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Flt3 Ligand Expands Lymphoid Progenitors Prior to Recovery of Thymopoiesis and Accelerates T Cell Reconstitution after Bone Marrow Transplantation

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Deficient thymopoiesis and retarded recovery of newly developed CD4+ T cells is one of the most important determinants of impaired immunocompetence after hemopoietic stem cell transplantation. Here we evaluated whether Fms-like tyrosine kinase 3 (Flt3) ligand (FL) alone or combined with IL-7 affects T cell recovery, thymopoiesis, and lymphoid progenitor expansion following bone marrow transplantation in immunodeficient mice. FL strongly accelerated and enhanced the recovery of peripheral T cells. An additive effect on T cell recovery was not observed after coadministration of IL-7. Lineage−sca-1+ c-kit+ Flt3+ lymphoid progenitor cell numbers were significantly increased in bone marrow of FL-treated mice before recovery of thymopoiesis. Thymocyte differentiation was advanced to more mature stages after FL treatment. Improved T cell recovery resulted in better immunocompetence against a post-bone marrow transplantation murine CMV infection. Collectively, our data suggest that FL promotes T cell recovery by enhanced thymopoiesis and by expansion of lymphoid progenitors. The Journal of Immunology, 2007, 178: 3551–3557.

Impaired T cell recovery following hemopoietic stem cell transplantation (HSCT) is currently considered to be the most important determinant of impaired immunocompetence in the late time period after HSCT (1). Especially, CD4+ T cell lymphocytopenia is associated with opportunistic infections (2). Therefore, strategies to improve T cell recovery are expected to reduce treatment-related mortality and morbidity associated with HSCT (3). T cell recovery after HSCT may occur either through thymic-dependent differentiation of bone marrow-derived progenitors into mature, naïve T cells (thymopoiesis) or by homeostatic peripheral expansion (HPE) of mature peripheral T cells infused with the graft or residual host T cells (4, 5). Thymopoiesis is considered important to generate a diverse TCR repertoire (6, 7). However, thymopoiesis is severely hampered in adult stem cell graft recipients, due to epithelial injury by chemo/radiotherapy, due to age-associated thymic involution, and by graft-vs-host disease (8, 9). IL-7 has been studied extensively in experimental bone marrow transplantation (BMT) models as a possible thymopoietic and T cell restorative agent (10–13). We and others have shown that IL-7 improves T cell recovery predominantly by peripheral expansion, whereas it marginally affects thymopoiesis (11, 13, 14). To enhance thymopoiesis, early acting cytokines such as stem cell factor and Flms-like tyrosine kinase 3 (Flt3) ligand (FL) are being explored (15). The FL receptor, flt3, is expressed on early hemopoietic progenitor cells, including lymphoid progenitors and a subset of double-negative (DN) thymocytes (16–18). FL may promote survival in vitro and expansion of Lin- sca-1+ c-kit+ (LSK) cells and lymphoid progenitors in vivo (18, 19). In addition, FL may enhance dendritic cell (DC)-driven homeostatic T cell expansion and may also improve thymopoiesis (15). Whereas expansion of DC by FL may drive peripheral T cell proliferation, the effects of FL on thymopoiesis and bone marrow lymphoid progenitors have been less well characterized. Here we show that FL accelerates T cell reconstitution and immune competence against murine CMV (mCMV). FL expanded LSKflt3+ progenitors in the bone marrow, and FL treatment resulted in enhanced thymopoiesis.

Materials and Methods

Mice

RAG-2<−/−> common cytokine γ-chain (γc)<−/−> mice on a mixed background (originally bred at the Netherlands Cancer Institute, Amsterdam, The Netherlands) were inbred on a BALB/c background and bred at the Erasmus MC Experimental Animal Center (Rotterdam, The Netherlands). RAG-2<−/−>γc<−/−> and C57BL/6-Cd45.2 RAG-1<−/−> mice (The Jackson Laboratory) were used as BMT recipients. Wild-type BALB/c or C57BL/6-Cd45.1 mice from the Erasmus breeding colony were used as respective bone marrow donors. Housing, care, and all animal experiments were done in accordance with Dutch legal regulations, which include approval by a local ethical committee.

