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Lymphoid Gene Upregulation on Circulating Progenitors Participates in Their T-Lineage Commitment

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Extrathymic T cell precursors can be detected in many tissues and represent an immediately competent population for rapid T cell reconstitution in the event of immunodeficiencies. Blood T cell progenitors have been detected, but their source in the bone marrow (BM) remains unclear. Prospective purification of BM-resident and circulating progenitors, together with RT-PCR single-cell analysis, was used to evaluate and compare multipotent progenitors (MPPs) and common lymphoid progenitors (CLPs). Molecular analysis of circulating progenitors in comparison with BM-resident progenitors revealed that CCR9+ progenitors are more abundant in the blood than CCR7+ progenitors. Second, although Flt3+ CLPs are less common in the BM, they are abundant in the blood and have reduced CD25+-expressing cells and downregulated c-Kit and IL-7Rα intensities. Third, in contrast, stage 3 MPP (MPP3) cells, the unique circulating MPP subset, have upregulated Il7r, Gata3, and Notch1 in comparison with BM-resident counterparts. Evaluation of the populations’ respective abilities to generate splenic T cell precursors (Lin−Thy1.2+CD25−IL7Rα+) after grafting recipient nude mice revealed that MPP3 cells were the most effective subset (relative to CLPs). Although several lymphoid genes are expressed by MPP3 cells and Flt3− CLPs, the latter only give rise to B cells in the spleen, and Notch1 expression level is not modulated in the blood, as for MPP3 cells. We conclude that CLPs have reached the point where they cannot be a Notch1 target, a limiting condition on the path to T cell engagement. The Journal of Immunology, 2015, 195: 156–165.

Inn hematopoiesis, T-lineage commitment is an important issue. Within the bone marrow (BM), commitment toward a given lineage proceeds through the loss of ability to generate other lineages (1). Several differentiation stages have been identified in relation to the cells’ developmental potential. Lin−Sca-1−c-Kit+ (LSK) hematopoietic stem cells (HSCs), which are subdivided into long-term and short-term (ST) HSCs, gradually lose their self-renewal potential; long-term HSCs can fully reconstitute hematopoiesis throughout the organism’s life span, whereas ST HSCs are only capable of reconstitution for the first 2 months of life. ST HSCs give rise to multipotent progenitors (MPPs) (2, 3), which can be subdivided according to their expression of VCAM-1 and Flt3 (Fig. 1) (1). Each subset has a distinct lineage potential: the VCAM-1+Flt3− stage 1 MPP (MPP1) can generate all blood cell lineages, the VCAM-1−Flt3− stage 2 MPP (MPP2) has lost megakaryocyte/erythroid potential, and the VCAM-1−Flt3+ stage 3 MPP (MPP3) has lost myeloid potential (1, 4) and gives rise to common lymphoid progenitors (CLPs), the source of B cells (5).

LSK populations, which contain HSCs and nonrenewing MPPs, have been detected in the blood and the thymus (6). The recent identification of lymphoid-primed MPPs (LMPs; Flt3+ MPPs) has been in favor of their involvement in the T cell lineage (7). However, a heterogeneity exists within this subset (according to VCAM1 expression) that needs to be evaluated by further purification and biological and molecular characterizations.

The respective contributions of LMPs and CLPs to the T cell pathway are still being hotly debated. More specifically, which BM progenitors harbor the full T cell potential, a multipotent or a lymphoid-restricted subset? It was claimed that CLPs are the earliest lymphoid-committed progenitor from which B- and T-lineage cells arise (8). However, the concept that CLPs are the progenitor population through which all T lymphocytes are derived has been challenged (8, 9).

