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ORIGINAL ARTICLE

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Tumor-specific granulocyte/macrophage colony-stimulating factor and interferon γ secretion is associated with in vivo therapeutic efficacy of activated tumor-draining lymph node cells

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Abstract In this study, cytokine release by tumor-draining lymph node cells sensitized in vitro (IVS-TDLN) was examined and correlated with therapeutic efficacy in adoptive immunotherapy. Mice bearing immunologically distinct MCA 207 and MCA 205 sarcoma tumors were utilized in criss-cross experiments. IVS-TDLN obtained from mice bearing 10-day subcutaneous (s.c.) tumors mediated immunologically specific regression of established 3-day pulmonary metastases, but demonstrated non-specific cytolytic reactivity against both tumors in a 4-h ^{51}Cr-release assay. By contrast, these IVS-TDLN cells were found specifically to secrete granulocyte/macrophage colony-stimulating factor (GM-CSF) and interferon γ (IFNγ) when restimulated in vitro with irradiated tumor cells. To determine the predictive value of tumor-specific cytokine release with in vivo therapeutic efficacy, a kinetic analysis of antitumor activities of TDLN obtained from animals bearing MCA 207 tumors for increasing lengths of time was performed. IVS-TDLN cells from mice bearing day-7, -10 and -14 s.c. tumors manifested tumor-specific release of GM-CSF and IFNγ, and mediated significant antitumor reactivity in vivo. In contrast IVS-LN cells from day-0 and day-21 tumor-bearing animals did not release significant amounts of GM-CSF and IFNγ, and were not therapeutically efficacious in vivo. Day-4 IVS-TDLN released high levels of GM-CSF and IFNγ non-specifically, and were not therapeutic in adoptive immunotherapy at doses effective for day-7 and day-14 IVS-TDLN cells. In other experiments, IVS cells generated from different lymph node groups in animals bearing 10-day established s.c. tumors were examined and found to have unique profiles of cytokine release. In these studies, the ability of IVS cells to release specifically both cytokines as opposed to one was associated with greater therapeutic efficacy on a per cell basis. Our findings suggest that the tumor-specific releases of GM-CSF and IFNγ are useful parameters to assess the in vivo therapeutic efficacy of immune lymphocytes.

Key words Sarcoma · Neoplasms · Adoptive immunotherapy · Cytokines

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Introduction

One of the difficulties in isolating or generating tumor-reactive lymphoid cells for adoptive immunotherapy in man has been the availability of reliable in vitro assays that can predict in vivo therapeutic efficacy. Unlike animal studies, where therapeutic efficacy of immune lymphoid cells can be ascertained with in vivo models, characterization of the antitumor reactivity of human lymphoid cells has depended upon in vitro analyses. Many investigators have utilized in vitro tumor lysis as a method to identify potential therapeutic cells. However, there are many examples of non-lytic cells capable of mediating tumor regression in adoptive immunotherapy [8, 10, 16, 23].

More recently, there have been reports suggesting that the elaboration of cytokines in tumor-bearing hosts plays a significant role in tumor regression. Barth et al. reported that the therapeutic efficacy of non-lytic TIL clones in a murine model was associated with the ability of these clones to secrete interferon γ (IFNγ) and tumor necrosis factor (TNF) in response to tumor in vitro [3]. The role of these cytokines as mediators of tumor regression in vivo was confirmed by the abrogation of the therapeutic effect of tumor-infiltrating lymphocytes (TIL) when neutralizing mAb was administered. In this context, TIL appeared to be a method to induce local elaboration of these cytokines at the sites of tumor, which resulted in tumor regression. In other studies, investigators have reported that the elaboration of various cytokines by genetically modified syngeneic tumor cells will result in reduced tumorigenicity and the induction of immunity to parental tumor antigens [12].

These observations document a significant role of host immunity induced by certain cytokines elaborated in the local tumor environment. In a comparison of several different cytokines, Dranoff et al. identified granulocyte/macrophage-colony-stimulating factor (GM-CSF) as one of the more potent immunoregulatory peptides, which when elaborated in the microenvironment of the tumor, elicited host immunity [7].

