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Immunosurveillance by Hematopoietic Progenitor Cells Trafficking through Blood, Lymph, and Peripheral Tissues

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SUMMARY

Constitutive egress of bone marrow (BM)-resident hematopoietic stem and progenitor cells (HSPCs) into the blood is a well-established phenomenon, but the ultimate fate and functional relevance of circulating HSPCs is largely unknown. We show that mouse thoracic duct (TD) lymph contains HSPCs that possess short- and long-term multilineage reconstitution capacity. TD-derived HSPCs originate in the BM, enter the blood, and traffic to multiple peripheral organs, where they reside for at least 36 hr before entering draining lymphatics to return to the blood and, eventually, the BM. HSPC egress from extramedullary tissues into lymph depends on sphingosine-1-phosphate receptors. Migratory HSPCs proliferate within extramedullary tissues and give rise to tissue-resident myeloid cells, preferentially dendritic cells. HSPC differentiation is amplified upon exposure to Toll-like receptor agonists. Thus, HSPCs can survey peripheral organs and can foster the local production of tissue-resident innate immune cells under both steady-state conditions and in response to inflammatory signals.

INTRODUCTION

Most differentiated cells found in mammalian blood have variable but limited life spans and must be constantly replenished. Blood cell homeostasis depends on a rare population of precursor cells, the hematopoietic stem cells (HSCs), which possess the unique capacity for self-renewal and multilineage differentiation. The function of HSCs and the partially lineage-committed progenitor cells that arise from them has been linked to their migratory properties, at least during fetal life, when the anatomic seat of hematopoietic activity changes several times (Cumano and Godin, 2007).

In postnatal mammalian life, hematopoietic stem and progenitor cells (HSPCs) reside mostly in specialized niches in bone marrow (BM) cavities that control HSPC survival, proliferation, self-renewal, and differentiation (Adams and Scadden, 2006). However, even in adulthood, HSPCs are not entirely sessile but contain a population of highly migratory cells. It is well established that some HSPCs recirculate constantly between BM and blood (Goodman and Hodgson, 1962; Wright et al., 2001b). Accordingly, normal blood from adult mice contains a small but stable population of several hundred HSPCs, which upon transplantation to irradiated recipients are capable of the long-term reconstitution (LTR) of hematopoietic activity (Fleming et al., 1993; Morrison et al., 1997). It has been speculated that the continuous trafficking of HSPCs between BM and blood is a mechanism to maintain full occupancy of HSPC niches in all BM cavities (Wright et al., 2001b). However, the exact trafficking pathways of blood-borne HSPCs and the physiological relevance of their postnatal migration remain largely unclear.

The daily turnover of HSPCs that enter and leave the bloodstream is believed to be high (Wright et al., 2001b). The BM is probably not the exclusive physiological source and destination of blood-borne HSPCs because HSPCs have also been recovered from extramedullary sites, like the liver (Cardier and Barbera-Guillem, 1997), spleen (Wright et al., 2001b), and muscle (McKinney-Freeman et al., 2002). Therefore, although we know little about the migratory dynamics of extramedullary HSPCs, it seems likely that circulating HSPCs visit anatomic regions other than the BM. A case in point is the trafficking of mature lymphocytes, which extravasate continuously into multiple lymphoid and nonlymphoid tissues. Most tissue-resident lymphocytes eventually return to the blood via lymphatics that drain into the thoracic duct (TD). This lymphocyte recirculation is essential for immunosurveillance because it maximizes the probability that lymphocytes encounter rare cognate antigens (von Andrian and Mackay, 2000).

Here, we have examined whether blood-borne HSPCs might follow similar extramedullary traffic patterns as lymphocytes. We demonstrate that efferent lymphatics

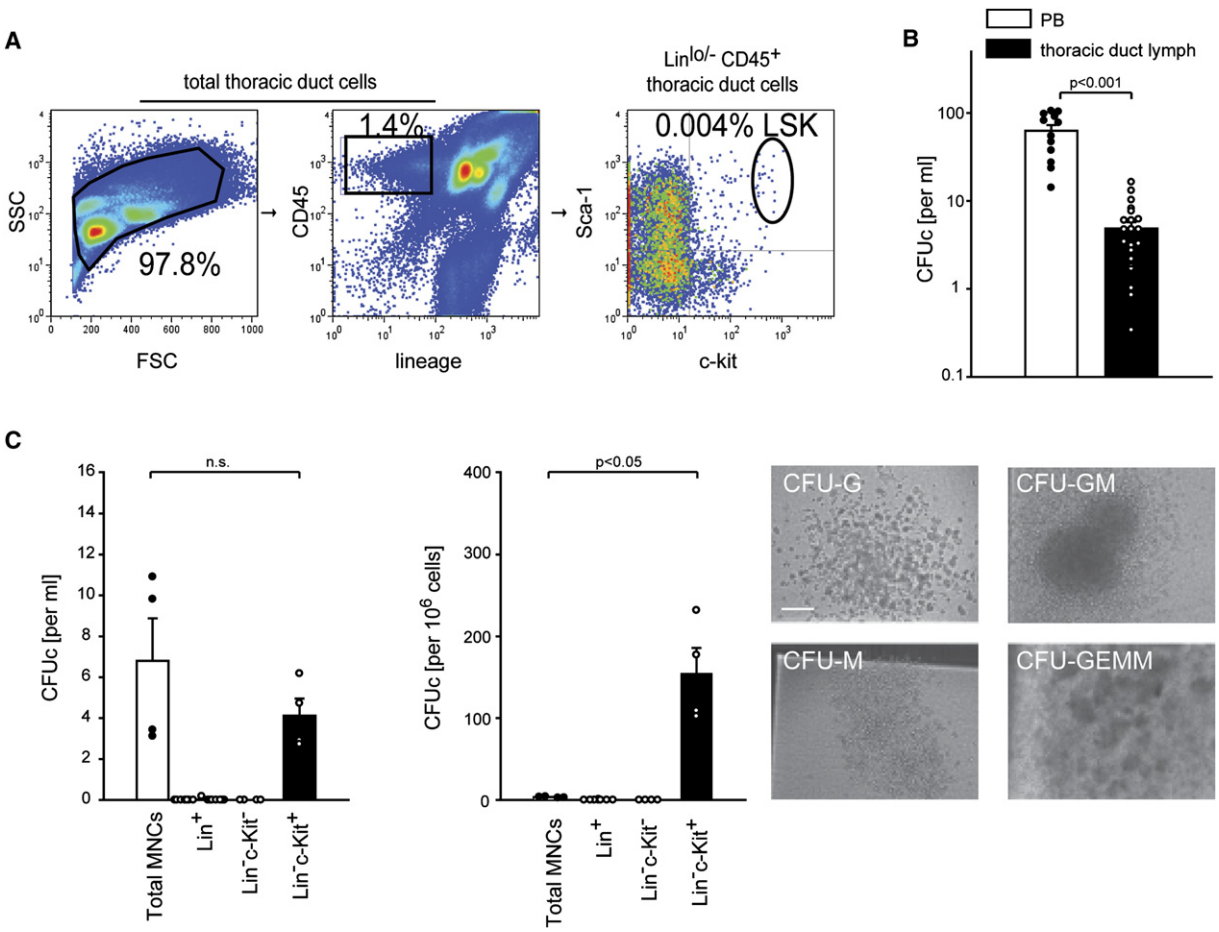

Figure 1. Clonogenic HSPCs Travel in TD Lymph

(A) FACS analysis of TD MNCs. Numbers show the frequency of gated cells among total MNCs. No events where detected in the LSK gate with isotype-matched control antibodies (data not shown).

(B) Concentration of CFU-Cs in normal mouse PB and TD lymph. Columns and error bars represent mean ± SEM, n = 12–23 assays (from 6–12 animals), and circles represent results from individual assays.

(C) Absolute (left) and relative frequencies (right) of CFU-Cs in total MNCs or sorted Lin^+, $Lin^-c\text{-}Kit^-$, and $Lin^-c\text{-}Kit^+$ TD cells. Mean ± SEM are presented (n = 4–16 assays, mean ± SEM). Right panel: Representative micrographs of $Lin^-c\text{-}Kit^+$ TD-derived CFU-Cs, defined by the presence of granulocytes, macrophages, erythroid cells and megakaryocytes as CFU-granulocyte (CFU-G), CFU-macrophage (CFU-M), CFU-granulocyte, macrophage (CFU-GM), CFU-granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM), respectively. Scale bars represent 50 μm.

contain a stable fraction of HSPCs that possess short- and long-term multilineage reconstitution capacity. TD HSPCs originate in the BM and traffic constitutively to multiple extramedullary, nonlymphoid tissues, where they reside for at least 36 hr until entering the draining lymphatics to return to the blood. This recirculation of HSPCs is regulated, in part, by the S1P receptor $S1P_1$ and might foster the local production of tissue-resident innate immune cells under both steady-state conditions and in response to infections.