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2 Abbreviations used in this paper: HSCT, hemopoietic stem cell transplantation; BMC, bone marrow cells; BMT, bone marrow transplantation; CLP, common lymphoid progenitor; DN, double negative; SP, single-positive; DC, dendritic cell; ETP, early T cell lineage progenitors; FL, Fms-like tyrosine kinase 3 ligand; HPE, homeostatic peripheral expansion; LSK cells, Lineage (Lin)−sca-1−c-Kit+ cells; mCMV, murine CMV; RQ-PCR, real time quantitative PCR; sTREC, signal joint TCR excision circle; TCD, T cell depleted.

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BMT
Bone marrow cells obtained from crushed femurs of donor mice were depleted of T cells by incubation with rat anti-mouse CD4 (YTS191, YTA312) and rat anti-mouse CD8 (YTS169) mAbs as described previously (11). The efficacy of T cell depletion was monitored by flow cytometry. Before transplantation, recipient animals were conditioned by 3 Gy total body irradiation (130 Cs γ source; Gammarad; Atomic Energy of Canada). Subsequently, these animals received 4 × 10^4 or 5 × 10^4 T cell-depleted (TCD) syn- or congeneic bone marrow cells (BMC) via tail vein infusion.

Cytokine administration
Recombinant human IL-7 was kindly provided by Dr. Michel Morre (Cytother). Recipient mice received IL-7 by s.c. injection at a dose of 1000 ng/injection three times a week from day 1 until the end of the experiment. Recipient human FL was kindly provided by A mengen, FL was administered s.c. from day 1 until the end of the experiments at a dose of 20 μg/mouse three times weekly.

mCMV
The Smith strain of mCMV (ATCC VR-1399) was propagated in second passage BALB/c mouse embryonic fibroblasts. Virus titers of virus stock preparations were determined by a plaque titer assay (20). Animals were infected by i.p. injection of 10^4 PFU of mCMV, a dose that was 100% lethal in untransplanted RAG-2^−/− mice.

Flow cytometric analysis
At serial time points after transplantation, absolute numbers of peripheral blood leukocytes were determined by a single-platform flow cytometric assay. mAbs used for flow cytometric analysis were FITC-conjugated anti-CD3ε, anti-CD4, and anti-CD8, anti-CD19, anti-CD45.1, anti-CD45, anti-CD25 (BD Biosciences); PE-conjugated anti-CD8, anti-CD19, anti-CD45.2, anti-Dx5, anti-CD45.1, anti-CD45.2, anti-CD4, anti-CD8, anti-Dx5, anti-CD45.2, and a biotin-conjugated lineage panel (anti-B220, anti-CD3, anti-Gr-1, anti-Mac-1, anti-Ter119; BD Biosciences); biotin-conjugated lineage panel (anti-B220, anti-CD3, anti-Gr-1, anti-Mac-1, anti-Ter119; BD Biosciences). Streptavidin-Cy, streptavidin-PE, streptavidin-allophycocyanin, or streptavidin-allophycocyanin-Cy7 (BD Biosciences) was used to detect biotinylated mAbs. Thymic DN were defined as lineage (Lin)−/CD4^+ /CD8^+ /CD45R^− cells (LSKflt3^−/−/AA4.1^−/−) in bone marrow samples were determined using previously published FACs criteria (21–23). In brief, bone marrow cells were stained with a mixture of biotin-conjugated lineage panel (BD Biosciences). Subsequently, cells were washed, and Lin− cells were visualized by streptavidin-conjugated allophycocyanin-Cy7. For LSK subsets cells were stained using FITC-conjugated anti-Sca-1, allophycocyanin-conjugated anti-c-kit and PE-conjugated anti-flt3. LSK cells were gated as Lin−/sca-1^−/−/flt3^−/− in bone marrow samples. For SP analysis, the bivariate correlation between the numbers of T, B, and NK cells in the peripheral blood and mCMV plasma viral load. Kaplan-Meier plots were used for survival after mCMV infection.

