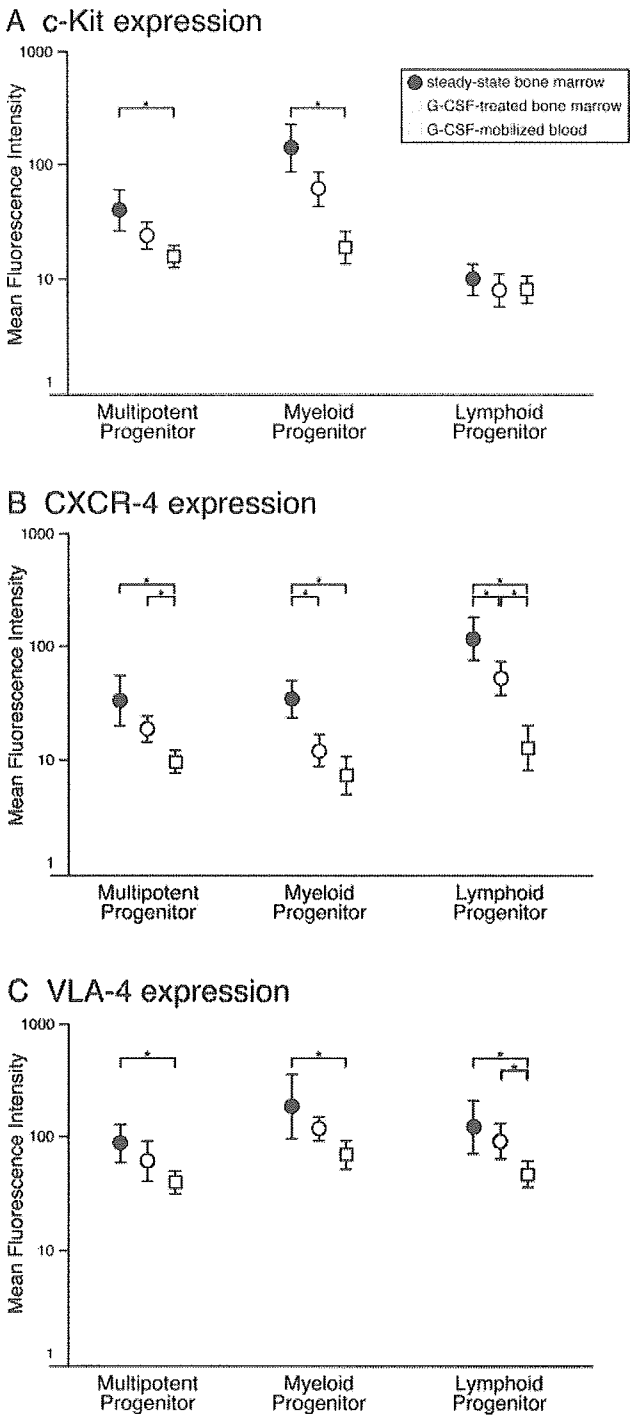


**FIGURE 2.** Comparative expression of c-Kit (*A*), VLA-4 (*B*), and CXCR-4 (*C*) on $CD34^+CD38^{-/low}Lin^-$ multipotent progenitors, $CD34^+CD38^+CD13^+$ myeloid progenitors, and $CD34^+CD38^+CD10^+$ lymphoid progenitors in the steady-state BM (●), G-CSF-treated BM (○), and G-CSF-mobilized PB (□). Circles and boxes indicate median MFI for these molecules among progenitors, and bars show SD. *, $p < 0.05$, significantly different compared with each tissue.

CXCR-4 and VLA-4 expression for all three progenitor types in BM declined after G-CSF administration; further decrease was observed in the G-CSF-mobilized progenitors. These results suggest that adhesion molecule expression on HPC in BM decreased during G-CSF administration in a lineage-independent manner. As a result, a fraction of cells with lesser expression of these molecules may be mobilized into the circulation.

*Mobilized lymphoid cells are restricted to B/NK cell lineage*

To test the differentiation capacity of mobilized cells possessing immature lymphoid phenotypes, doubly sorted $CD34^+CD10^+CD19^-$ and $CD34^+CD10^+CD19^+$ cells were cultured on methylcellulose with IL-7, SCF, IL-11, IL-3, GM-CSF, erythropoietin, thrombopoietin, and FL. One hundred $CD34^+Lin^-$ or $CD34^+CD38^+Lin^-$ cells purified from BM and G-CSF-mobilized PB gave rise to a variety of colonies including all types of myeloid lineages (13). In contrast, 500 $CD34^+CD10^+CD19^-$ and $CD34^+CD10^+CD19^+$ cells sorted from the steady-state BM and G-CSF-mobilized PB did not form any colonies after 14 days of culture under this condition (data not shown).

After 4 wk of culture on MS-5 or Sys-1 stromal layers in the presence of IL-7, SCF, IL-11, IL-3, IL-2, GM-CSF, and FL, BM $CD34^+Lin^-$ cells differentiated into $CD15^+$ myeloid cells, $CD19^+$ B lymphoid cells, and $CD56^+$ NK cells (Fig. 3, *A* and *B*). In contrast, early B cells sorted from BM and G-CSF-mobilized PB gave rise to $CD10^+CD19^+$ pro-B cells and $CD10^-CD19^+$ pre-B cells as well as NK cells but not myeloid cells (Fig. 3, *C*, *D*, *G*, and *H*). Pro-B cells from BM and G-CSF-mobilized PB also formed $CD19^+$ B cell-containing colonies, but neither myeloid nor NK cells were detected in cultured cells (Fig. 3, *E*, *F*, *I*, and *J*). Production of T cells was not observed in any of these cultures (data not shown).

Six to 8 wk after transplantation of FACS-sorted progenitors into irradiated NOD/SCID/$\beta2^{-/-}$ mice, animals were sacrificed to assess reconstitution of human hematopoiesis. FACS analysis of spleen and BM cells showed the presence of human $CD45^+$ cells in all mice transplanted with 50,000 $CD34^+Lin^-$ cells, $CD34^+CD38^+CD10^-CD19^+Lin^-$ pro-B cells, and $CD34^+CD38^+CD10^+Lin^-$ cells (including early B and pro-B cells). Strikingly, mice transplanted with $CD34^+CD38^+CD10^+Lin^-$ cells exhibited massive splenomegaly (5- to 10-fold enlargement) in contrast to mice receiving $CD34^+Lin^-$ cells, indicating that lineage-committed B lymphoid progenitors might proliferate rapidly in the spleen. In mice receiving human $CD34^+Lin^-$ cells, most $CD45^+$ cells in the spleen and BM were positive for CD19 (68 ± 7% and 45 ± 14%, respectively), but $CD45^+CD15^+$ and $CD45^+CD56^+$ cells also were found in both spleen and BM; thus, human $CD34^+Lin^-$ cells could differentiate into myeloid cells, B cells, and NK cells in these mice (Fig. 4*A*). In contrast, mice transplanted with 50,000 pro-B cells sorted from BM or G-CSF-mobilized PB exhibited only B lymphoid reconstitution (Fig. 4*B*). However, human $CD45^+$ cells could not be detected in animals transplanted with up to 3000 $CD34^+CD38^+CD10^+CD19^-Lin^-$ early B cells: the number of injected early B cells might not be sufficient to engraft mice. Because early B cell population was too tiny to sort cells enough for engraftment in xenogeneic hosts, we tested the differentiation potential of early B cells in vivo by injecting $CD34^+CD38^+CD10^+Lin^-$ cells, containing both $CD19^-$ early B and $CD19^+$ pro-B cells. Mice transplanted with $CD34^+CD38^+CD10^+Lin^-$ cells sorted from BM or G-CSF-mobilized PB reconstituted both $CD19^+CD56^-CD15^-$ B cells and $CD19^-CD56^+CD15^-$ NK cells in the BM and spleen (Fig. 4*C*). These in vitro and in vivo data revealed that G-CSF-mobilized $CD34^+CD38^+CD10^+CD19^+$ and $CD34^+CD38^+CD10^-CD19^+$ cells can rapidly differentiate in a B/NK and B lineage-restricted manner, which represents the same functional properties as in their BM counterparts, early B and pro-B cells.

*Lymphoid progenitors have no self-renewing capacity*

To test limited self-renewal activity, BM and G-CSF-mobilized early B and pro-B cells were plated in limiting dilution in LTC-IC assays and transferred to methylcellulose after 6 wk of culture as described previously (13). The estimated frequency of LTC-IC

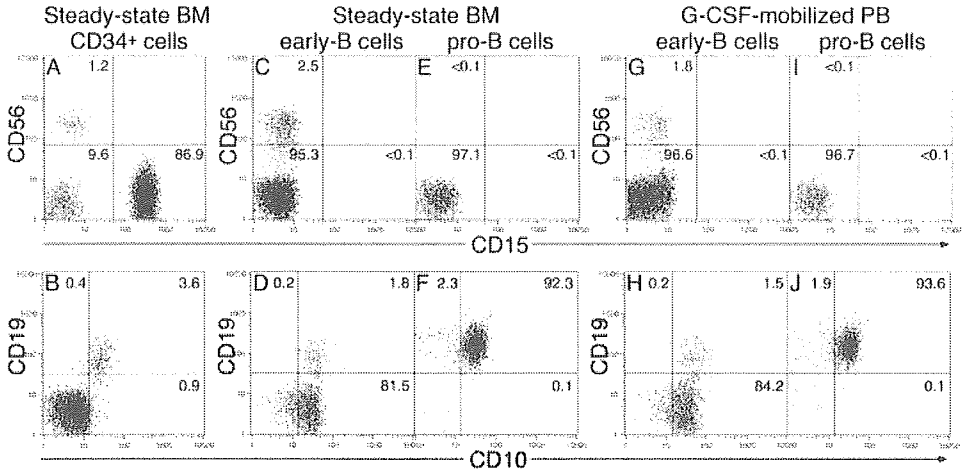


**FIGURE 3.** Differentiation potential of FACS-sorted progenitors in stroma-supported cultures. Cells cocultured on MS-5 and Sys-1 stromal cell layers with IL-7, SCF, IL-11, IL-3, IL-2, GM-CSF, and FL were harvested after 28 days, and FACS analysis was gated for viable $CD45^+$ cells. $CD34^+Lin^-$ BM cells differentiated into $CD15^+$ myeloid, $CD56^+$ NK cells (*A*), and $CD10^+CD19^-$ early B and $CD10^+CD19^+$ pro-B cells (*B*). Steady-state BM early B and pro-B cells gave rise to more differentiated lymphoid-restricted cells such as $CD10^+CD19^+$ pro-B and $CD10^-CD19^+$ pre-B cells and $CD56^+$ NK cells, but not myeloid cells (*C–F*). Similarly, $CD10^+CD19^-$ and $CD10^+CD19^+$ cells sorted from G-CSF-mobilized PB possessed B lymphoid-restricted differentiation potential in the stromal cell cultures (*G–J*).

was 1 in 20 for $CD34^+CD38^-Lin^-$ cells, but no LTC-IC activity was detected in early B and pro-B cells from either BM or G-CSF-mobilized PB (data not shown).

*Differentiation potential of early B cells in limiting dilution analysis*

We next evaluated B cell differentiation capacity of BM and G-CSF-mobilized early B cells at limiting dilution in culture on MS-5 stromal cell layers, which can support differentiation of B lymphoid progenitors from human $CD34^+$ cells (15). As shown in Fig. 3, early B cells were capable of differentiation into $CD10^+CD19^+$ pro-B cells and $CD10^-CD19^+$ pre-B cells after 4 wk of culture under this condition. In a limiting dilution assay, we estimated that one in eight BM early B cells and one in 10 G-CSF-mobilized early B cells could read out B cell differentiation in this culture condition (Fig. 5). Frequency of B cell development did not differ significantly between BM and G-CSF-mobilized early B cells.

*Mobilized lymphoid progenitors show lineage-specific gene expression profiles*

We tested expression of several genes in early B and pro-B cells sorted from the G-CSF-mobilized PB and BM by RT-PCR. Lymphoid lineage-specific genes such as IL-7R, TdT, Pax-5, and VpreB were expressed in early B and pro-B cells from both BM and G-CSF-mobilized PB (Fig. 6). Granulocyte lineage-related genes including G-CSFR, GM-CSF receptor, and myeloperoxidase were not detected in early B or pro-B cells from either BM or G-CSF-mobilized PB (Fig. 6).

