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ORIGINAL ARTICLE

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Biodistribution and tumor localization of lymphokine-activated killer T cells following different routes of administration into tumor-bearing animals

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Abstract Purpose: The efficiency of adoptive cellular immunotherapy of cancer might depend on the number of effector cells that reach the malignant tissues. In the present study, the biodistribution and tumor localization of ex vivo lymphokine-activated T killer (T-LAK) cells was investigated. **Methods:** T-LAK cells were labeled with ^{125}I-dU or the fluorescent dye tetramethylrhodamine isothiocyanate (TRITC) and transferred by intravenous, -cardiac, -portal or -peritoneal injection into normal (C57BL/6) mice or mice with syngeneic day-7 to day-12 B16 melanoma metastases established in various organs. The overall biodistribution of the T-LAK cells was measured by gamma counting and their tumor localization by fluorescence microscopy. **Results:** At 16 h after intravenous injection, the organ distribution of ^{125}I-dU-labeled T-LAK cells was identical in normal and tumor-bearing animals. Fluorescence microscopy of lung tissue from animals receiving TRITC-labeled T-LAK cells revealed, however, a fivefold higher accumulation of T-LAK cells in lung metastases than in the surrounding normal lung tissue (1174 and 226 cells/mm^2 respectively). Some pulmonary metastases were, however, resistant to infiltration. Very few intravenously injected cells redistributed to other organs or to tumors in these, since only 60 and 30 T-LAK cells/mm^2 were found within metastases of the adrenal glands and the liver respectively. However, following injection of T-LAK cells via the left ventricle of the heart, a threefold increase (from 60 to 169 cells/mm^2) in the number of transferred cells in metastases of the adrenal glands was observed. Moreover, following locoregional administration of T-LAK cells into the portal vein, tenfold higher numbers (from 30 to 400 cells/mm^2) were found in hepatic metastases than were observed following intravenous or intracardiac injection. In the liver, a surprisingly large number of intraportally injected T-LAK cells (approx. 1300/mm^2) were observed to accumulate in the perivascular spaces of the portal, but not the central veins. Even though some superficial ovarian and liver metastases were separated from the peritoneal cavity by only the peritoneal lining, no localization into these metastases was seen following intraperitoneal injection of the T-LAK cells. While treatment of tumor-bearing animals with T-LAK cells plus IL-2 reduced lung metastases by 76% as compared to treatment with IL-2 alone ($P < 0.03$), no significant reduction of liver metastases was seen. **Conclusions:** T-LAK cells are able to localize substantially into tumor metastases in various anatomical locations, but mainly following locoregional injection. This finding might have important implications for the design of future clinical protocols of adoptive immunotherapy based on T cells.

Key words T-LAK cells · B16 melanoma · Adoptive immunotherapy · Biodistribution · Route of injection

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Introduction

In several animal models, the systemic adoptive transfer of lymphokine-activated killer (LAK) cells and concomitant administration of interleukin-2 (IL-2) have been shown to effectively mediate the regression of disseminated micrometastatic tumors in the lungs and, to some extent, the liver [9, 17, 18, 21, 24, 30]. However, despite these promising preclinical results, the application

of adoptive cellular immunotherapy in the treatment of advanced cancer in humans has been less encouraging. The effect has been most notable in patients with melanoma or renal cell carcinoma where overall response rates between 20% and 25% have been reported [1, 23, 25, 27, 31]. Since LAK cells derived from human/murine natural killer (NK) or T lymphocytes usually show high cytolytic activity against a variety of autologous and allogeneic tumor cells ex vivo [10, 11, 19, 28], the low efficiency of the LAK cells in vivo might, in part, be due to an insufficient recruitment of the transferred effector cells to the tumor tissue.

The mechanism(s) behind the in vivo tumoricidal effect of transferred effector cells has not been clearly elucidated, but several possibilities exist. Once in the tumor tissue, the effector cells may interact directly with and kill tumor cells by cell-to-cell contact, or indirectly by release of different substances causing tumor cell and/or microvascular damage [28]. Alternatively, the effector cells may release cytokines, such as tumor necrosis factor, interferon γ and IL-2, which might lead to extravasation and activation of endogenous effector cells [13, 32]. However, regardless of which lytic mechanism(s) plays the predominant role, it seems reasonable to assume that a close contact between the transferred immune effector cells and their tumor targets is a prerequisite for initiation of the biological reactions leading to tumor eradication.

In the B16 melanoma model, we have recently demonstrated that, although intravenously injected, IL-2-activated natural killer (A-NK) cells as well as IL-2/phytohemagglutinins-activated T killer (T-LAK) cells indeed have the ability to localize selectively into pulmonary metastases [3, 16], their trafficking to metastases located in organs downstream from the lungs is inefficient. With respect to A-NK cells, a significant localization into liver metastases was seen, however, following locoregional delivery into the portal vein [3]. In the current study, we have analyzed whether a better delivery of adoptively transferred T-LAK effector cells to extrapulmonary metastases can likewise be obtained by injection of these cells locoregionally, i.e., closer to the tissues housing the metastases. By use of a subline of the B16-F1 melanoma cell line that induces high numbers of pulmonary, hepatic, adrenal gland, and ovarian metastases within each animal, we have analyzed the trafficking of intravenously, left ventricularly (heart), intraportally, and intraperitoneally injected T-LAK cells to pulmonary as well as extrapulmonary metastases.

Materials and methods

Animals and tumor cell lines

Female C57BL/6 mice, 8–12 weeks old, were purchased from Bomholtgaard (Rye, Denmark) and were maintained in a specific-pathogen-free environment. The B16-F1(3H) subline syngeneic to C57BL/6 mice was established from a hepatic metastasis arising from the B16-F1 melanoma and was maintained in RPMI-1640 medium (Gibco, Paisley, UK) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, 20 mM HEPES buffer, 0.8 g/l streptomycin and 1.6×10^5 U/l penicillin. Adherent cells were detached by exposure to 0.02% EDTA for 2–3 min and washed three times in RPMI-1640 medium. Cell viability, as judged by a trypan blue dye exclusion test, was always more than 95%. To establish pulmonary, hepatic, adrenal gland and ovarian metastases, mice were pretreated with 200 mg/kg cyclophosphamide (C-7397, Sigma Chemical Co., St. Louis, Mo.) i.p. on day −1 before tail vein injection of 0.8×10^6 B16-F1(3H) melanoma cells in 0.3 ml RPMI-1640 medium. All animal experiments were performed in compliance with *Principles of laboratory animal care* (NIH publication 85-23, revised 1985).

