Sequential Modulation of Growth Factors: A Novel Strategy for Adoptive Immunotherapy of Acute Myeloid Leukemia

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ABSTRACT
Evidence from allogeneic hematopoietic stem cell transplantation indicates a possible immune response against leukemia-associated antigens in patients with either acute myeloid leukemia (AML) or chronic myeloid leukemia (CML). However, autologous immune responses are less evident. We have developed a method using sequential modulation of growth factors (SMGF) to generate specific anti-AML T-cells from primary cultures of mononuclear cells (MNCs) from patients with AML. This culture method induces greater degrees of antigen presentation by inducing dendritic cell (DC) differentiation of AML in the presence of autologous lymphocytes, which are then expanded by interleukin (IL)-2 and costimulatory molecule ligation. MNCs consisting of 92.3% ± 5.1% AML blasts and 3.4% ± 3.2% CD3+ T-cells were obtained from AML patients (n = 12) and cultured in AIM-V medium with IL-4 and recombinant granulocyte-monocyte colony-stimulating factor. Recombinant IL-2 was added on day 8. On day 21, culture conditions were changed to anti-CD3/anti-CD28 monoclonal antibodies and IL-2. By day 42, 354 ± 182-fold CD3+ T-cell expansion had occurred. Cytotoxic T-lymphocyte assays demonstrated that these T-cells caused significant lysis of autologous leukemia cells and AML cell lines, but not of cells of other lineages, in an HLA class I-dependent manner. Specific Vβ subgroups (Vβ3, -7, and -12a), possibly representing T-cell clones specific to AML-specific antigens, were expanded in the cultures of cells from 3 AML patients. SMGF can be used to induce and expand autologous T-cells with HLA class I-dependent antileukemia potential from the peripheral blood of AML patients. Adoptive transfer of these expanded T-cells to patients is a possible therapeutic approach for further study.

KEY WORDS
Autologous immune response • Acute myeloid leukemia • Dendritic cells

INTRODUCTION
The role of the immune system in the eradication of residual leukemia after hematopoietic stem cell transplantation (HSCT) is well established. There is evidence that removal of T-lymphocytes from the allogeneic graft to reduce the risk of graft-versus-host disease (GVHD) is associated with an increased incidence of recurrence of leukemia after transplantation, whereas the occurrence of GVHD correlates with decreased risk of relapse [1-10]. Attempts have been made to enhance the antileukemic effect and reduce the risk of GVHD. These attempts have had limited success because the therapeutic window between the graft-versus-leukemia (GVL) effect and GVHD is difficult to define. Although allogeneic HSCT exhibits a stronger GVL effect than does autologous bone marrow transplantation (ABMT), detection of antileukemia-specific activity has also been observed spontaneously post-ABMT [11,12]. Increasing evidence indicates that at least one element of the curative potential of intensive chemotherapy is the patient immune system. Patients can spontaneously develop immunity to residual leukemia after chemotherapy; this immunity can be induced and measured in vitro [13,14]. Therefore, induction and expansion of autologous leukemia–specific T-cells might lead to successful elimination of residual acute myeloid leukemia (AML) without GVHD. These studies imply that in allogeneic HSCT, ABMT, and chemotherapy, immunological responses have key roles in the ultimate eradication of residual leukemia cells.
Autologous cytotoxic effector cells can be activated and expanded in untreated AML by high-dose interleukin (IL)-2 [15-18]. Bone marrow collected from newly diagnosed AML patients was used to derive T-cell clones generated in IL-2-containing media; these clones showed cytolytic activity against autologous and allogeneic AML blast cells. Leukemic blast cells were susceptible to autologous lymphokine-activated killer (LAK) cells [19-22]. Passive immunotherapy with autologous LAK cells is an attractive treatment option, but to eradicate residual leukemia, continued immunological memory in the form of induction and expansion of autologous anti-leukemia-specific T-cells is required. Both cellular and humoral immunity is suppressed in active AML. Immunosuppressive effects mediated by AML blasts may cause this effect. AML blasts suppress host immune responses by release of immunosuppressive soluble mediators such as transforming growth factor β [23-25], soluble IL-2 receptors [26-28], and soluble adhesion molecules [29-30]. AML blasts escape the host immune attack by mechanisms such as decreased expression of HLA and costimulatory molecules and down-regulation of CD3 [31-33]. These mechanisms lead to failure of antigen presentation and failure of immune response against AML blasts. Because of the immunosuppressive effects and the overwhelming growth of AML blasts, only small numbers of immunocompetent cells are present in AML patients before treatment. It is widely accepted that immunotherapy of AML will probably work best in a minimal residual disease setting after chemotherapy. Although chemotherapy eradicates most AML blasts, it also eradicates the few and potentially important AML-specific immunocompetent cells present at diagnosis. Our hypothesis is that just as T-cell depletion in allogeneic BMT increases the risk of relapse, chemotherapy-mediated depletion of immunocompetent T-cells may abrogate their potential beneficial effects.

Dendritic cells (DCs) that have been obtained from patients in complete remission (CR) and pulsed with leukemia-derived peptides or cell lysates have been used to obtain leukemia-specific T-cell effectors [34-36]. However, in many situations, the tumor antigen(s) are unknown or impossible to isolate. To avoid the need to identify and isolate leukemia-specific or -associated immunogenic peptides, cytokines have been used to induce DC differentiation from AML blasts. A DC phenotype with increased B7 expression can be induced after in vitro exposure of native AML blasts to several cytokine combinations. Such phenotypically altered AML cells appear to be more efficient than native blasts as antigen-presenting cells for the presentation of leukemia-restricted peptides to T-cells. AML DCs can be used to induce and activate leukemia-specific T-cells from allogeneic peripheral blood mononuclear cells (PBMCs) obtained from healthy bone marrow donors or autologous PBMCs obtained from patients with AML in CR [37-45].

