Different cytokine profiles released by CD4+ and CD8+ tumor-draining lymph node cells involved in mediating tumor regression

Atsushi Aruga, Etsuko Aruga, Mark J. Cameron, and Alfred E. Chang

Division of Surgical Oncology, Department of Surgery, University of Michigan, Ann Arbor

Abstract: We have previously demonstrated that the growth of weakly immunogenic murine sarcomas leads to the induction of immunologically specific pre-effector cells in tumor-draining lymph nodes (TDLN). The in vitro activation of TDLN cells with anti-CD3 monoclonal antibodies (mAbs) and interleukin-2 (IL-2) resulted in the acquisition of effector function as measured by tumor regression in the adoptive immunotherapy of pulmonary metastases. Further studies were performed to characterize the mechanisms associated with in vivo tumor reactivity mediated by activated TDLN cells. By positive selection, CD4+ and CD8+ T cells were purified and activated by the anti-CD3/IL-2 method. CD8+, but not CD4+, cells manifested tumor-specific granulocyte-macrophage colony-stimulating factor (GM-CSF) and interferon-γ (IFN-γ) release in vitro, and elicited tumor regression in vivo. By contrast, only activated CD4+ were found to release significant amounts of IL-2 in response to tumor antigen but did not mediate tumor regression in vivo. Mixing the two purified populations enhanced the antitumor activity of the CD8+ T cells. In culture, IL-2 was found to augment the relative amount of tumor-specific release of GM-CSF and IFN-γ by activated TDLN cells. We found that the tumor-specific release of GM-CSF and IFN-γ by activated lymphocytes was strongly associated with the in vivo therapeutic efficacy of these cells. Evidence in support of this included the following: (1) cytokine release of TDLN derived after different durations of tumor growth correlated with tumor reactivity in adoptive transfer studies, (2) cytokine release of T cells derived from different lymphoid organs corresponded with tumor reactivity in adoptive transfer, and (3) in vivo administration of neutralizing mAb to IFN-γ and GM-CSF significantly inhibited the antitumor reactivity of TDLN cells. These studies document the contributory roles of IFN-γ, GM-CSF, and IL-2 released by activated CD4+ and CD8+ T cells involved in tumor regression. *J. Leukoc. Biol.* 61: 507–516; 1997.

Key Words: *interferon-γ · interleukin-2 · tumor reactivity · tumor regression*

INTRODUCTION

We have previously reported that tumor-draining lymph nodes (TDLN) in animals bearing weakly immunogenic tumors harbor lymphoid cells with antitumor reactivity [1–6]. These TDLN cells were precursors that were not capable of mediating tumor regression in adoptive immunotherapy and required further in vitro activation to differentiate into functional effector cells. Hence, we have referred to TDLN cells as pre-effector lymphoid cells. One method to activate pre-effector lymphoid cells is to stimulate them with irradiated tumor cells in vitro in the presence of low concentrations of interleukin-2 (IL-2). In murine models, this in vitro sensitization (IVS) method generally required 9–10 days and resulted in a three- to fivefold expansion of lymphoblasts that were predominantly CD8+ T cells [1]. These IVS cells mediated immunologically specific tumor regression after adoptive transfer. Another method that can induce effector function in pre-effector cells is by the activation with immobilized anti-CD3 antibody followed by expansion in low concentrations of IL-2 [4–6]. This method allowed TDLN cells to expand 6- to 10-fold and did not require tumor stimulator cells that could be unavailable in a clinical setting.

Despite the polyclonal nature of stimulating pre-effector LN cells with anti-CD3, we have reported the ability to generate immunologically specific effector cells [4, 5]. However, it is apparent that only a fraction of the total pool of TDLN cells represent sensitized effector cells. Multiple anti-CD3 stimulation of a bulk population of lymphoid cells from immunized animals will result in the loss of antitumor

---

Abbreviations: TDLN, tumor-draining lymph nodes; mAbs, monoclonal antibodies; IL-2, interleukin-2; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN-γ, interferon-γ; IVS, in vitro sensitization; s.c., subcutaneous; HBSS, Hanks' balanced salt solution; IgG, immunoglobulin G; ELISA, enzyme-linked immunosorbent assay; CM, complete media; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; LAK, lymphokine-activated killer; APC, antigen-presenting cell; TIL, tumor-infiltrating leukocytes.

Correspondence: Alfred E. Chang, M.D., Division of Surgical Oncology, University of Michigan Medical Center, 1500 E. Medical Center Drive, Ann Arbor, MI 48109.

Received October 10, 1996; revised December 24, 1996; accepted December 26, 1996.

reactivity of the expanded cells, presumably due to the outgrowth of non-immune cells [7]. Hence, it is important to characterize the cellular mechanisms necessary in eliciting in vivo tumor reactivity in adoptive immunotherapy models in order to allow the development of more selective methods to isolate and expand functional effector cells. We have previously reported that anti-CD3/IL-2 activated TDLN cells are non-cytolytic in standard 4-h cytotoxicity assays and are distinctly different from TDLN cells cultured by the IVS method, which are cytolytic [1]. Therefore, alternative assays to assess the in vitro reactivity of potential effector cells was required. Recent investigations have reported that cytokine release may be a more important assay to predict the capability of effector cells in mediating in vivo tumor reactivity [8, 9]. In a prior study we found that tumor-specific interferon-γ (IFN-γ) and granulocyte-macrophage colony-stimulating factor (GM-CSF) release by IVS-cultured TDLN cells correlated with their ability to mediate tumor regression in vivo [10]. In this study we examined the role of IFN-γ, GM-CSF, and IL-2 release of anti-CD3/IL-2 lymphoid cells with respect to therapeutic efficacy in an adoptive transfer model. In addition, we have characterized the role of purified populations of $CD4^+$ and $CD8^+$ cells involved in tumor regression. The observations from this study extend our knowledge about the immune response associated with progressive tumors as well as providing insights into approaches for isolating competent immune cells from the tumor-bearing host.

MATERIALS AND METHODS

Mice

Female C57BL/6(B6) mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained in a specific pathogen-free environment. They were used at the age of 8 weeks or older. Principles of laboratory animal care (NIH publication No. 85-23, revised 1985) and animal protocols approved by the University of Michigan Laboratory of Animal Medicine were followed.

Tumor

The MCA 207 and MCA 205 are fibrosarcomas induced by 3-methylcholanthrene, syngeneic to B6 mice, and previously characterized to be immunologically distinct [11]. The studies performed with MCA 205 utilized a clone of the tumor, H12, and is hereafter referred to as MCA 205 in this article. Tumors were maintained in vivo by serial subcutaneous (s.c.) transplantation in syngeneic mice and used within the seventh transplantation generation. They were kindly provided by Dr. James C. Yang (National Cancer Institute, NIH, Bethesda, MD). Tumor cell suspensions were prepared from solid tumors by enzymatic digestion in 50 mL of Hanks' balanced salt solution (HBSS; GIBCO, Grand Island, NY) containing 40 mg of collagenase, 4 mg of DNase, and 100 units of hyaluronidase (Sigma Chemical Co., St. Louis, MO) for 3 h at room temperature as previously described [4].

TDLN cells

B6 mice were inoculated s.c. with 1.5×10^6 MCA 207 or MCA 205 tumor cells in the lower flank. At specific intervals after tumor inoculation the adjacent tumor-draining inguinal LN were harvested and single cell suspensions were prepared mechanically as described previously [4]. Typically, 10^7 lymphoid cells per TDLN were retrieved in mice bearing 10-day established s.c. flank tumors. In other studies, mesenteric lymph node, splenocyte, and thymus were similarly prepared for comparison with inguinal LN.

