A multistep adhesion cascade for lymphoid progenitor cell homing to the thymus

M. Lucila Scimone*, Iannis Aifantis†, Irina Apostolou‡, Harald von Boehmer‡, and Ulrich H. von Andrian*§

*The CBR Institute for Biomedical Research and Department of Pathology, Harvard Medical School, Boston, MA 02115; †Department of Medicine, University of Chicago, Chicago, IL 60637; and ‡Harvard Medical School, Dana–Farber Cancer Institute, Boston, MA 02115

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Homing of bone marrow (BM)-derived progenitors to the thymus is essential for T cell development. We have previously reported that two subpopulations of common lymphoid progenitors, CLP-1 and CLP-2, coexist in the BM and give rise to lymphocytes. We demonstrate that CLP-2 migrate to the thymus more efficiently than any other BM-derived progenitors. Short-term adoptive transfer experiments revealed that CLP-2 homing involves P-selectin/P-selectin glycoprotein ligand 1 interactions, pertussis toxin-sensitive chemoattractant signaling by CC chemokine ligand 25 through CC chemokine receptor 9, and binding of the integrins $\alpha 4\beta 1$ and $\alpha L\beta 2$ to their respective ligands, vascular cell adhesion molecule 1 and intercellular adhesion molecule 1. Preferential thymus-tropism of CLP-2 correlated with higher chemokine receptor 9 expression than on other BM progenitors. Thus, CLP access to the thymus is controlled by a tissue-specific and subset-selective multistep adhesion cascade.

cell trafficking | chemokines | integrin | lymphopoiesis | selectin

The cells that comprise the immune system are continually generated from self-renewing hematopoietic stem cells (HSC) localized in the fetal liver or the adult bone marrow (BM). Upon receiving differentiation signals, HSC lose their self-renewing capacity and commit to either the myeloid or lymphoid lineage (1). The earliest lymphoid-committed BM cells (early lymphocyte progenitors) retain high levels of the HSC-associated markers c-*kit* and Sca-1 but express lymphoid-specific genes like Rag1 and EBF and have minimal myeloid and erythroid potential (2). Although not experimentally proven, early lymphocyte progenitors are potential progenitors of the more differentiated common lymphoid progenitor (CLP) subset. CLP are lineage (lin)$^-$c-*kit*^{low}Sca-1^{low}IL-7Rα^+ and can only give rise to lymphoid (T, B, and natural killer) and dendritic cells (3). Although CLP are efficient at giving rise to T cells, they have not been found within the thymus, indicating that they may first differentiate in the BM into one or more phenotypically distinct subset(s) that subsequently home(s) to the thymus. The most primitive thymocytes described so far are contained within the double-negative 1 (lin$^-$CD44$^+$CD25$^-$) subset; these cells are CD4^{low}c-*kit*^{hi} and generate lymphoid but not myeloid cells (4, 5). However, double-negative 1 probably represents a mixture of progenitors, and the identity of the cellular link(s) between the BM and thymus is still obscure.

Using a transgenic mouse that expresses the human CD25 (hCD25) marker as a reporter driven by the pre-T cell receptor α-chain (pTα) promoter and enhancer, we showed recently that a subset of BM progenitors that arise from CLP can enter the thymus and give rise to T cells (6, 7). BM cells expressing pTα (and hCD25) are efficient lymphoid-committed progenitors. After adoptive transfer, they give rise to a single wave of T cells, indicating a limited self-renewal capacity similar to the previously described CLP (3). Early developmental stages in the BM of hCD25 transgenic mice, including HSC and early lymphocyte progenitors, do not express hCD25 (and pTα). hCD25 expression is first observed at the lin$^-$c-*kit*^{low}Sca-1^{low}IL-7Rα^+ developmental stage corresponding to the original CLP. However, only a fraction of CLP are hCD25$^+$ (and pTα^+), suggesting heterogeneity within this population (8). The BM-resident progenitors that express robust levels of hCD25 can be subdivided into three subsets: c-*kit*$^+$B220$^-$ (CLP-1), c-*kit*$^-$B220$^+$CD19$^-$ (CLP-2), and c-*kit*$^-$B220$^+$CD19$^+$ (pro-B cells). CLP-1 correspond phenotypically to the original CLP. This subset gives rise to CLP-2 *in vitro* (but not vice versa), which efficiently colonize the thymus after short-term adoptive transfer (6).

Circulating leukocytes are recruited to most organs by tissue- and leukocyte-specific multistep adhesion cascades (9). Bloodborne cells first tether and roll on endothelial cells using mainly selectin/selectin ligand interactions; this step is followed by the rapid activation of integrins, which is mediated by Gα_i protein-coupled receptor (GPCR) signaling. Activated integrins mediate firm arrest of rolling cells, which can then diapedese across the vascular wall into the tissue.