*IgH rearrangement in G-CSF-mobilized B lymphoid progenitors*

We examined the IgH rearrangement status in the mobilized B lymphoid progenitors, because Ig genes rearrangement status reflect well the differential stages in the B cell development pathway. In general, along with B cell differentiation pathway, Ig genes rearrangements proceed from $DJ_H$ at the early B cells or CLP stage through $VDJ_H$ at the pro-B cells stage and to L chain gene at the pre-B cells stage (17, 20, 23, 24).

In our experiments, $DJ_H$ and $VDJ_H$ rearrangements were undetectable in the most immature $CD34^+CD38^-Lin^-$ cells sorted from both BM and the G-CSF-mobilized PBPC. In contrast, a ladder of $DJ_H$ rearrangement bands ranging from 70 bp to 100 bp was observed in BM and G-CSF-mobilized $CD34^+CD10^+CD19^-$ early B cells (Fig. 7). $VDJ_H$ and $DJ_H$ rearrangements were detected in both BM and G-CSF-mobilized $CD34^+CD10^+CD19^+$ pro-B cells. The mobilized B lymphoid progenitors undergo IgH gene rearrangements in parallel with their BM counterparts.

## Discussion

In this study, using multicolor flow cytometry, we demonstrated that administration of G-CSF to human subjects induced mobilization into PB of tiny but significant cell populations possessing the same immature lymphoid phenotypes as those of B/NK and B lymphoid-committed progenitors that are well defined in the BM. These populations are phenotypically identified as $CD34^+CD10^+CD19^-Lin^-$ early B cells (or CLP) and $CD34^+CD10^+CD19^+Lin^-$ pro-B cells and do not circulate in PB under steady-state condition (14, 20). Importantly, however, a phenotypically defined population of mobilized blood cells may not necessarily have the same functional properties as its BM counterpart. Mobilized $CD34^+CD10^+CD19^-Lin^-$ cells and $CD34^+CD10^+CD19^+Lin^-$ cells had prominent B/NK and B lymphoid differentiation potential in vivo and in vitro, respectively. Neither of these two populations exhibited self-renewal or LTC-IC capacity, indicating that the mobilized cells had characteristics of B/NK and B lineage-committed progenitors. In addition, in a limiting dilution assay, the mobilized cells had a differentiation potential for B/NK lymphoid lineage equivalent to that of BM early B cells. Furthermore, mobilized B lymphoid progenitors expressed only B lymphoid lineage-affiliated genes, with no expression of myeloid-lineage restricted genes including G-CSFR. IgH genes rearrangements were detected in early B cells and pro-B cells sorted from the G-CSF-mobilized PBPC, respectively. Collectively, these results demonstrate that a phenotypically defined lymphoid progenitor in the G-CSF-mobilized PB had the same functional properties as its BM counterpart, indicating that BM-resident B lymphoid progenitors could be released into the periphery by administration of G-CSF.

HPC express various molecules such as VLA-4/VCAM-1, SDF-1/CXCR-4, and Kit-ligand, and anchor to the BM through adhesive contact with their respective ligands in the BM microenvironment. Several studies have demonstrated that following G-CSF

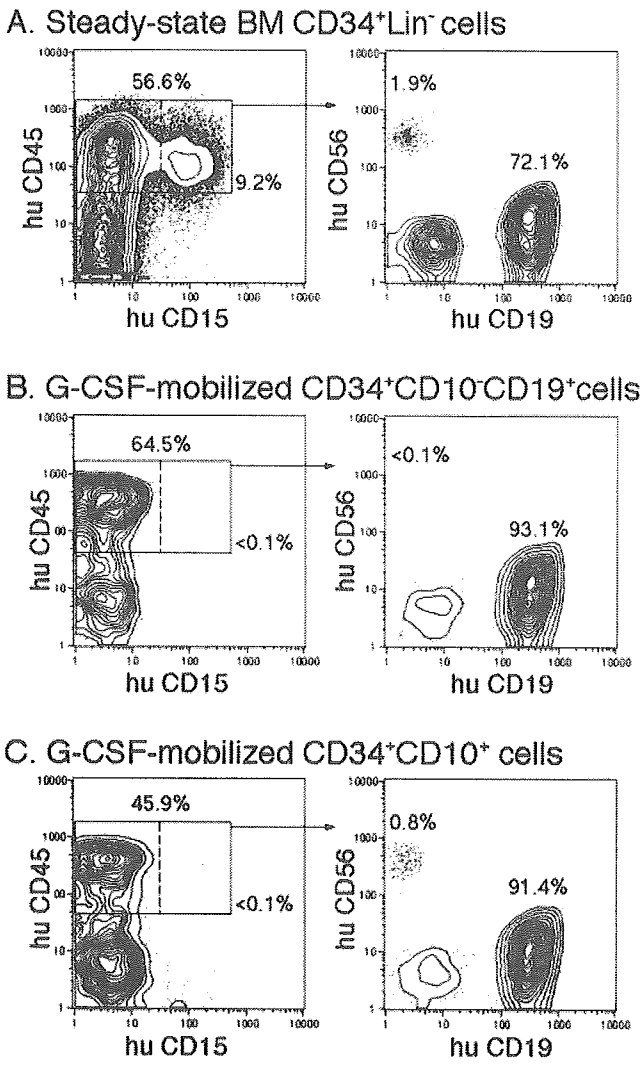


**FIGURE 4.** Engraftment and reconstitution potential of human different progenitors in NOD/SCID/$\beta 2^{-/-}$ mice. Six to 8 wk after i.v. injection of 50,000 $CD34^+Lin^-$ cells, $CD34^+CD38^+CD10^-CD19^+Lin^-$ pro-B cells, and $CD34^+CD38^+CD10^+Lin^-$ cells (including early B and pro-B cells) into irradiated mice, analysis was gated for viable human $CD45^+$ cells. Human $CD15^+$ myeloid, $CD56^+$ NK, and $CD19^+$ B cells were detected in the mice transplanted with BM $CD34^+Lin^-$ cells (*A*). In contrast, only B cells were generated in mice transplanted with G-CSF-mobilized $CD34^+CD38^+CD10^-CD19^+$ cells (*B*). Mice transplanted with $CD34^+CD38^+CD10^+Lin^-$ cells reconstituted both B and NK cells (*C*). Representative analyses of mice BM are shown.

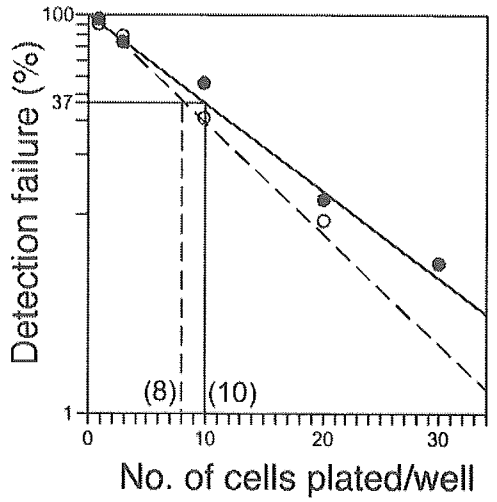


**FIGURE 5.** Limiting dilution analysis of early B cells from steady-state BM and G-CSF-mobilized PB. $CD34^+CD38^+CD10^+CD19^-$ cells plated by limiting dilution on MS-5 stromal layers were cultured in the presence of IL-7, IL-2, IL-11, SCF, and FL for 28 days. The differentiation potential of cells in individual wells was determined as $CD19^+$ mature B cells as shown in Fig. 3, *D* and *H*. We estimated that one in eight BM early B cells (○) and one in 10 G-CSF-mobilized $CD10^+CD19^-$ cells (●) could undergo B cell differentiation under this condition, indicating that G-CSF-mobilized $CD10^+CD19^-$ cells possess the same B cell differentiation potential as early B cells from BM. Numbers in parentheses represent limiting numbers.

administration, activated neutrophils and monocytes release proteolytic enzymes such as neutrophil elastase, cathepsin G, and MMP-9, which cleave and/or inactivate adhesion molecules expressed on the HPC (25–31). In fact, decreased expression of VLA-4 (32–35), CXCR-4 (28, 36), and c-Kit (30, 37, 38) on mobilized HPC has been reported during G-CSF administration in humans. Altered expression of adhesion molecules and consequent modification of their adhesion capacity might lead to release and migration of HPC into the circulation (7–11). Our study showed decreased expression of VLA-4 and CXCR-4 on circulating progenitors including MPP, myeloid, and lymphoid progenitors following G-CSF administration compared with that of BM, suggesting possible involvement of adhesion molecules in mobilization of at least three different types of immature progenitors. However, the extent to which each of these molecules contributed to mobilization of each hemopoietic lineage (11), or whether specific adhesion molecules are modulated in a lineage-dependent fashion, was not clear. Among the adhesion molecules that we examined, a dramatic decrease was observed in CXCR-4 expression on the lymphoid progenitors mobilized by G-CSF administration. SDF-1/CXCR-4 interactions also are involved in B lymphopoiesis, as substantiated by studies in CXCR-4-deficient mice that demonstrated reduced numbers of B lymphoid progenitors in the BM but abnormally high numbers of B lymphoid progenitors as well as the presence of mature B cells in blood and spleen (22, 39, 40). This suggests that CXCR-4 is required to retain B lymphoid progenitors within BM microenvironment for further maturation, as opposed to direct signaling to promote B cell development. These results agree with our findings that despite reduced expression in CXCR-4, the same differentiation capacity was preserved in mobilized lymphoid progenitors as their counterparts had in the steady-state BM. Thus, a decrease in CXCR-4 expression could be induced in a lineage-independent fashion following G-CSF, with resulting modulations contributing to migration of HPC without loss or alteration of differentiation capacity.

Importantly, hemopoietic growth factors can affect growth and/or properties of hemopoietic progenitors and cells. G-CSF has been characterized as a pivotal cytokine in proliferation, maturation, and survival in the myeloid lineage development pathway. Thus, G-CSF may affect potential or manifest characteristics of HPC during G-CSF mobilization, and HPC may have different abilities to develop and function. For example, mobilized $CD34^+$ cells have been reported to show decreased cell cycling compared with their BM counterparts (38, 41). In addition, numerous recent studies have demonstrated differentiation plasticity of committed progenitors, suggesting that hemopoietic progenitors retain a latent *trans* differentiation potential making them susceptible to diversion from their developmental fate (42–46). These observations suggest that circulating B lymphoid progenitors exposed to extremely high concentrations of G-CSF might show a different potential

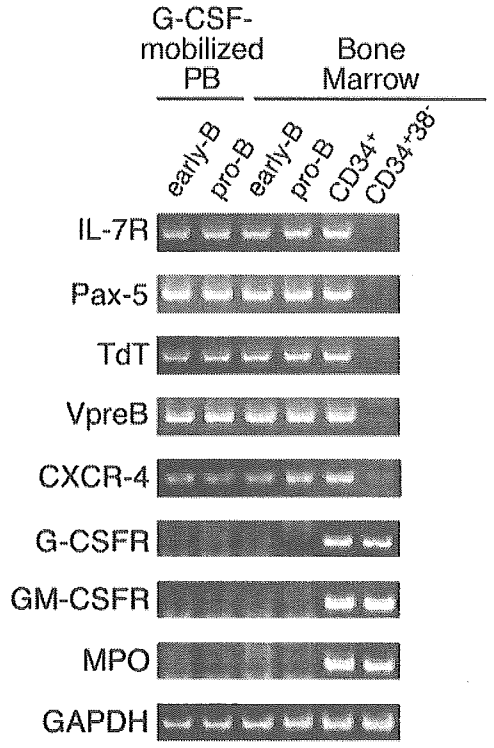


**FIGURE 6.** Differential expression of hematopoiesis-affiliated genes in BM and G-CSF-mobilized lymphoid progenitors as shown by RT-PCR analysis. BM $CD34^+$ cells were used as controls. In both BM and G-CSF-mobilized PB, lymphoid progenitors expressed lymphoid-affiliated genes but not myeloid-affiliated genes. MPO, myeloperoxidase; GM-CSFR, GM-CSF receptor.

