PI3K p110δ Is Expressed by gp38−CD31+ and gp38+CD31+ Spleen Stromal Cells and Regulates Their CCL19, CCL21, and LTβR mRNA Levels

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Abstract

The role of p110δ PI3K in lymphoid cells has been studied extensively, showing its importance in immune cell differentiation, activation and development. Altered T cell localization in p110δ-deficient mouse spleen suggested a role for p110δ in non-hematopoietic stromal cells, which maintain hematopoietic cell segregation. We tested this hypothesis using p110δWT/WT mouse bone marrow to reconstitute lethally irradiated p110δWT/WT or p110δD910A/D910A (which express catalytically inactive p110δ) recipients, and studied localization, number and percentage of hematopoietic cell subsets in spleen and lymph nodes, in homeostatic conditions and after antigen stimulation. These analyses showed diffuse T cell areas in p110δD910A/D910A and in reconstituted p110δWT/WT mice in homeostatic conditions. In these mice, spleen CD4+ and CD8+ T cell numbers did not increase in response to antigen, suggesting that a p110δ WT stroma defect impedes correct T cell response. FACS analysis of spleen stromal cell populations showed a decrease in the percentage of gp38−CD31+ cells in p110δD910A/D910A mice. qRT-PCR studies detected p110δ mRNA expression in p110δWT/WT spleen gp38−CD31+ and gp38+CD31+ subsets, which was reduced in p110δD910A/D910A spleen. Lack of p110δ activity in these cell populations correlated with lower LTβR, CCL19 and CCL21 mRNA levels; these molecules participate in T cell localization to specific spleen areas. Our results could explain the lower T cell numbers and more diffuse T cell areas found in p110δD910A/D910A mouse spleen, as well as the lower T cell expansion after antigen stimulation in p110δD910A/D910A compared with p110δWT/WT mice.

Introduction

Secondary lymphoid organs (SLO) are sites of highly organized lymphoid cell accumulation, supported by a network of stromal cells. This network facilitates effective encounter and interaction between antigen-presenting cells and lymphocytes, maximizing effectiveness of the immune response to pathogens. Lymph nodes (LN) and spleen are the best-studied SLO. The spleen has two well-defined areas. In the red pulp, macrophage-lined venous sinuses filter damaged erythrocytes from the blood and allow surveillance of blood-borne pathogens and large antigens. The white pulp is a compartmentalized lymphoid area that is specialized in antigen presentation [1]. Within the white pulp, T and B lymphocytes are segregated into specific areas. Around the central arteriole, T cells are located in the periarteriolar lymphoid sheath (PALS or T cell zone), surrounded by the B cell zone (B cell follicles) [2]. Specific chemokines that attract T and B cells to their respective areas maintain correct organization of the white pulp [1]. The marginal zone (MZ) separates the red and white pulp and contains mainly phagocytic macrophages (marginal metallophilic macrophages (MMM)), marginal zone macrophages (MZ M), marginal zone B cells (MZ B) and DC [2]. In LN, naïve lymphocytes extravasate from the bloodstream through specialized blood vessels known as high endothelial venules (HEV). B and T cell areas surround HEV; B cell follicles are located in the outer cortex and T cells in the diffuse lymphoid tissue of the inner cortex, also known as paracortex [3].

Stromal cells maintain the microarchitectural organization of SLO, allowing correct immune cell movement and interaction, necessary for a protective immune response to pathogens. SLO stromal cells are divided into four populations, defined by gp38 (podoplanin) and CD31 expression. gp38−CD31+ cells (fibroblastic...
tic reticular cells; FRC) form a conduit network for antigen transport and support of immune cell migration, gp38\(^{\text{CD31}}\) cells (lymphatic endothelial cells; LEC) build lymph vessels, gp38\(^{\text{CD31}}\) cells (blood endothelial cells; BEC) construct cortical vessels and capillaries, including HEV in LN, and gp38\(^{\text{CD31}}\) cells (double-negative stromal cells; DN) are a bulk population that includes follicular dendritic cells (FDC) and extrathymic Air-expressing cells [3], [4]. These four populations are well characterized in LN, FRC, FDC, and BEC are also detected in spleen, where they are likely to have similar characteristics [5]. In mouse spleen, gp38\(^{\text{CD31}}\) LEC are reported to form lymphatic vessels [6] that originate around central arteries in the white pulp, join other deep lymphatic vessels that drain into trabeculae, and exit from the spleen hilum [7]. LEC in spleen lymphatic vessels are thought to participate in T cell migration, since lymphocytes within these vessels are CD3\(^{+}\) [7]. FRC and FDC secrete cytokines and chemokines and express adhesion molecules that modulate immune cell migration, homeostasis and survival [8], [9], [10].