RESULTS

Lin^- Hematopoietic Cells Travel in the TD

We surmised that if HSPCs recirculate through extramyeloid tissues, then they, like differentiated lymphocytes, might become lymph borne. Indeed, lymph fluid collected from murine TD (see the Supplemental Experimental Procedures in the Supplemental Data available with this article online) contained up to 4% mononuclear cells (MNCs) that expressed the pan-leukocyte antigen CD45 but no other hematopoietic lineage markers (Figure 1A). This population included $Lin^-IL\text{-}7R\alpha^+c\text{-}Kit^+Sca\text{-}1^+$ (~0.003%–0.004% of all TD MNCs) and $Lin^-IL\text{-}7R\alpha^-c\text{-}Kit^+Sca\text{-}1^-$ cells (~0.01%–0.03% of all TD MNCs), resembling the phenotype of committed BM common lymphoid (CLP) (Kondo et al., 1997) and common myeloid (CMP) progenitor cells (Akashi et al., 2000), respectively (Figure S1). In addition, $CD45^+$ $Lin^-IL\text{-}7R\alpha^-$ TD cells included cells with a primitive HSC and/or multipotent progenitor (MPP) phenotype ($Lin^-IL\text{-}7R\alpha^-c\text{-}Kit^+Sca\text{-}1^+$ [LSK]) (Christensen and Weissman, 2001), which amounted to 0.001%–0.004% of

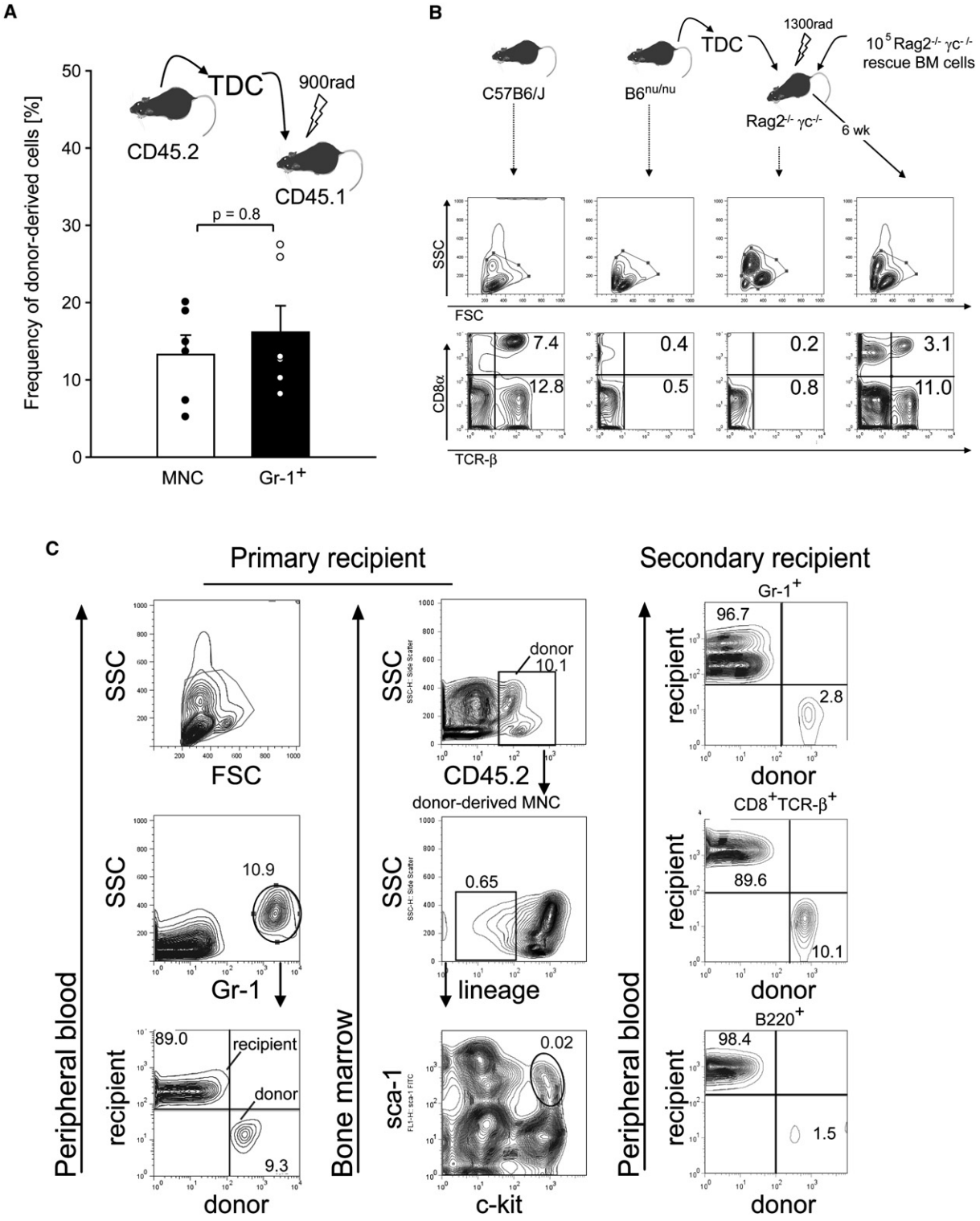

Figure 2. TD Lymph HSPCs Possess Long-Term Multilineage Repopulation Capacity

(A) TD-derived CD45.2$^+$ MNCs (2 × 10^7) were injected into sublethally irradiated CD45.1$^+$ recipients. Chimerism among total MNC and Gr-1$^+$ cells in PB was determined by FACS 6 weeks after transplantation (n = 6).

(B) TCR-β$^+$ T cells are found in PB of WT mice (first column) but not in nude (second column) or Rag2$^{-/-}$γc$^{-/-}$ mice (third column). Transfer of TD MNCs (2 × 10^7) from nude mice into lethally irradiated Rag2$^{-/-}$γc$^{-/-}$ mice restored T cells in Rag2$^{-/-}$γc$^{-/-}$ recipients (fourth column). Numbers show the frequency of CD8α$^+$ TCR-β$^+$ and CD8α$^-$ (mostly CD4$^+$) TCR-β$^+$ cells among MNCs. Data are representative of two experiments.

$CD45^+$ TD MNCs (Figure 1A and Figure S1). Lymph-derived LSK cells expressed several adhesion and traffic molecules, including CXCR4, CD44, PSGL-1, and LFA-1, as well as α4 and α5 integrins (Table S1). Methylcellulose-based colony-forming unit culture (CFU-C) assays of MNCs from blood and TD confirmed that both compartments contain clonogenic HSPCs (Figure 1B). Notably, no significant differences were observed in the number of CFU-Cs in peripheral blood collected 6, 12, or 24 hr after surgery or with control blood (without surgery), implying that the collection of thoracic duct lymph per se did not induce the enhanced mobilization of HSPCs (data not shown). Clonogenic cells mostly resided within the $Lin^-c\text{-}Kit^+$ population of TD cells, whereas Lin^+ or $Lin^-c\text{-}Kit^-$ TD cells gave rise to no or few colonies in CFU-C assays, respectively (Figure 1C). When lymph was collected from irradiated wild-type (WT) mice reconstituted with BM from green fluorescent protein (GFP)-transgenic donors (Wright et al., 2001a), virtually all of the colonies expressed GFP, indicating that the lymph-borne HSPCs are BM derived (Figure S2A). On average, we measured a TD lymph flow of 0.78 ± 0.05 ml/hr, and each ml of lymph contained 4.9 ± 0.8 CFU-C. Considering that TD lymph constitutes only ~50% of total body lymph flow, we estimate that ~200 clonogenic HSPCs pass through the lymph of a mouse every day. This number is substantial considering the fact that a single HSC can reconstitute the entire hematopoietic system of a mouse (Wagers et al., 2002) and that the infusion of as few as 30 of these cells is sufficient to save 50% of lethally irradiated mice and to reconstitute all blood cell types in the survivors (Spangrude et al., 1988).

Lin^- TD Cells Have BM Homing Capacity

To define whether lymph-borne Lin^- cells share the ability of blood-derived HSPCs to home to the BM, we injected fluorescently tagged Lin^- TD cells intravenously into mice, and their presence in recipient tissues was assessed by flow cytometry 24 hr later. Irrespective of whether recipients were preconditioned by irradiation, donor Lin^- cells were detectable in recipient BM and spleens (Figure S2B). Multiphoton intravital microscopy of skull BM (Mazo et al., 2005) in anesthetized recipients confirmed that Lin^- TD-derived cells can migrate into BM cavities within 12–24 hr after adoptive transfer (Figure S2C, Movies S1–S3).