Anti-CD3/IL-2 activation procedure

The 145-2C11 hybridoma cells producing hamster immunoglobulin G (IgG) monoclonal antibody (mAb) against the CD3ε chain of the murine TCR/CD3 complex were a gift from Dr. J. A. Bluestone, University of Chicago. Antibodies were prepared by injecting hybridoma cells into sublethally irradiated DBA/2 mice and collecting the ascites. The ascites was partially purified by 50% ammonium sulfate precipitation and the IgG content was determined by an enzyme-linked immunosorbent assay (ELISA). Twenty-four-well tissue culture plates were coated with anti-CD3 overnight at 4°C. TDLN cells (4×10^6) were cultured in 2 mL of complete media (CM) at 37°C in a 5% CO_2 incubator for 2 days. CM consisted of RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 0.1 mM non-essential amino acids, 1 μM sodium pyruvate, 2 mM fresh L-glutamine, 100 μg/mL streptomycin, 100 units/mL penicillin, 50 μg/mL gentimicin, 0.5 μg/mL Fungizone (all from GIBCO), and 5×10^{-5} M 2-mercaptoethanol (Sigma). After activation with anti-CD3, cells were washed once and cultured at 3×10^5 cells/well in 2 mL of CM containing 60 units/mL of human recombinant IL-2 in 24-well plates for 3 days. Human recombinant IL-2 was a gift from Chiron Therapeutics, Emeryville, CA. It has a specific activity of 18×10^6 international units/mg protein. In this study, results of all experiments are expressed in international units (IU). The anti-CD3/IL-2-activated LN cells were harvested, washed, and resuspended in HBSS for adoptive immunotherapy or in CM with irradiated tumors for cytokine release assay.

Immunofluorescence and flow cytometry

Immunophenotyping of lymphoid cells was carried out by indirect immunofluorescence. Briefly, 5 to 10×10^5 cells were incubated for 45 min at 4°C with 25 μL of appropriately diluted mAb in phosphate-buffered saline (PBS) containing 1% fetal bovine serum and 0.1% NaN_3. Rat mAb against the murine CD4 (GK1.5, L3T4), CD8 (2.43, Lyt-2.2), and hamster mAb against the murine CD3 (145-2C11) were used in ascites form for phenotyping of T cells (obtained from the American Type Culture Collection, Rockville, MD). Bound antibodies were detected by incubation with 20 μL of the fluorescein isothiocyanate (FITC)-labeled mAb to rat kappa-chain (MAR 18.5) or an anti-hamster IgG-FITC cocktail (PharMingen, San Diego, CA). Stained cell preparations were analyzed in a FACScan flow microfluorometer (Becton-Dickinson, Sunnyvale, CA). Fluorescence profiles were generated by analyzing 10,000 cells and displayed as logarithmically increasing fluorescence intensity versus cell numbers.

In vitro selection of $CD4^+$ and $CD8^+$ cells by magnetic beads

In vitro positive selection of $CD4^+$ or $CD8^+$ T cells was performed with anti-CD4 (L3T4) or anti-CD8 (Lyt 2) mAb followed by using anti-rat IgG-conjugated magnetic beads. Briefly, freshly harvested 2×10^7 TDLN cells were incubated with 200 μL of L3T4 (1:100 ascites) or Lyt 2 (1:200 ascites) mAb at 4°C for 30 min. After washing, 10^8 sheep anti-rat IgG-coated Dyna-beads M-450 (Dyna Inc., Great Neck, NY) at a 1:5 ratio of cells to beads were added into TDLN cells at 4°C for 30 min before placement in a magnetic holder. The supernatant, containing non-reacted cells, was depleted and positively isolated cells were incubated in 0.25% trypsin-ethylenediaminetetraacetate solution at 37°C for 15 min to detach the beads from the cells. These positively isolated cells were stimulated by the anti-CD3/IL-2 method described above and the efficacy of cell separation was evaluated by FACS analyses before the cells were used for in vivo adoptive transfer or in vitro cytokine release assays.

In vitro cytotoxicity assay

A long-term cytotoxicity assay was used to assess the cytolytic activity of TDLN cells over a 48-h period [12]. Freshly harvested tumor cells

were digested and single cells were cultured in a 96-well flat plate in 100 mL of CM for 24 h. After 24 h, tumor cells were subconfluent and TDLN after anti-CD3/IL-2 activation were added into the well with 100 μL of CM containing 4 units/mL of IL-2. Forty-eight hours later, wells were washed three times with PBS and fixed with fixing solution for 10 min. Fixing solution was made from 95% methanol plus glacial acetic acid in a 3:1 ratio. Plates were washed four times with water and 75 μL of crystal violet was added for 5 min. Staining solution was made from 0.5% crystal violet. The plates were then washed several times with water until the blank wells became clear. Plates were dried at room temperature overnight and dissolved with 100 μL of dissolving solution. Dissolving solution was made from 95% ethanol, double-distilled H_2O, and glacial acetic acid. Optical density of each well was read at 570 nm with an ELISA plate reader and cytotoxicity calculated by the following equation: % of cytotoxicity = [1 − (absorbance of tumor cells treated with effector cells/absorbance control)] × 100.

In this assay, only viable tumor cells were stained and measured by their optical density. The effector cells and nonviable tumor cells were removed when the wells were washed by PBS after 48 h.

Measurement of in vitro cytokine release

After anti-CD3/IL-2 activation, TDLN cells were restimulated with irradiated autologous tumor cells in CM. Tumor stimulator cells were irradiated to 7000 cGy by a ^{137}Cs source before using. LN cells (0.5×10^5/mL) and irradiated stimulators (2.5×10^5/mL) were co-cultured in 2-mL volumes in 24-well tissue culture plates. After 48 h, culture supernatants were collected for cytokine measurements in duplicate using commercially available ELISA kits. If detectable, background cytokine values produced by tumors alone were subtracted from the co-culture values when reporting results. For murine GM-CSF, a standard curve starting at 20 ng/mL with serial twofold dilutions was performed. For IFN-γ, tumor necrosis factor α (TNF-α), IL-4, and IL-2, standard curves starting at 1,000 IU/mL, 50 ng/mL, 1,000 IU/mL, and 20 ng/mL were established in a similar fashion, respectively. Experimental values were computed with the use of regression analysis.

Adoptive immunotherapy

B6 mice were inoculated intravenously (i.v.) with 2 to 2.5×10^5 MCA 207 or MCA 205 tumor cells to establish pulmonary metastases. Three days after tumor inoculation, mice were injected i.v. with TDLN cells and given intraperitoneal (i.p.) injections of IL-2 commencing on the day of cell transfer and continuing twice daily for 4 days. At approximately 3 weeks after tumor initiation, mice were randomized and killed for enumeration of pulmonary metastatic nodules. The metastases appeared as discrete white nodules on the black surface of lungs insufflated with a 15% solution of India ink and bleached by Fekete's solution [13]. Metastatic foci too numerous to count were assigned an arbitrary value of > 250. The significance of differences in numbers of metastatic nodules between experimental groups was determined by use of the non-parametric, Wilcoxon rank-sum test. Two-sided P values < 0.05 were considered significant. Each group consisted of at least five mice and no animal was excluded from the statistical evaluation.

