Non-cytotoxic and Sublethal Paclitaxel Treatment Potentiates the Sensitivity of Cultured Ovarian Tumor SKOV-3 Cells to Lysis by Lymphokine-activated Killer Cells

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Abstract. Background: The standard treatment of epithelial ovarian cancer is tumor debulking by surgery, followed by six cycles of chemotherapy consisting of cisplatinum and paclitaxel. However, this therapy protocol is not satisfactory, since about 50% of the treated patients eventually experience recurrence within a few years of follow-up. Thus, a more innovative treatment modality is urgently needed for patients with this malignancy. We hypothesized that pretreatment of ovarian cancer SKOV-3 cells at a non-cytotoxic to sublethal dose range of paclitaxel would result in increased sensitivity to LAK-mediated killing. Materials and Methods: MTT and trypan blue dye exclusion were used to determine the non-cytotoxic to sublethal range of paclitaxel against SKOV-3 cells. A 4-h 51Cr release cytotoxicity assay was used to evaluate the sensitivity of paclitaxel-treated and untreated SKOV-3 cells. Immunofluorescence/flow cytometric analysis was used for phenotypic changes of cells with or without paclitaxel treatment. Results: Our results with trypan blue dye exclusion and MTT assays showed that the non-cytotoxic to sublethal range was between 0.001 μM and 0.01 μM. Pretreatment of SKOV-3 cells with paclitaxel at these doses for 72 h revealed significantly enhanced LAK-mediated killing against SKOV-3 cells with the highest sensitivity achieved with cells treated with 0.001 μM paclitaxel, as compared with the baseline killing of untreated cells using LAK cell alone (p<0.05). The enhanced sensitivity of LAK-mediated killing appeared to be in part due to paclitaxel-induced expression of ICAM-1 on SKOV-3 cells.

Conclusion: This treatment approach may be useful for further development of an effective therapeutic mode for patients with ovarian cancer.

Human epithelial ovarian cancer is the fourth most lethal cancer in women with 75% of the patients presenting at an advanced stage of the disease (1). The standard treatment of advanced epithelial ovarian cancer is comprehensive tumor debulking, followed by six cycles of chemotherapy consisted of cisplatinum and taxanes. A second look operation is usually scheduled to determine the effectiveness of the treatments. However, the value of this second look surgery is controversial, since nearly 50% of treated patients with the so-called “pathologically tumor-negative findings” will experience recurrence within a few years of follow-up. Thus a more novel treatment modality is urgently needed for patients with advanced ovarian cancer.

Taxanes are cancer therapeutic agents such as paclitaxel (taxol) with potent antitumor activity against a number of human cancer types, including human ovarian cancer, breast, cervical, endometrial, esophageal, gastric, colon, bladder and lung (2). Paclitaxel was found in 1969 and was extracted from the western yew, Taxus brevifolia. Wani et al. (3) identified the chemical structure of paclitaxel and demonstrated its cytotoxic effect. The major mechanism of its antineoplastic action is the promotion of polymerization of tubulin dimers to form microtubules and stabilization of the microtubules by preventing depolymerization. Moreover, paclitaxel blocks the cell cycle at the metaphase/anaphase transition, causing inhibition of cell proliferation (4). Recently, paclitaxel has also been shown to induce apoptosis in an ovarian cancer cell line and its paclitaxel-resistant clone (5, 6).

IL-2 has been demonstrated to be able to augment the cytolysis capability of lymphokine-activated killer (LAK) cells both in vitro and in vivo against tumor cells (7-9). The
major problems of this kind of treatment modality are the toxicity induced due to a high dose of IL-2 infused along with LAK cells and the 15-20% of clinical responses (complete and partial responses) seen in patients with cancer refractory to conventional treatments (10). Unlike cytotoxic T-lymphocytes (CTL), each clonal population of which has its specificity for a specific peptide epitope and memory, LAK cells could kill tumor cells resistant to natural killer (NK) cells through a major histocompatibility complex (MHC)-nonrestricted fashion (11).