Because the thymus does not contain HSC with unlimited self-renewing capacity, it needs to recruit progenitors from the blood, a process that is thought to occur at the corticomedullary junction (10) and depend on the multistep engagement of adhesion molecules and chemoattractants. Once in the thymus, progenitors differentiate and mature to T cells, moving through different compartments in a coordinated migratory stream (11). Several adhesion molecules, such as lymphocyte function-associated antigen 1, $\alpha 4\beta 1$, $\alpha 5\beta 1$, and CD44 are required for the migration of developing thymocytes (12–20). In addition, a variety of chemokines, including CXC chemokine ligand (CXCL) 12, CC chemokine ligand (CCL) 5, CCL17, CCL19, CCL21, CCL22, and CCL25 have been found in different thymic compartments, and their receptors are differentially expressed on distinct thymocyte subsets. Thus, chemokines probably play a role not only during progenitor recruitment but also during thymocyte migration and/or maturation (21–25). Specifically, CXCL12 and CCL25 are thought to participate in the immigration of bloodborne progenitors to the thymus, although their precise role in this process is still unclear (26–28). CXCL12 is expressed on thymic fibroblasts within the cortex, and double-negative cells respond to CXCL12 *in vitro* (23). Although early experiments suggested that genetic deficiencies in CXCL12 or CXCR4 (a receptor for CXCL12) do not compromise T cell development (29, 30), a more recent study found that CXCR4$^{-/-}$ BM cells can enter the thymus but fail to differentiate and to migrate to the outer cortex (31, 32). Thus, the CXCL12/CXCR4 pathway is probably not essential for lymphoid progenitor entry into the thymus, but for subsequent differentiation. On the other hand, the role of the CCL25/CC chemokine receptor (CCR) 9 pathway in lymphoid progenitor homing is still somewhat unclear. BM-derived CCR9$^{-/-}$ cells have a reduced capacity to repopulate

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Abbreviations: BM, bone marrow; CLP, common lymphoid progenitor; PSGL-1, P-selectin glycoprotein ligand 1; CCR, CC chemokine receptor; CCL, CC chemokine ligand; ICAM, intercellular adhesion molecule; VCAM, vascular cell adhesion molecule; HSC, hematopoietic stem cell; pTα, pre-T cell receptor α-chain; hCD25, human CD25; GPCR, Gα_i protein-coupled receptor; CXCL, CXC chemokine ligand; PTX, pertussis toxin; lin, lineage; CFSE, carboxyfluorescein diacetate succinimidyl ester; TRITC, tetramethylrhodamine-5-isothiocyanate.

§To whom correspondence should be addressed at: The CBR Institute for Biomedical Research, 200 Longwood Avenue, Boston, MA 02115. E-mail: uva@cbr.med.harvard.edu.

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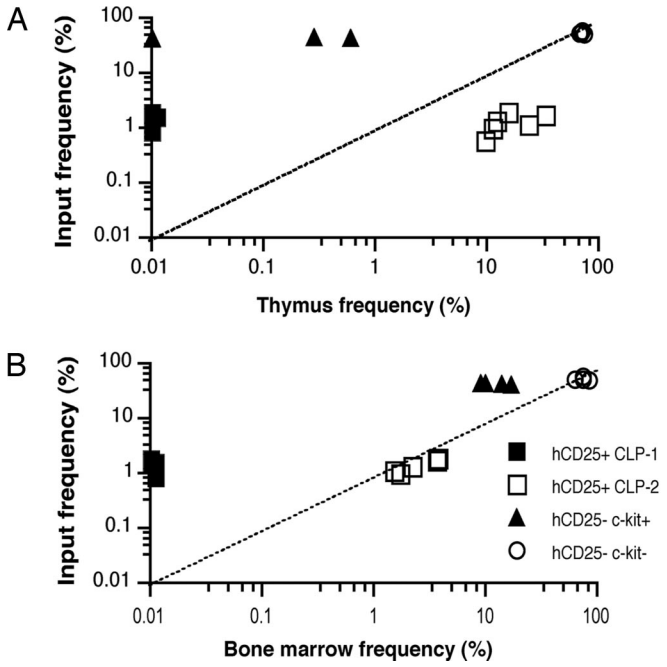

Fig. 1. CLP-2 are enriched in the thymus after adoptive transfer of lin$^-$ BM cells. lin$^-$ BM cells from pTα-hCD25 mice were adoptively transferred into Rag2$^{-/-}\gamma c^{-/-}$ mice. After 20 h, thymi (*A*) and BM (*B*) were harvested, and single-cell suspensions were stained for B220 and c-*kit*. Data are shown as the percentage of cells in the input population versus the percentage of those populations in the target organ.

the thymus in long-term transplantation assays; however, CCR9$^{-/-}$ mice have normal T cell development (26).

Exacting experiments to uncover the mechanisms of lymphoid progenitor homing from the BM to the thymus have been hampered by difficulties in identifying the relevant progenitor population and obtaining sufficient numbers of cells for *in vivo* experimentation. In this study, using the pTα-hCD25 mouse as a tool to track lymphoid progenitors after short-term adoptive transfer, we have dissected the predominant multistep cascade for CLP-2 recruitment to the thymus. We show that P-selectin/P-selectin glycoprotein ligand 1 (PSGL-1), $\alpha4\beta1$/vascular cell adhesion molecule (VCAM) 1, lymphocyte function-associated antigen 1/intercellular adhesion molecule (ICAM) 1, and CCL25/CCR9 are key components of this unique thymus-specific recruitment pathway.

Results

Differential Thymus-Tropism of Lymphoid Progenitor Subsets. We recently described the pTα-hCD25 mouse strain, in which two distinct subsets of lin$^-$ hCD25$^+$ CLP (CLP-1 and CLP-2) are found in the BM (6). CLP-1 are c-*kit*$^+$B220$^-$ and represent a fraction of the originally described CLP (3). CLP-1 give rise *in vitro* to CLP-2, which are c-*kit*$^-$B220$^+$ (6). In the BM, hCD25$^+$ CLP-1 and CLP-2 occur with similar frequency and together constitute $\approx$5% of lin$^-$ leukocytes (Fig. 7*A*, which is published as supporting information on the PNAS web site, and ref. 6). Both subsets contain bipotent precursors of T and B cells but differ in their ability to migrate to the thymus (6).