In this study, we investigated whether T-cells with anti-AML specificity can be expanded from the PB of AML patients with active disease. We reasoned that the blood of a patient at presentation would contain all of the elements necessary to elicit an immune response. Therefore, we designed a tissue culture protocol involving sequential modulation of growth factors (SMGF) to induce DC differentiation of AML blasts and subsequent antigen-specific T-cell proliferation.

**MATERIAL AND METHODS**

**Patient Samples**

PB samples were collected from patients who had given informed consent for diagnostic tests under the auspices of the institutional review board of the UCSD Medical Center. After Ficoll-Hypaque-gradient centrifugation, the mononuclear cells separated from patient blood were collected, washed, and cultured or cryopreserved immediately.

**Cells**

The HL60, U937, KG1a myeloid leukemia; Daudi, RPMI-8866 lymphoma; H345 breast cancer; and DMS273, H69 small cell lung cancer cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD). NB4 cells, which carry a 15:17 chromosomal translocation, were kindly provided by Dr. M. Lanotte [46]. Cell lines were cultured in RPMI-1640 containing 10% fetal bovine serum (Hazelton Dutchland, Danver, PA) in a humidified atmosphere of 5% CO2. Cells in log phase were freshly harvested from culture, washed 3 times, and resuspended in RPMI-1640 before use.

**SMGF Culture of AML PBMCs**

Primary AML patient mononuclear cells isolated from PB were suspended in culture medium (AIM-V medium [Gibco BRL, Gaithersburg, MD] containing 10% human AB serum) or culture medium supplemented with IL-4 (1000 U/mL) (kindly provided by Schering-Plough Research Institute, Kenilworth, NJ) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (50 ng/mL) (Immunex, Seattle, WA) at a concentration of 4 × 106 cells/mL and cultured in 6-well culture plates (Corning, Corning, NY). Every 4 to 5 days half of the culture medium was removed and the remaining medium supplemented with fresh medium containing growth factors. IL-2 (20 U/mL) (Collaborative Research, Bedford, MA) was added to the culture at day 8. At day 21, IL-2; anti-CD3 monoclonal antibody (MoAb), 10 ng/mL (clone: HIT3a, mouse immunoglobulin [Ig]G2a; BD PharMingen, San Diego, CA); and anti-CD28 MoAb, 5 μg/mL (clone: CD28.2; BD PharMingen) were added with fresh medium. The culture was continued for another 1 to 3 weeks with addition of fresh medium with IL-2/anti-CD3/anti-CD28 every 4 to 5 days. Viable cell numbers in the cultures were measured weekly by trypan blue exclusion. For some experiments, the T-cells were further cultured in medium containing IL-2 (20 U/mL) and 5% irradiated cryopreserved autologous AML DCs (AML PBMCs cultured for 8 days with IL-4/GM-CSF) for 1 to 3 weeks before cytotoxic T-lymphocyte (CTL) assay.

**Flow Cytometry Analysis**

The immunophenotypes of primary AML PBMCs were determined using flow cytometry. CD1a, CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD13, CD14, CD15, CD16, CD19, CD20, CD33, CD34, CD40, CD54, CD64, CD80, CD86, CD83, CD95, HLA-DR, and TdT expression were analyzed. For determination of DC differentiation of AML blasts, cells were stained with CD3, CD4, CD8, CD14, CD16, CD33, CD34, CD40, CD64, CD80, CD86, CD83, and HLA-DR at day 8 of culture. To evaluate T-cell priming and proliferation, CD3, CD4, CD8, CD16, CD33, and
CD56 expression were examined weekly after day 14 to the end of culture. MoAbs were purchased from Becton Dickinson (San Jose, CA).

**Morphology Study**
The morphologic change of AML cells in the cultures was examined with a phase-contrast transmission microscope at days 4 and 8. The cytopsin slides of AML cells from day 8 of SMGF culture were stained with Wright-Giemsa for microscopic examination.

**Cytotoxicity Assays**
The cytotoxic effects against autologous AML blasts, AML cell lines, lymphoma cell lines, breast cancer cell lines, and lung cancer cell lines of AML T-cells obtained from SMGF culture were analyzed in standard 4-hour $^{51}$Cr release assays (New England Nuclear, Boston, MA) as previously described [47]. T-cells used as cytotoxic effectors were obtained from SMGF culture and serially diluted (in 3 replicate wells) in 96-well plates. Target cells were incubated for 120 minutes at 37°C with $^{51}$Cr at 100 μCi/2 x 10^6 cells. The cells were washed 3 times, and 5 x 10^5 cells were added to each well. There were 6 wells containing only target cells (spontaneous-release control) and 6 wells containing target cells with 0.1 mL of 5% Triton X-100 (maximum-release control). The plates were incubated at 37°C in a 5% CO₂ humidified atmosphere for 4 hours, then samples were harvested and counted on 1450 MicroBeta TriLux (Wallac, Turku, Finland). The percentage of specific lysis was determined by the formula:

$$\text{(Experimental mean cpm – spontaneous release mean cpm)} \times 100$$

For the CTL blocking study, CTL analysis was performed after $^{51}$Cr-labeled target cells were incubated with 100 μg/mL of 251 (anti-CD33, mouse IgG1) (Medarex, Princeton, NJ), PM81 (anti-CD15, mouse IgM) (Medarex), and W6/32 (anti-HLA class I, 1:100 diluted ascites, mouse IgG2a) for 30 minutes at room temperature.

