Mobilization of CD34+ Progenitor Cells in Association with Decreased Proliferation in the Bone Marrow of Macaques after Administration of the Fms-Like Tyrosine Kinase 3 Ligand

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Fms-like tyrosine kinase 3 ligand (FLT3-L) is critical for the differentiation and self-renewal of CD34+ progenitor cells in primates and has been used therapeutically to mobilize progenitor and dendritic cells in vivo. However, little is known regarding the expansion of progenitor cells outside of peripheral blood, particularly in bone marrow (BM), where progenitor cells primarily reside. Evaluation of FLT3-L-mediated cell mobilization during lentivirus infections, where the numbers of CD34+ progenitor cells are reduced, is limited. We enumerated frequencies and absolute numbers of CD34+ progenitor cells in blood and BM of naive and SIV- or SHIV-infected macaques during and after the administration of FLT3-L. Flow cytometric analyses revealed that, while CD34+ cells increased in the circulation, no expansion was observed in BM. Furthermore, in the BM intracellular Ki67, a marker of cell proliferation, was downregulated in CD34+ progenitor cells but was upregulated significantly in the bulk cell population. Although the exact mechanism(s) remains unclear, these data suggest that CD34+ cell mobilization in blood was the result of cellular emigration from BM and not the proliferation of CD34+ cells already in the periphery. It is possible that the decreased progenitor cell proliferation observed in BM is evidence of a negative regulatory mechanism preventing hyperproliferation and development of neoplastic cells.

The cytokine receptor Fms-like tyrosine kinase 3 (FLT3) is expressed at high levels on both primitive and early lymphoid/myeloid CD34+ progenitor cells (3, 21). Interaction with its cognate ligand (FLT3-L), found in both soluble and membrane-bound isoforms, contributes to the regulation of self-renewal and differentiation potential of these cells (43, 44). However, dysregulation of FLT3/FLT3-L signaling can result in the development of various leukemias (1, 6, 22, 29), and increased serum levels of FLT3-L are often indicative of other hematologic and autoimmune abnormalities (17, 25, 39). Nonetheless, after both murine and human FLT3-L were cloned in the early 1990s (24), this hematopoietic cytokine was used effectively in vitro to expand and maintain CD34+ progenitor cells (26, 32, 33) and, in combination with other growth factors, was used to induce differentiation of myeloid lineage cells (4), dendritic cells (2, 15), natural killer (NK) cells (42), erythroid precursors (12), and even endothelial cells (41). In addition, FLT3-L was shown to specifically suppress apoptosis of CD34+ progenitor cells (27).

Early in vivo studies in mice demonstrated that FLT3-L administration not only mobilized and expanded murine CD34+ progenitor cells but also promoted expansion of human CD34+ cells transferred into SCID mice (8, 9, 24). In nonhuman primates FLT3-L was used to expand dendritic cell subsets (7, 30, 35), to treat radiation-induced myelosuppression (13, 14, 19), and as an adjuvant for various vaccines (23, 40). Although CD34+ cells primarily reside in the bone marrow (BM), examination of mobilization of these cells in vivo in nonhuman primates has been limited and typically restricted to analyses of blood (5, 18, 28). CD34+ cell mobilization and hematopoiesis is of particular interest in macaque models of lentivirus infections because, during both HIV and SIV infections, BM damage and reduced hematopoiesis is evident early after infection and is associated with decreased numbers and clonogenic potential of CD34+ progenitors, despite low levels of infection and virus replication in these cells (10, 16, 20, 34, 36, 37). Therefore, in the present study we quantified and characterized mobilization of CD34+ progenitor cells in BM in relation to that observed in peripheral blood by examining BM aspirates taken at various times during and after FLT3-L administration to naive and SIV- or SHIV-infected macaques.

