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The Blood Contains Multiple Distinct Progenitor Populations with Clonogenic B and T Lineage Potential

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The thymus is seeded by bone marrow-derived progenitors that circulate in the blood. Multiple cell types can be found in the thymus early after i.v. administration or in steady state, but most fail to satisfy the known characteristics of true T progenitors. Cells that do conform to classical definitions retain multilineage potential, but surprisingly, cannot make B cells. Because acquisition of the T lineage fate among noncommitted progenitors is a lengthy process, the absence of B cell potential in early thymocytes suggests that B and T lineages diverge prethymically. To test this suggestion, we screened numerous presumptive progenitor populations for T cell growth and differentiation potential, as well as for clonogenic T or B cell development. We find that blood and marrow each contain multiple distinct subsets that display growth and differentiation potential consistent with being canonical T progenitors. Assessment of clonogenic potential further shows that although all blood and marrow populations have high T cell cloning potential, no T/non-B cells are apparent. These data suggest that either true thymic reconstitution potential derives from a small T/non-B cell subset of one of these populations, or that most of the cells defined as canonical progenitors within the thymus do not, in fact, reside in the mainstream of T progenitor differentiation. The Journal of Immunology, 2007, 178: 4147–4152.

Under ordinary circumstances, T lineage differentiation occurs in the thymus. Remarkably, however, the thymus does not contain self-renewing stem cells (1, 2), but instead relies on the semicontinuous importation of bone marrow (BM)3-derived progenitors circulating in the blood (3). For many years, it was debated whether the thymus is reconstituted by cells that are already T lineage committed, and are thus specifically attracted to the thymus, or whether the thymus is seeded by multipotent progenitors that are induced to become T cells by exposure to conditions unique to the thymus. It now seems that, at least in the postnatal thymus, the truth lies somewhere in between. The earliest intrathymic progenitors have the potential to make T cells, as well as other hematopoietic lineages including dendritic cells and NK cells (4, 5), but T lineage differentiation is imposed by exposure to the thymic microenvironment (6), primarily through stimulation of Notch (7, 8) via ligands expressed on thymic stromal cells (9). Notably, what early intrathymic progenitors appear to lack is the potential to make B cells (4, 5), particularly among those that fulfill all the conventional criteria for T cell progenitors (4). It should be noted, however, that multiple types of cells can be found within the thymus, including cells with dual T cell/B cell potential (4, 10, 11), but in general their relative contributions to the T cell pool remain unclear.

Like numerous other cell types, commitment to the T lineage has been shown to be a protracted process (12). This finding, together with the fact that no detectable B cell progenitor activity can be found among canonical intrathymic progenitors (4, 5), suggests that divergence of B and T potential must occur extrathymically. To evaluate this possibility, we screened blood populations defined by presumptive progenitor markers (c-Kit, Sca-1, Flt3, and IL-7R) for growth capacity and clonality under T or B lineage differentiation conditions in vitro. Our findings indicate that multiple types of T cell progenitors are present in the blood, but none of them appear to lack B cell potential. The relevance of these findings in the context of prior publications is discussed.

Materials and Methods

Cell purification

Euthanized C57BL/6 male mice were used as sources of tissue. Our results were generally from mice 4–5 wk of age, although similar results have been found from mice of all ages studied (up to age 3 mo). Studies have been reviewed and approved by an appropriate institutional review committee. All steps were performed at 4°C. Blood was harvested by nicking the right ventricle, perfusing 5 ml of mouse-typotypic HBSS containing 5% FBS, 10 μg/ml DNase, and 10 U/ml heparin, and collecting blood from the thoracic cavity. Cells were washed once before lysing RBC using ACK lysis buffer (150 mM ammonium chloride, 1 mM potassium bicarbonate, and 0.1 mM EDTA). BM was harvested by flushing tibia and fibulas with HBSS/FBS/DNase/heparin solution, and homogenization by passage through a 20-gauge needle, followed by RBC lysis. Thymuses were removed and single cell suspensions prepared by passage through a cell strainer; small CD4+CD8+ double-positive (DP) thymocytes were depleted by centrifugation in an iso-osmotic solution of 13.6% Opti-Prep (Greiner BioOne). For all cell types, lineage depletion was performed by first treating cells with a mixture of Abs recognizing CD3, CD4, CD8, CD19, Mac-1, Gr1, B220, and an erythrocyte marker recognized by mAb Ter119, followed by depletion with anti-Ig-coated paramagnetic beads (Dyanal Biotech). During cell sorting, a second-pass lineage depletion was enforced by staining the residual cells with Red 613-conjugated anti-rat Ig (Caltag Laboratories). Depleted blood and BM cells were also stained with commercial Ab conjugates recognizing Flt3 (PE conjugate; eBioscience) and Sca-1 (allophycocyanin conjugate; BioLegend), as well as custom conjugates for IL-7R (clone SB-199, Alexa Fluor 488 conjugate) and c-Kit (ACK-2, biotin conjugate, followed by streptavidin-Alexa Fluor 660). Depleted thymocytes were stained with custom conjugates for c-Kit (ACK-2,
Identification and purification of presumptive T cell progenitor populations from blood.