**Colony-Forming Cell Assay**
Leukemia colony-forming cell (CFC) assay was performed as previously described [48]. Briefly, AML T-cells obtained from SMGF culture were serially diluted in 3 replicates in 96-well plates and mixed with HL60, NB4, U937 cells (5000 cell/well) in a final volume of 200 μL of RPMI-1640 culture medium supplemented with 10% fetal calf serum in 96-well U-bottom plates. The plates were incubated for 2 hours (AML cell lines) at 37°C in 5% CO₂. The cells in each well of AML cell lines were then suspended, mixed with 0.9 mL MethoCult H4230 (StemCell Technologies, Vancouver, BC, Canada) and 100 μL of conditioned medium of the respective cell line, and seeded into 35 mm culture dishes. Cultures were maintained for 7 days in a humidified atmosphere of 5% CO₂, and an inverted microscope was used to score cultures for leukemia cell colonies containing 50 or more cells.

**T-Cell Receptor Vβ Gene Repertoire Analysis**
The anchored reverse transcriptase polymerase chain reaction (RT-PCR) enzyme-linked immunosorbert assay (ELISA) technique [49,50] was used to determine the percent expression of each Vβ subgroup in the T-cell populations pre- and post-SMGF culture. Total RNA was extracted from 5 x 10^7 AML PBMCs or 5 x 10^6 T-cells of SMGF culture with the RNeasy Kit (Qiagen, Valencia, CA). First-strand complementary DNA (cDNA) was synthesized using 5 μg of the total RNA, an oligo-dT primer, and Superscript RT (Gibco BRL) as described [49,50]. The purified cDNA was poly-dG tailed using dGTP and terminal deoxynucleotase (Boehringer Mannheim, Indianapolis, IN) and purified using QIAquik purification columns (Qiagen). One fourth of the sample was subjected to primary anchored PCR amplification using an antisense oligonucleotide primer specific for the constant region of T-cell receptor (TCR) (C₆) and a 9:1 mixture of 2 anchor sense-strand primers. The PCR products were purified using QIAquik purification columns, and one third of this product was used as a template for a nested PCR. This second PCR reaction was the same as the primary anchored (AN) PCR except a 5’ biotinylated C₆-antisense primer was used that was upstream of the initial C₆ primer. The nested PCR product was purified using QIAquik purification columns and distributed onto ELISA wells that had been precoated with streptavidin (Sigma, St. Louis, MO). Oligonucleotides corresponding to the 32-Vβ [51] or 24-Vβ [49] subgroup-specific sense-strand sequences were labeled with digoxigenin and terminal deoxynucleotase (Boehringer Mannheim). The captured double-stranded PCR products were denatured with 0.1N NaOH and, after washing, were hybridized with each of the digoxigenin-labeled TCR Vβ oligonucleotide probes. The bound probes were detected with peroxidase-conjugated antidigoxigenin antibody (Boehringer Mannheim, Mannheim, Germany) and its substrate 3,3’,5,5’-tetramethylbenzidine peroxidase (Kirkegaard & Perry Laboratories, Gaithersburg, MD). The optical density (OD) at 450 nm was determined using an ELISA microplate reader (Molecular Devices, Menlo Park, CA). The relative expression of each subgroup was calculated by dividing the OD reading of each subgroup by the sum of the OD readings of all subgroups times 100.

**Statistical Analysis**
The significance level was determined by the Student t test when applicable.

**RESULTS**

**SMGF Culture for DC Differentiation of AML Blasts**
Our hypothesis was that the SMGF protocol would induce DC differentiation of AML blasts and activate the T-cells in AML PBMCs with IL-4/GM-CSF in the first stage (days 0-8) and induce AML-specific T-cells with IL-4/GM-CSF and low-dose IL-2 in the second stage (days 8-21). In the third stage, we expected to observe activation and expansion of AML-specific T-cells with IL-2/anti-CD3/anti-CD28 (days 21-35).

We studied 12 primary AML patient samples using SMGF. The clinical characteristics of the patients and the AML cell phenotypes before SMGF culture are shown in Table 1. PBMCs from AML patients at first presentation or...
relapse were obtained and immunophenotyped. The cells consisted of 92.3% ± 5.1% CD33+ or CD34+ AML blasts (range, 80.7%-98.7%) and 3.4% ± 3.2% CD3+ T-cells (range, 0.8%-13%). Unfractionated PBMCs were cultured in AIM-V medium supplemented with IL-4 and GM-CSF to induce DC differentiation of AML blasts. Cells cultured in medium alone were used as controls. After 8 days, the expression of CD64 on AML blasts cultured in GM-CSF/IL-4 was down-regulated and HLA-DR, CD80, and CD86 were up-regulated; a substantial proportion of cells (60%-70%) showed DC morphology (Figure 1). Figure 2 shows the flow cytometry results for cells from 1 AML subject (patient 4), showing up-regulation of CD80, CD86, and HLA-DR expression on AML cells. The geometric mean (±SD) of mean fluorescence intensity from 11 AML PBMCs after 8 days of culture with GM-CSF/IL-4 (Figure 2) is significantly up-regulated. However, AML PBMCs cultured with medium alone showed no significant changes (data not shown).

