Tumor-Infiltrating CD3$^-$ NK Cells Are More Effective than CD3$^+$ T Cells in Killing Autologous Melanoma Cells

Orly Azogui, Marie-Françoise Avril, Alvaro Margulis, Maryvonne Guillard, Bernard Caillou, and Michel Prade
INSERM Unite 333 (OA, MG), Departments of Dermatology (M-FA), Surgery (AM), and Anatomopathology (BC, MP), Institut Gustave Roussy, Villejuif, France

We have studied the phenotype and functional activity of tumor-infiltrating lymphocytes (TIL) derived from eight human melanomas cultured for up to 60 d in the presence of recombinant IL-2. In the early period of the cultures, TIL were predominantly T cells of CD8$^+$ phenotype and contained 10–30% of CD3$^-$ cells. Four of the five early TIL cultures tested in a cytotoxicity assay displayed a degree of MHC-unrestricted lysis on a series of autologous and allogenic melanoma cell lines as well as the K562 natural killer–sensitive target. With longer periods of time in culture, all TIL lines showed a decrease in lytic activity that was associated with the loss of CD3$^-$ cells. Thus, most of the killing of short-term TIL cultures appeared to be mediated by CD3$^-$ natural killer cells, whereas CD3$^+$ T cells were found to be weak anti-tumor effectors. Even though the CD3$^+$ T cells were not cytotoxic on K562 targets, their lytic activity (even weak) against melanoma cells appeared to be non-MHC restricted, and was blocked by anti-CD3 antibodies. In addition, cytotoxicity of the CD3$^+$ TIL cultures was compared to that of a CD3$^-$/NKH1$^+$ cell line purified from peripheral blood. It was found that natural killer cells were much more potent than CD3$^+$ TIL on the melanoma cell lines tested. *J Invest Dermatol 97:425–429, 1991*

Human solid tumors are infiltrated with a variety of mononuclear cells including monocytes and lymphocytes [1,2]. Despite the statistical evidence that a more favorable prognosis and tumor regression are associated with a greater level of lymphocyte infiltration [3,4], the role of the T cells in immune reactivity against tumors remains unclear.

Several investigators have reported that tumor-infiltrating-lymphocyte (TIL) cultures contain T lymphocytes that are capable of lysing autologous tumor cells [5–7]. TIL isolated from murine tumors have been found to destroy early pulmonary metastases when adoptively transferred to tumor-bearing hosts [8,9]. These anti-tumor effects have been shown to be mediated by specific MHC-restricted CTL. Recently, most studies have also reported that recombinant IL-2 induced proliferation of human TIL [10–13]. Only in some cases of melanoma-derived cultures (about 20% of reported attempts), functional investigations have shown that TIL can yield specific CTL with appropriate MHC restriction on autologous tumor cells [14–17]. TIL obtained from solid tumors of different origin mostly generated MHC-unrestricted cytotoxicity [7,12,18,19]. The fact that TIL have anti-tumor activity, the success of murine trials, and the ability of human TIL to grow in culture in the presence of rIL-2 have led to the use of TIL in human adoptive immunotherapy [20–22].

Attempts to adapt rIL-2–activated TIL to immunotherapy have run into two obstacles: the limited numbers of TIL that can be recovered from surgical specimens and the irreproducible yield of specific anti-tumor cytotoxic activity, probably due to the low frequency of specific CTL in initial infiltrates. If cultured human TIL are to be considered as a therapeutic modality for immunotherapy of cancer, it appeared to us important to characterize the effector cells that are more active in tumor killing. Although murine-specific TIL killers have been shown to be 100 times more potent than aymphokine-activated LAK cells in eliminating metastatic melanomas [23], the relative effectiveness of the NK component has not been well established.