Preparation of T-LAK cells

T-LAK cells were generated and maintained as previously described [15, 35]. Briefly, single-cell suspensions were prepared from murine spleens, filtered through a nylon mesh and washed twice with RPMI-1640 medium. Cells were transferred to T150 plastic flasks (Nunc, Roskilde, Denmark) at a concentration of 2×10^5 cells/ml in complete medium. Complete medium consisted of RPMI-1640 medium supplemented with 5% heat-inactivated FCS and 5% heat-inactivated normal human serum, 10 ml/l non-essential amino acids (Gibco), 50 mM 2-mercaptoethanol, 2 mM glutamine, 20 mM HEPES buffer, 0.8 g/l streptomycin and 1.6×10^5 U/l penicillin. Cells were activated with 100 Cetus U/ml human recombinant IL-2 (rIL-2, EuroCetus, Amsterdam, The Netherlands) and 0.4 μg/ml phytohemagglutinin P (PHA-P; Difco, Detroit, Michigan). After 2 days of incubation, clusters of T cells were transferred to 50-ml Nunc tubes and sediments were allowed to form for 5–7 min before the supernatant was gently removed. The cell sediment was resuspended at a concentration of 1×10^5 cells/ml in fresh complete medium containing 100 Cetus U IL-2/ml. Three days later, the cells were harvested, washed twice, and resuspended in RPMI-1640 medium for labeling. Routinely, the T-LAK cells were analyzed by a FACScan flow microfluorometer (Coulter Electronics Limited, Luton, UK), and were found to be more than 95% Thy1.2$^+$, more than 95% asialo-GM$_1^+$, more than 90% CD8 Lyt-2$^+$, and more than 90% CD3$^+$, but less than 5% NK1.1$^+$ and less than 2% CD4 L3T4$^+$, in agreement with our previous findings [16].

Labeling of T-LAK cells with ^{125}I-dU and TRITC

Samples containing 3×10^7 T-LAK cells in 50 ml complete medium were incubated for 20–22 h with 1 μCi ^{125}I-dU ([^{125}I]iododeoxyuridine, Amersham International Ltd., Amersham, Buckinghamshire, England). After labeling, cells were washed three times in RPMI-1640 medium and adjusted to 75×10^6 cells/ml. Microspheres (diameter, 15 ± 3 μm) of styrene-divinylbenzene copolymers, labeled with ^{57}Co (DuPont/NEN), were washed three times in RPMI-1640 medium before they were added to the ^{125}I-dU-labeled cells at a ratio of 1 to 600. At the time of injection, microspheres and T-LAK cells were kept in suspension by a small magnet inside a 1-ml syringe. This procedure prevented settling of the microspheres and ensured a constant ratio of microspheres to T-LAK cells.

To label T-LAK cells with tetramethylrhodamine isothiocyanate (TRITC), $(3–6) \times 10^8$ cells were incubated with 15 μg TRITC (Sigma Chemical Co., St. Louis, Mo.) in 50 ml RPMI-1640 medium (i.e., 0.3 μg/ml) for 30 min at 37 °C. After labeling, cells were washed twice in RPMI-1640 medium and adjusted to 75×10^6 cells/ml.

Injection of T-LAK cells

C57BL/6 mice with 10-day established tumors were injected with 15×10^6 TRITC- or ^{125}I-dU-labeled T-LAK cells in 200 μl RPMI-1640 medium, using the following routes of administration:

Intravenous injection

Intravenous (i.v.) injection of T-LAK cells was performed via the lateral tail vein.

Left-ventricular injection

Left ventricular (L.V.) injection was performed as previously described [2]. Briefly, mice were anesthetized with 0.01575 mg fentanyl citrate and 0.5 mg fluanisone (also called Hypnorm, from Janssenpharma, Birkerød, Denmark). The skin was shaved and cleansed with 70% alcohol, and a percutaneous cardiac puncture was performed 2 mm to the left of the sternum and 12 mm above the tip of the xiphoid process using a 27-gauge needle (0.4×25 mm) connected to the syringe via a Teflon tube. The tip of the needle was directed slightly in a caudal direction and angled about 25° relative to the sagittal plane. When lively pulsing blood was seen in the Teflon tube (indicating the correct location of the needle tip in the left ventricle), a total of 200 µl cell suspension was injected over a period of 35–45 s.

Intraportal injection

Before intraportal injections (v.p.), mice were anesthetized with Hypnorm. The skin was shaved, cleansed with 70% alcohol, and the anterior abdominal wall opened. A branch of the portal vein was identified and 200 µl cell suspension was injected over a period of 40–45 s from a 30-gauge needle connected to a syringe via a Teflon tube. The abdominal wall was closed in two layers with ligature (Look, Norwell, Mass.).

Intraperitoneal injection

Intraperitoneal injections (i.p.) of T-LAK cells were performed through the abdominal wall.

To support the NK cells, intraperitoneal injections of 6250 Cetus U IL-2 in 500 µl RPMI-1640 medium were given every 4 h, starting immediately after injection of the T-LAK cells.

Measurement of radioactivity

Mice injected with ^{125}I-dU-labeled T-LAK cells and ^{57}Co-labeled microspheres were sacrificed 16 h after injection and organs were placed in 70% ethanol. The ethanol was changed every day for 3 days. This procedure removes radioactivity not associated with whole DNA molecules [6, 14] and leaves the radioactivity in the microspheres unchanged. The organs were counted in a gamma counter (LKB-Wallace 1280, ultrogamma), with one channel recording ^{125}I-dU (15–35 keV) and another recording ^{57}Co (125–135 keV). Corrections were made for decay and spillover. Data are presented as the mean recovered radioactivity per organ as a percentage of the injected dose.

Distribution and enumeration of TRITC-labeled T-LAK cells in organs and blood

Animals were sacrificed 16 h after injection and representative samples of the lungs, livers, adrenal glands, kidneys, spleens, ovaries and brains were fixed in 4% formaldehyde for 24 h and thereafter placed in 30% sucrose for an additional 24 h. The tissues were snap-frozen in *n*-hexane at −70 °C and 8-µm cryosections were prepared from 15–20 different areas in each organ. TRITC-labeled cells were identified, using an Olympus fluorescence microscope with filter combinations for rhodamine (BP545). The density of transferred T-LAK cells in various organs was determined by counting the number of TRITC-labeled cells found in five to ten randomly chosen areas (measuring 0.25 mm^2) in each of the 15–20 sections made from the organ. The density was calculated as n/A, where n is the total number of fluorescent cells counted, and A is the sum (mm^2) of all the observed areas.