In SLO, B/T lymphocyte localization and subsequent segregation depend on chemokines secreted by non-hematopoietic stromal cells [3], [4]. In homeostasis, primary B cell follicles contain FDC, which participate in B cell compartment organization and in antigen presentation to B cells. The FRC recruit B cells by secreting CXCL13, which binds to CXCR5 on B cells [11]. The FRC subset forms a network that structures the T cell area [12], [13]; FRC secrete CCL19 and CCL21, chemokines that attract CCR7-expressing T cells and DC to facilitate antigen encounter [8], [14], [15]. FRC constitute the conduit system that allows small antigens and chemokines to migrate to SLO B and T cell areas. Large antigens are excluded from this conduit and are trapped by APC in the spleen MZ or the LN subcapsular sinus. This system extends mainly through the T cell area and also reaches B cell follicles, although less densely [16]. CCL19 and CCL21 are also expressed by BEC and LEC [17].

Members of the TNF family of cytokines have a central role in lymphoid organ development and organization. Lymphotixin-\(\alpha\) (LT\(\alpha\)), lymphotixin-\(\beta\) (LT\(\beta\)) and tumor necrosis factor (TNF) have varying levels of importance in the development of most SLO [18]. Although lymphotixin signaling is not necessary for spleen generation, it is needed for red and white pulp segregation, for functional development of spleen white pulp [13], and for appropriate homing and maintenance of B/T segregation [19]. The LT receptor (LT\(\beta\)R) is expressed mainly by irradiation-resistant stromal cells; triggering of LT\(\beta\)R on these cells induces CXCL13 expression in B cell areas and CCL19 and CCL21 in T cell areas, via activation of the “non-canonical” I KK\(\alpha\)/NIK-dependent NF\(\kappa\)B pathway [20]. LT\(\beta\)-deficient mice have disorganized T cell zones; these defects are more severe in spleens of LT\(\alpha\)- and LT\(\beta\)R-deficient than LT\(\beta\)-deficient mice [19]. Impaired signaling through LT\(\beta\)R reduces spleen CXCL13, CCL19 and CCL21 levels, leading to disorganization of white pulp areas [21]. LT\(\alpha\) also contributes to lymphangiogenesis [22].

p110\(^{\delta}\) is a catalytic subunit of class I\(\alpha\) PI3K, together with p110\(^{\alpha}\) and p110\(^{\beta}\). It shares a catalytic domain with the other PI3K and binds to a regulatory subunit (p85\(\alpha\) or \(\beta\), p55\(\alpha\), p50\(\alpha\) or p30\(\gamma\)). p110\(^{\delta}\) is expressed preferentially in leukocytes, whereas p110\(^{\alpha}\) and p110\(^{\beta}\) are ubiquitous [23]; p110\(^{\delta}\) is also expressed in neurons [24], in some cancer cell lines [25], [26], and in endothelial cell lines [26], [27], [28]. p110\(^{\delta}\) has a central role in immune cell processes, including differentiation, activation and development of B and T cells [29], [30], [31], [32], [33], regulatory T cells [34], macrophages [35] and mast cells [36]. p110\(^{\delta}\) is also essential for generation of immune responses, both primary and secondary (memory) [37], [38]. Analysis of spleen sections shows a severe reduction in MZ B cells in p110\(^{\delta}\)-deficient mice [31]. Lack of p110\(^{\delta}\) or its kinase activity greatly impairs germinal center (GC) formation in the spleen after immunization; when these GC form, their size and structure are atypical [30], [31], [32], [39]. These defects in cell segregation and organization in p110\(^{\delta}\)-deficient mouse SLO suggests that p110\(^{\delta}\) is expressed in non-hematopoietic stromal cells and that it contributes to the maintenance of cell segregation and organization.