Meanwhile, progenitors with restricted T-lineage potential have been described in the gut (10, 11), the BM (12), and the spleen (13–15). In fact, LinThy1.2+CD25−IL7Rα+ T cell precursors (pre-T cells) are naturally present in murine spleen (14, 15) and are also found in the spleen after BM transplantation (13). These pre-T cells arise before the generation of early thymic progenitors (ETPs) (L. Gautreau and S. Ezine, unpublished observations) (16) and accumulate in the spleen after thymectomy (15), representing
Figure 1. Schema depicting the development from LSK to MPPs and CLPs. Cell surface Ags indicate the phenotypes of the above-mentioned populations. +, present on the cell surface; -, not present on the cell surface.

the first stage of an early T cell pathway. Splenic and medullary progenitors generate T cells upon adoptive transfer, but additional NK cells develop from the latter population (12). We demonstrated that these splenic pre-T cells depend on Notch signaling for their generation (17). Similarly to thymic ETPs, their direct upstream progenitor is not known but might colonize the circulation.

Indeed, multiple progenitor types circulate, but the mechanisms regulating their mobilization from BM to blood are mostly unknown. Mobilization of HSCs into blood can be regulated by multiple adhesion molecules and cytokine receptors (18), but whether similar mechanisms also regulate the exit of progenitors into the circulation has not been examined. Moreover, chemokine receptors, such as CCR9 and CCR7, were described as being important for thymus settling and might also be used to exit the BM (19–21); however, it is unknown whether they are expressed on circulating progenitor cells.

In addition to surface Ags, several molecular markers of T cell commitment have been best described in the thymic environment and T cell lineage-specific transcription factors analyzed (22, 23). Notch1 (24) and Gata 3 (25) coexpression is specifically required among ETPs to proceed to the next stages (23); E2A was shown to control Notch1 expression (26). At this step, progenitors have completely lost expression of the stem cell gene Gata 2 (27), which controls Notch1 expression (26). At this stage, progenitors have entered the T cell lineage. Experiments for the BM and from six independent experiments for the blood, with at least three mice/experiment.

Materials and Methods

Mice

C57BL/6 (B6) Ly5.1, B6 Ly5.2, and B6 (nu/nu) mice were used at 6–8 wk (males and females). B6 Ly5.2 mice were purchased from Centre d’Elevage R. Janvier (Le Genest St. Isles, France). B6 (nu/nu) mice and B6 Ly5.1 mice were purchased from CDTA (Orleans, France) and were kept in specific pathogen–free conditions. All experiments were carried out in accordance with the guidelines of the French Ministry of Agriculture, under a personal license (number 75-1026).

Abs

The following mAbs were used for staining and cell sorting and were obtained from BD Pharmingen (San Diego, CA) and eBioscience (San Diego, CA): anti-CD3 (145-2C11), anti-CD4 (RM4-5), anti-CD8a (53-6.7), anti-CD8b (H35-172), anti-CD11b/Mac-1 (M1/70), anti-CD19 (1D3), anti-CD25 (IL-2Rα, PC61), anti-CD45.1/Ly5.1 (A20-1.7), anti-CD45.2/Ly5.2 (104-2.1), anti-CD90.2/Thy1.2 (53–2.1), anti-CD106 (VCAM-1; 429), anti–CD117/c-kit (SCF receptor; 2B8), anti–CD127/IL-7Rα (A7R34), anti–CD197 (CCR7; 4B12), anti–CD199 (CCR9; 242503), anti-NK1.1 (PK136), anti–Sca-1 (E13-161.7), anti–Ly-6G/Gr1 (RB6-8C5), anti–TcRβ (H57-597), anti–TcRδ (GL3), anti–Ly-76 (Ter119), anti–IgM (11/41), anti–CD21 (4E03), and anti–CD23 (B3B4). They were directly coupled to FITC, allophycocyanin, allophycocyanin-Cy7, allophycocyanin-eF780, PE, PE-Cy7, or PerCP-Cy5.5 or conjugated with biotin, the latter being revealed by streptavidin-allophycocyanin, streptavidin-PE-Cy7, or streptavidin-PE–Texas Red (BD Pharmingen).