The idea of chemo-immunotherapy has been advocated in the past with the rationale that after completion of surgery and chemotherapy, the micrometastatic lesion could be eradicated by administration of a nontoxic or sublethal dose of certain chemotherapeutic agents or other biological response modifiers, such as paclitaxel, followed by immunotherapy (or biotherapy), such as activated NK cells or LAK cells (12). To test the hypothesis of this chemo-immunotherapy protocol, we sought to determine whether cells of the ovarian cancer cell line SKOV-3, pretreated with paclitaxel at a nontoxic to sublethal dose range, would increase the killing effect of LAK cells as a prelude to the use of this protocol in the treatment of patients with epithelial ovarian cancer.

Materials and Methods

Cell line and culture conditions. The human ovarian cancer cell line SKOV-3 established from a 64-year-old Caucasian woman was originally purchased from the American Type Tissue Collection (Rockville, MD, USA). This cell line was obtained from Dr. Tse-Hou Wang, Department of Obstetrics and Gynecology, Chang Gung Memorial Hospital, Linkou, Taiwan, ROC). The cells were maintained as a monolayer culture in RPMI-1640 medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 µg/ml). All these culture media and supplements except FBS were purchased from GIBCO-Invitrogen Corp., Carlsbad, CA, USA. FBS was purchased from Sigma-Aldrich (St. Louis, MO, USA). When cultures reached confluence, cells were dissociated with 0.025% trypsin-EDTA (GIBCO-Invitrogen), followed by one cycle of washing in complete growth medium. Cells were then expanded to T-25 culture flasks or dishes (NUNC, Roskilde, Denmark) at 37°C in an incubator in an atmosphere containing 5% CO2/95% air and 100% humidity. Cells in some cultures were intermittently frozen in 20% FBS and 10% dimethyl sulfoxide in liquid nitrogen.

In cytotoxicity assays, two hematopoietic tumor cell lines, K562 (erythroleukemia) and Daudi (B-cell lymphoma), were used as NK and LAK reference cell lines respectively, because K562 cells are sensitive and Daudi are relatively resistant to NK cytolysis, while Daudi and K562 cells are both sensitive to LAK cytolysis. These two hematopoietic tumor cell lines were also maintained in RPMI-1640 plus 10% FBS. Eight different concentrations of the drug were prepared and added to complete growth medium containing a fixed number of SKOV-3 cells to make final drug concentrations of 0.0001, 0.0003, 0.001, 0.003, 0.01, 0.03, 0.1, and 0.3 µM. Triplicate cultures were set up in 6-well plates (3 cm in diameter/well) (NUNC, Roskilde, Denmark) for each paclitaxel concentration.

Detection of cell proliferation and viability. Two methods, namely the 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) (Sigma Aldrich) reduction assay and trypan blue dye exclusion were used to monitor the proliferation and viability of tumor cells upon exposure to different concentrations of paclitaxel for different incubation periods. For the MTT assay, 10^5 cells/well were seeded in 24-well plates (NUNC). SKOV-3 cells were grown in complete growth medium containing the concentrations of paclitaxel indicated above in a 37°C incubator for 1, 2, 3, 4 and 5 days. Reduction of the yellow MTT by cell mitochondrial activity gave purple formazan crystals. At the end of incubation, 1 ml of MTT (50 µg/ml in RPMI medium without FBS and phenol red) were added to each well and incubated for 90 min. After dissolving the resulting formazan product in dimethyl sulfoxide, absorbance was read on a BT 2000 Microkinetics Reader (Bio-Tek Instrument Inc., Winoski, VT, USA) at a wavelength of 570 nm. Trypan blue dye exclusion was used to assess the viability of SKOV-3 cells grown under different conditions as described above. Growth curves, each covering a total of five days of culturing without change of medium, were constructed from which the population doubling time and saturation density were determined.

Preparation of effector cells and reference cell lines. Fresh heparinized blood from healthy normal donors were obtained 1:1 with sterile phosphate-buffered saline (PBS), peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque (Pharmacia and Upjohn, Kalamazoo, MI, USA) density gradient centrifugation. LAK cells were prepared by culture of fresh PBMC at a concentration of 1x10^6 cells/ml in RPMI-1640 containing 1000 U/ml of human recombinant IL-2 (R&D Systems Inc., Minneapolis, MN, USA) for 3 days. SKOV-3 cells as well as two reference cell lines K562 and Daudi were also included in each cytotoxicity assay.

