### Title

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# Trafficking of Syngeneic Murine Lymphokine Activated Killer T Cells Following Intraperitoneal Administration in Normal and Tumor Bearing Mice[1]

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**Nongenetically restricted T cells may be important host effector cells in women with ovarian cancer receiving intraperitoneal (ip) IL-2 therapy. We developed an *in vitro* technique to produce murine lymphokine-activated killer T cells. Murine splenocytes were cultured in the presence of 1000 U/ml IL-2 for 10 to 15 days. Phenotypical analysis showed 95% of total cells to express the pan T phenotype Thy 1.2 and no NK cell phenotypes by Day 7 in culture. These cells were labeled with $^{51}Cr$ and their trafficking pattern after ip administration into normal and M5067 tumor bearing mice was examined. Various organs and tissues were collected at different timepoints and monitored for radioactivity. Within 4 hr., about 60% of the counts were associated with the bowel, peritoneum, and omentum of both normal and tumor bearing mice. About 15% of counts were associated with the blood, lung, kidney, spleen, and liver of both normal and tumor bearing mice.** 

## INTRODUCTION

Immunotherapy is a new experimental approach to treat patients with recurrent ovarian carcinoma [1–3]. Thymus derived (T) lymphocytes expressing the Pan T marker ($CD3^+$) infiltrate the solid tumor metastasis in the peritoneal cavity (pc) and have also been shown to be the predominant cells in the peritoneal exudate of these patients [4–6]. *In vitro* studies have revealed that upon treatment with Il-2, T cells from both the pc and infiltrating solid tumors undergo proliferation and activation and become nongenetically restricted LAK cells [7,8]. In addition, the numbers and cytolytic activity of T cells in the peritoneal exudate increase in women with ovarian cancer receiving ip, IL-2 immunotherapy [9]. The T cell could be a very important component of host antitumor resistance in women with this disease.

In the majority of patients, ovarian carcinoma remains confined to the pc throughout most of its course [10,11]. Thus, the pc is a reasonable site to consider the ip administration of adoptive cellular therapies [12]. A major problem in the various animal models of cellular therapies has been the trafficking of effector cells to the sites of the tumor [13]. Animal studies to delineate the basic parameters of recruitment and trafficking of T-LAK[3] cells in the pc could lead to a better understanding and application of adoptive cellular immunotherapy in these patients.

Several animal studies have been conducted in trafficking of leukocytes in the pc, Reynolds *et al.*, [14] demonstrated that syngeneic NK cell and nonactivated T cells remained free in the pc of rats for up to 24 hr after ip injection. Stevenson *et al.* [15] demonstrated that human peripheral blood monocytes, activated by 24-hr incubation *in vitro* with IFN-γ, rapidly adhere to the serosal surface of the peritoneal cavity when injected ip into cancer patients. *In vitro* techniques have been developed that enable human T-LAK cells to be expanded to large numbers using IL-2 [16–18]. However, no reports of the production of murine T-LAK cells or ip distribution of murine T-LAK cells has appeared. The present study was

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[3] Abbreviations used in this paper: T-LAK cell, lymphokine-activated killer T cell; pc, peritoneal cavity.



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conducted to examine the trafficking pattern of radiolabeled T-LAK cells in the pc of normal mice and mice bearing an ip tumor which serves as a model for ovarian cancer. The results of this study demonstrate that within 4 hr, the majority of the counts are associated with the bowel and remain localized in the pc of both normal and tumor bearing mice.

## MATERIALS AND METHODS

### *Mice*

Female C57BL/6 mice were obtained from the Charles River Breeding Labortories, Inc. (Wilmington, MA). The mice were maintained in a pathogen-free animal Biological Sciences Vivarium facility that provided food and water *ad libitum* and were used in the experiments at 12 weeks of age.

### *Tumor Cells and Continuous Cell Lines*

The M5076 is a murine reticulum cell sarcoma of ovarian origin [19,20] which was passed in the ascites form in C57BL/6 mice by ip inoculation of $1.0 \times 10^6$ tumor cells. This tumor grows as both solid tumor nodules and as ascites. The Yac-1 mouse lymphoma cell line (NK-cell sensitive) was obtained from the American Type Culture Collection (Rockville, MD) [21]. The Yac-1 cell line was maintained as a suspension culture in RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY), supplemented with 10% FCS (GIBCO) (RPMI-10%), and passed biweekly.