To characterize this differential thymus-tropism more thoroughly, we purified lin$^-$Ly5.1$^+$ BM cells and adoptive transfer into Ly5.2$^+$Rag2$^{-/-}\gamma c^{-/-}$ mice. Twenty hours after i.v. injection, few CLP-1 had migrated to the recipients' BM and none were recovered from the thymus (Figs. 1 and 7 *B* and *C*). By contrast, numerous CLP-2 homed to the thymus, where their frequency among Ly5.1$^+$ donor cells was 12.9 $\pm$ 2.1-fold (mean $\pm$ SEM) higher than in the input. CLP-2 also homed to the BM, but their frequency was more similar to their input concentration (1.96 $\pm$ 0.2-fold enrichment). Among the hCD25$^-$ fraction of donor cells, only the c-*kit*$^-$ subset was represented in the thymus. These hCD25$^-$c-*kit*$^-$ cells were exclusively B220$^+$; i.e., they were phenotypically very similar to the hCD25$^+$ CLP-2 population. However, it is unclear whether these thymus-tropic hCD25$^-$ cells are composed of different subsets and to what extent they contain lymphoid progenitors.

These findings confirm and expand our previous observations that hCD25$^+$ CLP-2 possess pronounced thymus-tropism. Indeed, for every 10^6 CLP-2 injected, we recovered from the thymus 3,932 $\pm$ 378 homed cells per milligram of wet weight; for hCD25$^-$c-*kit*$^-$ cells, the corresponding value was 626 $\pm$ 62 cells per milligram of wet weight per 10^6 cells injected. Thus, CLP-2 become much more concentrated in the thymus than any other BM-resident precursors. This remarkable efficacy of CLP-2 clearance from the circulation can explain why normal blood and lymphoid tissues contain very few CLP-2. Indeed, when the constitutive recruitment of endogenous circulating CLP-2 was abolished by subjecting pTα-hCD25 mice to thymectomy or by crossing them to nude mice, B220$^+$c-*kit*$^-$ lin$^-$ cells (but not HSC) accumulated in the spleen (data not shown and ref. 6). In light of this unique migratory capacity of CLP-2 and the fact that their pronounced lymphopoietic potential is well documented (6), we focused our further analysis on this subset.

Adhesion Molecule Patterns on Lymphoid Progenitor Subsets. To elucidate the molecular underpinnings of CLP-2 migration, we compared the traffic molecule profile of CLP-2 to that of CLP-1, pro-B cells [lin$^-$CD19$^+$hCD25$^+$ (7)], and early thymic immigrants [ETP, CD4$^-$CD8$^-$CD44$^+$CD25$^-$c-*kit*^{low}hCD25$^+$ (7)]. This analysis revealed only subtle differences in most common adhesion molecules (Table 1 and Fig. 8, which are published as supporting information on the PNAS web site). Both hCD25$^+$ CLP populations expressed comparable levels of integrins, PSGL-1 and CD44. Compared with CLP-1, L-selectin and E-selectin ligands were slightly lower on CLP-2, whereas P-selectin ligands were more abundant. Pro-B cells expressed low levels of L-selectin and αL and did not bind P- or E-selectin, but other traffic molecules were similarly expressed as on CLP-1 and CLP-2. Similarly, ETP expressed less L-selectin as well as P- and E-selectin ligands than both CLP subsets, whereas surface expression of αL, $\alpha4$, and $\beta1$ integrins was similar.

P-Selectin Is Required for Homing of Lymphoid Progenitors to the Thymus. The first essential event in intravascular multistep adhesion cascades is the tethering and rolling of fast-flowing leukocytes in postcapillary venules. The most important adhesion molecules for this step are the selectins and their carbohydrate ligands. To determine the *in vivo* role of these molecules, we performed short-term adoptive transfer experiments with neutralizing mAbs.

mAb blockade of E- or L-selectin did not affect CLP-2 homing to the thymus, but anti-P-selectin decreased by 67 $\pm$ 3% the number of hCD25$^+$ cells recovered from thymi (Fig. 2*A*). Combined inhibition of all three selectins had no further effect, suggesting that P-selectin is the only selectin involved in this process in the thymus. Interestingly, although HSC and memory T cells rely, in part, on selectins to home to the BM (33, 34), selectin inhibition did not reduce the number of lymphoid progenitors that homed to that tissue (Fig. 2*B*).

Most P-selectin ligands are presented by sialomucin-like glycoproteins, particularly PSGL-1 (35). Thus, we performed competitive adoptive transfer assays by comparing thymus-tropism of lin$^-$ BM cells from PSGL-1$^{-/-}$ and WT mice. As expected, WT lin$^-$ BM cells homed 2.6 $\pm$ 0.6 times better to the thymus than PSGL-1$^{-/-}$ cells (Fig. 2*C*). These results, which are in excellent agreement with a recent report (36), establish a role for the P-selectin/PSGL-1 pathway in the homing of lymphoid progenitors to the thymus.