Cell sorting and grafting

BM cells from B6 mice were incubated with unconjugated rat Abs against Ter119, Gr1, CD8a, CD4, and B220 (which are specific for erythroid cells, myeloid cells, mature T cells, and mature B cells, respectively). Positive cells were magnetically depleted with sheep anti-rat IgG-conjugated beads and sheep anti-mouse IgG-conjugated beads (Dynabeads M-450, Dynal Biotech, Oslo, Norway). The remaining cells (Lin−), enriched were labeled with Abs against c-Kit, Sca-1, and Lin Ags (NK1.1, TcRβ, TcRδ, CD8b, CD19, Ly-6G, and Ly-6C). Anti–CD115 and anti–CD135 Abs were used to isolate the different subsets of MPPs: MPP1 (LSK Flt3−VCAM-1−), MPP2 (LSK Flt3+VCAM-1+), and MPP3 (LSK Flt3−VCAM-1−). CLP populations are characterized by the addition of anti–IL-7Rα, their phenotype is Lin Sca-1−c-Kit−IL-7Rα−, and they can be separated using CCR7 expression into Flt3−CLPs and Flt3+ CLPs.

Cell sorting was performed on a FACSARia I with DIVA software (BD Biosciences, San Diego, CA). Sorted populations were grafted i.v. into sublethally irradiated (850 rad) B6 (nu/nu) recipients. The mice were sacrificed at different time points, and spleen and BM were analyzed.

Table I. Stem cell progenitors in BM and blood

<table>
<thead>
<tr>
<th>Progenitors</th>
<th>Tissues</th>
<th>Percentage (mean ± SEM)</th>
</tr>
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<tbody>
<tr>
<td>Lin−</td>
<td>BM</td>
<td>17.1 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>Blood</td>
<td>6.3 ± 1.2</td>
</tr>
<tr>
<td>LSK</td>
<td>BM</td>
<td>7.5 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>Blood</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>CLP</td>
<td>BM</td>
<td>3.3 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>Blood</td>
<td>3.0 ± 0.5</td>
</tr>
<tr>
<td>MPP1</td>
<td>BM</td>
<td>18.3 ± 3.0</td>
</tr>
<tr>
<td></td>
<td>Blood</td>
<td>6.9 ± 1.3</td>
</tr>
<tr>
<td>MPP2</td>
<td>BM</td>
<td>48.6 ± 5.8</td>
</tr>
<tr>
<td></td>
<td>Blood</td>
<td>9.8 ± 1.6</td>
</tr>
<tr>
<td>MPP3</td>
<td>BM</td>
<td>9.7 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>Blood</td>
<td>51.3 ± 5.1*</td>
</tr>
<tr>
<td>Flt3+ CLP</td>
<td>BM</td>
<td>42.0 ± 7.3</td>
</tr>
<tr>
<td></td>
<td>Blood</td>
<td>3.6 ± 0.5</td>
</tr>
<tr>
<td>Flt3− CLP</td>
<td>BM</td>
<td>10.9 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>Blood</td>
<td>41.7 ± 8.6*</td>
</tr>
</tbody>
</table>

Data represent the percentage of Lin− cells within total-nucleated cells, LSK (Lin− c-Kit−Sca-1−) cells within Lin− cells, CLPs (Lin− c-Kit−Sca-1−) within Lin− cells, MPP subsets (MPP1, MPP2 and MPP3) within LSK and CLPs subsets (CLP Flt3− and CLP Flt3+ ) within CLP. The mean was calculated from eight independent experiments for the BM and from six independent experiments for the blood, with at least three mice/experiment.

*p < 0.001, Student t test.
RT-PCR

Cell sorting was performed on a FACSria I with DIVA software and equipped with an automatic cell deposition unit (BD Biosciences). Single cells were collected in individual PCR tubes containing 5 μl 1× PBS and processed for multiplex RT-PCR, as described previously (22, 23, 29). They were lysed by freezing at -80°C, followed by heating to 65°C for 2 min. After cooling at 4°C, RNA was specifically reverse transcribed for 1 h at 37°C and then incubated for 3 min at 95°C for inactivation of reverse transcriptase. Next, cDNA generated by the reverse-transcription reaction was amplified by a seminested PCR. The first round of PCR consisted of a denaturation step at 95°C for 10 min, 15 amplification cycles (45 s at 95°C, 60 s at 60°C, and 90 s at 72°C), and a final step at 72°C for 10 min. This simultaneous amplification of all cDNA was followed by a second round of specific PCRs: the first-round PCR products were separated and amplified with specific primers (Supplemental Table I). The second round of PCR consisted of a denaturation step at 95°C for 10 min and then 54 amplification cycles (30 s at 95°C, 45 s at 70°C, and 60 s at 72°C, with the hybridization temperature decreased from 70 to 60°C every other cycle). The types and amounts of reagents used for reverse transcription and PCR amplifications were described previously (22, 29). PCR products were detected on a 1.5% agarose ethidium bromide gel.

