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Tumor-infiltrating lymphocytes as antitumor effector cells

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The presence of mononuclear cell infiltrates in and around tumors suggests that the immune response plays a role in the complex and still incompletely understood interactions between the host and growing cancer. A variety of other clinical and clinicopathologic observations support this impression. Thus, a relatively frequent occurrence of spontaneous regressions in some cancers [1, 2], increased incidence of malignancy in immunosuppressed hosts [3, 4], improved prognosis associated with T-cell infiltrates into tumors [5, 6], responses of certain tumors to tumor vaccines [7], and immunotherapy with adoptively-transferred activated immune cells [8] or with biologic response modifiers [9] — are all indications that the immune system may in some circumstances contribute to a control of tumor growth. Nevertheless, these phenomena are not generalized, especially in human solid cancer, and a controversy has evolved with respect to specific humoral and cellular responses directed at tumor-associated antigens (TAA) on human solid tumors. As long as the presence of unique and immunogenic antigens on tumor cells remains in doubt, so does the specificity of antitumor immune responses. As a result, two alternative, but not necessarily mutually exclusive, explanations for tumor-infiltrating mononuclear cells (MNC) have to be considered: one, that they represent the major histocompatibility complex (MHC)-unrestricted inflammatory cells responding not to TAA but to cytokines and inflammatory mediators generated at the site of tumor growth; and two, that they are cells specifically recruited to the tumor, which recognize and respond to TAA by the MHC-restricted activation, proliferation, and cytotoxicity.

Intuitively, it would appear that vigorous local antitumor responses, whether inflammatory in nature or mediated by tumor-specific effectors, have to be beneficial for the host. Suggestions have been made in the past, however, that tumor-infiltrating MNC may be a source of tumor growth-promoting factors [10]. This possibility has to be seriously considered, as evidence accumulates that activated MNC are a source of multiple cytokines, many of which have growth potentiating properties for tissue cells, and that tumor cells may express receptors for cytokines [11, 12]. Indeed, it may well be that the existing controversy about the prognostic significance of MNC infiltrates in human solid tumors can be at least partially explained by the complex effects of cytokines on tumor cells. An additional phenomenon that may explain a lack of clear-cut positive correlations between MNC in the tumor and prognosis is tumor-derived inhibition of immune responses [13].

Concerning the presence of MNC infiltrates at the tumor site, it seems important to establish whether they contain non-specific inflammatory cells, tumor-specific effectors or both. Therapy of cancer with tumor-infiltrating lymphocytes (TIL) is based on results obtained in murine tumor models, where tumor-specific T lymphocytes represent a major proportion of cells expanding in the presence of interleukin 2 (IL2) [14]. On the other hand, human TIL isolated from most solid tumors and cultured with IL2, with a notable exception of melanoma, are heterogeneous populations of mostly MHC-unrestricted effectors [15–17]. Since advantages of therapy with TIL as opposed to lymphokine-activated killer (LAK) cells derived from the

peripheral blood are not clear in man, considerable efforts have been made to provide both a rational basis for and practical approach to treatment of human solid tumors with TIL. These efforts have largely focused on isolation and phenotypic and functional characterization of fresh as well as *in vitro* expanded TIL. The overriding concern has been to confirm the presence of autotumor (AuTu)-specific effectors in human solid tumors and to develop strategies for their culture. The purpose of this chapter is to summarize these studies and to assess future directions to explore anti-tumor potential of TIL obtained from human solid tumors.

Isolation of TIL from tissue

Functional studies of human TIL are complicated by the fact that the cells have to be first isolated from surgically-removed tumor specimens. Difficulties associated with the process of tumor procurement and isolation involve requirements for adequate size, location, and freshness of tumor specimens, all of which are important for a recovery of sufficient numbers of viable cells; enzymatic and mechanical treatments of tumor tissues, which may induce changes in cellular function; separation of TIL from tumor and tissue cells on gradients, which may lead to selective losses of cellular subpopulations; and recovery of viable autologous tumor cells in sufficient numbers and adequate purity for *in vitro* assays of TIL functions. These difficulties are not trivial, and require that a carefully-orchestrated system of tumor procurement and processing be in place. Such a system involves close cooperation between surgeons, medical oncologists, pathologists and immunologists and, at our own institution, it is operating through a tissue bank functioning as a core facility [18]. Methods for enzymatic processing of tumors and TIL recovery have been developed, evaluated, and described earlier [19, 20]. It is necessary to bear in mind that the above difficulties in TIL isolation may be responsible for differences in phenotypic and functional characteristics of TIL between different laboratories.

Another consideration, frequently overlooked, is that TIL isolated from human tumors are invariably derived from large, and often advanced tumors. Clearly, this situation is not comparable to that in experimental murine systems, where TIL can be obtained within a few days of tumor cell injection. This difference in timing probably means that TIL populations from murine tumors are different in terms of composition, activation, and antitumor function from those obtained from human solid tumors.

In spite of these difficulties, TIL have been isolated from numerous primary and metastatic human tumors of different histologic types and evaluated for their phenotypic and functional properties (reviewed in [21, 22]). A helpful approach to isolation of human TIL has been to combine it with immunohistology *in situ*. Information about the cellular composition and precise localization of different cellular subsets in relation to tumor cell *in situ* can thus be combined with the data obtained with isolated TIL [23]. Immunohistology *in situ* can serve as a rough guide to quantitative and qualitative recovery of TIL: tumors intensely infiltrated with T cells should yield larger numbers of TIL per wet gram of tissue than scarcely infiltrated tumors [23]; tumors containing more $CD4^+$ than $CD8^+$ cells should be a source of TIL with the higher CD4/CD8 ratio than tumors enriched in $CD8^+$ cells [21]. At the same time, it is necessary to remember that immunohistology is performed on tumor tissues that are, at best, adjacent to those used for TIL isolation, and local differences in composition (e.g., between parenchyma and stroma; tumor periphery vs. tumor center) and intensity of infiltrates are known to exist [22]. Further, certain of lymphocyte surface antigens (e.g., CD56 antigen) appear to be sensitive to air drying and fixation necessary for immunostaining *in situ*, and immunohistology does not in such cases provide accurate information about cells expressing these antigens [24]. Finally, immunohistology is subjective, and a bias introduced by the examination of serial sections, each stained with a different monoclonal antibody, has to be considered while interpreting results. In general, however, *in situ* immunohistology of TIL has contributed insights into interactions between TIL and tumor cells, especially in regard to the expression of activation markers on TIL [25] and the expression of the MHC antigens on tumor cells [23, 26, 27].