We have previously reported that tumor-draining lymph nodes (TDLN) in animals bearing weakly immunogenic tumors harbor lymphoid cells with antitumor reactivity [5, 6]. These TDLN cells were precursors not capable of mediating tumor regression in adoptive immunotherapy, and required further in vitro activation in order 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) [5]. In murine models, this in vitro sensitization (IVS) method generally required 9–10 days and resulted in a three- to five-fold expansion of lymphoblasts, which were predominantly $CD8^+$ T cells. The adoptive transfer of these IVS-TDLN cells resulted in the complete regression of established macroscopic tumor associated with long-term survival in animal models [6]. Utilizing two immunologically distinct tumors induced chemically from the same murine host strain, we investigated the correlation between in vivo tumor reactivity and in vitro cytokine release mediated by IVS lymphoid cells. We found that the tumor-specific release of both GM-CSF and IFNγ by IVS cells in culture was the best determinant of in vivo therapeutic efficacy.

Materials and methods

Mice

Female C57BL6 (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 85-23, revised 1985) were followed, and animal protocols approved by the University of Michigan Laboratory of Animal Medicine.

Tumor

The MCA 207 and MCA 205 are 3-methylcholanthrene-induced fibrosarcomas, syngeneic to B6 mice. These tumors have been maintained in vivo by serial subcutaneous (s.c.) transplantation in B6 mice and were used within the seventh transplantation generation. They were kindly provided by Dr. James C. Yang (National Cancer Institute, NIH, Bethesda, Md.). These tumors have been previously characterized to be weakly immunogenic with distinct tumor-specific transplantation-rejection antigens [2]. Routinely, tumor cell suspensions were prepared from solid tumors by enzymatic digestion in 40 ml Hank's balanced salt solution (HBSS) [Grand Island Biological Co. (Gibco), Grand Island, N. Y.] containing 40 mg collagenase, 4 mg DNase and 100 units hyaluronidase (all obtained from Sigma Chemical Co., St. Louis, Mo.) for 3 h at room temperature as previously described [23].

IL-2

Human recombinant IL-2 was a gift from Chiron Therapeutics, Emeryville, Calif. It has a specific activity of 6×10^6 Cetus units/mg protein. One Cetus unit is equivalent to 6 international units (IU). In this study, amounts are expressed in international units in all of the experiments.

Tumor-draining lymph node

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 lymph nodes were harvested, and single-cell suspensions prepared mechanically as described previously [23]. In other studies, axillary and mesenteric lymph nodes were similarly prepared for comparison with inguinal nodes. Axillary and mesenteric nodes represent further removed nodal sites compared with inguinal nodes in relation to lower-flank tumors. Single-cell suspensions were cultured by the IVS procedure.

IVS procedure

The procedure for generating IVS lymphoid cells has been previously described [6]. Briefly, 4×10^5 responding lymph node cells and 4×10^5 irradiated (60 Gy) MCA 207 or MCA 205 tumor stimulator cells were cultured in 2.0 ml complete medium (CM) containing 60 units/ml IL-2 in 24-well tissue-culture plates (Costar, Cambridge, Mass.). CM consisted of RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 0.01 mM non-essential amino acids, 1 μM sodium pyruvate, 2 mM fresh L-glutamine, 100 μg/ml streptomycin, 100 μ/ml penicillin, 50 μg/ml gentamycin, and 0.5 μg/ml fungizone (all reagents from Gibco). The cultures were incubated at 37 °C in 5% CO_2 and were fed with 1.0 ml CM containing 60 IU/ml IL-2 on days 5 and 7. The IVS cells were routinely harvested on day 9 when they grew to a high density. These cells were washed three times before being resuspended in Hanks' balanced salt solution (HBSS) for adoptive immunotherapy or in CM for cytotoxicity and cytokine release.

Generation of LAK cells

Lymphokine-activated killer (LAK) cells were generated by in vitro culture with high concentrations of IL-2. Briefly, 10^8 normal B6 splenocytes were placed into 150-cm^2 (750 ml) flasks in 50 ml CM with 6000 IU/ml IL-2. The flasks were incubated at 37 °C, 5% CO_2 for 72 h. The LAK cells were then harvested in sterile centrifugation tubes and washed three times in HBSS before resuspension in HBSS for i.v. injection or in CM for in vitro assays.

Adoptive immunotherapy

B6 mice were inoculated i.v. with 2×10^5–2.5×10^5 MCA 207 or 205 tumor cells to establish pulmonary metastases. Three days after tumor inoculation, mice were infused i.v. with IVS cells and given i.p. injections of IL-2 (60000 IU) continuing twice daily for 4 days, commencing on the day of cell transfer. Approximately 3 weeks after tumor initiation, mice were randomized and sacrificed for enumeration of pulmonary 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 Fekette's solution [23]. 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 using the non-parametric Wilcoxon rank-sum test. Two-sided P valaues below 0.05 were considered significant. Each group consisted of at least five mice, and no animal was excluded from the statistical evaluation.