Real-time quantitative PCR and statistical analysis

Real-time quantitative PCR was performed by adding 12 μl 2× SYBR Green PCR Master Mix (Applied Biosystems) to each well containing 4 μl of the first PCR products and 8 μl a primer mix with 0.25 μM each specific primer in a 24-μl reaction volume, using the ABI Prism 7900 HT Sequence Detection System (Applied Biosystems). After a denaturation step at 95°C for 10 min, 60 amplification cycles (30 s at 95°C, 30 s at 60°C, and 45 s at 72°C) were performed. Slope values were determined for the exponential PCR phase using Sequence Detection System software (version 2.2; Applied Biosystems).

The PCR efficiency was assessed for the linear phase of the reaction using LinRegPCR software (version 7.0). Statistical tests of the technique’s validity and data analysis (a t test and an ANOVA) were performed using GraphPad Prism for Windows software (version 5; GraphPad, San Diego, CA).

Statistical analysis

Statistical analyses of gene-expression data and the absolute number of pre-T cells were performed using GraphPad Prism software. All data are representative of three or more independent experiments. The significance

**FIGURE 2.** BM subset isolation. (A) Sorting of BM subsets. BM cells enriched for progenitors by magnetic beads depletion were labeled for lineage (NK1.1, TCRβ, TCRδ, CD8β, CD19, Ly-6G, and Ly76), c-Kit, Sca-1, VCAM-1, Flt3, and IL-7Rα Ago. Numbers indicate the percentage of each population. (B) Sorting of identical populations as above in the blood.

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of differences was determined by an unpaired Student \( t \) test. Significance threshold was set to \( p < 0.05 \).

Results
Selective progenitors in the blood: MPP3 and Flt3\(^+\) CLP subsets are the main circulating progenitors
The origin of the circulating progenitors and, specifically, pre-T cells, is unclear. Therefore, we analyzed BM progenitors (MPPs and CLPs) and their counterparts in the blood. All progenitors were selected from the Lin\(^-\) (NK1.1\(^-\), TCR\(\beta\)^-\(,\), TCR\(\delta\)^-\(,\), CD8\(^\beta\)^-\(,\), CD19\(^-\), Ly-6G\(^-\) and Ly76\(^-\)) Sca1\(^+\)-c-Kit\(^+\), VCA M1 and Flt3 (MPP subsets) and IL7R\(^+\) for CLPs (Figs. 1, 2). Analysis of Flt3 expression enabled us to identify two distinct subsets of CLPs (30) (Fig. 2).

By screening the blood for the presence of MPPs and CLPs (Fig. 2B, Table I), we found that the MPP3 subset was more abundant than the MPP1 and MPP2 subsets. We also detected the presence of the Flt3\(^-\) CLP subset, which is poorly represented in BM. Hence, in contrast to the situation in the BM compartment, MPP3 and Flt3\(^-\) CLP subsets were the most common circulating progenitors, and, thus, have open access to the thymus and the spleen.

Subsequently, we wondered whether one of the differences between resident and circulating MPP and CLP subsets is related to the expression of genes coding for the chemokine receptors Ccr7 and Ccr9, as shown for thymic-settling progenitors (19–21).

Characterization of circulating progenitors: modulation of Ccr9 expression in circulating MPP3 cells and Flt3\(^-\) CLP progenitors
In adult T cell development, the chemokine receptors CCR7 and CCR9 have been implicated in thymus colonization and homing of progenitors (19–21); however, it is not known whether they are involved in exit of BM to the blood. Thus, we wondered whether the expression of CCR receptors could be a characteristic of circulating progenitors.