Phenotypic characteristics of freshly-isolated TIL

Data on phenotypic properties of fresh TIL from human solid tumors are limited, mainly due to the paucity of recovered cells [21]. Also, mononuclear cells isolated by enzymatic digestion of tumors and density gradient centrifugation represent mixtures of T lymphocytes and tumor cells with different proportions of macrophages, polymorphonuclear leukocytes, eosinophils and large granular lymphocytes [19]. In order to isolate TIL for phenotypic or functional analyses, further purification steps are necessary. We have used differential density gradients followed by sorting by flow cytometry to obtain highly purified preparations of T ($CD3^+$ or $CD2^+$) cells from various human solid tumors [28, 29]. These T lymphocytes expressed the CD3-TCR complex as determined by reactivity with anti-CD3 and WT31 monoclonal antibodies [29]. The expression of the beta chains of the T cell receptor (TCR) was comparable on the surface of TIL-T and normal PBL-T [29]. These and other studies showed that $CD3^+$ T lymphocytes are the major component of TIL in most human solid tumors [30], except perhaps in melanoma, where considerable proportions of B lymphocytes have been detected in some cases [J.M. Kirkwood, personal communication]. A recent finding that T γ/δ cells are a major subpopulation in TIL obtained from B-cell and non-Hodgkin's lymphomas and, possibly, from the tumors of the GI system [31], is interesting but needs confirmation. Our recent studies of TIL from primary and metastatic liver tumors showed that T γ/δ cell represent a minor (1–5%) population of TIL-T recovered from these tumors [32].

Two-color flow cytometry has been extensively used for phenotypic analysis of fresh TIL suspensions recently. The FACS analysis of TIL is facilitated by a possibility for setting the gate on lymphoid cells thus excluding a high background due to the presence of non-lymphoid cells and cell fragments. Using this approach, we studied phenotypic characteristics of TIL suspensions obtained from human squamous cell carcinomas of the head and neck (SCCHN) [33], ovarian carcinomas [34] and primary and metastatic liver tumors [17, 32]. In each case, a different question was addressed. Thus, in SCCHN we determined that the T cell composition of TIL resulting in a low CD4/CD8 ratio, due in part to a significant increase in the percentage of $CD8^+$ cells ($p < 0.01$), and in part to a decrease in $CD4^+$ cells ($p < 0.01$), at the time of surgery was associated with the absence of extracapsular spread and cervical metastases and thus with a better prognosis [33]. This study suggested that the content of $CD8^+$ T cells, in at least some human tumors, may have prognostic significance [33]. Several other studies indicated that the CD4/CD8 ratio in fresh TIL isolated from metastatic melanomas [35, 36], renal cell carcinomas [37] and primary breast [38] and lung [39] cancer is significantly reduced as a result of enrichment in $CD8^+$ cells. In contrast, in non-malignant inflammatory exudates the majority of T cells have the $CD4^+$ phenotype [40, 41]. In another series of SCCHN, attempts were made to look for the presence of lymphoid suppressor cells among TIL and lymph node lymphocytes (LNL) isolated from tumor-involved nodes. A population of $CD8^+CD11b^+$ 'suppressor' cells was shown to be significantly increased in lymph nodes and TIL of patients with laryngeal and bladder carcinomas by Cozzolino and colleagues [42, 43]. No such increases in $CD8^+CD11b^+$ cells were observed by us in TIL ($n = 12$) or LNL from tumor-involved ($n = 15$) or -uninvolved nodes ($n = 28$) of patients with SCCHN [44]. Table 1 shows the cellular composition of fresh TIL obtained by us from ovarian carcinomas. Two points are worth emphasizing: (a) among

Table 1. Phenotypic characteristics of freshly-isolated tumor-infiltrating lymphocytes from human ovarian carcinomas.

Marker	Positive (mean ± SD)[a] n = 11
$CD3^+$	58 ± 14
$CD3^+CD25^+$	14 ± 6
$CD3^+HLA\text{-}DR^+$	53 ± 12
$CD3^+CD56^+$	12 ± 11
$CD3^-CD56^+$	16 ± 14
$CD8^+$	28 ± 13
$CD8^+CD11b^-$	13 ± 9
$CD4^+$	39 ± 12

[a] TIL obtained from ovarian carcinomas by a mild enzymatic digestion and density-gradient centrifugation were stained with labeled monoclonal antibodies, and the frequency of positive cells was determined by two-color flow cytometry.

Table 2. Phenotypic analysis of freshly isolated tumor-infiltrating lymphocytes obtained from human primary and metastatic liver tumors[a]

Markers	Primary n = 10	Metastatic n = 5
$CD3^+$	70 ± 6	79 ± 2
$CD4^+$	44 ± 5	38 ± 8
$CD8^+$	32 ± 3	34 ± 10
$CD56^+$	25 ± 7	21 ± 3
$CD3^+CD56^+$	14 ± 5	11 ± 3
$CD3^+CD25^+$	14 ± 3	14 ± 1
$CD3^+$HLA-DR^+	46 ± 8	39 ± 3

[a] Data are percentages of positive cells (means ± SE) as determined by two-color flow cytometry.