Given the lack of data on p110\(^{\delta}\) in SLO stromal cells, and on its role in homing and maintenance of B/T segregation, we studied p110\(^{\delta}\) expression and function in murine spleen and LN. We found p110\(^{\delta}\) is expressed in gp38\(^{\text{CD31}}\) and gp38\(^{\text{CD31}}\) spleen stromal cell subpopulations, where it regulates LT\(\beta\)R expression as well as CCL19 and CCL21 production; this suggests a role for p110\(^{\delta}\) in the control of T cell migration to appropriate spleen areas through the regulation of homeostatic chemokine production by stromal cells.

**Methods**

**Mice**

p110\(^{\delta}\)\(^{\text{WT/WT}}\) and p110\(^{\delta}\)\(^{\text{D910A/D910A}}\) mice were lethally \(\gamma\) irradiated (single dose, 10 Gy). After 3–4 h, mice were reconstituted by intravenous injection (tail vein) of total bone marrow from p110\(^{\delta}\)\(^{\text{WT/WT}}\) mice. Six weeks after reconstitution, mice were sacrificed, and spleen and LN collected. Half were frozen for immunofluorescence studies, and the remainder used to prepare single-cell suspensions for populations counts and flow cytometry analysis.

**Bone marrow reconstitution assays**

p110\(^{\delta}\)\(^{\text{WT/WT}}\) and p110\(^{\delta}\)\(^{\text{D910A/D910A}}\) mice were lethally \(\gamma\)-irradiated (single dose, 10 Gy). After 3–4 h, mice were reconstituted by intravenous injection (tail vein) of total bone marrow from p110\(^{\delta}\)\(^{\text{WT/WT}}\) mice. Six weeks after reconstitution, mice were sacrificed, and spleen and LN collected. Half were frozen for immunofluorescence studies, and the remainder used to prepare single-cell suspensions for populations counts and flow cytometry analysis (see Supplement S1).

**Immune response induction with heat-inactivated *Candida albicans***

Heat-inactivated *Candida albicans* cells (10\(^{6}\)) were injected into p110\(^{\delta}\)\(^{\text{WT/WT}}\), p110\(^{\delta}\)\(^{\text{D910A/D910A}}\,\text{reconstituted p110}^{\delta}\)\(^{\text{WT/WT}}\), p110\(^{\delta}\)\(^{\text{D910A/D910A}}\,\text{reconstituted p110}^{\delta}\)\(^{\text{WT/WT}}\) and p110\(^{\delta}\)\(^{\text{D910A/D910A}}\ mice (see Supplement S1 for details). Mice were sacrificed 5 days post-injection, and spleen and LN collected. Half were frozen for immunofluorescence studies, and the remainder used to prepare single-cell suspensions for populations counts and flow cytometry analysis (see Supplement S1).

**Immunofluorescence of SLO sections**

Frozen sections of spleen and LN from p110\(^{\delta}\)\(^{\text{WT/WT}}\), p110\(^{\delta}\)\(^{\text{D910A/D910A}}\,\text{reconstituted p110}^{\delta}\)\(^{\text{WT/WT}}\) and reconstituted p110\(^{\delta}\)\(^{\text{D910A/D910A}}\ mice were analyzed by immunofluorescence staining to study distribution and location of immune cell (Thy1.2\(^{+}\) and CD3\(^{+}\) T cells, MOMA\(^{+}\) MMM, B220\(^{+}\) B cells, CD11c\(^{+}\) DC, see Supplement S1).

**Hematoxylin-eosin staining of spleen sections**

Frozen sections from p110\(^{\delta}\)\(^{\text{WT/WT}}\), p110\(^{\delta}\)\(^{\text{D910A/D910A}}\,\text{reconstituted p110}^{\delta}\)\(^{\text{WT/WT}}\) and reconstituted p110\(^{\delta}\)\(^{\text{D910A/D910A}}\ mice were hematoxylin/eosin stained to analyze lymphoid follicle area (see Supplement S1).
Flow cytometry analysis of immune cell populations

Secondary lymphoid organ cells from $p110^{\text{WT/WT}}, p110^{\text{D910A/D910A}}$, and reconstituted mice were processed and stained for flow cytometry analysis (see Supplement S1).