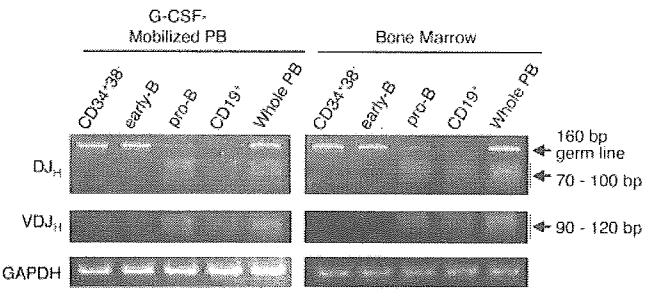


**FIGURE 7.** PCR analysis of $DJ_H$ and $VDJ_H$ genes rearrangement on DNA from BM and G-CSF-mobilized lymphoid progenitors. $CD34^-CD19^+$ cells were used as controls. None of the Ig genes rearrangements was observed in $CD34^+CD38^-$ cells. Partial $DJ_H$ rearrangement initiated at the stage of $CD34^+CD10^+CD19^-$ early B cells followed by the rearrangement of $VDJ_H$ genes at $CD34^+CD10^+CD19^+$ pro-B cells along with B cell development pathway. G-CSF-mobilized B lymphoid progenitor displayed the same pattern of Ig rearrangement status as its BM counterpart.

than their BM counterparts under physiological conditions, or might be *trans*-differentiated from other lineages. In our analysis, expression of B lineage-specific differentiation programs was preserved, and no myeloid genes were activated in G-CSF-mobilized lymphoid progenitors. By limiting dilution assay, we also demonstrated that the B cell differentiation potential of G-CSF-mobilized lymphoid progenitors was equivalent to that of their BM counterparts. Thus, G-CSF can mobilize B lymphoid progenitors without loss or alteration of the original characteristics of B lymphoid progenitors in BM. For that reason, B lymphoid progenitors, as opposed to all $CD34^+$ cells or myeloid cells, represent a good population for analysis of mechanisms of G-CSF-induced mobilization, because, lacking the receptor, lymphoid progenitors would be less affected by G-CSF signals during mobilization.

Recent insights have increased understanding of the important role of the BM microenvironment, or niche, in retention and development of HPC within the BM. Regulation of cell-fate determination and trafficking of the primitive HPC may be governed by complex interactions between HPC and the surrounding BM niche (47, 48). As discussed above, SDF-1/CXCR-4 signaling is crucial for retention of B lymphoid progenitors within the BM, which can support further B cell development within the BM microenvironment (22, 39, 40). However, whether G-CSF can change BM microenvironments themselves to promote or inhibit HPC mobilization remains largely unknown. Our findings indicated that G-CSF can mobilize cell populations that do not possess G-CSFR from the BM into the circulation. Accordingly, G-CSF-mobilized blood cells can include a variety of populations such as mesenchymal stem and progenitor cells, which can differentiate into nonhematopoietic cells such as vascular endothelial cells, cardiac muscle cells, and hepatocytes. Such mobilized blood cells conceivably might serve as a therapeutic agent in the treatment of various degenerative disorders as opposed to BM cells as a stem cell source (49). Up to now, G-CSF has been the HPC mobilizer of choice in clinical settings, based upon its potency and safety. However, poor mobilization has been reported in ~10–20% of healthy donors, representing a major problem (50, 51). To address these unresolved issues, further investigation of mechanisms of G-CSF-induced mobilization may lead to more effective and safer mobilization methods and agents, and clarify the usefulness of G-CSF-mobilized PB cells as an alternative source of a variety of cells for regenerative medicine.

In summary, our data provide further evidence for an indirect effect of G-CSF on human HPC mobilization by demonstrating mobilization of lymphoid progenitors. Lineage-independent modulation of adhesion molecules such as VLA-4 and CXCR-4 might be involved in G-CSF-induced mobilization. These findings suggest that G-CSF can mobilize not only HPC but also nonhematopoietic cells residing in the BM by indirect effects involving multiple *trans*-acting signals that affect cell interactions with the marrow microenvironment.

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## Disclosures

The authors have no financial conflict of interest.

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# The Immunosuppressive Agent FK506 Enhances the Cytolytic Activity of Inhibitory Natural Killer Cell Receptor (CD94/NKG2A)-Expressing CD8 T Cells

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Tacrolimus (FK506) is a potent immunosuppressive agent that inhibit transcription of cytokines such as IL-2 in T cells. The C-type lectin superfamily inhibitory NKR CD94/NKG2A-expressing cells can monitor the global status of HLA class I on tumor and leukemic cells through the recognition of HLA-E and induce cytolytic attack without an inhibitory signal against HLA class I-decreased target cells. We found that there was no effect of FK506 on the expansion of inhibitory NKR CD94/NKG2A-expressing T cells from granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood mononuclear cells (G-PBMCs). However, cytolytic activity levels of purified CD94-expressing cells from 7-day cultures with FK506 were much higher than those from 7-day cultures without FK506. These data suggested that FK506 did not inhibit cytolytic activities of inhibitory NKR-expressing T cells and that there was a possibility of cytolytic activities being enhanced through the induction of cytotoxic molecules such as NKG2D and granzyme during a seven-day culture with FK506.

**Keywords:** FK506, CTL, Natural killer cell, GVL.

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Inhibitory natural killer cell receptors (NKRs) on not only NK cells but also T cells negatively regulate NK cell and T cell functions through their binding to MHC class I molecules (*1–6*). The C-type lectin superfamily inhibitory NKR CD94/NKG2A-expressing cells can monitor the global status of HLA class I on tumor and leukemic cells through the recognition of HLA-E and induce cytolytic attack without an inhibitory signal against HLA class I-decreased target cells (*7, 8*). In our previous studies, we were able to expand CD94/NKG2A-expressing $CD8^+$ cells with HLA-unrestricted cytolytic activity from granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood mononuclear cells (G-PBMCs) and other blood mononuclear cells after stimulation with immobilized anti-CD3 mAb and IL-15 (*9, 10*).

The mechanisms by which different immunosuppressive drugs interfere with T cell activation and proliferation are diverse (*11*) and interfere with NK cells and NK-like cells are to some extent not well defined. Tacrolimus (FK506) is a potent immunosuppressive agent that binds to FK506-binding protein and then inhibits the activity of serine/threonine phosphatase calcineurin, resulting, in prevention of transcription of cytokines such as IL-2 in T cells (*12*). In a clinical setting, the combination of tacrolimus and MTX after unrelated donor marrow transplantation significantly decreased the risk of acute GVHD compared to the effect of the combination of cyclosporine (CSP) and MTX, with no significant increase in toxicity, infection, or leukemia relapse (*13, 14*). In an in vitro setting, it has been shown that FK506 did not inhibit NK cell killing activities and ADCC activities (*15*) and also that FK506 did not impair lymphokine-activated killer (LAK) cytotoxicity (*16*). Furthermore, there were calcineurin-dependent and independent TCR-mediated granule exocytosis pathways in CTL clones (*17*). In this study, we analyzed the effect of FK506 on the cytolytic activity of inhibitory NKR-expressing T cells expanded by stimulation with immobilized anti-CD3 mAb and IL-15.

Peripheral blood stem cell donors were administered rhG-CSF (Lenograstim, 1.2 million units (MU)/ 10 μg, Chugai or Filgrastim, 1 MU/ 10 μg, Kirin-Sankyo, Japan) by subcutaneous injection at a dose of 10 μg/kg once daily for 4 to 5 days. Leukapheresis was performed from day 4 of rhG-CSF administration, and G-PBMCs were obtained from the first leukapheresis. G-PBMCs ($1\times10^6$/ ml) were cultured with immobilized anti-CD3 mAb (OKT3, 1 μg/ml, Ortho Biotech, Raritan, NJ) and IL-15 (5 ng/ml, R & D Systems, Minneapolis, MN) in RPMI-1640 with 5% of human AB serum in T25 culture flasks for 7 days, and purified $CD94^+$ cells were obtained by magnetic cell sorting (MACS) using magnetic microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) as described previously (*9*). The phycoerythrin (PE)-conjugated monoclonal antibody (mAb) HP-3D9 (anti-CD94) was obtained from Ancell (Bayport, MN, USA), and Z199 (anti-NKG2A), ON72 (anti-NKG2D) and C1.7 (anti-CD244) were obtained from Immunotec (Marseilles, France). FITC-conjugated anti-CD3 and anti-CD8 mAb were purchased from Pharmingen (San Diego, CA), and anti-CD56 mAb and anti-granzyme A mAb were obtained from Becton Dickinson (BD, San Jose, CA). Anti-CD3 mAb OKT3 was obtained from Or-

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**TABLE 1.** Proportions and absolute numbers of CD94/NKG2A-expressing cells before and after stimulation

| | $CD94^+/CD3^+$ | $CD94^+/CD8^+$ | $NKG2A^+/CD3^+$ | $NKG2A^+/CD8^+$ |
|---|---|---|---|---|
| Before | 2.2±0.7 | 1.1±0.7 | 0.7±0.3 | 0.2±0.1 |
| | 0.088±0.029 | 0.044±0.031 | 0.029±0.011 | 0.0086±0.0053 |
| IL15 | 32.8±13.2 | 30.0±13.5 | 21.3±9.6 | 20.1±10.4 |
| | 6.29±3.32 | 5.77±3.22 | 4.31±2.47 | 3.83±2.25 |
| IL15+FK | 34.4±10.8 | 27.7±9.6 | 18.7±7.6 | 16.8±7.7 |
| | 4.65±1.54[a] | 3.77±1.45[a] | 2.88±0.92[a] | 2.52±0.92[a] |

The upper columns show the percentages of CD94 and NKG2A-expressing cells (means ± SDs, nine separate experiments), and the lower columns show absolute numbers of cells before and after culture ($\times 10^6$). Cultures were started from $10 \times 10^6$ mononuclear cells. Slight differences were noted when the values of IL15 and IL15+FK506 were compared.

[a] $P<0.1$.

tho Biotech (Raritan, NJ). Intracellular granzyme A was stained using cytofix/cytoperm reagent according to the manufacturer's instructions (BD). The fluorescence intensity of the cells was analyzed using a FACS Calibur (BD). Statistical analysis was performed using Student's *t* test.

We found that the proportion and absolute number of CD94/NKG2A-expressing $CD3^+/CD8^+$ T cells in G-PBMCs were increased after 7 days of immobilized anti-CD3 mAb (OKT3) stimulation with IL-15 regardless the existence of FK506 (0.3 ng/ml, Tacrolimus, Fujisawa, Osaka, Japan) (Table 1). In our culture condition using immobilized OKT3 and IL-15, CD3-negative NK cells could not proliferate and OKT3 stimulation was required to induce CD94/NKG2A expression on CD8 T cells. There was a slight decrease in the absolute numbers of CD94/NKG2A–expressing T cells in G-PBMCs culture with FK506 compared with those in culture without FK506 ($P<0.1$). However, the proportions of activating NK cell receptor NKG2D-expressing $CD8^+$ T cells and intracellular granzyme A expression in CD94/NKG2A-expressing cells were much more increased in G-PBMCs cultured with FK506 (Table 2). In order to analyze the effect of FK506 on the cytolyitc activity of purified CD94-expressing cells (CD94>90%, CD8>80% and CD56<5%) using magnetic microbeads from 7-day cultures tested against $^{51}Cr$-labeled K562 cells ($5\times10^3$) by standard 4-hour $^{51}Cr$ release assays without prior sensitization, at first, we evaluated the cytolytic activity of CD94-expressing cells with or without FK506 for 4 hr culture during $^{51}Cr$ release assays and found there were no significant difference between cytolytic activity cultured with FK506 and the activity cultured without FK506 (40.7±16.2 and 42.2±16.0, seven separate experiments, effector target ratio, 10:1). However, unexpectedly, cytolytic activity levels of purified CD94-expressing cells from 7-day cultures with FK506 were much higher than those from 7-day cultures without FK506 (Table 3). Furthermore, we evaluated the cytolytic activity of CD94-expressing cells after 7 days of culture without an immunosuppressant, with cyclosporine (10 ng/ml, Novartis, Tokyo, Japan) or with FK506 using the same samples and found that there was significant enhancement of the activity when cells were cultured with FK506 (55.0±20.2, eight separate experiments; effector target ratio, 10:1) compared with the activity when cells were cultured without an immunosuppressant (43.3±17.3, $P<0.01$) and compared with the activity when cells were cultured with cyclosporine (46.5±17.0, $P<0.05$). Therefore, this effect may be specific for FK506 at least under this culture condition. These data suggested that FK506 did not inhibit cytolytic activities of inhibitory NKR-expressing T cells and that there was a possibility of cytolytic activities being enhanced through the induction of cytotoxic molecules such as NKG2D and granzyme during 7-day culture with FK506. CD94/NKG2A-expressing T cells have cytolytic activity like NK cells that is not restricted by HLA and TCR; therefore, this killing function might be calcineurin-independent and not affected by the immunosuppressive agent FK506. On the other hand, it has been reported that FK506 suppressed alloantigen-specific T cells and abrogated CTL activation in mice and also in humans (*18–20*). Therefore, FK506 can strongly inhibit antigen-specific T cell immune functions but might spare antitumor activity mediated by NK cells and also inhibitory NKR-expressing T cells. The effects of FK506 on T cells and inhibitory NKR-expressing T cells might have some roles on the regulation of graft-versus-host disease and graft-versus-leukemia/tumor effect.