SMGF Culture for Generation of T-Cells

Fresh medium with GM-CSF/IL-4 and IL-2 (20 U/mL) was added at day 8. At 21 days, flow cytometric analysis results showed the proportion of CD3+ cells was increased variably from 7% to 72%. Cells with DC morphology were alive and well in the SMGF cultures. IL-2, anti-CD3, and CD28 MoAb were added with fresh medium at 21 days. Accelerated CD33+ AML blast decrease and CD3+ T-cell increase was observed. Pure T-cells in the culture were obtained within 2 to 3 weeks. According to flow cytometric analysis results, 99.5% ± 0.4% of the cells were CD3+, CD16-, CD56- T-cells with 40.6% ± 22.9% CD4+ cells and 57.8% ± 22.8% CD8+ cells. The changes in the cell populations of 1 representative patient (patient 1) for 12 experiments (expressed as the percentage of different cell phenotypes during SMGF culture) is shown in Figure 3.

Results from 12 experiments showed that the duration of culture needed to generate more than 99% T-cells in SMGF culture differed among samples from different AML patients, ranging from 21 days to 42 days (average, 31.3 ± 6.1 days). Figure 4 summarizes patterns of T-cell development in SMGF culture. The average percentage of CD4+ T-cells increased faster than that of CD8+ T-cells in the first and second phases. Average percentages were 1.5% ± 1.5% CD4+ versus 2.1% ± 1.9% CD8+ T-cells on day 0, 4.0 ± 4.2% CD4+ versus 2.7% ± 2.8% CD8+ T-cells on day 8, and 17.1% ± 17% CD4+ versus 11.5% ± 7.7% CD8+ T-cells on day 21. CD8+ T-cell proliferation was then accelerated in the third phase, with an average percentage of 57.8% ± 22.8% CD8+ versus 40.6% ± 22.9% CD4+ T-cells when more than 99% of T-cells were obtained in the culture at an average of 31 days. These results suggest the enhancement effect of IL-2 and IL-4 on activation and proliferation of CD4 T-cells in the first and second phases; these T-cells then contributed to the development of cytotoxic T-cells by providing a priming signal to the AML antigen-presenting cells. The data of the first 6 experiments indicated that at day 42, CD3+ T-cell expansion was 354 ± 182 times, CD4+ T-cell expansion was 445 ± 505 times, and CD8+ T-cell expansion was 228 ± 258 times. The T-cells in SMGF culture could be continuously expanded beyond 60 days by changing medium and re-adding IL-2/anti-CD3/anti-CD28. Based on the magnitude of T-cell expansion observed by day

![Figure 1. Morphology of AML cells cultured in GM-CSF/IL-4.](image)

Wright-Giemsa staining of cytopsin slides showed AML cells with dendritic morphology after 8 to 10 days.

### Table 1. Characteristics of AML Patients

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age, y</th>
<th>Sex</th>
<th>FAB Subtype</th>
<th>CD33+, %</th>
<th>CD34+, %</th>
<th>CD3+, %</th>
<th>CD4+, %</th>
<th>CD8+, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>30</td>
<td>F</td>
<td>M5</td>
<td>96</td>
<td>0.1</td>
<td>2.1</td>
<td>0.1</td>
<td>2.0</td>
</tr>
<tr>
<td>Patient 2</td>
<td>33</td>
<td>M</td>
<td>M2</td>
<td>86</td>
<td>0.3</td>
<td>13</td>
<td>5.2</td>
<td>7.5</td>
</tr>
<tr>
<td>Patient 3</td>
<td>68</td>
<td>F</td>
<td>M5</td>
<td>95</td>
<td>0.1</td>
<td>2.4</td>
<td>2.4</td>
<td>1.0</td>
</tr>
<tr>
<td>Patient 4</td>
<td>57</td>
<td>M</td>
<td>M5</td>
<td>99</td>
<td>0.2</td>
<td>0.8</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>Patient 5</td>
<td>65</td>
<td>M</td>
<td>M7</td>
<td>12</td>
<td>92.0</td>
<td>3.4</td>
<td>3.0</td>
<td>2.8</td>
</tr>
<tr>
<td>Patient 6</td>
<td>68</td>
<td>M</td>
<td>M2</td>
<td>2</td>
<td>91.6</td>
<td>2.5</td>
<td>0.8</td>
<td>2.1</td>
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<tr>
<td>Patient 7</td>
<td>29</td>
<td>M</td>
<td>M4</td>
<td>94</td>
<td>57.5</td>
<td>2.6</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Patient 8</td>
<td>73</td>
<td>M</td>
<td>M1</td>
<td>53</td>
<td>87.6</td>
<td>2</td>
<td>1.8</td>
<td>0.9</td>
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<tr>
<td>Patient 9</td>
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<td>M</td>
<td>M5</td>
<td>98</td>
<td>0.1</td>
<td>2.4</td>
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<td>1.0</td>
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<tr>
<td>Patient 10</td>
<td>49</td>
<td>F</td>
<td>M4</td>
<td>91</td>
<td>0.5</td>
<td>3.2</td>
<td>2.2</td>
<td>0.6</td>
</tr>
<tr>
<td>Patient 11</td>
<td>60</td>
<td>M</td>
<td>M5</td>
<td>97</td>
<td>10.9</td>
<td>2.2</td>
<td>1</td>
<td>0.8</td>
</tr>
<tr>
<td>Patient 12</td>
<td>62</td>
<td>M</td>
<td>M1</td>
<td>81</td>
<td>73.6</td>
<td>2.2</td>
<td>0.1</td>
<td>2.3</td>
</tr>
</tbody>
</table>