By performing single-cell RT-PCR analyses according to a previously described method (22, 23, 29), we evaluated Ccr7 and Ccr9 expression in BM-resident and circulating subsets (Fig. 3). This technology was particularly appropriate because of the low cell number involved. Hence, single-cell analysis of Ccr7 and Ccr9 expression in the BM and the blood was performed on between 23 and 72 cells. Both chemokine receptors were detected in MPP2 cells (4% positive cells), but they were significantly expressed in MPP3 cells (11%, Fig. 3A). The values continued to increase: \( \sim \)35% Ccr7\(^+\) and Ccr9\(^+\) for the Flt3\(^+\) CLP stage. In contrast, in the next step (Flt3\(^-\) CLP subset), the frequency of Ccr7 expression dropped to 8% (Fig. 3A). Similar data were obtained by flow cytometry analysis (Supplemental Fig. 1). We noticed that both chemokine receptors were expressed on independent cells; however, Ccr7\(^+\)Ccr9\(^+\)-expressing cells were detected among Flt3\(^-\) CLPs only (12% Ccr7\(^+\)Ccr9\(^+\)).

In the blood, in contrast to the BM, Ccr9\(^+\) MPP3 cells are increased (55%) in comparison with Ccr7\(^+\) MPP3 cells (18%, Fig. 3B), and double-expressing Ccr7\(^+\)Ccr9\(^+\) cells were detected (15%). Among circulating CLPs, Flt3\(^-\) CLPs reached the same expression frequency for both receptors (\( \sim \)10% positive cells) (Fig. 3B), and no double-expressing cells were detected.

When comparing BM and circulating progenitors, we observed a significant increase in the percentage of cells expressing Ccr7 or Ccr9 only in the circulating MPP3 subset. In contrast, Ccr9 gene expression was significantly downregulated in circulating Flt3\(^-\) CLPs.

Thus, CCR expression analysis showed that Ccr7 and Ccr9 identify independent subsets in the BM and blood, and their expression is modulated when they reach the blood environment. Most importantly, the percentage of MPP3 Ccr9\(^+\) cells is sizable in the circulation.

Selective upregulation of lymphoid gene expression on circulating MPP3 cells
The MPP3 and Flt3\(^-\) CLP subsets are present in the circulation, but whether their molecular profile is similar to their counterparts in the BM is unknown. Therefore, to analyze their lineage-commitment status, we evaluated the expression of several key genes involved in stem cell, myeloid, and T cell lineage commitment (Fig. 4). Analysis was performed using single-cell RT-PCR analysis, as previously described (22, 23, 29).

As expected, the percentage of cells expressing the Gata2 gene (involved in homeostasis and proliferation of stem cells) (27) decreased significantly in the BM from MPP1 (57%) to MPP2 (46%) to MPP3 (16%) cells (Fig. 4A). In contrast, the number of cells expressing Pcl1, a B/myeloid gene (28), was constant (70–95%) in all MPP subsets. The expression of both genes remained stable in circulating MPP3 cells (Fig. 4A).

Lymphoid commitment is best characterized by E2a, Il7r, and Rag1 expression (26, 31, 32). As depicted in Fig. 4B, the number...
of cells expressing the E2a gene, which is involved in B–T commitment, was greatest in resident MPP3 cells: ~68% of cells scored positive in comparison with 40% of MPP1 and MPP2 cells. In contrast, Il7r and Rag1 were expressed in only a small fraction of MPP3 cells: 18% were Il7r+ (8+ cells of 44 single cells analyzed) or Rag1+ (20 of 111 single cells analyzed).

Analysis of Gata3 and Notch1 expression yielded contrasting results; the expression frequency of Gata3 decreased during the MPP1 to MPP2/3 transition, whereas that of Notch1 increased significantly (Fig. 4C). Within the MPP3 subset, 9% of cells were Gata3+, and 67% were Notch1+.

Interestingly, additional changes in lymphoid gene expression occurred in the blood (Figs. 4, 5). In circulating MPP3 cells, the expression frequency of E2a was decreased (to 37%), and Rag1 expression was lost. Quantitative analysis revealed that Pu1, Il7r, and Notch1 were increased significantly (10–20-fold) in circulating MPP3 cells (Fig. 5). We noted that Notch2 frequency and quantitative expression remained unchanged (Supplemental Fig. 2).