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**TABLE 2.** Proportions of cytotoxic molecule-expressing cells before and after stimulation

| | $CD244^+/CD8^+$ | $NKG2D^+/CD8^+$ | $CD94^+/GR^+$ | $NKG2A^+/GR^+$ |
|---|---|---|---|---|
| Before | 4.1±3.9 | 11.6±9.6 | 5.2±1.9 | 2.7±2.6 |
| IL15 | 20.0±7.8 | 21.0±7.8 | 3.2±1.1 | 3.2±2.0 |
| IL15+FK | 25.3±11.0 | 43.6±9.8[a] | 8.8±10.4[b] | 14.2±8.0[b] |

Significant differences were noted when the values of IL15 and IL15+FK506 were compared (means ± SDs, five separate experiments).

[a] $P<0.01$.

[b] $P<0.05$.

GR, intracellular granzyme.

**TABLE 3.** Cytolytic activity of CD94-expressing cells against K562

| E:T | 5:1 | 10:1 | 20:1 |
|---|---|---|---|
| IL15 | 18.9±15.9 | 29.2±19.0 | 40.7±18.4 |
| IL15+FK | 42.1±24.9[a] | 56.1±26.1[a] | 65.2±22.4[b] |

Values indicate the specific lysis of K562 by CD94-expressing cells measured by 4 hours $^{51}$Cr release assay (means ± SDs, five separate experiments). Significant differences were noted when the values of IL15 and IL15+FK506 were compared.

[a] $P<0.05$.

[b] $P<0.01$.

E:T, effector target ratio.

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□ CASE REPORT □

# Successful Reduced-intensity Stem Cell Transplantation (RIST) for a Patient with Malignant Lymphoma and an Ileostomy

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## Abstract

**A 56-year-old man was admitted for treatment of non-Hodgkin's lymphoma (NHL). He had undergone a partial small bowel and colon resection and had ileostomy due to bowel perforation induced by chemotherapy. After the operation, his disease status was in partial remission (PR), and reduced-intensity allogeneic stem cell transplantation (RIST) was therefore performed for further improvement of disease status. The conditioning regimen consisted of fludarabine and busulfan. Graft-versus-host disease (GVHD) prophylaxis was performed using cyclosporin and short-term methotrexate. The occurrence of serious infection during the period of neutropenia was prevented by the administration of amphotericin B, fluconazole and acyclovir. This case report provides important information on the appropriate strategy for treating patients who have ileostomy.**
**(Internal Medicine 44: 476–479, 2005)**

*Key words:* non-Hodgkin's lymphoma (NHL), chemotherapy, bowel perforation, reduced intensity allogeneic stem cell transplantation (RIST), ileostomy

## Introduction

Reduced-intensity stem cell transplantation (RIST) can be performed in patients with hematological diseases or solid tumors who are considered ineligible for conventional myeloablative conditioning (such as high-dose chemotherapy and total body irradiation) (1–8). In general, because of the reduced regimen-related toxicity, this procedure is thought to be safer than conventional myeloablative conditioning in patients of advanced age and/or with various medical complications, though life-threatening severe graft-versus-host disease (GVHD) often occurs in patients who have undergone RIST (9). The therapeutic significance of RIST regimens is mainly based on the graft-versus-tumor (GVT) effect mediated by immunocompetent cells such as T cells. We have recently encountered a patient with ileostomy who received RIST for the treatment of non-Hodgkin's lymphoma (NHL). He had undergone an operation for bowel perforation that occurred during chemotherapy and had an ileostomy. Here, we report a successful RIST in a patient with ileostomy due to perforation caused by chemotherapy for NHL.

## Case Report

A 56-year-old Japanese man was admitted to Hokkaido University Hospital for treatment of NHL, diffuse large B-cell lymphoma, in November 2000. He had experienced body weight loss, appetite loss, constipation and abdominal discomfort in September 2000. He had undergone computed tomography (CT) in November 2000 and a giant mass had been detected in his right lower abdomen (Fig. 1a). The tumor mass extended from the pancreas head to the pelvic cavity, involving the duodenum, right ureter, small intestine and ascending colon. He had then undergone open abdominal biopsy and was diagnosed as having NHL of the diffuse large B-cell type (DLBCL).

18-F-fluorodeoxyglucose-positron emission tomography ($^{18}$FDG-PET), performed in our hospital in November 2000,

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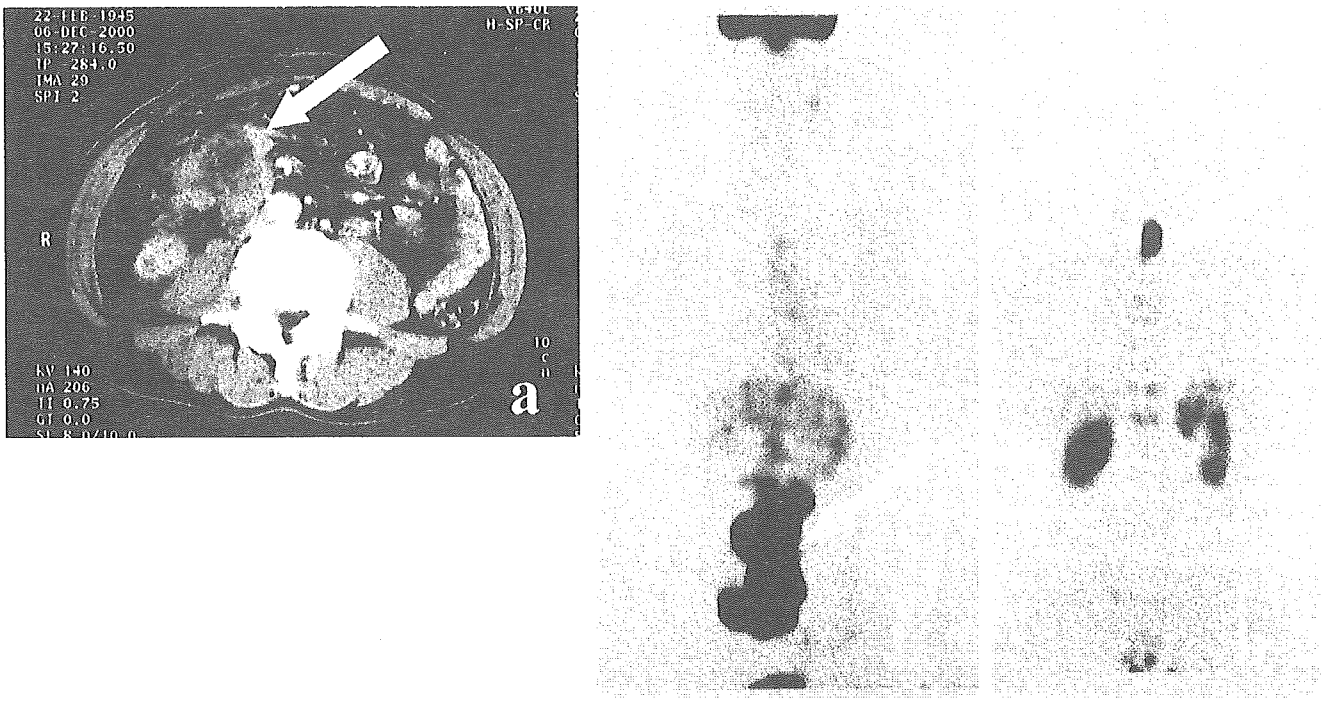


**Figure 1. Abdominal computer tomography (CT) and 18-F-fluorodeoxyglucose-positron emission tomography ($^{18}$FDG-PET) images taken in November 2000. (a): Abdominal CT image. White arrow (⇨) shows a giant mass in the right lower abdomen. (b): $^{18}$FDG-PET image before chemotherapy. A right lower abdominal lesion (b-1) and mediastinal lesion (b-2) were detected (⇨).**

revealed mediastinal and abdominal lymphoadenopathy (Fig. 1b-1, b-2), and a diagnosis of clinical stage IVB was made. The patient received combination chemotherapy consisting of cyclophosphamide (CY) at 750 mg/m$^2$ on day 1, vincristine (VCR) at 1.4 mg/m$^2$ (max dose, 2 mg/body/d) on day 1, doxorubicin (ADR) at 50 mg/m$^2$ on day 1 and prednisolone (PSL) at 40 mg/m$^2$/d on days 1–5 (CHOP). Six courses of combination chemotherapy were completed in March 2001. However, the response to the treatment was not satisfactory. He received salvage chemotherapy consisting of CY, VCR, ADR, PSL (CHOP, at the same doses as those stated above) and etoposide (VP-16) at 100 mg/m$^2$ on day 1 (CHOP-E), but the tumor remained even after 2 courses of CHOP-E. He therefore received another salvage chemotherapy regimen consisting of CY at 650 mg/m$^2$ on day 1, ADR at 25 mg/m$^2$ on day 1, VP-16 at 120 mg/m$^2$ on day 1, cytarabine at 300 mg/m$^2$ on day 8, bleomycin at 5 mg/m$^2$ on day 8, VCR at 1.4 mg/m$^2$ on day 8, methotrexate at 120 mg/m$^2$ on day 8, and PSL at 60 mg/m$^2$/d on days 1–15 (ProMACE-CytaBOM). After receiving two courses of ProMACE-CytaBOM, he complained of right lower quadrant (RQO) pain with fever. Blumberg sign was positive, but rebound tenderness and muscular guarding were negative. Although chest radiography did not reveal the existence of free air under the diaphragm, he was judged to be suffering from panperitonitis due to perforation of the intestine. A partial small bowel and colon resection was immediately performed, and he had an ileostomy. However, at surgery, the site of perforation could not be identified. Macroscopically, the remains tumor scar had involved the blood vessel, ureter and ileocecum were red color due to ischemia. Microscopic examination of the resected specimens revealed scarring, but no lymphoma cell involvement was detected. Therefore, we rediagnosed the patient as having panperitonitis due to ischemia caused by a reduction of tumor size.