Mean ± SD

|            | 75.2 ± 32.9 | 34.6 ± 39.9 | 3.4 ± 3.2 | 1.5 ± 1.5 | 2.0 ± 1.9 |

*Mean ± SD of the major cell populations of CD33+ or CD34+.
Growth Factor Modulation—New Adoptive Therapy for AML

42, an estimated total of $10^9$ CD8+ T-cells could be obtained from 10 mL peripheral blood of a patient presenting with a white blood cell count of 50,000/µL consisting of 1% T-cells.

Cytotoxicity of T-Cells Obtained Using SMGF Culture of AML Cells

T-cells obtained from 10 of 12 SMGF cultures of AML PBMCs showed similar cytotoxic effects (Table 2). In two cultures, insufficient cells were available for cytotoxicity assays. Standard 4-hour $^{51}$Cr-release assays demonstrated that the T-cells induced and expanded from primary AML patients by SMGF culture with GM-CSF/IL-4 caused significant lysis of autologous leukemia cells and AML cell lines HL-60 (Figure 5), NB4, and U937 (Table 2). There were no significant cytotoxic effects on the cell lines of lymphoma (Daudi, RPMI-8866), breast cancer (H345), small cell lung cancer (DMS273) (Figure 5), AML (KG1a), lung cancer (H69), natural killer cell–sensitive K562 cells, or Epstein-Barr virus immortalized lymphocytoid cell lines (EBV-LCL) (data not shown).

Cultures were set up with cells from selected AML patients to compare unmanipulated AML cells with AML DCs that were induced in SMGF culture because of their ability to stimulate autologous T-cells. Three culture methods were used: group 1, culture medium only; group 2, IL-2/anti-CD3 added at the beginning of the culture and IL-2/anti-CD3/anti-28 added when T-cells reached 90% of total cultured cells; and group 3, 3-step SMGF culture. In groups 2 and 3, addition of anti-CD3/anti-CD28 to the culture was stopped after 100% T-cells were obtained. T-cell cultures were maintained with culture medium supplemented with IL-2 (20 U/mL) and 10% irradiated autologous AML DCs. In group 2, AML blasts were not induced to differentiate into DCs and no anti-CD28 was added in the early phase of the culture to block costimulation of B-7 by AML blasts, so these AML blasts were expected to be able to function as unmanipulated AML cell controls to stimulate autologous T-cells. Group 2 cultured cells were also used to analyze whether the CTLs that existed de novo were in an anergic state and were activated by the anti-CD3/anti-CD28 MoAb. No T-cells grew in group 1. Groups 2 and 3 both obtained pure T-cell cultures in 4 of 4 experiments. For group 3, cultures in 4 of 4 experiments maintained high CTL activity.

Figure 2. Induction of costimulatory molecules CD80, CD86, and HLA-DR on AML blasts cultured in the presence of GM-CSF and IL-4. Primary AML PBMCs were cultured in AIM-V medium supplemented with GM-CSF/IL-4. CD80, CD86, and HLA-DR expression were analyzed by flow cytometry. Mean fluorescence intensity (MFI) changes preculture (gray peak) and on day 8 (black peak) of culture of 1 representative experiment is shown. The average MFIs from 11 AML PBMCs are listed below the figure.

Figure 3. Loss of CD33+ cells and gain of CD3+ (predominately CD8+) T-cells during culture in sequentially modulated growth factors. Cells from primary AML patients were cultured in 3-phase SMGF cultures, including DC differentiation induction of AML blasts with GM-CSF/IL-4, T-cell priming with GM-CSF/IL-4/IL-2, and T-cell expansion with IL-2/anti-CD3/anti-CD28. The changes in the cell populations of cells from 1 representative of 12 experiments (patient 1) are expressed as the percentage of different cell phenotypes during SMGF culture.

<table>
<thead>
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<th>MFI: Day0/Day8; Geometric mean (−SD, +SD):</th>
<th>P &lt; .006</th>
<th>.002</th>
<th>.008</th>
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<td>10(5, 20)/69 (32, 150)</td>
<td>26(5, 81)/238 (120, 832)</td>
<td>289 (56, 1122)/1455 (537, 2951)</td>
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</tbody>
</table>

**Figure 2.** Induction of costimulatory molecules CD80, CD86, and HLA-DR on AML blasts cultured in the presence of GM-CSF and IL-4. Primary AML PBMCs were cultured in AIM-V medium supplemented with GM-CSF/IL-4. CD80, CD86, and HLA-DR expression were analyzed by flow cytometry. Mean fluorescence intensity (MFI) changes preculture (gray peak) and on day 8 (black peak) of culture of 1 representative experiment is shown. The average MFIs from 11 AML PBMCs are listed below the figure.