Flow cytometry analysis of spleen stromal cells

Stromal cells were extracted using an established protocol [40]. Briefly, mouse spleens were removed, pierced with fine forceps, and placed in ice-cold RPMI-1640 (5 min, on ice). Spleens were dissected, RPMI-1640 removed, and replaced with 2 ml of a fresh enzymatic mixture composed of dispase (0.8 mg/ml; Gibco) and...
collagenase IV (0.2 mg/ml; Roche). Tubes were incubated (37°C, 20 min), the cell suspension removed and placed in a fresh tube with ice-cold FACS buffer (3% FBS, 2 mM EDTA in PBS). The remaining spleen was re-incubated with 2 ml fresh enzyme mix (37°C, 10 min), after which the cell suspension was removed and added to fresh tube above. The remaining spleen was reincubated (37°C, 15 min) in 2 ml fresh enzyme mix with vigorous pipetting every 5 min, the cell suspension was removed, placed in the same tube, whose contents were then filtered through a 100 μm nylon mesh. Cells were counted and viability assayed using trypan blue.

Cells were stained with CD45 (30-F11, Biolegend), TER119 (TER119, eBioscience), gp38 (8.1.1, eBioscience) and CD31 (MEC 13.3, BD Biosciences) in 100 μl (30 min, 4°C) before analysis on a Cytomix (Beckman Coulter).

Stromal cell enrichment and cell sorting
Stromal cells were harvested as above. After spleens were fully digested, cells were centrifuged, counted, and the single cell suspension depleted of non-hematopoietic stromal cells using CD45 microbeads in the autoMACS system (Miltenyi) and

Figure 2. Absolute numbers of spleen and LN total cells, CD4+ and CD8+ T cells before and after antigen stimulation. Spleens and LN were extracted from p110δWT/WT, p110δD910A/D910A, and reconstituted mice in homeostatic conditions (t = 0) and after antigen stimulation (five days post-injection of inactivated C. albicans, t = 5 d). Whole organ cell suspensions were counted to determine total cell number (A, D) and stained to determine CD4+ T (B, E) and CD8+ (C, F) cell numbers by flow cytometry (n=6 mice/condition). Mean ± SD. doi:10.1371/journal.pone.0072960.g002
incubated (20 min, 4°C), CD45-labeled cells were depleted using the autoMACS Depletes program. Purified stromal cells were counted and stained before sorting on a FACSAria III (BD Biosciences).

qRT-PCR analysis of gene expression

Total RNA was extracted from spleen, LN, and sorted cell populations isolated from p110βWT/WT, p110βD910A/D910A, and reconstituted mouse in homeostatic conditions (t = 0) and after antigen stimulation (five days post-injection of inactivated C. albicans, t = 5 d). B cell (A) and DC (B) were stained and cell numbers determined by flow cytometry (n = 6 mice/condition). Mean ± SD. doi:10.1371/journal.pone.0072960.g003

SLO analysis after bone marrow reconstitution and antigen stimulation

To test whether p110βD910A/D910A mouse SLO structural defects in homeostasis are corrected after antigen stimulation, we performed similar studies in bone marrow-reconstituted mice. We studied spleen and LN immune responses simultaneously using heat-inactivated C. albicans, which generates concurrent local and systemic immune responses ([41], [42], Figure S2). We injected heat-inactivated C. albicans into mice 6 weeks after reconstitution, and sacrificed mice after five days (Figure S2, Supplement S1).