Similar analyses of BM-resident CLP subsets confirmed the CLP lymphoid commitment (Fig. 4D): Rag1 was widely expressed (73% of cells were Rag1+; data not shown), and the number of E2a+ cells was stable in all CLP subsets (Fig. 4D). Interestingly, in the BM, most Flt3+ CLPs express Cd25 (~60%) in comparison with Flt3− CLPs, whereas in the blood, <10% of circulating CLPs express Cd25+. Cd25 gene expression was absent in MPP subsets (data not shown), in contrast to a previous report by Lai and Kondo (33).

Quantitative studies revealed that the expression of Gata3 mRNA molecules in Flt3+ CLPs is increased among some circulating cells (Fig. 5C). Surprisingly, the level of expression of Il7r, a hallmark of CLPs, was remarkably downregulated on circulating CLPs (Fig. 5B) and also was observed at the protein level (Fig. 2B).
Moreover, Notch1 was expressed at the same frequency (20% positive cells) and the same level in resident and circulating Flt3-CLP subsets (Fig. 5C).

Therefore, we observed several important lymphoid gene expression modulations, at the qualitative and quantitative levels, between BM-resident and circulating progenitors.

**MPP3 subset gives rise to extrathymic pre-T cells**

The data obtained through the molecular analysis of circulating progenitors led us to more closely examine the impact of these progenitors on the ability to generate pre-T cells.

Intravenous transfer of BM progenitors (Figs. 1, 2A) allows them to gain additional modifications in circulation. To address this question, we sorted these cells from B6 mice and injected them i.v. into sublethally irradiated nude hosts. We then screened the donor-type progeny in the spleen and the BM between 8 and 30 d posttransplant (Fig. 6A). Splenic pre-T cells were detected in nude mice (14, 15) and are present before ETP generation following BM transfer (L. Gautreau and S. Ezine, unpublished observations) (16). Thus, they can be considered the first step toward commitment to the T cell pathway.

Very few donor cells were generated from CLP-grafted recipients, regardless of the number of grafted cells, the injection route, and the day of analysis (Figs. 6A, 7). Therefore, the Flt3+ CLP subset was better able to give rise to a few splenic pre-T cells (~400–800 cells, detected at day 30 only) than was the major CLP Flt3- subset (Fig. 6B). In the spleen, CLPs were able to generate IgM+ B cells, marginal zone B cells, and follicular B cells (Supplemental Fig. 3).

We found that Mac1+Gr1+ cells were present in the BM and spleen of MPP1 and MPP2 recipients but were absent in MPP3- and CLP-grafted recipients (Supplemental Fig. 3). In contrast, marginal zone and follicular B cells were produced primarily by CLPs rather than the MPP3 subset (Supplemental Fig. 3B).

When MPP subsets were sorted as indicated in Fig. 2A and grafted, pre-T cell generation peaked at day 15 for all three subsets (Fig. 6A, 6C). The MPP3 subset was the most competent in terms of the production of splenic pre-T cells (Fig. 6C). Our data show that 1.6 x 10^6, 0.4 x 10^6, and 0.1 x 10^6 cells were recovered from the spleens of MPP3-, MPP2-, and MPP1-recipient hosts, respectively. The values at days 10 and 15 emphasize each subset’s intrinsic capacity. MPP3 cells colonize the spleen and give rise to pre-T cells more effectively compared with the other progenitors and irrespective of the number of cells grafted (Fig. 7). However, the MPP2 to MPP3 transition must be very rapid in vivo because, in some individual experiments, similar levels of pre-T cells were attained when these progenitors were grafted (data not shown). Later, at day 30, pre-T cells decreased as a result of the major generation of mature cells (B, myeloids).

Our data revealed that, in our setting, only the MPP3 subset has an intrinsic T cell potential, which is shaped in the bloodstream.

**Discussion**

Our results showed that MPP3 cells represent the BM compartment with intrinsic T cell potential. In comparison with their immediate progenitors (CLPs), their priming, which is initiated in the BM, is...
accomplished in the blood. Although CLP populations express lymphoid genes, are present in the blood, and colonize the spleen to generate B cells, they are devoid of T cell activity when grafted in athymic mice.