### *Generation of Murine T-LAK*

Intact spleens were aseptically removed from the C57BL/6 mice. The spleens were then bathed in RPMI-10% and manually minced with scalpel blades until a single-cell suspension was achieved. Red blood cells were selectively lysed by a hypotonic solution of Tris-buffered ammonium chloride [22]. The splenic cell suspension was centrifuged (1500 rpm for 5 min) into a pellet and resuspended in 10 ml of 0.14 *M* $NH_4Cl$ and 17.0 m*M* Tris at pH 7.2 for 2 min at room temperature. The cells were then washed twice by alternate centrifugation and resuspension with RPMI-10%. The resulting cell suspension was then macrophage depleted by absorption onto FCS-treated plastic tissue culture flasks for 45 min at 37°C [23]. The nonadherent cells were suspended in complete media which consists of RPMI-10% supplemented with 2 m*M* glutamine (Sigma Chemicals, St. Louis, MO), 10 U/ml penicillin (Sigma), 0.1 mg/mi streptomycin (Sigma), 0.1 m*M* nonessential amino acids (GIBCO), 1 m*M* sodium pyruvate (GIBCO), $5 \times 10^{-5}$ *M* 2-mercaptoethanol (J. T. Baker, Phillipsburg, NJ), and 1000 U/ml recombinant human IL-2 kindly supplied by the Cetus Corporation, (Emeryville, CA). The LAK cell cultures were incubated at a density of $2.0 \times 10^6$ cells/ml in T-150 $cm^2$ plastic flasks (Corning Glassware, Corning, NY) at 37°C in an atmosphere of 5% $CO_2$. After 4 days the cells were passed every 48 hr in complete media at a density of $0.5 \times 10^6$ cells/ml.

### *Phenotypic Analysis by Fluorescence-Activated Cell Sorting*

All phenotyping was done with a FACScan (Beckton–Dickinson Immuncytometry System, Mountain View, CA) equipped with an argon laser and interfaced with Consort 30 software. The FACScan was calibrated by the AutoComp program and optimized for each cell culture tested by forward and side scatter analysis. Peak separation was obtained on a log-fluorescence scale. Positive and negative gates were set by the operator using unlabeled cells or cells labeled only with second-step reagents. Antibodies and final dilutions used were Anti-Thy 1.2 fluorescein conjugate at 1:20 (Beckton–Dickinson), Rabbit Anti-Asialo GM1 fluoroscein conjugate at 1:20 (Wako Chemicals, Richmond, VA.). The NK1.1 antibody was generated in our laboratory by injecting hybridoma cells (HB191) (American Type Culture Collection) into Balb-c mice (Charles River). IgG antibody was purified from ascites fluid using the MAb-Trap G (Pharmacia, Piscataway, NJ) system [24,25]. The cells were incubated with the Anti-NK1.1 at 1:4 dilution for 30 min at 4°C and then were washed with PBS and goat anti-mouse IgG2a fluorescein conjugate at 1:100 (Southern Biotechnology Associates, Inc., Birmingham, AL) was incubated with the cells for 30 min at 4°C. Cells were stained for analysis as follows: $1 \times 10^6$ were resuspended in 100 $\mu$l of the appropriate dilution of antibody in cold RPMI-3% for 30 min at 4°C. The cells were then washed twice with 5 ml of cold PBS. After washing, cells were fixed in 1% paraformaldehyde in PBS and stored in the dark at 4°C until FACS analysis.