MATERIALS AND METHODS

Animals and FLT3-L administration. Adult pig-tailed macaques (Macaca nemestrina) of both sexes were used in the present study and included three SIV-naïve macaques (97P009, JC2, and UA2), three macaques infected with SHIV-89.6P (97P045, 98P012, and AV1C), and three macaques infected with SHIVmac239-derived mutants (98P016, 99P032, and CT19), as described previously (30). Animals were inoculated subcutaneously with human recombinant FLT3-L, a gift from Amgen Corp., for 7 consecutive days (100 µg/kg/day), according to a protocol developed by Teleshova et al. (35). Blood samples were analyzed on days 0, 4 or 5, 8, 12, and 15 (except for animals that were sacrificed at day 12) after initiation of the FLT3-L inoculations. Analyses of BM aspirates were done prior to the start of the study and at various times during and after FLT3-L inoculations, as described previously (30) (Fig. 1). Before each procedure macaques were anesthetized with an intramuscular injection of ketamine-HCl (10 mg/kg). Macaques were housed in BSL2 isolation facilities at the University of Alabama at Birmingham (UAB) in accordance with institutional and Animal Welfare Act guidelines. Before the study was initiated, it was re-
Cell collection and processing. Venous blood was collected into EDTA-treated tubes for whole blood flow cytometric analysis and for complete blood counts (CBCs) and differentials. BM was obtained from the medullary cavity of the proximal humerus with 15-gauge BM aspiration needles (Medical Device Technologies, Gainesville, FL) previously flushed with heparin; precipitated yellow fat layers at the top of these EDTA-treated tubes were removed from the BM. BM mononuclear cells (BMMCs) were isolated by density gradient centrifugation through lymphocyte separation media (ICN Biomedicals, Inc., Aurora, OH); any residual fat precipitated at the top of the gradient was aspirated before removal of the BMMCs. Contaminating red blood cells in blood were lysed hypotonically using an ammonium chloride solution. MCs were resuspended in phosphate-buffered saline with 2% fetal bovine serum (FBS) for subsequent flow cytometry staining or were cryopreserved in a dimethyl sulfoxide-FBS solution in liquid nitrogen vapor.

Flow cytometric analyses. Percentages of CD34\(^+\) cells in EDTA-treated whole blood or single-cell suspensions of BMMCs isolated from BM aspirates were determined by using a cross-reactive PE-conjugated anti-CD34 antibody (clone 563) that accurately identifies most primitive progenitor cells in macaques (31). In some experiments, intracellular Ki67 was measured by using a BD Cytofix/CytoPerm kit and a fluorescein isothiocyanate-conjugated anti-Ki67 antibody (clone B56). Isotype-matched control antibodies for each fluorochrome were always included. MCs were gated based on forward- and side-scatter characteristics and included nucleated red cells among BMMCs. All antibodies and reagents were purchased from BD Biosciences/Pharmingen (San Jose, CA) unless otherwise noted; acquisitions were performed on a BD-LSRII. Absolute numbers of CD34\(^+\) cells were calculated based on CBCs and percentages of lymphocytes in differentials for blood or on numbers of total MCs in BM, which included nucleated red cells, multiplied by their corresponding frequencies, as determined by flow cytometry.

Quantification of serum FLT3-L. Serum concentrations of FLT3-L were determined by using a cross-reactive human Quantikine ELISA kit (R&D Systems, Minneapolis, MN), according to the manufacturer’s suggested protocol; the lower limit of detection for this assay was 7 pg/ml.

Statistical analyses. All graphics and statistical tests were done by using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA). Mann-Whitney U tests and nonparametric Spearman and Wilcoxon matched-pairs tests were used where indicated; P values of <0.05 were assumed to be significant.

### FIG. 1. Frequencies (A) and absolute numbers (B) of CD34\(^+\) progenitor cells in BM aspirates of macaques inoculated with FLT3-L. The frequencies of CD34\(^+\) cells in single-cell suspensions of BMMCs were determined by flow cytometry and absolute numbers were determined by multiplying these frequencies by the number of MCs collected per ml of BM aspirate. Total numbers of BMMCs (C) are shown for comparison. Horizontal lines indicate medians. Macaque infection status: naive, 97P009 and JC2; SHIV-89.6P infected, 97P045, 98P012, and AV1C; SIVmac239 infected, 98P016, 99P032, and CT19.

