Development and Migration of Plasma Cells in the Mouse Lymph Node

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SUMMARY

In this study, we imaged the differentiation and migratory behavior of nascent plasma cells (PCs) in mouse lymph nodes by intravital microscopy. Pre-PCs exhibited a unique migration pattern characterized by long, linear paths that were randomly oriented. Although chemotaxis via Gαi coupled-receptors has been implicated in PC migration, treatment with Pertussis toxin (Ptx), which ablates these signals, did not prevent movement of pre-PCs while it arrested other lymphocytes. In vitro, pre-PCs displayed processive amoeboid locomotion on surfaces coated with integrin ligand, whereas fully differentiated PCs moved slowly or were arrested. Both PC arrest and differentiation occurred in the medullary cords. Ptx treatment before PC differentiation blocked their accumulation in the medullary cords but pre-PCs still differentiated in other lymph node regions. Taken together, we suggest pre-PCs undergo a persistent random walk to find the medullary cords, where localized chemokines help retain these cells until they undergo differentiation and arrest in situ.

INTRODUCTION

Plasma cells (PCs) play important roles in the acute response to infection and the long-term protection of the host by acting as antibody factories. These terminally differentiated B cells can be divided into two subsets. Short-lived PCs are produced early in the immune responses, starting on day 3 after immunization; they provide a first wave of lower-affinity antibodies but die shortly thereafter. Meanwhile, long-lived late PCs are produced by T cell-dependent germinal centers (GCs) that coalesce around day 6 after immunization and can continue to generate PCs for weeks. These late PCs generate higher-affinity antibodies through affinity maturation. Acute ablation of the GC by anti-CD40 treatment halts generation of new PCs and prevents further improvements of antibody affinity.

PCs are identified by syndecan-1 surface expression, have an extensive rough endoplasmic reticulum (ER), and are enriched within the red pulp of the spleen, in the medullary cords of lymph nodes, and in the bone marrow. The precur-ors of PCs (pre-PCs, also called plasmablasts) are dividing cells that are found in B cell follicles in addition to red pulp and medullary cords, but not in the bone marrow. Plasma cells and pre-PCs are collectively referred to as antibody-secreting cells (ASCs) or antibody-forming cells (AFCs) on the basis of their ability to secrete antibody, which is often class-switched.

PC differentiation is dependent on a key transcriptional repressor, Blimp-1, which inhibits many B cell lineage (Pax5) and GC-specific (Aicda, Bcl6) genes. By repressing Pax5, Blimp-1 allows expression of XBP1, a transcription factor essential to survive ER stress associated with massive antibody secretion. Kallies et al. inserted a DNA construct consisting of an internal ribosome entry site followed by a green fluorescent protein (GFP) cDNA into exon 6 of the Pdm1 locus to generate a Blimp-1 reporter mouse (Blimp-1-GFP) and showed Blimp-1 expression as an early marker of PC development. Pre-PCs expressed lower levels of Blimp-1-GFP than fully differentiated PCs and were heterogeneous for syndecan-1 expression.

Within secondary lymphoid organs, both short- and long-lived PCs localize to medullary cords in lymph nodes and red-pulp regions of the spleen, where they are thought to secrete antibody. Within these anatomic locations, PCs are largely sessile. PC migration to these regions has never been visualized directly, but chemokine receptors are thought to play a role because expression of CXCR4, CCR6, and EBI2 increases and CXCR5 is reduced during PC differentiation. In vitro trans-well assays have shown chemotaxis of spleen red pulp PCs to the chemokines S1p and CXCL12, which are ligands of S1p1/S1p3 and CXCR4 receptors, respectively. Consistent with this idea, CXCL12 is expressed in red pulp and medullary cords, and genetic ablation experiments showed that CXCR4-deficient PCs failed to accumulate in red pulp or bone marrow but were
enriched in blood and normal in lymph nodes compared to CXCR4-sufficient cells (Hargreaves et al., 2001). These results suggest a role for CXCL12 in PC homing (Hargreaves et al., 2001; Wehrli et al., 2001).