In the current study, cytotoxicity and immunophenotype of human melanoma-derived TIL were determined after 14 and 60 d of culture with high doses of rIL-2. We have observed that the highest anti-tumor cytotoxicity correlates with the presence of the CD3$^-$ natural killer (NK) cells in short-term TIL cultures. Moreover, a comparison of target sensitivities indicates that NK cells purified from peripheral blood are considerably more effective than autologous CD3$^+$ TIL in killing melanoma cells.

MATERIALS AND METHODS

Tumors and Cell Suspensions Six metastatic (T1 to T6), one primary (TP), and one local recidive (TR) of melanoma were resected from seven patients at the Institut Gustave Roussy. T3 and T5 were resected from the same patient. One non-metastatic lymph node (G2) was obtained as a control. Briefly, each tumor was minced into small pieces and digested during 3 h in RPMI culture medium (Seromed) containing 0.01% hyaluronidase type V, 0.002% DNase type 1, 0.1% collagenase type IV (Sigma), and anti-

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Reprint requests to: Orly Azogui, Laboratoire d'Hémato-Immunologie, INSERM U333, Institut Gustave-Roussy, 39 rue Camille Desmoulins, 94805 Villejuif Cedex, France.

Abbreviations:
- CTL: cytotoxic T lymphocyte
- LDCC: lectin-dependent cytotoxic cells
- MHC: major histocompatibility complex
- NK: natural killer cells
- PHA: phytohemagglutinin
- rIL2: recombinant interleukin 2
- TIL: tumor infiltrating lymphocytes

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biotics. Suspensions of TIL and tumor cells were washed twice and resuspended in RPMI 1640 medium with 10% AB serum for TIL expansion or RPMI with 10% FCS (Gibco Laboratories) for amplification of the autologous tumor cells.

Tumor Cell Lines Two tumor cell lines used in this study were kindly provided by J. F. Doré (Mel Do and Mel Br) [24]. The others were derived from the patients described above. Two of these cell lines (Mel 5 and Mel TR) were established in long-term cultures according to the known techniques [25]. The other two, Mel6 and Mel TP, were only kept in short-term cultures. Briefly, 5×10^5 to 10^6 viable cells from Mel5, Mel6, MelTP, and MelTR cell suspensions were distributed into 25-cm² tissue culture flasks in RPMI 1640 supplemented with 10% FCS, 2% L-glutamine (200 mM), 1% sodiumpyruvate (100 mM), and antibiotics. After 1 week, the medium was changed and unattached cells were removed. Every 2 weeks, the cells were detached using trypsin-EDTA (Gibco) and transferred into new flasks kept at 37°C in an atmosphere containing 10% CO_2. After several passages, contaminating fibroblasts disappeared and the nature of the cell lines checked by electron microscopy. The four lines showed a typical melanocyte morphology under electron microscope, i.e., contained melanosomes and stained positively with Fontana-Masson (melanin content) and the characteristic protein S100.

Short-Term TIL Cultures Tumor-infiltrating T lymphocytes were obtained directly from freshly resected melanomas. They proliferated in the presence of viable tumor cells and IL-2 added in the initial suspension. Tumor cells can be recognized from lymphoid cells by the size, morphology, and the brownish cytoplasmic pigmentation. After enzymatic digestion, the whole-cell suspension, containing approximately 2×10^5 T cells, was seeded in 24-well tissue culture plates (Falcon, NJ) with 1 ml of medium supplemented with 10% AB serum and 500 Units of rIL-2 (Roussel-Uclaf, Romainville, France). Half the volume of each well was replaced every 3 d with fresh medium and the density of cells maintained at 0.5 to 1×10^6 cells/ml during 2 weeks. After this period, all the tumor cells disappeared and the cultures were 100% lymphoid cells.