A. Analysis of lineage-negative blood cells stained for the markers that identify MPP or CLP cells in BM (namely, c-Kit, Sca-1, IL-7R, and Flt3). Numerous populations are clearly identifiable, including cells that match the conventional definitions for marrow-derived MPP (c-Kit^{high}Sca-1^{low}IL-7R^{low}Flt3^{-}), or CLP (c-Kit^{low}Sca-1^{low}IL-7R^{low}Flt3^{-}), as well as others that do not match conventional definitions (namely, cells that, like CLP, are c-Kit^{low}Sca-1^{low}Flt3^{-}, but that lack IL-7R expression). All of these populations can be purified to homogeneity, and can be distinguished from each other, as shown in B.

Stromal cocultures

OP9-control or OP9-DL1 cells (8) were inoculated with lymphoid progenitors when they reached 80% confluency. For measurement of clonal growth in 96-well plates, stromal cells were sublethally irradiated (20 Gy). Growth medium for lymphocyte cocultures was IMDM containing 20% FBS (Invitrogen Life Technologies), 100 ng/ml gentamicin, 5 ng/ml IL-7 (PeproTech), and 5 ng/ml Flt3L (PeproTech). For analysis of differentiation into DP cells, IL-7 concentrations were reduced to 2 ng/ml. All cultures were initiated using 30% preconditioned medium (i.e., medium from lymphostromal cocultures previously established in bulk). At various intervals after culture, part of the supernatant medium was replaced with fresh medium, and/or cultures were harvested by vigorous pipetting. Harvested cells were counted on a hemocytometer, and where appropriate, were reincubated into stromal cocultures using the same starting conditions described. Clonal growth was evaluated by microscopic examination.

Stromal-free B cell cultures

It has been shown that FBS-containing stromal cocultures may actually inhibit the development of B cell potential in more mature progenitors, such as common lymphoid progenitors (CLP) (13). Consequently, B cell potential was evaluated not only using OP9 control stromal cocultures, but using defined serum-free medium as well (13). The medium for such assays was X-VIVO medium (Fisher Scientific) containing 1% detoxified BSA (StemCell Technologies), 2 mM l-glutamine, 50 μM 2-ME, 100 U/ml penicillin-streptomycin, 100 ng/ml Flt3 ligand, 20 ng/ml stem cell factor, and 1 ng/ml IL-7. Fresh medium was added on days 5 and 10 of culture, and growth was analyzed using a stereomicroscope on day 11.

Results

Identification of multiple blood-borne populations that correspond to known progenitor phenotypes in BM

To determine whether the blood contained minor subsets of cells that were enriched in T lineage potential, we stained lineage-depleted blood cells with a panel of markers that identify various types of progenitors in marrow. Not surprisingly, we were able to identify cells (Fig. 1A) corresponding to the phenotype of BM-derived multipotent progenitors (MPP) defined as c-Kit^{low}Sca-1^{low}Flt3^{low}IL-7R^{low} (14–16), as well as BM stem cells (data not shown). Surprisingly, we were also able to clearly identify cells corresponding to BM-derived CLP (defined as c-Kit^{low}Sca-1^{low}Flt3^{low}IL-7R^{low}) (17), even though others have indicated that such a cell could not be found in blood (18). In addition, we find another putative (by prognostic surface markers) progenitor population that appears very similar to CLP, except that it lacks IL-7R (Fig. 1). Other studies have revealed a similar population in BM (13), and for our study, we refer to these cells as “CLP-like.” All of these populations could be purified to homogeneity (Fig. 1B), indicating that they are truly distinct from one another.

Blood MPP, CLP, and CLP-like cells all proliferate extensively under in vitro conditions that support T cell progenitor growth

