Cytolytic activity and regulatory functions of inhibitory NK cell receptor–expressing T cells expanded from granulocyte colony-stimulating factor–mobilized peripheral blood mononuclear cells

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Inhibitory natural killer cell receptor (NKR)–expressing cells may induce a graft-versus-leukemia/tumor (GVL/T) effect against leukemic cells and tumor cells that have mismatched or decreased expression of HLA class I molecules and may not cause graft-versus-host disease (GVHD) against host cells that have normal expression of HLA class I molecules. In our study, we were able to expand inhibitory NKR (CD94/NKG2A)–expressing CD8+ T cells from granulocyte colony-stimulating factor (G-CSF)–mobilized peripheral blood mononuclear cells (G-PBMCs) by more than 500-fold using stimulation by an anti-CD3 monoclonal antibody with interleukin 15 (IL-15). These expanded and purified CD94-expressing cells attacked various malignant cell lines, including solid cancer cell lines, as well as the patients’ leukemic cells but not autologous and allogeneic phytohemagglutinin (PHA) blasts in vitro. Also, these CD94-expressing cells prevented the growth of K562 leukemic cells and CW2 colon cancer cells in NOD/SCID mice in vivo. On the other hand, the CD94–expressing cells have low responsiveness to alloantigen in mixed lymphocyte culture (MLC) and have high transforming growth factor (TGF)–β1– but low IL-2–producing capacity. Therefore, CD94-expressing cells with cytolytic activity against the recipient’s leukemic and tumor cells without enhancement of alloresponse might be able to be expanded from donor G-PBMCs.

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of NKRs, and ligands may be useful for induction of the GVL effect during allo SCT.20-22

Although granulocyte colony-stimulating factor (G-CSF)–mobilized peripheral blood mononuclear cell (PBMC) grafts contain at least 10 times more T cells than do standard bone marrow grafts, the incidence and severity of acute graft-versus-host disease (aGVHD) after allogeneic peripheral blood stem cell transplantation (PBSCT) are not higher than those observed with allogeneic marrow. Also, there is a possibility that allogeneic PBSCT prevents leukemia relapse by induction of the GVL effect.23-25 It was previously reported that G-PBMC leukapheresis products contain large numbers of CD14+ cells, which suppress donor T-cell proliferation in a dose-dependent fashion.26,27 Also, we have shown that the induction of a costimulatory molecule, CD28, responsive complex, in CD4+ cells appears to be suppressed by the presence of CD14+ cells in G-PBMCs.28 Therefore, it seems useful to use G-PBMCs as a source of lymphocytes in order to manipulate cells for cell therapy to modulate GVHD and GVL. In this study, we expanded NKR-expressing T cells from donor G-PBMCs and investigated their cytolytic characteristics and regulatory functions.

Materials and methods

Donors and G-CSF mobilization

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. PBMCs before administration of G-CSF (PrefG-PBMC) and G-PBMC samples were cryopreserved to enable simultaneous testing.

Immunofluorescent staining for flow cytometric analysis and monoclonal antibodies

The phycoerythrin (PE)–conjugated monoclonal antibody (mAb) HP-3D9 (anti-CD94) was obtained from Ancell (Bayport, MN), and Z199 (anti-NKG2A), ONT7 (anti-NKG2D), Z231 (anti-NKp44), and C1.7 (anti-CD244) were obtained from Immunotec (Marseilles, France). Fluorescein isothiocyanate (FITC)–conjugated anti-CD3, anti-CD8 mAb, and anti-HLA-A, -B, -C mAb (G46-2.6) were purchased from Pharmingen (San Diego, CA). Anti-CD35 mAb and anti-granzyme A mAb were obtained from Becton Dickinson (BD, San Jose, CA). Anti–HLA class I mAb BRA-23/9 and W6/32 were obtained from NeoMarkers (Fremont, CA), and rat anti–HLA class I mAb (YTH862.2) was obtained from Serotec (Oxford, England). Anti-CD3 mAb OKT3 was obtained from Ortho Biotech (Raritan, NJ). Anti-NKG2C and anti-NKG2D mAb were obtained from R&D Systems (Minneapolis, MN). Intracellular granzyme A was stained using cytofix/cytoperm reagent according to the manufacturer’s instructions (Becton Dickinson). The fluorescence intensity of the cells was analyzed using a FACS Calibur (Becton Dickinson). Statistical analysis was performed using Student t test.

Immunomagnetic cell sorting

Purified CD14+ cells (> 95% CD14+, as determined by flow cytometric analysis), CD8+ cells (> 90% CD8+), and CD94+ cells (> 90% CD94+) were obtained by magnetic cell sorting (MACS) using magnetic microbeads according to the manufacturer’s instructions (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany).