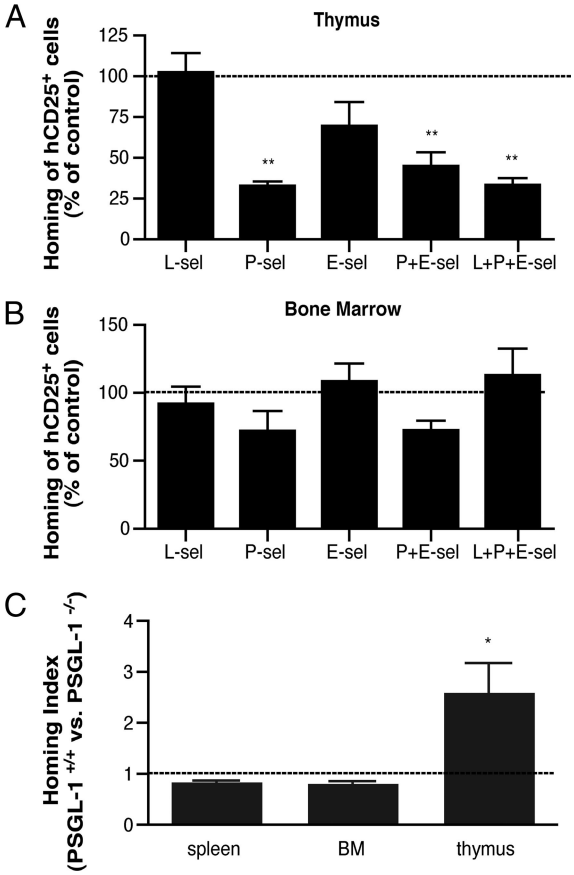

Fig. 2. P-selectin and PSGL-1 mediate lymphoid progenitor homing to the thymus. lin$^-$ BM cells from pTα-hCD25 mice were pretreated with anti-L-selectin or control mAbs and adoptively transferred into Rag2$^{-/-}\gamma c^{-/-}$ mice. Anti-P-selectin, anti-E-selectin, or control mAbs were injected into recipient mice 15 min before transferring lin$^-$ BM cells. After 20 h, thymi (*A*) and BM (*B*) were harvested, and single-cell suspensions were stained for Ly5.1 and hCD25 ($n = 6$ mice). (*C*) CFSE-labeled lin$^-$ BM cells from PSGL-1$^{-/-}$ mice together with TRITC-labeled lin$^-$ BM cells from WT controls were mixed in a 1:1 ratio and injected i.v. into Rag2$^{-/-}\gamma c^{-/-}$ cells. After 20 h, spleen, thymi, and BM were harvested, and single-cell suspensions were analyzed for the presence of TRITC$^+$ and CFSE$^+$ lin$^-$ BM cells ($n = 5$ mice).

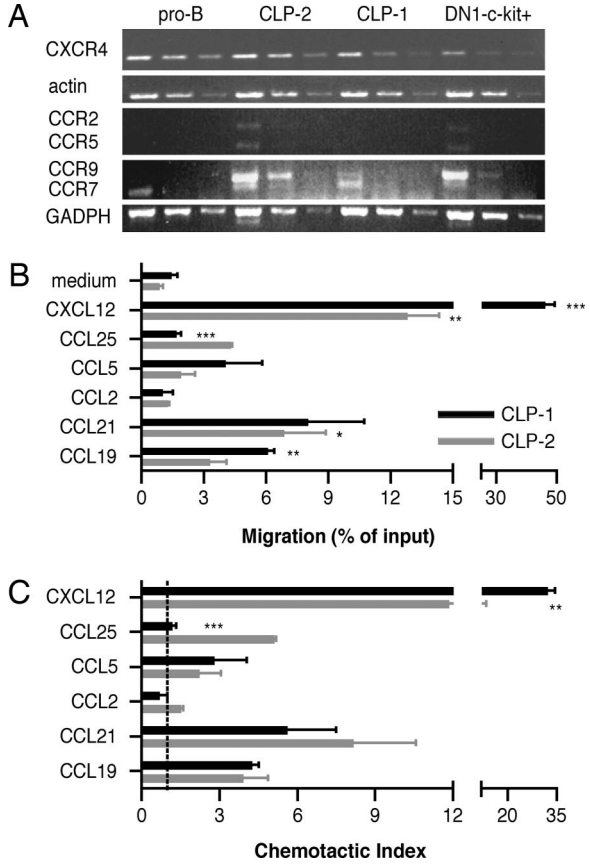

Fig. 3. Chemokine receptor expression and chemotaxis of lymphoid progenitors. (*A*) From left to right: BM pro-B cells, CLP-2, CLP-1, and early thymic immigrants (double-negative 1 c-*kit*$^+$hCD25$^+$) were sorted and analyzed by semiquantitative RT-PCR for the expression of multiple CCRs. cDNA quantities were normalized according to GADPH or actin expression over three subsequent 5× dilutions. (*B* and *C*) lin$^-$ BM cells from pTα-hCD25 mice were added to inserts that were placed in wells containing medium alone or the following chemokines: CCL5, CCL2, CCL19, CCL21, CXCL12, or CCL25. Responding cells were harvested 2 h later, stained for hCD25, B220, and c-*kit*, and analyzed by flow cytometry. (*B*) Migration as percentage of the input. (*C*) Chemotactic index. ($n = 3$ experiments.)

Lymphoid Progenitor Homing Requires Gα_i Protein Signaling. The second step in the adhesion cascade is the engagement of a GPCR, which results in the activation of integrins that mediate firm adhesion. Signals that activate leukocyte integrins are highly diverse, including bacterial products, components of the complement cascade, arachidonic acid metabolites and other lipids, and chemokines.

Receptors for chemokines and most other chemoattractants are seven-transmembrane-spanning GPCR, whose signaling is efficiently and irreversibly blocked by pertussis toxin (PTX) (37). To determine whether thymus-homing progenitors require a PTX-sensitive GPCR, pTα-hCD25 mice were systemically treated with PTX, and BM cells were collected 16 h later. PTX-treated or control BM cells were injected into Rag2$^{-/-}\gamma c^{-/-}$ mice. Twenty hours later, the frequency of homed PTX-treated CLP-2 in the thymus was reduced to 51 ± 8% compared with control cells (Fig. 9, which is published as supporting information on the PNAS web site). By contrast, PTX had no effect on CLP-2 homing to the BM.