Metastatic lesions were easily identified by light microscopy because of the high content of melanin produced by the melanoma cells. The area of the metastatic tissue was measured by light microscopy using a 20× objective with a reticle containing 100 squares (at 20×, the reticle covers 0.25 mm^2). The number of infiltrating TRITC-labeled cells in individual metastatic lesions was counted by fluorescence microscopy. The average density of T-LAK cells in the metastatic tissue was calculated as:

$$\frac{(\text{number of TRITC-labeled cells in 10–20 metastases})}{(\text{total area of the 10–20 metastases})}\times$$

For the estimation of TRITC-labeled T-LAK cells found in the blood, 100-µl samples were drawn from the left ventricles of the hearts of ether-anesthetized animals. The red blood cells were lysed with ammonium chloride/potassium buffer at room temperature for 3 min, washed twice in RPMI-1640 medium and resuspended to 100 µl in 4% formaldehyde. The number of TRITC-labeled cells was counted and the average number of cells/ml blood was calculated.

Adoptive immunotherapy

To evaluate the antitumor effect of transferred T-LAK cells, 20×10^6 T-LAK cells were injected i.v. into animals bearing 7-day pulmonary and hepatic metastases. The animals received 20 000 Cetus U Peg-IL-2 (kindly provided by the Chiron Corporation, Emeryville, Calif.) in 0.5 ml twice a day for 4 days, starting immediately after the T-LAK cell injection. Organs were removed on day 15 of tumor growth. The tumor burden of the lung was measured as the percentage area of the lung surface covered by B16 metastases. The tumor burden of the liver was measured as the percentage area of B16 metastases seen in six to eight representative sections from each liver.

Statistical analysis

The results presented are expressed as the mean value (±SD) of five mice in each experimental group. Statistical significance was assessed by Student's two-tailed *t*-test.

Results

Organ distribution of ^{125}I-dU-labeled T-LAK cells 16 h after i.v., L.V. and v.p. injection

To evaluate the organ distribution of T-LAK cells administered by different routes, ^{125}I-dU-labeled T-LAK cells together with ^{57}Co-labeled microspheres were injected via a lateral tail vein (i.v.), the left ventricle of the heart (L.V.), a branch of the portal vein (v.p.), or via the abdominal wall, into the peritoneal cavity (i.p.). On the basis of their efficient entrapment in the first capillaries encountered, radiolabeled microspheres were, as previously described [2, 34], added to the inoculum to enable us to estimate the tissue distribution of the T-LAK cells immediately after injection. Organs were removed at 16 h and measured for radioactivity. The data are presented as the radioactivity recovered per organ as a percentage of the administered dose.

Following i.v. injection into normal mice, almost all (more than 99%, Table 1) of the microspheres were

Table 1 Percentage recovery of lymphokine-activated killer T (*T-LAK*) cells 16 h after i.v., left vertricular (*L.V.*) and intraportal (*v.p.*) injection into normal and tumor-bearing mice. 15×10^6 ^{125}I-dU-labeled T-LAK cells mixed with 2.5×10^4 ^{57}Co-labeled microspheres were injected i.v., L.V., and v.p. into C57BL/6 mice ($n = 5$) bearing 10-day-old B16 metastases in lungs, liver, adrenal glands, and ovaries. Organs were removed 16 h following injection of the T-LAK cells and their radioactivity was measured in a gamma counter. Data are presented as the recovered radioactivity as a percentage of the injected dose

Site	i.v. injected cells recovered ± SD (%)			L.V. injected cells recovered ± SD (%)			v.p. injected cells recovered ± SD (%)		
	Microspheres all mice	T-LAK cells		Microspheres all mice	T-LAK cells		Microspheres all mice	T-LAK cells	
		Tumor-bearing mice	Normal mice		Tumor-bearing mice	Normal mice		Tumor-bearing mice	Normal mice
Lungs	99.95 ± 10.9	33.7 ± 7.8	35.0 ± 9.1	1.8 ± 0.7	17.1 ± 2.3	15.4 ± 1.0	0.4 ± 0.2	9.3 ± 2.0	9.1 ± 1.4
Heart	0	0	0	2.1 ± 0.8	0.3 ± 0.1	0.4 ± 0.1	0	0.1 ± 0.1	0.1 ± 0.1
Spleen	0	3.1 ± 0.9	2.8 ± 0.4	1.3 ± 0.5	3.5 ± 1.1	3.9 ± 0.6	0	0.9 ± 0.1	0.9 ± 0.1
Kidneys	0	0.9 ± 0.1	2.0 ± 0.1	11.4 ± 2.6	2.3 ± 0.5	2.9 ± 0.2	0	0.6 ± 0.1	0.7 ± 0.1
Adrenal glands	0	0	0.1 ± 0.0	0.5 ± 0.2	0.3 ± 0.1	0.3 ± 0.1	0	0	0
Ovaries	0	0.1 ± 0.0	0.1 ± 0.0	1.4 ± 0.4	0.4 ± 0.1	0.5 ± 0.2	0	0.2 ± 0.1	0.2 ± 0.1
Intestine	0	2.1 ± 0.2	3.1 ± 0.6	17.2 ± 1.6	13.3 ± 3.0	17.6 ± 3.4	1.3 ± 1.4	6.3 ± 2.4	5.1 ± 1.0
Liver	0	10.6 ± 2.2	13.6 ± 1.0	2.1 ± 1.0	26.5 ± 1.7	24.2 ± 0.8	89.4 ± 14.8	80.0 ± 9.9	78.0 ± 9.8
Brain	0	0	0	5.9 ± 2.7	0.5 ± 0.4	0.7 ± 1.3	0	0.1 ± 0.1	0
Bone, skin, muscles	1.3 ± 0.6	10.9 ± 3.1	13.0 ± 2.0	30.0 ± 9.8	18.5 ± 5.2	14.8 ± 1.8	2.8 ± 4.4	11.4 ± 3.4	13.1 ± 3.4
Total	101.4	61.6	67.1	73.7	82.6	80.7	93.8	108.9	107.2

arrested in the pulmonary microcirculation. However, only approximately 34% of the injected T-LAK cells were found in this organ after 16 h. At this time, 10%–15% of the T-LAK cells were found in the liver and about 3% in the spleen. Practically no T-LAK cells were found in the ovaries and the adrenal glands.

In order to analyze whether tissue distribution of the i.v. injected T-LAK cells would be critically influenced by the presence of metastatic tissue at different histological sites, these experiments were repeated in animals with 10-day established B16 melanoma metastases in the lungs, liver, adrenal glands and ovaries. However, as seen in Table 1, the percentage recovery of T-LAK cells from the lungs as well as from various other organs of tumor-bearing hosts was not significantly different from that of the corresponding organs of normal animals, indicating that the overall organ distribution of the T-LAK cells was not affected by the presence of tumors.