Results
FL accelerates and enhances T cell recovery after T cell-depleted BMT
To evaluate whether FL improves T cell recovery, T, B, and NK cell-deficient RAG-2^−/− mice received 3 Gy of irradiation reverse primers, 200 nM probe, and 10 μl of DNA sample. Primers and probes for the detection of mCMV were based on the mCMV glycoprotein B sequence (GenBank accession number M735191, forward primer 5′- AGGGCTTGGAGAGGACCTACA-3′, reverse primer 5′- GCCCGTGCGGACGCTAGTC-3′, and probe FAM-5′- AGCTAGACGA CGCCCAAAGAACGA-3′-TAMRA). Thermal cycling started with UNG activation for 2 min at 50°C, followed by an inactivation step of 10 min at 95°C. Thereafter, 42 cycles of amplification were run consisting of 15 s at 95°C and 1 min at 60°C. To monitor for the loss of DNA and/or inhibition, a fixed amount of internal control virus phocine herpesvirus type 1 was used throughout the whole process and quantified as described previously (26). Quantification was based on an external standard curve using mCMV, which was treated exactly like the material of interest. The mCMV glycoprotein B DNA concentration in the unknown samples was calculated using the data from the standard curve.

Statistical analysis
Statistical analysis of the data was performed using the SPSS software package (SPSS, Inc.). Differences between the different cytokine treatments were analyzed using the Mann-Whitney U test. Spearman’s ρ test was used to analyze the bivariate correlation between the numbers of T, B, and NK cells in the peripheral blood and mCMV plasma viral load. Kaplan-Meier plots were used for survival after mCMV infection.

Table I. B, NK, and DC cell recovery

<table>
<thead>
<tr>
<th>Lymphocyte Subset</th>
<th>Recovery per μl of Blood after Treatment by</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>PBS</td>
</tr>
<tr>
<td>T cells</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>2 (1–6)</td>
</tr>
<tr>
<td>28</td>
<td>1 (0–7)</td>
</tr>
<tr>
<td>B cells</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>72 (11–376)</td>
</tr>
<tr>
<td>28</td>
<td>247 (50–736)</td>
</tr>
<tr>
<td>NK cells</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>92 (60–180)</td>
</tr>
<tr>
<td>28</td>
<td>119 (68–308)</td>
</tr>
<tr>
<td>DCs</td>
<td>21</td>
</tr>
<tr>
<td>28</td>
<td>56 (32–141)</td>
</tr>
</tbody>
</table>

*Median numbers (cells per microliter of blood (range in parentheses) of T cells (CD45^+CD3^+), B cells (CD45^+CD19^+), NK cells (CD45^−CD3^−CD5^+), and DCs (CD45^−CD11c^−MHC II^+ and/or CD11c^+MHC II^+ and/or CD11c^+MHC II^+ and/or CD11c^+MHC II^+ and/or CD11c^+MHC II^+).
followed by a syngeneic TCD BMT containing $4 \times 10^4$ BMC. Mice were treated with PBS, FL, IL-7 alone, or FL in combination with IL-7 from day 1 onwards. Absolute numbers of bone marrow-derived newly developed T cells were quantified in peripheral blood at weekly intervals (Fig. 1). PBS-treated control mice showed a very slow T cell recovery. Median numbers of CD3$^+$ T cells measured 2 (range, 1–6) and 38 (range, 0–543) per $\mu$L of blood at days 21 and 56 post-BMT, respectively. In contrast, mice treated with FL showed an accelerated and enhanced recovery of CD3$^+$ T cells. Median numbers of CD3$^+$ T cells were 7 (range, 2–8) and 424 (range, 53–1364) per $\mu$L of blood at days 21 and 56 post-BMT, respectively. The combination of FL and IL-7 did not result in a further acceleration of T cell recovery as compared with FL alone. At all time points evaluated, except for day 49, no statistical difference could be demonstrated between FL and IL-7 as compared with FL alone with respect to absolute T cell numbers. Repopulation of CD4$^+$ and CD8$^+$ T cells was improved in a similar way (results not shown). In addition, B cells, NK cells, and DC repopulated faster in FL-treated mice (Table I). Splenic T cell numbers and percentages were evaluated at days 29 and 64 after BMT. FL-treated mice showed both higher percentages and absolute CD3$^+$ T cell numbers as compared with PBS- or IL-7-treated mice. Addition of IL-7 to FL did not exert an additive effect (results not shown).