$CD3^+$ TIL, up to 25% and 65% expressed CD25 or HLA-DR antigens, respectively (activation markers), and (b) these TIL preparations contained variable and sometimes considerable proportions of $CD3^-$ $CD56^+$ natural killer (NK) cells and of $CD3^+CD56^+$ T lymphocytes. Similar results were obtained with TIL from primary liver and colon tumors metastic to liver by us (Table 2) and from lung carcinomas [45], melanomas [46] and renal cell carcinomas [47, 48] by others. Finally, when phenotypic markers were compared on TIL freshly isolated from primary liver tumors with those from metastatic liver tumors (Table 2), no significant differences were detected.

More recent studies have extended our knowledge of the state of activation of TIL-T in human solid tumors. Lymphocyte differentiation from naive to primed or memory cells induced by antigen-specific stimulation is accompanied by a change in the CD45 gene product from a high molecular weight molecule (CD45R, CD45RA) to the low molecular weight forms (CDw29, CD45RO, UCHL1). This unidirectional change in the phenotype as a T cell progresses from a naive to activated or primed state can be detected with monoclonal antibodies, because antigen-activated cells express high densities of CDw29 and CD45RO (UCHL-1) and express no or low levels of CD45R or CD45RA antigens, which are markers found on naive cells [49, 50]. Table 3 shows that in fresh TIL isolated from liver and ovarian tumors, both $CD4^+$ and $CD8^+$ lymphocyte subpopulations contained a predominance of cells expressing the 'committed' $CDw29^+$ phenotype. However, in comparison to the $CD4^+$ population, $CD8^+$ cells had a significantly lower ratio of primed to naive T cells. In TIL from liver tumors, this low ratio seemed to reflect an enrichment in 'naive' T cells in the $CD8^+$ population. Cardi et al [36] and Alexander et al. [47] also reported that TIL from metastatic melanoma and renal cell carcinoma, respectively, predominantly expressed the 'committed' CDw29 phenotype. The presence of T cells expressing an 'antigen-committed' phenotype in human tumors implies that these TIL are primed to tumor antigens. Sorted $CD3^+CDw29^+$ TIL from RCC proliferated well in response to AuTu and IL2 in preliminary experiments performed by Finke and colleagues [51].

The phenotypic analysis of fresh TIL in sus-

Table 3. Naive and primed T cell subpopulations in freshly isolated tumor-infiltrating lymphocytes obtained from human ovarian and liver tumors[a]

Markers	Ovarian carcinoma n = 5	Liver tumors n = 9
$CD4^+$ TIL		
A. $CD4^+CDw29^+$	25 ± 7.6 p = 0.02	36 ± 10 N.S.
B. $CD4^+CD45R^+$	11 ± 2.2	17 ± 10
RATIO A/B	7 ± 5	7 ± 4.9
$CD8^+$ TIL		
A. $CD8^+CDw29^+$	44 ± 9.3	32 ± 9.6
B. $CD8^+CD45R^+$	8 ± 3.8 p = 0.02	7 ± 4.4 p = 0.003
RATIO A/B	2 ± 1.0[b]	2 ± 1.8[b]

[a] TIL were isolated from tumor tissues and stained with labeled monoclonal antibodies specific for the CD4 or CD8 antigens and for naive ($CD45R^+$) or primed ($CDw29^+$) lymphocyte subsets. Doubly-stained lymphocytes were quantitated by two-color flow cytometry. The data are mean percentages ± SD of naive or primed lymphocytes or ratios of primed to naive T cells within the $CD4^+$ or $CD8^+$ lymphocyte subpopulations.

[b] Differences in the ratios of primed/naive T lymphocytes between $CD4^+$ and $CD8^+$ TIL are significant at $p < 0.05$ (paired Student's t test). The ratios of primed/naive T lymphocytes for $CD4^+$ and $CD8^+$ PBL of patients with liver cancer (n = 4) were 1.5 and 1.0, respectively.

pension by two- or three-color flow cytometry allows for a more precise quantitation of TIL subsets than earlier immunofluorescence or immunohistologic studies. With the availability of monoclonal antibodies to activation antigens on T cells, it has been possible to identify differences between TIL, LNL, and autologous PBL [33, 36, 44]. On the whole, newer phenotypic data lend support to a notion that human TIL contain T lymphocytes that are activated and immunologically committed as well as a substantial proportion of NK cells.

Functional properties of fresh TIL

It has been recognized for a long time that both proliferative and cytotoxic responses of TIL freshly isolated from human tumors are impaired (reviewed in [21, 22]). Fresh TIL, especially those from metastatic tumors, are functionally deficient as evidenced by inability to lyse autologous or allogeneic NK-sensitive or NK-resistant tumor cell targets in 4 h ^{51}Cr release assays [52], to mediate antibody-dependent cellular cytotoxicity [53], and by reduced responsiveness of TIL to exogenous IL2, mitogens, and alloantigens [28, 29, 54]. Further, we have shown earlier [28, 55] that the clonogenic potential of TIL-T was significantly reduced as determined by limiting dilution analysis (LDA), which permits calculations of the frequency (f) of proliferative T lymphocyte precursors (PTL-p). Only about 1/20 TIL-T was able to proliferate in a culture system in which 1/2 to 1/1 normal PBL-T were clonogenic [29]. These observations could not be easily explained and appeared contradictory with respect to the presence of activated and 'committed' T cells in TIL as determined by flow cytometry (see above). It was, therefore, necessary to find a plausible explanation for this refractory behavior of human TIL. The following three hypotheses were considered: (a) an intrinsic defect of the TIL-T; (b) the presence of suppressor lymphocytes among TIL; (c) inhibition caused by the tumor microenvironment.