HSPCs with Short- and Long-Term Multilineage Reconstitution Capacity Travel in the TD Lymph

So that the functional engraftment capacity of lymph-borne HSPCs could be explored, TD MNCs from $CD45.2^+$ donors were transplanted into sublethally irradiated $CD45.1^+$ congenic recipients. After 6 weeks, ~15% of $Gr\text{-}1^+$ peripheral blood granulocytes were donor derived (Figure 2A), indicating that lymph-borne HSPCs possess myeloid differentiation potential (Morrison and Weissman, 1994; Wright et al., 2001b). To test whether they also give rise to T cells, we harvested TD MNCs from athymic $B6^{nude/nude}$ mice, which lack mature T cells, although their HSPCs can give rise to T cells when transplanted to thymus-sufficient animals. $B6^{nude/nude}$ TD MNCs were injected into lethally irradiated $Rag2^{-/-}\gamma c^{-/-}$ mice, which possess a functional thymic anlage but are genetically incapable of generating lymphocytes (Goldman et al., 1998). Remarkably, 6 weeks after transplantation, $Rag2^{-/-}\gamma c^{-/-}$ recipients of $B6^{nude/nude}$ TD MNCs had a near-normal T cell compartment (Figure 2B). To address whether lymph-borne HSPCs contain multipotent HSCs with persistent self-renewal capacity, we transplanted $CD45.2^+$ TD MNCs into lethally irradiated $CD45.1^+$ recipients, which were sacrificed 12 weeks later. $CD45.2^+$ cells were then sorted from the first recipients' BM and transplanted into another $CD45.1^+$ recipient (Figure 2C). Ten weeks after the secondary transplantation, the recipient showed multilineage reconstitution by $CD45.2^+$ cells. Hence, normal murine lymph contains long-term multilineage repopulating HSPCs that meet the phenotypic and functional criteria of true HSCs.

HSPCs Do Not Require Lymphoid Structures for Recirculation

HSPCs travel between blood and lymph similar to naive lymphocytes. Hence, both populations might share transit routes guiding them from blood into lymphatics. Naive lymphocytes must home to lymph nodes and Peyer's patches (PPs) to gain access to lymph vessels (von Andrian and Mackay, 2000). Consequently, MNCs were dramatically reduced in lymph of lymphotoxin (LT)-α deficient mice (Figure 3A), which lack these lymphoid tissues (De Togni et al., 1994). However, HSPCs were still present in the TD lymph of LT-$\alpha^{-/-}$ mice, as assessed by fluorescence-activated cell sorting (FACS) (data not shown) or CFU-C assays (Figure 3B). Thus, HSPCs, unlike lymphocytes, do not require secondary lymphoid organs (SLOs) to recirculate but might traffic directly to the lymph from extramedullary nonlymphoid tissues.

HSPCs Continuously Travel from the BM to Various Extramedullary Tissues

To define where and how HSPCs might leave the blood and pass through extramedullary tissues into the lymph, we first surveyed different murine tissues for the presence of HSPCs by using CFU-C assays. Clonogenic

(C) $CD45.2^+$ TD-MNCs (2×10^7) were transplanted into sublethally irradiated $CD45.1^+$ primary recipients. Twelve weeks later, chimerism among PB granulocytes ($Gr\text{-}1^+$) was determined (left column). Analysis of $CD45.2^+$ cells in BM (middle column) demonstrates the persistence of a Lin^- subset, which includes LSK cells (indicated by circle in bottom panel). Twelve weeks after the primary transplantation, the $CD45.2^+$ $CD45.1^-$ cells were sorted out of the primary recipient's BM and transplanted into a lethally irradiated $CD45.1^+$ secondary host (right column). Chimerism among PB granulocytes ($Gr\text{-}1^+$), T cells ($CD8^+TCR\text{-}\beta^+$), and B cells ($B220^+$) was assessed by FACS 12 weeks after the secondary transplantation. Numbers show the frequency of cells in the individual quadrants.

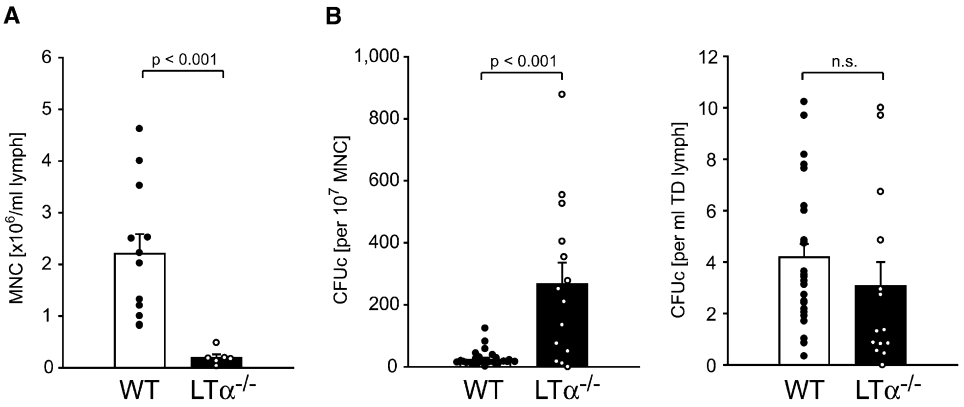

Figure 3. Secondary Lymphoid Organs Are Not Required for Recirculation of HSPCs across Extramedullary, Nonlymphoid Tissues
CFU-C assays were performed with TD MNCs collected from WT or LT-$\alpha^{-/-}$ mice. (A) shows the concentration of MNC in lymph (n = 6–12 animals); (B) shows the relative (left) and absolute (right) frequencies of CFU-Cs among lymph-borne MNCs (n = 14–28 assays). Error bars represent the mean ± the SEM.

progenitors were detectable in many tissues, including the lung, liver, small intestine, kidneys, and blood, but CFU-Cs were rare or absent in cardiac and skeletal muscle, skin, and LNs (Figure S3). It should be noted that we counted the number of colonies 7 days after the plating of the cells in methylcellulose to detect primitive HSPCs with large burst size. Therefore, our data do not account for the presence of late-outgrowth (more committed) progenitors, that have been reported to reside within some tissues, including peripheral LNs (MacVittie and McCarthy, 1977). Virtually all clonogenic tissue HSPCs were BM derived because colonies uniformly expressed GFP in chimeric wild-type recipients of GFP^+ BM (data not shown). Moreover, the transplantation of liver-derived CFU-Cs into irradiated recipients resulted in multilineage reconstitution, indicating that at least in some organs, clonogenic cells possess multilineage reconstitution capacity (data not shown). On the basis of the yield of CFU-Cs in surveyed organs, we estimate the total number of HSPCs resident in extramedullary nonlymphoid tissue to be at least twice as large as the average number of HSPCs circulating in murine blood (Wright et al., 2001b).

Next, we generated pairs of parabiotic mice, in which one partner ubiquitously expressed GFP. Consistent with previous reports (Wright et al., 2001b), crosscirculation was established by day 3 after surgical joining, and the host:partner (i.e. GFP^+:GFP^-) ratio of both mature leukocytes and CFU-C in blood plateaued at ~3:2 (corresponding to 40% chimerism) between days 10 and 14 (data not shown). In contrast, CFU-C chimerism in the BM reached only ~5% after 14 days of parabiosis (Figure 4A). Similar levels of BM chimerism were reported in mice joined for up to 16 weeks (Wagers et al., 2002). In extramedullary tissues of parabiotic mice, the highest level of HPC chimerism was in the spleen (40 ± 7%), and then the lungs (31 ± 6%), liver (24 ± 9%), and kidneys (15 ± 5%), implying that within these organs, tissue-resident HSPCs are constantly replenished by blood-borne HSPCs and are turned over within a matter of days. Partner-derived CFU-Cs were undetectable in the small intestine or brain. We also detected marked CFU-C chimerism (27 ± 1%) in the lymph of parabiotic mice, indicating that at least some homed HSPCs are not permanently retained in extramedullary tissues but recirculate freely between lymph and blood (Figure 4B).

To define the kinetics at which HSPCs enter the lymph after having accessed extramedullary tissues, we surgically separated some pairs of mice after 14 days of parabiosis. After separation, blood chimerism decreased rapidly, whereas lymph-borne HSPC chimerism was unchanged during the first 36 hr and declined only thereafter (Figure 4C). Given this delayed drop in HSPC chimerism, we estimate that the mean dwell time of HSPCs that have homed to peripheral tissues is at least 36 hr. This suggests that many organs are sites of active HSPC traffic from blood to tissue to lymph, but certain organs recruit few, if any, circulating HSPCs, at least over the 2 week time interval tested. Of note, a tracer (1% Evans Blue) injected directly into femur BM did not appear in any juxtaposed LN (data not shown) but became rapidly visible in draining LNs after intracutaneous injection. This is consistent with the long-held notion that mammalian BM lacks lymphatic drainage. Thus, HSPCs in the BM egress directly into the blood, whereas lymph-borne HSPCs have most likely departed from tissues other than the BM.