Table 1 Specificity of tumor regression mediated by in-vitro-sensitized tumor-draining lymph node (*IVS-TDLN*) cells in adoptive immunotherapy. Ten days after MCA 205 or MCA 207 tumor s.c. inoculation, TDLN were harvested for IVS culture. Samples containing 4×10^5 TDLN cells were cultured with 4×10^5 irradiated tumor cells in the presence of IL-2 (60 IU/ml) for 9 days. Mice were inoculated with 2×10^5 MCA 205 or MCA 207 tumor cells i.v. Three days later, mice received IVS-TDLN cells i.v. along with a concomitant administration of IL-2 (60000 IU) i.p. twice daily for a total of eight doses. Lungs were harvested on day 19. *IL-2* interleukin-2

IVS effector cells*	Adoptive immunotherapy			
	No of cells transferred	IL-2	Mean no. pulmonary metastases (SEM)	
			MCA 207	MCA 205
	–	–	>250	>250
	–	+	>250	>250
MCA 207 TDLN	1.2×10^7	+	0*	225 (8)
MCA 205 TDLN	1.2×10^7	+	238 (12)	10 (4)*

* $P < 0.01$ compared to other groups

Immunofluorescent staining and flow cytometry

Analysis of cell-surface phenotypes of IVS cells was carried out by indirect immunofluorescent staining. Briefly, $(5-10) \times 10^5$ cells were incubated for 45 min at 4 °C with 25 µl appropriately diluted mAb in phosphate-buffered saline containing 2% fetal bovine serum and 0.1% NaN_3. Rat mAb against the murine CD4 (GK1.5, L3T4) and CD8 (2.43, Lyt-2.2) were used in ascites form phenotyping of murine T cells (obtained from the American Type Culture Collection, Rockville, Md.). A rat mAb specific for the murine IL-2 receptor (IL-2R) (7D4) was used a primary antibody for the detection of the expression of IL-2R. Bound antibodies were detected by incubation with 20 µl of the fluorescein-isothiocyanate-labeled mAb to rat κ chain (MAR 18.5, PharMingen, San Diego, Calif.). Stained cell preparations were analyzed in a FACScan flow microfluorometer (Becton Dickinson, Sunnyvale, Calif.). Fluorescence profiles were generated by analyzing 10000 cells and displayed as logarithmically increasing fluorescence intensity versus cell numbers.

Cytotoxicity assay

A standard 4-h ^{51}Cr-release assay was used to assess the cytotoxic reactivity of IVS cells. Briefly, $(5-10) \times 10^6$ tumor target cells were labeled with ^{51}Cr ($Na^{51}CrO_4$; 50 mCi; New England Nuclear, Boston, Mass.) for 1 h. Labeled target cells (10^4) were incubated with various numbers of effector cells in 0.2 ml CM in plates containing 96 U-bottomed wells and incubated at 37 °C for 4 h. After incubation, the supernatants were collected by the Titertek collecting system (Flow Laboratories, McLean, Va.) for gamma counting. The percentage lysis was calculated as [(experimental ^{51}Cr release – spontaneous release)/(maximal release – spontaneous release)] × 100.

Measurement of in vitro cytokine release by IVS cells

Release of GM-CSF, IFNγ, TNFα and IL-4 by IVS lymphoid cells in response to tumor cell stimulation was assessed. IVS cells (1×10^6/ml) and irradiated tumor cells (5×10^5/ml) were co-cultured in 2.0-ml volumes in 24-well tissue-culture plates. Tumor cells were irradiated with 6000 cGy by a ^{137}Cs source (500 cGy/min, Gamma Cell 1000 Irradiator, Atomic Energy of Canada). After 24 h, culture supernatants were collected and cytokine measurements assessed in duplicate using commercially available enzyme-linked immunosorbent assays (ELISA) (Pharmingen, San Diego, Calif.). For murine GM-CSF, a standard curve starting at 12.5 ng/ml with serial twofold dilutions was performed. For IFNγ, TNFα and IL-4; standard curves starting at 1000 IU/ml, 50 ng/ml, and 1000 IU/ml respectively were established in a similar fashion. Experimental values were computed with the use of regression analysis.