Miescher and colleagues provided convincing evidence that the impaired clonogenic potential of TIL-T is a result of intrinsic defect in these cells [28, 29]. Stimulation of sorted $CD2^+$ TIL in low-density cultures by either PHA-P or anti-CD3 monoclonal antibody showed that the antigen-dependent activation pathway was impaired, although structurally-intact TCR complexes were apparently present on the cell surface as measured by flow cytometry. The depressed proliferative response of $CD2^+$ TIL could not be reversed *in vitro* by phorbol esters alone or in combination with ionomycin, which bypass the TCR [29]. The impaired proliferative potential of TIL was unequally distributed among $CD4^+$ and $CD8^+$ subsets of TIL, with $CD8^+$ cells showing a significantly greater reduction in the clonogenic potential in comparison to $CD4^+$ TIL [55]. Thus, both in LDA and low-density cultures, $CD8^+$ TIL were preferentially unresponsive to proliferative stimuli [55]. We have previously shown that TIL possess a significant cytolytic potential in spite of their reduced proliferative capacity [23, 54]. This was defined by activity in the lectin-dependent cytotoxicity (LDCC) assay which allows detection of cytolytic potential irrespective of antigenic specificity of effector cells and acts by triggering the lytic machinery via the TCR. Up to 67% of TIL clones, which proliferated in the presence of 50 U/ml of IL2 mediated LDCC. In contrast, LAK activity was only infrequently observed [29].

The presence of lymphoid suppressor cells among TIL was not supported by the LDA performed by us, as our data were consistent with a single-hit Poisson model [28, 29], which assures that only one cell was titrated and that second-party cells did not interfere with growth. Earlier mixing experiments in which TIL were added in various proportions to mitogen-activated autologous PBL [56] also failed to provide evidence for suppression of proliferation. More recently, in a series of mixing experiments utilizing lymph node lymphocytes (LNL) from tumor-draining nodes of patients with SCCHN and autologous PBL, we again were unable to demonstrate suppression of *in vitro* responses of PBL to mitogens, IL2 and autologous tumor cells by LNL from tumor-draining LN [57]. Thus, we could not confirm that suppressor activity was present and mediated by a lymphoid cell in the tumor-draining lymph nodes as reported by Cozzolino et al. for patients with cancer of the larynx and bladder [43] and by Mukherji et al. for those with melanoma [58]. It is, of course, possible

that suppressor activity in tumor tissues may be mediated by the non-lymphoid cell.

Substantial evidence has accumulated to date for the ability of human tumors to produce factors capable of suppressing functions of immune cells [59]. Several likely candidates for a tumor-derived immunoinhibitory factor have been identified including prostaglandins [60], transforming growth factor beta (TGF-beta) [61], the p15E retrovirus-related glycoprotein [62], and the immunosuppressive factor from the colon carcinoma cells [63]. Recently, Robb [64] has shown that cell surface gangliosides inhibit proliferation of mitogen-activated PBL by interfering with the activity of IL2. There are many ways in which tumor cells may influence anti-tumor immune responses, and it is not clear at this time to what extent tumor-derived inhibitory factors are responsible for poor expansion of $CD8^+$ antitumor effectors at the tumor site. A better understanding of inhibitory factors produced by human solid tumors and means to reverse these immunosuppressive effects are necessary to achieve successful *in vitro* activation of TIL.

Of the three hypotheses advanced to explain functional deficiencies of fresh TIL from human solid tumors, two may be consistent with the available experimental evidence. Human TIL, especially $CD8^+$ T cells, appear to have an intrinsic defect in the antigen-dependent activation pathway which leads to proliferation. At the same time, cytotoxic potential of these TIL appears to be intact. Inability of fresh TIL to produce cytokines (IL2, IFNγ, IL1β and TNFα) upon *in vitro* stimulation with PHA and bacterial LPS is consistent with this intrinsic defect (personal observations). It is possible that tumor-derived immunoinhibitory factors, in particular TGF-beta, are responsible for the defect. The phenotypic expression of activation markers, HLA-DR and CDw29, on TIL suggests that TIL may be blocked in their functions after reaching the activated state *in situ*.

Proliferative responses of TIL to high doses of exogenous IL2

TIL cultured in the presence of IL2 acquire the ability to kill tumor targets and to proliferate trations (500–1,000 Cetus U/ml) is the best agent for overcoming inhibitory effects of the tumor milieu on TIL. We recently studied paired TIL and A-PBL from 44 primary human solid tumors for proliferation and antitumor cytotoxicity during growth in the presence of IL2. TIL achieved significantly greater expansion fold (median 197×) than A-PBL (37×), but 7 out of 44 TIL were unresponsive to rIL2 by proliferation, while all A-PBL responded. Also, in some cases, TIL proliferated only after a lag of several days. In terms of antitumor cytotoxicity, which was monitored serially against autologous and/or allogeneic tumor targets, TIL and A-PBL were similar on a per cell basis [21, 67]. Since, however, TIL proliferated better, total lytic units of cytotoxicity were higher for TIL than A-PBL cultures [53]. Similar results were reported for TIL from RCC, colon and ovarian carcinomas, and melanoma [16, 68, 69, 70].