Induction of CD94/NKG2A on CD8+ T cells by stimulation with immobilized anti-CD3 monoclonal antibody and IL-15

For coating with anti-CD3 mAb, 24-well flat-bottom plates or tissue culture flasks were preincubated with OKT3 (1 μg/mL) in 100 mM Tris [tris(hy-
stimulated by phorbol myristate acetate (PMA) (10 ng/mL) and ionomycin (500 ng/mL) after 1 day were estimated. TGF-β1 was measured by using a human TGF-β1 enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems), and other cytokines were measured by using a LiquiChip Human Cytokine System (Qiagen, Tokyo, Japan) according to the manufacturer’s instructions.

**NOD/SCID mice**

Female 5- to 8-week-old NOD/SCID mice were purchased from Clea (Tokyo, Japan). Breeding and maintenance were performed in a micro-isolator under sterile conditions. K562 cells or CW2 colon cancer cells with or without purified CD94-expressing cells expanded from G-PBMCs were suspended in 0.5 mL phosphate-buffered saline (PBS) and injected subcutaneously into the right flanks of the NOD/SCID mice. NOD/SCID mice did not receive irradiation or anti-ASGM1 antibody.

**Results**

**Induction of CD94/NKG2A expression on CD8+ T cells in G-PBMCs by immobilized anti-CD3 monoclonal antibody stimulation with IL-15**

We found that the proportion of CD94/NKG2A-expressing CD3+ / CD8+ T cells in G-PBMCs was increased after immobilized anti-CD3 mAb stimulation (Table 1). Although there was no difference between the proportions of CD94/NKG2A-expressing T cells in PBMCs obtained from 7 donors before administration of G-CSF (PreG-PBMCs) and after administration of G-CSF (G-PBMCs) without stimulation, the proportions of CD94/NKG2A-expressing T cells derived from G-PBMCs after 7 days of stimulation with immobilized anti-CD3 mAb both with and without IL-15 were significantly higher than the proportions of CD94/NKG2A-expressing T cells derived from PreG-PBMCs (Table 1). We also found that the proportions of CD94/NKG2A-expressing CD8+ T cells that had been purified from G-PBMCs before culture were increased by immobilized anti-CD3 mAb stimulation with IL-15 (Table 2). The addition of 3 × 10⁶ purified CD14+ cells derived from the same G-PBMCs to purified CD8+ T cells induced much more CD94/NKG2A expression on those purified CD8+ T cells. This effect of purified CD14+ cells tended to be inhibited by the use of a membrane (Table 2). These results suggest that CD14+ cells play an important role in the induction of CD94/NKG2A expression on T cells and that this effect might require at least partial contact between responder cells and CD14+ cells.

Furthermore, it was revealed that CD94/NKG2A expression on purified CD8+ T cells from G-PBMCs could be induced in our culture system. TCR engagement has been reported to play an important role in the induction of inhibitory NKRs on CD8+ T cells. Several cytokines, such as IL-12 and IL-15, are known to be CD94/NKG2A-inducible cytokines. It is possible that IL-15 induces inhibitory NKRs on CD8+ T cells derived from G-PBMCs during T-cell activation by the immobilized anti-CD3 mAb.

**Expansion of CD94+ expressing cells from G-PBMCs**

PreG-PBMCs and G-PBMCs contained almost equal numbers of CD94/NKG2A-expressing T cells before stimulation. CD94/ NKG2A-expressing CD8+ T cells from both PreG-PBMCs and G-PBMCs were expanded by more than 100-fold after 7 days of culture. Moreover, a significantly greater number of CD94/NKG2A-expressing T cells was obtained from G-PBMCs than from PreG-PBMCs (Table 3). CD94+ cells (> 90% CD94+) were obtained by MACS using magnetic microbeads, and more than 80% of CD94+ expressing cells coexpressed CD8 (data not shown). The CD94-depleted cells contained only low CD94+ expressing cells (mean fluorescence intensity [MFI], CD94-depleted cells vs CD94-expressing cells, 24.7 ± 7.2 vs 234.1 ± 30.5, n = 7).

Moreover, these CD94+ cells contained granzyme A, which is an important enzyme for induction of apoptosis of target cells in the cytoplasm. Also, CD94-expressing cells expanded from G-PBMCs had a large repertoire of TCR-Vβ, as revealed by reverse transcriptase–polymerase chain reaction (RT-PCR) analysis using 26 kinds of TCR-Vβ primer pairs (data not shown). These expanded CD8+ T cells expressed NKG2D and CD244 but did not express CD158a, CD158b, NKB1, CD161, nor NKp44 (data not shown).