In vivo neutralization of GM-CSF and IFN-γ by mAb

Inhibition of GM-CSF and IFN-γ was performed with the i.v. administration of the neutralizing mAb to both cytokines. Briefly, rat anti-IFN-γ mAb (R4-6 A2) obtained from Dr. Keith Bishop (University of Michigan, Ann Arbor, MI) and rat anti-GM-CSF mAb (MP1-22E9) obtained from Dr. Robert Coffman (DNAX, Palo Alto, CA) were utilized as ascites developed from the hybridoma lines. Hybridoma cells were cultured in CM, injected i.p. (3×10^7 cells) into cyclophosphamide-pretreated DBA mice after 600 cGy whole-body irradiation. Approximately 9 days later, ascites were harvested and centrifuged to remove the cells. The efficacy of neutralizing activity was measured by ELISA assay where 1:100 ascites could neutralize at least 20 ng/mL of GM-CSF or 1000 units/mL of IFN-γ. In the adoptive transfer model, 0.2 mL of ascites was administered via caudal vein daily for 4 days after cell transfer. Control groups were given rat Ig i.v. at the same dose and intervals as the neutralizing mAb.

RESULTS

Role of CD4+ and CD8+ TDLN cells in the regression of MCA 207 tumor

To document the role of CD4+ and CD8+ T cells in the regression of MCA 207 tumor, we depleted those T cell subsets with mAb after adoptive transfer of activated TDLN cells. MCA 207 TDLN cells were harvested 9 days after s.c. inoculation and activated by the anti-CD3/IL-2 method. Shortly after adoptive transfer of 10^7 TDLN cells, anti-CD4 or anti-CD8 mAb was administered i.v. Selected groups of mice were given IL-2 i.p. As shown in **Table 1**, depletion of CD8+ cells completely abrogated the antitumor regression mediated by the whole population of activated TDLN cells with or without the administration of exogenous IL-2. Depletion of CD4+ cells in the absence of IL-2 administration diminished the tumor regression mediated by the remaining CD8+ TDLN cells. With the administration of IL-2, the depletion of CD4+ cells had no effect on the antitumor reactivity mediated by the transferred TDLN cells. This experiment demonstrated the requisite role of CD8+ T cells involved in MCA 207 tumor regression as well as the facilitory contribution of CD4+ cells.

To more fully evaluate the reactivity of CD4+ and CD8+ T cell subsets, we proceeded to purify each population from whole TDLN for further analysis. Freshly harvested MCA 207 TDLN obtained 10 days after tumor inoculation contained a heterogeneous population of lymphoid cells. Immunophenotyping by flow cytometry of fresh TDLN revealed 28% Thy 1.2+ lymphoid cells that were 14% CD4+ and 18% CD8+ (data not shown). After anti-CD3/IL-2 activation the cells were predominantly Thy 1.2+ (99.8%) and consisted of (9%) of CD4+ and (90%) CD8+ cells (**Fig.**

TABLE 1. Therapeutic Efficacy of TDLN Cells After In Vivo Depletion of CD4+ and CD8+ T Cells

Group	TDLN cells[a]	In vivo IL-2[b]	In vivo antibody[c]	Mean no. of lung metastases (SEM)
A	−	−	−	>250
B	−	+	−	>250
C	+	−	Rat Ig	6 (2)[d]
D	+	−	anti-CD4	125 (7)[e]
E	+	−	anti-CD8	>250
F	+	+	Rat Ig	0[e]
G	+	+	anti-CD4	8 (3)[d]
H	+	+	anti-CD8	>250

[a] Nine days after MCA 207 tumor s.c. inoculation, TDLN were harvested for anti-CD3/IL-2 activation. Mice were injected with 2×10^5 tumor cells i.v. and received 10^7 TDLN cells 3 days later.

[b] IL-2, 30,000 IU i.p. twice daily after cell transfer.

[c] Anti-CD4 or anti-CD8 ascites (0.2 mL) were injected i.v. after cell transfer. Lungs were harvested on day 20.

[d] $P < 0.0001$ compared with group A, B, E, and H; $P < 0.001$ compared with group D.

[e] $P < 0.001$ compared with group A, B, E, and H.

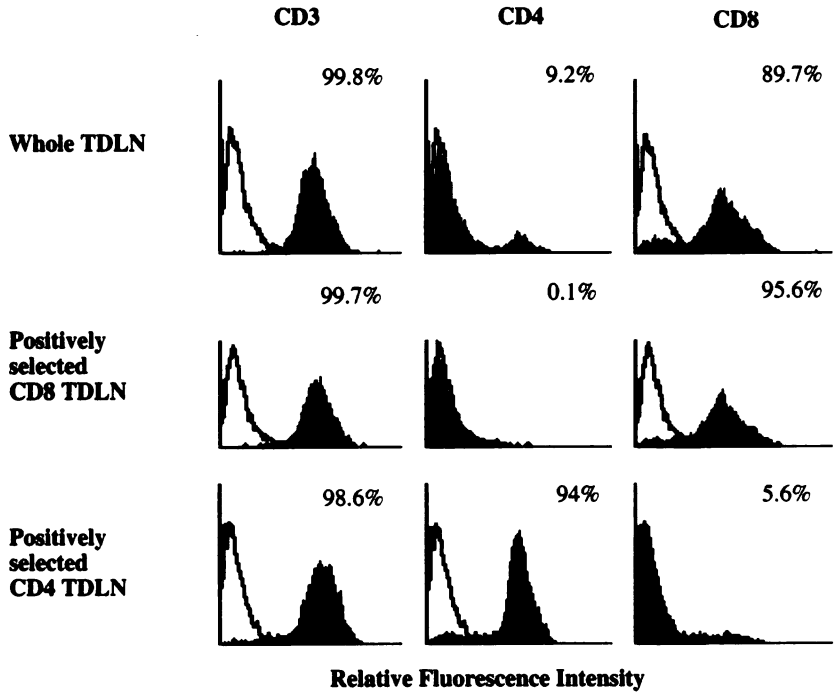

Fig. 1. Flow cytometric analysis of TDLN after activation with anti-CD3/IL-2. The whole-cell population was analyzed in conjunction with CD4-and CD8-selected subpopulations.

1). CD4+ and CD8+ T cell subpopulations were selected by an immunomagnetic bead technique from whole TDLN and subsequently activated by the anti-CD3/IL-2 method. Flow cytometry demonstrated a relatively homogeneous population of cells: the L3T4-selected cells were 94% CD4+ and the Lyt-2 selected cells were 96% CD8+ (Fig. 1).

The therapeutic efficacy of these cells was assessed in the adoptive immunotherapy of pulmonary metastases (**Table 2**). The adoptive transfer was performed in the absence of exogenous IL-2 administration to determine the relative role of CD4+ cells. The whole TDLN population significantly mediated tumor regression. CD4+ cells were not efficacious compared with CD8+ cells, which significantly reduced the mean number of pulmonary metastases. Importantly, when both CD4+ and CD8+ cells were mixed together, tumor regression was significantly improved compared with CD8+ cells alone and indicated a cellular interaction between the two cell populations.

TABLE 2. Therapeutic Efficacy of MCA 207 TDLN After Positive Selection for CD4+ and CD8+ T Cells

		Adoptive immunotherapy[b]	
Group	Effector cells[a]	IL-2	Mean no. pulmonary metastases (SEM)
A	–	–	>250
B	Whole TDLN	–	0[c]
C	CD4+ TDLN	–	>250
D	CD8+ TDLN	–	110 (9)[d]
E	CD4+ plus CD8+ TDLN	–	0[c]

[a] Ten days after MCA 207 tumor s.c. inoculation TDLN were harvested for anti-CD3/IL-2 activation.