As indicated above, rIL2 at a concentration of 1000 Cetus U/ml induced most, but not all, TIL from primary tumors to proliferate. When TIL obtained from primary (n = 18) and metastatic (n = 29) human liver tumors were cultured in the presence of IL2 and TNFα, we found that TIL originating from primary tumors expanded significantly better, faster, and mediated higher antitumor cytotoxicity and maintained it longer than those from metastatic tumors (Figure 1). It should be noted that cellular composition or the proportions of activated T cells as determined by

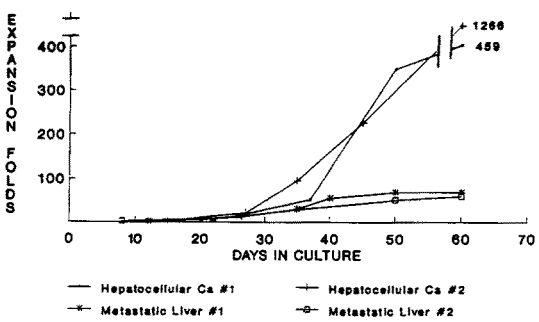

Fig. 1. Human TIL obtained from human liver tumors and cultured in the presence of rIL2 (100 Cetus U/ml) and TNFα (1000 Cetus U/ml). TIL recovered from primary liver tumors (i.e., hepatocellular carcinoma) proliferated significantly better in long-term cultures ($p < 0.05$) than those from tumors metastatic into liver.

two-color flow cytometry (Table 2) were not different in TIL from primary vs. metastatic tumors. Our results indicated that TIL obtained from metastatic disease were significantly less responsive to rIL2 in bulk cultures than those from primary cancers [17, 71]. Even when expansion was achieved in the presence of IL2, TIL from metastatic liver cancers failed to mediate antitumor cytotoxicity. It is likely that tumor-induced inhibition cannot be reversed by rIL2 in TIL originating from metastatic tumors.

Antitumor cytoxicity of TIL in IL2 cultures

The central question regarding TIL proliferating in culture in the presence of IL2 concerned the nature of cytotoxic antitumor effector cells. Flow cytometry analyses indicated that TIL cultures were as heterogeneous as LAK-cell cultures comprising $CD3^+CD56^-$, $CD3^+CD56^+$, and $CD3^-CD56^+$ cells in various proportions [67, 72]. Perhaps the only exception were TIL cultures derived from melanoma, where more homogeneous populations of $CD3^+CD8^+$ effectors with AuTu cytotoxicity outgrew in some cases [70]. Cultures of TIL obtained from SCCHN and ovarian tumors were sorted on FACStar and the separated cells tested for cytotoxicity against AuTu and allogeneic tumor targets to determine which cells were responsible for cytotoxicity [16, 67]. Similar to LAK cell cultures, two relatively small populations of $CD3^-CD56^+$ NK cells (10–15%) and $CD3^+CD56^+$ MHC-unrestricted T cells (8–12%) mediated antitumor cytotoxicity in these TIL cultures. The vast majority (70–80%) of IL2-activated T cells ($CD3^+CD56^-$) had no antitumor activity at all. In our hands, TIL from several solid tumors other than melanomas, when induced with exogenous IL2, generated effector populations that were identical to autologous LAK cells [21].

At the same time, most of these cultures of TIL from primary SCCHN, ovarian and colon carcinomas, and liver tumors showed variable, and sometimes high, levels of AuTu cytotoxicity. Furthermore, AuTu cytotoxicity was generally higher in TIL than A-PBL cultured in IL2 [16]. Since $CD3^+CD56^+$ effectors represented at least one T lymphocyte population involved, and since AuTu specificity is difficult to demonstrate in IL2 cultures, because of 'promiscuous' behavior of effector cells, we considered that clonal analysis may help to determine if tumor-specific T cells were present among TIL. Taking advantage of the availability of fresh autologous tumor cells, we recently performed LDA and clonal analysis of proliferating T cells derived from fresh TIL in 18 cases of human primary and metastatic liver cancer [32]. In cytotoxicity assays against AuTu cells, cytolytic T lymphocyte precursors (CTL-p) were identified only in TIL and were not found among cells from fresh A-PBL. The frequency of CTL-p for AuTu in fresh TIL isolated from primary liver tumors was 0.02–0.13, and 12/81 clones obtained were selectively reactive against AuTu. In contrast, only 1/66 TIL clones obtained from metastatic tumors showed AuTu reactivity. Interestingly, most (7/9) AuTu-cytotoxic clones that we obtained were $CD4^+$. This result is consistent with previous observations of the preferential outgrowth of $CD4^+$ clones from human TIL, but not from patients' PBL. It also emphasizes that $CD4^+$ T lymphocytes may mediate antitumor effects by lytic and, perhaps, non-lytic mechanisms as well, as suggested by Fossatti and colleagues [73]. Most important, these data indicated that local immune responses against AuTu exist in primary liver tumors and that TIL, but not A-PBL, contained AuTu effectors, some of which outgrew in IL2-containing cultures. It is thus possible to tentatively conclude that TIL in primary liver tumors may be enriched in AuTu-reactive effector cells.

There was an impelling reason for searching for CTL among TIL from human solid tumors. Murine experiments convincingly demonstrated that cytolytic T lymphocytes can be generated from tumors [14, 74] as well as lymphoid organs of tumor-bearing animals [75]. In humans, CTL have been generated from PBL, LNL and TIL obtained from patients with metastatic melanoma [35, 76–78], providing evidence in favor of the presence of CTL in at least this human solid tumor. Based on these findings, adoptive immunotherapy with TIL and IL2 was initiated in patients with some cancers unresponsive to conventional therapies [79]. Yet, it was

not clear that TIL offered any therapeutic advantage over LAK cells in these cancer patients. A formal demonstration of AuTu-specific T cells in TIL and means to proliferate them in culture from most human solid tumors was needed to provide a rationale for this therapeutic approach.