**Characteristics of cytolytic activities of CD94+ expressing cells expanded from G-PBMCs against K562 leukemic cells**

We investigated the characteristics of cytolytic activity of CD94+ expressing cells expanded from donor G-PBMCs. The cytolytic activity level of purified CD94+ expressing cells detected by a standard 4-hour ⁵¹Cr release assay against HLA class I–deficient K562 cells was found to be always higher than that of CD94-depleted cells and also higher than that against autologous PHA blasts (Figure 1A). Furthermore, the cytolytic activity level of CD94+ expressing cells against allogeneic PHA blasts was the same as that against autologous PHA blasts (Figure 1B). HLA class I expression is inducible on K562 cells by IFN-γ. The MFI s of HLA class I molecules on untreated K562 and IFN–γ–treated K562 cells were 521 ± 146 (n = 5) and 901 ± 25.5 (n = 6), respectively. Although we did not show the surface expression level of HLA-E on HLA class I–expressing cells, we could show HLA-E mRNA induction in K562 cells I–expressing K562 cells in an RT-PCR experiment (data not shown). Therefore, the leader peptide of HLA class I stabilizes HLA-E expression and subsequently may be able to induce a higher level of HLA-E expression on HLA class I–expressing cells. The cytolytic activity of CD94+ expressing cells against IFN–γ–treated K562 cells was attenuated compared with

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<th>Before</th>
<th>After anti-CD3 stimulation</th>
<th>Anti-CD3 and IL-15</th>
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<tr>
<td></td>
<td>PreG</td>
<td>G-PBMC</td>
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<tr>
<td>CD94+ /CD3+</td>
<td>5.7 ± 3.1</td>
<td>4.6 ± 1.9</td>
<td>8.4 ± 4.4</td>
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<td>CD94+ /CD8+</td>
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<td>NKG2A+ /CD3+</td>
<td>1.8 ± 1.4</td>
<td>1.8 ± 0.7</td>
<td>3.0 ± 1.9</td>
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<td>NKG2A+ /CD8+</td>
<td>0.5 ± 0.3</td>
<td>0.4 ± 0.5</td>
<td>2.3 ± 1.3</td>
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</table>

Values indicate the percentage of CD94 or NKG2A+ expressing cells (means ± SDs, n = 7). Significant difference were noted when comparing the value of PreG and G-PBMC after stimulation with and without IL-15 (*P < .01; †P < .05, ‡P < .1).
Comparing the value of PreG and G-PBMC after stimulation (*P < .05, †P < .01, ‡P < .005), we noted significant differences when comparing the value of before and after stimulation; CD8* cell only and addition of purified 3 × 10^6 CD14* cells without membrane; and the contact inhibition by the membrane.

Table 2. Induction of CD94/NKG2A expression on purified CD8+ cells from G-PBMC

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Values indicate the percentage of CD94 or NKG2A-expressing cells after anti-CD3 stimulation in the presence of IL-15 (means ± SDs, n = 7). Significant differences were noted when comparing the value of before and after stimulation; CD8* cell only and addition of purified 3 × 10^6 CD14* cells; without membrane; and the contact inhibition by the membrane.

**Table 3. Expansion of CD94/NKG2A-expressing cells from paired PreG- and G-PBMC**

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Cultures were started from 2.5 × 10^6 mononuclear cells. Values indicate absolute number of cells before and after culture. Significant differences were noted when comparing the value of PreG and G-PBMC after stimulation (*P < .05, †P < .01).

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Discussion

In this study, we found that the proportion of CD94/NKG2A-expressing CD3+CD8+ T cells in G-PBMCs was increased after immobilized anti-CD3 mAb stimulation with IL-15. We also found that CD14+ cells derived from G-PBMCs play an important role in the induction of CD94/NKG2A expression on purified CD8+ T cells. Therefore, CD8+ T cells derived from G-PBMCs could express CD94/NKG2A after stimulation. Also, we were able to expand CD94-expressing CD8+ T cells from donor G-PBMCs by more than 500-fold. The absolute number of CD94-expressing T cells after stimulation from G-PBMCs was significantly higher than that from PreG-PBMCs. It is possible that a greater number of CD14+ cells in G-PBMCs than in PreG-PBMCs can stimulate the first signal through TCR. This TCR engagement has been reported to play an important role in the induction of inhibitory NKRs on CD8+ T cells.6 Although we showed a CD94-inducing effect of CD14+ cells in a contact-dependent manner, other factors such as cytokines may be implicated in this effect. Also, it is not clear enough whether G-CSF has an effect on progenitor cells of CD94/NKG2A-expressing cells. Nevertheless, G-PBMCs, which are easy to obtain and store at the time of PBSC collection from the donor, may be a useful source for the expansion of inhibitory NKR-expressing cells.