Table 2 Cytolytic activity of TDLN after IVS. The effector cells were the IVS-TDLN cells generated for the experiment described in Table 1. Lymphokine-activated killer (*LAK*) cells were established from normal splenocytes incubated in 6000 IU/ml IL-2, and were used as positive controls for tumor lysis. Fresh tumor cells were utilized as targets in a 4-h ^{51}Cr-release assay. Results show the percentage lysis assessed at various effector: target ratios

Effector cells	Target	Lysis (%)			
		100:1	20:1	4:1	0.8:1
MCA 207 TDLN	MCA 207	22	20	16	2
	MCA 205	15	13	11	6
MCA 205 TDLN	MCA 207	30	18	9	8
	MCA 205	40	25	23	15
LAK	MCA 207	38	29	19	9
	MCA 205	33	28	12	0

Results

In vivo tumor specificity of IVS-TDLN cells did not correlate with in vitro cytolytic reactivity

The specificity of tumor regression mediated by IVS-TDLN was assessed by the adoptive immunotherapy of two immunologically distinct tumors in a criss-cross experiment. TDLN harvested 10 days after the s.c. inoculation of 1.5×10^6 MCA 205 or MCA 207 tumor cells were cultured by the IVS method. After IVS culture, 1.2×10^7 IVS cells were adoptively transferred to mice bearing 3-day pulmonary tumors along with the concomitant i.p. administration of 60000 IU IL-2 twice daily for eight doses. As summarized in Table 1, IL-2 administration alone did not result in tumor regression. However, the transfer of IVS-TDLN cells plus IL-2 mediated immunologically specific tumor regression.

The phenotypic characteristic of TDLN cells were determined before and after IVS culture by fluorescence-activated cell sorting. Fresh TDLN comprised approximately 30%–40% $Thy1.2^+$ T cells with an approximately 2:1 ratio of CD4:CD8-positive cells. After IVS culture, the cell population was at least 90% $Thy1.2^+$ cells with virtually all the cells bearing CD8.

The in vitro cytolytic reactivity of the cells analyzed in adoptive immunotherapy (Table 1) was also evaluated using a 4-h ^{51}Cr-release assay. Both IVS-TDLN populations mediated lytic reactivity against the tumor with which they were primed in vivo (Table 2). However, there was significant lysis of the unrelated tumor as well, albeit at a lower level. LAK cells generated from normal splenocytes by incubating in 6000 IU/ml IL-2 mediated non-specific lysis of both tumor targets as expected. The specificity of in vivo tumor regression mediated by IVS-TDLN was not

Table 3 Tumor-specific cytokine release before and after IVS culture. TDLN cells were harvested from mice inoculated with MCA 205 or MCA 207 tumor cells as described in Table 1. IVS cultures were established as described in Table 1. Cytokine release was measured from the culture supernatant of TDLN cells restimulated with irradiated tumor cells for 24 h. Values reflect the amount of released cytokine after subtraction of low background amounts measured from irradiated tumor cells and TDLN cells alone. *IFNγ* interferon γ. *GM-CSF* granulocyte/macrophage-colony-stimulating factor

		Cytokine release			
		IFNγ (IU/ml)		GM-CSF (pg/ml)	
TDLN cells	IVS	MCA 207	MCA 205	MCA 207	MCA 205
MCA 207	Before	0	0	0	0
	After	76	0	780	0
MCA 205	Before	0	0	0	0
	After	0	48	90	790

Table 4 Kinetics of in vivo efficacy of MCA 207 TDLN after IVS. Mice were inoculated s.c. with MCA 207 tumor cells. At different intervals after tumor inoculation, inguinal TDLN were harvested for IVS culture as described in Table 1. The tumor reactivity of the IVS-TDLN cells was assessed in the adoptive immunotherapy of 3-day established MCA 207 pulmonary metastases as described in Table 1

Days after s.c. tumor inoculation	IL-2	Adoptive immunotherapy: mean no. of pulmonary metastases (SEM)
–	–	>250
–	+	>250
0	+	>250
4	+	241 (7)
7	+	6 (4)*
10	+	5 (4)*
14	+	29 (22)*
21	+	176 (53)**

* $P < 0.01$ compared to all other groups
** $P < 0.01$ compared to groups with more than 250 mean pulmonary metastases

reflected by the tumor specificity of in vitro lysis. Of interest, LAK therapy at comparable cell doses was not effective in mediating tumor regression either (see later).