To bypass the pulmonary capillary network, which seems to represent a major obstacle for the subsequent circulation of i.v. transferred T-effector cells, administration of T-LAK cells was performed directly into the arterial system via the left ventricle (L.V.) of the heart. On the basis of the distribution of microspheres following L.V. injection, more T-LAK cells were expected to reach extrapulmonary organs following this route of administration (Table 1) than after the i.v. route. Indeed, at 16 h more cells were found in livers, adrenal glands, and ovaries of L.V. injected animals than in the organs of those receiving the T-LAK cells by the i.v. route. As seen after i.v. injection, the presence of metastases in these organs did not change the overall organ distribution of the T-LAK cells. While higher numbers of L.V. injected T-LAK cells were found in the pulmonary tissue (approx. 17%) than were expected from the

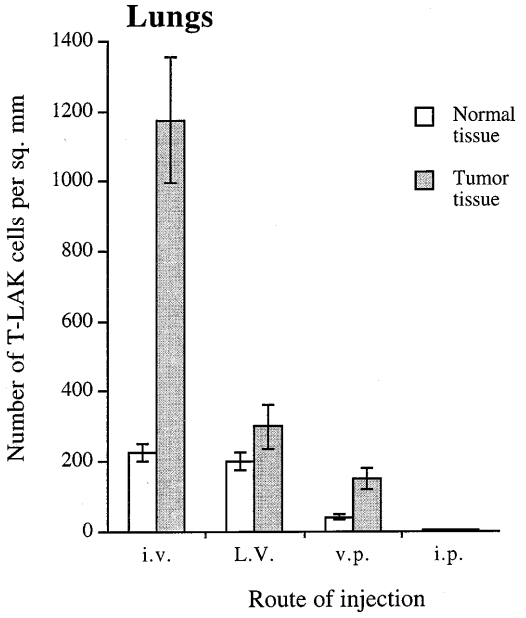

Fig. 1 Number of tetramethylrhodamine-isothiocyanate (TRITC)-labeled lymphokine-activated killer T (*T-LAK*) cells/mm^2 normal and metastatic lung tissue 16 h following different routes of administration. Mice received i.p. injections of 6250 Cetus U IL-2 every 4 h. *Bars* SD. Statistical analysis (by Student's two tailed *t*-test): number of i.v. versus number of left vertricularly (*L.V.*) transferred T-LAK cells in metastatic tissue: $P < 0.001$. Number of L.V. versus number of intraportally (*v.p.*) transferred T-LAK cells in metastatic tissue: $P < 0.001$. Number of i.v. versus number of L.V. transferred T-LAK cells in normal tissue: not significant (NS). Number of L.V. versus number of v.p. transferred T-LAK cells in normal tissue: $P < 0.001$

percentage of microspheres retained in this organ (1.8%, i.e., the blood supply of the lungs via the bronchial arteries), substantially fewer cells than expected were

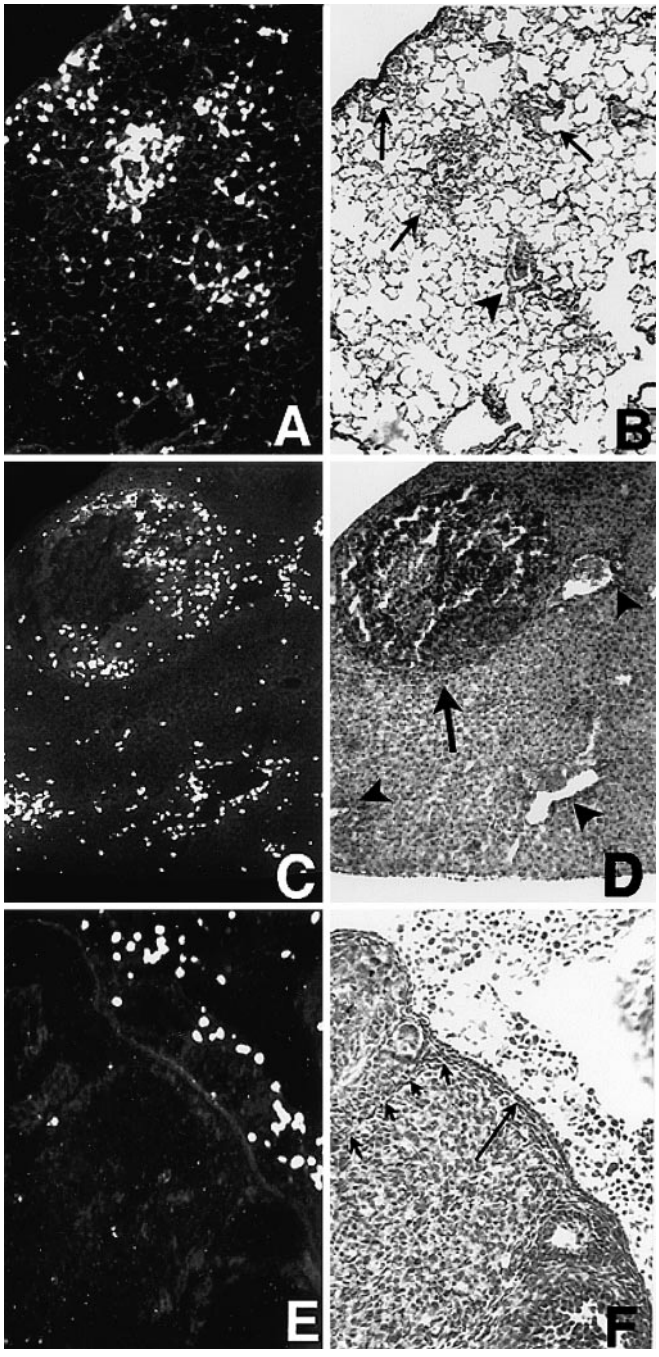

Fig. 2A–F Fluorescence- and light-microscopical images of lungs, liver, and ovaries following administration of 15×10^6 T-LAK cells by various routes supported by i.p. injections of 6250 U interleukin-2 (*IL-2*) every 4 h. **A** Fluorescence micrograph of lung tissue 16 h after i.v. administration of TRITC-labeled T-LAK cells. Note the significant accumulation of T-LAK cells (*white dots*) not only in the metastatic tissue (*black arrows*, **B**), but also in the subendothelial area of a larger vessel located in normal lung tissue (*arrowhead*, **B**). **B** Hematoxylin staining of the section shown in **A**. **C** Fluorescence micrograph of liver tissue 16 h after intraportal (v.p.) injection of TRITC-labeled T-LAK cells, showing accumulation of T-LAK cells (*white dots*) in a metastatic lesion (*black arrow*, **D**). Note the substantial accumulation of T-LAK cells also in the portal areas of the normal liver tissue (*arrowheads*, **D**). **D** Hematoxylin staining of the section shown in **C**. **E** Fluorescence image of ovary tissue 16 h after intraperitoneal injection of TRITC-labeled T-LAK cells. Even though substantial accumulation of T-LAK cells (*white dots*) is seen near the metastatic tissue, few, if any, of the T-LAK cells are able to penetrate the germinal epithelium. **F** Hematoxylin staining of the section shown in **E**. Metastatic tissue is located in the *top left-hand corner (above and to the left of small black arrows)*. The surface of the ovaries is indicated by a *large black arrow*

found in organs such as the kidneys, heart and brain (Table 1). Apparently, the T-LAK cells were not efficiently trapped in these organs, but were able to traverse them to enter the venous circulation, from where they subsequently reached the lungs.