[b] The tumor reactivity of the activated TDLN cells was assessed in the adoptive immunotherapy of 3-day established MCA 207 pulmonary metastases as described in Table 1. Lungs were harvested on day 20.

[c] $P < 0.001$ compared with other groups.

[d] $P < 0.01$ compared with A and C.

In vitro immunological reactivity of activated CD4+ or CD8+ TDLN cells

We have previously reported that anti-CD3/IL-2-activated TDLN cells were non-cytolytic in a 4-h cytotoxicity assay [5]. However, in a 48-h cytotoxicity assay, we found that purified CD8+ cells mediated significant MCA 207 tumor lysis with markedly diminished activity observed with purified CD4+ cells (**Fig. 2**). The cytolytic activity of CD8+ cells was immunologically specific (data not shown).

The release of IFN-γ, GM-CSF, and IL-2 by purified CD4+ and CD8+ TDLN cells was assessed. MCA 207 TDLN cells obtained 10 days after inoculation were positively selected for CD4+ and CD8+ T cells and activated

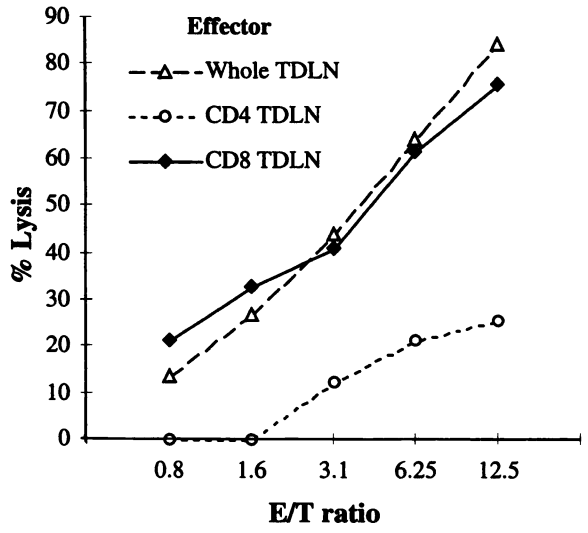

Fig. 2. Cytotoxicity of MCA 205 or MCA 207 TDLN against the relevant tumor target in a 48-h assay.

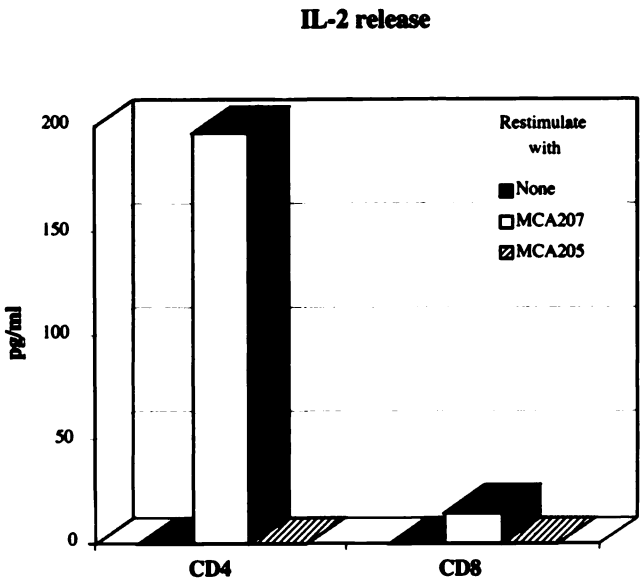

Fig. 3. Tumor-specific release of IL-2 by CD4+ MCA 207 TDLN cells.

by the anti-CD3/IL-2 method. We found that activated CD4+ TDLN released significantly greater amounts of IL-2 compared with CD8+ cells in response to MCA 207 tumor antigen and not to the irrelevant MCA 205 tumor (**Fig. 3**). By contrast, activated CD8+ TDLN cells demonstrated tumor-specific release of IFN-γ and GM-CSF compared with CD4+ cells, which had significantly diminished IFN-γ secretion in response to MCA 207 antigen, and released both IFN-γ and GM-CSF in a non-specific manner in response to MCA 205 (**Fig. 4**) [12]. The non-specific release of these cytokines by CD4+ cells suggested a lymphokine-activated killer cell (LAK)-like phenomenon. We have previously reported that LAK cells, which secreted IFN-γ and GM-CSF nonspecifically in response to tumor, were significantly less efficient in mediating tumor regression on a per cell basis compared with sensitized T cells, which released those cytokines in a tumor-specific manner [10]. The in vivo tumor reactivity of MCA 207 and MCA 205 TDLN cells at the whole population level was found to be immunologically specific in criss-cross experiments summarized in **Table 3**. We did not evaluate the in vivo antitumor reactivity of CD4+ cells at significantly higher dose levels required to observe nonspecific LAK activity.

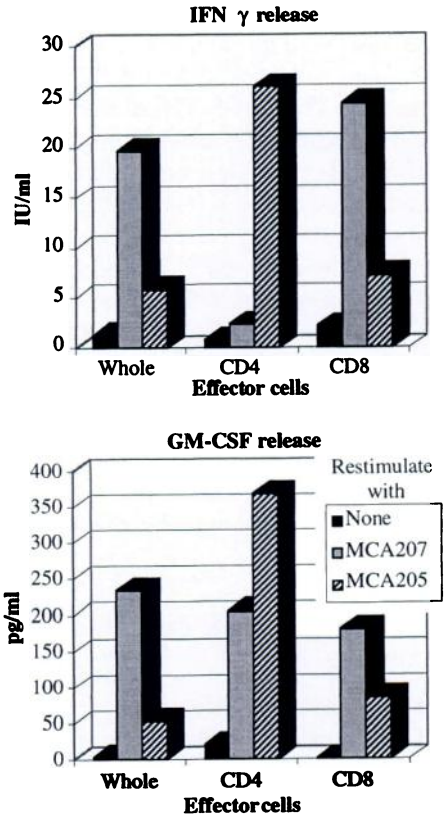

Fig. 4. Tumor-specific release of IFN-γ and GM-CSF by activated MCA 207 TDLN before and after positive selection for CD4+ and CD8+ cells. The cytokine release assay was performed in the presence of 4 IU/mL IL-2. Tumor-specific release of cytokines was observed from the whole and CD8+-selected population.

In a separate experiment we evaluated the effect of exogenously added IL-2 to the in vitro release of GM-CSF and IFN-γ by activated TDLN cells. As shown in **Figure 5**, the presence of increasing concentrations of IL-2 resulted in greater release of both GM-CSF and IFN-γ over the 24-h culture period. This observation, in conjunction with our adoptive transfer studies, suggested that CD4+ cells provide help to CD8+ cells elaborating IL-2, which in turn, enhances GM-CSF and IFN-γ release in response to tumor antigen.

Neutralization of GM-CSF and IFN-γ by mAb administration

The role of GM-CSF and IFN-γ released from anti-CD3/IL-2-activated TDLN cells was examined with the use of neutralizing mAb administered to mice after the adoptive transfer of cells. Neutralizing antibodies were administered daily for 4 days after cell transfer with control animals receiving rat Ig. Animals that received activated TDLN along with rat Ig demonstrated significant tumor regression compared

TABLE 3. Specificity of Tumor Regression Mediated by Anti-CD3/IL-2-Activated TDLN Cells in Adoptive Immunotherapy

	Adoptive immunotherapy[b]			
			Mean no. pulmonary metastases (SEM)	
Source of TDLN[a]	No. of cells transferred	IL-2	MCA 207	MCA 205
None	–	–	>250	>250
None	–	+	>250	>250
MCA207	10^7	+	0[c]	235 (12)
MCA 205	10^7	+	>250	0[c]

[a] Ten days after MCA 207 or MCA 205 tumor inoculation TDLN were harvested for anti-CD3/IL-2 activation.