Long-lived PCs egress from lymph nodes, and homing to the bone marrow is critical for their long-term survival. In both s1p1- and β2-integrin-deficient conditions (Kabashima et al., 2006; Pabst et al., 2005), PCs are unable to exit the lymph nodes. S1p expression is high in blood and low in secondary lymphoid organs, providing a gradient that may be used for egress (Rosen and Goetzl, 2005; Schwab and Cyster, 2007). Intercellular adhesion molecule-1 (ICAM-1) is highly expressed in medullary cords, which are the exit sites of PCs from lymph nodes.

From these reports and others, an image of newly minted pre-PCs leaving the germinal center to the medullary cords along a chemokine highway has emerged. However, the current model of pre-PC chemotaxis to the medullary cords poses a few conceptual challenges. Although CXCL12 is a candidate for attracting PCs to the medullary cords, it is not clear how pre-PCs would first escape from the GC, where CXCL12 is also used to partition GC B cells between the light and dark zones (Allen et al., 2004). After exit from the GC, a long and stable chemokine gradient would have to be produced to attract cells to structures that may lie millimeters away. Finally, for many existing paradigms for chemokine function (Allen et al., 2004; Bajenoff et al., 2006), regions of abundant cognate chemokines often lead to high cell motility, although in the case of plasma cells, their localization in the medullary cords seems to correlate with an arrested phenotype.

We used direct intravital imaging of pre-PCs with two-photon laser scanning microscopy (TPLSM) to understand their migration. Surprisingly, pre-PCs exhibit a randomly oriented highly linear migration within the follicle and T zone that does not depend on Gαi signaling. Processive amoeboid locomotion with rare direction changes provides an efficient means for pre-PCs to reach the medulla without a need to evolve a distinct chemotactic system. Upon reaching the medullary cords, chemokine and adhesive contacts may serve to confine and retard PCs within the medullary cords while they mature and arrest.

RESULTS

Generation and Characterization of Blimp-1-YFP Mice

To study plasma cells in vivo, we used bacterial artificial chromosome transgenic mice (Rutishauser et al., 2009) that express yellow fluorescent protein (YFP) cDNA under the control of regulatory sequences from the Prdm1 locus (Turner et al., 1994), which encodes the Blimp-1 protein. These Blimp-1-YFP+ mice showed normal B cell development in the spleen, bone marrow, and peritoneum when compared to wild-type B6 mice (Figure S1A). Consistent with the Blimp-1-GFP reporter mouse (Kallies et al., 2004), analysis of lymphoid organs showed that only a small number of B cells (which varied 0.05% to 0.5% in a given mouse) expressed the Blimp-1-YFP transgene in the spleen or bone marrow in the steady state, and these cells expressed syndecan-1+, CD19+, B220+, CD43+, CXC5R5, and CXC4+ surface profiles consistent with plasma cells (Figures S1B and S1C available online). As reported by others (Kallies et al., 2004), some ASCs were syndecan-1+Blimp-1-YFP- (Figure S1B), possibly because of transgene variegation effects (Williams et al., 2008).

To examine the development of antigen-specific plasma cells in vivo, we produced Blimp-1-YFP mice that also carry the B1.8th immunoglobulin (lg) heavy-chain allele (Blimp-1-YFP-B1.8th mice), which encodes a nitrophenyl (NP) hapten-specific B cell receptor when combined with Igδ (Shih et al., 2002). Naive (CD45.2+Blimp-1-YFP+B1.8th) B cells were adoptively transferred into congenic (CD45.1+) B6 recipients, boosted with NP-OVA or without (control), and analyzed for short- or long-lived PC production at early (day 4) or late (day 10) times, respectively, as well as an in-between (day 7) time point during the response. Transferred B cells produced a small subset of YFP+ cells that accumulated in draining lymph nodes, peaking at day 7 (Figure 1A). Although both YFPhi and YFPlow populations were detectable at all three time points, the YFP+ cells increased after day 4. On day 7, within the GC compartment of the transferred population (2% of total), a rare fraction (0.09%) of YFP+ cells could be detected (Figure 1B), which were YFPlow cells. Transferred B cells were subdivided into naive, GC, YFPhi, and YFPlo populations and analyzed for surface phenotypes (Figure 1C). The YFP+ cells were consistent with a PC surface phenotype, namely syndecan-1+CD19+CD25+CXCR5low, whereas half of YFPlo cells were syndecan-1+. These surface phenotypes for the characterized subsets were consistent with analyses on day 10. On day 4, YFP+ cells showed no CD19 downregulation (Figure 1C), consistent with a short-lived plasma cell phenotype (Kallies et al., 2004). Heterogeneous expression of syndecan-1 by the Blimp-1-YFP+ cells is consistent with these cells containing transitional pre-PCs (Kallies et al., 2004).