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3 weeks after anti-CD3/anti-CD28 depletion. However, in 3 of 4 experiments, group 2 cultures lost CTL activity 3 weeks after anti-CD3/anti-CD28 depletion. Figure 6 shows the results from 1 of 4 experiments. These data suggest that IL-2/anti-CD3/anti-CD28 added at the beginning of the AML PBMC culture can also lead to pure T-cell cultures and that these T-cells can exert an immediate killing effect on AML cells. Because these T-cells lost killing activity 3 weeks after anti-CD3/anti-CD28 depletion, this killing effect was most probably anti-CD3 MoAb activated and mediated. These data indicate that DC induction of AML blasts is important to prime AML-specific CTLs, that there may be de novo AML-specific CTLs in a small number of primary AML PBMCs, and that AML-specific CTLs can be expanded by IL-2/anti-CD3/anti-CD28.

To study the specificity of the cytolytic T-cell reaction, we performed experiments with blocking MoAb. Results of 6 experiments showed that the anti-HLA class-I MoAb W6/32, but not PM81 (anti-CD15) or 251 (anti-CD33), totally blocked the cytotoxic effect of AML T-cells in SMGF, suggesting that the leukemia-specific cytotoxicity was major histocompatibility complex (MHC) class I dependent and cytotoxic T-cell mediated (Figure 7). Human serum IgG (1 mg/mL) and mouse IgG2a (100 µg/mL) did not block the cytotoxicity (data not shown).

To rule out the possibility that residual mouse anti-CD3 MoAb redirected killing of AML cells through CD64, we further cultured T-cells obtained from the 3-group culture for 3 weeks without anti-CD3/anti-CD28 MoAb. CTL assay (Figure 8) demonstrated that AML T-cells obtained by SMGF culture maintained strong cytotoxic activity against AML cells after 3 weeks of culture without anti-CD3/anti-CD28 MoAb. This cytotoxic effect could be totally blocked by anti-HLA class-I MoAb W6/32. However, AML T-cells obtained by anti-CD3/IL-2 culture lost cytotoxic activity against AML cells after 3 weeks of culture without anti-CD3 MoAb. Anti-CD3 MoAb (10 ng/mL) could reestablish the cytotoxicity of these T-cells. As expected, the anti-CD3 MoAb–directed cytolysis of AML cells was only partially blocked by anti–class I HLA MoAb W6/32.

**Leukemia Colony-Forming Cell Depletion**

The cytotoxic effect of AML cells on leukemia colony-forming cells (L-CFCs) was analyzed by colony assay. AML T-cells obtained from patient 6 were incubated with

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**Table 2. HLA Class I Phenotype of AML Patients and AML Cell Lines**

<table>
<thead>
<tr>
<th>Patients</th>
<th>HLA Class I Phenotype</th>
<th>Specific Lysis by AML T-Cells</th>
<th>against</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>Cw</td>
</tr>
<tr>
<td>Patient 1</td>
<td>25, 31</td>
<td>18, 48</td>
<td>4, –</td>
</tr>
<tr>
<td>Patient 2</td>
<td>2, 31</td>
<td>35, 51</td>
<td>4, –</td>
</tr>
<tr>
<td>Patient 3</td>
<td>3, 24</td>
<td>7, 62</td>
<td>3, 7</td>
</tr>
<tr>
<td>Patient 4</td>
<td>2, 28</td>
<td>8, 44</td>
<td>5, 7</td>
</tr>
<tr>
<td>Patient 6</td>
<td>1, 2</td>
<td>7, 8</td>
<td>7, –</td>
</tr>
<tr>
<td>Patient 7</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Patient 8</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Patient 9</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Patient 10</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Patient 11</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Cell lines</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HL-60</td>
<td>1</td>
<td>B57, Bw4</td>
<td>6</td>
</tr>
<tr>
<td>NB4</td>
<td>11, 35</td>
<td>60, w6</td>
<td>4, 3</td>
</tr>
<tr>
<td>U-937</td>
<td>3, w19</td>
<td>5, 18</td>
<td>1, 3</td>
</tr>
</tbody>
</table>

*ND indicates not determined.
†E:T ratio, 20:1.
⁺CTL from patient 6 against patient 4 AML cells. Other patients represent autologous CTL and AML samples.
§Cell lines HL60 and NB4 were HLA-typed using standard serological techniques at the UCSD Immunogenetics Laboratory. The HL60 typing was confirmed by molecular typing methods. The U937 HLA type is that reported in the literature [53].
Growth Factor Modulation—New Adoptive Therapy for AML

**Figure 5.** CTL activity against AML cells (primary and HL60) and lack of activity against nonmyeloid cancer cells. Standard 4-hour 
\(^{51}\)Cr-release assays used the T-cells obtained from SMGF culture of AML-PBMCs (from patient 1) as effectors. Ten experiments showed similar cytotoxic effects against autologous AML and AML cell line cells without effects against various cell lines other than AML cell lines.

5000 cells/well of HL–60, NB4, or U937 cells in triplicate in 96-well round-bottom plates at different effector:target-cell (E:T) ratios for 2 hours. The cells in each well were then suspended and mixed with 0.9 mL MethoCult H4230 and seeded into 35-mm culture dishes for colony assay. The results showed that AML T-cells from SMGF culture were very effective L-CFC killers. These T-cells were so toxic to leukemia cells that most L-CFCs were depleted by AML T-cells even at an E:T ratio of 1:1 with a 2-hour incubation (Figure 9). These results may explain the accelerated elimination of AML cells in the third phase of SMGF culture.