In vitro cytokine release by IVS-TDLN cells

We proceeded to examine the release of cytokines by TDLN cells as a means to assess in vitro immunological reactivity. TDLN were harvested 10 days after MCA 205 or MCA 207 tumor inoculation and cultured by the IVS method. TDLN cells before and after IVS were assessed for cytokine release by culturing these cells with irradiated MCA 205 or MCA 207 tumor cells for 24 h. The culture supernatants were harvested and assayed for IFNγ, GM-CSF, IL-4 and TNFα. Freshly harvested TDLN cells did not secrete cytokines after tumor stimulation (Table 3). However, after IVS culture, MCA 207 IVS-TDLN cells demonstrated specific GM-CSF and IFNγ release when restimulated by MCA 207, and not with MCA 205. By contrast,

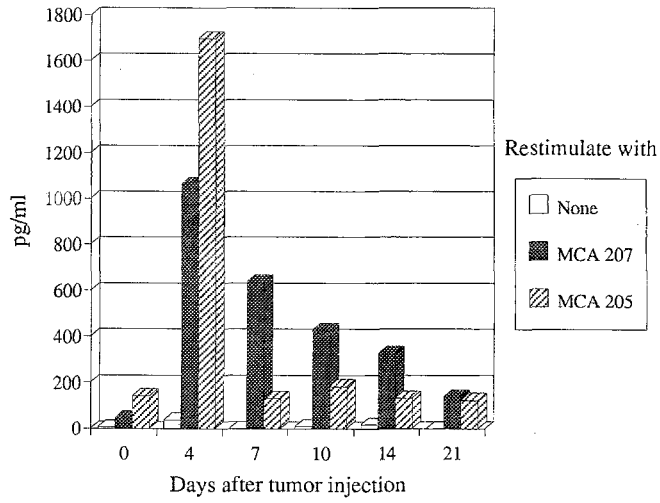

Fig. 1 Kinetics of granulocyte/macrophage-colony-stimulating factor (GM-CSF) secretion from in-vitro-stimulated tumor-draining lymph node (IVS-TDLN) cells obtained from mice bearing MCA 207 tumors for different lengths of time. Culture supernatants were collected 24 h after restimulation with irradiated MCA 207 or MCA 205 tumor cells. Values reflect the amount of released cytokine after subtraction of amounts measured from tumor cell alone

MCA 205 IVS-TDLN cells released GM-CSF and IFNγ when restimulated with MCA 205, but not MCA 207. IVS-TDLN cells did not release deflectable IL-4 or TNFα after tumor restimulation.

We next examined the kinetics of pre-effector cell development in TDLN. MCA 207 tumor cells were inoculated s.c. and TDLN harvested every few days for cryopreservation up to day 21 after tumor inoculation. Upon collecting all the TDLN from the different interval times, the cells were thawed, washed, and cultured by the IVS method. After IVS culture, these IVS-TDLN cells were assessed for therapeutic efficacy by adoptive transfer (1.2×10^7 cells/animal) into mice with established 3-day MCA 207 pulmonary metastases (Table 4). TDLN harvested on days 0 and 4 had no therapeutic reactivity compared to control groups. IVS cells generated from TDLN harvested on days 7, 10 and 14 after tumor inoculation mediated significant regression of pulmonary metastases. By day 21, there was diminished pre-effector cell activity in the TDLN.

In kinetic studies, we also evaluated the tumor-specific cytokine release of the IVS-TDLN cells. After culture in IVS, TDLN cells were restimulated in vitro with irradiated MCA 207 or MCA 205 tumor and the supernatant was harvested 24 h later for cytokine (GM-CSF and IFNγ) determinations (Fig. 1, 2). There were no significant amounts of GM-CSF or IFNγ released by day-0 IVS-TDLN. Day-4 IVS-TDLN cells secreted high amounts of both cytokines non-specifically. Tumor-specific cytokine release was observed in TDLN harvested up to 14 days after tumor inoculation and diminished by day 21 to levels similar to those of day-0 TDLN (i.e., normal lymph nodes). These results suggested that a correlation existed between in vitro tumor-specific GM-CSF and IFNγ release and the in