### TABLE 1. Hematologic parameters of macaques inoculated with FLT3-L*  

| Treatment and animal | No. of PBMC/µl of blood | FLT3-L concn (pg/ml of serum)b | % CD34\(^+\) PBMC | No. of CD34\(^+\) cells/ml of bloodc |
|---------------------|-------------------------|-------------------------------|-------------------|-----------------------------------|
|                     | Day 0 | Peak (day) | Day 0 | Peak (day) | Day 0 | Peak (day) | Day 0 | Peak (day) |
| Naive               |       |           |       |           |       |           |       |           |
| 97P009              | 3,717 | 7,463 (8) | 170  | 1,373 (4) | 0.19  | 0.25 (12) | 7,062 | 17,165 (8) |
| JC2                 | 5,077 | 5,059 (8) | 92   | 1,139 (4) | 0.2   | 6.88 (8)  | 10,154| 348,059 (12)|
| UA2                 | 9,633 | 9,902 (8) | ND   | ND         | 0.1   | 0.21 (15) | 9,633 | 20,664 (15) |
| SHIV-89.6P infected |       |           |       |           |       |           |       |           |
| 97P045              | 2,552 | 7,080 (12) | 114  | 887 (4)   | 0.09  | 0.81 (12) | 2,297 | 57,348 (12) |
| 98P012              | 3,628 | 8,203 (4) | 24   | 770 (4)   | 0.14  | 1.39 (8)  | 5,079 | 81,496 (8)  |
| AV1C                | 2,383 | 3,202 (8) | 76   | 1,201 (4) | 0.17  | 1.77 (12) | 4,051 | 38,321 (12) |
| SIVmac239 infected  |       |           |       |           |       |           |       |           |
| 98P016              | 2,633 | 4,775 (12) | 85   | 892 (8)   | 0.13  | 0.24 (12) | 3,423 | 11,460 (12) |
| 99P032              | 2,640 | 4,738 (8) | 18   | 816 (4)   | ND    | ND         | ND    | ND         |
| CT19                | 2,970 | 7,228 (5) | 83   | 1,105 (12)| 0.13  | 0.51 (8)  | 5,171 | 34,512 (12) |
| Median              | 3,628 | 7,080 (8) | 84   | 999 (4)   | 0.135 | 0.66 (12) | 5,125 | 36,417 (12) |

*“Day 0” refers to the day of initiation of FLT3-L administration. “Peak” indicates the greatest value of the indicated parameter during the period of monitoring (relative to day 0). ND, not determined.

b Previously reported (30).

c Values were calculated by using flow cytometry and CBCs.
RESULTS AND DISCUSSION

FLT3-L administration results in a cell-specific expansion of CD34+ progenitor cells in peripheral blood but not in BM. As we previously reported (30), subcutaneous administration of FLT3-L, using a 7-day regimen, resulted in increased serum levels of the cytokine (Table 1), which peaked around day 4 postinoculation and was followed by a 2-fold (median, P = 0.0156 [Wilcoxon matched-pairs test]) increase in total PBMC (Table 1). By comparison, increases in the numbers of circulating CD34+ cells were as great as 30-fold (P = 0.0078), a change also reflected in the frequencies of CD34+ cells (P = 0.0078). These findings are similar to those of other studies in macaques that demonstrated FLT3-L-induced mobilization of CD34+ cells in the absence of large increases in lymphocytes (28). It is interesting, however, that the peak of expansion of CD34+ cells occurred on day 12, which was 5 days after FLT3-L had been discontinued (day 7) and 8 days after peak serum levels of FLT3-L (day 4); neither the frequencies nor the absolute numbers of CD34+ cells correlated with serum FLT3-L concentrations (data not shown). This result suggested that the cells might first be mobilizing in the BM prior to emigration into the periphery. Also of interest, at the beginning of the present study, numbers of circulating CD34+ cells were significantly lower in infected compared to naive animals (P = 0.0357 [Mann-Whitney U test]), but this difference appeared to have no influence on their mobilization during FLT3-L administration.