**Analysis of T-Cell Repertoire in AML Patient T-Cells**

We compared the TCR V\(\beta\) repertoires expressed by the T-cells of healthy individuals (n = 13) to those of the AML patients (n = 3) pre- and post-SMGF culture (Figure 10). The preculture T-cells of each AML patient were found to have a TCR V\(\beta\) repertoire that was unique and aberrant compared to that found in preculture T-cells of healthy individuals. The TCR subgroups V\(\beta\)5b, -5c, and -19 were most highly represented in patient 2; the subgroups V\(\beta\)5b, -5c, -6Ba, and -10 were overexpressed in patient 4. We observed an overexpression of V\(\beta\)5b and -5c by the T-cells of patient 6 compared to the repertoire expressed by the T-cells of healthy individuals.

The expansion and reduction of the percentages of V\(\beta\) subgroups from 3 patients after SMGF culture are shown in Figure 11. If the specific V\(\beta\) subgroup was expanded, we divided postculture % V\(\beta\) subgroup by preculture % V\(\beta\) subgroup to obtain the multiple of expansion; if the specific V\(\beta\) subgroup faded, we divided preculture % V\(\beta\) subgroup by postculture % V\(\beta\) subgroup to obtain the magnitude of reduction. The data clearly demonstrated the specific selection of certain clones and reductions in others in SMGF culture. Some clones, such as V\(\beta\)3 and V\(\beta\)12a, were commonly expanded in 3 patient samples; some, such as V\(\beta\)6Ba, V\(\beta\)6bc, V\(\beta\)7, and V\(\beta\)14, expanded in 2 of 3 samples; some expanded individually. The extinguished clones acted the same. It is possible that during culture some existing AML-specific CTL clones expand, and some less-expressed clones are induced and then expanded. Selected AML-reactive T-cells may recognize more than one AML antigen or epitope simultaneously.

**DISCUSSION**

AML continues to be a difficult disease to cure. The mainstay of therapy has been chemotherapy, including high-dose chemotherapy with HSCT. Allogeneic HSCT is curative in a large proportion of patients, in part through a GVL effect mediated by the donor immune system. This assertion is supported by evidence from syngeneic HSCT, the association of GVHD with durability of remissions following allogeneic HSCT, and the efficacy of donor lymphocyte infusions in inducing remissions in patients who relapse after allogeneic HSCT.

The immune system in patients with AML has failed to recognize and destroy the malignant cells. The reasons for this failure are surely multiple and complex, and include: (1) insufficient antigen presentation by the malignant cells, (2) insufficient T-cell recognition of the malignant cells, and (3) insufficient GVL effect induced by the donor immune system.
immune suppression by soluble or cellular factors, and (3) insufficient numbers of specific lymphoid cells to react to the rapidly growing clone of malignant cells. The existence of leukemia-associated antigens is suggested by several lines of evidence such as the GVL effect noted in HSCT [6], the identification of specific antigens recognized by cytolytic T-cell clones [10], and over-expression of certain gene products detected by microarray technology [52].

While investigating the possibility that T-cells with anti-AML specificity can be expanded from the PB of AML patients with active disease, we have developed a strategy of 3-stage sequential modulation of growth factors. The first phase is initiation of AML blast differentiation to DCs, the second phase is priming of T-cells, and the third stage is expansion of T-cells.

We have shown that a DC phenotype with increased B7 and HLA-DR expression can be induced and up-regulated after in vitro exposure of native AML blasts to cytokine combinations of IL-4/GM-CSF. AML-specific CTLs can be induced and expanded thereafter. Successful induction and expansion of AML-specific T-cells from all 12 primary AML patients with diseases of various French-American-British (FAB) subtypes indicates the potential power of this strategy in adoptive immunotherapy of AML.

Primary AML patient MNCs in 3-phase SMGF culture produced expanded CTLs that caused significant lyses of autologous leukemia cells and AML cell lines HL-60, NB4, and U937 but had no effect on cell lines of AML (KG1a), lymphoma (Daudi, RPMI-8866), EBV-LCL, breast cancer (H345), natural killer cell–sensitive K562 cells, or small cell lung cancer (DMS273). Anti–HLA class I MoAb W6/32 totally blocked the CTL effect. The T-cells obtained from the SMGF culture were CD3+, either CD4+ or CD8+, and CD16– or CD56–. These results indicate that the AML-specific cytotoxic effect of AML T-cells was dependent on recognition of MHC class I molecules and was nonspecific LAK cell activity.

Engagement of CD64 by the Fc portion of the anti-CD3 MoAb (IgG2a subclass) is at least partially responsible for T-cell activation, because the Fc portion of the MoAb acts as a CD3 cross-linking agent. We explored the possibility that the anti-CD3 MoAb enhanced CTL activity by mediating antibody-dependent cell-mediated cytolysis (ADCC) because, although the level of anti-CD3 was in the

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**Figure 7.** Blockade of CTL activity by MoAb to class I HLA. CTL assay results after 3HCr-labeled target cells were incubated with 50 µg/mL MoAb PM81 (anti-CD15), 251 (anti-CD33), or MoAb W6/32 (anti-HLA class-I) (1:100 diluted ascites) for 30 minutes. Results of 1 representative (cells from patient 4) of 6 experiments are shown.