After the operation, we found a new disease lesion and he therefore received one course of ProMACE-CytaBOM. However, the response to the treatment was poor. We discussed the eligibility of allogeneic stem cell transplantation, and we reached the conclusion that he was eligible to receive RIST for the following reasons: 1) advanced age, 2) skin and gut infection risk due to ileostomy and 3) possible cardiac side effects because of the high dose of ADR (750 mg/body) administered. His brother was found to be an HLA-A, -B, -DR-matched and ABO-matched donor. He was therefore offered the option of RIST after extensive discussion of the potential benefits, risks and alternatives. The conditioning regimen consisted of fludarabine (FL, 30 mg/m$^2$ for 6 days) and busulfan (BU, 4 mg/kg for 2 days). RIST involving an infusion of granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood stem cells ($5.08\times10^6$/kg) from the brother was performed in November 2001. Treatment with intravenous cyclosporin (CsA, 3 mg/kg/day) starting on day-1 and methotrexate (MTX) at 10 mg/m$^2$ on days 1, 3 and 6

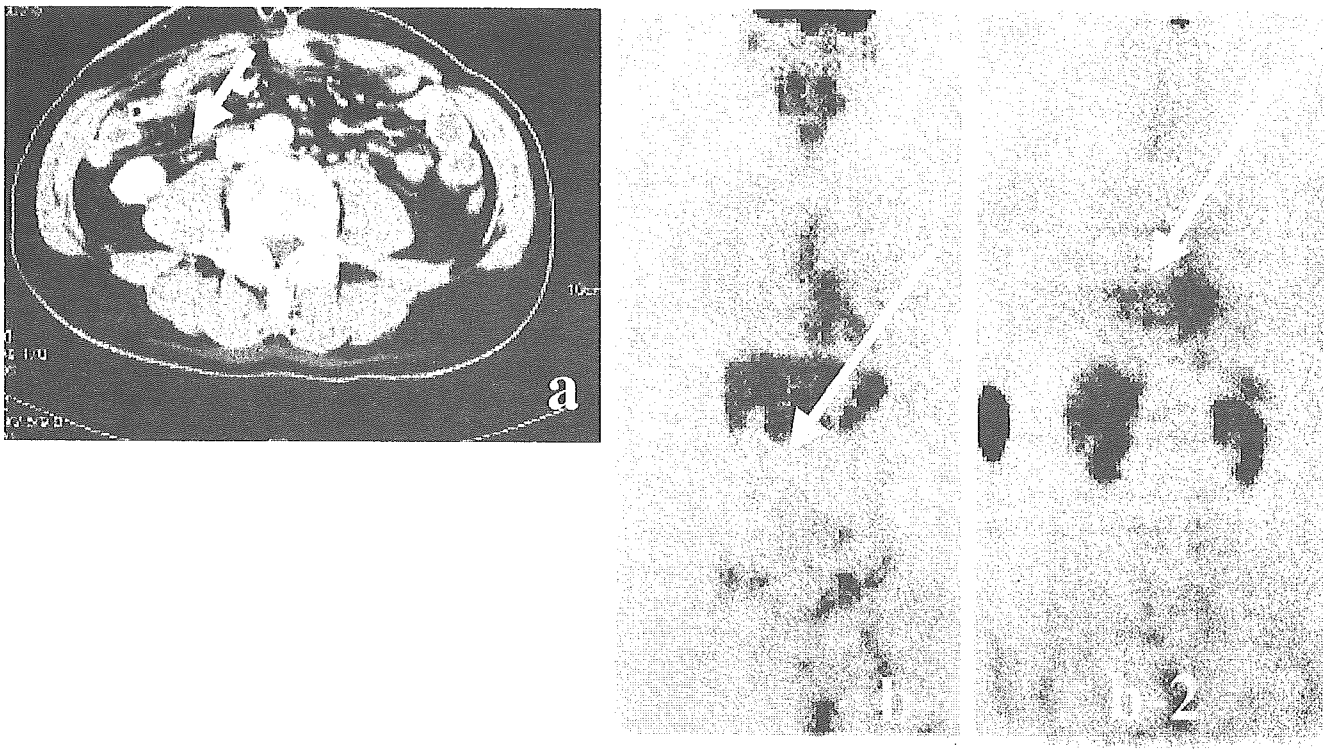


**Figure 2. Abdominal CT and $^{18}$FDG-PET images after RIST taken in May 2002. (a): Abdominal CT image after RIST. The giant mass in the right lower abdomen was not detected after RIST (⇨). (b): $^{18}$FDG-PET image after RIST. The right lower abdominal lesion (b-1) and mediastinal lesion (b-2) were not detected (⇨).**

was performed to prevent graft-versus-host disease (GVHD). To prevent gut infection and to decontaminate the ileostomy, we used 2,400 mg/kg/day amphotericin B syrup and intravenous nutrition. Acyclovir was administered at a dose of 1,000 mg/day until day 35 for prophylaxis. Fluconazole and levofloxacin prophylaxis was employed from day 14 at a dose of 200 mg/day p.o. during the period of treatment with immmunosuppresive agents. Engraftment was prompt, with a neutrophil count of $>0.5\times10^9/l$ on day 17 and with a platelet count of $>50.0\times10^9/l$ on day 15. We examined donor-type chimerism in peripheral blood mononuclear cells by a procedure that was reported in detail previously (10). Complete donor-type chimerism was observed on day 28. The patient had a peak bilirubin level of 1.0 mg/dl and a peak creatinine level of 1.5 mg/dl attributed to high CsA serum levels before GVHD developed. The dose of CsA was tapered, and CsA treatment was stopped on day 60 with no evidence of GVHD. The patient had no bacterial infection or other complications during the period of neutropenia. His condition was good until day 100. However, chronic GVHD developed in the liver (peak bilirubin level of 9.0 mg/dl on day 187). CsA and prednisolone were administered, and the chronic GVHD gradually improved. He was discharged on July 26, 2002. As of September 30, 2003, the patient is in complete remission of NHL with stable engraftment. Abdominal CT and $^{18}$FDG-PET post-RIST images taken in May 2002 are shown in Fig. 2.

## Discussion

Generally, it is important to prevent active infection in patients undergoing conventional allogeneic stem cell transplantation (allo-SCT). However, successful RIST has been reported in patients complicated with active infection (11). Moreover, patients with mild organ dysfunction were eligible in previous studies on RIST (4, 5, 12).

We performed RIST in an NHL patient with ileostomy. The reasons for performing RIST are as follows: 1) after the operation, his disease was progressive and he received one course of ProMACE-CytaBOM, but the response to the treatment was poor, 2) his age was advanced for conventional allogeneic stem cell transplantation, 3) we were concerned about the risk of skin and gut infection because he had ileostomy, and 4) we were concerned about cardiac side effects because of the high dose of ADR administered. It was important to prevent the occurrence of serious infection, especially intestinal infection, in the present case. If he had not undergone RIST, his disease would have rapidly progressed. Therefore, we suggest that RIST is a preferable treatment for patients who are ineligible for conventional allogeneic stem cell transplantation. There has been no report on an effective method for preventing infection in patients undergoing

RIST.

Here, fluconazole and itraconazole were administered to prevent the occurrence of fungal infection, and acyclovir was administered to prevent the occurrence of herpes virus infection. Amphotericin B syrup, which has been used in patients undergoing conventional bone marrow transplantation and in intravenous nutrition for fasting cure, was used to prevent the occurrence of intestinal infection. Therefore, no active intestinal infection occurred in our patient.

Bowel perforation in lymphoma patients has been noted by many investigators (13), and the incidence of perforation in patients with small intestinal malignant lymphoma has been reported to be about 10% (14). It has also been reported that spontaneous gastrointestinal perforation can occur in the stomach or bowel wall in patients undergoing systemic chemotherapy and/or steroid treatment even if there is no tumor (15). Bowel perforation has been reported to have been caused by chemotherapeutic toxicity, immunosuppression, protein malnutrition, and steroid therapy (16–18 ). The perforation in the present patient was thought to have been caused by intestinal ischemia associated with a reduction of tumor size due to chemotherapy.

In conclusion, we encountered an NHL patient with ileostomy in whom RIST was successfully performed. This case report provides important information on the appropriate strategy for treating patients who have an ileostomy.

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Case Report

# Successful Reduced-Intensity Hematopoietic Stem Cell Transplantation in Myelodysplastic Syndrome with Severe Coronary Artery Disease

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**Abstract**

A 60-year-old Japanese man with myelodysplastic syndrome (MDS) and effort angina was referred to our clinic for treatment of MDS. The patient was transfusion-dependent and displayed coronary artery disease (CAD) with 99% obstruction of the left anterior descending coronary artery. Treatment comprised reduced-intensity hematopoietic stem cell transplantation with administration of fludarabine phosphate (180 mg/m$^2$) and busulfan (8 mg/kg), followed by allogeneic peripheral blood stem cell transplantation from an HLA-matched sister. The regimen was well tolerated, and engraftment occurred rapidly without any therapy-related complications, including cardiovascular attack. Sex chromosome analysis by fluorescence in situ hybridization revealed complete donor chimerism on day 29 for bone marrow cells and on day 59 for peripheral blood leukocytes. The patient became transfusion-independent on posttransplantation day 8. As of 22 months postoperatively, he remains well, with 100% Karnofsky performance status, a limited type of chronic graft-versus-host disease, and no recurrence of disease. The clinical course of the patient suggests that this preparative regimen allows safe allogeneic stem cell transplantation for MDS patients with severe CAD.

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*Key words:* Reduced-intensity hematopoietic stem cell transplantation; Myelodysplastic syndrome; Coronary artery disease

## 1. Introduction

Myelodysplastic syndrome (MDS) represents a heterogeneous group of hematopoietic disorders predominantly affecting elderly individuals (median age, 69 years), characterized by abnormal cellular maturation resulting in cytopenias and a variable risk of progression to acute leukemia [1,2]. Allogeneic hematopoietic stem cell transplantation (HSCT) from an HLA-identical sibling is a highly effective treatment for patients with advanced MDS. However, treatment outcomes can be negatively affected in elderly patients and when organ damage is present. Such damage includes heart failure, liver dysfunction, and diabetes mellitus. Patients more than 55 years old or with some kinds of organ damage are thus ineligible for HSCT irrespective of whether an HLA-identical donor is available or not [3].

The usefulness of reduced-intensity hematopoietic stem cell transplantation (RIST) is being explored for grafting patients with hematological or non-hematological malignancies not normally considered for allogeneic HSCT because of poor organ function or advanced age [4-7]. Compared to conventional preparative regimens for allografting, these regimens produce less toxicity, but which types and what degrees of organ damage can be eligible remains to be elucidated. We describe herein successful RIST in a patient with MDS and severe coronary artery disease (CAD).

## 2. Case Report

A 60-year-old Japanese man was admitted to Akita University Hospital in May 2003 because of transfusion-dependent pancytopenia and chest pain during exercise. Results of peripheral blood tests on presentation were: leukocytes, 3.2 × 10$^9$/L; hemoglobin, 8.6 g/dL; and platelets, 24 × 10$^9$/L. Diagnoses of MDS and refractory anemia were made based on bone marrow examination, in accordance

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**Figure 1.** Coronary angiography (CAG) revealing 99% stenosis of the left anterior descending coronary artery (arrow).

with the French-American-British classification [8]. Bone marrow examination revealed normocellular marrow, with erythroid hyperplasia and 0.8% myeloblasts. Erythroblasts showed a megaloblastic change, and a pseudo-Pelger anomaly was observed in neutrophils. Megakaryopoiesis was hypoplastic with no obvious dysplasia. Chromosomal analysis of bone marrow cells revealed 46,XY,add(3)(p13)[3]/46,XY[17]. The International Prognostic Scoring System score was 1.0, and the patient was classified in the intermediate-1 risk group [9]. He had been dependent on weekly erythrocyte and platelet transfusions since November 2002. A donor search for allogeneic RIST was performed, and his sister proved to be HLA-identical. General work-up before RIST revealed ischemic changes on an electrocardiography (ECG)-Monitored Exercise Test. Although the ECG at rest was normal and the ejection fraction (EF) was 75% on echocardiography, coronary angiographies (CAG) revealed 99% stenosis of the left anterior descending coronary artery (Figure 1). Stress/Rest (99 m) Technetium-MIBI single-photon emission tomography (SPECT) confirmed ischemic changes from the anterior wall to the apex of the left ventricle. A diagnosis of effort angina was made. Percutaneous transluminal coronary angioplasty was attempted, but proved unsuccessful. Coronary artery bypass surgery could not be performed due to severe thrombocytopenia. Based on the Prognostic Gradient of Ischemic Responses During an ECG-Monitored Exercise Test [10], the patient was assessed as being at intermediate risk. Written informed consent was obtained prior to RIST, as required and approved by the Committee of Akita University School of Medicine for the Protection of Human Subjects.

The patient was administered 30 mg/m$^2$ fludarabine phosphate (Flu) on days –8 to –3 and 4 mg/kg busulfan orally on days –6 and –5. He received granulocyte-colony stimulating factor (G-CSF)–mobilized peripheral blood stem cells from his sister on day 0, containing 4.7 × 10$^6$/kg CD34$^+$ cells. For prophylaxis of acute graft-versus-host disease (GVHD), cyclosporin A (CyA) and short-term methotrexate were administered: continuous intravenous CyA, 3 mg/kg starting on day –1 and methotrexate at 10 mg/m$^2$ on day +1 and 7 mg/m$^2$ on days +3 and +6. Irradiated, leukocyte-filtered, packed erythrocytes were transfused to maintain hemoglobin levels at >8.0 g/dL. G-CSF was not administered. Blood pressure and heart rate were controlled by an experienced cardiologist. The patient received treatment with diltiazem (100 mg/day), isosorbide mononitrate (40 mg/day), and metoprolol (40 mg/day) before and after HSCT. Engraftment was rapid; neutrophils rose to >0.5 × 10$^9$/L by day +15, platelets to >50 × 10$^9$/L by day +13, and reticulocytes to >50 × 10$^9$/L by day +22. The final erythrocyte transfusion was given on day +6, and the final platelet transfusion was administered on day +7. No symptoms or signs of cardiac toxicity, including chest pain, were recognized after transplantation. There were no ischemic changes on monitored ECG during conditioning and infusion of the graft. Acute GVHD was not seen. On day +62, peripheral blood count revealed: leukocytes, 4.6 × 10$^9$/L; hemoglobin, 11.3 g/dL; and platelets, 220 × 10$^9$/L. Myelography demonstrated complete remission. Fluorescence in situ hybridization demonstrated complete donor chimerism of bone marrow cells on day +29 and of blood leukocytes on day +59. The patient was discharged from the hospital on day +91. However, he was readmitted to the hospital because of idiopathic interstitial pneumonia 5 months after RIST [11], and immunosuppressive steroid treatment achieved a complete response. ECG at rest was normal and EF was 71% on echocardiography after HSCT. At the time of this writing, 22 months after transplantation, the patient was doing well, with 100% Karnofsky performance status, a limited type of chronic GVHD, and no recurrence of disease.