To characterize nascent pre-PCs, we sorted Blimp-1-YFP+, Fas+GL7+ cells and compared their cellular structures to sorted populations of naive, GC B cells, and PCs by using transmission electron microscopy. The cellular morphology of the pre-PC was more consistent with GC cells than mature PCs (Figure 1D), although they showed some expansion in their ER, consistent with early stages of plasma cell differentiation (Geuze and Slot, 1980).

Blimp-1-YFP+ Distribution in the Lymph Node

We characterized PC development during an immune response by determining the distribution and expression of Blimp-1-YFP+ cells within the lymph node. Using TPLSM on days 4, 7 and 10, we obtained 3D-tiled stacks from both the follicular and medullary sides of fixed popliteal lymph nodes, which represented 50%–75% of the total lymph node volume. We identified Blimp-1-YFP+B1.8th (green) distribution within the medullary cords (cyan, labeling various macrophages by nonspecific uptake of quantum dots), follicular dendritic cell (FDC) network of the GCs (red labeling by NP-Tomato surface adhesion), and the remaining B cell follicles and T cell zone, collectively called BT zones. We found Blimp-1-YFP+ cells concentrated in the medulla but present throughout the BT zones as well, with a few cells within GCs on days 7 and 10 (Figures 2A and 2B). In contrast, naive B cells (blue), expressing cyan fluorescent protein (CFP) constitutively, were more commonly distributed within B cell follicles than in the medulla. The few rare Blimp-1-YFP+ cells found in GCs appeared to be positioned along the periphery of the structure (Movie S1, Figure S2). We compared pre-PC distribution with
respect to the FDC network of the GC and found them to be more peripherally localized than naive B cells. In a previous study in which Blimp-1 expression was identified in the GC, there was no mention of a peripheral localization (Angelin-Duclos et al., 2000). However, differences in the techniques from these two studies could account for the discrepancy.

Figure 1. Characterization of Blimp-1-YFP+ Cells

Purified, naive B1.8hiBlimp-1-YFP+ B cells were transferred into immunized congenic CD45.1+C57BL/6 recipients. Mice were boosted subcutaneously with NP-OVA or without (control) 1 day after transfer. Lymphocytes were analyzed 4, 7, and 10 days after boost by flow cytometry. Numbers reflect the relative percentages of cells within the gates.

(A) Transferred cells (CD45.1+) generated rare Blimp-YFP+ cells on days 4, 7, and 10 but not in control mice.

(B) Day 7 transferred cells were subdivided into YFPhi, YFPlo, and YFP- populations, and the GC compartment (Fas+/GL7+) was assessed. Some YFPlo cells were GC+ but none were YFPhi.

(C) Transferred populations were subdivided into naive B cells (gate I: YFP-/Fas-/GL7-), GC B cells (gate II: YFP+/Fas+/GL7+), YFPlo (gate III), and YFPhi (gate IV) and analyzed for surface expression of CD19, CD138, CXCR4, and CXCR5. For comparison, CD19 and CD138 profiles are shown for day 4 and day 10 populations. Flow-cytometry analysis shows samples pooled from duplicate mice in a single experiment, but is representative of at least three independent experiments.

(D) Naive (B220+), GC (Fas+/GL7+), plasma cell (B220+, syndecan-1+), and pre-PC (Blimp-1-YFP+, Fas+/GL7+) B cells were all sorted (98% purity) and imaged by transmission electron microscopy.
YFP+ were highly motile in the follicle and largely sessile in the medulla (Figure 3A). Blimp-1-YFP+ cells in vivo (green). Before imaging, mice received IV transfers of naive lymph nodes were pooled per condition. Error bars represent standard error of the mean (SEM). Scale bars represent 200 m in all images.

