid tissue (as those used in the present study).

Although our data do not support a role for PI3K p110 $\delta$  activity in TCR-independent chemokine-induced responses by memory T cells, TCR-driven T cell trafficking has previously been shown to be enhanced by the concomitant delivery of chemokine-mediated signals (5, 29). The complete abrogation of TCR-dependent T cell migration in the absence of PI3K p110 $\delta$  activity as observed in this study suggests that this activity may precede and may be required for the subsequent contribution of chemokine-initiated signaling pathways (mediated by either by DOCK2 or p110 $\gamma$ ). In this context, a direct cross-talk between TCR- (mediated by zeta-associated protein 70 [ZAP-70]) and chemokine receptor-mediated signaling has recently been shown to be essential for chemokine-induced T cell migration (30).

We have recently shown that CD28-mediated signals are required for primed T cells to access nonlymphoid tissue (22). As murine endothelium expresses CD80 (but not CD86) molecules (31),



we sought to discriminate the relative contribution of TCR- and CD28-dependent PI3K activation to the regulation of primed T cell recruitment. In line with our previous observation, TCR- and CD28-mediated effects on T cell migration can act independently. However, our data suggest that both receptors rely on P110 $\delta$  activity to exert their effects. Therefore, it is likely that inhibition of PI3K P110 $\delta$  activity would prevent antigen-dependent recruitment of T cells by human endothelium, which does not express either CD80 or CD86 molecules.

Finally, our results show that selective PI3K p110 $\delta$  inhibitors have the potential to reduce access and retention of specific T cells into different target tissues. Small molecule inhibitors against p110 $\delta$  are currently being developed with the aim of alleviating autoimmune and inflammatory diseases (32). Our observation that pharmacological p110 $\delta$  inhibition prevents TCR-dependent T cell recruitment to skin grafts may be relevant to autoimmune diseases such as psoriasis and paves the way for a wider application of this strategy for a variety of T cell-mediated pathologies, including autoimmunity and transplantation. These inhibitors may prevent antigen-dependent T cell migration and subsequently cell-cell interactions without inducing overt immune suppression, thus representing a novel and desirable target for the regulation of undesired T cell inflammation.

## Methods

**Mice.** C57BL/6 mice aged 4–8 weeks were purchased from Olac Harlan and used at the age of 8 weeks. CD28<sup>Y170F</sup> and p110 $\delta$ <sup>D910A</sup> mice were generated as previously described (10, 26, 33, 34). Experiments with animals were performed under the Home Office authority (PPL 70/5872) and approved by the local ethics committee, Hammersmith Hospital.

**mAbs and intravital dyes.** APC-conjugated anti-mouse CD4 was obtained from Caltag Laboratories. Anti-mouse CD28 37.51, hamster Ig and rabbit anti-hamster Ig were purchased from BD Biosciences. All the other antibodies used in this study were purchased from BD Biosciences. The p110 $\delta$ -selective inhibitor IC87114 was kindly provided by Joel Hafflick (ICOS Corp., Bothell, Washington, USA). The cell linker PKH26 and CFSE were purchased from Sigma-Aldrich.

**ECs.** Murine microvascular ECs were purified and cultured from murine lung tissue as previously described (31). For functional assays, the ECs were used between passages 4 and 6 and treated with 300 U/ml murine IFN- $\gamma$  (PeproTech) for 72 hours to induce MHC class II expression (data not shown) prior to use in experiments.

**T cells.** Memory CD4<sup>+</sup> T cells specific for the male-specific minor transplantation antigen HY peptide epitope NAGFNSNRANSSRSS and restricted by H2-A<sup>b</sup> (35) were obtained from WT, p110 $\delta$ <sup>D910A</sup>, and CD28<sup>Y170F</sup> mice by 2 fortnightly i.p. immunizations of female mice with male splenocytes, as previously described (36), and further expanded by stimulation with male C57BL/6 splenocytes and rIL-2 (20 U/ml; Roche). T cell specificity was determined by [<sup>3</sup>H]thymidine incorporation and IFN- $\gamma$  production (by intracellular staining; data not shown). T cells were harvested 2 weeks after stimulation, purified by isolation on a Ficoll-Paque gradient (Amersham Biosciences), and incubated in medium alone overnight. The phenotype of T cells at the time of injection is shown in Supplemental Figure 1. They are designated HY-specific CD4<sup>+</sup> memory T cells herein.

**Lymphocyte migration assays and chemotaxis assays.** The transendothelial migration experiments were carried out using HUVEC monolayers ( $2 \times 10^4$  cells/well) grown on fibronectin-coated (10  $\mu$ g/ml; Sigma-Aldrich) Costar Transwell tissue culture well inserts (diameter 6.5 mm) mounted with polycarbonate membranes with a 3- $\mu$ m-pore size (Costar Ltd.), as previously

described (25). T cells ( $5 \times 10^5$ /well) were added into each insert and left to migrate through the monolayers. The number of migrated T cells was determined by hemocytometric counting of the cells present in the well media at different time points over the next 24 hours. Results are expressed as percentage of transmigrated cells.