We next examined the frequencies and absolute numbers of CD34+ cells in the BM of each of the animals at various times during FLT3-L administration. Not unexpectedly, the baseline frequencies of CD34+ cells among MCs were significantly greater (P = 0.0002 [Mann-Whitney U test]) in BM (median, 6.51%) compared to peripheral blood (median, 0.135%), which is similar to frequencies previously reported for rhesus macaques (31). However, in contrast to what was observed in peripheral blood, there was no significant increase in either the frequencies (Fig. 1A) or absolute numbers (Fig. 1B) of CD34+ cells in BM aspirates during or after FLT3-L administration. Moreover, serum levels of FLT3-L did not correlate with absolute numbers of CD34+ cells in BM, which is in contrast to the linear relationships between serum FLT3-L and DC subsets in BM that we previously reported in these animals (30). Collectively, these data suggest that, during FLT3-L administration, CD34+ cells may not expand in BM prior to mobilization in blood, thus indicating that the cells in blood are likely to be FLT3-L-induced emigrants of cells already present in
BM and not newly generated CD34+ progenitor cells. Regardless, it is clear that FLT3-L administration can adequately mobilize CD34+ cells in the periphery, even in infected animals where these cell numbers are reduced, without increasing viral load, as previously described (30).

Intracellular Ki67 is downregulated in CD34+ BM cells but upregulated in bulk MCs. Since the BM is a primary reservoir for CD34+ progenitor cells in the body, we explored further the lack of increase of these cells in BM during FLT3-L administration by assessing proliferation of CD34+ progenitor cells in the BM and, more specifically, by quantifying intracellular expression of the nuclear proliferation antigen, Ki67 (Fig. 2A). Interestingly, basal levels of Ki67 in CD34+ cells were significantly greater than that of the bulk MC population (Fig. 2B) with no obvious differences in Ki67 expression between naive and SIV- or SHIV-infected animals. However, during FLT3-L administration, the frequency of CD34+ cells expressing Ki67 was reduced (Fig. 2C) and the frequency of Ki67+ CD34+ BM cells was correlated inversely with the number of CD34+ cells per ml of BM aspirate (R = −0.645, P < 0.0038) (Spearman correlation). These data suggested that, while FLT3-L can mobilize CD34+ cells in the peripheral blood, a negative regulatory mechanism exists in the BM, possibly involving an autocrine/paracrine feedback loop at the FLT3/FLT3-L axis, as was described recently for human progenitor cells in vitro (38). Similarly, Donahue et al. (11) reported that while most CD34+ in BM were in the S/G2 phase of cell division, there was little change in cell cycle in response to treatment with another growth factor, granulocyte colony-stimulating factor, despite significant expansion of CD34+ cells in blood. In contrast to what was observed in CD34+ cells, Ki67 was upregulated significantly in the bulk BMMC population (Fig. 2D), which might be a consequence of the large increases in DCs and/or monocyte precursors that we and others have found not only in BM but also in blood and mucosal and lymphoid tissues during FLT3-L administration to macaques (30, 35). Thus, it is possible that FLT3-L administration results in differentiation and subsequent proliferation of more committed precursors without inducing proliferation of more primitive CD34+ cells. Since in macaques CD38 coexpression on CD34+ cells identifies more committed cell lineages (31), it would be of interest to include an analysis of this cell surface antigen in future, more comprehensive studies.

Here we demonstrated that, whereas FLT3-L administration can be used to expand CD34+ progenitor cells in peripheral blood of both naive and SIV- or SHIV-infected macaques, it results in downregulation of proliferation of CD34+ cells in BM, leading to the hypothesis that mobilized CD34+ cells emigrate from BM to blood. Alternatively, these data could be interpreted to show that the CD34+ progenitor cell population already in the circulation proliferated, and the observed expansion in blood was not influenced by emigration of such cells from the BM. Even so, the lack of expansion and proliferation of CD34+ cells in BM during FLT3-L administration is suggestive of intense regulatory pressure to prevent hyperproliferation in this tissue.

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