## 3. Discussion

General guidelines for HSCT include a left ventricular EF >50% to 55% [12,13], but guidelines for CAD have not been proposed so far. Several factors are responsible for the occurrence of cardiac complications after HSCT. These include the cardiotoxic effects of radiotherapy, antineoplastic and immunosuppressive drugs, abnormal immunological reactions associated with GVHD [14], and infectious agents. The number of deaths related to cardiac toxicity from HSCT remains low. However, caution is required when cyclophosphamide or anthracyclines such as mitoxantrone are used in patients with possible underlying heart damage, such as elderly patients or those with aplastic anemia or systemic sclerosis [15]. Life-threatening and often fatal cardiotoxicity can occur in patients conditioned with high-dose cyclophosphamide. This complication can occur even in patients with normal cardiac function on ECG [15]. In addition, patients receiving high-dose cyclophosphamide need hydration and forced saline diuresis to prevent bladder toxicity [16], and such hydration may worsen cardiac function when the recipient has cardiac complications. RIST for the present patient did not include cyclophosphamide, and such a protocol may be more feasible for elderly patients with cardiac complications.

The eligibility of patients with CAD for HSCT remains unclear. The treatment of systemic sclerosis with autologous SCT was carefully analyzed in 59 patients and found to be less useful than anticipated. Of the 19 deaths reported as

having some cardiac component, only 2 were suspected to be directly related to cyclophosphamide. Another 2 events, 1 after mobilization and the other 2 days posttransplantation, were determined at autopsy to be attributable scleroderma myocarditis and acute CAD, respectively [15], emphasizing the need for careful assessment.

Immunosuppressants such as CyA that damage endothelial cells may have adverse effects on severe CAD. However, in our case, there were no symptoms or signs of cardiac toxicity, including chest pain, during CyA administration. It has been reported that systemic immunosuppression therapy of CyA combined with prednisolone inhibited in-stent restenosis in renal transplant patients with CAD treated with stenting [17]. For treating patients with RIST, it is necessary to perform stent implantation to treat coronary artery stenosis.

According to recent research, regenerative medicine with bone marrow stem cells has been used for the myocardial regeneration of ischemic myocardium [18,19]. Although the effects of hematopoietic stem cells derived from the donor on myocardial regeneration are of great concern, myocardial biopsy was not done before and after RIST.

Our success in treating this patient with RIST has important implications for the management of patients with CAD. Allogeneic myeloablative HSCT might not have been considered the treatment of choice for such patients, even if an HLA-identical donor was available, as the probability of treatment-related death might be considered too high. This is the first report that RIST could be done for a patient with CAD. Although this is a case report and the observation time is 22 months, we consider that RIST may at least have potential in hematological malignancy patients with CAD. Our findings in a CAD patient may encourage physicians to consider RIST, which may improve the prognosis of patients with severe CAD. Identification of factors predictive of the outcomes for this approach is necessary for RIST in patients with CAD.

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# Phagocytosis of codeveloping megakaryocytic progenitors by dendritic cells in culture with thrombopoietin and tumor necrosis factor-α and its possible role in hemophagocytic syndrome

Kunie Saito, Makoto Hirokawa, Kayo Inaba, Hiroshi Fukaya, Yoshinari Kawabata, Atsushi Komatsuda, Junsuke Yamashita, and Kenichi Sawada

**Tumor necrosis factor-α (TNF-α) and thrombopoietin (TPO) have been shown to induce the differentiation and proliferation of CD34$^+$ cells toward dendritic cells (DCs) in the presence of multiacting cytokines. We hypothesized that the costimulation of TPO and TNF-α generates megakaryocytic progenitors and DCs together from human CD34$^+$ cells and that the interaction of these cells may indicate a physiologic and/or a pathologic role of DCs in megakaryopoiesis. When highly purified human CD34$^+$ cells were cultured for 7 days with TPO alone, the generated cells expressed megakaryocytic markers, such as CD41, CD42b, and CD61. The addition of TNF-α with TPO remarkably decreased the number of megakaryocytic progenitor cells without affecting the cell yield. Almost half of the cells thus generated expressed CD11c, and most of them were positive for CD4 and CD123. Furthermore, CD11c$^+$ cells were found to capture damaged CD61$^+$ cells and to induce autologous T-cell proliferation, although the cytokine productions were low. We also confirmed an engulfment of CD61$^+$ cells and their fragment by CD11c$^+$ cells in bone marrow cells from patients with hemophagocytic syndrome. These findings suggest that DCs generated under megakaryocytic and inflammatory stimuli are involved in megakaryopoiesis and the subsequent immune responses to self-antigens. (Blood. 2006;107:1366-1374)**



## Introduction

One of the earliest mediators of the acute phase response of infection, inflammation, and/or tissue damage is tumor necrosis factor-α (TNF-α).[1] TNF-α, a proinflammatory cytokine, is mainly produced by activated macrophages and lymphocytes,[2,3] and it has a multifunctional effect on various cell types.[4-6] For example, TNF-α acts as a positive and negative regulator on myeloid-cell proliferation and differentiation during hematopoiesis.[7-10] It is also well documented that TNF-α enhances the proliferation of human CD34$^+$ cells while also promoting the development of dendritic cells (DCs) in the presence of stem-cell factor (SCF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and/or interleukin-3 (IL-3).[11,12]

Thrombopoietin (TPO) is a primary and sole factor for megakaryopoiesis,[13] because the genetic elimination of *c-mpl* or *tpo* leads to profound thrombocytopenia in mice due to a greatly reduced number of megakaryocytic progenitors and mature megakaryocytes.[14] TPO also acts in synergy with IL-3 and SCF on hematopoietic stem cells to induce cell-cycle progression and to increase the both primitive and committed hematopoietic progenitor cells of all lineages.[15-17] Furthermore, TPO cooperates with FLT3 ligand (FLT3-L) and SCF in the generation of DC precursors from human CD34$^+$ cells,[18-20] and human DCs generated from CD34$^+$ cells following incubation with SCF, GM-CSF, and TNF-α either with or without TPO express the TPO receptor c-mpl.[21] As a result, both TPO and TNF-α enhance the proliferation of CD34$^+$ cells and differentiation toward DCs in the presence of multipotent cytokines such as SCF, FLT3-L, and IL-3.

We recently showed that nonerythroid cells were cogenerated from human CD34$^+$ cells during erythroid differentiation in the presence of IL-3/SCF/erythropoietin with TNF-α and expressed DC phenotypes. The CD11c$^+$ DCs physically and selectively associate with developing damaged and immature self-erythroid cells and then phagocytose them.[22] This phenomenon may not be limited to the erythroid lineage. We hypothesized that TNF-α in the course of the inflammatory response by viral and microbial infection facilitates DC development during hematopoiesis, thus leading to phagocytosis of damaged self-progenitor cells by DCs. To confirm this hypothesis, we examined the effect of TNF-α and TPO on CD34$^+$ cells.

We herein provide evidence that TNF-α inhibits the generation of megakaryocytic progenitors from CD34$^+$ cells in the presence of TPO while inversely increasing the number of CD4$^+$ CD11c$^+$ CD123$^+$ nonmegakaryocytic cells that show a DC phenotype. We also found that DCs physically associate with immature

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megakaryocytic cells during differentiation and then phagocytose damaged cells. Next, DCs acquire an ability to induce autologous T-cell proliferation in vitro However, they produce cytokines, which are more tolerogenic than immunogenic, and the T cells activated by them also do not produce a significant amount of immune cytokines. Interestingly, DCs with an immature phenotype in bone marrow from hemophagocytic syndromes are also found to capture $CD61^+$ cells. These findings may suggest the possibility that the phagocytosis of damaged cells by DCs under daily life conditions with either a weak or strong inflammatory response could thus play a pivotal role in regulating the immune responses against hematopoietic progenitor cells.

## Materials and methods

### Reagents

Bovine serum albumin (BSA), Iscove modified Dulbecco medium (IMDM), and propidium iodide (PI) were purchased from Sigma (St Louis, MO). Fetal calf serum (FCS), penicillin, and streptomycin were obtained from Flow Laboratories (McLean, VA). Insulin (porcine sodium, activity 26.3 U/mg; United States Pharmacopoeia) was obtained from Calbiochem of Behring Diagnostics (La Jolla, CA). Fluorescein isothiocyanate (FITC)–labeled monoclonal antibodies (mAbs) specific for CD4 (SK7), CD8 (SK1), CD19 (4G7), CD20 (L27), and CD34 (8G12) and phycoerythrin (PE)–labeled antibody for CD34 (8G12) were purchased from Becton Dickinson (Mountain View, CA). The PE-CD120a (TNFR-I: 16803.1) and PE-CD120b (TNFR-II: 22235) were obtained from R&D Systems (Minneapolis, MN), and PE-CD11c (B-ly6) and PE-CD83 (HB15a) were from Immunotech (Marseilles, France). PE-CD14 (TUK4), FITC-CD14 (TUK4), PE-CD41 (5B12), PE-CD42b (AN51), mouse anti–human glycophorin A (GPA: JC159), normal mouse serum, goat anti–rabbit IgG conjugated to peroxidase-labeled dextran polymer, diaminobenzidine (DAB)–substrate chromogen system, and fuchsin-substrate chromogen system were from DAKO Japan (Kyoto, Japan). Cyanin-labeled CD4, FITC-CD86 (FUN-1), PE-conjugated CD61 (VI-PL2), CD80 (L307.4), CDw123 (IL-3Rα; 9F5), c-mpl (BAH-1), major histocompatibility complex (MHC) class I (-A, -B, -C) (B9.12.1) and MHC class II (-DR) (B8.12.2), and unconjugated antimannose receptor were purchased from PharMingen (San Diego, CA). Anti–single-stranded DNA (anti-ssDNA) antibody was donated by Dr T. Sugiyama (Akita University, Japan).[23] Anti-CD3 (OKT3) was purified from ascites.

TNF-α was purchased from R&D Systems (Minneapolis, MN). TPO, IL-3, and SCF were kind gifts from the Kirin Brewery (Tokyo, Japan) and granulocyte colony-stimulating factor (G-CSF) from Chugai Pharmaceutical (Tokyo, Japan). Vitamin $B_{12}$ and folic acid were purchased from Sankyo Pharmaceutical (Tokyo, Japan). $^3$H-thymidine was purchased from Perkin Elmer (Yokohama, Japan).

### Cell preparations

Human $CD34^+$ cells and $CD34^-$ cells were purified from healthy volunteers, who had signed informed consent forms approved by the Akita University School of Medicine Committee for the Protection of Human Subjects, and stored in liquid nitrogen until use as previously described.[24] The thawed $CD34^+$ cells were suspended in IMDM containing 30% FCS and 100 U/mL DNAse and then were washed twice with IMDM containing 20% FCS. Next, the cells were seeded in 50 mL polystyrene flasks (Corning Costar, Cambridge, MA) at $2 \times 10^5$ to $5 \times 10^5$ cells per milliliter in IMDM containing 5% heat-inactivated pooled human AB plasma, 1% BSA, 10 μg/mL insulin, 10 μg/mL vitamin $B_{12}$, 15 μg/mL folic acid, 50 nM β-mercaptoethanol, 50 U/mL penicillin, and 50 U/mL streptomycin in the presence or absence of 100 ng/mL TPO and/or at the indicated doses of TNF-α for the various periods at 37°C in a 5% $CO_2$/5% $O_2$ incubator.