**Figure 2. Blimp-1-YFP+ Distribution in the Lymph Node**
Recipient mice were prepared for imaging as in Figure 1 to induce B1.8hiBlimp-1-YFP+ cells in vivo (green). Before imaging, mice received IV transfers of naive CFP+ B cells (blue) as controls and subcutaneous injection of QDot705 (that label phagocytes concentrated in the medullary cords, in cyan) plus NP-tomato (to label FDCs in NP-specific GC light zones in red). (A) Examples of flattened 3D stacks taken by TPLSM of the follicle and medulla sides of whole fixed popliteal lymph nodes from days 4, 7, and 10 are shown. Automated analysis of the images was used to determine Blimp-1-YFP cell distributions within medullary cords, GCs, and other regions (collectively referred to as BT zones) per lymph node and summarized in (B) as percent total. The same analysis was made for naive CFP B cells for comparison. At least four lymph nodes were pooled per condition. Error bars represent standard error of the mean (SEM). Scale bars represent 200 m in all images.

**Dynamics of Blimp-1 Cell Migration**
We imaged the migratory behavior of Blimp-1-YFP+ cells in the lymph node by intravitral time-lapse TPLSM. In vivo, Blimp-1-YFP+ cells in the follicle were similar in size and shape to blasting GC B cells, which are larger and more polarized than naive B cells (Schwickert et al., 2007) (Movie S2). In general, Blimp-1-YFP+ were highly motile in the follicle and largely sessile in the medullary cords (Figure 3A). Blimp-1-YFP+ cell migration seemed to be similar on days 4, 7, and 10 (Movie S2, Figure 3B), with cells in the medullary cords slightly more motile on day 4 than on days 7 or 10, which correlated with their transition from pre-PC to PC in the medulla. Outside the medullary cords, Blimp-1-YFP+ cell average track velocity was lower than naive cells (Figure 3B) although some cells reached similar speeds.

Pre-PC migration was highly linear over long distances and less confined as compared with naive B cells, which migrate randomly in the follicle. To determine whether pre-PCs were more processive than naive B cells, we calculated the average mean squared displacement (MSD) for cell tracks over time. As modeled in Figure 3C, the MSD for random walk scales linearly with time, whereas the MSD for confined motion reached a plateau. Random walks with long free-path lengths or persistent random walks generate exponential MSD plots that accelerate with increased path lengths (see Codling et al., 2008; Zygourakis, 1996 for further discussion). We calculated the MSD for Blimp-1-YFP+ cells, segmented by day and region, and compared them to control naive B cells in those fields. The MSD plots for naive cells had initial slopes that were linear and reached a maximum displacement after ~30 min consistent with limited follicle size (Figure 3D). Nonmedullary Blimp-1-YFP+ cells showed higher displacements than naive B cells, despite having slower speeds (Figure 3B). Based on fitting the average MSD plots to an equation for 3D persistent random walk (see Experimental Procedures), pre-PCs showed an average 3 min persistence time, compared to 1 min for naive cells. Confinement of Blimp-1-YFP+ cells in the medullary cords increased at later time points (Figure 3D).

Despite a high degree of linearity in pre-PC movement, analysis of the track displacement showed no overall directionality at any time point (Figure 3E). We could not detect a bias in pre-PC displacements along any axis, or when compared to naive B cells in the same fields. In movies that contained regions of medulla or GC, pre-PCs showed no bias toward or away from these elements (Movies S2). Moreover, we anecdotally observed Blimp-1-YFP+ pre-PCs crawling linearly until reaching the capsule of the lymph node, changing direction, and continuing in a new direction, in what appeared to be a blind search for the medulla (Movie S3).