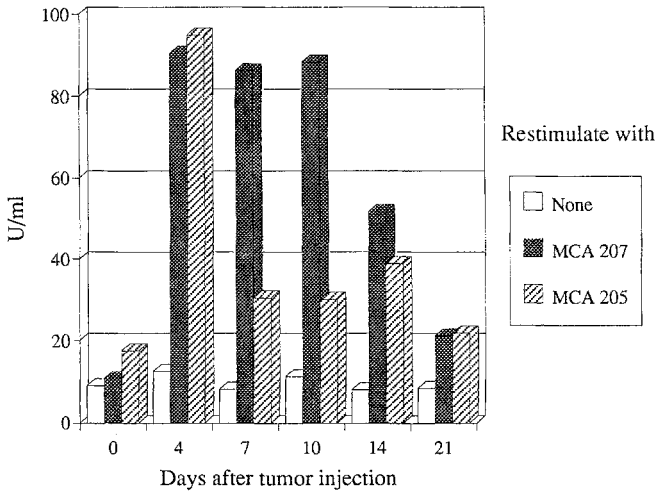

Fig. 2 Kinetics of IFNγ secretion from IVS-TDLN cell obtained from mice bearing MCA 207 tumors for different lengths of time. Culture supernatants were collected as described in Fig. 1. There was negligible IFNγ secretion from tumor cells alone

vivo therapeutic efficacy of IVS-TDLN cells. In addition, it was evident that host- or tumor-related factors appeared to play a role in down-regulating the activity of pre-effector TDLN cells during the course of progressive tumor growth.

LAK phenomenon

The non-specific release of cytokines demonstrated by day-4 TDLN was suggestive of a LAK-like phenomenon. Therefore, we proceeded to examine the cytokine-release profile and therapeutic efficacy of standard LAK cells in our models. The in vitro cytokine release mediated by LAK cells generated from normal splenocytes was evaluated. As demonstrated by in vitro cytotoxicity assays, LAK cells were found to lyse MCA 205 and MCA 207 in a non-specific manner (Table 2). In both of two experiments, LAK cells were also found to release GM-CSF and IFNγ non-specifically when stimulated by irradiated MCA 205 and MCA 207 tumor in vitro (Table 5).

The therapeutic efficacy of LAK cells was examined in a dose-escalation fashion in the treatment of 3-day established MCA 207 pulmonary metastases (Table 6). At a dose of IVS-TDLN cells (i.e., 1.2×10^7) that significantly

Table 5 Non-specific cytokine release of LAK cells. LAK cells were generated from splenocytes incubated in 6000 IU/ml IL-2 for 3 days. Cytokine release was measured in culture supernatants as described in Table 4. LAK cells cultured in the absence of tumor cells did not secrete either cytokine

	Cytokine release			
	IFNγ (IU/ml)		GM-CSF (pg/ml)	
Experiment	MCA 207	MCA 205	MCA 207	MCA 205
1	103	228	478	416
2	204	128	495	297

Table 6 Minimal in vivo therapeutic efficacy of murine LAK in the therapy of MCA 207 pulmonary metastases. LAK cells were generated as described in Table 5. The tumor reactivity of LAK cells was assessed in the adoptive immunotherapy of 3-day established MCA 207 pulmonary metastases as described in Table 1

No. of LAK	IL-2	Mean no. of pulmonary metastases (SEM)	
		Expt. 1	Expt. 2
–	–	>250	>250
–	+	>250	>250
0.6×10^7	+	>250	–
1.2×10^7	+	249 (1)	–
2.4×10^7	+	245 (5)	–
4.8×10^7	+	203 (7)	240 (9)
10.0×10^7	+	–	54 (15)*

* $P < 0.01$ compared to all other groups

reduced the number of pulmonary metastases, LAK cells were ineffective. Only at much greater numbers of LAK cells (i.e., 10^8) was antitumor reactivity identified. These numbers are consistent with the antitumor efficacy of LAK cells in previous reports [15, 21]. These observations suggested that the *non-specific* release of cytokines by effector cells in response to tumor stimulation is not as predictive as *tumor-specific* release in determining their therapeutic potential in vivo. Nevertheless, at sufficiently high numbers of transferred cells, LAK-like cells can mediate tumor regression.