We analyzed total, CD3+CD4+, and CD3+CD8+ T cell number in p110βWT/WT, p110βD910A/D910A, and bone marrow-reconstituted mouse spleens in homeostasis and after antigen stimulation (Figure 2A–C). After stimulation, total cell numbers increased in spleens from p110βWT/WT but not from p110βD910A/D910A mice (Figure 2A). CD4+ and CD8+ T cell numbers increased similarly in p110βWT/WT mouse spleen after stimulation, but not in p110βD910A/D910A mice (Figure 2B, C), suggesting defective T cell expansion in p110βD910A/D910A mice. Total spleen cell, CD4+ and CD8+ T cell numbers increased after stimulation compared to homeostatic conditions in reconstituted p110βWT/WT, but not in p110βD910A/D910A recipient mice (Figure 2A–C), indicating that spleen stromal cells in p110βD910A/D910A mice might not contribute appropriately to T cell expansion in response to heat-inactivated C. albicans. We analyzed total, CD3+CD4+ and CD3+CD8+ cell number in p110βWT/WT, p110βD910A/D910A, and bone marrow-reconstituted mouse LN in homeostasis and after antigen stimulation (Figure 2D–F). LN from p110βWT/WT and p110βD910A/D910A mice showed an increase in total cell number, which was smaller in p110βD910A/D910A than in p110βWT/WT mice (Figure 2D). A similar increase was observed for CD4+ and CD8+ T cells in LN (Fig. 2E, F), indicating that p110βWT/WT and p110βD910A/D910A mouse LN respond to C. albicans stimulation, although the response was slightly lower in p110βD910A/D910A than in p110βWT/WT mice. After mouse reconstitution, total LN cell numbers increased after antigen stimulation in p110βWT/WT, and to a lesser extent in p110βD910A/D910A recipients (Figure 2D).

Results

Analysis of SLO after bone marrow reconstitution assays in homeostatic conditions

To determine whether defects in the MZ and in MZ B cells in p110βD910A/D910A mouse spleen ([30], Figure S1, Supplement S1) were due solely to anomalies in p110βD910A/D910A hematopoietic cell populations or also to non-hematopoietic stromal cell defects, we used bone marrow reconstitution assays in p110βWT/WT and p110βD910A/D910A mice and analyzed SLO in homeostatic conditions. Lethally irradiated p110βWT/WT and p110βD910A/D910A mice were reconstituted with total bone marrow from p110βWT/WT donors. Six weeks after reconstitution, mice were sacrificed for immunofluorescent staining of spleen and LN sections to detect immune cell populations (Figure 1). We also analyzed total cell numbers and lymphoid cell populations of spleen and LN by flow cytometry (Figure 2).
Figure 4. FACS analysis of stromal cell populations in spleen from p110^WT/WT and p110^D910A/D910A mice. Spleens from p110^WT/WT and p110^D910A/D910A mice were processed and stained with anti-CD45, -TER119, -CD31, and -gp38 mAb. A) Representative gating strategy for the analysis of stromal cell populations. Stromal cells were gated via the exclusion of dead, CD45-, and TER119-positive cells. B) Quantification of the percentage and absolute number of stromal cell populations in spleens of p110^WT/WT and p110^D910A/D910A mice (n = 3 experiments/spleen, 6 mice/group). Student's t-test, *p < 0.05.

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also found an increase in total number of gp38 cells (Figure 4A). Analysis of CD45 function of these cell types \[30\], \[31\], \[32\], \[43\].

To better define the defects identified in whole spleen extracts, we separated the spleen gp38/CD31-defined stromal cell subsets by cell sorting and analyzed chemokine and TNF family mRNA expression in extracts of each population. Analysis showed a reduction in CCL19 mRNA levels only in p110\(_{D910A/D910A}\) mice compared to p110\(_{WT/WT}\) mice. Expression of CCL21 and to a lesser extent, that of CCL19 were lower in total RNA extracts from p110\(_{D910A/D910A}\) and p110\(_{D910A/D910A}\) mice. Expression of CCL21 and to a lesser extent, that of CCL19 were lower in total RNA extracts from p110\(_{D910A/D910A}\) than from p110\(_{WT/WT}\) mouse spleens (Figure 6A); there were no differences in LN from either genotype (Figure 6B). Analysis of mRNA levels of TNF family proteins or their receptor LTBR showed no differences in spleen or LN (Figure 6A, B).

To further define the defects identified in whole spleen extracts, we separated the spleen gp38/CD31-defined stromal cell subsets by cell sorting and analyzed chemokine and TNF family mRNA expression in extracts of each population. Analysis showed a reduction in CCL19 mRNA levels only in p110\(_{D910A/D910A}\) mice compared to p110\(_{WT/WT}\) mice. Expression of CCL21 and to a lesser extent, that of CCL19 were lower in total RNA extracts from p110\(_{D910A/D910A}\) than from p110\(_{WT/WT}\) mouse spleens (Figure 6A); there were no differences in LN from either genotype (Figure 6B). Analysis of mRNA levels of TNF family proteins or their receptor LTBR showed no differences in spleen or LN (Figure 6A, B).