**In Vivo Requirements for Blimp-1 Pre-PC Migration**
Despite the absence of directional movement by pre-PCs, changes in CXCR4 and CXCR5 chemokine receptor expression on PCs and reports of chemotaxis in vitro suggested that chemokinesis might have a role in coordinating pre-PC motility in the lymph node (Hargreaves et al., 2001; Wehrli et al., 2001). To determine whether G protein-coupled receptor (GPCR) signaling might be involved in pre-PC motility in vivo, we treated mice with a high dose of Pertussis toxin (Ptx), which ablates G signaling and most chemokine-directed movement (Kaslow and Burns, 1992) in B cells. Treatment with Ptx completely stopped naive B cell migration (Figure 4B), which served as a positive control; cells extended small filopodial projections (Movies S4 and S5) but were completely confined based on their MSD plots. Despite having slower speeds (Figure 3B). One explanation for this small reduction may be the presence of large numbers of arrested lymphocytes creating barriers and added resistance to Blimp-1 cell migration. We saw similar trends in migration at all three
time points. Medullary Blimp-1-YFP+ cells at later time points that were already arrested remained arrested. We confirmed that Blimp-1-YFP+ cells were capable of chemotaxis in response to CXCL12, as had been previously reported for pre-PCs. Both Blimp-1-YFP+ cells and naive cells migrated toward CXCL12 in an in vitro transwell migration assay (Figure S3A). Pretreatment of these cells with Ptx completely blocked transwell migration. S1p chemokine signaling has also been implicated in PC chemotaxis and migration from the medulla to the bone marrow. S1p signals through S1p1 and S1p3 GPCRs, which use the Ptx-sensitive Ga12,13 for signaling. However, S1p3 can also utilize the Ptx-insensitive Gaq for signaling (Spiegel and Milstien, 2003). To block these additional pathways, we also gave Ptx-treated mice FTY720, which downregulates S1p1 and S1p3 receptors and prevents signaling. Mice treated with both Ptx and FTY720 showed no significant differences in pre-PC movement when compared to mice treated with Ptx (Figure S3B). These experiments indicate that although Blimp-1+ cells are capable of (in vitro) chemotaxis, their migration in vivo is only partially dependent on chemokine cues.

Pre-PC Migration Is Adhesion Mediated

We conducted in vitro live-cell imaging studies to determine what factors mitigate Blimp-1-YFP+ cell motility to allow arrest in the medulla. Glass substrates were coated with the adhesion molecule ICAM-1 and with or without chemokine ligands CXCL12 or CXCL13. We focused on ICAM-1 because of the established role of b2 integrins in PC egress from lymph nodes and the presence of high levels of ICAM-1 on many lymph node cells (Pabst et al., 2005). In vitro-activated Blimp-1-YFP+ cells were imaged in combination with naive dsRed+ B cells as controls. We used interference reflection microscopy (IRM) to image the cell contacting the substrate in order to measure adhesion and differentiate crawling versus fluid flow movement. Both cell types were capable of migrating on these substrates (Figure 5A, Movies S6 and S7). Naive cells migrated rapidly in response to either chemokine but also on ICAM-1 alone if BSA was present (Figure 5C), and chemokines were required for naive cell migration in the presence of fatty-acid-free BSA (Figure S5).

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Ptx treatment blocked naive cell migration (Figures 5B and 5D) and also reduced their ability to adhere to the substrate (Figure 5E). In contrast, Blimp-1-YFP+ cells, which migrated slower than naive cells (Figure 5C), only required ICAM-1 for migration (Figure S5). Blimp-1-YFP+ cell adhesion and migration on ICAM-1 substrates were unaffected by Ptx (Figures 5B, 5D, and 5E, Movie S8).

Based on the IRM image captured during cell migration, we could visualize the cell footprint, which informs on the binding to ICAM-1, as well as the mode of locomotion. Blimp-1+ cells, which are larger cells, had a larger contact surface than naive B cells, even when normalized to the cell size (Figures 5F and 5G). In time-lapse movies, we observed that (Movie S9) naive B cells had a dynamic and transient IRM image, with frequent rounds of detachment and reattachment to the substrate. In contrast, Blimp-1+ cells showed a stable IRM pattern, which spread evenly as they migrated in a processive amoeboid fashion.