Sphingosine 1-Phosphate Receptor $S1P_1$ Regulates the Egress of HSPCs from Tissues into Lymphatics

Next, we sought to determine whether chemoattractants guide HPC recirculation. Most chemoattractants that control leukocyte migration signal through Gαi-coupled receptors that can be inhibited by pertussis toxin (PTX). Indeed, as early as 2–12 hr after a single PTX injection, we observed a dramatic drop in the number of lymph-borne CFU-Cs (Figure 5A). In blood, the number of CFU-Cs

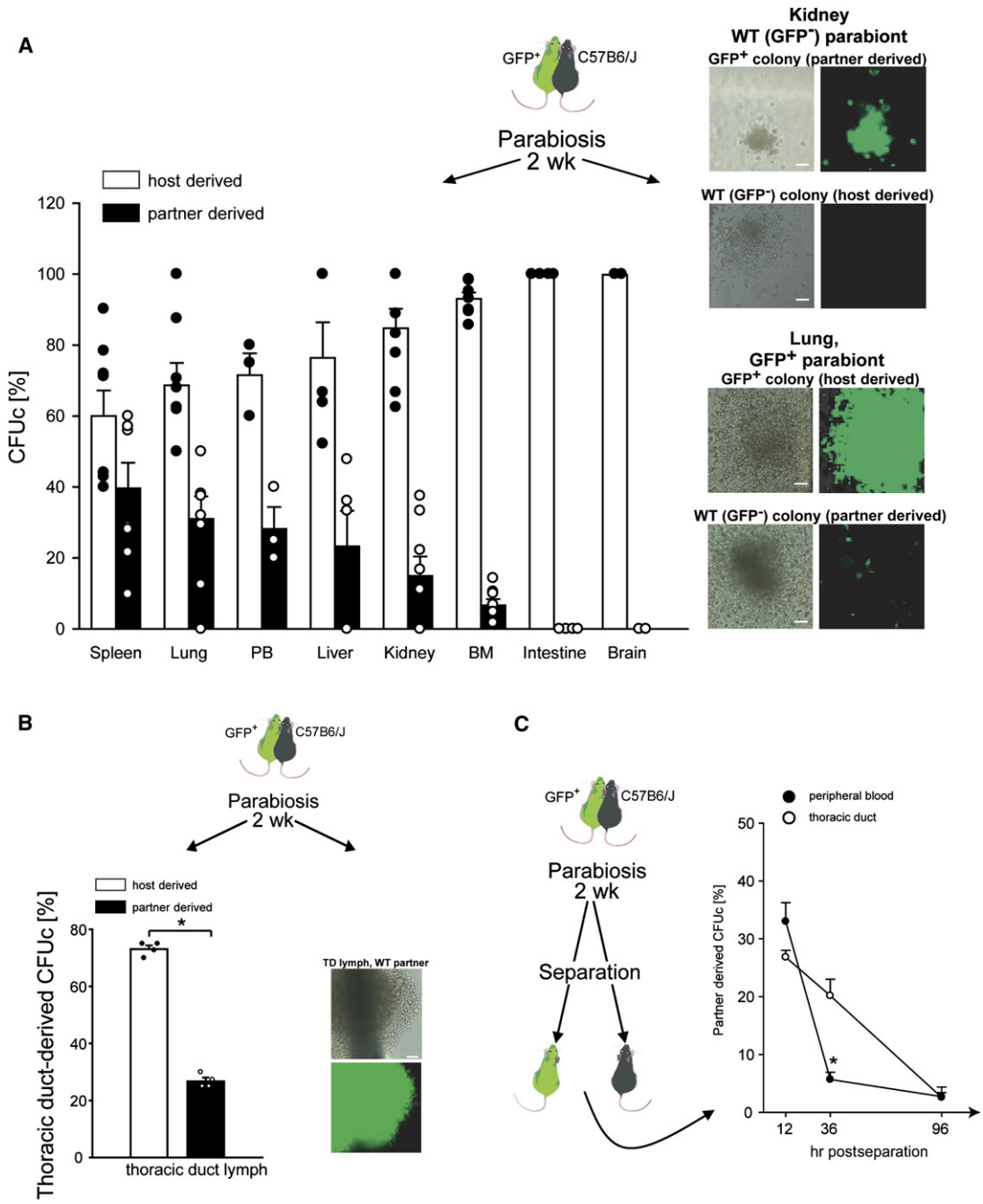

Figure 4. Recirculation of HSPCs through Multiple Extramedullary, Nonlymphoid Tissues

(A) We parabiosed WT and β-actin-GFP transgenic mice and analyzed them by isolating MNCs from the indicated tissues and testing them for GFP^+ and GFP^- CFU-C content (bar graph). Micrographs show representative brightfield (left) and fluorescence (right) images of host- and partner-derived CFU-Cs. n = 2–8, mean ± SEM.

(B) CFU-C chimerism in TD lymph of parabiotic mice (n = 4, mean ± SEM).

(C) CFU-C chimerism in PB (closed circles) and TD lymph (open circles) at different time points after surgical separation of parabiotic mice (n = 4–8 experiments, mean ± SEM).

was not significantly altered during this time interval; however, their number increased markedly at 24–96 hr after PTX injection (Papayannopoulou et al., 2003; data not shown). Thus, Gαi-coupled chemoattractants might play a role in the long-term recruitment of HSPCs from blood to tissues. However, our parabiosis experiments predict that the dwell time of HSPCs within tissues is greater than 36 hr. This implies that a PTX-induced drop in

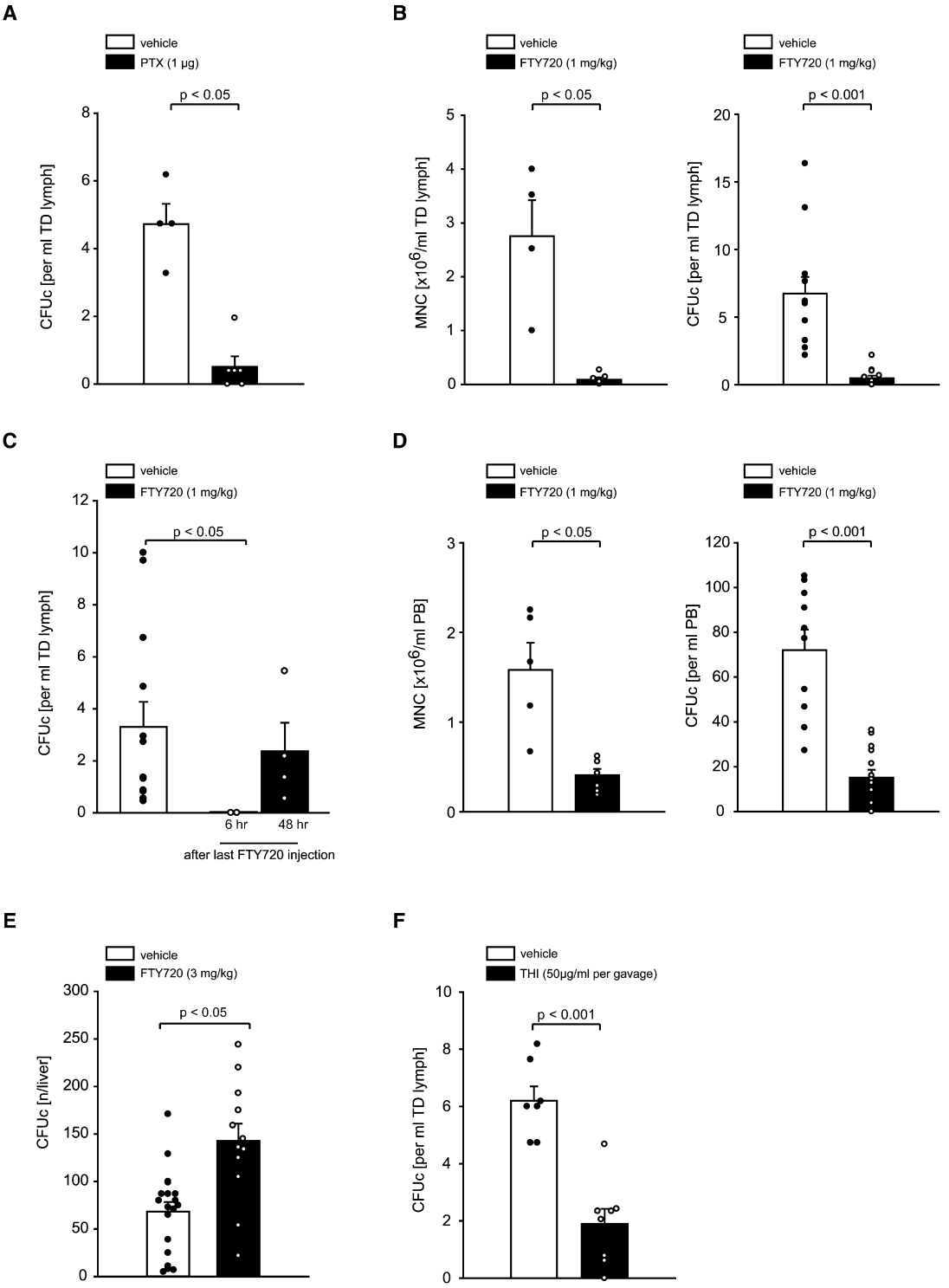

Figure 5. Egress of HSPCs from Extramedullary Tissues into Lymph is PTX Sensitive and Depends on S1P

(A) Effect of PTX on lymph-borne CFUc. Lymph was collected for 8 hr from mice that were previously treated for 2 hr with vehicle or 1 μg PTX, and CFU-C concentration was measured. n = 4–6 assays.