Discussion

The immune response is controlled by lymphoid and stromal cell function and location in SLO [4]. The PI3K p110\(\delta\) is expressed preferentially by leukocytes, although it is also detected in other cell types [24], [25], [26], [27], [28]. MZ B cell numbers are extremely low in p110\(\delta\)-deficient mouse spleen [31], and lack of p110\(\delta\) or its kinase activity severely impairs germinal center (GC) formation in the spleen after immunization [30], [31], [32], [39]. We tested whether this isoform is expressed in SLO stromal

Figure 5. p110\(\delta\) mRNA expression in spleen stromal cell populations from p110\(_{WT/WT}\) and p110\(_{D910A/D910A}\) mice. Total RNA was extracted from sorted p110\(_{WT/WT}\) and p110\(_{D910A/D910A}\) spleen stromal cell subsets (n=5 mice/ genotype). Lymphoid cells (CD45\(^d\)) were sorted as control. Expression of p110\(\delta\) mRNA was analyzed by qRT-PCR. Normalized quantities (mean \(2^{-\Delta\Delta Ct}\)) of p110\(\delta\) mRNA are shown.

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Results were similar for LN CD4\(^d\) and CD8\(^\dagger\) T cells, suggesting that LN stroma supports the T cell immune response to heat-inactivated C. albicans.

To determine whether other spleen cell types involved in the immune response to heat-inactivated C. albicans were affected, we analyzed B cell (B220\(^d\)) and dendritic cell (DC, CD11c\(^d\)) numbers in p110\(_{WT/WT}\), p110\(_{D910A/D910A}\), and bone marrow-reconstituted mouse spleens in homeostasis and after antigen stimulation (Figure 3A, B). B cell numbers were increased in p110\(_{WT/WT}\) but not in p110\(_{D910A/D910A}\) mouse spleen (Figure 3A). DC cell numbers showed a similar increase in p110\(_{WT/WT}\) spleen after stimulation, but not in spleens from p110\(_{D910A/D910A}\) mice (Figure 3B), suggesting defective B cell and DC expansion in p110\(_{D910A/D910A}\) mice after antigen stimulation compared to homeostatic conditions in reconstituted p110\(_{WT/WT}\) and p110\(_{D910A/D910A}\) recipient mice (Figure 3A, B). These results suggest that spleen stromal cells lacking p110\(\delta\) activity contributed to correct B cell and DC expansion in response to heat-inactivated C. albicans. The defect in spleen B cell and DC expansion in p110\(_{D910A/D910A}\) mice after antigen stimulation is probably due to the role of p110\(\delta\) in the function of these cell types [30], [31], [32], [43].

FACS analysis of spleen stromal cell populations in p110\(_{WT/WT}\) and p110\(_{D910A/D910A}\) mice

To evaluate the effect of lack of p110\(\delta\) activity on the percentages and numbers of the four stromal cell subsets defined by gp38 and CD31 in spleen (FRC, LEC, BEC, DN), we used FACS to analyze p110\(_{WT/WT}\) and p110\(_{D910A/D910A}\) mouse spleen cells (Figure 4A). Analysis of CD45\(^d\) TER119\(^d\) splenocytes showed a significant decrease in the percentage of gp38\(^d\) CD31\(^d\) cells (BEC) in p110\(_{D910A/D910A}\) compared to p110\(_{WT/WT}\) mice (Figure 4A). We also found an increase in total number of gp38\(^d\) CD31\(^d\) (FRC) and gp38\(^d\) CD31\(^d\) (DN) cells in p110\(_{D910A/D910A}\) compared to p110\(_{WT/WT}\) mice (Figure 4B).

p110\(\delta\) mRNA expression in spleen stromal cell populations

To test whether p110\(\delta\) mRNA was expressed in spleen stromal cells, the four stromal cell subsets defined by gp38/CD31 expression were sorted from p110\(_{WT/WT}\) and p110\(_{D910A/D910A}\) mouse spleens and p110\(\delta\) expression analyzed by RT-PCR. As a positive control, CD45\(^d\) (lymphoid) cells were also sorted. Although lymphoid cells express higher p110\(\delta\) mRNA levels, gp38\(^d\) CD31\(^d\) cells (LEC) and to a lesser extent, gp38\(^d\) CD31\(^d\) cells (BEC) also expressed p110\(\delta\) mRNA, whereas gp38\(^d\) CD31\(^d\) (FRC) cells did not (Figure 5). Within the LEC population, p110\(\delta\) mRNA levels were notably reduced in p110\(_{D910A/D910A}\), whereas they were similar in BEC and lymphoid cells (Figure 5).
cells, and whether expression mediates cell location and compartmentalization in these organs.