Blimp-1+ cell velocity was heterogeneous in vitro (Figure 5C) and in vivo (Figure 3B). However, the cell speed was inversely related to YFP+ expression, a measure of their maturation state (Kallies et al., 2004). YFPlo cells were highly motile, whereas YFPhi cells appeared slow or arrested (Figure 6A). This relationship was also detected in in vivo track analysis (Figure 6B). Because this behavior is seen on ICAM-1 alone, it suggests PC arrest is cell autonomous.

Proper PC Localization in Medulla Requires Chemokine

On day 4 after immunization, large numbers of early pre-PCs develop and migrate, with ~80% accumulation in the medullary cords as compared to 10% of naive B cells. However, Ptx treatment (24 hr before visualization) reduced PC accumulation in the medulla to 40% while leaving naive B cells distributed normally (Figures 7A and 7B, Movie S5). BT zones normally contain YFPlo cells consistent with pre-PC; however, after treatment, BT zones were enriched in YFPhi cells consistent with PCs (Figure 7C). At later time points, most PCs were already arrested in the medullary cords, and Ptx treatment had no effect on their distribution (data not shown). We conclude that chemokine signaling is required for Blimp-1+ cell accumulation in the medulla.

DISCUSSION

PC retention in secondary lymphoid organs and bone marrow is critical for their long-term survival. Using a newly generated Blimp-1-YFP mouse, we were able to image pre-PCs as they differentiate and migrate to become PCs. To understand their migration, we compared their behavior to naive B cells, which are found throughout the lymph node. Naive B cells migrate rapidly in the B cell follicle in random directions, with short linear excursions punctuated by frequent turns (Miller et al., 2002). This frequent random turning is thought to be mediated by chemokine signals, particularly CXCL13, presented on surfaces of
stromal FDC networks, such that the scale for the free path between turns is set by the spacing of natural branch points in the stromal network (Bajenoff et al., 2006; Okada and Cyster, 2006). The exploration of this elaborate stromal network allows for a high-density search inside the B cell follicle (Miller et al., 2002). Consistent with the proposed role of chemokines in naive B cell movement in lymph nodes, Ptx treatment caused them to arrest.

In vivo, pre-PCs displayed a greater displacement velocity than naive B cells, and their pattern of migration was similar to a random walk but with a very long free-path length between turns. We describe this as a “random sprint.” Pre-PCs showed no directional bias away from the GC or follicle or toward the medullary cords, as might be expected for chemotaxis. This is consistent with the minor effect on migration by blocking chemokine signals with Ptx and FTY720 treatment. The pre-PCs only appear to turn when they encounter obstacles like the subcapsular sinus (Movie S4), suggesting that their migration to the medulla may be stochastic. The long, linear paths followed by pre-PC are shared by positively selected thymocytes moving toward the thymic medulla (Witt et al., 2005). However, the movement of the positively selected thymocytes is highly directed and chemotactic, whereas this is not the case for the movement of pre-PCs. Because the motility coefficient for a random walk process scales linearly with the free-path length or time persistence (Zygourakis, 1996), random motility with a long free path is an ideal strategy to search for the medullary cords, a compartment that may be hundreds of microns from the GC (Friedrich, 2008).