(B) Effect of FTY720 on lymph-borne MNCs and CFUc. The effect of treatment with S1P receptor antagonist FTY720 (6 hr) on MNC concentration (left panel, n = 4–7) and CFU-C content (right panel, n = 12–14) in lymph is shown.

(C) Effect of FTY720 in LTx-$\alpha^{-/-}$ mice. CFU-C assays were performed with thoracic duct MNC collected from LT-α-deficient mice treated with vehicle or FTY720. Lymph MNCs were collected for 8 hr from FTY720-pretreated mice starting at 6 or 48 hr after the last drug administration. The number of CFU-Cs per ml of lymph is shown.

lymph-borne CFU-Cs should only become manifest after ~36 hr if migration across the blood-tissue interface is the only step affected by PTX. The much more rapid time course of PTX-induced CFU-C disappearance from lymph implies an essential role for a Gαi-coupled signal that directs tissue-resident HSPCs into draining lymphatics.

Egress of tissue-resident lymphocytes into lymphatics is controlled by $S1P_1$, a Gαi-coupled receptor for sphingosine 1-phosphate (S1P) (Massberg and von Andrian, 2006; Rosen and Goetzl, 2005). We found that murine HSPCs express $S1P_1$, as well as $S1P_2$, $S1P_3$, and $S1P_4$ messenger RNA (mRNA) (Figure S4A). Although quantitative real-time polymerase chain reactin (PCR) showed that the $S1P_1$ mRNA expression levels are lower on HSPC subsets compared to B and T cells (Figure S4B), they were much higher than on myeloid cells, and both progenitor subsets migrated toward S1P in vitro (Figure S4C). Thus, we asked whether S1P signaling might influence HSPC recirculation. Most S1P receptors, including $S1P_1$, are blocked by the immunosuppressant FTY720, which induces lymphocyte sequestration in SLOs, causing profound lymphopenia in blood and lymph (Chiba et al., 1998; Mandala et al., 2002; Matloubian et al., 2004). Remarkably, the treatment of mice with FTY720 for 6 hr nearly abolished lymph-borne CFU-Cs (Figure 5B). Among the multiple S1P receptors expressed by HSPCs, $S1P_1$ appeared to play the predominant role for this effect because SEW2871, which selectively targets $S1P_1$ (Jo et al., 2005), transiently reduced the appearance of lymph-borne CFU-Cs to a similar extent as FTY720 (Figure S4D). The disappearance of lymph-borne CFU-Cs in response to S1P receptor inhibition did not require HSPC sequestration in SLOs because FTY720 blocked HSPC recirculation also in LT-$\alpha^{-/-}$ mice (Figure 5C). HSPCs reappeared in the lymph of LT-$\alpha^{-/-}$ mice within 2 days after the discontinuation of FTY720 treatment (Figure 5C), suggesting that FTY720 does not affect the viability of HSPCs. Likewise, FTY720 did not alter the capacity of HSPCs to form colonies in CFU-C assays (data not shown).

Notably, FTY720 not only depleted lymph-borne HSPCs but also induced a significant (74 ± 5%) drop of CFU-Cs in the blood (Figure 5D). On the basis of our parabiosis experiments, a decrease of HSPC migration across the blood-tissue interface would translate into a decrease in lymph CFU-Cs only after ~2 days. This suggests that the drop of blood CFU-Cs alone cannot account for the disappearance of CFU-Cs from the lymph observed within 6 hr after FTY720 treatment. The treatment of mice with FTY720 over 7 days resulted in a significant increase in the number of HSPCs residing within extramedullary tissues (Figure 5E). Hence, our data imply that S1P receptors, particularly $S1P_1$, regulate the egress of HSPCs from tissue into the draining lymphatic vasculature. The number of HSPCs in the BM of FTY720-treated mice was not significantly altered compared to vehicle-treated animals (Figure S4E). Hence, the FTY720-induced disappearance of HSPCs from blood was due to the inhibition of HSPC recirculation from extramedullary tissues. Consistent with this notion, we did not observe a significant difference in the number of HSPCs mobilized to the blood in response to G-CSF between mice treated with FTY720 or vehicle (data not shown).

S1P is abundant in blood and lymph but low in lymphoid and nonlymphoid tissues because of rapid interstitial degradation by tissue-resident S1P lyase (Schwab et al., 2005). Extravascular lymphocytes in LNs enter the lymph in response to steep S1P gradients. This egress step can be inhibited by the pharmacological blockade of S1P lyase with 2-acetyl-4-tetrahydroxybutylimidazole (THI) (Schwab et al., 2005). To test whether S1P degradation through S1P lyase is also required for HSPC recirculation, we collected lymph from THI-treated mice. As reported previously (Schwab et al., 2005), THI treatment resulted in profound lymphopenia in blood and lymph (data not shown). Concomitantly, lymph-borne HSPCs were markedly reduced (Figure 5F), indicating that S1P lyase is required for HSPC passage through tissues.

Tissue-Resident HSPCs Give Rise to Myeloid Cells in Peripheral Tissues

To test whether recirculating HSPCs might give rise to mature hematopoietic cells locally within extramedullary tissues, we implanted HSPC-enriched Lin^-GFP^+ TD-derived cells (5×10^5) or HSC-enriched BM-derived Lin^- $c\text{-}Kit^+Sca\text{-}1^+$ cells (5×10^4) under the left kidney capsule of recipient mice (Figures 6A and 6B). Medium without cells was injected into the contralateral (right) kidney. Six days later, a sizeable number of GFP^+ cells was detected within the kidney implanted with Lin^-GFP^+ TD cells and, especially, after the implantation of HSC-enriched BM-derived $Lin^-c\text{-}Kit^+Sca\text{-}1^+$ cells (Figures 6A and 6B). A significantly lower but stable number of GFP^+ cells was observed in the medium-injected control kidney ($p < 0.05$, Figure 6A) but also within the blood and the BM, as assessed by CFU-C assay after the implantation of $Lin^-c\text{-}Kit^+Sca\text{-}1^+$ cells (Figure 6C). Confocal microscopy showed that virtually all GFP^+ cells in injected kidneys co-expressed the pan-hematopoietic marker CD45 (suppl. Figure 5A). The majority (>50%) of the GFP^+ cells coexpressed myeloid lineage markers (Figure 6A and Figures S5A and S5B) and ~10% coexpressed B220,

(D) Effect of FTY720 on blood-borne MNCs and CFUc. The effect of treatment with FTY720 (6 hr) on MNC concentration (left panel) and CFU-C frequency (right panel) in PB is shown.

(E) Effect of FTY720 on hepatic CFUc. Liver MNCs were collected from mice treated with vehicle or FTY720 (3 mg/kg) for 7 days. The total number of CFU-C per liver is given.

(F) Effect of THI on lymph-borne CFUc. The CFU-C content in TD lymph of control mice (vehicle) and animals that had received the S1P lyase inhibitor THI for 3 days (n = 7–8) is shown.

Results are shown as mean ± SEM.