Human AuTu-specific T cells may be expected to exhibit the following functional characteristics in *in vitro* assays: an ability to proliferate in response to AuTu cells and/or to lyse AuTu cells, inhibition of these responses by antibodies to the TCR complex, class I MHC restriction, and a lack of reactivity with autologous normal tissue cells. Nevertheless, it should be remembered that AuTu-specific T cells may show reactivity with MHC-matched allogeneic tumors of the same histologic type due to the recognition of a common TAA or even with those MHC-unmatched tumors of different histologic types that express an antigen containing shared determinants, as described by Finn et al. [80]. In IL2-containing cultures, discrimination between AuTu-specific and 'promiscuous' activated T cells becomes difficult and may require repeated stimulations with AuTu to encourage selective proliferation of the former prior to measurements of effector cell function. In Figure 2 we present cytolytic profiles of three $CD3^+CD8^+$ T cell clones derived from TIL of a patient with gastric carcinoma metastatic to liver and cultured for 4 months in the presence of 50 Cetus U/ml of IL2 with repeated AuTu stimulations. Again, these data give support to our hypothesis that AuTu-specific cytolytic effectors can be obtained from TIL in some human solid tumors.

The question remains why it has been so difficult to outgrow *bonafide* $CD8^+$ cytolytic T cells from most human TIL. As indicated above, several groups have succeeded in outgrowing CTL populations and clones with specificity for AuTu from melanoma TIL [76–78]. Since most human TIL are T lymphocytes [21, 28], often comprising a preponderance of $CD8^+$ cells, it is curious that mostly $CD4^+$ lymphocytes outgrow from TIL in the presence of low or high concentrations of IL2 [23]. It appears that $CD8^+$ T cells present in human TIL do not respond or respond poorly to IL2 and do not proliferate, i.e., are inhibited or intrinsically defective, as suggested earlier [55]. It can thus be hypothesized that most human

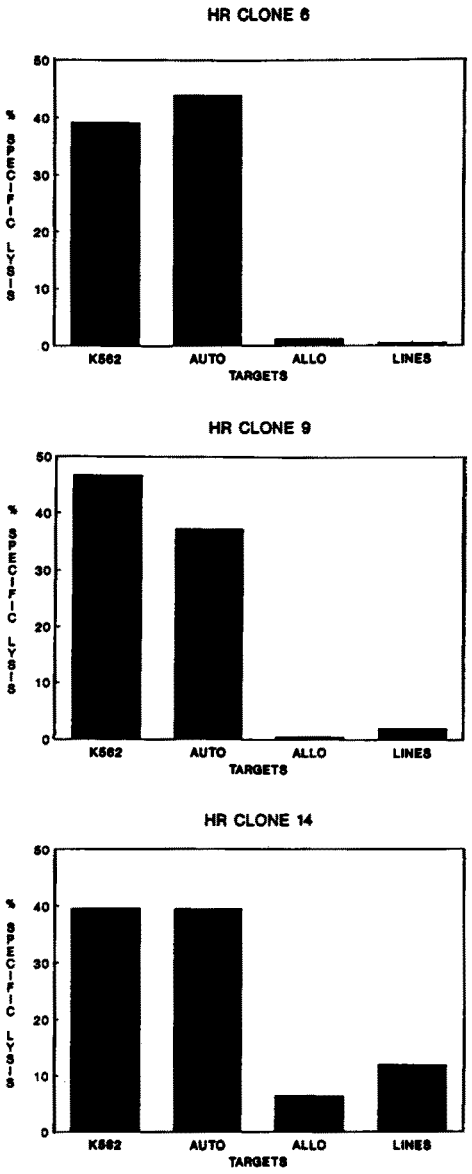

Fig. 2. Cytolytic activities of three T cell clones (all are $CD3^+CD8^+$) derived from TIL of a patient with gastric carcinoma metastatic to liver. TIL were cloned by limiting dilution and then cultured for 4 months in the presence of 50 Cetus U/ml of rIL2. The clones were stimulated (every 2 weeks) with irradiated autologous tumor. Cytotoxicity of each of the clones was measured against NK-sensitive K-562 targets, NK-resistant autologous tumor and seven different allogeneic (fresh) liver tumor targets as well as against NK-resistant tumor cell lines SK-HEP, LS-174 (colon carcinoma) and PCI-88 (melanoma). The clones were cytotoxic against autologous but not any of the allogeneic targets. Their reactivity against K-562 targets reflects 'promiscuous killing' not a lack of specificity. Monoclonal antibodies to the CD3 antigen blocked killing of autologous targets but not of K-562 targets. (Y. Shimizu, et al., unpublished data).

solid tumors contain CTL as well as NK cells and other MHC-unrestricted cytotoxic cells, and the conditions utilized for *in vitro* cultures may select for proliferation and/or activation of one effector cell type or another. Further, were appropriate *in vitro* conditions found for activating $CD8^+$ T cells in fresh TIL and for their growth, TIL from most primary human tumors could be a good source of therapeutically effective $CD3^+CD8^+$ cytotoxic lymphocytes.

Outgrowth of $CD3^+CD8^+$ antitumor effectors from TIL.

Evidence for the presence of CTL in human solid tumors has been reviewed above. However, optimal *in vitro* conditions for their outgrowth and expansion have not been yet defined. Earlier studies indicated that the presence and interactions of several cytokines may play an important role in generation and successful culture of CTL [81]. For example, in mice, endogenously secreted IFNγ was shown to be obligatory for induction of LAK and T lymphocyte killer activity but not for cytotoxicity mediated by natural killer cells [82]. We have recently demonstrated by *in situ* hybridization that fresh TIL isolated from ovarian carcinomas do not express a message for IFNγ and do not produce this cytokine [83]. More recent studies with LNL from SCCHN patients also showed that freshly isolated LNL from both tumor-involved and tumor-uninvolved LN are unable to produce cytokines (IL2, IL1β, TNFα, and IFNγ) when activated *in vitro* with LPS or PHA [57]. Since exogenous IL2 alone only rarely induces outgrowth of $CD3^+CD8^+$ effectors from TIL populations, we wondered if a combination of cytokines might not be more successful for this purpose. We chose a combination of IL2 and TNFα, because of our hypothesis that human TIL are functionally inhibited by tumor-derived factors related to or identical to TGFβ. It has been demonstrated earlier that TGFβ suppressed the generation of alloreactive CTL in the human MLC [84]. Tumor-derived TGFβ might similarly suppress CTL generation from TIL cultured in the presence of rIL2. Further, TNFα is known to selectively promote the generation and outgrowth of allospecific CTL in the MLC and to reverse their inhibition induced *in vitro* by TGFβ [84]. It could be expected that TNFα may similarly reverse tumor-induced inhibition of $CD8^+$ T lymphocytes in TIL preparations obtained from human solid tumors.

