Lymphokine-Activated Killer Cell Functions in Patients With Leukemic B-Lymphoproliferative Diseases

By D. van der Harst, A. Brand, S.A.P. van Luxemburg-Heys, E.M.C. Kooy-Winkelaar, and J.J. van Rood

Nine patients with leukemic B-lymphoproliferative diseases (B-LPD) were evaluated for development of in vitro recombinant interleukin-2 (rIL-2)-activated killer (LAK) cells. B-cell cultures were established from peripheral blood mononuclear cells (PBMNCs) containing 63% ± 29% malignant cells. Short-term cultures were tested after 5-day activation with 500 U rIL-2/mL. Long-term cultures were maintained for 4 to 6 weeks by weekly addition of 500 U rIL-2 and autologous irradiated feeder cells. In the first week, the cells decreased considerably in the long-term cultures but thereafter cells proliferated (mainly T cells) on the average 300-fold (range 30- to 1,000-fold). In the short-term cultures, there was a 38% reduction of malignant B cells. In long-term cultures, B cells were reduced from 63% to 8%; three cultures still contained greater than 15% B cells. The CD16-positive cell percentage was comparable in both types of cultures and ranged from 2% to 17%. Effector cells lysing the natural killer (NK)-sensitive cell line K562 could be induced in all patients. Except in patients with chronic lymphocytic leukemia (CLL) and high malignant cell numbers, NK activity was already restored after 5 days. Optimal NK activity was obtained after 1.5 to 2.5 weeks. LAK cells killing NK-resistant lymphoma cell lines showed optimal activity after 2 to 3 weeks of culture. However, LAK cells killing greater than 10% of autologous malignant cells were obtained in only one third of the patients. The discrepancy between strong cytolytic activity against the NK-sensitive (K562) target cells obtained in all patients and the cytotoxic activity against NK-resistant cell lines contrasts with the poor development of LAK cells against autologous tumor cells. This discrepancy does not appear to be explained by soluble inhibitory factors released during the tumor cultures, as allogeneic LAK cells were not inhibited by supernatants from patients' cultures. Further investigations are warranted to reveal cell-mediated inhibition by tumor cells or suppressor cells.

ADAPTIVE CELLULAR immunotherapy using recombinant interleukin-2 (rIL-2) and in vitro rIL-2-activated killer (LAK) cells is an exciting new approach in cancer treatment. Which cancer type benefits most from this therapy and whether there is a relation between the degree of LAK cell activation and clinical response is currently being investigated in several studies in patients resistant to standard treatment protocols. No clinical information is available about leukemia, although a few lymphoma patients have been treated with promising results. Leukemia patients pose unique problems when adoptive cellular immunotherapy is considered because the malignant cells circulate in the peripheral blood and may interfere with immune manipulation in vivo and in vitro. Leukemia patients, especially those with lymphoproliferative malignancies, have impaired immune functions during active disease and in remission during maintenance therapy. Whether immune deficiency is the cause or the effect of malignancy is not clear. Patients with depressed natural killer (NK) functions are more apt to develop lymphoma as are patients receiving T cell or IL-2 suppressive drugs to prevent organ graft rejection.

The presence of leukemic cells appears to contribute at least partly to impaired immune response, as NK activity may normalize in remission after withdrawal of maintenance therapy. As far as comparisons can be made between different techniques, in vitro LAK generation with rIL-2 also achieves better results in remission than in relapse, unless tumor-depleted mononuclear cells are cultured in vitro. The presence or absence of malignant cells during in vitro culture may also account for disagreement about the kinetics of optimal LAK cell generation. Oshimi et al reported optimal generation of LAK cells after 5 days, when patients were studied in remission or by culture of tumor-depleted mononuclear cell fractions, respectively. In contrast, an optimum of LAK cell effectiveness was found by Adler et al after 3 weeks when patients with acute nonlymphocytic leukemia (ANLL) and 20% to 50% leukemic blasts in the peripheral blood were tested. No data are available about lymphoproliferative leukemias, which generally have even more severely suppressed immune functions and in which the malignant cells may even proliferate in response to rIL-2 and NK cells.