T lineage differentiation is characterized by extensive proliferation, with each canonical (4) intrathymic progenitor giving rise to ~1 million cells (19). Importantly, although multiple intrathymic T cell progenitors can be identified, those that are c-Kit^{low} (similar to BM-CLP) have very limited proliferative capacity (4). To evaluate whether the c-Kit^{low} (CLP and CLP-like) cells found in the blood (Fig. 1) most resembled canonical or atypical progenitors, we cultured them under conditions known to support immature T cell proliferation in vitro (OP9-DL1 stromal cells, see Ref. 8). As controls, parallel populations from BM, as well as MPP and canonical intrathymic progenitors (c-Kit^{high} double negative (DN)1) were used. Blood CLP exhibited substantial growth potential, even slightly greater than that of canonical DN1 thymocytes, with similar kinetics, although their proliferative capacity was slightly but consistently less than that of BM CLP (Fig. 2). These findings are consistent with what would be expected if BM CLP were progenitors to blood CLP (and then to DN1). In contrast, MPP cells from either BM or blood displayed slower initial growth kinetics than DN1, but were clearly on course to surpass the proliferative capacity of DN1 by late in the culture period. Again, this behavior is consistent with a precursor/progeny relationship for BM and blood.
MPP and intrathymic progenitors, and is consistent with BM data published by others (20). Like CLP, BM MPP appeared to have slightly more proliferative capacity than that of blood MPP, again consistent with a precursor-progeny relationship. Overall, the proliferative capacities and proliferation kinetics of these populations suggest that the blood-borne cells are downstream of the equivalent of marrow cells, and very importantly, that all of them have T cell potential. Unexpectedly, CLP-like cells from marrow and blood also displayed extensive proliferation potential under T lineage conditions, rivaling that of conventional CLP, and very similar to that of DN1. This is consistent with cells of a similar phenotype from BM that were evaluated for B cell potential (13). All populations that gave clonal growth also generated cells expressing conventional T lineage markers with predictable kinetics (Fig. 3). Together, these data show that multiple putative progenitor populations from blood can proliferate in a manner consistent with being upstream of canonical intrathymic progenitors.

Clonal assessment of T progenitor activity from defined blood and BM populations

Proliferation studies (Fig. 2) indicate that multiple distinct blood populations have proliferative capacities consistent with being
Classical T cell progenitors. Although bulk cultures have the advantage of allowing analysis at early time points, the liability is that outgrowth of minor (unidentified) subpopulations may be misinterpreted as representing the whole population. Consequently, we plated single CLP, CLP-like, and MPP cells from blood or BM on OP9-DL1 stromal monolayers, and examined them for growth after 12 or more days, using DN1 thymocytes as a control (Fig. 4A). c-Kit\textsuperscript{high} DN1 thymocytes (DN1a plus DN1b; see Ref. 4) exhibited an absolute cloning efficiency of just over 50%, indicating that the single cell culture system worked relatively well. MPP from either blood or BM both exhibited T cell cloning efficiencies nearly identical with that of DN1 thymocytes, consistent with their status as a robust, multipotent cell. In contrast, CLP from either blood or BM were quite similar to each other, but were only \(\sim 60\%\) as efficient as DN1 thymocytes in generating T clonal growth. CLP-like cells from BM appeared slightly better at generating T cell clones than those from blood, although for unexplained reasons, there was significantly more variation in these populations than in CLP or MPP, which could account for the differences between BM and blood for this cell type.

BM CLP are putative intermediates between BM MPP and thymocytes, yet like their blood counterparts were less efficient at generating T cell clones than either their putative precursors (MPP) or progeny (DN1). To determine whether this decrease in efficiency represents the divergence of T vs B lineage committed cells, we cultured MPP, CLP, and CLP-like cells from blood or BM under B cell conditions, using either OP9 stromal cocultures or stromal-free serum-free conditions (13). All populations from blood were roughly equivalent to their BM counterparts in B cell assays (Fig. 4B). MPP were 40–50% efficient at generating B cells in either stromal or stromal-free conditions, but more mature progenitors from either blood or marrow did not grow well in stromal cocultures, consistent with the data of other studies for BM (13). In stromal-free cultures (Fig. 4C), CLP from either blood or marrow were about half as efficient as MPP at making B cells, which is very similar to their relative efficiency under T cell conditions (Fig. 4A). CLP-like cells from either BM or blood had B cell efficiencies that were somewhat reduced compared with CLP, consistent with previous studies using BM cells (13).

**Discussion**

The nature of cells that seed the postnatal thymus remains unclear. The thymus contains no self-renewing cells (1, 2), and therefore depends on BM-derived progenitors that are unlikely to arrive by any means other than the blood circulation. In the marrow, numerous multipotent stem and progenitor cell types and precursor-progeny relationships can be identified (21). BM CLP are generally believed to represent the divergence of lymphoid and myeloid lineages (17). However, the earliest progenitors in the thymus are not T committed, and can make NK and dendritic cells, among others, but not B cells (4, 5). Because T lineage commitment is a prolonged process (12), the inability of classical intrathymic progenitors to give rise to B cells suggests that T/B lineage divergence occurs extrathyMICally, and calls into question whether CLP are the progenitors to T cells. It has previously been reported that the blood does not contain CLP, and that all T lineage potential must therefore derive from MPP that circulate in the blood (18). On the contrary, we were clearly able to observe cells with a CLP phenotype (c-Kit\textsuperscript{low}Sca-1\textsuperscript{low}IL-7R\textsuperscript{+}Flt3\textsuperscript{+}) among lineage-negative cells in the blood (Fig. 1A). Such cells could be purified, and, when cultured under conditions that support the development of immature T cells (22), can proliferate in a fashion that is consistent with that of canonical intrathymic progenitors, and acquire CD4 and CD8 lineage markers (Fig. 3). Thus, not only can CLP be found in the blood, but unlike a similar c-Kit\textsuperscript{low} population found in the thymus (4), such cells have proliferative capacity consistent with that of a classical T cell progenitor (19).