Stimulation of Long-Term Cultured TIL As the IL-2-activated T cells did not grow after 14 d in culture, 10^4 TIL per well were distributed in 96-well U-bottomed microplates in RPMI medium supplemented with 10% AB serum, 200 μ/ml of rIL-2, PHA (0.5 μg/ml, Wellcome), and in the presence of feeder cells composed of irradiated PBMC from three healthy volunteers (6×10^4 cells/well) and one allogeneic irradiated EBV-transformed B cell line (2×10^4 cells/well). The IL-2 dose of 200 units (i.e., 75 ng/ml) used in our culture system was determined by preliminary experiments so as to be optimal for the growth of T cells. Each culture phenotype was controled by fluorescence staining before and after stimulation with the feeder cells. The CD4/CD8 ratio was not affected by the presence of allogeneic feeder cells.

Phenotypic Analysis Phenotypes of cultured TIL were determined after 14 and 60 d of culture, in order to correlate the cytolytic activity with a particular lymphoid subset. Briefly, lymphocytes were incubated with MoAb as OKT3(CD3), OKT4(CD4), OKT8(CD8), BMA031 (TcR$\alpha\beta$), 3B8(NKH1), OKT11(CD2) at 4°C for 30 min. Cells were then washed and stained with FITC-conjugated goat anti-mouse for 30 min at 4°C and washed twice in PBS. All cells were fixed in 1% paraformaldehyde and stored at 4°C until they were analyzed. Fluorescence was evaluated using an EPICS C flow cytometer (Coulter). A minimum of 3000 cells per sample were analyzed.

Cytotoxicity Assay TIL and NK cytotoxicity was performed using a chromium-51 release assay. Between 2 and 5×10^5 target cells were labeled with 200 μCi of sodium chromate for 90 min at 37°C, washed 3 times in RPMI, and resuspended in order to distribute 1×10^3 cells/well in duplicate volumes of 150 μl in V-bottomed microtiter tray. In the LDCC assay, PHA was added to the effector cells immediately before placing target cells in microwells. After 4 h incubation the plates were centrifugated and 100 μl of supernatant were removed for measurement of ^{51}Cr release in a gamma counter. All experiments have been done twice and each data point represents the mean of duplicates with a standard error always inferior to 15%. Specific lysis was calculated according to the following formula:

$$\text{percent of specific lysis} = \frac{\text{Experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100.$$

Table I. Phenotypes of TIL Activated with PHA and Expanded During 14 d in the Presence of rIL-2

TIL from Patient	Percent Positive Cells					
	CD2	CD3	TcR$\alpha\beta$	CD4	CD8	NKH1
T1	99	96	55[a]	1	95	1
T2	97	91	84	7	77	4
T3	97	97	78	14	78	ND
T4	95	95	83	26	51	2
T5	99	97	95	39	42	3
T6	97	96	84	42	59	11
TR	92	90	87	10	92	4
TP	99	93	88	55	31	4
G2	99	99	95	49	26	4

[a] This represents a weak cell-surface labeling of BMAO31 on all cells.

Purification of CD3⁻ NK Cells by Immunorosetting Natural killer cells were purified from blood by immunorosetting method [26]. PBMC were isolated on Ficoll hypaque density gradient and monocytes removed by two cycles of adherence on Petri culture dishes (45 min at 37°C). T and B cells were removed from cell suspension by incubation of 25×10^6 non-adherent cells with a mixture of monoclonal antibodies including anti-CD3 (OKT3), −CD4 (OKT4), −CD8 (OKT8), −CD19 (B4), and −TcR$\alpha\beta$ (BMA031). All the antibodies were used in excess (1/100 to 1/1000). After 45 min at 4°C of incubation with 1 ml of suspension containing antibodies coupled with magnetic beads, rosetted (T and B cells) and non-rosetted (NK cells) cell fractions were separated using a magnet. Purity of cells were determined by immunofluorescence and cytofluorometric analysis. Purified NK cells were expanded on the same feeder layer used for TIL cultures, in the presence of PHA-P (Wellcome) and rIL-2. Ninety percent of the cells were found to be CD3 negative and NKH1 (3B8) positive.