Chemokine Receptor Expression and Chemotactic Responsiveness of CLP, Pro-B Cells, and Early Thymic Immigrants. The effect of PTX hinted at the presence of one or more thymic chemoattractant(s) that facilitate(s) lymphoid progenitor recruitment. We reasoned that the differential ability of CLP-2 versus CLP-1 to home to the thymus could be a consequence of their differential responsiveness to such chemoattractant(s). Therefore, to identify candidate pathways, we analyzed mRNA expression levels for several chemokine receptors using semiquantitative RT-PCR on sorted CLP-1, CLP-2, pro-B cells, and hCD25$^+$ ETP (Fig. 3*A*; see also Fig. 10, which is published as supporting information on the PNAS web site). Both CLP subsets contained similar mRNA levels for CCR7, the receptor for CCL19 and CCL21, and for CXCR4, the receptor for CXCL12 (Fig. 3*A*). However, at the surface protein level both receptors were preferentially expressed on CLP-1 (Table 1 and Fig. 8). Moreover, chemotaxis assays showed that a larger fraction of CLP-1 than CLP-2 responded to CXCL12 (Fig. 3*B*). This difference became even more apparent when data were expressed as chemotactic index (Fig. 3*C*). CLP-2, unlike CLP-1, expressed detectable mRNA for CCR2 and CCR5, but they responded inefficiently to CCL2 and CCL5, their respective ligands. Importantly, CLP-2 expressed higher mRNA and surface protein levels of CCR9, the receptor for CCL25. Indeed, of all chemokines tested in chemotaxis assays, only CCL25 induced a significantly stronger response in CLP-2 (4.6 ± 0.7-fold) than in CLP-1. Of note, early hCD25$^+$ ETP and CLP-2 expressed the same chemokine receptor pattern, at least at the mRNA level, which is consistent with a close lineage

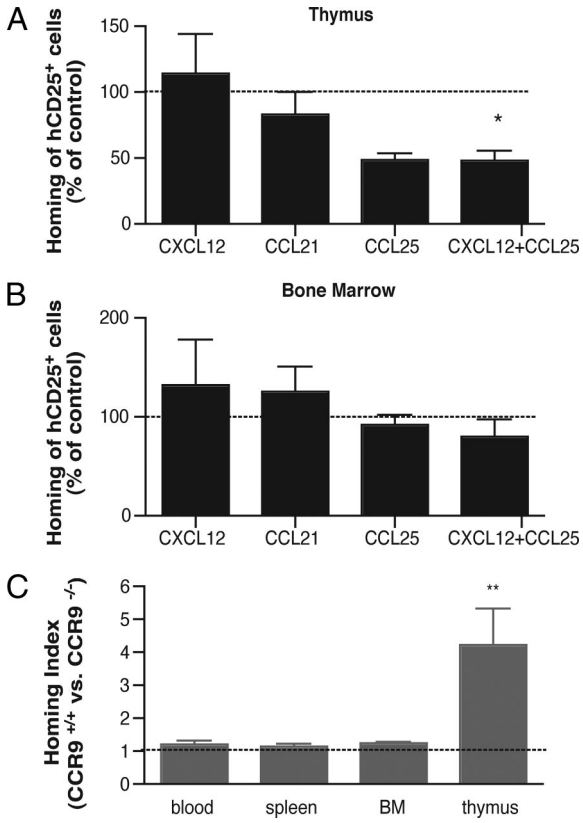

Fig. 4. CCR9/CCL25 is involved in lymphoid progenitor homing to the thymus. lin$^-$ BM cells from pTα-hCD25 mice were i.v injected into Rag2$^{-/-}\gamma c^{-/-}$ mice pretreated with anti-CXCL12, anti-CCL21, anti-CCL25, or control mAbs. After 20 h, thymi (*A*) and BM (*B*) were harvested, and single-cell suspensions were stained for Ly5.1 and hCD25 ($n = 8$ mice). (*C*) CFSE-labeled lin$^-$ BM cells from CCR9$^{-/-}$ mice together with TRITC-labeled lin$^-$ BM cells from WT controls were mixed in a 1:1 ratio and injected i.v. into Rag2$^{-/-}\gamma c^{-/-}$ cells. After 20 h, spleen, thymi, and BM were harvested, and single-cell suspensions were analyzed by flow cytometry for the presence of TRITC$^+$ and CFSE$^+$ lin$^-$ BM cells ($n = 6$ mice).

relationship between these two populations. Compared with CLP-2, hCD25$^+$ ETP responded similarly to CXCL12, but less well to CCL19 and CCL21, and they responded poorly to CCL25 (data not shown).

CCL25/CCR9 Mediates CLP Recruitment to the Thymus. Having identified several chemokines to which CLP-2 were responsive, we tested the effect of neutralizing mAbs to each pathway on CLP-2 homing to the thymus. To this end, mAbs to CXCL12 and/or CCL25 or CCL21 were injected i.v. into Rag2$^{-/-}\gamma c^{-/-}$ mice 15 min before transferring hCD25$^+$lin$^-$ BM cells (Fig. 4*A*). Only anti-CCL25 significantly decreased (by 52 ± 5%) the accumulation of hCD25$^+$ cells in the thymus, whereas anti-CCL21 had no effect. Anti-CXCL12 was also ineffective whether given alone or in combination with anti-CCL25. Homing of hCD25$^+$ progenitors to the BM was not significantly altered by any mAb (Fig. 4*B*), suggesting that CCL25 provides a thymus-specific recruitment signal. In addition, we performed competitive homing assays using lin$^-$ BM cells from WT and CCR9$^{-/-}$ mice, the only known receptor for CCL25 (Fig. 4*C*). These experiments revealed that WT progenitors have a 4.2 ± 1.1-fold advantage over their CCR9$^{-/-}$ counterparts in short-term homing to the thymus. Taken together, these findings strongly suggest that the CCL25/CCR9 pair is critical for the recruitment of bloodborne lymphoid progenitors to the thymus.