Reconstitution assays have been used to analyze and confirm specific p110δ functions in memory T cells; lethally irradiated WT mice were reconstituted with purified memory T cell subsets (CD62L<sup>hi</sup> central memory T cells and CD62L<sup>lo</sup> effector memory T cells) from p110δ<sup>D910A/D910A</sup> and p110δ<sup>WT/WT</sup> mice [35]. Using reconstitution assays with total bone marrow from

Figure 6. qRT-PCR analysis of homeostatic chemokines and TNF family members in spleen, LN and spleen stromal cell subsets from p110δ<sup>WT/WT</sup> and p110δ<sup>D910A/D910A</sup> mice. Total RNA was extracted from p110δ<sup>WT/WT</sup> and p110δ<sup>D910A/D910A</sup> spleen, LN, and sorted spleen stromal cell subsets (n = 5 mice/genotype). Expression of CCL19, CCL21, LTα, LTβ and LTβR was analyzed by qRT-PCR in spleen (A), LN (B), and stromal cell subsets (C). Normalized quantities (mean 2<sup>-ΔΔCt</sup>) of mRNA are depicted. Student’s t-test, *p<0.05, **p<0.01, ***p<0.001.

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p110\textsuperscript{WT/WT} mice, we tested whether stromal cells have a role in SLO reconstitution (p110\textsuperscript{WT/WT}, reconstituted p110\textsuperscript{D910A/D910A}, p110\textsuperscript{WT/WT} reconstituted p110\textsuperscript{D910A/D910A}, mice). Immunohistochemical analysis of p110\textsuperscript{D910A/D910A} and reconstituted p110\textsuperscript{D910A/D910A} recipient mouse spleen showed reduced T cell staining and more diffuse T cell areas than in p110\textsuperscript{WT/WT} or p110\textsuperscript{RTA/WT} reconstituted mice. In addition, in p110\textsuperscript{D910A/D910A} mice reconstituted with p110\textsuperscript{WT/WT} bone marrow, spleen CD4\textsuperscript{+} and CD8\textsuperscript{+} T cell numbers did not increase in response to heat-inactivated \textit{C. albicans}, suggesting that a p110\textsuperscript{D910A/D910A} stroma defect impedes a correct immune response. We thus hypothesized a role for p110 in stromal cell function in the spleen.

SLO stromal cells are divided into four populations as defined by gp38 and CD31 expression, LEC (gp38\textsuperscript{CD31+}), BEC (gp38\textsuperscript{CD31+}), and double negative cells (gp38\textsuperscript{CD31-}) [3], [4]. FACS analysis of spleen stromal cell populations showed a significant decrease in the percentage of gp38\textsuperscript{CD31+} cells in p110\textsuperscript{D910A/D910A} mice, which paralleled an increase in total gp38\textsuperscript{CD31-} and gp38\textsuperscript{CD31-} cells. This result suggested that p110 is expressed differently in each spleen stromal population. As there are no reports of p110 expression in SLO stromal cell subsets, we sorted the four subpopulations from p110\textsuperscript{WT/WT} and p110\textsuperscript{D910A/D910A} spleen and tested for p110 mRNA expression by qRT-PCR. In addition to its expression in lymphoid cells, p110 was detected in spleen LEC and BEC subsets. p110 mRNA levels in LEC were significantly lower in p110\textsuperscript{D910A/D910A} than in p110\textsuperscript{WT/WT} spleen.