By evaluating the source of extrathymic pre-T cells in this setting, we were able to gain insights into the T cell specificities of the MPP and CLP populations. As evidenced by their hierarchy in the hematopoietic tree, MPPs produce the downstream populations: the MPP1, MPP2, and MPP3 subsets are generated sequentially, with CLPs as the terminally lymphoid-restricted product. However, MPP3 cells may have been more effective in producing T cells than CLPs, but the reasons for such an advantage were never clarified. Our setting allowed the identification of a very early T cell capacity, in the absence of thymic influence, which is concentrated in MPP3 cells. Moreover, the pre-T cells (Lin' Thy1.2'CD25'IL7Rα') generated are not innate lymphoid cells, because these innate lymphoid cells are detected a month postgraft by analysis of RORG and Gata3 coexpression (data not shown).

It could be argued that CLPs did not conserve T cell activity because of a lack of survival signals, but the generation of B cells in the spleen is in opposition to this hypothesis. CLPs could be unresponsive to peripheral signals: they might have reached a stage in which Notch1 signaling is not sufficient to activate target genes. If any T cell potential exists in CLPs, it might take some time to be acquired and/or requires unknown signals to be defined. Thus, CLPs might enter the thymus in a different niche compared with MPPs and, most probably, never contribute to the ETP stage. This will reconcile numerous studies aimed at comparing ETP and CLP potentialities (9).

In our study, we compared BM-resident progenitors with their counterparts in the blood. Of the three MPP subsets, as defined by their surface markers, only MPP3 (Flt3'VCAM1') populations

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**FIGURE 6.** Extrathymic pre-T potential of CLP and MPP subsets after graft to nude mice. (**A**) Flow cytometry analysis of donor-derived pre-T cells (Lin' Ly5.1'Thy1.2'CD25') in the spleen from recipient nude mice grafted (1–5 × 10^3 cells) with Flt3' LSK, CLP, or MPP cells. (**B**) Absolute numbers of pre-T cells recovered in the spleen after graft of CLP subsets. (**C**) Absolute numbers of pre-T cells recovered in the spleen after graft of MPP1, MPP2, or MPP3 subset. At least three mice were collected per time point for each condition in three or four independent experiments. Error bars show SD. **p < 0.01, Student t test.
were found in the blood, and they represent a minor subset in the BM; similarly, only the Flt3\(^-\) CLP subset was present in the blood. In the periphery, c-Kit and IL-7R intensities were lower than on BM progenitors, and so, identification of circulating CLPs has been missed by others. Thus, in unmanipulated mice, MPP3 cells and Flt3\(^-\) CLPs circulate. This indicates that physiologic mobilization is a selective event whose trigger signal is unclear. G-CSF/Flt3L treatment generates only LSK and MPP3 cells in the blood (data not shown).

Among the genes tested, a discrepancy was observed for the T cell–prone gene Notch1 between resident and circulating progenitors. Expression was quantitatively upregulated on the circulating MPP3 subset in comparison with their resident counterparts. Single-cell analysis revealed that the percentage of Notch1\(^+\) MPP3 cells was stable between the BM and the blood, whereas the mRNA quantity increased ∼20-fold. This was not observed for CLP populations; the percentage of Notch1\(^+\) cells decreased, and quantitative levels remained constant. Our data show that resident and circulating progenitors modulate specific factors.

Circulating MPP3 cells maintained expression of the Pu1 gene (∼80% of Pu1\(^+\) cells with also a quantitative increase), which establishes the close link between myeloid and T cell potentialities (34–36).

Although a number of studies detected MPPs in the blood, they failed to characterize these progenitors (6). Our study provides valuable information on the circulating MPP3 subset. After initiation of lymphoid commitment in the BM, circulating populations were found to have differentiated further (as evidenced by the decreased percentage of E2A\(^+\) cells and loss of Rag1 expression).