Earlier studies with ovarian TIL in our laboratory indicated that a combination of IL2 and TNFα was favorable for an initial outgrowth from TIL of $CD8^+$ T lymphocytes enriched in AuTu-reactive effectors [83]. More recently, we demonstrated that a combination of IL2 and TNFα was also advantageous for selective outgrowth of $CD3^+CD8^+$ T lymphocytes with AuTu reactivity in long term cultures of ovarian TIL (Figure 3). At days 30–40 of growth, total lytic units of autotumor cytotoxicity per culture increased from a mean of 59 to 2,155, and the percentage of $CD3^+CD8^+$ T lymphocytes rose to 98% in some of the cultures [34]. The mechanisms responsible for the preferential *in vitro* outgrowth of CTL in the presence of TNFα from human TIL preparations remain unknown. Studies from Palladino's group indicate that well-known immunoinhibitory effects of TGFβ may be reversed by the addition of TNFα [84]. An alternative explanation may be that TNFα and IL2 induce production of growth factors necessary for expansion of $CD8^+$ TIL but absent in the tumor milieu. The combination of TNFα and IL2 was also effective in promoting $CD3^+CD8^+$ T cell growth from TIL obtained from primary

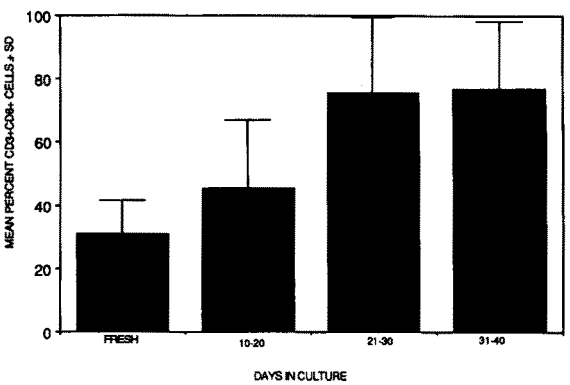

Fig. 3. Phenotype of TIL obtained from human ovarian carcinomas (n = 13) and expanding in the presence of rIL2 (100 Cetus U/ml) and TNFα (1000 U/ml) in long-term cultures. Differences in the percentages of $CD3^+CD8^+$ cells between fresh TIL and cultures tested on any of the days indicated were significant at $p < 0.02$.

heptocellular carcinomas but not from metastatic liver cancers [71]. Also, it was much less effective in the case of TIL and LNL obtained from patients with SCCHN. It is thus unclear at this time whether this combination of cytokines will produce the desired effects in TIL from all human solid tumors. Even in TIL preparations from ovarian carcinomas, where the combination was very effective in generating CTL lines, the feasibility of maintaining these $CD3^+CD8^+$ TIL as AuTu-specific lines remains to be determined.

Another interesting aspect of the combination of TNFα and IL2 was that it proved to be advantageous for achieving selective outgrowth of AuTu-reactive $CD3^+CD8^+$ TIL without the addition of irradiated AuTu cells as stimulators. It has been argued that the presence of AuTu in IL2 cultures is essential for CTL growth [85], possibly because of the ability of tumor cells to present antigens. Although there is evidence that melanoma cells are capable of processing and presenting their own surface antigens to immune effector cells [86], it is not clear that other tumor cells can do so as well. Clearly, in view of the usual shortage of AuTu cells, it is advantageous to have a system for outgrowing and culturing CTL without AuTu cells present.

Spontaneous enrichment in autologous melanoma-reactive T lymphocytes has been reported in TIL cultures from human melanomas by Rosenberg's group [70]. Others have achieved outgrowth of CTL from melanomas using IL2, feeder cells, and *in vitro* sensitization with autologous irradiated tumor cells [35, 76–78]. Establishment of cytotoxic T cell lines from patients with pancreatic cancer by continuous stimulation of tumor-draining lymph node cells with allogeneic pancreatic tumor cell lines has been recently reported [87]. One of these lines recognizes a specific antigen, a glycosylated mucin molecule containing many repeating subunits via a T cell receptor but in an MHC-unrestricted manner [87]. We have recently succeeded in establishing $CD3^+$ autotumor-specific T cell clones from human liver tumors [32]. Tumor-specific T cell clones have also been obtained from human breast, ovarian and glial tumors [88, 89, 90]. It thus appears that the presence of CTL in human solid tumors other than melanomas has been confirmed.