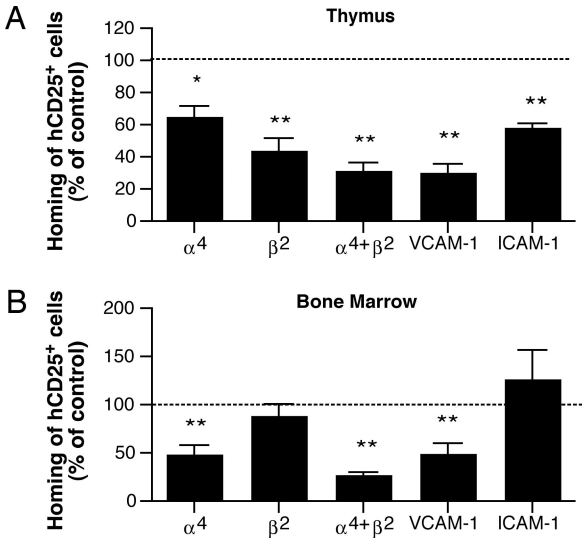

Fig. 5. A nonredundant role of $\alpha 4\beta 1$ and $\alpha L\beta 2$ in lymphoid progenitor homing to the thymus. lin$^-$ BM cells from pTα-hCD25 mice were pretreated with anti-α4, anti-β2, both, or control mAbs and adoptively transferred into Rag2$^{-/-}\gamma c^{-/-}$ mice treated or not with anti-VCAM-1 or anti-ICAM-1 mAbs. Twenty hours later, thymi (*A*) and BM (*B*) were harvested, and cell suspensions were stained for Ly5.1 and hCD25 ($n = 4$ mice).

The Role of Integrins. Intravascular firm adhesion of rolling leukocytes is mediated almost exclusively by integrins, particularly members of the $\beta 2$ and $\alpha 4$ subfamilies (9). Both CLP populations express high levels of $\alpha L\beta 2$ and $\alpha 4\beta 1$ but almost no $\alpha M\beta 2$ and very little $\alpha 4\beta 7$ (Table 1). Pretreatment of lin$^-$ BM cells with anti-α4 or anti-β2 (50 μg/ml for 15 min at 37°C) significantly reduced CLP-2 homing to the thymus ($P < 0.05$ and $P < 0.01$, respectively), and combined inhibition of α4 and β2 had a moderate additive effect (Fig. 5*A*). Thymic endothelial cells express ICAM-1 and VCAM-1, which function as counterreceptors for $\alpha L\beta 2$ and $\alpha 4\beta 1$, respectively (38). Indeed, mAb inhibition of ICAM-1 or VCAM-1 reduced CLP-2 homing to the thymus by 39 ± 4% and 70 ± 5%, respectively. In contrast to the thymus, homing of lymphoid progenitors to the BM was supported only by $\alpha 4\beta 1$ and VCAM-1, but not $\alpha L\beta 2$ or ICAM-1 (Fig. 5*B*).

Discussion

There is broad consensus that the thymus must be continuously supplied with BM progenitors that are released into the blood. It is also widely accepted that these circulating thymus-tropic progenitors must use specific adhesion molecules and chemoattractants to home to the thymus and to migrate within it. However, the identity of the molecules that constitute the thymic "vascular ZIP code" has remained uncertain because of a number of factors. First, it has been difficult to track and isolate relevant lymphoid progenitors in mice. Several populations of BM progenitors generate T cells *in vitro* or *in vivo* after intrathymic injection or long-term adoptive transfer (6, 39–41). However, the capacity of a candidate progenitor to generate mature T cells can only be assessed several weeks after transfer. This strategy will yield a positive readout not only upon transfer of progenitors that home directly to the thymus but also of earlier precursors that first migrate to the BM or elsewhere to generate thymus-seeking progeny. Second, several chemokines and adhesion molecules are expressed in the BM and/or other tissues as well as in the lumen of thymus microvessels and/or the parenchyma itself. This expression pattern makes it difficult to pinpoint the precise site(s) where traffic molecules contribute to thymopoiesis in long-term adoptive transfers. Thus, a rigorous dissection of the cascade of intrathymic traffic molecules

that function at the blood–tissue interphase is best achieved by short-term adoptive transfer of tagged BM precursors whose accumulation in the thymus can be directly quantified.

The question is, then, which BM progenitors are most appropriate for such homing studies? At a minimum, the relevant cells should meet three criteria: (*i*) they must possess T cell potential, (*ii*) they must be able to home to the thymus, and (*iii*) they must be able to exit the BM and enter the blood. There are several subsets, including HSC, early lymphocyte progenitors, and CLP, that give rise to T cells and have been proposed to seed the postnatal thymus (6, 39–41). Among those, HSC are known to traffic continuously into and out of the BM (42), and cells with HSC-like (i.e., lin$^-$c-*kit*^{hi}) phenotype are found in both blood and thymus (41). Thus, it has been proposed that circulating HSC-like cells, rather than CLP-like cells, may be the first thymic immigrants (2, 39, 41). However, recent BM transplantations show that thymic reconstitution precedes the recurrence of the HSC-like cells, suggesting that they are not needed to reconstitute the thymus (43). In agreement with these data and previous work by others (44), we found that lin$^-$c-*kit*$^+$ BM cells migrate poorly to the thymus. Thus, HSC-like cells meet only two of the three criteria outlined above.