**FL affects the distribution of thymocyte subsets**

We next addressed the question to what extent administration of FL affects thymopoiesis. RAG-1$^{−/−}$ mice irradiated with 3 Gy received a TCD BMT containing $4 \times 10^4$ BMC followed by FL or PBS administration from day 1 until days 14, 21, or 28. Thymopoiesis was evaluated by flow cytometry of thymocyte subsets and RQ-PCR of sjTRECs obtained from thymi harvested at days 14, 21, or 28 post-BMT. Thymic cellularity, numbers of donor-derived thymocytes, and numbers of sjTREC-positive thymocytes per thymus were comparable between FL- and PBS-treated mice at days 14 and 21. At day 28, the median total number of donor-derived cells harvested from thymi of FL-treated mice was $320 \times 10^4$.
range, 0.3–3300 × 10^4) as compared with 3.4 × 10^4 (range, 0.1–46 × 10^4) in PBS-treated mice, and the total median number of sjTREC-positive thymocytes per thymus was 39 × 10^4 (range, 0.0–392 × 10^4) and 1.6 × 10^4 (range, 0.0–7.7 × 10^4) in FL- and PBS-treated mice, respectively (Table II). At days 21 and 28, mature thymocytes were significantly more abundant in FL-treated mice. FL-treated mice showed significantly increased numbers of both intermediate single-positive (ISP) and mature SP thymocytes, whereas these mature thymocytes were nearly absent in PBS-treated mice at day 21 (Fig. 2). At day 28, FL-treated showed increased numbers of total donor-derived thymocytes, including significantly more DN and SP thymocytes (results not shown).

In addition, the relative distribution of the different DN subsets was assessed. FL-treated mice showed a shift toward more mature DN thymocytes with high percentages of DN3 and DN4 thymocytes, whereas thymi of PBS-treated mice contained predominantly DN1 thymocytes (Fig. 3). The relative distribution of the various subsets indicates that thymopoiesis is more advanced after FL treatment.

**FL increases the number of lymphoid progenitors in bone marrow**

Because the concurrent accelerated T, B, and NK cell recovery and the more advanced thymopoiesis in FL-treated mice suggested a prethymic effect of FL, we next evaluated whether FL expands lymphoid progenitors following BMT. The current concept of lymphoid development holds that T cell development occurs via a differentiation process starting in the bone marrow with LSKflt3^- cells, containing multipotent self-renewing hemopoietic stem cells via LSKflt3^+, also known as multipotent progenitors, containing early lymphoid progenitors and lymphoid-primed multipotent progenitors. LSKflt3^- cells are the most likely candidates to migrate to the thymus to become early T lineage progenitors (ETP) or to become preferentially B cell lineage-restricted CLP in the bone marrow (19, 21, 27). Total body-irradiated (3 Gy) RAG-1^-/- mice...
received a syngeneic TCD BMT containing $4 \times 10^4$ BMC followed by FL or PBS administration from day 1 to day 28. Bone marrow was harvested at days 14, 21, and 28, and bone marrow progenitors were quantified by flow cytometry. The results are shown in Fig. 4. LSKflt3$^+$ cells in PBS-treated mice expanded in time after BMT, and FL treatment resulted in an increased expansion at all time points evaluated. CLP also expanded in time following BMT in PBS-treated mice and FL resulted in increased CLP cell numbers at day 28 only. No effect was observed on LSKflt3$^+$ cells. Collectively, these data indicate that FL expands BM progenitors with lymphoid differentiation potential.