### *Cytolytic $^{51}Cr$ Release Assay*

Cytolytic assays employing the nonadherent YAC-1 target cell were performed in 96-well round-bottomed microcytotoxicity plates, whereas cytolytic assays for the adherent M5076 target cell were performed in 96-well flat-bottom microcytotoxicity plates [26]. Radiolabeling of the nonadherent Yac-1 cells was accomplished by the addition of 20 $\mu$l of $^{51}Cr$ at a concentration of 1 mCi/liter with specific activity of 183 mCi/mg (ICN, Irvine, Ca) in 1 ml of serum-free media (RPMI-0%) for 1–2 hr at 37°C in 5% $CO_2$. After 1–2 hr the cells were washed three times with cold RPMI-10%. A constant number of labeled target cells ($2 \times 10^4$ in 20 $\mu$l) was added to microtiter wells with various numbers of effector cells. Radiolabeling

of adherent target cells was accomplished by the addition of 20 μl of $^{51}Cr$ to 1.0 × $10^6$ cells in 10 ml of RPMI-10%. The radiolabeled cell suspension was then dispensed at 100 μl/well in the 96-well flat-bottomed titer plates. After incubation for 24 hr at 37°C, the labeled cells were washed with cold RPMI-10% to remove the free isotope. Effector cells were added to the tumor cells at various E/T ratios. For both target cells, the ratios tested were performed in triplicate and the averages for three wells were calculated. Both nonadherent and adherent target cell cultures were incubated for 4 hr at 37°C in 5% $CO_2$. The release of $^{51}Cr$ was measured by the uptake of cell-free supernatants with Titerteks supernatant collection system (Skatron, Lier Norway) and quantitated in an 1272 Clinigamma counter (LKB Pharmacia, Piscataway, NJ). The total $^{51}Cr$ releasable (90–95% of total counts) was determined by lysing the cells with 100 μl of 3% (w/v) sodium dodecyl sulfate solution. Spontaneous $^{51}Cr$ release was 1–3% per hr. Percentage lysis was determined by the following formula:

$$\frac{\text{Experimental Release - Spontaneous Release}}{\text{Release - Spontaneous Release Lysis}} \times 100 = \% \text{ Specific Total.}$$

*Trafficking of T-LAK Cells following Intraperitoneal Administration*

Murine T-LAK cells cultured for 10 to 12 days were centrifuged into a pellet and resuspended in RPMI-0% at cell density of 30 × $10^6$ cells/ml. The cells were labeled by coincubating 1 ml of suspension with 200 μCi of $^{51}Cr$ (1 mCi/ml; specific activity, 293.76 mCi/mg; New England Nuclear, Boston, MA) for 1 hr at 37°C in 5% $CO_2$. The cells were then washed three times with warm RPMI-10%, resuspended in warm PBS, and transferred to a syringe at a cell density of 20 × $10^6$ cells/ml. The labeled cells were then injected into the peritoneal cavity of normal and 8-day tumor bearing mice. Spontaneous release was derived by incubation of this cell suspension with PBS for 6 hr at 37°C in 5% $CO_2$ and was approximately 5.0% per hr. Animals were sacrificed at 0, 1, and 4 hr after injection. The pc of each mouse was washed with 10 ml of warm PBS and individual organs were then removed. The whole bowel was removed with the mesentery. All pelvic, abdominal, and diaphragmatic portions of the peritoneum were collected and pooled together. All counts were measured in a Beckman biogamma counter (Fullerton, Ca). The blood count was adjusted to indicate the total counts in an estimated total blood volume of 2 ml. Four to five animals/group were sacrificed at the given timepoints. Each animal's individual organs were removed and counted separately. The total radioactivity recovered from each mouse was calculated, and the percentage contributed from each organ was also determined for each separate animal. The numbers displayed in Tables 2 and 3 under "X" are the averages obtained from these 4–5 animals' organs.

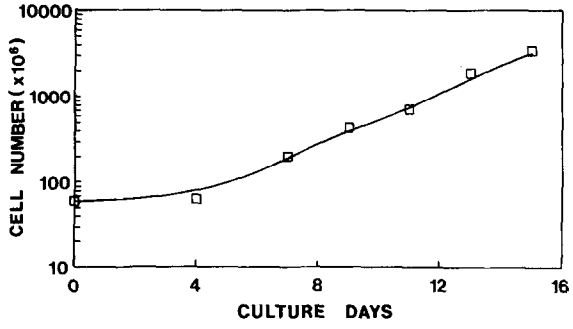


**FIG. 1.** *In vitro* generation and cell proliferation of murine T-LAK cells. Spleen cells (60 × $10^6$) from C57BL/6 mice were cultured in the presence of IL-2 (1000 U/ml) for a total of 15 days. Cell numbers were determined at the initial day of culture and at various intervals.