One progenitor subset that can efficiently seed the thymus and give rise to T cells is the CLP-2 (6, 44). After adoptive transfer, CLP-2 home much more efficiently to the thymus than any other BM progenitor subset we have tested. However, to be viable candidates, CLP-2 must be able to enter the blood. In normal mice, CLP-2 are very scarce in blood, which has prompted the argument that they may not reflect a major link between BM and thymus (41). However, the physiological paucity of circulating CLP-2 may not necessarily reflect an inability to leave the BM; a depletion of bloodborne leukocytes could also result from their rapid clearance in a target tissue. A case in point are naïve T cells, which are abundant in blood as they recirculate between blood and lymphoid organs but rapidly disappear from the circulation when they are prevented from leaving lymphoid tissues after homing (45). Based on published data (45), we calculate the hourly rate at which naïve T cells home to peripheral lymph nodes after i.v. injection as 135 ± 8 cells per 10^6 cells injected per milligram of tissue wet weight; the hourly traffic of B cells to the spleen amounts to 189 ± 17 cells per 10^6 cells injected per milligram of wet weight. Remarkably, CLP-2 accumulated in the thymus of Rag2$^{-/-}\gamma c^{-/-}$ mice with similar or even slightly higher efficiency (197 ± 19 cells per 10^6 cells injected per milligram of wet weight).

The above calculations are consistent with the idea that the pronounced thymus-tropism of CLP-2 combined with their likely inability to recirculate, rather than inefficient release from the BM, are responsible for their shortage in blood. This hypothesis predicts that CLP-2 should accumulate in the periphery when they are prevented from homing to the thymus. Indeed, as reported, the spleens of pTα-hCD25 mice with a normal thymus contained a sizeable population of HSC but almost no CLP-2, whereas spleens of athymic pTα-hCD25xnude mice contained significantly more hCD25$^+$B220$^+$ cells (6). Thus, the lack of thymus resulted in a significant splenic accumulation of BM progenitors with demonstrable thymus-homing capacity, but not of progenitors that home poorly to the thymus.

It should be emphasized that the above considerations do not rule out the existence of other T cell progenitors that may also migrate from the BM to the thymus. Indeed, multiple distinct BM progenitor populations are T cell-competent and could contribute to intrathymic T cell development (46). However, the CLP-2 in the pTα-hCD25 mouse model is presently the only phenotypically and functionally well defined subset that meets the prerequisite criteria of usefulness for short-term homing assays. Using this approach, we provide evidence for a thymus-specific adhesion cascade composed of a sequence of distinct adhesion molecules and chemoattractants that mediate CLP-2 homing to the adult thymus (Fig. 6).

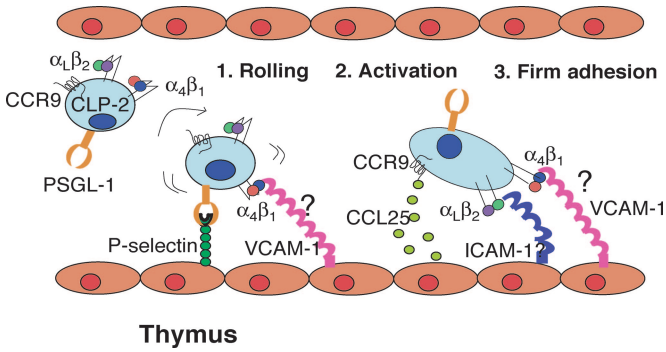

Fig. 6. Homing to the thymus is mediated by a unique cascade of adhesion and signaling events. Cells expressing PSGL-1 or other P-selectin ligands and probably also $\alpha4\beta1$ can initiate the adhesion cascade to home to the thymus. Subsequently, a Gα_i-coupled activating stimulus is required to activate the integrins $\alpha4\beta1$ and $\alpha L\beta2$, which allow the firm adhesion of the progenitors through interactions with VCAM-1 and ICAM-1, respectively. This activation depends on signaling through the CCL25/CCR9 pair. A still-unknown Gα_i-independent mechanism is partially involved in the homing of lymphoid progenitors to the thymus. The unique combination of these events efficiently results in the selective recruitment of lymphoid progenitors.

Consistent with a recent report (36), we found that P-selectin, a known rolling receptor, is the principal selectin involved. The main P-selectin ligand, PSGL-1, is also required, because PSGL-1$^{-/-}$ progenitors homed less efficiently to the thymus than their WT counterparts. However, given that anti-P-selectin did not completely abrogate CLP-2 homing to the thymus, it is likely that additional molecular interactions (possibly including $\alpha4\beta1$/VCAM-1) contribute to rolling. Rolling cells must adhere firmly before they can transmigrate across the vessel wall. Firm adhesion is almost exclusively mediated by integrins that need to switch from an inactive, closed conformation to an open, high-affinity state. A major mechanism for integrin activation is signaling triggered by chemokines, which bind to specific GPCR on leukocytes. We show that both $\alpha L\beta2$ and $\alpha4\beta1$ integrins are required for efficient homing of progenitors to the thymus. In contrast, progenitor homing to the BM depended almost exclusively on $\alpha4$ integrins. ICAM-1 and VCAM-1, the counterreceptors for $\alpha L\beta2$ and $\alpha4\beta1$, respectively, are expressed in thymus corticomedullary vessels, where the entry of lymphoid progenitors is thought to occur (10, 38). Given the additive effect of combined inhibition of these pathways, our results suggest that both $\alpha L\beta2$/ICAM-1 and $\alpha4\beta1$/VCAM-1 can mediate arrest of rolling progenitors in thymus microvessels.