In vitro migration of naive and Blimp-1 cells revealed a more mechanistic explanation for the in vivo observations. Naive B cell contacts with the ICAM-1 surface were highly dynamic and rapidly contracted. Their migration and even adhesion to ICAM-1 was Ptx sensitive, suggesting a requirement for $G_{ai}$ signaling. In contrast, Blimp-1+ cells adhered to ICAM-1 and displayed slow and steady migration in the presence of Ptx and the absence of chemokine signals. Both of these two modes of migration have recently been described for naive T cells by Jacobelli et al., (2009). T cells can migrate either with an intermittent amoeboid locomotion dependent upon myosin IIa contraction or with a processive amoeboid “sliding” mode involving continuous ICAM-1 adhesion linked to retrograde actin flow. Naive B cells primarily use the intermittent, low adhesion mode allowing for frequent direction changes, whereas pre-PCs use the processive adhesion-dependent mode, leading to persistence of direction over tens of microns. This search strategy is effective because the long free-path length is well matched to the distances that the pre-PCs need to travel to reach the medullary cords. Pre-PCs may use FRC and FDC branched networks as scaffolds for adhesion and migration. However, their long free paths suggest that pre-PCs do not utilize these networks exclusively. ICAM-1 is probably not the only adhesion molecule that pre-PCs can use for migration in lymph nodes. In the absence of $\beta_2$ integrins, PCs still accumulate in the medullary cords but are unable to exit the tissue for the bone marrow (Pabst et al., 2005). We would propose that the ICAM-1-dependent arrest of PC allowed maturation in the medullary cords prior to a slower, integrin-dependent egress process that allows trafficking to the bone marrow.
The random sprint serves pre-PCs by facilitating their rapid exit from the GC. Pre-PCs might encounter a number of compartments on these random sprints including subcapsular region, T cell zone, other follicles, cortical sinuoids, and the medullary cords. Movement between compartments in tissues has typically been guided by chemokine gradients with CCR7 for T cell zones and thymocyte movement to the medulla and S1p in in vitro transwell assays may be modeling the in vivo retention of these cells in the medulla and red pulp rather than their directed migration to these regions. Indeed, Ptx treatment inhibited accumulation but not migration to the medulla, suggesting that retention in the medullary cords is chemokine dependent. PC arrest and differentiation were mislocalized but not inhibited by Ptx, confirming that in vivo, these processes are also cell autonomous. Myeloid cells in the medullary cords may provide survival signals such as BAFF and April (Belnoue et al., 2008; Mohr et al., 2009) but only for PCs that manage to find the medullary cords before they arrest. Further work is needed to determine whether cell-cell interactions contribute to PC migration and arrest as well.

### Experimental Procedures

#### Animals, Immunization, and Transfer

For adoptive-transfer experiments, wild-type C57BL/6, B6.SJL (CD45.1), CFP*, and dsRed+ mice (Jackson Laboratories) 7–12 weeks of age were used with protocols approved by the Institutional Animal Care and Use Committee. CD45.1+ hosts were immunized with an intraportalon (IP) immunization of 50 μg/mouse of OVA (Sigma) emulsified in Alum (Pierce) to enrich T cell help 2 weeks prior to cell transfer. B1.8* B cell line (with or without CFP* transgene) were used for donor cells. Splenic naive B cells were purified by CD43 depletion with MACS beads (Miltenyi Biotec) and transferred intravenously (IV) to hosts (5–10 x 10^6/mouse). After 24 hr, hosts received a subcutaneous (S/C) boost in the footpads with 100 μg of NP(15)-OVA (Biosearch). Lymphocytes were harvested and analyzed 4 to 10 days later by flow cytometry on a LSR II (BD Biosciences).

For imaging experiments, hosts were imaged on days 4, 7, and 10 after boost with NP-OVA. One day prior to imaging, naive control B cells purified from CFP* B6 mice were adoptively transferred into the mouse recipient as motility controls. For the labeling of medullary cord macrophages, 1 pmole of QTracker 705 nontargeted quantum dot (Invitrogen) was injected in the hind footpad. In addition, 2.5 μg of NP[11]Tomato or NP-Phycoerythrin (PE) (Biosearch) was injected in the hind footpad to label the light zone of the GC structures. NP-tomato was made in house, as a tomato-GST construct, expressed in e.coli, purified by Glutathione column (Amersham), and conjugated with NP-SE (Biosearch). For in vivo experiments, Pertussis toxin (Ptx, 400 μg/kg Calbiochem) was administered IV, and FTY720 (1 mg/kg) was delivered IP 12–24 hr prior to imaging (Huang et al., 2007).