## RESULTS

### *I. Generation of T-LAK Cells from the Spleens of Normal Mice*

Murine splenocytes were grown in presence of 1000 U/ml IL-2. Total viable cell numbers were determined every 48–72 hr when cultures were expanded. The growth pattern of a representative culture over a 15-day period is shown in Fig. 1. There is a decline of about 50% in viable cell number over the first 1–4 days in culture. However, these cells begin to proliferate between 4 and 6 days and maintain a constant 30–35-hr division time. Most cultures were terminated after approximately 2 weeks. Cells would not proliferate in the absence of IL-2.

### *II. Expression of Lymphocyte Surface Markers by T-LAK Cells*

Many T-LAK cell cultures were subjected to phenotyping at various intervals during the culture period. The data derived from typing the T-LAK cell cultures are shown in Table 1. The results shown are the averages of three separate cultures for each specified timepoint. The SD among the experiments did not exceed 10% of the mean. In every culture studied the T cell expressing Thy 1.2 became the predominant cell type by 5–6 days and remained the predominant cell for the remainder of the culture period. The asialo-GM1 phenotype, which detects activated T cells also increased with time. In contrast, the NK cell marker NK 1.1 was low and remained low throughout the culture period.

TABLE 1
**Phenotype of Murine Spleen Cells**[a]

| Days in culture | Percentage of total cells staining | | |
|---|---|---|---|
| | Thy 1.2 | GM1 | NK1.1 |
| 0 | 44 | 10 | 2 |
| 3 | 60 | 22 | 7 |
| 7 | 95 | 33 | 1 |
| 10 | 96 | 30 | 8 |
| 14 | 97 | 39 | 4 |

[a] Spleen cells of C57Bl/6 mice were cultured in the presence of IL-2 (1000 U/ml) for a total of 14 days. The various phenotypic markers expressed by the cells at different days in culture were determined using specific antibodies and flow cytometric analysis.

### *III. In Vitro Cytolytic Activity of T-LAK Cells*

At varius intervals, T-LAK cells were tested for their capacity to induce cytolysis of the NK cell sensitive Yac-1 and M5076 tumor cells *in vitro*. Four hour $^{51}Cr$ release assays were conducted as described under Materials and Methods. Cells from these cultures were tested at intervals over a 16-day period, in triplicate at various E/T ratios. For clarity only the E/T ratio of 25:1 is presented. Figure 2 represents the averages of four separate cell cultures for each timepoint. The SD between experiments was less than 10%. Both the Yac-1 and M5076 cell lines showed similar sensitivity profiles. From Day zero (unstimulated) to Day 4 cells expressed an increase in cytolytic activity. Then after 8 days the cells reached peak cytolytic activity (60–70%) which was maintained until Day twelve. By Day sixteen, the ability of the T-LAK cells to kill the tumor cells began to decrease.

### *IV. Distribution of $^{51}Cr$-Labeled T-LAK Cells following Intraperitoneal Injection in Normal and Tumor Bearing Mice*

Ten- to twelve-day T-LAK cells were radiolabeled and injected into normal and tumor bearing C57Bl/6 mice as described under Materials and Methods. Animals were sacrificed at various timepoints and individual organs, tissues, and peritoneal washes collected and samples counted in a gamma counter. The counts in each sample are expressed as a percentage of total recoverable counts. The data from all animals tested were pooled and are shown in Tables 2 and 3. Clearly, after 4 hr the counts in the peritoneal wash (free cells) from normal and tumor bearing mice decreased to 4.7% and 17.0%, respectively. Thus, the radioactivity had localized to positions in the peritoneal cavity. Conversely, there was a marked increase in the radioactivity which became associated with the bowel. In addition, a slight increase in counts was also observed to associate with the peritoneum and omentum for both groups of mice. In contrast, small amounts of radioactivity were recovered from the liver, lung, and other organs; however, the level and distribution remained relatively constant during the 4-hr experiment and was, with one exception, blood, comparable between tumor bearing and normal animal.