Adoptive immunotherapy with tumor-infiltrating lymphocytes

Initial studies from Rosenberg's group indicated that TIL derived from murine tumors and grown in rIL2 were 50–100 times more effective to erradicating established micrometastesis in a murine sarcoma model [91]. Culture techniques have been developed to allow expansion of large numbers of TIL adoptive immunotherapy, and it has been possible to treat patients with as many as 2×10^{11} TIL or greater [66]. With hopes of enhancing effectiveness of adoptive immunotherapy in man, several phase I trials utilizing TIL from human solid tumors have been recently initiated [79, 92, 93]. The preliminary results of these trials with TIL expanded *in vitro* in high [79] or low doses of IL2 [93] have been mildly encouraging. Although the feasibility and practicality of administering IL2-expanded TIL to patients with metastatic disease has been confirmed in these trials, responses were relatively few and of limited duration [79, 93]. For example, in a phase I trial reported by Rosenberg [79] in patients with metastatic melanoma, of 20 patients selected for TIL therapy and pretreated with cyclophosphamide (25 mg/kg), a single administration of TIL (median number, 2×10^{11}), and rIL2 (1×10^5 Cetus U/kg every 8 hours), 11 had objective responses lasting from 2–12 months. In another study with TIL performed by Kradin et al. [93], 28 patients (13 with malignant melanoma, 7 with renal cell carcinoma (RCC), and 8 with non-small-cell lung cancer) were treated with TIL (about 10^{10}) and continuous infusions of rIL2 ($1–3 \times 10^6$ Cetus U/ml 24 h). Twenty-nine percent of the patients with RCC and 23% of those with melanoma achieved objective tumor responses lasting 3–14 months. These early studies indicated that human TIL administered following previous immunosuppression or without such immunosuppression might be effective in inducing clinical responses in chemoresistant cancers. However, it remains unclear if the same or higher responses could be achieved with IL2 alone or in combinations with other cytokines. Furthermore, the mechanisms responsible for those responses that were achieved are not understood. For example, while studies in Rosenberg's laboratory with ^{111}In-

dium-labeled TIL indicated that TIL homed to the tumor, Kradin et al. could not demonstrate the presence of labeled TIL within tumors by gamma-imaging [93]. Adoptive immunotherapy with TIL in the preliminary phase I trials is in its early stages, and the encouraging results that have been reported need to be followed by more systematic and in depth evaluation of this form of therapy. Clearly, improvements are necessary both in practical (e.g., enormous numbers of cells infused, need for high doses of IL2) and economic (i.e., high cost associated with cell preparation methodology) aspects of this form of therapy. Additional studies and clinical trials are needed to optimize *in vitro* methodology, dosages, numbers and frequency of administration, and impact, if any, on survival. At this time, it seems reasonable to suggest that therapy with more homogeneous populations of human TIL would facilitate studies of the mechanisms involved. The most rational approach to adoptive therapy in humans with TIL should now focus on developing means for consistent generation and expansion from TIL of autotumor-specific, preferably highly cytotoxic, T cells which could then be administered to patients in lower numbers with an appropriate mixture of cytokines.

Future directions and summary

Much has been learned about TIL in human solid tumors recently. Still, a number of crucial questions about functional characteristics and antitumor efficacy of these cells *in vivo* remain unanswered. Current efforts are directed at the generation of homogeneous populations of cytotoxic TIL with specificity for AuTu. Much remains to be learned about selective activation *in vitro* of TIL with combinations of AuTu and different cytokines or with T-cell activating monoclonal antibodies and cytokines [94, 95, 96]. Since it has been documented that antitumor responses can be mediated by $CD8^+$ as well as $CD4^+$ TIL, approaches are needed to capture both of these TIL subsets, expand them and investigate their effectiveness *in vitro* and *in vivo*. Methods are becoming available for performing such separations using immunoaffinity on antibody-coated surfaces and/or columns, and it will be important to study the effects of various mixtures of cytokines ± AuTu on captured $CD8^+$ and $CD4^+$ subsets of human TILs.

Although subpopulations of $CD8^+$ and $CD4^+$ TIL can be already captured and cultured *in vitro*, little is known about their growth requirements and, especially, requirements for growth factors. It is still necessary to grow them in the presence of irradiated feeder cells and conditioned media. Better definition of growth requirements and differentiation factors for antitumor effectors derived from TIL are needed.

The issue of antigen presentation in TIL cultures and conditions optimal for AuTu sensitization need to be addressed. While some tumors, e.g., melanoma, have abilities to process and present antigens and to produce IL1 [86], it is by no means certain that other tumor cells can function as antigen-presenting cells.

Long-term expansion in culture of selected lines or clones of TIL without a loss of antitumor reactivity to achieve numbers of cells needed for therapy may be a difficult problem to overcome. So far, it has been difficult to maintain autotumor-reactive effectors for longer than 4–6 months in culture. This may be important, if therapy with more than one course of TIL is considered.

A better understanding and identification of tumor-derived inhibitory factors and improved understanding of the mechanisms through which these factors affect the immune system would clearly facilitate *in vitro* manipulations aimed at overcoming such tumor-derived inhibition in order to obtain functionally active effectors from human TIL.

There continues to be a great need for suitable animal models for local and systemic adoptive immunotherapy in which lines and clones of antitumor effectors developed from TIL can be evaluated for therapeutic effectiveness.

In addition, lines or clones of $CD4^+$ or $CD8^+$ T cells which recognize or respond to AuTu antigens represent useful biologic probes for studies of TAA on human tumors. Already, Hersey [97], Knuth [98] and Finn et al. [80] are using such lines and clones as antigen-specific probes for TAA. Not only physical/chemical properties of TAA can be investigated using such biologic probes but also interactions be-

tween tumor and effector cells and the importance of accessory and adhesion molecules on the surfaces of tumor and effector cells can be better defined.

Our knowledge of TIL as antitumor effectors remains incomplete. It is still unproven that TIL represent clonally-restricted populations of autotumor-specific T cells. *In situ* molecular biology studies are necessary to clarify the antigenic repertoire of T cells found in human solid tumors. The potential of TIL in anti-cancer therapy depends on successful generation *in vitro* of tumor-reactive or, better, tumor-specific cytolytic lines or clones of TIL and their effective delivery to the tumor site in patients with metastatic disease.

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