[b] Mice were inoculated with 2.5×10^5 MCA207 or 4×10^5 MCA 205 tumor cells i.v. Three days later, mice received activated TDLN cells i.v. along with the concomitant administration of IL-2 (60,000 IU) i.p. twice daily for a total of eight doses. Lungs were harvested on day 20.

[c] $P < 0.0001$ compared with other groups.

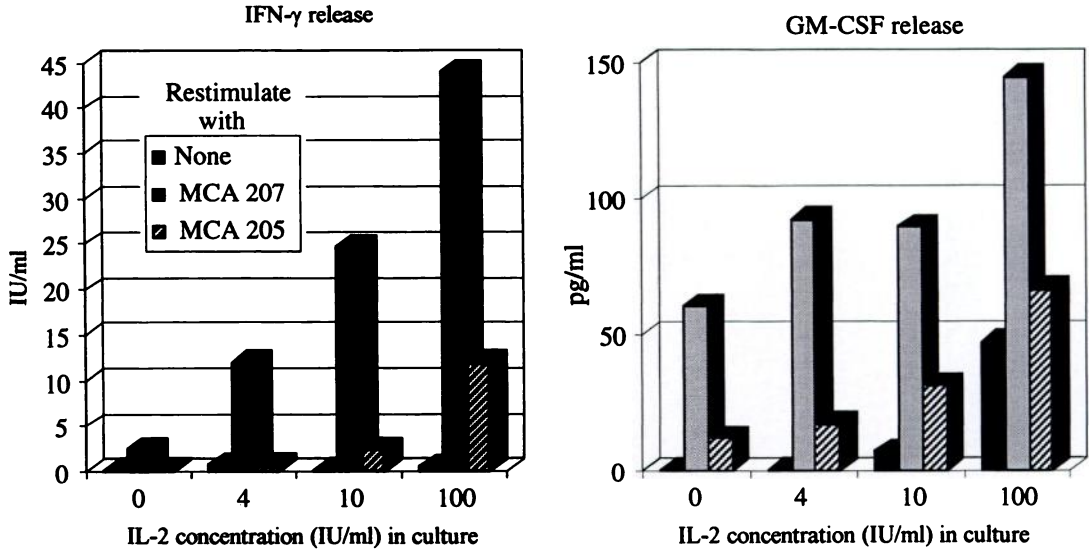

Fig. 5. Tumor-specific release of IFN-γ and GM-CSF by activated MCA 207 TDLN is increased by the addition of IL-2 in culture.

with mice that received rat Ig only (**Table 4**). When anti-GM-CSF or anti-IFN-γ mAb were administered after cell transfer, in vivo therapeutic efficacy was diminished; however, there was no statistical difference between the rat Ig-administered group and either the anti-GM-CSF or anti-IFN-γ mAb-administered group. Importantly, when both neutralizing mAb were administered, in vivo therapeutic efficacy was significantly diminished. These results confirm that both GM-CSF and IFN-γ released from TDLN cells have an important role in the tumor destruction associated with this therapy.

Kinetics of pre-effector cell induction in TDLN

The correlation of GM-CSF and IFN-γ release by activated lymphoid cells and their subsequent therapeutic efficacy was further examined in this tumor model. The kinetics of pre-effector cell induction in TDLN was examined by inoculating MCA 207 tumor subcutaneously and harvesting TDLN every few days for cryopreservation up to 21 days.

TABLE 4. Therapeutic Efficacy of MCA 207 TDLN After Administration of GM-CSF and IFN-γ-neutralizing mAb

Group	MCA 207 TDLN[a]	Antibody	Mean no. pulmonary metastases (SEM)[b]
A	–	Rat Ig	>250
B	–	anti-GM-CSF + anti-IFN-γ	>250
C	+	Rat Ig	24 (8)[c]
D	+	anti-GM-CSF	50 (12)[c]
E	+	anti-IFN-γ	60 (11)[c]
F	+	anti-GM-CSF + anti-IFN-γ	135 (14)[c,d]

[a] Ten days after MCA 207 or MCA 205 tumor s.c. inoculation TDLN were harvested for anti-CD3/IL-2 activation.

[b] Mice were injected with 2.5×10^5 MCA 207 tumor cells i.v. and received 10^7 effector cells three days later. Antibody (0.2 mL) was injected i.v. daily for 4 days after cell transfer. Lungs were harvested on day 15.

[c] $P < 0.0001$ compared with other groups.

[d] $P < 0.01$ compared with groups C, D, and E.

Upon collecting all the TDLN from different time intervals, the TDLN were thawed, washed, and cultured by the anti-CD3/IL-2 method. After activation, TDLN cells were restimulated in vitro with irradiated MCA 207 or MCA 205 tumor and supernatant harvested for cytokine determinations (**Fig. 6**). There were insignificant amounts of IFN-γ or GM-CSF released by day 0, 4, and 21 TDLN cells. These TDLN cells were also assessed for therapeutic efficacy by adoptive transfer into mice with established 3-day MCA 207 pulmonary metastases (**Table 5**). TDLN harvested on days 0, 4, and 21 had no therapeutic activity compared with control groups. TDLN cells harvested on day 10 after tumor inoculation mediated significant regression of pulmonary metastases. These results suggested that a correlation existed between in vitro tumor-specific IFN-γ and GM-CSF release with the in vivo therapeutic efficacy of TDLN cells. The mechansim associated with loss of antitumor function of TDLN cells during progressive tumor growth may be related to an acquired defect in T cell signaling, which has been reported to occur with prolonged tumor exposure [14]. In a separate report we have documented a significant decrease in TCRζ chain expression by TDLN cells in late tumor-bearing animals, which persisted despite subsequently anti-CD3/IL-2 in vitro activation [15].

Distribution of pre-effector cells in different lymphoid organs

We proceeded to investigate whether a correlation was present between tumor-specific cytokine release of lymphoid cells obtained from different sites and their in vivo therapeutic efficacy. B6 mice were inoculated in the lower flank with MCA 207 tumor cells and 10 days later had inguinal, mesenteric LN, spleen, and thymus removed for anti-CD3/IL-2 activation. After activation, cells were assessed for in vitro cytokine release and for in vivo tumor reactivity as previously described. The therapeutic efficacy of each cell population

was found to vary, with inguinal TDLN cells being the most effective followed by splenocytes (**Table 6**). Mesenteric LN and thymus cells did not mediate significant tumor regression nor did these cells release significant amounts of cytokines (**Fig. 7**). In contrast, tumor-specific release of IFN-γ and GM-CSF was associated with in vivo therapeutic efficacy, which was greatest with inguinal TDLN cells followed by splenocytes.