Locoregional injection via the portal system led to an almost complete retention of the T-LAK cells in the liver, with no appreciable redistribution to other organs except the lungs, where about 9% of the injected cells were found at 16 h (Table 1).

Survival of T-LAK cells following i.v., L.V. and v.p. injection

Interestingly, a total of only 60%–70% of the radiolabeled T-LAK cells could be recovered 16 h after i.v. injection (Table 1), suggesting the death of 30%–40% of the cells at this time. Following L.V. injection, the total recovery of T-LAK cells at 16 h was approximately 80%, i.e., higher than what was seen after i.v. injection. The total recovery of intraportally transferred T-LAK cells was around 100% (Table 1).

Localization into metastatic tissue of various organs by T-LAK cells injected by different routes

In order to evaluate the ability of T-LAK cells to localize into B16 metastases growing in various organs, T-LAK cells were labeled prior to injection with the fluorochrome TRITC. Localization of i.v., L.V., v.p. and i.p. injected T-LAK cells into normal and metastatic tissue of lungs, livers, adrenal glands and ovaries was measured by counting fluorescent cells in sections of the various organs.

Lungs

Sixteen hours after i.v. transfer of TRITC-labeled T-LAK cells into tumor-bearing animals, five- to sixfold higher numbers of T-LAK cells were found in pulmonary metastases than in the surrounding normal tissue (Figs. 1, 2A, B). This substantial localization of i.v. injected T-LAK cells into the pulmonary metastases (1174 cells/mm^2) was significantly higher than the average density of T-LAK cells in pulmonary metastases observed following L.V. (298 cells/mm^2) or v.p. (147 cells/mm^2) injection (Fig. 1). Interestingly, in the normal

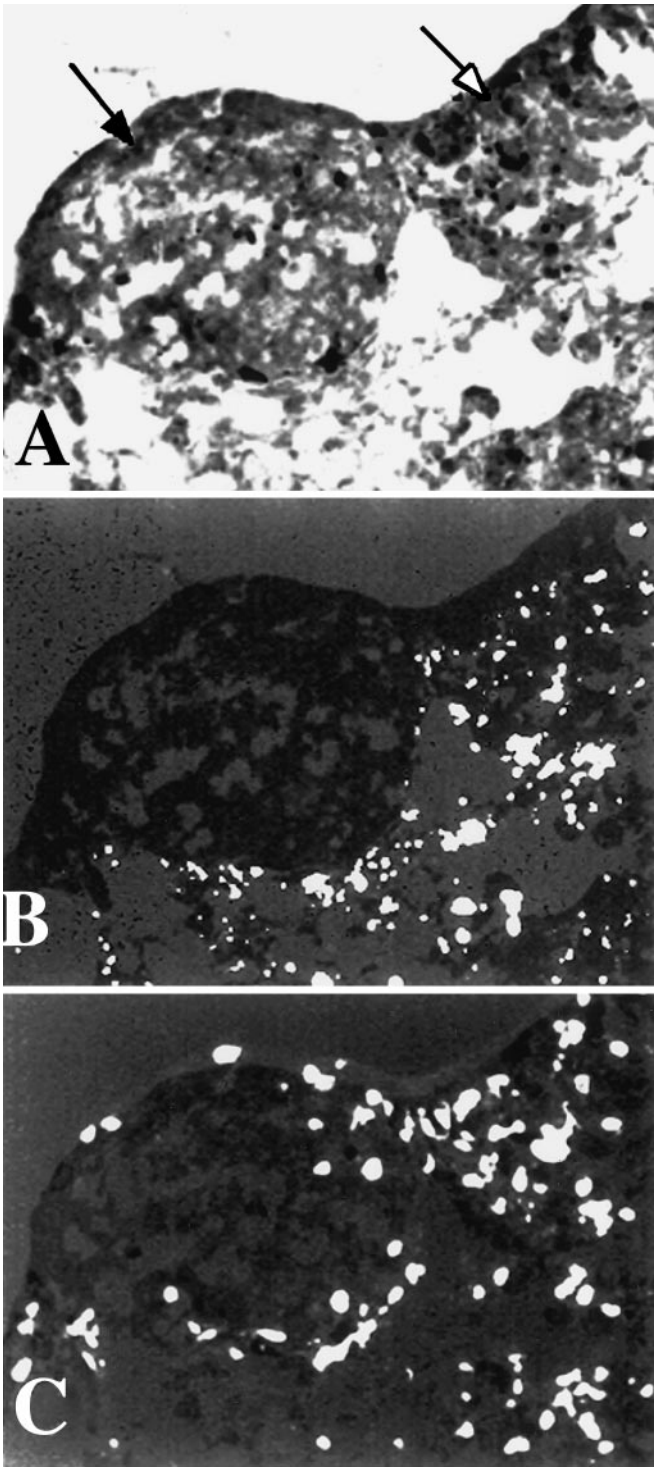

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Fig. 3A–C Infiltration-resistant and -permissive B16 melanoma metastases on the lung surface. Samples containing 15×10^6 TRITC-labeled T-LAK cells were injected 16 h prior to removal of the lungs. **A** The infiltration-resistant metastases grew as large, spherical nodules protruding from the lung surface (*black arrow*) in contrast to the more irregular and flat metastases of the infiltration-permissive type (*white arrow*). **B** Fluorescent micrograph of the area in **A**, showing lack of uptake of 0.2-μm fluorescein-isothiocyanate-labeled micro-beads by the infiltration-resistant metastases. In contrast, the infiltration-permissive lesion contains numerous microbeads (*white dots*) indicating an abundant presence of blood vessels. The microbeads were injected i.v. just prior to removal of the lungs. **C** Fluorescent micrograph of the area shown in **A** and **B**, demonstrating lack of uptake of TRITC-labeled T-LAK cells by the infiltration-resistant metastases. In contrast, the infiltration-permissive lesion contains numerous TRITC-labeled T-LAK cells (*white dots*)

lung tissue, a large number of the i.v. injected T-LAK cells was observed just beneath the endothelial lining of the pulmonary veins (Fig. 2A).

T-LAK cells injected by the i.p. route showed no appreciable accumulation into pulmonary metastases (fewer than 7 cells/mm^2), indicating that only few of the T-LAK cells were able to leave the peritoneal cavity.