After ip administration $^{51}Cr$-labeled T-LAK cells showed patterns of distribution which were very similar in both normal and tumor bearing animals. The majority of the recoverable radioactivity remained in the peritoneal cavity and the labeled cells rapidly attached to the serosal surface of the bowel within 1–2 hr. The total recovered radioactivity was constant in the different experiments and ranged from 50–90%.

## DISCUSSION

Studies in women with ovarian carcinoma reveal that T cells predominate the peritoneal ascites and the cells infiltrating solid tumor nodules on the peritoneal serosa [4–6]. Animal studies of the trafficking patterns of activated T cells in the pc could be important for understanding the mechanisms involved and improving the clinical trials with biological response modifiers in women with this disease.

The present study was initiated to generate and examine the ip trafficking of murine T-LAK cells in normal and tumor bearing mice. A method for the *in vitro* generation of large numbers of murine T-LAK cells was developed. Phenotyping at regular intervals during culture revealed that T cells predominate these cultures by day 5 and remain the major cell type for the duration of the culture. In support of the idea that the culture is mostly T cells, there is little or no expression of the NK phenotype NK1.1. *In vitro* cytotoxicity studies revealed that these T cells are nongenetically restricted and will lyse NK-cell sensitive YAC-1 cells and the M5076 ovarian tumor cells *in vitro* in a 4-hr $^{51}Cr$ release assay. Thus,

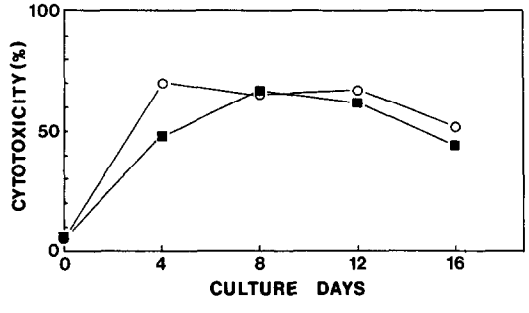


**FIG 2.** *In vitro* cytotoxic activity of murine T-LAK cells against two murine tumor cell lines, in a 4-hr $^{51}Cr$-release assay. Cytotoxic activity of T-LAK cells was tested at various culture days against radiolabeled YAC-1 lymphoma (○) and M5076 reticulum cell sarcoma (■).

**TABLE 2**
**Distribution of Labeled T-LAK Cells in Normal C57BL/6 Mice following Intraperitoneal Injection**

| | % total recovered $^{51}Cr$ activity (cpm/organ) | | | | | |
|---|---|---|---|---|---|---|
| Time | 0 hr | | 1 hr | | 4 hr | |
| Organ | $x$ | SD | $x$ | SD | $x$ | SD |
| Bowel | 8.8 | 13.8 | 34.1 | 11.8 | 57.1 | 29.2 |
| P. wash | 79.3 | 27.4 | 34.0 | 25.2 | 4.7 | 6.1 |
| Blood | 0.8 | 1.4 | 1.7 | 1.0 | 1.3 | 0.8 |
| Liver | 0.8 | 0.4 | 3.9 | 2.9 | 3.7 | 3.5 |
| Spleen | 0.2 | 0.2 | 3.3 | 4.1 | 1.1 | 0.8 |
| Peritoneum | 1.5 | 0.8 | 4.3 | 5.9 | 4.0 | 3.2 |
| Omentum | 0.9 | 0.8 | 4.1 | 5.3 | 6.8 | 7.5 |
| Lung | 0.2 | 0.2 | 0.4 | 0.3 | 0.4 | 0.3 |
| Kidney | 0.3 | 0.3 | 0.6 | 0.3 | 0.8 | 0.4 |
| Total number of animal | ($n$ = 4) | | ($n$ = 5) | | ($n$ = 5) | |

these cells appear phenotypically and functionally similar to human $CD_3^+CD16^-$ T-LAK cells [4–6].