RESULTS

Surface Phenotype of PHA Stimulated TIL Cultures Expanded During 14 D with R-IL2 Six metastatic (T1 to T6), one primary tumor (TP), and one local relapse (TR) of melanoma were resected from seven patients (T3 and T5 were derived from the same patient). T lymphocytes from a non-metastatic lymph node(G2) were used under the same culture conditions. Because the number of TIL was unsufficient for direct analysis of their surface phenotype, cells were stimulated at day 0 with PHA and cultured for 14 d in rIL-2. Although the percentages found by these measurements may not reflect the actual composition of the initial infiltrate, they indicate the presence of the identified T-cell subsets in the starting cell suspension.

Staining with monoclonal antibodies BMA031 and OKT3 revealed that most of these cultures contained predominant cell population that expresses a classical phenotype CD3/TcR-$\alpha\beta$ (Table I). However, CD4/CD8 ratio was heterogenous as judged by the percentage of CD4⁺ cells observed in the different cultures. In fact, the percentage of CD4⁺ cells varies from 26–55% in four of eight TIL cultures and G2, whereas it is very low (1–14%) in the four others. All the TIL cultures express a majority of CD8⁺ cells except in TIL TP and the lymph node G2. These results indicate that the tumors are infiltrated by both CD4 and CD8 cells.

Phenotype of Activated-TIL Expanded in a Short- and Long-Term Culture with rIL-2 Alone Cells expanded in the presence of PHA do not represent the in vivo activated population.

Table II. Phenotypes of rIL2-Activated TIL Expanded in Short-Term (14-d) and Long-Term (60-d) Cultures

TIL from Patient	Days of Culture	Percent Positive Cells					
		CD2	CD3	TcRαβ	CD4	CD8	NKH1
T1	14	98	83	84	2	91	1
T2	14	98	59	44	10	75	21
T3	14	92	72	55	14	44	ND
	60	97	97	96	64	36	8
T4	14	95	79	59	6	65	11
	60	95	95	93	7	73	50
T5	14	91	92	94	18	78	4
	60	93	93	92	57	27	7
T6	14	91	68	63	2	77	5
	60	97	97	94	5	87	61
TR	14	95	53	52	12	64	31
	60	96	96	94	22	78	12
TP	14	99	86	87	87	9	12
	60	99	96	91	99	1	4
G2	14	95	91	95	78	25	2

Therefore, cultures were established from the same cell suspension, in parallel, in the absence of PHA. In order to analyze the cell-surface phenotype of in situ-activated TIL, the initial suspensions were amplified in the presence of high doses (500 μ/ml) of rIL-2 alone. Flow cytometry studies were performed at 14 and 60 d after culture initiation. Our objectives were to compare phenotypes of TIL cultured with viable tumor cells (14 d) with the same cells grown in long-term cultures (60 d).

As shown in Table II, analysis of the differential expression of the CD2 and CD3 molecules indicated that most of the short-term (14 d) TIL cultures contain 10–30% of $CD2^+CD3^-$ cells defined as NK cells on the basis of the absence of the CD3 antigen [27,28]. Considering the CD4/CD8 ratio, it is predominantly the $CD8^+$ cells that are growing in rIL-2 alone, except for the primary tumor TP and the normal lymph node G2 in which IL2 favored the $CD4^+$ T cells. By contrast, analysis of the long-term culture (60 d) phenotypes revealed that $CD3^-$ cells disappeared and cultures became totally $CD3^+$/TcRαβ. A marked increase of the $CD4^+$ cells was observed in three cases, whereas in three other cases it remained unchanged. The percentage of $NKH1^+$ cells increased significantly on the long-term $CD3^+$ TIL in two of six cases tested.

Taken together, these data show that cultured TIL in the presence of rIL-2 alone promotes the outgrowth of $CD8^+$-activated T cells, despite the presence of $CD4^+$ cells in the initial suspensions (see Table I). In most cases, in the presence of a high dose of rIL-2, the $CD3^-$ NK cells grow and represent 10–30% of lymphocytes only in the early period of TIL cultures.