51Chromium release cytotoxicity assay. A standard 4-h 51Cr release cytotoxicity assay was performed as described elsewhere (13). In brief, monolayer cells adhered to flasks or dishes that had been pretreated with paclitaxel (at the nontoxic and sublethal dose range) were collected and washed after light trypsinization. The cytotoxicity assay used was a 4 h 51Cr release assay using a single cell suspension of target cells that had been prelabeled with 100 µCi of Na251CrO4 (Perkin Elmer, Boston, MA, USA) for 90 min at 37°C. Excess free Na251CrO4 was washed off with complete growth medium by centrifugation at 400 xg at room temperature for 5 min. In a 96-well U-bottom microplate (NUNC), 5x10^3 target cells were mixed with two-fold serially diluted effector cells to produce different effector (E) to target (T) ratios, ranging from 100:1 to 3.125:1, and the mixers were incubated at 37°C for 4 h. Maximum and spontaneous (or minimum) releases of 51Cr were determined by replacing effector cells with 2% sodium dodecyl sulfate (SDS) (Sigma, St. Louis, MO, USA) and complete growth medium alone, respectively. The plate was then centrifuged for 5 min at 800 xg and 50 µl supernatants were carefully harvested and transferred to wells of Lumaplate-96 (Packard, Meriden, CT,
USA), and counted on a TopCount-NXT® Microplate Scintillation and Luminescence Counter (Packard, Downers Grove, IL, USA). γ-Ray emission from each well was detected by the single photomultiplier tube of the counter. Percentage cytotoxicity was calculated using the following equation (E-S)/(M-S) x 100, where E = experimental count per minute, S = spontaneous counts per minute, and M = maximum counts per minute. Results were also expressed in lytic units (LU) for inter-experimental comparison and LU were calculated as previously described (14). In this study, one LU was defined as the number of effector cells required for 20% lysis of 5x10³ target cells and was calculated using a computer program.

Monoclonal antibodies. A panel of mAbs were used for immunophenotyping including anti-HLA-A, B, C (clone W6/32; BD PharMingen, San Diego, CA, USA), AE1 (642-01; Signet, Dedham, MA, USA), AE3 (464-01; Signet), MAK-6 (mixture of two mAbs to cytokeratins including CK type 14, 15, 16, 19 for one mAb and to CK type 8 and 18 for another mAb; Zymed, San Francisco, CA, USA), CK7 (OV-TL12/30; DAKO), CK20 (Ks20.8; DAKO), EMA (E29; DAKO), E-cadherin (67A4; Biodesign, Saco, ME, USA), Fas (UB2; MBL, Nagoya, Japan), FasL (sc-19681; Santa Cruz Biotechnology, Santa Cruz, CA, USA), EGFR (R1; Santa Cruz Biotechnology), Hsp70 (SPA-810; Stressgen, Victoria, Canada), CD58 (LFA-3) (MEM-63; Serotec, Raleigh, NC, USA), and CD54 (ICAM-1) (LB-2; BD PharMingen).

Surface/cytoplasmic immunofluorescence and flow cytometric analysis. Monodispersed cells harvested from cell cultures were dispensed into test tubes (3x10⁵ cells/ml/tube) and centrifuged at 400 xg to pellet the cells. Test antibodies (5 Ìg/ml) were added and the mixture incubated at 4°C for 30 min. Indirect immunofluorescence-based detection of surface antigen was performed with live cells incubated with various monoclonal antibodies, including appropriate positive and negative controls (see below), followed by FITC-conjugated goat anti-mouse IgG secondary antibodies for another 30 min. At the end of incubation, cells were washed and fixed in 0.3 ml 1% paraformaldehyde and allowed to stand at 4°C overnight. Cells were then analyzed with a FACScan flow cytometer at an excitation wavelength of 490 nm and emission wavelength of 520 nm. For cytoplasmic antigens, the procedure was essentially the same as that for the detection of surface antigens, except that before addition of primary monoclonal antibodies, the harvested cell pellet was first treated with 1% paraformaldehyde at 4°C for 30 min and then with cold acetone for 5 min (15). This procedure allows the reagents and washing solution to penetrate into the cytoplasm through the cell membrane. Positive control (anti-HLA-A, B, C, clone W6/32, BD PharMingen) and negative controls (PBS, isotype-matched irrelevant monoclonal antibodies or normal mouse Ig) in place of primary antibody were included in each experiment for testing either surface or intracellular antigens.