#### In Vitro Imaging and Transwell

Blimp-1* naive B cells were purified and stimulated with LPS (25 ng/mL), IL-4 (5 ng/mL), IL-5 (15 ng/mL), and BAFF (10 ng/mL) for 3–4 days. Naive dsRed+ B cells were purified and incubated in RPMI+10% FBS overnight before imaging or transwell experiments. For transwell experiments, cells were incubated in the upper chamber of 5 μm Costar transwells for 2 hr with CXCL12 (1 μg/mL, R&D Systems) and were counted on a FACScalibur. For imaging, cells were imaged in Lab-Tek chamber slide systems (Nunc) with an adapted protocol kindly provided by the laboratory of Ronen Alon at the Weizmann Institute of Science (Rehovot, IL). Chemokines were spotted on clean glass surface for 2 hr at 37°C, washed, and then spotted with 5 μl of transmembrane ICAM-1 at 400 molecules/μm² for 1 hr at room temperature. The glass surface was blocked with 10% bovine serum albumin (BSA) or 10% fatty-acid-free (FBS) with or without Ptx (50 ng/ml) for 2 hr. Cells were resuspended in HBSS supplemented with 10 mM HEPES, 2 mg/ml BSA (FAB or Regular), and imaged at 37°C with a Zeiss LSM710 and standard confocal settings for YFP and dsRed fluorophores.
Immunity

Pre-Plasma Cell Migration in the Lymph Node

TPLSM Imaging and Analysis

For the preparation of imaging, mice were anesthetized with KXA (Ketamine, Xylazine, Acepromazine) solution (4 μl/g). We exposed the popliteal lymph node by shaving the leg and dissecting the skin and fatpad in an aseptic manner. A metal stabilization plate and coverslip were used to immobilize the lymph node. The mouse was comforted with regular injections of KXA, temperature control stage, and nose cone with O2 supplement.

For intravital TPLSM, an upright BioRad microscope was used as before (Schwickert et al., 2007) with a 40× Nikon objective and also repeated on a Zeiss LSM-710 inverted microscope with Mai-Tai Sapphire Laser for two-photon excitation with a 25× objective. Time lapse Z stacks were taken at 30 s intervals on either system, with similar measurements for cell speeds and migratory behavior. Tiled 3D stacks of the lymph node were collected on the Zeiss system.

Collected imaging series were analyzed for cell position and dynamics with Volocity 4.2 (Improvision), Excel 2003 (Microsoft), and GraphPad 4.0 (Prism). Movies were annotated with Adobe Affect Effects. Cell migrations were tracked automatically with Volocity, annotated for region, and pruned manually. Only continuous tracks that persisted at least 6 min in the field were used for analysis. For cell distributions within a lymph node, cell bodies were identified by RGB thresholding to cells. Rather than segmenting and counting individual cells, which were tightly packed particularly in the medullary cords, we determined the volume (in pixels) corresponding to a cell population, subdivided it by region in the lymph node, and presented it as a percentage of the total volume of the cell population found in the lymph node. Both sides of the lymph node were totaled for each measurement and repeated for four to six lymph nodes per condition. At least four lymph nodes were pooled per condition. Error bars represent SEM. (C) The average YFP intensity for all Blimp-1-YFP cells collected was calculated and plotted by region and Ptx condition. t test comparisons of the conditions were made with p values labeled above or with *** (p < 0.001).

Cell Staining and Flow Cytometry

Flow-cytometry measurements were conducted on a BD LSR II and analyzed with FlowJo 7.2. Cell populations were stained with BD Pharmagen antibodies unless otherwise stated: CD19, B220, CD138, CXCR5, CXCR4, Fas, GL7, and CD45.1.

Electron Microscopy Preparation and Imaging

Cells were washed with serum-free media or appropriate buffer and then fixed with a modified Karnovsky’s fix of 2.5% glutaraldehyde, 4% paraformaldehyde, and 0.02% picric acid in 0.1M sodium cacodylate buffer at pH 7.2. Following a secondary fixation in 1% osmium tetroxide, 1.5% potassium ferrocyanide, samples were dehydrated through a graded ethanol series, cleared with acetone, and then infiltrated and embedded in an epon analog resin. Ultrathin sections were cut with a Diatome diamond knife (Diatome) on a Leica Ultracut S ultramicrotome (Leica). Sections were collected on copper grids, further contrasted with lead citrate, and viewed on a JSM 4400X electron microscope (JEOL) operated at 80 kV. Images were recorded on Kodak 4489 Electron Image film and then digitized on an Epson Expression1600 Pro scanner.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and nine movies and can be found with this article online at doi:10.1016/j.immuni.2010.06.015.

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