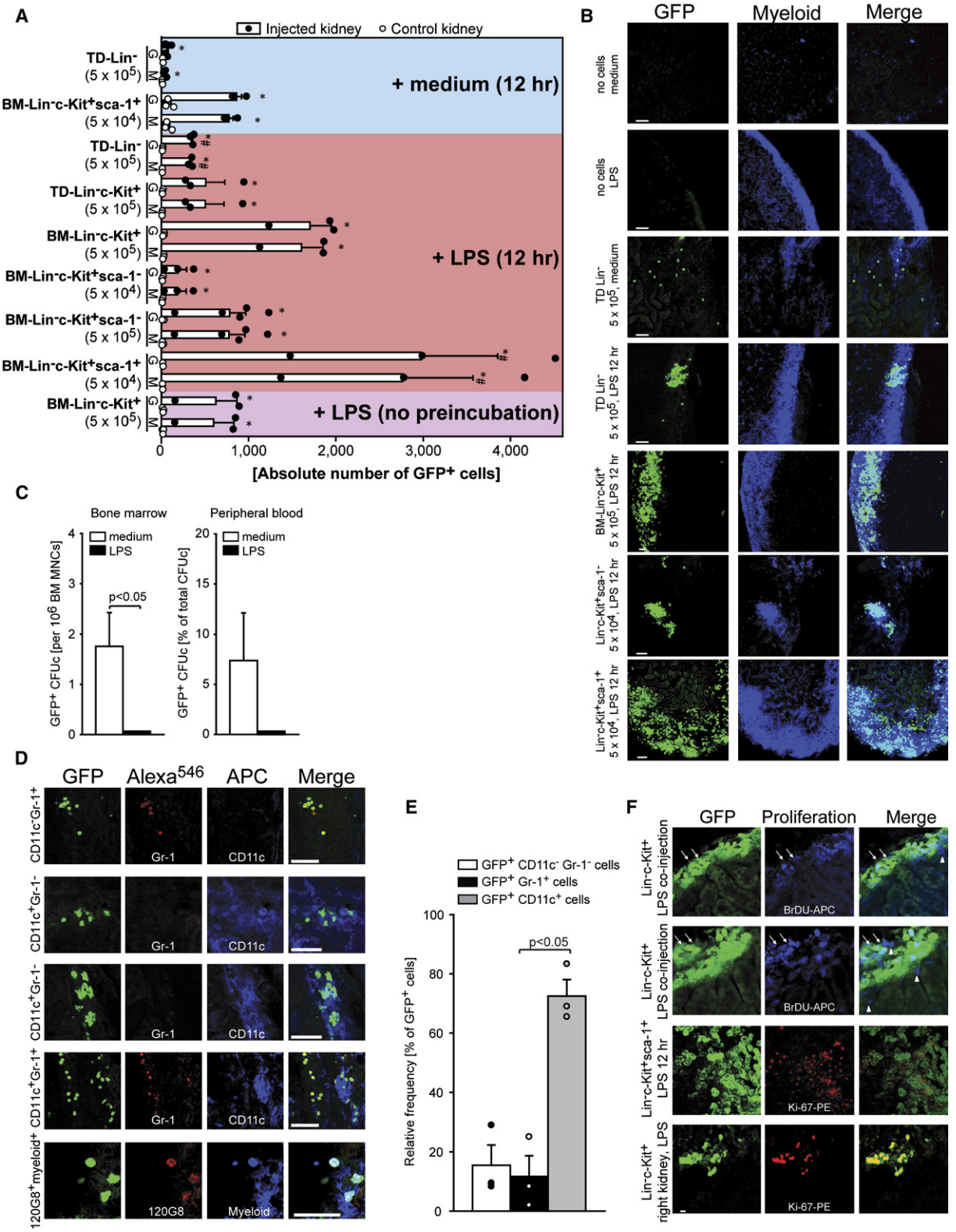

Figure 6. HSPCs Proliferate and Give Rise to Myeloid Cells in Peripheral Tissues Particularly in Response to TLR Ligands

(A and B) Distinct TD- or BM-derived HSPC-enriched populations were implanted under the left kidney capsule of WT mice with medium or LPS with or without preincubation for 12 hr as indicated. Medium or LPS without cells was injected under the contralateral control kidney capsule. (A) shows quantitative analysis of the total number of GFP$^+$ cells ("G") and of GFP$^+$ myeloid cells ("M") in the left (empty bars and filled circles) and in the right

whereas no CD3- (T cell lineage) expressing GFP$^+$ cells were detected (Figure S5A). The absolute numbers of GFP$^+$ cell with myeloid phenotype were 26- and 14-fold higher in the kidneys injected with Lin$^-$GFP$^+$ or Lin$^-$ c-Kit$^+$Sca-1$^+$GFP$^+$ cells, respectively, compared to the contralateral cell-free medium-treated kidneys. This implies that after deposition within tissues, HSPCs have the capacity to differentiate locally into myeloid lineages. Cell fusion is not likely to account for this process because the coexpression of CD45.1 and GFP was never observed when GFP$^+$ CD45.2$^+$ HSPC-enriched cells were implanted into CD45.1$^+$ kidney capsules (data not shown).

Tissue-Resident HSPCs Respond to TLR Ligands

HSPCs express Toll-like receptors (TLRs) and their coreceptors, including TLR4, MD-2, and CD14, required for recognition of bacterial lipopolysaccharide (LPS) from Gram-negative bacteria (Nagai et al., 2006). In vitro, TLR signaling drives the differentiation of HSPCs into myeloid lineages. Migratory HSPCs that encounter TLR ligands within extramedullary tissues might thus act as a highly versatile local source to rapidly replenish innate immune cells during infection. We therefore incubated distinct subsets of TD- or BM-derived HSPC-enriched cell populations for 12 hr with LPS (10 μg/ml) and implanted them with LPS underneath the left kidney capsule. LPS (10 μg/ml) without HSPCs was injected into the contralateral (control) kidney (Figures 6A and 6B). Six days after implantation, large numbers of GFP$^+$ cells were present in all kidneys injected with GFP$^+$ HSPCs subsets (Figures 6A and 6B). These cells were typically located in clusters, each comprising up to ~400 cells. Similar findings were obtained when we instilled LPS beneath the kidney capsule immediately prior to the implantation of HSPCs that were not previously incubated with LPS (Figure 6A). The highest numbers of GFP$^+$ cells were observed after implantation of HSC-enriched Lin$^-$c-Kit$^+$sca-1$^+$ cells (10^4), whereas HSC-depleted Lin$^-$c-Kit$^+$sca-1$^-$ cells (enriched in CMPs) gave rise to comparably lower numbers of GFP$^+$ cells.

In the presence of LPS, more than 95% of the GFP$^+$ cells coexpressed myeloid lineage markers (Figures 6A and 6B). Most cells (>70% of all GFP$^+$ cells) expressed the dendritic cell marker CD11c (Figures 6D and 6E, Figure S5C). A small subset (~10%) of GFP$^+$ cells expressed Gr-1 at intermediate to high levels, indicative of monocyte and/or macrophage and/or granulocyte differentiation, whereas very few (<1%) GFP$^+$ myeloid cells were stained with 120G8 mAb, which recognizes plasmacytoid dendritic cells (Figures 6D and 6E, Figure S5C, and data not shown). Most of the GFP$^+$ clusters contained either CD11c$^+$ or Gr-1$^+$ cells, and < 10% of the clusters contained a mixture of both subsets. A sizeable fraction (>15%) of GFP$^+$ cells expressed neither CD11c nor Gr-1, suggesting that some cells remained either undifferentiated or gave rise to other leukocyte subsets.

In all instances, the numbers of all GFP$^+$ cells and of myeloid GFP$^+$ cells were significantly higher after the implantation of HSPCs together with LPS compared to kidneys injected with the same subset of GFP$^+$ HSPCs without LPS (Figure 6A), suggesting their local proliferation after TLR ligand engagement. When recipient mice were treated with bromodeoxyuridine (BrDU) after the implantation of HSPC-enriched GFP$^+$ cells, the majority of the cells of each of the GFP$^+$ clusters had incorporated BrDU into their nucleus (Figure 6F). Similar findings were obtained when clusters were stained for the proliferation marker Ki-67 (Figure 6F), implying that in the presence of TLR ligands HSPCs proliferate locally. The engagement of LPS also reduced the exit of clonogenic HSPCs out of peripheral tissues because the number of donor-derived GFP$^+$ CFU-Cs was significantly reduced in both peripheral blood and BM after the implantation of LPS-pretreated cells (Figure 6C). Along these lines, the number of GFP$^+$ cells in the control kidneys (injected with medium or LPS without cells), was significantly higher in mice injected with medium as compared to LPS-treated mice. Although the overall number of donor-derived cells was low in the control kidneys injected with LPS without HSPCs, we detected GFP$^+$Ki-67$^+$ myeloid clusters in these kidneys (Figure 6F), providing additional in vivo evidence for the concept of HSPC migration to peripheral tissues contributing to myeloid defense in inflamed organs.

LPS Interferes with S1P-S1P$_1$ Signaling in Recirculating HSPCs

Finally, we asked whether TLR stimulation might modulate the capacity of HSPCs to migrate toward S1P gradients. We sorted Lin$^-$ or Lin$^-$c-Kit$^+$ TD cells and measured S1P$_1$ mRNA levels after incubation with LPS or medium for 12 hr. Compared with medium-treated cells, TD-derived Lin$^-$ and Lin$^-$c-Kit$^+$ TD cells that had been exposed to LPS, had 2- to 3-fold-reduced S1P$_1$ transcript levels (Figure 7A, $p < 0.05$). The downregulation of S1P$_1$ in response to LPS was paralleled by the failure of LPS-treated HSPCs to chemotax toward gradient of S1P, whereas migration toward CXCL12/SDF-1α was enhanced (Figure 7B).

(control, open circles) kidney. "*" indicates $p < 0.05$ versus control kidney and "#" indicates $p < 0.05$ versus medium. Mean ± SEM is shown; n = 3 experiments. (B) shows representative confocal images showing the expression of GFP and reactivity with a mixture of mAbs to myeloid markers (CD11c, CD11b, F4/80, Gr-1) 6 days after implantation. Scale bars represent 50 μm.

(C) Detection of GFP$^+$ (donor-derived) CFU-C within the BM (left) and PB (right) after the implantation of Lin$^-$c-Kit$^+$Sca-1$^+$ cells preincubated with medium or LPS for 12 hr. Mean ± SEM is shown; n = 3 experiments.

(D and E) HSPC-enriched Lin$^-$c-Kit$^+$ GFP$^+$ cells were implanted into the left kidney capsule of WT recipient mice together with LPS. (D) shows representative micrographs showing the expression of GFP, Gr-1, CD11c, or the PDC marker 120G8 in the kidney 6 days later. Scale bars represent 50 μm. (E) shows relative subset frequencies among GFP$^+$ cells in serial kidney sections. Mean ± SEM is shown; n = 3 experiments.