As the limiting factor determining T cell recovery in transplants with low numbers of BMC may be a reduced thymic engraftment by lymphoid progenitors, we evaluated whether transplantation with higher numbers of progenitors would result in a comparable acceleration of T cell recovery as exerted by FL. T cell recovery appeared both accelerated and enhanced in mice transplanted with a higher number of BMC ($5 \times 10^6$) as compared with mice receiving a BMT containing $4 \times 10^4$ BMC. Median numbers of peripheral T cells measured 274 (range, 78–1826)/μl blood vs 85 (range, 1–275)/μl blood for FL- and PBS-treated mice, respectively. Both CD4$^+$ and CD8$^+$

Table III. Peripheral blood T cell subsets in mCMV-infected mice

<table>
<thead>
<tr>
<th>Mice Treated with</th>
<th>PBS</th>
<th>FL</th>
<th>Fold Increase</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3$^+$</td>
<td>85 (1–275)</td>
<td>274 (78–1826)</td>
<td>3.2</td>
<td>0.08</td>
</tr>
<tr>
<td>CD4$^+$</td>
<td>64 (1–189)</td>
<td>195 (93–1227)</td>
<td>3.0</td>
<td>0.05</td>
</tr>
<tr>
<td>CD8$^+$</td>
<td>20 (1–87)</td>
<td>91 (18–577)</td>
<td>4.6</td>
<td>0.05</td>
</tr>
</tbody>
</table>

$^a$ Median numbers of T cell subsets 48 days post-BMT (cells per microliter of blood; range in parentheses) are shown of PBS ($n = 5$)- or FL-treated ($n = 5$) RAG-2$^{−/−}$ γc$^{−/−}$ BMT recipients infected with mCMV.
T cell numbers were higher in FL-treated mice (Table III). A significant inverse correlation between absolute T cell numbers and plasma viral load was observed at days 42 and 48 post-BMT ($R = -0.727, p < 0.001; R = -0.821, p < 0.001$).

**Discussion**

Recovery of naive T cells may be severely impaired in recipients of hematopoietic stem cell grafts (1, 5). Because low numbers of T cells after HSCT are associated with opportunistic infections, approaches to improve T cell recovery may be of clinical importance. We and others observed that the potential thymopoietic cytokine IL-7 preferentially affects HPE of T cells (11, 13, 14). In the present study, we evaluated whether administration of FL after experimental BMT may affect T cell recovery, thymopoiesis, lymphoid progenitor expansion, and immunocompetence. Here, we show that T, B, and NK recovery was significantly accelerated and enhanced in FL-treated mice. A significant expansion of especially LSKflt3 progenitors was observed in the bone marrow of FL-treated mice before recovery of thymopoiesis, suggesting that FL may improve T cell recovery by improving thymopoiesis and by effects exerted at the bone marrow level.