We evaluated patients with 10% to 90% high-, intermediate-, and low-grade lymphoma cells in the peripheral blood on their ability to generate LAK cells, using bulk cultures of peripheral blood mononuclear cells (PBMCs). Proliferation, residual B cells, and cytotoxic potential against NK-sensitive and NK-insensitive target cell lines and autologous and allogeneic lymphoma cells were evaluated.

MATERIALS AND METHODS

Patients

Nine untreated patients with leukemic B-LPD either at initial presentation or in relapse underwent leukapheresis. The mononuclear cells were used for both effector cell preparations and target cell suspensions. From one patient with a hairy cell leukemia (patient 9) showing 8% hairy cells in the peripheral blood, the tumor target cells were prepared from the spleen. Table 1 shows diagnosis and hematologic data. Mononuclear cells from blood collected by leukapheresis in preservative-free heparin (50 IU/mL) were sepa-
rated by Ficoll-Isopaque gradient centrifugation and cryopreserved PBMNCs according to the protocol of Rosenberg et al: 10^6 mononuclear cells/mL were cultured in 2-mL cluster wells for 5 days at 37°C in 5% CO2 well-humidified incubator in RPMI 1640 (GIBCO, Grand Island, NY) + 10% heat-inactivated selected human serum + 50 μg gentamycin/mL + 3 mmol/L glutamin and 500 μg/mL 2-mercaptoethanol (a gift from Ortho, Raritan, NJ). As a control, PBMNCs were cultured in culture medium without rIL-2.

**Tumor cocultured cytotoxic cells (TCC).** Prolonged culture of effector cells using feeder/stimulator cells was used for generation of tumor-infiltrating lymphocytes (TILs) 23; 10^6 mononuclear cells were incubated with 10^5 autologous mononuclear cells irradiated with 2000 rad in 1-mL cluster wells. Cells were cultured in Isaacs’s modified Dulbecco’s medium (IMDM, GIBCO) supplemented with gentamycin and 10% heat-inactivated selected human serum. After 3-day incubation, rIL-2 (500 U/mL) was added. After 6 days, 1 x 10^6 autologous irradiated stimulator cells were added, and this was repeated weekly. At biweekly intervals, half of the medium was refreshed with 500 U rIL-2/mL in IMDM, diluting to a concentration of 0.1 x 10^6 cells/mL.

**Preparation of Target Cells**

*Cryopreserved fresh target cells.* Thawed viable mononuclear cells were washed twice in IMDM before radioactive labeling. For patient 5, the non-T fraction after E rosette sedimentation was used; for patient 9, splenic lymphocytes (80% hairy cells) were used as target cells.

*Other target cells.* In each experiment, a mycoplasma-free K562 erythroleukemia line was used as control. Four lymphoma cell lines were included as target cells: BB2 and HH1 both derived from the spleen from patient 9; BB1 was a spontaneously growing population of leukemic cells in the peripheral blood. From patient 5, a lymph node from a patient with Burkitt-type lymphoma. Cell lines were growing continuously during the period of the experiments in RPMI 1640 + 10% fetal calf serum.

**3Cr-labeling**. After washing, the target cells were labeled with 100 μCi Na251CrO4 (Dupont, NEN products, Boston MA; specific activity 100 to 350 mCi/mg) for 1 hour in a 37°C water bath. After incubation, excess washing fluid was added, centrifuged at 200 g for 10 minutes. This was repeated twice, and the pellet was resuspended in a concentration of 0.5 x 10^6/mL.

**Cell-Mediated Lympholysis**

Effector cell (0.1 mL) and target cell (0.1 mL) suspension was added to a round-bottomed microtiter plate in duplicate. Microtiter plates were centrifuged for 1 minute at 900 g and incubated for 4 hours at 37°C. For spontaneous release, 0.1 mL target cell suspension and 0.1 mL culture medium per well (in triplicate) were incubated for the same period. For maximal release, 0.1 mL target cell suspension and 0.1 mL Zapomin solution (10 mL RPMI 1640 and 20 drops Zaponin, Coulter Electronics Ltd., Luton, GB) were incubated for 4 hours at 22°C. After incubation, the microtiter plates were centrifuged for 5 minutes at 900 g and the supernatant was removed with the Flow Supernatant harvester. Samples were counted for 1 to 3 minutes in a y-counter (Packard Multi-Prias B5301, Downers Grove, IL). The percentage of lysis was expressed as follows:

\[
\text{Percentage of lysis} = \frac{\text{Experimental Mean cpm} - \text{Spontaneous Released Mean cpm}}{\text{Maximum Release Mean cpm} - \text{Spontaneous Released Mean cpm}} \times 100
\]

The spontaneous release did not exceed 25% (unless otherwise indicated).