Cytotoxic Activity of 14-D rIL2-Activated TIL on Autologous and Allogeneic Short-Term Culture Tumor Cell Lines Cytotoxicity assays were performed with five TIL cultures at day 14 on autologous and allogeneic tumor cell targets maintained in culture for 1 to 2 months. The results expressed in Table III show that T3, T6, TP, and TR cultures exhibit a significant non-MHC restricted lysis of melanoma and the NK-sensitive K562 targets. T5 TIL and T lymphocytes from G2 were only weakly lytic for all targets. The fact that four of five cultures displayed a broad range of lytic activity including the NK-sensitive target K562 may be related to the presence of CD3— NK cells as demonstrated in Table II. It is important to emphasize that the TIL culture T5 with the lowest lytic activity on melanoma and K562 targets did not contain $CD3^-$ cells.

Cytotoxic Activity of 60-d rIL2-Activated TIL on Autologous and Allogeneic Melanoma Cell Lines After 15 to 20 d in culture, TIL were frozen until the autologous tumor cells were established as long-term melanoma cell lines. Once the melanoma cells began proliferating vigourously (approximately 6 months for Me15 and MelTR), autologous TIL were thawed, passed on feeder cells in the presence of high doses of rIL-2 for 6 weeks, and their cytolytic activity assayed on autologous and allogeneic tumor cell

Table III. Autologous and Allogeneic Cytotoxicity Mediated by 14-d Cultures of rIL2-Activated Melanoma TIL

TIL from Patient		Percent of Lysis				
		Melanoma Cell Lines Used as Targets				
		Mel5	Mel6	MelTR	MelTP	K562
T3	50:1	<u>45</u>[a]	73	47	25	80
	5:1	nd	47	23	12	nd
T5	50:1	<u>8</u>	nd	34	4	38
T6	50:1	21	<u>66</u>	51	30	60
	5:1	nd	<u>25</u>	21	13	nd
TR	50:1	nd	74	<u>76</u>	50	64
TP	50:1	53	73	67	<u>50</u>	70
	5:1	nd	34	35	<u>25</u>	nd
G2	50:1	8	8	1	2	32
	5:1	nd	4	1	2	nd

[a] Percent of autologous killing is indicated in underlined characters.

lines and K562. The results summarized in Table IV show a decline of cytolytic activity for all TIL cultures on all melanoma cells, including K562. This correlates with the disappearance of $CD3^-$ cells from the long-term TIL cultures (see Table II), suggesting that the short-term cytotoxic activity was in part due to the NK fraction of TIL cultures.

In order to verify the lytic potential of $CD3^+$ TIL cultures and the ability of each target to be lysed, the assay was performed in the presence of PHA in a lectin-dependent cytotoxic assay (LDCC). In parallel, the sensitivity of melanoma targets to $CD3^-NKH1^+$ cells enriched from peripheral blood was tested and compared to TIL cultures. The results expressed in Table IV show that most cultures retain potentially cytotoxic populations that are not capable of recognizing melanoma targets spontaneously, except for TIL TP, which completely loose their cytotoxic activity. By contrast, the lytic activity of the purified $CD3^-$ cells indicates that each melanoma cell line has the same characteristic sensitivity to lysis regardless of the source of cytotoxic NK cells.

All these data strongly suggest that effector cells are heterogenous and modulate with the duration of culture. The comparative study between TIL and the purified $CD3^-$NK cell line demonstrates that the latter lyse melanoma targets more effectively than the former. This is seen with the significant lysis effected by peripheral NK cells both with and without PHA in the assay.