Bone marrow aspirate was obtained from 3 patients (patients 1 to 3; Table 1) with hemophagocytic syndrome who had signed informed consent forms approved by the Akita University School of Medicine Committee for the Protection of Human Subjects. The diagnosis was made according to the diagnostic criteria for hemophagocytic lymphohistiocytosis.[25] Smear preparations of bone marrow were air dried and stored at −80°C.

**Table 1. Characteristics of each of 3 patients with hemophagocytic syndrome***

| | Patient no. | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| Sex/age, y | M/70 | M/58 | F/67 |
| Underlying diseases | DIC‡ | HIV/AIDS§ | DLBCL‖ |
| **Clinical findings** | | | |
| Nonremitting high fever | + | + | + |
| Hepatosplenomegaly | + | + | + |
| **Peripheral blood** | | | |
| White cells (neutrophils), × $10^9$/L | 2.7 (1.6) | 0.8 (0.5) | 1.0 (0.6) |
| Hemoglobin, g/L | 85 | 65 | 61 |
| Platelet, × $10^9$/L | 96 | 31 | 6 |
| **Biochemistry¶** | | | |
| Triglycerides, mg/dL | ND | 120 | 106 |
| Fibrinogen, mg/dL | 97 | 78 | 481 |
| Ferritin, μg/L | 1746 | ND | 2861 |
| sIL-2R, U/mL | 3870 | ND | 6280 |
| FDP, μg/mL | 53.2 | 38.6 | 8 |
| Hemophagocytosis in bone marrow† | + | + | + |
| Outcome | Remission | Rapidly fatal | Remission |

sIL-2R indicates soluble interleukin-2 receptor; FDP, fibrin degeneration products; DIC, disseminated intravascular coagulation syndrome; HIV, human immunodeficiency virus; AIDS, acquired immunodeficiency syndrome; DLBCL, diffuse large B-cell lymphoma; ND, not determined.

*According to the diagnostic guidelines developed by the Histiocyte Society, the diagnosis of hemophagocytic lymphohistiocytosis (HLH) requires the presence of all 5 major criteria: *(1)* fever, *(2)* splenomegaly, *(3)* cytopenia involving 2 or more cell lines, *(4)* hypertriglyceridemia or hypofibrinogenemia, *(5)* hemophagocytosis. Either criterion *(a)* low or absent natural killer cell activity or a combination of criteria, *(b)* ferritin above 500 mg/L, and *(c)* sIL-2R above 2400 U/mL may substitute for 1 of the major criteria.[25]

†Details are shown in Table 3.

‡Chronic DIC after surgery for aortic abdominal aneurysm.

§Diagnosed as AIDS at acute onset of *Pneumocystis carinii* pneumonia. Cytomegalovirus antigenemia assay was also positive.

‖DLBCL with bone marrow invasion. Some cases of DLBCL have a prominent background of reactive T cells and histiocytes.

¶Reference ranges for normal values are 30 to 150 mg/dL for triglycerides, 160 to 360 mg/dL for fibrinogen, below 234 μg/L for ferritin, 135 to 483 U/mL for sIL-2R, and less than 10 μg/mL for FDP.

### Flow cytometry

The cells collected from the cultures were washed twice with phosphate-buffered saline (PBS) containing 3% FCS, 2 mM EDTA, and 0.05% $NaN_3$ (staining medium) and stained with FITC- and PE-labeled mAbs, and they then were analyzed using a FACS Calibur (Becton Dickinson). To determine the TNF receptor (TNFR) expression, the cells were incubated at 37°C for 2 hours without TNF-α before staining with specific mAbs, because TNFRs were down-regulated by TNF-α in the culture medium (data not shown).

### Enzymatic immunohistochemistry

The cells were spun onto slides using a Cytospin 3 (Shandon Lipshaw, Pittsburgh, PA), and smear specimens of bone marrow were fixed in 100% methanol, dried, and examined by enzymatic immunohistochemistry as described.[26] After blocking endogenous peroxidase activity with 3% hydrogen peroxide, the preparations were incubated with mouse mAbs, followed by goat anti–mouse IgG conjugated to peroxidase-labeled dextran polymer, and then were visualized with a DAB-substrate chromogen system. For double staining, the preparations were further incubated with rabbit antihuman antibody, followed by goat anti–rabbit IgG conjugated to alkaline phosphatase-labeled dextran polymer using a fuchsin-substrate

chromogen system. ssDNA-specific rabbit polyclonal IgG antibodies were used to recognize hexadeoxynucleotides with various base sequences in apoptotic cells,[2,3] and the stained specimens were incubated with goat anti–rabbit IgG conjugated to peroxidase-labeled dextran polymer. Specimens that were incubated with normal serum with secondary antibody served as negative controls.

### Confocal microscopy

For confocal microscopy, cells generated after 5 days of culture with TPO and TNF-α were fixed using PermaFluor Aqueous Mounting Medium (Thermo Shandon, Pittsburgh, PA), permeabilized with BD FACS Permeabilizing Solution (Becton Dickinson), and then stained with PE-CD11c and FITC-CD61 as described previously.[22] Cells engulfing $CD61^+$ cells were observed using a CLSM 510 confocal laser scanning microscope (Carl Zeiss Microscope Systems, Oberkochen, Germany) equipped with a 40 ×/65 μm (FITC) or 40 ×/72 μm (PE) oil-immersion objective lens at zoom 5. Fluorochromes were excited using an argon laser at 488 nm for FITC and helium neon at 543 nm for PE. Detector slits were configured to minimize cross talk between channels. Z-sliced optical sections were collected with an optimal interval of 0.45 mm and processed using Carl Zeiss software and Adobe Photoshop 6.0 software (Adobe Systems, San Jose, CA).

### T-cell proliferation assays

Autologous T lymphocytes were prepared as a negative fraction from a nylon-fiber syringe[27] of thawed $CD34^-$ cells. Cells generated in the culture with TPO plus TNF-α or TNF-α alone for 7 days were used as a stimulator after γ-irradiation at 30 Gy (3000 rad) and then were cocultured with autologous responder T cells ($1 \times 10^3$ cells per well) in RPMI 1640 medium containing 10% heat-inactivated human AB serum in round-bottom 96-well microculture plates. Anti-CD3 mAb was added into some cultures as positive controls. $^3$H-thymidine (1 μCi per well [37 kBq/well]) incorporations for the last 18 hours of 5 days of culture were measured by a liquid scintillation counter (TopCount NXT; PerkinElmer LAS, Boston, MA), and the results were expressed as the mean ± SD of triplicate cultures.

### Cytokine measurement

Culture supernatants were obtained from autologous mixed lymphocyte reactions (MLRs) and culture of $CD34^+$ cells at $4 \times 10^5$ in 2 mL complete medium with TPO (100 ng/mL) and TNF-α (100 ng/mL) for 7 days in 24-well flat-bottomed Falcon tissue culture plates (Becton Dickinson, Franklin Lakes, NJ). Cytokine activities were assessed with cytometric bead array system (Pharmingen) according to the manufacturer's protocol.

## Results

### TNF-α inhibits the generation of megakaryocytic progenitors while increasing the nonmegakaryocytic cells from $CD34^+$ cells supported by TPO

Human $CD34^+$ cells (98.5% ± 1.0% purity; mean ± SD) were cultured with TPO in the presence of various concentrations of TNF-α ranging from 0 to 250 ng/mL. Seven days later, the total number of recovered cells was counted, and CD41, CD42b, and CD61 expressions, specific markers for the megakaryocytic lineage, were examined using a cytofluorometer. The total number of cells increased 1.4-fold during the 7 days of culture with TPO alone, and 89%, 43%, and 87% of the generated cells expressed CD41, CD42b, and CD61, respectively, thus indicating that most of them consisted of megakaryocytic progenitors. The addition of TNF-α significantly decreased the number of $CD41^+$, $CD42b^+$, and $CD61^+$ cells in a dose-dependent manner while conversely increasing $CD41^-$, $CD42b^-$, and $CD61^-$ cells (Figure 1A). A low dose of TNF-α (0.25 ng/mL) tended to increase the cell yield but reduced the proportion of $CD41^+$, $CD42b^+$, and $CD61^+$ cells. Then

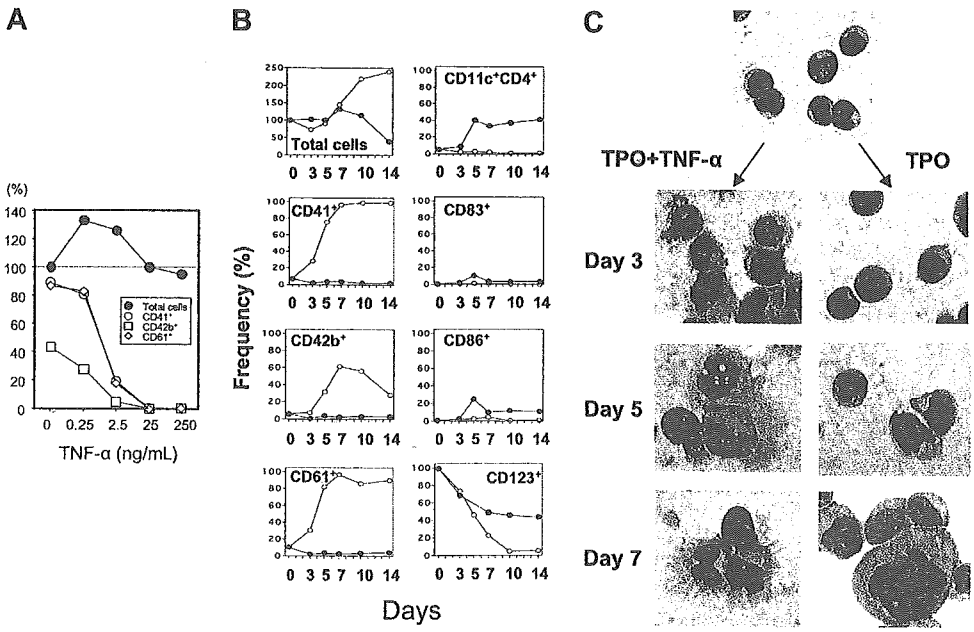


**Figure 1. TNF-α inhibits the generation of megakaryocytic progenitors but increases the number of nonmegakaryocytic cells from $CD34^+$ cells supported by TPO.** (A) Human $CD34^+$ cells at $7.5 \times 10^5$/mL were cultured with 100 ng/mL TPO in the presence of various concentrations of TNF-α ranging from 0 to 250 ng/mL. Seven days later, the total numbers of recovered cells (●) were counted, and CD41 (○), CD42b (□), and CD61 (◇) expressions were examined by a cytofluorometer. The cell yields were represented as the percent relative to the total number of cells without TNF-α. Marker-positive cells were also shown as the percent of the total number of cells in an individual group. The result shown is representative of 3 independent experiments. (B) $CD34^+$ cells were cultured as in panel A in the presence (●) or absence (○) of 100 ng/mL TNF-α. Cell counts and phenotype analyses were performed at various time points. The results shown are representative of 3 independent experiments. (C) Morphologic differences in the cells differentiated by TPO with and without TNF-α as in panel B. Cytospin specimens were stained with May-Grünwald-Giemsa solution at the indicated time points (× 1000).