(F) Representative confocal images showing expression of GFP, incorporation of BrDU, and expression of Ki-67 6 days after implantation of the indicated HSPC populations. Scale bars represent 10 μm. Arrows indicate GFP$^+$ BrDU$^+$ cells, and arrow heads show GFP$^-$ BrDU$^+$ cells.

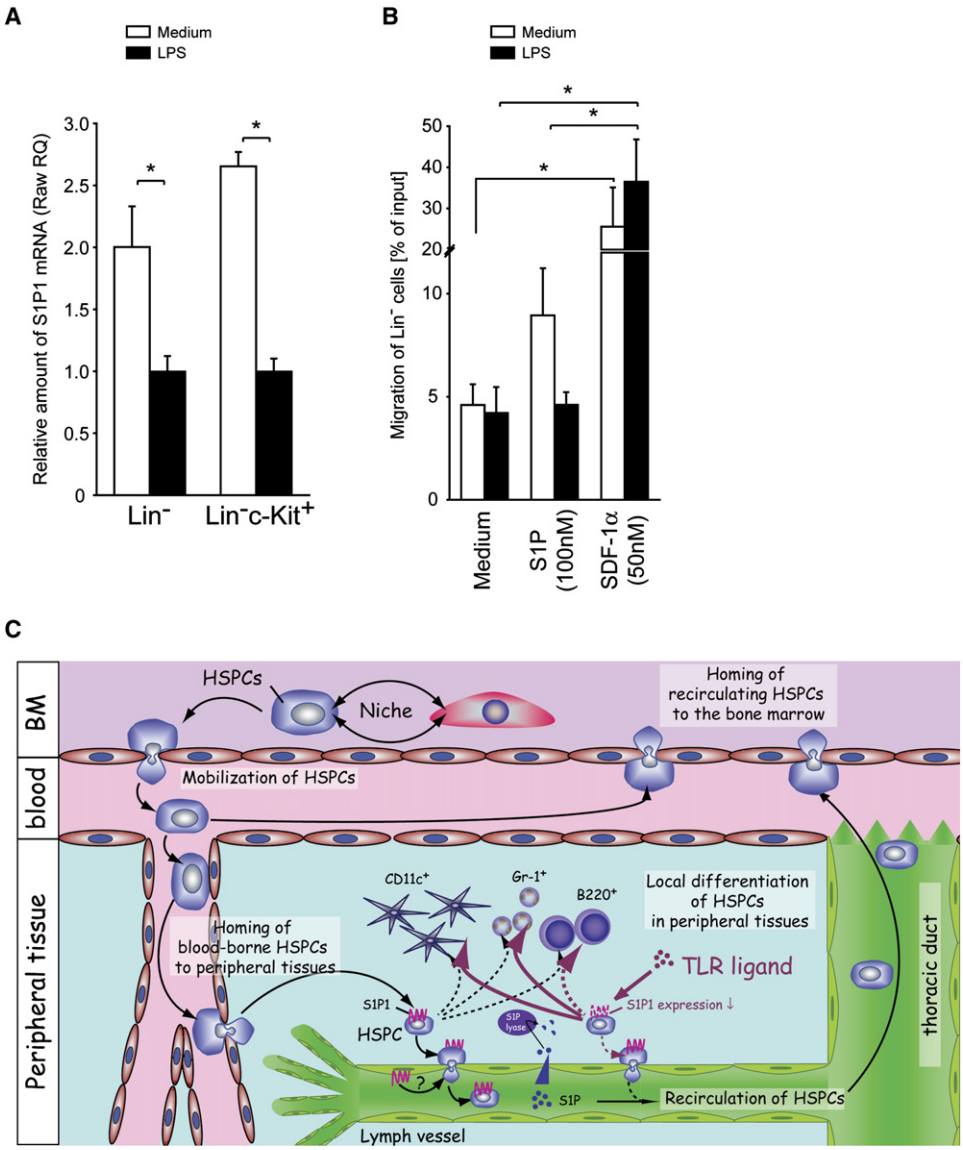

Figure 7. A TLR Ligand Reduces the Capacity of HSPCs to Migrate to Sphingosine 1-Phosphate

(A) Quantitative PCR analysis of $S1P_1$ mRNA expression by medium- or LPS-treated Lin^- or $Lin^-c\text{-}Kit^+$ TD-derived cells. The amount of $S1P_1$ mRNA is expressed as relative amount of LPS-treated cells normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Experiments were performed in quadruplicate, and "*" indicates $p < 0.05$. Results are shown as mean ± SEM.

(B) Lin^- BM-derived cells pretreated for 12 hr with LPS or medium were tested for their ability to migrate to 100 nM S1P or to 50 nM CXCL12/SDF-1α. Migration to the lower chamber was determined by flow cytometry and is given as percentage of the number of input cells (n = 3 experiments per group). "*" indicates $p < 0.05$ versus SDF-1α. Results are shown as mean ± SEM.

(C) Schematic model illustrating the trafficking of migratory HSPCs under physiological conditions and during an inflammation (for details, see main text).

Hence, LPS recognition not only triggers local proliferation and differentiation but also leads to the retention of HSPCs within extramedullary tissues (Figure 6C), at least in part by interfering with S1P-$S1P_1$-dependent signaling.

DISCUSSION

Our data suggest that extramedullary tissues are constitutively surveyed by a pool of recirculating hematopoietic progenitors that are highly versatile and can respond rapidly to distress signals from the local microenvironment (Figure 7C). Naive T and B lymphocytes, another population of recirculating cells, gain access to the lymphatic vasculature after homing to SLOs, which they reach through specialized postcapillary venules, the high endothelial venule (HEV) (von Andrian and Mackay, 2000). However, HSPCs can be recovered from TD lymph of lymphotoxin-α-deficient mice that lack HEVs and SLOs (De

Togni et al., 1994). This implies that blood-borne HSPCs do not require SLOs to access lymphatics. Accordingly, we recovered few, if any, CFU-Cs from pooled LNs. Although HSPCs entering the lymph in peripheral nonlymphoid tissues must pass through at least one draining LN before reaching the TD, lymph-borne HSPCs that give rise to early CFU-Cs (within 7 days of culture) are apparently not retained in the LN stroma but continue to travel through efferent lymph conduits into the blood. This lack of HSPC accumulation in LNs is most likely due to the fact that HSPCs lack CCR7 (Wright et al., 2002), which is essential for homing to LNs (Debes et al., 2005; Forster et al., 1999; Stein et al., 2000). However, HSPCs express multiple other traffic molecules, including PSGL-1, VLA-4 (CD49d/CD29), LFA-1 (CD11a/CD18), CD44, and the chemokine receptor CXCR4, which are likely to contribute to HSPC migration to nonlymphoid tissues, particularly sites of inflammation (Table S1) (Wright et al., 2002).

Although the recruitment mechanism(s) responsible for HSPC trafficking from blood into extramyeloid tissues remain(s) to be elucidated, our experiments have uncovered a pivotal role for S1P during the subsequent departure of tissue-resident HSPCs into lymphatics. S1P plays a key role in enabling lymphocyte egress from the thymus into the blood and from SLOs into the lymph (Cyster, 2005; Massberg and von Andrian, 2006; Rosen and Goetzl, 2005). Our study demonstrates that S1P and its receptors, in particular $S1P_1$, also regulate HSPC recirculation. HSPCs disappeared from blood and TD lymph when WT or lymphotoxin-α-deficient mice were treated with FTY720. In principle, this effect would be expected with any treatment that blocks HSPC trafficking from one compartment to another, be it from the BM to the blood, from blood to extramyeloid tissues, or from tissues into the lymph. However, our parabiosis experiments revealed that HSPCs need at least 36 hr to transit from the blood across peripheral tissues into the draining lymph, so treatment effects that block HSPC homing from blood to tissues would only result in reduced lymph-borne CFU-Cs after this lag time. In contrast, both SEW2871 and FTY720 treatment depleted HSPCs from the lymph within only 6 hr, indicating that S1P, acting primarily through $S1P_1$, controls HSPC exit from nonlymphoid tissues into the draining lymph vessels. This interpretation is also consistent with the observation that prolonged FTY720 treatment resulted in HSPC accumulation in peripheral tissues, whereas the number of blood-borne HSPCs was reduced. Because HSPCs express $S1P_1$ mRNA and chemotax toward S1P gradients in vitro (Figures S4A–S4C and Figure 7), it is likely that HSPC-expressed $S1P_1$ mediates HSPC egress from extramedullary tissue. However, it should be noted that lymphatic endothelial cells also express $S1P_1$ (Singer et al., 2005; Wei et al., 2005), which raises the possibility that S1P might exert additional control over HSPC trafficking via its action on lymphatic endothelial cells.