Cytotoxic Activity of 14- and 60-d Cultures of TIL TR on the Same Melanoma Cell Lines Used as Targets Because both TIL and melanoma cells were kept simultaneously in culture, the lower cytotoxicity observed in some effector/target combination

Table IV. Cytotoxic Activity Mediated by Long-Term Cultures of rIL2-Activated TIL on Autologous and Allogeneic Melanoma Cell Lines

TIL from Patient	LDDC[a]	Percent of Lysis of Targets (E/T = 30/1)				
		MEL5	MELTR	MELBR	MELDO	K562
T3	−	<u>22</u>	28	31	36	15
	+	<u>35</u>	68	28	55	33
T5	−	<u>0</u>	0	3	3	3
	+	<u>27</u>	81	32	57	49
T6	−	8	19	15	18	4
	+	22	72	37	62	47
TR	−	16	<u>14</u>	17	20	10
	+	34	<u>64</u>	33	57	33
TP	−	1	3	0	3	2
	+	10	15	11	10	3
NK cell-line	−	73	84	33	85	74
	+	78	97	47	81	77

[a]PHA was added to the effector cells before placing target cells in the assay. Percent of autologous killing is indicated in underlined characters.

Table V. Comparison of Cytotoxic Activity Mediated by Short-Term and Long-Term TIL TR on Melanoma Cell Lines

TIL from Patient		Percent of Lysis			
		MelTR	MelDo	Mel5 (ST)[a]	Mel5 (LT)
TR (14 d)	40:1	54	57	44	40
	4:1	34	30	11	19
TR (60 d)	40:1	27	22	20	18
	4:1	16	11	17	6
NK cell line	40:1	80	82	85	73
	4:1	83	82	82	55

[a] The sensitivity of Mel5 was assayed after 2 months (ST) and 6 months (LT) in culture.

may reflect subpopulation selection of either TIL (less cytotoxic) or melanoma cells (less sensitive). We therefore studied short (14-d) and long-term (60-d) cultures of TIL TR on the same established melanoma cell-lines MelTR (autologous), MelDo and Mel5 (allogeneic). In this assay, the sensitivity of Mel5 to TIL TR and the NK cell line was compared after short (ST)- and long-term (LT) cultures. The results, expressed in Table V, confirm the decrease with time of cytotoxicity of TIL TR effector cells on all melanoma cells used as targets. Moreover, the same pattern of lytic activity was observed on the two different cultures of Mel5. These results suggest that the loss of cytotoxic activity is due to selection of a subpopulation that is less cytotoxic on all melanoma targets.

Blockage of TIL Lytic Activity by Anti-CD3 MoAb The effect of anti-CD3 on cytolysis was investigated in order to determine if the lytic activity of TIL is mediated by the CD3 molecules. The effector cells were incubated with either anti-CD3, or anti-NKH1 (3B8) MoAb used as antibody control. These cells were tested against the autologous and allogeneic melanoma ^{51}Cr-labeled cell lines and K562 targets. Table VI summarizes the results of these experiments. In all TIL cultures, cytotoxicity was negative on K562 targets. In four cases, the anti-melanoma lysis was effectively inhibited by antibodies directed against CD3, whereas anti-NKH1 (3B8) had no inhibitory effect in the same experiments using TIL and $CD3^-$NK cell line. These data show that the cytolytic activity of long-term cultured TIL was mediated by $CD3^+$ T cells, in contrast with short-term TIL culture TR in which the cytolytic activity was of NK type, as it was not inhibited by the presence of anti-CD3 antibodies (data not shown). The broad spectrum of the inhibition reflects the non-MHC-restricted nature of the cytotoxic TIL activity. The fact that K562 can be killed in the presence of anti-CD3 antibodies is explained by the Fc receptors on the K562 and confirms the presence of $CD3^+$ cytotoxic effectors in long-term TIL cultures.

DISCUSSION

TIL are currently under intensive investigation, due to their presence within tumor tissue and presumed homing to that tissue. They have been identified as activated T cells with either autologous tumor-specific cytotoxicity [16,17] or MHC-unrestricted cytolytic activity [12,32,33]. Given their antitumor reactivity and their capacity to grow in the presence of rIL-2, TIL have been used in recent cancer treatment protocols that incorporate adoptive immunotherapy [20-22].