**Analysis of Target and Effector Cells**

Analysis was made on a FACS/TM analyzer or FACS Scan (Becton Dickinson, Mountain View, CA) with the following antibodies: anti-Leu-4 (CD3), anti-Leu-3a (CD4), anti-Leu-2a (CD8), anti-Leu-7 (HNK-1), anti-common ALL antigen (CALLA) (CD10), anti-Leu-11b (CD16), anti-Leu-12 (CD19), anti-Leu-16 (CD20), anti-Leu-19 (NKH-1) and anti-Tac (Becton Dickinson). Oligospecific antibodies against M, G, A, k and λ were purchased from Dakopatts (Birmingham, AL).

**RESULTS**

**Patient Data**

As shown in Table 1, patients 5 and 9 had a minor population of leukemic cells in the peripheral blood. From patient 9, target cells were prepared from the spleen; from patient 5, E rosette-depleted PBMNCs were tested as target cells. Five patients were never treated for their disease, and
observed in most of the control cultures without rIL-2 (Table 2). During the 5-day cultures in rIL-2, the total cell number decreased, and the cell recovery after 5 days varied between 15% and 60%. There was a relative increase in T and/or NK cells, which was observed in most of the control cultures without rIL-2 (Table 2). In the long-term (TCC) cultures, using autologous irradiated feeder cells, the cell numbers initially decreased, similar to LAK cultures, but after 3 to 4 weeks, the recovery was 30- to 1,000-fold. T-cell, NK-cell, and B-cell percentages representing the residual number of malignant cells before and after culture are shown in Table 3.

Cell Proliferation and Phenotype Analysis

During the 5-day cultures in rIL-2, the total cell number decreased, and the cell recovery after 5 days varied between 15% and 60%. There was a relative increase in T and/or NK cells and a relative decrease in B cells, which was not observed in most of the control cultures without rIL-2 (Table 2). In the long-term (TCC) cultures, using autologous irradiated feeder cells, the cell numbers initially decreased, similar to LAK cultures, but after 3 to 4 weeks, the recovery was 30- to 1,000-fold. T-cell, NK-cell, and B-cell percentages representing the residual number of malignant cells before and after culture are shown in Table 3.

Cell-Mediated Lympholysis

Five-day cultures. Figure 1 shows that one patient (patient 6) developed substantial LAK activity against autologous tumor cells; in an effector/target ratio of 20:1 four patients developed ~10% lysis of autologous lymphoma cells. Four patients showed no measurable lysis (~10%). From seven of these cultures, cytotoxicity against K562 was detected (Fig 2). As shown in Table 2, these short-term LAK cultures still contain 24% to 78% B cells, which are not expected to contribute to cytotoxicity. When the effector/target (E/T) ratio was corrected for the residual number of malignant cells after 5-day culture, the cytotoxicity against NK-sensitive cells became normal in five patients (patients 4, 5, 6, 7, and 9), was decreased in patients 1, 2, and 3, and was absent in patient 8 (Table 4).

The corrected E/T target ratio in which cytotoxicity against autologous cryopreserved tumor cells was tested was very low. When this was compared with normal donors tested in the same low E/T ratios, three patients (patients 5 through 7) showed lysis of autologous tumor cells in the same or higher range as compared with normal donors, three patients (patients 3, 8, and 9) lysed their own tumor cells less than normal donors, and three patients (patients 1, 2, and 4) could not kill their tumor cells, whereas at least two of these three tumors could be killed by normal donors’ LAK cells.