**Figure 8.** The AML-specific cytotoxicity of T-cells from SMGF culture is not anti-CD3 MoAb mediated. Group 2 and group 3 T-cells harvested from AML PBMC cultures described in Figure 5 were cultured for 3 weeks with IL-2 and irradiated autologous AML DC and analyzed for cytotoxicity against AML cells. A, Cytotoxicity against AML cells without blocking antibody; B, blocking with anti–HLA class I MoAb W6/32; C, incubation with anti-CD3 MoAb; D, incubation with anti-CD3 MoAb and blocking with W6/32. Data are representative of 4 experiments.
low nanomolar range, some of the killing might have been
due to ADCC. This hypothesis was negated, however, by
the finding that high concentrations of human IgG and
mouse IgG2a did not block the CTLs of AML T-cells.
Comparative study of AML-specific cytotoxicity of T-cells
from SMGF culture and T-cells from IL-2/anti-CD3 MoAb
added at the beginning of the culture further ruled out this
possibility. Although the addition of anti-CD3 MoAb could
redirect and enhance the killing of AML cells, this killing
could not be blocked by anti–HLA class I MoAb W6/32.
When T-cells were cultured without adding fresh MoAb for
more than 2 weeks, there was no detectable remaining cell-
bound MoAb, yet the T-cells still had demonstrable
cytolytic activity. This finding demonstrated that the T-cells
mediated lysis that was not ADCC. However, even higher
levels of killing could be achieved through the addition of
anti-CD3. The level of CD64 on AML cells may be impor-
tant to the potency of CTL generation through T-cell acti-
vation in the induction phase of the cultures.

The cytotoxicity was not MHC restricted, because pri-
mary AML patient cells, HL-60 cells, NB4 cells, and U937
cells do not share the same HLA phenotype (Table 2).
Although recognition of HLA class I molecules is essential
for cytotoxicity of AML-specific T-cells in SMGF culture,
strict HLA identity does not seem to be required. There was
no significant cytotoxicity against the myeloid cell line
KG1a (which is at a more primitive differentiation stage),
selected solid tumor cell lines, or nonmyeloid leukemia
lines. MHC-unrestricted killing of various tumor cells by
CTLs has been reported, including autologous and allo-
geneic CML cells (by bcr-abl–specific CD8+ CTLs) [54],
acute leukemia cells [55], breast and pancreatic cancer cells
(by MHC-unrestricted MUC-1–specific CTLs) [56-58], and
melanoma cells [59]. Human CD8+ T-cells raised in
response to herpes simplex virus (HSV) lysed autologous
and allogeneic HSV-infected target cells via a non–MHC-
restricted pathway [60]. In these studies, CTLs recognized
specific antigens because specific antigens were used to
induce the antigen-specific CTLs in a T-cell–priming sys-
tem. In our SMGF culture system, we obtained MHC-
dependent AML-specific CTLs directed to unknown target
antigens. The mechanism involved in the killing of AML
cells by these T-cells will require further study. It is possible
that distinct populations of CTLs that can kill autologous or
allogeneic AML cells or both may exist in the bulk cultures.
To investigate this possibility, we are attempting to clone
individual T-cell clones.

Figure 9. Effect of CTL on L-CLCs from 3 cell lines. AML T-cells
(from patient 6) of SMGF culture were incubated for 2 hours with
5000 cells/well of HL-60, NB4, and U937 cells in triplicate in 96-well
round bottom plates at different E:T ratios. L-CLC assay was per-
formed as described in “Material and Methods.”

Figure 10. Pre-SMGF culture TCR Vβ repertoires expressed by the T-cells of healthy individuals (n = 13) compared to those of AML patients (Pt.) (n = 3).
The results of TCR repertoire analysis suggest that oligopeptide-specific cytotoxic T-cell clones against several AML-associated or AML-specific antigens may be generated in these cultures. It is possible that unidentified AML antigens can be isolated using the AML-specific CTLs of the SMGF culture as a probe. We plan to study more patients prospectively to further define the HLA and other antigenic variables that operate in this system.

The TCR Vβ analysis revealed that specific T-cell subgroups were expanded by the SMGF culture conditions. Specifically, the Vβ3 and -12a subgroups that were expanded in the cultures of all 3 AML patients could represent T-cell clones that are expanded in response to AML-specific antigens. T-cell proliferation by direct stimulation of growth factors would result in a polyclonal TCR Vβ repertoire as expressed preculture by the T-cells of the 3 AML patients. Selected expansion and extinction of specific Vβ subgroups post-SMGF culture suggests that AML patients may have cytotoxic T-cell clones that specifically recognize autologous leukemic cells.

The circulating T-cells of patients with chronic lymphocytic leukemia (CLL) have also been shown to exhibit abnormal TCR Vβ repertoires [61-64]. The overexpressed T-cell clones observed in these patients may also reflect an ineffective response to leukemia-associated antigens. Results have shown evidence for T-cell clonal expansion of specific Vβ subgroups that are expressed by CD8+ T-cell clones that recognize autologous tumor cells in vitro [61]. Similarly, our studies suggest that the SMGF culture may result in expansion of specific Vβ subgroups expressed by cytotoxic T-cell clones that specifically recognize autologous leukemic cells.

In conclusion, we have demonstrated that HLA class I–dependent AML-specific CTLs can be induced and/or expanded from the PB of AML patients with active disease by a 3-phase sequential modulation of growth factors. The advantage of this approach to the generation of AML-reactive CTLs is that no cell separation or purification is needed. Rather, a sequential change in the combination of growth factors during culture of primary AML cells for a period of 30-40 days results in the generation and expansion of immunocompetent AML-specific cytotoxic T-cells. It may be possible to adoptively transfer such expanded autologous cells to patients in remission as immunotherapy to eradicate residual disease following induction chemotherapy.

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REFERENCES