For time-lapse microscopy migration assays, 35-mm dishes were coated with recombinant ICAM-1 (2  $\mu$ g/ml; R&D Systems) in PBS and incubated at 4°C overnight. The plate was subsequently blocked with PBS containing 2.5% BSA at 37°C for 1 hour and washed with 0.5% BSA/PBS. WT and p110 $\delta$ <sup>D910</sup> T cells were serum starved in migration medium for 2 hours and subsequently seeded on the ICAM-1-coated dishes at a concentration of  $1 \times 10^6$ /ml/dish and incubated at 37°C for 5–30 minutes. Plates were washed once to remove nonadherent cells. The migration of WT and p110 $\delta$ <sup>D910</sup> T cells was observed by time-lapse microscopy using Tempus software (Kinetic Imaging Ltd.). Images of WT and p110 $\delta$ <sup>D910</sup> T cells were acquired with a KPM1E/K-S10 CCD camera (Hitachi Denshi) using Kinetic Imaging software (Andor Technology) every 15–30 seconds for 25–50 minutes. The path of each cell was tracked for the whole of the time-lapse sequence using Tempus Meteor software (Andor Technology). Analysis of migration speed was then carried out using Mathematica 6.0 (Wolfram Research Institute) notebooks (37).

For chemotaxis assays, T cells were seeded ( $5 \times 10^5$  to  $10 \times 10^5$ /well) in the upper chamber of a 5- $\mu$ m-pore polycarbonate Transwell (Costar). A 0.5-ml volume of chemotaxis medium (RPMI 0.5% FCS) containing CXCL10 (300 ng/ml; PeproTech), CXCL12 (50 ng/ml; PeproTech), or CCL5 (100 ng/ml; PeproTech) was added to the bottom chamber of the Transwell, while 0.2 ml of cell suspension was added to the top chamber. Transwells were incubated for 6 hours at 37°C with 5% CO<sub>2</sub>. The number of migrated cells was evaluated as described above.

**Skin grafting.** Skin grafting was conducted by the method of Billingham and Medawar (38) using tail skin grafted onto the lateral thorax. Grafts were harvested 24 hours after i.v. injection of T cells at the indicated time points.

**Recruitment of circulating T cells into tissues.** Labeled T cells ( $10^7$ /mouse) were injected i.v. or i.p. ( $3 \times 10^6$ /mouse). For labeling, PKH26 and CFSE were added at a final concentration of 5  $\mu$ M and 1  $\mu$ M, respectively. T cells were incubated at 37°C for 30 minutes, then washed 3 times with PBS before injection. After 24 hours, mice were sacrificed and tissues were sampled and embedded in optimal cutting temperature compound (O.C.T.; Agar Scientific Ltd.), snap frozen, and stored until analysis. Tissue infiltration by T cells was assessed by wide-field fluorescence microscopy. In the peritoneal recruitment and infiltration models, labeled T cells were injected either i.v. or i.p. into mice that had received an i.p. injection of IFN- $\gamma$  (600 U/ml; PeproTech) 48 hours earlier. Infiltration of the peritoneal membrane by labeled T cells and the enrichment of labeled T cells in the peritoneal lavage were assessed 24 hours later by wide-field fluorescence microscopy and by cytofluorimetric analysis, respectively. In experiments assessing chemokine-induced migration, syngeneic female mice received an i.p. injection of CXCL12 (1,200 ng) 2 hours prior to being injected with labeled T cells ( $10^7$ /mouse).

**Wide-field fluorescence microscopy and flow cytometry.** Peritoneal membranes or frozen tissue sections were laid onto Polysine Microscope slides (VWR International), left to dry overnight, and then mounted in Vectashield mounting medium for fluorescence with DAPI (Vector Laboratories), to visualize the nuclei. Slides were visualized with a Coolview 12-cooled CCD camera (Photonic Science) mounted over a Zeiss Axiovert S100 microscope equipped with Metamorph software (Zeiss).  $\times 10$  and  $\times 20$  NA 0.6 objectives and standard epi-illuminating fluorescein and rhodamine fluorescence filter cube were used, and 12-bit image data sets were generated. Tissue infiltration was quantified by randomly selecting ten  $\times 10$ -magnified fields



and assessing the number of fluorescent cells in each field, as previously described (25). Quantification of T cell infiltrates observed by wide-field fluorescence microscopy was performed using a specifically designed software to run in the LabView (version 7.1; National Instruments) environment. This automatic cell counting algorithm is based on a combination of background subtraction, multiple thresholding, and morphological processing approaches (22), which allows identification of single fluorescent cells within the tissue.

The numbers of infiltrating cells obtained (10/sample) were then averaged and assessed statistically. Infiltration is expressed as the mean of fluorescent cells per  $\times 10$  field in a given experimental condition  $\pm$  SD.

The presence of labeled cells in the peritoneal lavage was analyzed by flow cytometry using a FACSCalibur (BD) and FlowJo version 7.1.2 software (Tree Star Inc.).

**Statistics.** In the in vitro experiments, comparisons between groups were made using 2-tailed Student's *t* test. In the in vivo experiments, the Mann-Whitney *U* test was used. All reported *P* values are 2-sided. *P* values of less than 0.05 were considered significant.

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