Some pulmonary metastases did not become infiltrated at all. These non-infiltrated metastases were often growing around tubular structures (vessels or bronchioles) deep in the lung parenchyma or on the lung surface, where they, in contrast to the disc- or cone-shaped infiltration-permissive lesions, formed large, spherical tumors (Fig. 3A). Typical for these infiltration-resistant tumors was their apparent lack of functional blood vessels, as demonstrated by their poor perfusion with i.v. injected, fluorescently labeled microbeads (Fig. 3B). In contrast, fluorescent microbeads were always observed in lesions infiltrated by T-LAK cells (Fig. 3B, C).

Liver

In accordance with the results based on radiolabeled T-LAK cells, significantly more TRITC-labeled T-LAK cells were found in the hepatic parenchyma following L.V. injection than following i.v. injection (Fig. 4). Also, more T-LAK cells were found in the hepatic metastases following L.V. injection than there were after i.v. injection (35 cells/mm^2 and 26 cells/mm^2 respectively), but this difference was not statistically significant. Following locoregional delivery of T-LAK cells via a branch of the portal vein, substantially higher numbers of transferred cells were found not only in the hepatic parenchyma, but also in the hepatic metastases. Compared to the i.v. and L.V. routes of administration, 11- to 15-fold higher numbers of T-LAK cells were found in the hepatic metastases 16 h after the v.p. injection (Fig. 4). The density of T-LAK cells in individual hepatic metastases varied considerably. In some of the metastases (especially those growing around or near the portal areas), the T-LAK cells were distributed homogeneously throughout the lesion (Fig. 2C, D) but, in most other metastases, the marginal regions seemed to be the major area for accumulation of the T-LAK cells. Equivalent to the accumulation of T-LAK cells around pulmonary veins in the lungs, substantial numbers (1300 cells/mm^2) of

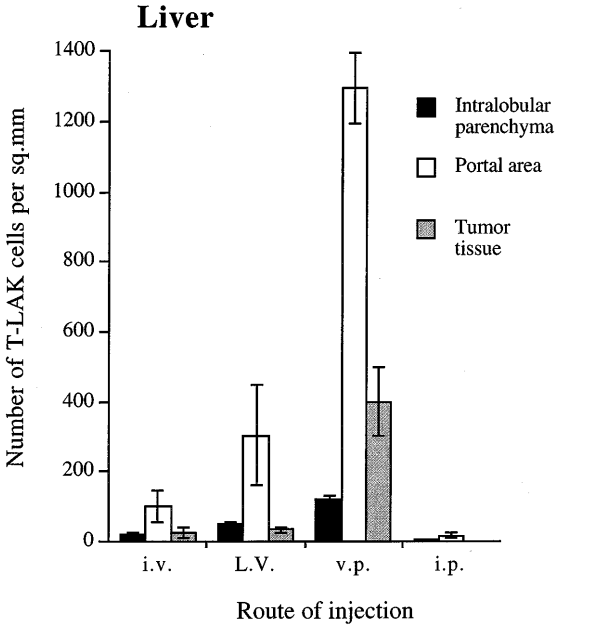

Fig. 4 Number of T-LAK cells/mm^2 normal intralobular parenchyma, portal area and metastatic liver tissue at 16 h following injection of 15×10^6 TRITC-labeled T-LAK cells by different routes. Mice received i.p. injections of 6250 Cetus U IL-2 every 4 h. *Bars* SD. Statistical analysis (by Student's two-tailed *t*-test): number of i.v. versus number of L.V. transferred T-LAK cells in metastatic tissue: NS. Number of L.V. versus v.p. transferred T-LAK cells in metastatic tissue, portal areas or intralobular parenchyma: $P < 0.001$. Number of i.v. transferred T-LAK cells in portal areas versus number of L.V. transferred T-LAK cells in portal areas: $P < 0.001$. Number of i.v. versus L.V. transferred T-LAK cells in the intralobular parenchyma: $P < 0.005$

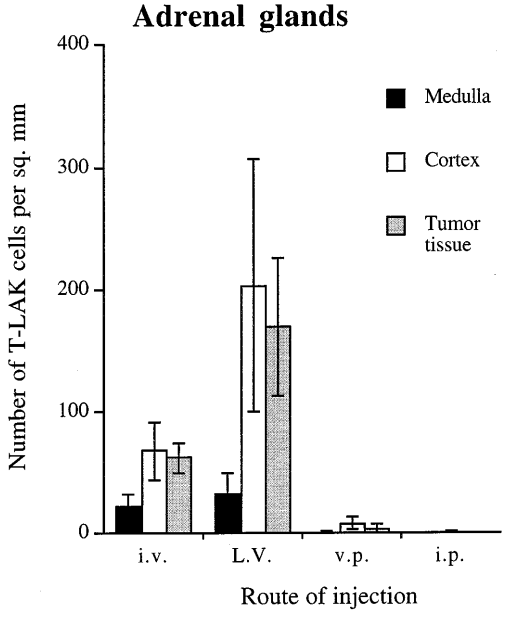

Fig. 5 Number (cells/mm^2) of T-LAK cells in the medulla, cortex and metastatic tissue of the adrenal glands 16 h after injection of 15×10^6 TRITC-labeled T-LAK cells by different routes. Mice received i.p. injections of 6250 Cetus U IL-2 every 4 h. *Bars* SD. Statistical analysis (by Student's two-tailed *t*-test): number of i.v. versus number of L.V. transferred T-LAK cells in normal cortex or metastatic tissue: $P < 0.001$. Number of i.v. or L.V. transferred T-LAK cells in metastatic tissue versus number of i.v. or L.V. transferred T-LAK cells in normal cortex: NS. Number of L.V. versus number of L.V. transferred T-LAK cells in normal medulla: $P < 0.001$

v.p. administered T-LAK cells were seen in the portal areas, surrounding the portal veins, the hepatic arteries and bile ducts (Fig. 2D). In contrast, no tropism of T-LAK cells towards the perivascular space of the central veins was found.

Many hepatic metastases were located on the surface of the liver, only separated from the peritoneal cavity by the liver capsule and the peritoneal lining. Nevertheless, T-LAK cells injected into the peritoneal cavity were not able to traverse this barrier to gain access to the liver metastases.

Adrenal glands

The numbers of metastatic lesions in the adrenal glands ranged from one to three. These metastases appeared almost exclusively as spherical nodules located in the cortex, close to the medullo-cortical junction. Following i.v. injection, T-LAK cells were found in low numbers in the normal cortex as well as in the metastatic tissue (68 and 62 cells/mm^2, respectively, Fig. 5). Injection of T-LAK cells via the L.V. route resulted in threefold higher numbers of cells in both the cortex and the metastases (204 cells/mm^2 and 170 cells/mm^2, respectively). However, in contrast to pulmonary and hepatic metastases, where the localization of T-LAK cells in the tumors was substantially higher than in the surrounding normal tissue, no significant difference was seen between the number of T-LAK cells accumulating in the adrenal cortex and that in the metastatic tissue. Regardless of the route of administration used, approximately threefold higher numbers of transferred cells were located in the cortex than in the medulla. As seen from Fig. 5, T-LAK cells injected by the intraperitoneal route were not able to reach the adrenal glands or metastases located within them.