Distribution of pre-effector cells in different lymph nodes

The regional draining lymph node has been known to be a critical lymphoid organ during the immunological response to antigenic stimulation [19, 22]. We have previously demonstrated that the distribution of tumor-reactive pre-effector cells can vary, depending upon the source of lymphoid cells in the tumor-bearing host. We proceeded to investigate the correlation between tumor-specific cytokine release of IVS cells obtained from different lymph node sites of MCA 207 tumor-bearing hosts 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, axillary and mesenteric lymph nodes removed for IVS culture. Normal splenocytes were used as control cells. After IVS culture, cells were assessed for in vitro cytokine release after tumor restimulation or in vivo tumor reactivity by the adoptive immunotherapy of pulmonary metastases as previously described. In order to quantify tumor-specific cytokine release, a specific release ratio (SRR) was calculated:

$$\text{SRR} = \frac{\text{total cytokine release against specific tumor} \quad \text{tumor alone} \quad \text{effector cells alone}}{\text{total cytokine release against non specific tumor} \quad \text{tumor alone} \quad \text{effector cells alone}}$$

As summarized in Table 7, tumor regression was mediated by all three IVS-LN populations at the highest cell dose transferred (1.2×10^7). However, dose titration revealed that the therapeutic potency of each cell popula-

Table 7 Correlation between in vitro tumor-specific cytokine release and in vivo efficacy of different IVS-LN cells from MCA 207 tumor-bearers. Mice were inoculated s.c. with MCA 207 tumor cells in the lower flank and underwent removal of lymph nodes (*LN*) 10 days later for IVS culture as described in Table 1. Cytolytic activity (lytic units, *LU*) was determined by 20% lysis of 10^4 target cells in 10^6 effector cells. Mice were inoculated with MCA 207 tumor cells i.v. to establish pulmonary metastases; 3 days later, IVS-LN cells were adoptively transferred i.v. with the concomitant administration of IL-2 as described in Table 1. The percentage reduction of metastases was calculated for each IVS-LN cell dose with respect to control animals that received IL-2 alone. *SRR* specific release ratio of cytokine

Source of	SRR		Cytolytic activity (LU)		Reduction of metastases (%)			
IVS LN cells	GM-CSF	IFNγ	MCA 207	MCA 205	12×10^6	6×10^6	3×10^6	1.5×10^6
Inguinal LN	17.2	29	9	9.5	100*	88*	31*	18
Axillary LN	1.3	40	9.5	13.7	98*	23	5	0
Mesenteric LN	<1	64	1.9	62.1	80*	2	0	0
Normal spleen	<1	3	<1	82	0	–	–	–

* $P < 0.01$ compared to control animals that received IL-2 alone

tion varied, the inguinal IVS-LN cells being the most effective followed by the axillary and mesenteric IVS-LN cells in decreasing order. Normal IVS cultured splenocytes had no therapeutic activity at the highest cell dose, and were found to have negligible tumor-specific release of GM-CSF and IFNγ. Tumor-specific release of both cytokines appeared to be associated with the greatest in vivo therapeutic efficacy as observed with inguinal IVS-LN cells. High tumor-specific release of IFNγ with minimal tumor-specific release of GM-CSF was associated with low to intermediate reduction of pulmonary metastases, as observed with axillary and mesenteric IVS-LN cells.

Discussion

One of the major obstacles confronting investigators in the generation of tumor-reactive lymphocytes for adoptive immunotherapy is the unavailability of reliable in vitro assay methods to identify therapeutically functional effector cells. We and others have demonstrated that the use of standard 4-h chromium-release assays is not predictive of whether a particular cell population can mediate tumor regression in vivo [8, 10, 16, 23]. More recently, several reports have indicated that cytokine secretion by TIL appears to correlate with the in vivo tumor regression mediated by those cells. Barth et al. isolated several murine TIL clones from various methylcholanthrene(MCA)-induced tumors which were $CD8^+$ and non-cytolytic [3]. He was able to demonstrate that the secretion of IFNγ and TNFα upon restimulation of these TIL clones was associated with their antitumor reactivity in vivo. Moreover, the therapeutic efficacy of the cells was attenuated by neutralizing monoclonal antibodies to these cytokines. Goedegebuure et al. demonstrated that the release of IFNγ and GM-CSF by TIL during activation and expansion with anti-CD3 and IL-2 appeared to correlate with their in vivo antitumor reactivity in the MCA 105 tumor model [9]. TIL isolated from different culture conditions were non-specifically lytic to various tumor targets in their study. They found that the elaboration of GM-CSF and IFN-γ by TIL during culture was associated with the magnitude of tumor regression manifested by the TIL after adoptive transfer. There have been few clinical studies of adoptive immunotherapy to determine whether one can extrapolate these observations to humans. In one of the largest clinical experiences with TIL therapy at the National Cancer Institute, Schwartzentruber et al. reported that the release of GM-CSF by TIL in response to autologous tumor appeared to be an important independent predictor of tumor response in melanoma patients [18].