The receptor for FL, flt3, is highly expressed on hematopoietic progenitor cells as well as on mature DCs in thymus, spleen, and epidermis (16–19, 28). Upon FL administration in various murine models, flt3 progenitor cells, their progeny, and flt3 DCs are expanded (18, 29). Recent studies have characterized LSKflt3 progenitor cells as a subset with lymphomyeloid differentiating potential and loss of self-renewal capacity (22, 30). Especially, the LSKCD34 subset, also called multipotent progenitors, was associated with reconstitution of lymphopoiesis (19). Collectively, these observations have suggested that FL may be an important cytokine to be applied in immunodeficiencies characterized by severe T cell depletion. Increased thymic output as well as HPE by FL was recently demonstrated in an experimental murine BMT model (15). Although both DC-driven HPE and a higher thymic output may explain enhanced peripheral blood T cell numbers, an earlier recovery of T cells in FL-treated mice, as observed in the present study is less likely explained by HPE. Several findings suggest that FL-mediated acceleration of T cell recovery in our study may also result from expansion of lymphoid progenitors before thymic seeding. First, before recovery thymopoiesis, we observed expansion of LSKflt3 progenitors by FL. Second, apart from an accelerated and enhanced T cell recovery, also NK cells and B cells recovered more rapidly in FL-treated mice, suggesting an effect exerted at the level of a common progenitor with lymphoid potential. Third, higher percentages of more mature DN3 and DN4 thymocytes and higher numbers of all donor-derived thymocyte subsets were observed in FL-treated mice. In addition, mature SP thymocytes could be detected in FL-treated mice, whereas these were nearly absent in PBS-treated control mice. Fourth, transplantation of mice using grafts with a higher number of progenitor cells resulted in a comparable acceleration of lymphoid reconstitution as in mice receiving grafts with a low numbers of BMC followed by FL treatment. Such an earlier presence of mature thymocytes may suggest that thymopoiesis is affected at a very early stage or that thymic seeding has occurred earlier. Our explanation is supported by recent findings of Sambandam et al. (31) showing that FL mice had normal numbers of bone marrow and blood LSK progenitors but decreased numbers of ETP and DN2 thymocytes, suggesting that thymic seeding or expansion of the earliest thymocytes is critically dependent on FL-mediated signaling. However, at present we cannot directly determine whether FL improves thymic seeding and/or directly stimulates the ETPs in vivo, given that no discernible assay for thymic seeding is available.

Transplantation of grafts with limited numbers of progenitors may occur clinically in adult recipients of cord blood or unrelated donor marrow (32, 33). Such transplants are frequently complicated by a retarded recovery of T cells and high incidence of opportunistic infections (34–37). FL could be envisaged to have a role in such conditions by expansion of lymphoid progenitors.

The results of the FL experiments show improved protection against an in vivo challenge of mCMV. Opportunistic mCMV has extensively been studied and has provided important insights into major determinants of antigenicity on one hand and protective immunity on the other hand (38, 39). Although Abs and NK cells may limit the dissemination of viral infection (40–42), the cytotoxic CD8+ and CD4+ helper T cell responses have been demonstrated as pivotal for viral clearance and prevention of recurrent infection and lethality (42, 43). Indeed, in the experiments reported here, FL conferred enhanced immunocompetence toward a mCMV infection. FL prevented mCMV-associated mortality, especially in those mice that effectively recovered their T cell compartments. By using both RQ-PCR and flow cytometry, we were able to monitor and correlate viral load and immune recovery in individual mice. All mice that effectively recovered their T cell compartments between days 28 and 56 were able to clear the virus and survived, whereas mice with insufficient recovery at these time points showed a continuing increase of viral load. An inverse correlation of viral load and T cell numbers was observed, emphasizing the well-established role of T cell-mediated immunity against a CMV infection (42, 43). FL enhanced both CD8+ and CD4+ T cell numbers. Given the importance of CD8+ T cells, clinical studies have been performed using adoptive transfer of CMV-specific CD8+ T cells that were shown to revert CMV reactivation, but CD4+ T cells appeared necessary for sustained protection (44). To provide a sustained cytotoxic and helper T cell response, the expansion of progenitor cells, as reported in the present study or the adoptive transfer of lymphoid progenitors (45) might be preferred. The importance of a sustained T cell response is underscored by the observation that recurrent CMV reactivation is still a major complication after HSCT associated with considerable morbidity and mortality (46).

In conclusion, expansion of LSKflt3 progenitor cells and improved thymopoiesis by FL improves T cell recovery and immune competence after transplantation with a low number of BMC. These results may provide a rationale for clinical studies in recipients of HSCT with retarded T cell recovery, mainly due to transplantation of limited numbers of progenitor cells.

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**Disclosures**

The authors have no financial conflict of interest.

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