Ovaries

Regardless of the route of administration, low numbers of T-LAK cells were found in the normal (fewer than 20 cells/mm^2) and the metastatic tissue (fewer than 5 cells/mm^2) of the ovaries. In animals receiving T-LAK cells by the intraperitoneal route, high numbers of cells were associated with the peritoneal serosa associated with the ovaries, but no cells were able to enter the ovarian metastases, even when located on the surface of this organ (Fig. 2E, F). Apparently, the germinal epithelium and/or the peritoneal lining functioned as efficient barriers to further migration of T-LAK cells.

Distribution of T-LAK cells into spleen, kidneys, brain and blood

In the spleen, the T-LAK cells were primarily found scattered throughout the white pulp. Only slightly more cells were found in this organ following L.V. injection than after i.v. injection (39.1 compared to 33.0 cell/mm^2, respectively). In the kidney, approximately six- to sevenfold higher numbers were found in the cortex (i.e., in the glomeruli) than in the medulla. Significantly more cells were found in this organ following L.V. injection than after i.v. injection (66.3 versus 22.0 cells/mm^2 respectively). The few homogeneously scattered T-LAK cells found in the brain tissue following i.v. and L.V. injection (1.3 and 3.1 cells/mm^2 respectively) indicated that the blood-brain barrier efficiently prevented influx of these cells from the blood.

Blood samples were drawn at 16 h and examined for the presence of TRITC-labeled cells. Regardless of the route of administration, very few T-LAK cells were found in the blood (approximately 1000–1300 cells/ml at 16 h, i.e., less than 0.01% of the injected dose/ml).

Therapeutic effect of i.v. injected T-LAK cells

To evaluate the therapeutic efficacy of systemically transferred T-LAK cells against 7-day established pulmonary and hepatic B16 melanoma metastases, 15×10^6 T-LAK cells were injected i.v. into animals. The T-LAK cells were supported by two daily i.p. injections of 20 000 Cetus U Peg-IL-2 for 4 days starting immediately following the T-LAK cell injection. The tumor burden, with respect to the lungs, was measured as the percentage of the lung surface area covered by tumor and, with respect to the liver, as the percentage of tumor tissue area compared to normal tissue seen in six to eight representative sections of each liver. The injection of IL-2 alone significantly reduced the tumor burden of both lungs and liver (Table 2). While the combination of T-LAK cells and IL-2 resulted in a further significant reduction of the lung metastases as compared to animals treated with IL-2 alone, T-LAK cells plus IL-2 did not induce a significant reduction of liver metastases as compared to IL-2 alone. Thus, a significant effect of the i.v. injected T-LAK cells was only observed in the lungs, consistent with the finding that i.v. injected T-LAK cells infiltrate lung metastases much better than liver metastases.

Discussion

Assuming that the extent to which adoptively transferred effector cells accumulate within or at least near the vicinity of malignant tissue is a critical factor for efficacy of adoptive immunotherapy, we have studied the role of the route of injection for the ability of ex vivo activated T cells to reach and infiltrate metastases at various anatomical locations.

In the current study, we found that 16 h after systemic injection of ^{125}I-labeled T-LAK cells, 50% of the recovered cells were still in the lungs. This supports the hypothesis that an efficient entrapment of transferred effector cells occurs in the capillary bed of this organ [3, 16]. We speculated that a higher retention of cells in organs downstream from the lungs would occur if the lung capillaries were by-passed by injecting the T-LAK cells into the arterial system via the left ventricle of the heart. This was found to be only partly true. Even though greater numbers of T-LAK cells were indeed found in most extrapulmonary organs following L.V. injection than were found after i.v. injection, the recovery of T-LAK cells in organs such as the kidneys, heart, brain and ovaries was less than 50% of the initial delivery of cells, indicating that many of them rapidly pass through these organs to enter the venous circulation, from where they are subsequently transported to and retained in the lungs. This is consistent with the finding that the recovery of T-LAK cells in the pulmonary tissue following L.V. injection was approximately six to eightfold higher than expected on the basis of the arterial supply of this organ. When cells were injected into the portal vein, a very high retention of T-LAK cells (80%) was found in the livers after 16 h. Consequently, cells that are able to pass through the vasculature of the

Table 2 Therapeutic efficacy of i.v. injected T-LAK cells. To induce metastases in lungs and liver, mice (five per group) were pretreated with 200 mg/kg cyclophosphamide i.p. on day −1 before tail-vein injection of 0.8×10^6 B16-F1(3H) melanoma cells. On day 7 of tumor growth, mice received i.v. injections of phosphate-buffered saline (PBS) [control and interleukin-2 (*IL-2*) groups] or 20×10^6 T-LAK cells (*T-LAK + IL-2* group). Mice received 0.5 ml PBS (control) or 20 000 Cetus U Peg-IL-2 in 0.5 ml PBS twice a day for 4 days following T-LAK cell injection (*IL-2* and *T-LAK + IL-2* groups). Data are presented as the percentage area (SD) covered by tumor. In lungs, the tumor area was measured on the lung surfaces. In the liver, the area of tumors was measured in six to eight representative sections of each liver. The percentage reduction in tumor area (lung metastases): IL-2 versus control: 58% ($P < 0.01$); T-LAK + IL-2 versus control: 90% ($P < 0.01$); T-LAK + IL-2 versus IL-2: 76% ($P < 0.03$). Percentage reduction in tumor area (liver metastases): IL-2 versus control: 48% ($P < 0.01$); T-LAK + IL-2 versus control: 82% ($P < 0.01$); T-LAK + IL-2 versus IL-2: 46% ($P > 0.3$)

Site	Area covered by tumor (%) following treatment with:		
	Control	IL-2	T-LAK + IL-2
Lungs	16.6 (3.7)	6.9 (3.14)	1.7 (0.9)
Liver	48.1 (9.7)	16.1 (14.9)	8.7 (2.6)

gastro-intestinal tract to enter the portal circulation will become efficiently arrested in the liver. These findings show that the lungs and liver are the main obstacles to the free circulation of transferred T-LAK cells. The low numbers of T-LAK cells found in the blood, regardless of route of administration, confirms the low circulatory potential of these cells.