We have further investigated the association of cytokine release by effector T cells and their therapeutic efficacy in vivo. Rather than utilizing TIL, we examined the in vitro reactivity of TDLN secondarily stimulated by tumor in an IVS culture method we have previously described [5, 6]. To evaluate tumor specificity we employed two immunologically distinct MCA-induced sarcomas that were derived in the same mouse strain. Tumor regression mediated by IVS-TDLN was exquisitely specific; however, the cytolytic reactivity was not. By contrast, the release of GM-CSF and IFNγ by competent IVS-TDLN was highly tumor-specific.

We also observed that the ability to secrete GM-CSF or IFNγ in a non-specific manner was not as predictive as the specific release of these cytokines for the mediation of tumor regression in vivo. For example, LAK cells were found to elaborate high levels of GM-CSF and IFNγ in a non-specific manner in vitro. Yet on a per cell basis, LAK cells were not as potent as IVS-TDLN cells that secreted cytokines in a tumor-specific fashion. Moreover, we found that day-4 IVS-TDLN secreted high amounts of GM-CSF and IFNγ non-specifically and were therapeutically ineffective compared to day-7–14 IVS-TDLN, which released these cytokines in a tumor-specific manner. As a corollary, cells that did not secrete significant amounts of GM-CSF or IFNγ in response to in vitro tumor stimulation (i.e., normal splenocytes or day-21 TDLN) had no efficacy in adoptive immunotherapy.

The ability of effector cells to secrete both cytokines in a specific fashion appeared to be better than their release of a single cytokine. As noted in Table 7, the tumor-specific release of IFNγ was demonstrated by IVS-cultured LN cells from inguinal, axillary and mesenteric node groups. At a relatively high dose of adoptively transferred cells (i.e., 1.2×10^7), tumor regression was mediated by all three cell populations. However, only inguinal lymph node cells that

were derived from the closest nodal group draining the tumor inoculum demonstrated the specific release of both cytokines, and also had the most potent therapeutic effect in vivo on a per cell basis.

Both GM-CSF and IFNγ have been found to be important cytokines in the host response to progressive tumors. Utilizing gene-transfer techniques several investigators have demonstrated that the elaboration of GM-CSF and IFNγ by genetically modified tumors will enhance T cell sensitization of the host. Using a poorly immunogenic tumor, Dranoff et al. reported that GM-CSF elaboration by the B16 melanoma was successful in establishing systemic immunity to wild-type tumor in a naive host [7]. Our laboratory, utilizing a subline of the B16 tumor, B16-BL6, demonstrated the ability to induce antitumor-reactive cells in lymph nodes draining a clone of the B16-BL6 engineered to secrete GM-CSF, in contrast to wild-type tumor, which did not [1]. The antitumor reactivity of the lymph node cells was assessed in adoptive immunotherapy experiments. The role of GM-CSF in these models appears to be related to an increased influx of antigen-presenting cells to the local tumor environment as well as the draining lymph nodes. Other investigators have demonstrated the activation of dendritic cells by GM-CSF, which may also be important in antigen processing and presentation [4, 13, 17]. By similar mechanisms, the ability of adoptively transferred mature effector cells to elaborate GM-CSF in response to autologous tumor may be important in the recruitment of hot cells to respond at the site of progressive tumor.

The interferons have been postulated to have a variety of immunomodulatory effects important in tumor immunity. These include macrophage activation, naturall killer cell activation, and up-regulation of tumor antigens and MHC molecules [11]. Another novel role IFNγ may serve is to induce tumor cells to become "non-professional" antigen-presenting cells. Restifo et al. observed that MCA 101, a poorly immunogenic murine sarcoma, developed the capability of processing exogenous antigen by genetically modifying a tumor line with IFNγ cDNA [14]. This was associated with up-regulation of tumor immunogenicity since TIL derived from IFNγ-transduced MCA 101 were therapeutically effective compared to TIL generated from the wild-type tumor. The release of IFNγ by adoptively transferred effector T cells may promote tumor regression via similar mechanisms.

We are currently evaluating the predictive effect of tumor-specific cytokine release with the functional in vivo antitumor activity of other effector populations. These include TDLN cells activated by anti-CD3 [23], and bacterial superantigens [20]. These studies will also allow us to define the role of T cell subpopulations (CD4, CD8 and Vβ specific) in tumor reactivity in vitro and in vivo.

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