Effect of Supernatants From Patients’ LAK Cultures on Development of Normal LAK Activity

The supernatants from the LAK cultures from seven patients were added (50/50 vol/vol) to the culture medium...
Fig 2. Cytotoxicity of patients' 5-day LAK cells against the NK-sensitive target cell line K562 at different E/T ratios not corrected for the residual number of malignant cells after culture. Patient 8 not shown (0% lysis at all E/T ratios).

of normal donor PBMNCs together with 500 U rIL-2/mL. None of the seven different supernatants inhibited generation of LAK activity from cells of a normal donor against NK-sensitive (K562) and NK-resistant (HL-60) cell lines and cryopreserved leukemia cells (Table 5).

![Graph](image)

**Fig. 2.** Cytotoxicity of patients' 5-day LAK cells against the NK-sensitive target cell line K562 at different E/T ratios not corrected for the residual number of malignant cells after culture. Patient 8 not shown (0% lysis at all E/T ratios).

**Fig. 3.** Kinetics of cytotoxicity in long-term TCC cultures tested against K562 at an E/T ratio of 20:1. Median value of nine patients.

<table>
<thead>
<tr>
<th>Target</th>
<th>E/T</th>
<th>CM Lysis (%)</th>
<th>SUP1</th>
<th>SUP2</th>
<th>SUP3</th>
<th>SUP4</th>
<th>SUP5</th>
<th>SUP8</th>
<th>SUP9</th>
</tr>
</thead>
<tbody>
<tr>
<td>K562</td>
<td>6:1</td>
<td>67</td>
<td>57</td>
<td>63</td>
<td>81</td>
<td>65</td>
<td>77</td>
<td>65</td>
<td>65</td>
</tr>
<tr>
<td>HL60</td>
<td>25:1</td>
<td>52</td>
<td>41</td>
<td>42</td>
<td>49</td>
<td>48</td>
<td>49</td>
<td>49</td>
<td>NT</td>
</tr>
<tr>
<td>T1*</td>
<td>25:1</td>
<td>35</td>
<td>36</td>
<td>32</td>
<td>31</td>
<td>29</td>
<td>31</td>
<td>33</td>
<td>33</td>
</tr>
<tr>
<td>T2*</td>
<td>25:1</td>
<td>12</td>
<td>NT</td>
<td>10</td>
<td>10</td>
<td>8</td>
<td>8</td>
<td>NT</td>
<td>19</td>
</tr>
</tbody>
</table>

Abbreviations: CM, control medium; SUP, control medium + 50% supernatant; NT, not tested. *T1, T2 cryopreserved leukemia target cells containing 90% blasts with a viability of 85% after thawing.

**Long-Term Cultures: TCC**

Figure 3 shows the kinetics of development of (cytotoxic) cells in long-term cultures. These prolonged cultures were tested weekly or biweekly, and almost all patients showed a comparable pattern. The lysis of NK-sensitive target cells (K562) peaks between 1.5 and 2.5 weeks of culture, starting 3 days after addition of rIL-2. After 2 weeks, the lytic capacity toward K562 usually decreases. Lytic activity against autologous tumor cells remained in the same low range as after 5-day culture, but lysis of NK-resistant lymphoma cell lines increased after 2 weeks, when lysis

**Table 5. Supernatant From 5-Day LAK Cultures From Seven Patients' LAK Cultures Added to PBMNCs From a Normal Donor**

<table>
<thead>
<tr>
<th>Target</th>
<th>E/T</th>
<th>CM Lysis (%)</th>
<th>SUP1</th>
<th>SUP2</th>
<th>SUP3</th>
<th>SUP4</th>
<th>SUP5</th>
<th>SUP8</th>
<th>SUP9</th>
</tr>
</thead>
<tbody>
<tr>
<td>K562</td>
<td>6:1</td>
<td>67</td>
<td>57</td>
<td>63</td>
<td>81</td>
<td>65</td>
<td>77</td>
<td>65</td>
<td>65</td>
</tr>
<tr>
<td>HL60</td>
<td>25:1</td>
<td>52</td>
<td>41</td>
<td>42</td>
<td>49</td>
<td>48</td>
<td>49</td>
<td>49</td>
<td>NT</td>
</tr>
<tr>
<td>T1*</td>
<td>25:1</td>
<td>35</td>
<td>36</td>
<td>32</td>
<td>31</td>
<td>29</td>
<td>31</td>
<td>33</td>
<td>33</td>
</tr>
<tr>
<td>T2*</td>
<td>25:1</td>
<td>12</td>
<td>NT</td>
<td>10</td>
<td>10</td>
<td>8</td>
<td>8</td>
<td>NT</td>
<td>19</td>
</tr>
</tbody>
</table>

Abbreviations: CM, control medium; SUP, control medium + 50% supernatant; NT, not tested. *T1, T2 cryopreserved leukemia target cells containing 90% blasts with a viability of 85% after thawing.