DISCUSSION

A variety of methods have been reported to isolate lymphoid cells from the tumor-bearing host for ex vivo culture and subsequent use in adoptive immunotherapy. One of the first clinical applications was the harvesting of peripheral blood lymphoid cells by leukapheresis for subsequent activation in IL-2 to generate LAK cells [16]. LAK cells were found to be highly cytotoxic in vitro against tumor targets in a non-MHC-restricted manner [17]. Although these cells mediated regression of established disease in animal models, it required relatively large numbers of transferred cells and was limited to the treatment of micrometastatic disease. In addition, clinical studies involving LAK cells plus IL-2 have demonstrated minimal benefit over the use of IL-2 alone [18]. Alternative approaches have focused on T cells with MHC-restricted reactivity to tumor. One such approach involves the isolation of tumor-infiltrating lymphocytes (TIL),

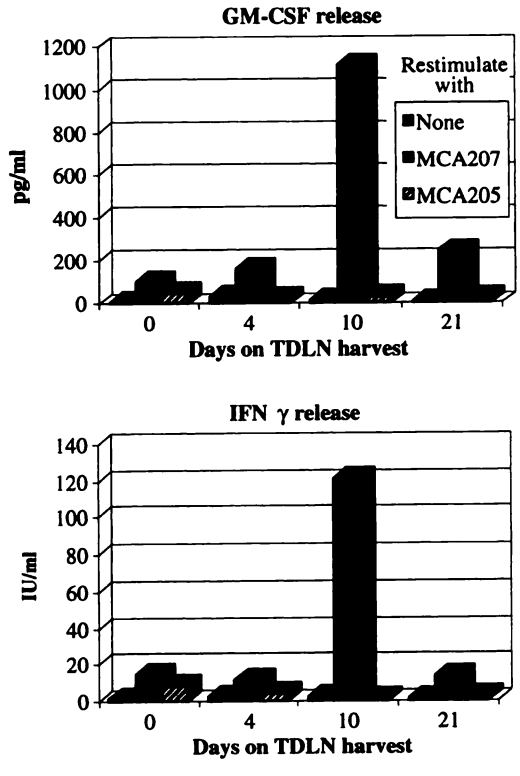

Fig. 6. Duration of MCA 207 tumor growth influenced the cytokine release profile of activated TDLN. Only day 10 MCA 207 TDLN released IFN-γ and GM-CSF cytokines and correlated with in vivo antitumor efficacy (see Table 5).

TABLE 5. Therapeutic Efficacy of MCA 207 TDLN Obtained on Different Days after Tumor Inoculation

Days after s.c. tumor inoculation[a]	Adoptive immunotherapy[b] IL-2	Mean no. pulmonary metastases (SEM)
–	–	>250
–	+	>250
0	+	>250
4	+	173 (39)
10	+	0[c]
21	+	>250

[a] Mice were inoculated s.c. with MCA 207 tumor cells. At different intervals after tumor inoculation inguinal TDLN were harvested for anti-CD3/IL-2 activation.

[b] The tumor reactivity of the activated TDLN cells was assessed in the adoptive immunotherapy of 3-day-established MCA 207 pulmonary metastases as described in Table 1.

[c] $P < 0.0001$ compared with all other groups.

which are expanded in IL-2 [19, 20]. In animal models, these approaches are more potent than LAK cell therapies and appear to have clinical efficacy in a select group of patients [21, 22]. As another approach to generate tumor-specific T cells, we have examined the antitumor reactivity of human vaccine-draining LN cells secondarily activated in vitro [23, 24]. Characterizing the requisite cell populations that are necessary for mediating tumor regression in vivo and defining in vitro parameters that correlate with therapeutic potential would be critical in the further development of clinical cellular therapies.

Several reports have indicated that cytokine secretion by TIL appears to correlate with therapeutic efficacy in animal models. Barth et al. examined non-cytolytic CD8+ TIL cell lines derived from a weakly immunogenic murine sarcoma and demonstrated that IFN-γ and TNF-α release in response to tumor stimulation was associated with their antitumor reactivity after adoptive transfer [9]. Goedegebuure et al. reported that the release of IFN-γ and GM-CSF by TIL during culture with anti-CD3 and IL-2 appeared to correlate with their therapeutic efficacy in the treatment of MCA 105 tumors [10]. In clinical TIL studies, Schwartzentruber et al. reported that the release of GM-CSF by TIL in response to autologous tumor appeared to be an independent predictor of tumor response in melanoma patients [22].

This study extends our earlier reported observations that the tumor-specific release of GM-CSF and IFN-γ by TDLN cells secondarily activated in culture was associated with therapeutic efficacy [10]. In contrast to the previous report, which involved in vitro activation of TDLN cells with tumor antigen, we examined an alternate method to activate TDLN cells utilizing anti-CD3 mAb, a pan-T cell-stimulating reagent. In addition, we have examined the release of GM-CSF and IFN-γ by CD4+ and CD8+ TDLN cells with their tumor reactivity in vivo. Isolation of these T cell subsets involved positive selection techniques resulting in purified cell populations. We found that positively selected CD8+ TDLN cells were capable of mediating tumor regression in the absence of CD4+ cells. CD8+ cells were found to release GM-CSF and IFN-γ in a tumor-specific manner. By contrast, isolated

TABLE 6. Therapeutic Efficacy of Lymphoid Cells From Different Sites in MCA 207 Tumor-Bearing Mice

Group	Source of lymphoid cells for activation[a]	Adoptive Immunotherapy: IL-2	Adoptive Immunotherapy: Mean no. of pulmonary metastases (SEM)[b]
A	–	+	181 (18)
B	Inguinal LN	+	0[c]
C	Spleen	+	64 (18)[d]
D	Mesenteric LN	+	154 (9)
E	Thymus	+	187 (12)

[a] Mice were inoculated s.c. with MCA 207 tumor cells in the lower flank and underwent removal of LN 10 days later for anti-CD3/IL-2 activation.

[b] The tumor reactivity of the activated TDLN cells were assessed in the adoptive immunotherapy of 3-day established MCA 207 pulmonary metastases as described in Table 1.

[c] $P < 0.01$ compared with all other groups.

[d] $P < 0.05$ compared with groups A, D, and E.

$CD4^+$ cells did not mediate tumor regression upon adoptive transfer. $CD4^+$ cells manifested reduced levels of IFN-γ release and nonspecific release of GM-CSF. The important role that GM-CSF and IFN-γ has in mediating tumor regression was documented in several other experiments. We were able to show that the induction of pre-effector TDLN cells over a prolonged duration of tumor growth diminished. This was directly correlated with loss of the ability of activated TDLN cells to release both cytokines in response to tumor. In addition, the ability to generate therapeutic effector cells from different lymphoid organs was also associated with the functional ability of the activated cells to release GM-CSF or IFN-γ in a tumor-specific manner. Finally, the administration of neutralizing mAb to either cytokine reduced the antitumor effect of adoptively transferred immune cells, with neutralization of both cytokines having a greater effect than either cytokine alone. It should be noted that we did not completely abrogate tumor regression with neutralization of these cytokines, which suggested either incomplete neutralization or more likely that other mechanisms were involved in the antitumor response.

The role of $CD4^+$ cells was also characterized in our studies. We confirmed by in vivo depletion studies that $CD4^+$ cells were important in the adoptive immunotherapy model. This cell population appeared to be necessary as a source of IL-2, since their absence could be replaced by the exogenous administration of the cytokine. In cytokine release assays, we found that purified $CD4^+$ cells released significantly greater amounts of IL-2 in response to tumor stimulation compared with $CD8^+$ cells. Moreover, IL-2 was found to augment the release of GM-CSF and IFN-γ when added exogenously to activated TDLN cells co-cultured with irradiated tumor cells. This latter observation suggests one mechanism by which $CD4^+$ cells interact with $CD8^+$ cells in mediating tumor regression in vivo.