Since the T-LAK cells circulate poorly, their only way of reaching tumors in various organs is either by reaching the tumor in the first passage or, for those cells that become retained in the tumor-bearing organs, by migration from the normal tissue towards the malignant tissue. Kinetic studies following systemic transfer of A-NK cells [3, 4] and tumor-sensitized T lymphocytes [15] have shown that the accumulation into pulmonary metastases is a time-dependent process. While few effector cells were found in pulmonary metastases within the first 2–4 h after transfer, significant numbers were detected after 8–12 h. Similar kinetic studies with T-LAK cells have given the same results, indicating that only a minority of the infiltrating T-LAK cells reach the pulmonary metastases in the first hours following injection. In other words, the accumulation of T-LAK cells seen in pulmonary metastases at 16 h is not caused by retention of the cells during their first passage of the tumor vasculature. Owing to the poor or slow circulation of the T-LAK cells, it is probably not caused by retention during subsequent passages of the tumor vasculature either. Against this background, it is very likely that the effector cells are first arrested in the capillaries of the normal lung parenchyma and that they subsequently extravasate and migrate to the tumor. If this is the case, one should expect to see the best tumor localization in those organs that retain high numbers of transferred cells in the tumor-surrounding normal tissue, i.e., the lungs and the liver. Moreover, while some infiltration should be seen in tumors in the adrenal glands (which are partially able to retain effector cells), low infiltration should be expected in tumors of the ovaries, brain and kidneys, which only retain a few effector cells. Indeed, this seemed to be the case, since quantification of fluorescently labeled T-LAK cells in tumors of various organs revealed that significant infiltration of pulmonary and hepatic metastases occurred following i.v. or v.p. injection. Following L.V. injection, some infiltration of metastases in the adrenal glands was seen, but no infiltration of ovarian metastases was detected (since no tumors formed in the kidneys and brain, the infiltration of metastases in these organs could not be assessed). Taking all these results together, it appears that increased infiltration of tumors can be achieved if the effector cells are injected locoregionally rather than systemically. However, significant tumor infiltration occurs only when the tumor-bearing organ is able to retain at least some of the effector cells. Furthermore, a significant antitumor effect was seen only when substantial infiltration of the tumors was achieved, as, in lung metastases following i.v. injection of the T-LAK cells, for example. Better tumor localization into and antitumor responses against liver metastases following v.p. injection than after i.v. injection of A-NK cells have recently been demonstrated by Hagenaars et al. in a rat model [12].

In ovarian cancer, the tumor is often separated from the peritoneal cavity only by the peritoneum and a thin layer of ovarian tissue [29, 36]. It is therefore possible that adoptively transferred effector cells would localize better into ovarian malignancies if they were injected by the i.p. route rather than the i.v. route [20]. In a study by Cappuccini et al. [7], the trafficking patterns of intraperitoneally transferred T-LAK cells was examined with ^{51}Cr as the cell label. In this study, a rapid adherence of the T-LAK cells to the serosal surface was demonstrated. By tracing i.p. injected TRITC-labeled cells, we also found a substantial adherence of T-LAK cells to the peritoneal serosa. This adherence might explain why we found so few i.p. injected T-LAK cells at any of the extraperitoneal locations and why few, if any, of the T-LAK cells were able to invade metastases located on the surface of the ovaries or the liver. These findings point towards the peritoneum as an efficient barrier through which the effector cells are not able to migrate. On the other hand, previous studies from our group have shown substantial infiltration of B16 metastases if these are located *in* the peritoneal cavity [8]. Therefore, treatment of patients with ovarian cancer, for example, with i.p. injection of effector cells might be most effective in those situations where the malignant tissue has penetrated the peritoneum and established itself in the peritoneal cavity.

Interestingly, the overall survival of the T-LAK cells varied with the route of injection. Survival of T-LAK cells is worst following i.v. injection, better following L.V. injection and optimal following v.p. injection, correlating with the number of cells reaching the lungs after injection by these routes. An explanation for the apparent damage/death of transferred cells in pulmonary tissue cannot be given in the current study, but it is possible that pure mechanical forces applied to the T-LAK cells during their passage through the lung microvasculature might damage or kill the cells. Weiss et al. [33] have shown that, following inoculation of tumor cells with a mean diameter of 16.5 μm, many of the tumor cells are damaged as a consequence of deformation-induced lethal mechanical trauma in the microcirculation. Since IL-2-activated effector cells are also quite large (14–18 μm) as well as generally more rigid and less deformable than non-activated lymphocytes [26], it is possible that the same mechanism is responsible for the damage to the T-LAK cells. Alternatively, some effector cells might be killed by endogenous NK cells as suggested by Brubaker et al. [5].

Analyzing sections of lung and liver tissues revealed a characteristic location of the fluorescent T-LAK cells. Following transfer of T-LAK cells via the portal vein, up to 30% of the cells were found in the portal areas, most often located beneath the endothelial lining of the portal vein, hepatic artery and, to a lesser extent, the bile

duct. Substantially fewer cells were found in the sinusoids and hardly any around the central veins. A similar preferential location of T-LAK cells in the subvascular spaces of pulmonary vessels was observed following i.v. injection of the cells. Immunohistochemical staining with anti-CD8 antibody confirmed this distribution pattern in normal hepatic and pulmonary tissue even 48 h following locoregional transfer of the cells (Kjærgaard, J., unpublished observation). The characteristic accumulation in the perivascular spaces of veins in the lungs and liver in the mouse is apparently attributable only to T-LAK cells, since similar accumulation was not observed following transfer of murine A-NK cells [22]. However, since as many as 30% of all T-LAK cells found in the liver are located in these perivascular spaces, it will be important to elucidate whether they are permanently "trapped" at this location (and therefore unable to reach the malignant tissues) or whether they represent a pool of potentially tumor-seeking T-LAK cells, temporarily residing in this area.

In conclusion, adoptively transferred T-LAK cells show a remarkable ability to migrate to and localize into metastases of the lungs and liver, and, to a certain extent, metastases in the adrenal glands. However, as is the case with adoptively transferred A-NK cells, the circulatory potential of these cells is low, mainly because of an efficient entrapment of the T-LAK cells in the lungs and the liver. Collectively, our results indicate that these effector cells must be injected locoregionally in order to ensure significant infiltration into the malignant tissues and to mediate significant antitumor responses. This can be fairly easily accomplished in the case of the lungs and liver, which are the primary target organs for metastasis of almost all types of cancers, by injecting the cells i.v. and v.p. respectively. In this regard, we are currently investigating to what extent the localization of T-LAK cells into metastases in various locations correlates with therapeutic efficacy. When more is learned about the molecular events leading to entrapment of the T-LAK cells in the perivascular spaces of vessels in the lungs and the liver, it might be possible, e.g., by manipulation of the expression of various adhesion molecules on the T-LAK cells and/or various tissues, to obtain a better circulation of these cells and to direct them specifically to selected target tissues.

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