The trafficking of $^{51}Cr$-labeled cells after ip injection in both normal and tumor bearing animals was examined. While not shown, we initially employed [$^3$H]thymidine-labeled T-LAK cells in these studies. We found these cells localized rapidly in the peritoneal cavity and the count distributions did not change over the 24–48-hr period. Because of these results we changed to $^{51}Cr$-labeled cells which are more easily detected. We found the radioactivity rapidly associates with selected organs within the peritoneal cavity. Injected cells almost totally disappeared from the peritoneal washing by 2 hr and most of the counts were associated with the bowel, peritoneum, and omentum but absent in blood, lung, kidney, etc. By far the majority of counts were associated with the whole bowel. These data support the concept that passively transferred syngeneic T-LAK cells rapidly adhere to and remain in the pc. In addition, no difference in trafficking was noted between normal animals and animals bearing 8-day ip tumors. It should be noted that the tumor is at an early stage, and laparotomy reveals many small metastases throughout the peritoneal cavity and free tumor cells in the ascites. These results are somewhat similar to those obtained by Stevenson *et al.* [15] in humans who found autologous peripheral blood monocytes, activated *in vitro* for 24 hr with IFN-γ, and injected ip into human patients with bowel tumors rapidly adhere to peritoneal serosa. But contrast with the results of Reynolds *et al.* [14] who found autologous rat splenic NK cells, and non-activated splenic T cells, remained free and unattached for up to 24 hr when injected into the pc of normal animals. It may be that once activated, macrophages and T-LAK cells rapidly attach to the walls of the peritoneal cavity [14].

A small number of counts appears in the blood of these

**TABLE 3**
**Distribution of Labeled T-LAK Cells in 8-Day Tumor Bearing C57BL/6 Mice following Intraperitoneal Injection**

| | % total recovered $^{51}Cr$ activity (cpm/organ) | | | | | |
|---|---|---|---|---|---|---|
| Time | 0 hr | | 1 hr | | 4 hr | |
| Organ | $x$ | SD | $x$ | SD | $x$ | SD |
| Bowel | 20.8 | 15.0 | 44.3 | 26.6 | 46.4 | 23.5 |
| P. wash | 47.0 | 23.5 | 29.9 | 22.2 | 17.7 | 12.0 |
| Blood | 2.8 | 6.2 | 6.6 | 2.5 | 10.1 | 5.6 |
| Liver | 4.0 | 2.0 | 3.6 | 1.6 | 5.3 | 1.8 |
| Spleen | 2.1 | 1.3 | 1.6 | 1.2 | 2.2 | 1.0 |
| Peritoneum | 3.8 | 1.3 | 4.2 | 3.1 | 5.2 | 2.6 |
| Omentum | 5.0 | 2.0 | 6.5 | 3.4 | 9.2 | 5.3 |
| Lung | 2.1 | 1.8 | 1.2 | 0.9 | 1.5 | 0.7 |
| Kidney | 2.6 | 1.6 | 1.1 | 2.6 | 1.2 | |
| Total number of animal | ($n$ = 4) | | ($n$ =5) | | ($n$ = 5) | |

animals; however, there appears to be more counts in the blood of tumor bearing versus normal animals. In addition, while the data is not shown, there are also counts in the urine from these animals. This finding suggests T-LAK cells in tumor bearing animals may be dying and releasing counts more rapidly than in normal animals. This phenomenon does not appear to be spontaneous cell death, for while the data are not shown, control cells incubated at 37°C for 1–4 hr in PBS or peritoneal washings do not release isotope in excess of spontaneous release. Furthermore, in separate experiments of which the data are not shown, the T-LAK cells recovered in the peritoneal wash from normal mice 4 hr after ip injection, were 90% viable. Additional studies will have to be conducted to establish the fate of these radiolabeled cells. The majority of the radioactivity is associated with the bowel; however, this may not be selective binding because calculations indicate this tissue represents up to 60% of the surface area of the pc. In addition, the coiling and folding may trap cells, thus making a more appropriate substrate for binding.

These studies demonstrate that T-LAK cells after ip injection, rapidly adhere to the serosal surface(s) and remain localized in the pc in both normal and tumor bearing animals. Ovarian tumors are localized in these sites; thus passively transferred autologous T-LAK cells make logical candidates for use in cell immunotherapies in tumors localized to the pc. They can more easily be generated by *in vitro* techniques from the peripheral blood than lymphocytes from the ascites or infiltrating tumors of women with this disease. These cells could be employed alone or in conjunction with other cytokines to increase the numbers of effector $CD_3^+$ T cells in these sites.

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