The potential role of GM-CSF and IFN-γ involved in the immune rejection response may relate to their influence on host components. We and others have demonstrated that the adoptive transfer of immune lymphoid cells can mediate tumor regression in animals previously subjected to whole body irradiation (500 cGy), which eliminated a major contribution of the host lymphoid compartment in the rejection of tumor [25–28]. A potential radiation-resistant cell that may play a significant role in the immune response are monocytes/macrophages. In two separate studies, the in vivo inhibition of macrophage function by carragenan or trypan blue abrogated the therapeutic efficacy of adoptively transferred lymphoid cells in animal models [27, 28]. As a corollary, we have reported that the administration of anti-Ia mAb will also inhibit the antitumor effects mediated by adoptively transferred T cells suggestive of a contributory role for host antigen-presenting cells (APC) [3]. Both IFN-γ and GM-CSF have been shown to activate macrophages to become tumoricidal [29–31]. In addition, APC has been reported to be down-regulated in the tumor-bearing host [32, 33]. In this context, GM-CSF has been recently shown to reconstitute the immunostimulatory capacity of tumor-derived APC [33].

Other potential mechanisms related to the antitumor reactivity associated with IFN-γ release may be secondary to its effects on tumor. IFN-γ has direct tumoricidal effects on tumor cells [34]. It is also associated with the enhanced expression of MHC and/or tumor-associated antigens that could make tumors more susceptible to T cell eradication

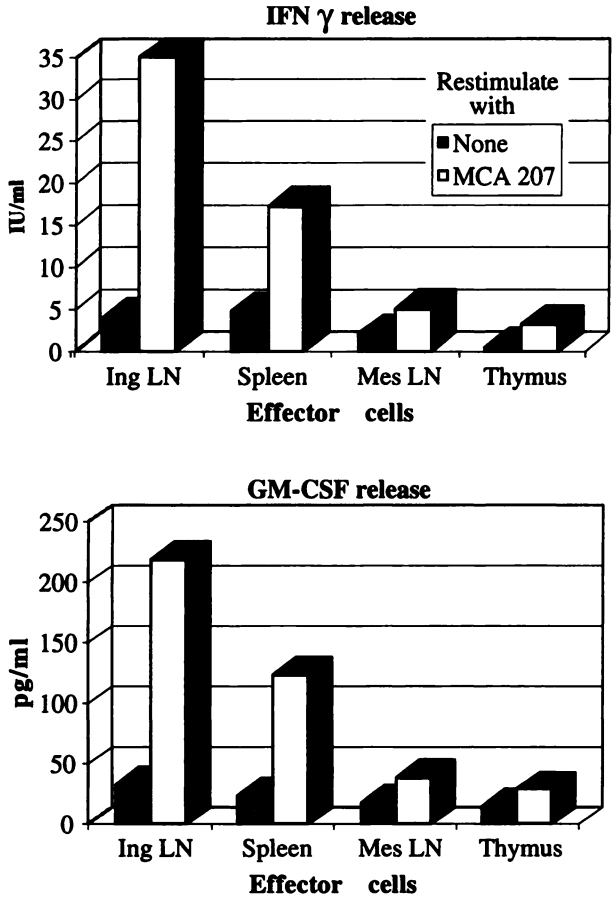

Fig. 7. Tumor-specific release of activated cells derived from different lymphoid organs in animals inoculated with MCA 207 tumor 10 days earlier. Cytokine release correlated with in vivo antitumor reactivity (see Table 6).

[35, 36]. Another intriguing mechanism that may be occurring is the modulation of tumor cells by IFN-γ to become non-professional APC that can present tumor antigen to host immune components [37].

In summary, we have demonstrated that sensitized CD8+ T cells are necessary in initiating tumor regression in an adoptive immunotherapy model. The elaboration of GM-CSF and IFN-γ are involved in this antitumor response and may prove to be important functional endpoints for the identification of cellular reagents used in adoptive immunotherapy.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health Grants CA59327 and CA57815; and the Gillson Longenbaugh Foundation. Dr. Chang is an American Cancer Society Faculty award recipient.

The authors would like to thank Dr. James Mulé for his helpful review of this manuscript and Susan Sackrider for her excellent assistance in its preparation.