It is now acknowledged that CCR9 expression is consistent with the specificity of thymus settling (37). We show in this study, by single-cell analysis, that the frequency of Ccr9\(^-\) expressing progenitors is increased from MPP2 cells to CLPs among BM-resident cells. This highlights a novel marker as a lymphoid-commitment signal: ∼12% of MPP3 cells and 35% of CLPs are Ccr9\(^+\). Moreover, Ccr9 expression was independent of Flt3 signaling, in contrast to a previous report (19), because Flt3\(^+\) and Flt3\(^-\) CLP subsets were positive for CCR9. Lai and Kondo (33) identified a CCR9\(^+\) MPP subset in the BM that was able to rapidly colonize the thymus in comparison with their counterpart CCR9\(^-\) subset; however, they did not provide a mechanism for the difference. We show in this study that Ccr9\(^+\) MPP3 progenitors are

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**FIGURE 7.** Pre-T cells generated according to the number of grafted progenitors. The absolute number of pre-T cells generated is shown at the indicated time points when MPPs (A) or CLPs (B) were grafted. Gating on Lin\(^-\) populations to identify pre-T cells (Lin\(^-\)Ly5.1\(^+\)Thy1.2\(^+\)CD25\(^+\)) required to pool three to six spleens from grafted mice. Symbols represent the number of pre-T cells in these pools; three or four independent experiments at each time points are shown.
the unique MMP subset in the blood; furthermore, Notch1 expression is quantitatively increased ~20-fold in circulation. Thus, this mechanism might direct blood progenitors rapidly toward the T cell pathway, when needed. This suggests that the priming initiated in the BM (CCR9 activation) continues in the blood (Notch1, IL-7Rα, and Gata3 upregulation) and that these progenitors do not represent leakage of BM cells into the circulation. Carrying Flt3+ CLPs were identified by other investigators in the blood (38). The absence of T cell potential in the Flt3+ CLP subset was shown to be due to a low dose of Notch1, but it was restored when Id proteins were inhibited (39). Gata3, a T and NK cell–specific factor, was upregulated on few CLP cells, as a possible indicator of FLT contribution to one or the other lineage. IL-7Rα intensity is maintained in circulating multipotent progenitors but is downregulated in CLPs. Altogether, Gata3 and IL-7Rα might denote an NK cell fate within these circulating CLPs. Therefore, continuing differentiation is taking place in the blood. Indeed, downregulation of Flt3 in CLPs accompanies NK cell developmental restriction (40, 41).

Most studies in the literature evaluated the role of the LMPP subset, which encompasses the Flt3-expressing MPP2 and MPP3 subsets (7). They were shown to express lymphoid genes, Rag1 and Rag2, and CCR9 (37, 42). In this study, single-cell analysis showed that these subsets are heterogeneous and different: with regard to Gata2, E2a, and Gata3 expression, MPP2 cells exhibit a profile that is more similar to the MPP1 subset. As the main circulating subset, the MPP3 population has a weak self-renewal capacity (as evidenced by a low frequency of Gata2 expression) and is partially committed to the lymphoid pathway (as demonstrated by E2a, Itf7, Notch1, and Rag1 expression). Thus, the heterogeneous LMP subset requires more study in two distinct populations of MPP2 and MPP3.

By choosing athymic mice for our graft experiments, we removed the thymic influence on BM precursor cells. We found that, in this context, CLPs were unable to give rise to splenic pre-T cells. In line with another study (9), our data show that CLPs, regardless of their Flt3 expression status (30), are B committed and, thus, give rise to mature B cells. We hypothesized that the CLPs require another signal (i.e., in addition to CCR9 and Notch1 expression). CLPs’ inability to generate extrathymic pre-T cells in this setting may be explained by the lack of commitment in the absence of thymic influence. It was reported that Flt3^CD27^ CLPs are the major source of T cells (8). However, we did not detect this subset in the blood, because the main circulating CLP subset had lost Flt3 expression. Hence, the main MMP3 cells contain full T cell capacity.

In conclusion, our results show that the MMP3 population represents the reservoir of T cell progenitors in the BM and among circulating progenitors, owing to the quantitative upregulation of Notch1 expression. Our findings provide another source of T cell progenitors in the blood and open the way for the manipulation of circulating progenitors as a source of T cells.

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Disclosures

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