The purpose of this investigation was to describe the evolution with time of immunophenotype and cytotoxic activity of TIL derived from melanoma-bearing patients. In the early culture period (14 d), we found that four of five rIL-2-expanded TIL cultures were highly cytotoxic on melanoma cells and the NK-sensitive K562 target. At that stage, phenotypic analysis showed the presence of 10-30% CD3− NK cells that have been shown to mediate MHC-unrestricted cytotoxicity [27,28]. In agreement with previous studies [15,16], $CD8^+$ cells were found to be preferentially expanded in rIL-2 alone despite the presence of $CD4^+$ cells in the initial suspensions (see Tables I and II). With longer periods of time, all TIL cultures showed a decline in lytic activity on all melanoma and K562 targets, which was associated with the loss of $CD3^-$ cells. Thus, most of the killing in short-term cultures appeared to be mediated by TIL-NK cells.

The association between the highest anti-melanoma lytic activity and the presence of the $CD3^-$ NK populations in melanoma-derived TIL cultures is in agreement with previous studies that reported that IL-2 enhances the lytic activity of local NK cells [29,30] and TIL [13]. In addition, the comparison of $CD3^+$ TIL cultures and a $CD3^-NKH1^+$ cell line purified from peripheral blood shows that activated NK cells are significantly more effective killers than 60-d cultured $CD3^+$ TIL on melanoma targets.

The $CD3^+$ T cells that overgrow the long-term culture appear to be much weaker anti-tumor effectors than NK cells. This time-dependent change in activity was not related to variations of the tumor targets, which remained equally sensitive to lysis (Table V). It seems to be due to selection of a $CD3^+$ population that can no longer "see" the melanoma targets, but retains the capacity to lyse in the presence of lectin (Table IV). Several reports have shown that melanoma-derived TIL can yield specific MHC-restricted CTL [16,17]. However, the specific activity observed by the authors was obtained in only one of four cases of melanoma-TIL, probably because of the low frequency of specific CTL in the initial infiltrates or a profile varying for individual tumors. In our experiments, the broad spectrum of cytotoxicity observed on melanoma targets demonstrates the MHC-unrestricted lytic activity of the $CD3^+$ TIL we have cultured (Table IV). However, there is some evidence for the presence among TIL cultures of CTL capable of lysing melanoma targets in a different manner than NK effectors. K562 targets were not

Table VI. Effect of Anti-CD3 Blocking on Melanoma Lysis by Long-Term Culture of rIL2-Activated TIL

TIL from Patient	Antibody Added	Percent of Lysis (E/T = 30/1) on Melanoma Cells Used as Targets				
		MEL5	MELTR	MELBR	MELDO	K562
T3	None	22	28	31	36	15
	OKT3	4	5	2	4	45
	3B8	21	32	30	34	11
T5	None	0	0	3	3	3
	OKT3	4	5	3	4	57
	3B8	0	1	2	4	3
T6	None	8	19	15	18	4
	OKT3	0	0	0	5	66
	3B8	6	21	14	19	6
TR	None	16	14	17	20	10
	OKT3	3	7	2	3	56
	3B8	16	12	18	19	10
Activated NK cells	None	73	84	33	85	74
	OKT3	78	73	35	83	74
	3B8	62	81	33	82	72

killed and the cytotoxicity, albeit weak, was blocked by anti-CD3 antibodies on all the melanoma cell lines tested (Table VI). Whether this corresponds to a melanoma-specific reactivity of an immunodominant antigen could not be formally established from these studies. However, the inhibition of cytotoxicity and the absence of lysis on the K562 targets suggest that TIL cultures may include low levels of MHC-unrestricted specific CTL, as has recently been reported in the pancreatic adenocarcinoma [31]. In that model, it is shown that tumor-reactive CTL with apparent tumor specificity can kill without MHC restriction. Further investigation of the mechanisms of cellular anti-tumor effectors will lead to a better understanding of the role of the TIL in tumor recognition, rejection, and possibly nonspecific recruitment of other effector cells into the tumor site.

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