REFERENCES

1. Chou, T., Chang, A. E., Shu, S. (1988) Generation of therapeutic T lymphocytes from tumor-bearing mice by in vitro sensitization. Culture requirements and characterization of immunologic specificity. *J. Immunol.* **140**, 2453–2461.
2. Chou, T., Bertera, S., Chang, A. E., Shu, S. (1988) Adoptive immunotherapy of microscopic and advanced visceral metastases with in vitro sensitized lymphoid cells from mice bearing progressive tumors. *J. Immunol.* **141**, 1775–1781.
3. Ward, B. A., Shu, S., Chou, T., Perry-Lalley, D., Chang, A. E. (1988) Cellular basis of immunologic interactions in adoptive T cell therapy of established metastases from a syngeneic murine sarcoma. *J. Immunol.* **141**, 1047–1053.
4. Yoshizawa, H., Sakai, K., Chang, A. E., Shu, S. (1991) Activation by anti-CD3 of tumor-draining lymph node cells for specific adoptive immunotherapy. *Cell. Immunol.* **134**, 473–479.
5. Yoshizawa, H., Chang, A. E., Shu, S. (1991) Specific adoptive immunotherapy mediated by tumor-draining lymph node cells sequentially activated with anti-CD3 and IL-2. *J. Immunol.* **147**, 729–737.
6. Geiger, J. D., Wagner, P. D., Cameron, M. J., Shu, S., Chang, A. E. (1993) Generation of T cells reactive to the poorly immunogenic B16-BL6 melanoma with efficacy in the treatment of spontaneous metastases. *J. Immunother.* **13**, 153–165.
7. Crossland, K. D., Lee, V. K., Chen, W., Riddell, S. R., Greenberg, P. D., Cheever, M. A. (1991) T cells from tumor-immune mice nonspecifically expanded in vitro with anti-CD3 plus IL-2 retain specific function in vitro and can eradicate disseminated leukemia in vivo. *J. Immunol.* **146**, 4414–4420.
8. Goedegebuure, P. S., Zuber, M., Leonard-Vidal, D. L., Burger, U. L., Cusack, J. C., Chang, M. P., Douville, L. M., Eberlein, T. J. (1994) Reactivation of murine tumour-infiltrating lymphocytes with solid-phase anti-CD3 antibody: in vitro cytokine production is associated with in vivo efficacy. *Surg. Oncol.* **3**, 79–89.
9. Barth, R. J., Mulé, J. J., Spiess, P. J., Rosenberg, S. A. (1991) Interferon γ and tumor necrosis factor have a role in tumor regressions mediated by murine CD8+ tumor-infiltrating lymphocytes. *J. Exp. Med.* **173**, 647–658.
10. Aruga, A., Shu, S., Chang, A. E. (1995) Tumor-specific GM-CSF and INF-γ secretion is associated with in vivo therapeutic efficacy of activated tumor-draining lymph node cells. *Cancer Immunol. Immunother.* **41**, 317–324.
11. Barth, R. J., Bock, S. N., Mulé, J. J., Rosenberg, S. A. (1990) Unique murine tumor-associated antigens identified by tumor infiltrating lymphocytes. *J. Immunol.* **144**, 1531–1537.
12. Finke, J. H., Rayman, P., Alexander, J., Edinger, M., Tubbs, R. R., Connelly, R., Pontes, E., Bulowski, R. (1990) Characterization of the cytolytic activity of CD4+ and CD8+ tumor-infiltrating lymphocytes in human renal cell carcinoma. *Cancer Res.* **50**, 2363–2370.
13. Wexler H. (1966) Accurate identification of experimental pulmonary metastases. *J. Natl. Cancer Inst.* **36**, 641–645.
14. Mizoguchi, H., O'Shea, J. J., Longo, D. L., Loeffler, C. M., McVicar, D. W., Ochoa, A. C. (1992) Alterations in signal transduction molecules in T lymphocytes from tumor-bearing mice. *Science* **258**, 1795–1798.
15. Fu, E. J., Arca, M. J., Haim, J. M., Krinock, R., Rado, J., Cameron, M. J., Chang, A. E., Sondak, V. K. (1997) Tumor-induced suppression of antitumor reactivity and depression of TCR expression in tumor-draining lymph node lymphocytes: possible relationship to the Th2 pathway. *J. Immunother.* in press.
16. Rosenberg, S. A., Lotze, M. T., Muul, L. M., Leitman, S., Chang, A. E., Ettinghausen, S. E., Matory, Y. L., Skibber, J. M., Shiloni, E., Vetto, J. T., Seipp, C. A., Simpson, C., Reichert, C. M. (1985) Observations on the systemic administration of autologous lymphokine-activated killer cells and recombinant interleukin-2 to patients with metastatic cancer. *N. Engl. J. Med.* **313**, 1485–1492.
17. Grimm, E. A., Robb, R. J., Roth, J. A., Neckers, L. M., Lachman, L. B., Wilson, D. J., Rosenberg, S. A. (1983) Lymphokine-activated killer cell phenomenon. Evidence that IL-2 is sufficient for direct activation of peripheral blood lymphocytes into lymphokine-activated killer cells. *J. Exp. Med.* **158**, 1356–1361.
18. Rosenberg, S. A., Lotze, M. T., Yang, J. C., Topalian, S. L., Chang, A. E., Schwartzentruber, D. J., Aebersold, P., Leitman, S., Linehan, W. M., Seipp, C. A., White, D. E., Steinberg, S. M. (1993) Prospective randomized trial of high dose interleukin-2 alone or in conjunction with lymphokine activated killer cells for the treatment of patients with advanced cancer. *J. Natl. Cancer Inst.* **85**, 622–632.
19. Rosenberg, S. A., Spiess. P., Lafreniere, R. (1986) A new approach to the adoptive immunotherapy of cancer with tumor infiltrating lymphocytes. *Science* **233**, 1318–1321.
20. Spiess, P. J., Yang, J. C., Rosenberg, S. A. (1987) In vivo antitumor activity of tumor infiltrating lymphocytes expanded in recombinant interleukin-2. *J. Natl. Cancer Inst.* **79**, 1067–1075.
21. Rosenberg, S. A., Yannelli, J. R., Yang, J. C., Topalian, S. L., Schwartzentruber, D. J., Weber, J. S., Parkinson, D. R., Seipp, C. A., Einhorn, J. H., White, D. E. (1994) Treatment of patients with metastatic melanoma with autologous tumor infiltrating lymphocytes and interleukin 2. *J. Natl. Cancer Inst.* **86**, 1159–1166.
22. Goedegebuure, P. S., Douville, L. M., Li, H., Richmond, G. C., Schoof, D. D., Scavone, M., Eberlein, T. J. (1995) Adoptive immunotherapy with tumor infiltrating lymphocytes and interleukin-2 in patients with metastatic malignant melanoma and renal cell carcinoma: A pilot study. *J. Clin. Oncol.* **13**, 1939–1949.
23. Chang, A. E., Yoshizawa, H., Sakai, K., Cameron, M. J., Sondak, V. K., Shu, S. (1993) Clinical observations on adoptive immunotherapy with vaccine-primed T lymphocytes secondarily sensitized to tumor in vitro. *Cancer Res.* **53**, 1043–1050.
24. Chang, A. E., Aruga, A., Cameron, M. J., Sondak, V. K., Normolle, D. P., Fox, B. A., Shu, S. (1996) Adoptive immunotherapy with vaccine-primed lymph node cells secondarily activated with anti-CD3 and IL-2. *J. Clin. Oncol.*, in press.
25. Schwartzentruber, D. J., Hom, S. S., Dadmarz, R., White, D. E., Yannelli, J. R., Steinberg, S. M., Rosenberg, S. A., Topalian, S. L. (1994) In vitro predictors of therapeutic response in melanoma patients receiving tumor-infiltrating lymphocytes and interleukin-2. *J. Clin. Oncol.* **12**, 1475–1483.
26. Chang, A. E., Shu, S., Chou, T., Lafreniere, R., Rosenberg, S. A. (1986) Differences in the effects of host suppression on the adoptive immunotherapy of subcutaneous and visceral tumors. *Cancer Res.* **46**, 3426–3430.
27. Shu, S., Fonseca, L. S., Kato, H., Zbar, B. (1983) Mechanisms of immunological eradication of a syngeneic guinea pig tumor: participation of a component(s) of recipient origin in the expression of systemic adoptive immunity. *Cancer Res.* **43**, 2637–2643.
28. Mulé, J. J., Rosenstein, M., Shu, S., Rosenberg, S. A. (1985) Eradication of a disseminated syngeneic mouse lymphoma by systemic adoptive transfer of immune lymphocytes and its dependence upon a host component(s). *Cancer Res.* **45**, 526–531.
29. Kleinerman, E. S., Knowles, R. D., Lachman, L. B., Gutterman, J. U. (1988) Effect of recombinant granulocyte/macrophage colony-stimulating factor on human monocyte activity in vitro and following intravenous administration. *Cancer Res.* **48**, 2604–2609.
30. Arenzana-Seisdedos, F., Virelizier, J. L., Fiers, W. (1985) Interferons as macrophage-activating factors. Preferential effects of interferon γ on the interleukin 1 secretory potential of fresh or aged human monocytes. *J. Immunol.* **134**, 2444–2448.
31. Hamann, U., Krammer, P. H. (1985) Activation of macrophage tumor cytotoxicity by the synergism of two T cell derived lymphokines: immune interferon (IFN-γ) and macrophage cytotoxicity inducing factor 2 (MCIF2). *Eur. J. Immunol.* **15**, 18–24.
32. Watson, G. A., Lopez, D. M. (1995) Aberrant antigen presentation by macrophages from tumor-bearing mice is involved in the down regulation of their T cell responses. *J. Immunol.* **155**, 3124–3134.

33. Nestle, F. O., Burg, G., Fah, J., Nickoloff, B. J. (1997) In vivo and in vitro characterization of human tumor associated dendritic cells: deficiency in T cell co-stimulatory molecules and impaired antigen-presenting function. *Am. J. Pathol.*, in press.
34. Tuttle, T. M., McCrady, C. W., Inge, T. H., Salour, M., Bear, H. D. (1993) γ-Interferon plays a key role in T cell induced tumor regression. *Cancer Res.* **53**, 833–839.
35. Cameron, R. B., McIntosh, J. K., Rosenberg, S. A. (1988) Synergistic antitumor effects of combination immunotherapy with recombinant interleukin-2 and a recombinant hybrid α-interferon in the treatment of established murine hepatic metastases. *Cancer Res.* **48**, 5810–5817.
36. Weber, J. S., Jay, G., Tanaka, K., Rosenberg, S. A. (1987) Immunotherapy of a murine tumor with interleukin-2. Increased sensitivity after MHC class I gene transfection. *J. Exp. Med.* **166**, 1716–1733.
37. Restifo, N. P., Spiess, P. J., Karp, S. E., Mulé, J. J., Rosenberg, S. A. (1992) A nonimmunogenic sarcoma transduced with the cDNA for interferon γ elicits $CD8^+$ T cells against the wild-type tumor: correlation with antigen presentation capability. *J. Exp. Med.* **175**, 1423–1431.