Statistical analysis. Student's t-test was employed to determine the difference between the group of LAK-mediated specific lysis against untreated SKOV-3 cells and each of the groups of LAK-mediated specific cytolysis of target cells pretreated with different concentrations of paclitaxel. Significant differences were considered at p<0.05.

Results

Effect of paclitaxel concentration on SKOV-3 cell proliferation as determined by MTT assay. MTT was used to determine the non-cytotoxic to sublethal dose range of paclitaxel (Figure 1A). A slight stimulatory effect of SKOV-3 cell proliferation was noted with the 3 lower concentrations of paclitaxel (0.0001, 0.0003, 0.001 ÌM) used, with the most pronounced level at 0.001 ÌM, while the inhibitory effect was observed with the drug between 0.003 and 0.3 ÌM in a dose-dependent manner.
Nontoxic to sublethal dose range of pacitaxel against SKOV-3 cells as determined by trypan blue exclusion. To determine the nontoxic and sublethal concentrations of pacitaxel to SKOV-3 cells, concentrations of paclitaxel ranging from 0.0001 µM to 0.3 µM were incubated with SKOV-3 cells for 5 days, and the total number of trypan blue uptake cells and trypan blue excluded viable cells were counted every 24 h. The results showed that the non-cytotoxic dose range was between 0.0001 µM and 0.001 µM and the sublethal dose was 0.003-0.01 µM (Figure 1B). In the growth curve study with trypan blue scoring for viable cells, the non-cytotoxic to sublethal dose range became much clearer (Figure 2), which was by and large consistent with the results shown in Figure 1B. Thus, we selected the four paclitaxel concentrations (0.1, 0.03, 0.01, 0.001 µM) for the cytotoxicity experiments described below.

Comparison in the sensitivity of paclitaxel-treated and non-treated SKOV-3 cells to LAK-mediated cytolysis. By the ^51^Cr release cytotoxicity assay, hematopoietic tumor cell lines, Daudi and K562 serving as control reference cell lines, were both killed efficiently by LAK cells to a similar extent, as expected (Figure 3). The specific cytolysis of LAK against untreated SKOV-3 cells was about 22% at an E:T ratio of 100:1, which was markedly enhanced after the cells were pretreated with paclitaxel at the non-cytotoxic to sublethal doses (Figure 4). Specifically, at the E:T ratio of 100:1, the specific lysis reached a maximum of 56% and a minimum of 36%. The highest enhancement of LAK cytolysis was observed with cells treated with the drug at the doses of 0.001 to 0.01 µM, and a modest enhancement with cells treated with the drug at 0.1 µM. Statistically, the LAK cytolytic activities were significantly boosted when tested on SKOV-3 cells pretreated with 0.001 to 0.01 µM paclitaxel at E:T ratios of 100:1-3.125:1 (p<0.05), except for the situations where the lysis was obtained with the target SKOV-3 cells pretreated with 0.1 µM paclitaxel at E:T ratios of 6.25:1 and 3.125:1. The boosted LAK cytolytic activities were compared with LAK baseline activities.
Figure 3. LAK-mediated cytolytic responses of two reference control cell lines K562 (erythroleukemia) and Daudi (B cell lymphoma) as determined by $^{51}$Cr-release cytotoxicity assay. Results are expressed as % specific lysis (mean±SD). Values in LU (see Materials and Methods) for the two target lines are also indicated.