A role for CCR9/CCL25 in the colonization of the thymus by lymphoid progenitors has been suggested; CCR9$^{-/-}$ BM cells showed a delay in thymus reconstitution after BM transfer compared with WT cells (26). However, because this analysis was done 3 weeks after BM transfer, the defect in T cell development could be the result of a requirement for CCR9 during progenitor entry or during intrathymic migration, development, or survival of thymocytes. Our experiments clearly show that the CCR9/CCL25 pathway is partially required for the recruitment of progenitors, most likely by triggering integrin activation on rolling cells. By examining the expression pattern of chemokine receptors and their functionality on CLP-1 and CLP-2, we determined that both expressed CCR9, but only CLP-2 responded to CCL25. This difference between the two CLP subsets can explain why only CLP-2 home to the thymus. Of note, CLP-1 expressed more CXCR4 and responded better to CXCL12 than CLP-2. It has been suggested that CXCL12, which is highly expressed in BM, may retain immature B cells in that organ (47). It is tempting to speculate that CLP-1 are also better retained in the BM than CLP-2 because of their enhanced responsiveness to CXCL12. In agreement with this hypothesis, pro-B cells expressed similar levels of CXCR4 as CLP-1,

at both the mRNA and protein levels, and responded equally well as CLP-1 to CXCL12 in chemotaxis assays (data not shown). On the other hand, the similarity of the chemokine receptor repertoires on CLP-2 and on ETP supports the view that these two populations are directly connected.

Although lymphoid progenitors in BM and thymus expressed CXCR4 and responded to CXCL12, we could not block CLP-2 homing to the thymus by neutralizing CXCL12. This failure to block is in agreement with two recent reports that have suggested a role of CXCL12/CXCR4 in the expansion and differentiation of T cell progenitors, but only after their entry into the thymus (31, 32). Nevertheless, it seems likely that $G\alpha_i$-independent chemoattractants contribute to CLP-2 migration to the thymus, because $G\alpha_i$ inhibition by PTX (as well as anti-CCL25 treatment) reduced CLP-2 homing only by ≈50%. Thus, although the sequence of molecular traffic signals identified here probably represents a major recruitment mechanism for thymic progenitors, the incomplete effectiveness of our interventions at each step suggests that additional or alternative multistep adhesion cascades may exist.

In summary, we describe a unique, tissue-specific multistep adhesion cascade mediating the homing of lymphoid-committed progenitors to the thymus. This cascade involves P-selectin/PSGL-1 interactions, signaling of CCL25 through its receptor, CCR9, and, finally, activation-induced binding of the integrins $\alpha4\beta1$ and $\alpha L\beta2$ to their respective ligands, VCAM-1 and ICAM-1. The exclusive ability of CLP-2 to home to the thymus correlates *in vitro* with their chemotactic response to CCL25 and high expression of CCR9.

Materials and Methods

Adoptive Transfer. BM cells were harvested from WT, pTα-hCD25, CCR9$^{-/-}$, or PSGL-1$^{-/-}$ femora and tibiae; mature blood cells were depleted by using biotinylated mAb to Ter119, DX5, Ly6C/G, CD3, CD8, CD11c, and CD19 followed by streptavidin-conjugated magnetic beads (Dynal, Oslo). lin$^-$ cells ($1–2 \times 10^7$) were injected i.v. in nonirradiated Rag2$^{-/-}\gamma c^{-/-}$ (Ly5.2$^+$) mice. In competitive homing assays, lin$^-$ BM cells from knockout strains were fluorescently tagged before transfer using carboxyfluorescein diacetate succinimidyl ester (CFSE), whereas WT cells were stained with tetramethylrhodamine-5-isothiocyanate (TRITC; Molecular Probes). Labeled cells were resuspended to $2–4 \times 10^7$ cells per ml and injected i.v. After 20 h, recipients were killed and thymi and BM were harvested and analyzed by FACS. To block recipient-expressed traffic molecules, 100 μg of specific or isotype control mAb per mouse were injected i.v. 15 min before transfer of lin$^-$ BM cells. To block CLP-expressed molecules, lin$^-$ BM cells were incubated with 50 μg/ml mAb for 15 min before injection.

Chemotaxis Assays. Chemotaxis assays were performed in tissue culture-treated 24-well plates with 5-μm pore-size inserts (Costar, Cambridge, MA). lin$^-$ BM cells (5×10^5 to 1×10^6) from pTα-hCD25 mice were resuspended in RPMI medium 1640 with 10% FCS and loaded onto the inserts. Lower chambers were loaded with 600 μl of medium without additives or with CXCL12 (10 nM); CCL2, CCL5, CXCL5, CCL19, and CCL21 (100 nM each); or CCL25 (300 nM).

RT-PCR. cDNA was synthesized from total mRNA from sorted populations, and the reverse transcription was equilibrated by using an actin or GADPH semiquantitative PCR (7). To detect CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CCR8, CCR9, and CX3CR1, commercial PCR amplification kits were used (Maxim Biotech, San Francisco).

Statistical Analysis. All data are presented as mean ± SEM. Significance was set at $P < 0.05$.

Supporting Information. Additional details can be found in *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site.

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