T homing and compartmentalization in SLO requires chemokine secretion by stromal cells. FRC secrete the homeostatic chemokines CCL19 and CCL21 [3], which are also produced by LEC and BEC [17]. Analysis of their expression in total RNA extracts of p110\textsuperscript{D910A/D910A} spleen showed significantly lower levels of CCL21 and, to a lesser extent, of CCL19 than in p110\textsuperscript{WT/WT} or p110\textsuperscript{D910A/D910A} LN. In p110\textsuperscript{D910A/D910A} spleen marginal zone, CCL21 production is significantly impaired in BEC, and reduced CCL21 production in all populations. This CCL19 and CCL21 expression defect in the stromal cells could give rise to the abnormal B/T cell segregation observed in p110\textsuperscript{WT/WT} mouse spleen. LT\alpha, LT\beta, and TNF participate to some degree in the development of most SLO [18]. Lymphotoxin signaling is necessary for red and white pulp segregation, as well as for correct B/T cell homing and maintenance of segregation [19]. We found no differences in spleen or LN LT\alpha and LT\beta expression between p110\textsuperscript{WT/WT} and p110\textsuperscript{D910A/D910A} mice. When we analyzed mRNA in specific spleen stromal cell populations, however, expression of LT\alpha and LT\betaR expression were significantly lower in p110\textsuperscript{D910A/D910A} LEC, and somewhat less so in BEC compared to those of p110\textsuperscript{WT/WT} mice; no differences were observed in LN LT\beta expression. LT\alpha, LT\beta, and LT\betaR defects differed in SLO [44], [45], [46], [47]. The p110\textsuperscript{D910A/D910A} spleen phenotype is similar to that of mice in which LT\beta-LT\betaR interaction is blocked by a soluble LT\betaR-IgG1 fusion protein [48], and includes loss of MZ and of T/B cell segregation, although segregation was normal in LN. Low LT\betaR expression in LEC and BEC appears to be the primary cause of these spleen defects in p110\textsuperscript{D910A/D910A} mice, together with low CCL19 and CCL21 production, which affects T/B cell migration and compartmentalization. The need for LT\beta for B/T cell segregation in spleen white pulp, whereas TNFR-I is necessary for B/T cell segregation in LN [49], is consistent with the lesser defects in p110\textsuperscript{D910A/D910A} LN compared with spleen.

In summary, we found p110 expression by gp38\textsuperscript{CD31+} and gp38\textsuperscript{CD31-} spleen stromal cells. Lack of p110 activity in these populations correlated with lower LT\betaR, CCL19 and CCL21 mRNA levels. These findings could explain the lower T cell numbers and more diffuse T cell areas observed in p110\textsuperscript{D910A/D910A} mice, and the lower T cell expansion after antigen stimulation observed in p110\textsuperscript{D910A/D910A} compared with p110\textsuperscript{WT/WT}.

Supporting Information

Supplement S1 Supporting Materials and Methods, Results and References. (DOC)

Figure S1 Distribution of immune cell types from p110\textsuperscript{WT/WT} and p110\textsuperscript{D910A/D910A} spleen marginal zone. Histological sections from p110\textsuperscript{WT/WT} and p110\textsuperscript{D910A/D910A} spleens were immunofluorescent stained for marginal zone immune cell types. (A) MZB (B220\textsuperscript{+} surrounding MOMA\textsuperscript{+} cells around spleen follicles) and MMM (MOMA\textsuperscript{+} (n = 4 mice/genotype). (B) MZM (SIGNR\textsuperscript{+}) and MMM (MOMA\textsuperscript{+}) (n = 4 mice/genotype). Bar = 200 \mu m. (TIF)

Figure S2 Immune response in p110\textsuperscript{WT/WT} mice injected with heat-inactivated \textit{C. albicans}. p110\textsuperscript{WT/WT} mice received i.p. injections of heat-inactivated \textit{C. albicans} for the indicated times (0, 2, 5, 7, 9 and 21 d) to stimulate an immune response. Total CD4\textsuperscript{+} T cells from p110\textsuperscript{WT/WT} spleens (A) and LN (B) were counted between (t = 0) and several times after \textit{C. albicans} injection (n = 6–10 mice). Mean \pm SD. (TIF)

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Author Contributions

Conceived and designed the experiments: TMZ RS VM ACC DFB. Performed the experiments: TMZ RS VM SPF DFB. Analyzed the data: TMZ RS VM COS ACC DFB. Contributed reagents/materials/analysis tools: COS KO. Wrote the paper: TMZ RS DFB. Help with image quantification: SG. qRT-PCR studies: LA. Advice, protocols and helpful suggestions: RM LM EG AF ASF. Editorial assistance: CM.
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