**Table 4. Comparison at 15:1 or 7:1 E/T Ratio of Cells After 5-Day LAK Culture From Patient Group and Normal Donors**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Patients' tumor cells Lysis (%)</th>
<th>K562 Lysis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patient LAK</td>
<td>ND LAK</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>39</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>43</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>22</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>41</td>
<td>5</td>
</tr>
<tr>
<td>7</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>5</td>
<td>11</td>
<td>6</td>
</tr>
<tr>
<td>8</td>
<td>6</td>
<td>15</td>
</tr>
<tr>
<td>9</td>
<td>4</td>
<td>20</td>
</tr>
</tbody>
</table>

Targets: (autologous) fresh tumor cells and the NK-sensitive cell line K562. The E/T ratio for the patients was corrected for the residual number of tumor cells after culture (ie, 50% tumor cells after 5 days at 20:1 E/T is a net E/T of 10:1); n = 9. Patients 1-4, 6, and 7 tested at E/T ratio 7:1 in all experiments; comparison with ND LAK also at E/T ratio 7:1. Patients 5, 8, and 9 tested at E/T ratio 15:1 in all experiments; comparison with ND LAK also at E/T ratio 15:1.

Abbreviation: ND, normal donor.
against K562 was already decreasing. The lysis of allogeneic lymphoma cells as compared with lysis of lymphoma target cell lines is shown in Table 6. The B cells in those prolonged cultures were reduced as compared with the number of B cells in 5-day LAK cells (Table 3), but in three patients was still greater than 15% at 3 to 4 weeks.

**DISCUSSION**

Clinical adoptive cellular immunotherapy protocols for solid tumors use bulk cultures of PBMCs. A similar approach in leukemia would be attractive, especially for B-cell malignancies, because low- and intermediate-grade B-cell malignancies cannot be brought into remission. Relapsed intermediate- and high-grade lymphomas are often resistant to reinduction therapy. Although phase II and III studies have been performed in patients with solid tumors, such studies are contraindicated in leukemia because the presence of IL-2 receptors on leukemic cells and growth induction owing to factors produced by IL-2-activated accessory cells may occur.

We evaluated nine patients with various types of leukemic B-cell lymphomas of which some with extremely high leukemic cell numbers (patients 1 through 4), not eligible for clinical adoptive cellular immunotherapy. The experiments were performed with cryopreserved PBMCs, which accounts for a considerable cell loss of 40% to 85% during the 5-day cultures. This is hardly at variance with the cell loss of 40% to 75% obtained with normal donors' cryopreserved cells on subsequent activation with rIL-2. During 5-day cultures, there was a relative increase in cells with a T-cell or NK-cell phenotype from 12% to 27% and a relative decrease of 36% in the malignant B cells. When this was compared with control cultures without rIL-2, there was almost no decrease in malignant cells (Table 2), although the data could be masked owing to cell death, mainly as a result of B cells in these control cultures.

During long-term cultures despite weekly addition of irradiated autologous PBMCs, in six of nine cultures B cells disappeared (mean 7% residual B cells) and in three were still present (greater than 15%). There was a sufficient cell proliferation up to 4 weeks of 300-fold (range 30- to 1,000-fold) for eventual adoptive cellular immunotherapy; after 4 to 6 weeks, cell proliferation rapidly decreases and the cultures die. Cytotoxic effector cells lysing the K562 cell line developed in six of nine patients after 5-day culture, reached an optimum number in the long-term culture assays at 2 weeks, and were as effective as PBMCs of healthy individuals after in vitro stimulation with rIL-2. In particular, patients with high leukemic cell numbers had slower development of optimal NK function. Although it is remarkable that adequate cytotoxic effector cells lysing NK-sensitive target cells can be obtained from cultures initially consisting mainly of leukemic cells, a major point of concern was the lytic potential against autologous lymphoma cells.