An S1P gradient between lymphoid tissue and lymph fluid is required for lymphocyte egress from LN (Schwab et al., 2005). Interstitial tissue fluids contain low levels of S1P, whereas extracellular S1P concentrations in lymph and plasma are high (Cyster, 2005; Schwab et al., 2005). Although plasma S1P is mainly produced by hematopoietic cells, high lymph S1P levels are maintained by a nonhematopoietic, radiation-resistant source (Pappu et al., 2007). The S1P gradient between LNs and lymph fluid is maintained by the S1P-degrading enzyme S1P lyase (Schwab et al., 2005), which is abundant in most tissues and cell types except in platelets and erythrocytes (Pappu et al., 2007). Our present findings suggest that S1P lyase is similarly essential for the egress of HSPCs from nonlymphoid extramedullary tissues. S1P gradients are also required for the migration of bilateral heart progenitors in zebrafish (Kupperman et al., 2000). Hence, guidance signals provided by extracellular lipids, such as S1P, might represent a conserved navigation mechanism for migrating stem and progenitor cell populations that is utilized by vertebrates and invertebrates alike.

What is the functional relevance of HSPC trafficking in adults? The recirculation of HSPCs between the BM and blood is believed to be important for the maintenance of hematopoietic homeostasis in dispersed BM cavities (Wright et al., 2001b). However, the role of the migratory pool of HSPCs that percolate constitutively through extramedullary tissues and then return to the blood via the TD is less obvious. Our kidney capsule transplantation experiments show that even in the absence of inflammation, recirculating HSPCs divide locally in peripheral tissues and differentiate into mature myeloid cells. Homed HSPCs might thus help to constitutively replenish the diverse population of tissue-resident leukocytes that perform multiple essential functions in peripheral organs, including, e.g., the removal of dead cells and debris.

Given the diverse activities of leukocytes in the context of tissue damage and immune defense, it would seem plausible that HSPCs might possess the ability to sense and respond to situations, such as tissue damage and infections, that require the rapid influx of large numbers of innate immune cells. Indeed, HSPCs express TLR2 and TLR4 (Nagai et al., 2006), which sense pathogen-associated molecular patterns, such as peptidoglycan and lipoteichoic acid from Gram-positive bacteria and LPS from Gram-negative bacteria, respectively (Hoshino et al., 1999). It was shown in vitro that the incubation of both primitive and committed HSPCs with TLR ligands triggers HSPC proliferation and rapid myeloid differentiation. On the basis of this discovery, it was hypothesized that this mechanism could provide a means for boosting innate immunity during life-threatening infections, where microbial components either access the circulation and reach the BM or where dispersed stem and progenitor cells sense foreign materials outside the marrow compartment and become locally converted to innate effector cells (Nagai et al., 2006). In fact, we show the existence of a migratory pool of HSPCs that constitutively survey peripheral tissues. Our experiments indicate that once these cells sense microbial danger signals, they proliferate

vigorously and boost the local supply of innate effector cells. Thus, the migratory pool of HSPCs might act as a source of highly versatile stem and progenitor cells that can respond to danger signals locally within tissues before the information has spread to the BM. Notably, TLR stimulation also blocks HSPC egress from inflamed tissues and abolishes HSPC chemotaxis to S1P in vitro. Hence, TLR-induced changes in S1P receptor expression regulate the dwell time of HSPCs in inflamed tissues and thereby act as a rheostat that can boost local leukocyte generation.

In conclusion, we show here that HSPCs constitutively survey extramedullary nonlymphoid tissues. Our results raise the possibility that in the absence of infection, migratory HSPCs contribute to the continuous restoration of specialized hematopoietic cells that reside in peripheral tissues. During tissue infection, the migratory pool of HSPCs might act as an immediate and highly adaptive source of progenitor cells that proliferate locally and generate innate immune effector cells. It will be important to determine how HSPC traffic within peripheral tissues is influenced by and might modulate the course of infectious diseases and of other acute or chronic inflammatory conditions.

EXPERIMENTAL PROCEDURES

Thoracic Duct Cannulation

Thoracic duct cannulation was performed as described previously (Ionac, 2003). Where indicated, mice were treated with PTX (50 μg/kg iv, 2 hr prior to cannulation), FTY720 (1 mg/kg ip, 6 hr prior to cannulation), SEW2871 [20 mg/kg 6 hr prior to cannulation (Wei et al., 2005)], or THI (drinking water with 10 g/L glucose plus 50 μg/ml THI for 3 days prior to cannulation).

Parabiosis

Six- to eight-week-old sex- and age-matched C57BL/6 WT and β-actin/GFP mice (Wright et al., 2001a) were joined at the flanks as described (Wright et al., 2001b). Where indicated, mice were surgically separated prior to the collection of TD lymph.

Colony-Forming Unit Assays

MNCs or sorted Lin^+, $Lin^-c\text{-}Kit^-$ and $Lin^-c\text{-}Kit^+$ cells isolated from TD lymph, BM, and peripheral blood (PB) were assessed for CFU-C content by the culturing of 6×10^6 cells in methylcellulose-containing MethoCult M3434 medium (StemCell Technologies [Vancouver, Canada]) as reported (Papayannopoulou et al., 2003).

Primary and Secondary Transplantation Assays

TD MNCs (2×10^7) collected from $CD45.2^+$ C57BL/6 or nude mice were injected into sublethally (900 rad) or lethally (1300 rad) irradiated $CD45.1^+$ or $Rag2^{-/-}\gamma c^{-/-}$ mice. Where indicated, 10^5 rescue BM cells from $Rag2^{-/-}\gamma c^{-/-}$ donors were coinjected with TD MNCs. After 6 weeks, blood was obtained from the retro-orbital plexus, and the presence of donor-derived myeloid cells ($CD45.2^+Gr\text{-}1^+$) or T cells (TCR-β^+) was assessed by flow cytometry. For secondary transplantation, 2×10^7 $CD45.2^+$ TD MNCs were isolated and transplanted into sublethally irradiated (900 rad) $CD45.1^+$ congenic recipients. Twelve weeks after reconstitution, recipients with pronounced chimerism of peripheral blood granulocytes were sacrificed, and donor-derived ($CD45.1^-$) BM cells were sorted with a FACSAria cell sorter (BD Biosciences) and transplanted into lethally irradiated $CD45.1^+$ secondary recipients. Ten weeks later, chimerism among peripheral blood granulocytes, T cells, and B cells ($CD19^+$ $B220^+$) was assessed by FACS analysis.

In Vivo Assessment of HSPC Differentiation

TD-derived $GFP^+CD45.2^+Lin^-$ (5×10^5), $GFP^+CD45.2^+Lin^-c\text{-}Kit^+$ (5×10^5), BM-derived GFP^+ $Lin^-c\text{-}Kit^+$ (5×10^5), $Lin^-c\text{-}Kit^+sca\text{-}1^-$ (5×10^4 or 5×10^5), or $Lin^-c\text{-}Kit^+sca\text{-}1^+$ (5×10^4) were implanted under the left kidney capsule of $CD45.1^+$ congenic recipient mice. Where indicated, the cells were preincubated with medium containing stem cell factor (R&D, 20 ng/ml), Flt-3 ligand (R&D, 100 ng/ml) in the absence or presence of LPS (Sigma, 10 μg/ml), and CD14-Fc (R&D, 1 μg/ml) for 12 hr before implantation or were not pre-exposed to LPS but injected into the kidneys together with LPS (10 μg/ml). The right kidney was used as a control and was injected with medium with or without LPS (Sigma, 10 μg/ml) and CD14-Fc (R&D, 1 μg/ml). Where indicated, the recipient mice received 1 mg/day BrDU i.p (BD Biosciences [San Jose, CA]) on days 4, 5, and 6 after implantation. Six days after the implantation of the cells, mice were perfusion fixed and the kidneys were harvested and quantitatively analyzed by confocal laser scanning microscopy. To this end, five serial sections (each 30 μm thick) were obtained from the anterior aspect of the kidney and stained with fluorescently tagged anti-GFP (Rockland [Gilbertsville, PA]), anti-CD45, anti-CD45.1, anti-CD45.2, anti-Gr-1(Ly6G/C), anti-CD11c, anti-B220, anti-CD3 (all from BD Biosciences), mouse anti-human Ki-67 antibody (B56, crossreacting with mouse Ki-67, BD Biosciences) and anti-pDC/IPC (clone 120G8.04, Dendritics [France]) as indicated. So that myeloid differentiation could be detected, sections were stained with a panel of monoclonal antibody (mAb) against myeloid lineage markers [CD11b, CD11c, F4/80, Gr-1(Ly6G/C)] and then APC-conjugated streptavidin (BD Biosciences). BrDU in situ detection was performed according to the manufacturer's recommendations with biotin-labeled antibody recognizing BrDU (BrDU in situ detection kit, BD Biosciences) and APC-conjugated streptavidin (BD Biosciences).

Statistical Analyses

Quantitative results are expressed as means ± the standard error of the mean (SEM) unless otherwise indicated. The Mann-Whitney test was used for the determination of the significance of differences between means. Statistical significance was assumed at $p < 0.05$.

Supplemental Data

Supplemental Data include Experimental Procedures, five figures, one table, and three movies and can be found with this article online at http://www.cell.com/cgi/content/full/131/5/994/DC1/.

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