Figure 4. LAK-mediated cytolytic responses of SKOV-3 cells pretreated with 0 (control), 0.001, 0.01 and 0.1 μM paclitaxel for 72 h. The maximum cytolysis was found on SKOV-3 cells pretreated with 0.001 and 0.01 μM paclitaxel, within the predetermined sublethal dose range of drug (see Figure 1). The LAK cytolytic activities were significantly boosted when tested on SKOV-3 cells pretreated with 0.001 to 0.01 μM paclitaxel at the range E:T ratios of 100:1-3.125:1 tested (p<0.05), except for the target SKOV-3 cells pretreated with 0.1 μM paclitaxel at the E:T ratios of 6.25:1 and 3.125:1 tested. Results are expressed as % specific lysis (mean±SD). Values in LU are also indicated in the figure.
Effect of paclitaxel on the phenotypic expression of epithelial cell and tumor-associated markers by SKOV-3 cells. The cytotoxicity results of SKOV-3 cells pretreated with 0.03 µM paclitaxel, which were nearly identical to those of cells treated with 0.1 µM paclitaxel, was not included in the Figure 4. The results are also expressed as LU as indicated in Figures 3 and 4 for overall comparative purposes. The extent of increased sensitivity of tumor cells treated with lower concentrations of paclitaxel was clearly reflected by a graded increase in LU (Figure 4).

Of note, the dose of 0.003 µM of the drug did cause a cytostatic effect based on the data of growth curves as assessed with trypan blue dye exclusion for viable cells (Figure 1B), since after seeding, the number of viable cells did not increase at day 3 (72 h), at which point in time the $^{51}$Cr release cytotoxicity experiment was conducted.

Because of similarity in the lytic curves, the cytotoxicity results are pooled from 0.03 and 0.1 µM paclitaxel-treated cells as Group 1 and those from 0.001 and 0.01 µM paclitaxel-treated cells as Group 2, and compared with those of the untreated control cells, with all of the E:T ratios being considered. The $p$-values calculated are between 0.003 and 0.00005 and between 0.0008 and 0.0000004, respectively, as depicted in Figure 5. When results of Group 1 and Group 2 are compared, the $p$-value is between 0.03 and 0.0002, with the exception that the comparison is made at the E:T ratio of 100:1, the $p$-value of which is >0.05.

Discussion

In this study we demonstrated that paclitaxel pretreatment of SKOV-3 cells at the non-cytotoxic and sublethal range could enhance LAK cell-mediated killing. SKOV-3 cells were however not sensitive to NK cells. This observation was reproducible with LAK cells prepared from more than one allogeneic individual. Our cytotoxicity data are valid, since we demonstrated the selective cytotoxicity against K562 cells but little cytotoxicity against Daudi cells as targets by NK cells, and the efficient killing of both Daudi and K562 targets by LAK cells. In addition, this phenomenon was not seen with normal PBMC as test targets following non-cytotoxic to sublethal doses (data not shown).

Epithelial ovarian cancer is a leading cause of cancer mortality in women worldwide. Chemotherapy is effective in reducing tumor burden in a majority of patients, however, only about 20% of advanced disease patients will survive tumor free. The recurrence of the disease probably arises from the incomplete killing of the residual tumor by the
The exact mechanism underlying the increased susceptibility of SKOV-3 to LAK cytosis after treatment with paclitaxel at the nontoxic and sublethal concentrations remains to be elucidated. In the context of the cellular differentiation status aforementioned, a number of factors that may influence susceptibility of target cells to MHC-non-restricted killing are represented by the differentiation stages of tumor cells. A decrease in LAK cell sensitivity is detectable after induction of a more differentiated phenotype in colon carcinoma cell lines (23) and in melanoma cell lines (13) by treatment with differentiation agents. It also appears to be

<table>
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<th>Antigen</th>
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Results are expressed as % positive cells (mean fluorescence intensity); – not tested.
related to an increased expression of certain adhesion molecules such as E-cadherin, ICAM-1 and LFA-3 (16, 21-24) and/or heat-shock protein such as Hsp70 (19, 20) after chemical or physical induction. We therefore sought to look for the induction or increased expression of these putative molecules before and after paclitaxel treatment. Our immunofluorescence/flow cytometry analysis failed to detect a correlation between the expression of any of these candidate surface molecules with one exception that might have caused an increase in the sensitivity of SKOV-3 cells pretreated with paclitaxel. This exception was ICAM-1 which was induced on the cell surface of SKOV-3 cells following paclitaxel treatment at a lower range of concentrations (Table I). These results suggest that the increased LAK sensitivity of paclitaxel-treated SKOV-3 cells may be in part, if not altogether, attributable to the induction of ICAM-1 on ovarian cancer cells and indicate that post-binding events between ICAM-1 on the tumor cells and LFA-1 on LAK cells also play a role in the increased sensitivity to LAK-mediated cytolysis observed after paclitaxel treatment at the non-cytotoxic to sublethal dose range. Considering the heterogeneous nature of LAK cells (11, 37, 38), one might envision subpopulations of LAK cells possessing different affinities for the target SKOV-3 cells. Thus the expression of ICAM-1 on the target cells might have increased the target/LAK interactions for cells with lower affinity long enough for a lethal hit to be delivered. The participation of ICAM-1 induced on tumor target cells in augmenting the sensitivity of LAK-mediated cytolysis in our study is indeed consistent with the observations made in other human tumor systems (13, 21-23), although the agent used for the induction/enhancement of ICAM-1 in each of these studies was not the same.