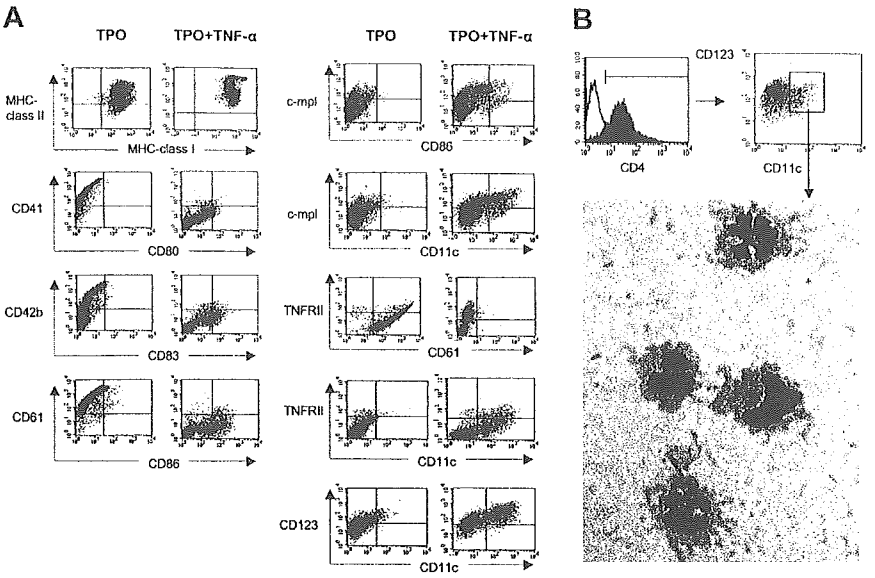


**Figure 2. Phenotypic analyses of nonmegakaryocytic cells.** (A) Cells generated after 7 days cultured as described for Figure 1 were assessed for their phenotype with cytofluorometer using various combinations of mAbs. (B) The purification of $CD11c^+$ $CD123^+$ cells and their morphology. Cytospin specimens of sorted $CD11c^+$ cells were stained with May-Grünwald-Giemsa solution (arrow). Original magnification, × 1000.

half-maximal dose of TNF-α to inhibit the generation of megakaryocytic cells ranged from 0.25 to 2.5 ng/mL.

### Characterization of nonmegakaryocytic cells

To understand the kinetics of nonmegakaryocytic-cell development in our system, purified $CD34^+$ cells were cultured for 14 days with 100 ng/mL TPO in the presence or absence of 100 ng/mL TNF-α, and the surface phenotypes were monitored at the indicated time points (Figure 1B). The number of total cells recovered from the cultures with and without TNF-α were comparable to each other for 7 days. During this period, the proportions of $CD41^+$, $CD42b^+$, and $CD61^+$ cells dramatically increased only when TNF-α was not added. The cell yield substantially increased in the culture with TPO alone but decreased with TPO plus TNF-α thereafter. Very few cells expressed CD41, CD42b, and CD61 in the presence of TNF-α throughout the culture period, thus suggesting that TNF-α affects $CD34^+$ cell development by inhibiting the generation of megakaryocytic progenitors. Interestingly, $CD11c^+$ cells were inversely generated as early as at day 5, and some of them expressed CD86 and CD83. Then morphologic changes of cells also showed the development of cells with DC features, such as dendrites and eccentric nuclei (Figure 1C).

To further characterize the DC-like cells in detail, the surface phenotypes were monitored on day 7 (Figure 2A). All cells became positive for MHC class II in the presence of TNF-α. A significant number of $CD11c^+$ cells and $CD86^+$ cells coexpressed c-mpl and TNFRII. In addition, $CD61^+$ cells expressed not only c-mpl but also TNFRII. None of the $TNFRI^+$, $CD3^+$, $CD8^+$, $CD19^+$, $CD20^+$, and $CD56^+$ cells was present in either of the culture conditions (data not shown).

CD123 (IL-3 receptor-α), a marker of plasmacytoid DCs or interferon-producing cells (IPCs),[28] was detected in 70% ± 19% of the total cells and 97% of $CD11c^+$ cells (Figure 2A). Chen et al[20] demonstrated that TPO and FLT3-L allow human $CD34^+$ cells to differentiate into $CD11c^-$ $CD123^{high}$ IPCs, $CD11c^+$ immature DCs, and $CD14^+$ monocytes. Therefore, we examined the CD123 expression on $CD11c^+$ DCs using purified cells gated on CD4, which is expressed on DCs.[28] $CD4^+$ cells consisted of 92% of bulk cells at day 7, and all of them were positive for CD123 (Figure 2B). The sorted $CD11c^+$ $CD123^+$ cells showed a uniform picture characterized by abundant dendrites and eccentric nuclei.

### Megakaryocytic cells and DCs are closely associated together during their development in the presence of TNF-α

DCs are known to develop from proliferating precursor cells in aggregates. We stained cells with megakaryocyte-specific and DC-associated markers to visualize the localization of megakaryocytic progenitors and DCs (Figure 3). The $CD34^+$ cells proliferated

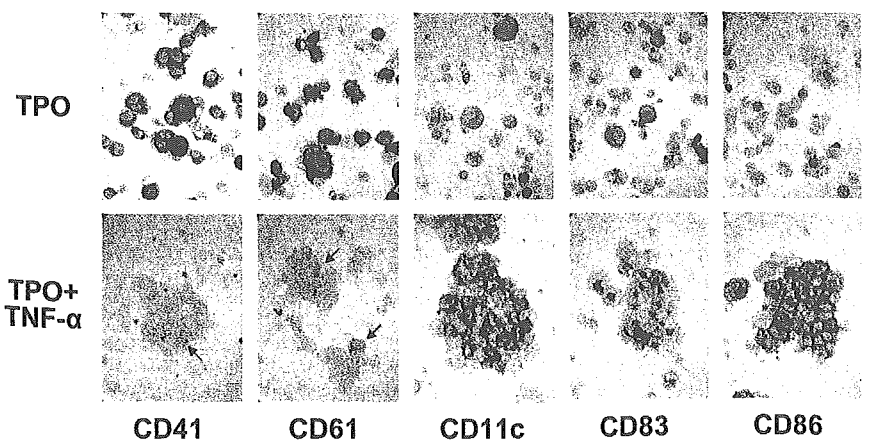


**Figure 3. DCs develop in aggregates along with megakaryocytic cells in the presence of TPO and TNF-α.** The aggregates formed were collected by gentle pipetting after 5 days of culture with TPO in the presence or absence of TNF-α as in Figure 2. The cytospin specimens were stained with megakaryocyte-specific and DC-associated markers. Original magnification, × 40.

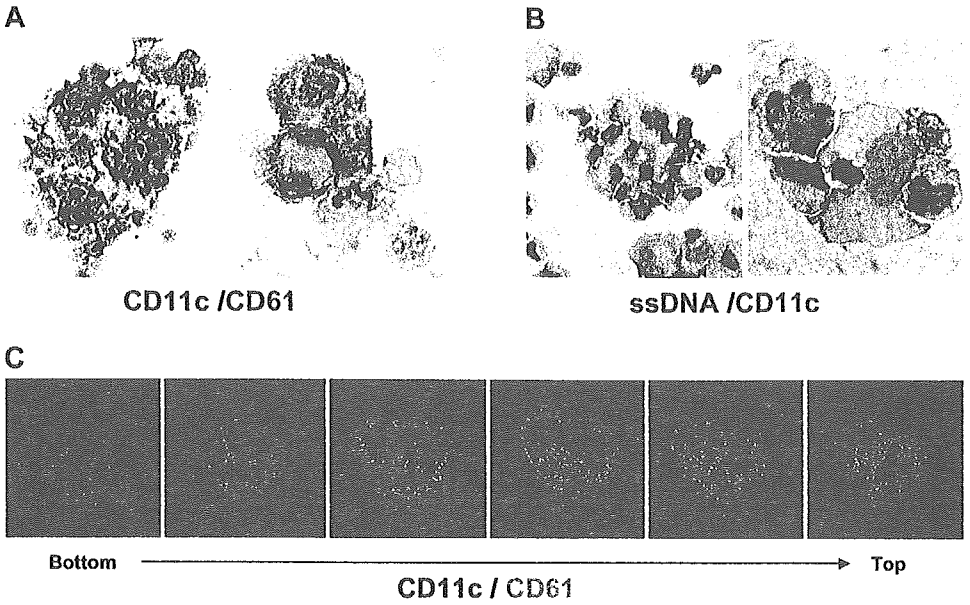


**Figure 4. Physical association and phagocytosis of CD61$^+$ cells by CD11c$^+$ cells.** Five days after culture with 100 ng/mL TPO and 2.5 ng/mL TNF-α, the cells were cytospun and stained with anti-CD11c (brown) and anti-CD61 (red) (A) or with anti-CD11c (red) and anti-ssDNA (brown) (B); original magnification, × 1000. (C) Cells placed on coverslips were fix-permeabilized, stained with FITC-CD61 (green) and PE-CD11c (red), and then were observed by laser scanning confocal microscopy. A serial 3-dimensional reconstruction of the cells demonstrates the phagocytosis of CD61$^+$ cells (green) by CD11c$^+$ cells (red).

as single cells without forming aggregates in the absence of TNF-α, and they expressed CD41 and CD61. In the presence of TNF-α, the aggregates were found to consist of CD11c$^+$, CD83$^+$, and CD86$^+$ cells, and they contained fragments that were positive for CD41 or CD61 (Figure 3, arrows).

Immature DCs are known to be capable of capturing apoptotic and necrotic cells,[29,30] thus leading to antigen presentation, which thus induces immunity and tolerance.[31,32] Therefore, it is possible that developing immature DCs in aggregates engulf damaged megakaryocytic progenitor cells. To address this question, CD34$^+$ cells were cultured for 5 days with 100 ng/mL TPO and 2.5 ng/mL TNF-α, half-maximal dose of inhibition, to maintain the shape of megakaryocytic progenitor cells. Cytospin specimens of the aggregated cells were stained with CD11c in combination with anti-CD61 (Figure 4A) or anti-ssDNA (Figure 4B). CD11c$^+$ cells were often shown to be associated with CD61$^+$ megakaryocytic progenitor cells in aggregates, and many ssDNA$^+$ cells were present in the aggregates. When the aggregates were dispersed with gentle pipetting, large parts of CD11c$^+$ cells associate with (63% ± 5%) or engulfed (15% ± 4%) CD61$^+$ cells. In addition, ssDNA was also found in the cytoplasm of CD11c$^+$ cells, which associated with ssDNA$^+$ cells. These observations indicate that DCs capture dying megakaryocytic progenitors. This was also confirmed with confocal microscopy, which showed that the CD11c$^+$ cells phagocytosed CD61$^+$ cells (Figure 4C).

### Autologous T-cell activation by DCs generated in the presence of TPO and TNF-α

We hypothesized that DCs phagocytosing self-megakaryocytic cells might thus modify self-proteins and, as a result, they can present antigen to autologous T cells. We therefore assessed autologous T-cell proliferation using cells generated during a 7-day culture with TPO plus TNF-α or TNF-α alone (Figure 5). CD3-dependent T-cell proliferation induced by these DCs served as positive controls. TNF-α alone was able to induce DC development from CD34$^+$ cells, although the cell yield was much lower than the culture with TPO and TNF-α (data not shown). DCs generated in the culture with TPO plus TNF-α, but not TNF-α alone, were found to potently activate autologous T cells, although T-cell proliferation was much lower than that induced by anti-CD3 mAb.

When T cells were cocultured with DCs at a 2:1 ratio, low but significant amounts of IL-2 (6.5 ± 1.7 pg/mL) and IL-6 (12.5 ± 2.4 pg/mL) were detected. However, no IFN-γ, IL-4, and IL-10 were detected. In contrast, large amounts of IFN-γ (825 ± 70 pg/mL), TNF-α (233 ± 21 pg/mL), and IL-10 (416 ± 68 pg/mL) as well as IL-6 (193 ± 35 pg/mL) were produced when T cells were stimulated via CD3 with DCs, although no IL-2 activity was detected at this time point.

### Cytokine secretion by developing cells during the culture

Cytokines secreted during the culture are believed to have an important autocrine and paracrine effect on developing cells both in

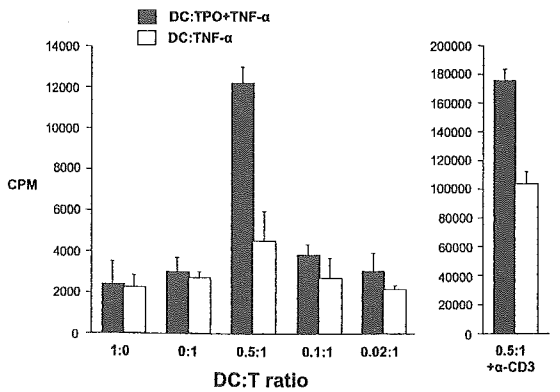


**Figure 5. Autologous T-cell proliferation by DCs generated in the presence of TPO and TNF-α.** The cells prepared by culture for 7 days with 100 ng/mL TNF-α in the presence (■) or absence (□) of 100 ng/mL TPO were used at graded doses as stimulator cells after γ-irradiation. In some MLRs, anti-CD3 mAb was added. Proliferation was assessed by adding $^3$H-thymidine (1 μCi per well [37 kBq/well]) during 72 to 90 hours of culture. Results represent the mean ± SD of triplicate cultures.