In addition to conventional CLP, the c-Kit\textsuperscript{low}Sca-1\textsuperscript{low} component of lineage-negative blood contained another population that was Flt3\textsuperscript{+}, but IL-7R\textsuperscript{–} (CLP-like); similar cells have been found by others in marrow (13). Like MPP (Fig. 2) (18) and CLP, such cells could be purified to homogeneity by cell sorting (Fig. 1B), and exhibited substantial proliferative capacity when cultured under T cell conditions. Such cells represent interesting candidates for thymus homing progenitors because they lack IL-7R, and thus are similar in this respect to the earliest intrathymic progenitors defined by our group and others (4, 23). However, they also differ...
from early intrathymic progenitors in being c-Kit\textsuperscript{low} (Fig. 1), whereas the earliest intrathymic progenitors are c-Kit\textsuperscript{high} (4, 5, 23). Furthermore, such CLP-like blood cells exhibit high clonogenic potential for both T and B lineages (Fig. 4), which is inconsistent with any intrathymic progenitor population (4, 5). As previously discussed (4), there are similar intrathymic progenitors (DN1c) that are c-Kit\textsuperscript{low} and IL-7R\textsuperscript{+}, and these can give rise to both T and B cells, but do not generate T cells efficiently. As the only clearly bipotent population in the thymus, these c-Kit\textsuperscript{low}IL-7R\textsuperscript{+} intrathymic cells are the likely source of B cells in the absence of Notch (7), but do not appear to be mainline T cell progenitors (4). Thus, there remains a gap between CLP-like cells in the blood and similar cells found in the thymus, in terms of loss of extensive T proliferation potential in the latter.

To ensure that growth of blood populations under T cell conditions did not represent outgrowth of a minor contaminant, we performed single-cell cloning assays using the same in vitro system. All populations exhibited cloning potential that exceeded the frequency of minor contaminants (Fig. 1). MPP from either blood or BM exhibited T cell cloning efficiencies indistinguishable from that of DN1 thymocytes, while CLP (from blood or marrow) were only ∼60% as efficient. The reason why CLP, which (at least from BM) are putative intermediates between MPP and DN1 thymocytes, should be less efficient than either at generating clones under T cell conditions is not clear, although our estimates for BM CLP are similar to estimates reported by others (24, 25). One possibility is that CLP is a heterogeneous population that includes subsets with either T or B potential. If one compares cloning potential of CLP cells (from BM or blood) to MPP, the sum total of B cell and T cell cloning frequencies is not greater than 100% (Fig. 4, A and C), supporting this possibility. We are currently searching for presumptive markers that may segregate CLP into subpopulations, or identify a population with T cloning potential that rivals DN1 or MPP, although none are yet apparent. The most obvious marker, L-selectin, is homogeneously expressed on c-Kit\textsuperscript{low} cells from lineage-negative BM (26), so it is unlikely to provide significant enrichment. Consistent with this finding, preliminary studies from our lab show that although L-selectin\textsuperscript{+} CLP and CLP-like cells are indeed enriched in T cell cloning potential, they do not approach the efficiencies seen with MPP or DN1 (data not shown).

Our objective in screening for putative T progenitor populations from blood was to resolve the apparent discrepancy between the lineage potential of early T progenitors within the thymus, and what is known about the T lineage commitment process. As mentioned, previous studies from our laboratory (4) and another (5) have shown that early thymocytes possessing the classical attributes of a T cell progenitor lack B cell potential. Because it has been shown that T lineage commitment is a protracted process (12), it is unlikely that divergence occurs after entry into the thymus, even though exposure to the thymic microenvironment does repress development of other non-T lineages (4, 6), mainly through the opening of vascular gates (3). As has been previously pointed out (29), it is possible, if not likely, that multiple types of progenitors home to the thymus and contribute to the T cell pool. It remains to be determined whether they all contribute equally to the intrathymic and/or peripheral T cell pool, whether any of them have a bias for one T lineage over another (CD4, CD8, TCR-γδ, NK cell-T cell, etc.), or whether they perform other functions (such as conditioning the microenvironment or controlling vascular gates). At the present point, there is no apparent continuity between intrathymic and extrathymic progenitors in terms of their phenotypes or lineage potentials, and thus the identity and nature of the thymic seeding cell, if there is only one, remains elusive.

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Disclosures

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