A comment must be made on the relation between the increased surface ICAM-1 expression and the increasing concentrations of paclitaxel used for the treatment of SKOV-3 cells (Table I). This observation appears to be contradictory to the results which showed that the increased sensitivity to LAK-mediated cytolysis was inversely related to the concentration of paclitaxel employed (Figure 4). In our preliminary study, soluble ICAM-1 was purposely added to the mixture of LAK cells and SKOV-3 cells in standard 4-h 

\[^{51}\text{Cr}\] cytotoxicity assays in which soluble ICAM-1 was allowed to compete with surface ICAM-1 on paclitaxel-treated SKOV-3 cells, resulting in a reduction in LAK cell-mediated activity. Taken together, these results suggest that factors other than ICAM-1 may have also been involved in the increased LAK-mediated cytolysis against SKOV-3 cells pretreated with paclitaxel.

The main cell killing mechanism of paclitaxel at high cytotoxic doses (~10 \(\mu M\)) lies in the depolymerization of the microtubules (4) and was also likely through apoptotic events (6, 39). This SKOV-3 cell line is known to be relatively sensitive to paclitaxel-induced apoptosis, although it could be induced to paclitaxel-resistant subclones by increasing paclitaxel dosages in culture (5, 6). Here, we should emphasize the non-cytotoxic to sublethal dose range of paclitaxel we employed (0.001-0.01 \(\mu M\)), which may act as a sensitizer or a “stress signal” to render the tumor cells vulnerable to being killed by LAK cells.

The overall enhancement of LAK cytolysis against SKOV-3 cells in this study needs further elucidation. The application of adoptive immunotherapy could play a role in ovarian cancer patients who have undergone standard surgical debulking followed by chemotherapy in order to truly eradicate the occult lesions. The participation of paclitaxel at non-cytotoxic to sublethal dosages with the adoptive therapy using LAK cells may improve the current conventional therapy with surgery followed by chemotherapy. It is noteworthy that a recent investigation found that LAK cytolytic activity against HER2/neu-positive tumor cells was specifically augmented with a low dose of trastuzumab (anti-HER2/neu mAb) which was likely mediated through the mechanism of antibody-dependent cellular cytotoxicity (40). This investigation, as well as our present study, point to the notion that clinical trials of LAK cell adoptive immunotherapy against selective breast/ovarian tumors in combination with either pretreatment with trastuzumab, paclitaxel or both may be warranted.

The original laboratory procedures for LAK activation defined by Muul et al. (41) were used to facilitate the clinical studies of Rosenberg and others (7-9), involving the in vitro culture of large numbers of peripheral blood mononuclear cells in high concentrations of IL-2. To this end, PBMCs were usually obtained from cancer patients through leukapheresis procedures (7-10, 42). For in vitro analytical studies, the culture of PBMCs for LAK cell preparation was carried out in plastic culture flasks (12, 13, 40) or roller bottles (43). Thus, from the technical standpoint, the means of a large scale preparation of NK or LAK cells from cancer patients in a cost-effective way has been the subject of ongoing attention in the field of cancer adoptive cellular immunotherapy.

Paclitaxel at nontoxic to sublethal doses may augment LAK cell activity against ovarian cancer. Obviously, additional work is needed to elucidate the mechanism involved in the augmentation of LAK cell killing in the future. We feel strongly that adoptive cellular immunotherapy using autologous LAK cells should be regarded as a valuable cancer therapeutic option for maximizing the full anticancer potential of the human immune system by combination with a chemotherapeutic agent such as paclitaxel.

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