After 5-day culture in rIL-2, lysis of autologous target cells greater than 10% (mean 21%) was observed in three of nine patients, although after correction for residual contaminating B cells of the effector population, the E/T ratios tested were extremely low. In the same low E/T ratios, however, normal donors' LAK cells showed lysis of greater than 10% in seven of nine cases. The long-term cultures were tested for cytotoxic potential weekly, and after 2 weeks, optimum lysis toward NK-sensitive targets was observed. Killing of autologous targets of more than 10% (mean 13%) could be measured in only three patients in E/T ratios of 20:1. IL-2-activated cells from normal donors showed lysis of greater than 10% (mean 34%) of the lymphoma targets in 40 of 52 experiments, although there was a large variation in lysis of the lymphoma targets by normal donors' LAK cells (range 0% to 97% lysis in E/T ratio of 20:1). Although insensitivity of leukemia target cells was suggested by Adler et al.28 for myeloid leukemia target cells, this does not appear to explain the low autologous lymphoma lysis. We did not determine whether the lymphoma target cells became more susceptible for LAK lysis after interferon treatment. We agree with Adler et al.28 that manipulation of the target cells (eg, activation by phytohemagglutinin in short-term cultures) renders them significantly more sensitive for lysis (data not shown) than normal lymphocytes treated in the same manner; this may provide tools for determination of hidden or cell cycle-dependent antigens on the tumor cell surface important for lysis susceptibility.

Another important factor that may contribute to the low autologous target cell lysis is the malignant cells present in the culture. Soluble factors sometimes observed to be released by tumor cells19–24 could be ruled out because none of the seven culture supernatants tested inhibited development or the lytic potential of LAK cells of a normal donor tested against NK-sensitive and NK-insensitive target cells. Cold target inhibition by the leukemic cells may have played a role in the 5-day cultures but probably did not in the prolonged cultures unless the weekly addition of irradiated excess leukemic cells consumed the autologous killer cells in a specific manner. As non-major histocompatibility complex-restricted cytotoxicity is considered an aspecific phenomenon, the LAK activity against NK-resistant targets was expected to be inhibited as well, which was not the case. LAK cell activity against NK-resistant targets became optimal between 2 and 4 weeks of prolonged culture and clearly reached their optimum later than effector cells killing NK-sensitive targets K562. Lower lysis of autologous tumor cells by interferon or rIL-2–activated PBMCs during active disease was observed by Oshimi et al.19 in 10 of 11 lymphoma patients and by Adler et al28 in 10 of 19 patients with acute

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**Table 6. Lysis of Autologous and Allogeneic Lymphoma Cells and Lymphoma Cell Lines by TCC Cells After 2.5 to 4 Weeks of Culture as Compared With Normal Donor LAK Cells (E/T = 20:1)**

<table>
<thead>
<tr>
<th>Targets</th>
<th>Autologous Lymphoma Cells</th>
<th>Allogeneic Lymphoma Cells</th>
<th>Lymphoma Cell Lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of experiments/lysis by TCC</td>
<td>17/8.2 ± 5</td>
<td>31/6.7 ± 6</td>
<td>37/39 ± 24</td>
</tr>
<tr>
<td>No. of experiments/lysis by ND-LAK</td>
<td>—</td>
<td>47/34 ± 22</td>
<td>52/88 ± 24</td>
</tr>
</tbody>
</table>

Values are no. of experiments/mean ± SD.

Abbreviation: ND, normal donor.
myeloid leukemia. Such decreased lysis of autologous tumor cells does not occur when patients in remission are tested or when large granular lymphocyte fractions are processed for effecter cell functions. Oshimi et al. speculate that not only enrichment of precursor cytotoxic cells but also depletion of specific suppressor cells, which appear to be present in excess especially in PBMCs of patients with lymphocytic leukemia, may be considered. Such suppressor monocytes can also be removed by nylon wool filtration. Further studies of cell-mediated inhibition of autologous LAK cells and removal of these cells are necessary.

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REFERENCES

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