These expanded and purified CD94-expressing cells had CD8 expression but not CD56 expression on their surfaces. Also, these CD94-expressing cells contained granzyme A in the cytoplasm and had a large repertoire of TCR-Vβ as revealed by RT-PCR analysis using 26 kinds of TCR-Vβ primer pairs. Furthermore, these expanded CD8 T cells did not express other killer cell immunoglobulin-like receptors (KIRs) such as CD158a, CD158b, or NKB1 or NK cell–activating markers such as CD161 or Nkp44, but they did express NK cell–activating receptors NKG2D and CD244. Therefore, these cells have both inhibitory receptors (CD94/NKG2A) and activating receptors (NKG2D). The cytolytic activity of CD94-expressing cells depends at least partially on NKG2D-activating NKR, because anti-NKG2D mAb suppressed this activity. However, it is possible that other receptors that were not analyzed in this study are involved in the killing activity.

HLA-E, a CD94/NKG2A ligand, preferably bound to a peptide derived from the signal sequences of most HLA-A, -B, -C, and -G and was also up-regulated by these peptides.31 We investigated the characteristics of cytolytic activities of CD94-expressing cells using IFN-γ-induced HLA class I molecule–expressing K562 cells that had increased mRNA of HLA-E. HLA-C signal peptide was found to suppress the cytolytic activity of CD94-expressing cells against IFN-γ-induced HLA class I molecule–expressing K562 cells. Also, anti-NKG2A mAb and some anti–HLA class I mAbs partially restored the cytolytic activity of CD94-expressing cells against HLA class I molecule–protected K562 cells. In addition, results of analysis of the cytolytic activities of CD94-expressing cells against 10 malignant cell lines, including 3 solid cancer cell lines, indicated that this killing activity roughly depended on the expression of HLA class I molecules on the cell surface. However, the cytolytic activity of CD94-expressing cells does not depend entirely on the expression of HLA class I molecules. The cytolytic activity of CD94-expressing cells may be regulated by the balance among the expression levels of HLA class I, HLA-E itself, and certain molecules on target cells.

We also investigated the cytolytic activities of CD94-expressing cells against 17 patients’ primary leukemic cells. Donor and allogeneic CD94-expressing cells could attack patients’ CML cells and AML cells but could not attack some patients’ leukemic cells such as ATL cells, which had high expression levels of HLA class I molecules. Also, the addition of anti–HLA class I mAb induced
restoration of the cytolytic activity of CD94-expressing cells against PHA blasts and ALL (Ph1) but not ATL cells. Although these CD94-expressing cells attacked HLA class I low-intermediate patients’ leukemic cells, the killing activity varied, depending on the type of leukemia. Patients’ leukemic cells have different expression levels of HLA class I, and they may have different expression levels of other regulatory molecules for the killing activities of CD94-expressing cells. Therefore, not only HLA class I molecules on leukemic cells but also other molecules such as adhesion molecules and stimulatory NKR ligands such as MHC class I chain–related protein (MIC) and activating molecules on effector cells might be important for the regulation of these killing activities.

In vivo analysis revealed that CD94-expressing cells prevented the growth of K562 leukemic cells and also CW2 colon cancer cells in NOD/SCID mice. These models suggest that CD94-expressing cells may therefore have a graft-versus-leukemia/tumor effect.

In addition, the CD94-expressing cells exhibited low proliferative capacity in MLC and high TGF-β1 productivity with attenuated IL-2 productivity. These cells therefore have low responsiveness to alloantigens and may also have a suppressive effect on HLA class I–induced alloresponse.

We previously reported increased expression of CD158 and CD94/NKG2A on T cells in chronic GVHD patients with good prognosis and showed that these inhibitory NKR-expressing cells have a suppressive effect on allogeneic response in MLC. Therefore, inhibitory NKR expression during allogeneic stimulation after allo SCT may play an important role in modulation of GVHD. Based on clinical and experimental data, we speculate that these inhibitory NKR-expressing cells have a GVL/T effect against leukemic cells and tumor cells that have decreased expression levels of HLA class I molecules and do not enhance GVHD against host cells that have normal expression levels of HLA class I molecules.

Elucidation of cytolytic characteristics, proliferative characteristics, and cytokine productivity of inhibitory NKR-expressing cells might provide clues about how to control the delicate balance between GVHD and GVL. It may be possible to use expanded CD94-expressing cells from donor G-PBMCs, which contain a large number of T cells, for allogeneic cell therapy instead of naive donor lymphocyte infusion to induce the GVL effect without enhancing GVHD. Donor G-PBMCs, which are an alternative stem cell source for allogeneic stem cell transplantation, might also be a useful source of lymphocytes for expanding NKR-expressing cells for cell therapy for some patients whose leukemic cells and tumor cells have escaped from allogeneic recognition by usual cytotoxic T cells because of the low expression level of HLA class I molecules.

**Acknowledgments**

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References


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