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Defective lymphokine production by most CD8+ and CD4+ tumor-specific T cell clones derived from human melanoma-infiltrating lymphocytes in response to autologous tumor cells *in vitro**

Human melanomas are infiltrated by tumor-reactive T lymphocytes. However, the ability of these cells to elicit a specific anti-tumor response *in vivo* remains to be established. Because lymphokine production is critical for T cell functions, we have analyzed the capacity of melanoma-specific tumor-infiltrating lymphocyte (TIL) clones to produce major lymphokines: interleukin-2 (IL-2), interferon-γ (IFN-γ) and interleukin-4 (IL-4), as well as tumor necrosis factor (TNF), in response to direct antigen presentation by autologous and allogeneic tumor cells. We report here that, upon stimulation by autologous melanoma cells, all TIL clones secreted TNF but only a few of them produced significant amounts of IL-2, IL-4 or IFN-γ. Nonetheless, all these clones consistently produced two or three of these last lymphokines upon stimulation with phorbol myristate acetate and calcium ionophore, as well as IL-2 upon CD3 stimulation, showing the existence of three lymphokine profiles among them: Th1, Th0 and a profile characterized by IL-2 and IL-4, but not IFN-γ secretion. Stimulation of TIL clones by allogeneic melanoma lines sharing the appropriate HLA-peptide complexes revealed that defective IL-2 production seemed to be a constant feature for some clones, while it was, for other clones, dependent on the antigen-presenting tumor cells. For this last type of clone, we further showed that defective IL-2 induction resulted from an LFA-3 defect of some melanoma cells or from distinct yet undefined defects of other melanoma lines. Our data suggest that defective lymphokine secretion may be an essential component of the *in vivo* failure of melanoma-reactive TIL to control tumor development. Interestingly both CD4+ and CD8+ TIL clones from one patient were fully activated by the autologous melanoma cells *in vitro*, supporting a potential role of such TIL in spontaneous or induced tumor rejection.

1 Introduction

Human tumor-infiltrating lymphocytes (TIL) specifically reacting with tumor-associated antigens have been observed in a number of human tumor types, most frequently in melanomas [1–5]. Most studies have in fact described CD8+ tumor-reactive TIL, the tumor specificity of which was established by showing their restricted lytic activity. These TIL, as well as PBL-derived CTL clones, provided unique tools through which the existence of common melanoma epitopes presented on HLA class I molecule could be established [6–8]. Interestingly, one such epitope was recently shown to be restrictedly expressed by tumor cells from adult melanoma patients [9, 10], providing the first rational basis for therapeutic interventions based on stimulating CTL responses against tumor antigens. On the basis of HLA class II-restricted proliferation and TNF production to tumor cells, we recently demonstrated the existence of CD4+ melanoma TIL, specific for shared melanoma epitopes (Gervois et al., in preparation). Provided these tumor peptides are not expressed by normal cells, which we have established so far for lymphoblasts, this will dramatically widen the field of immunotherapeutic intervention against melanomas by allowing manipulation of the helper T cell response, as well as the CTL response, against these tumors. Helper function and more specifically Th1-restricted lymphokine production were shown to play a critical role in the regression of some animal tumors [11, 12]. Nonetheless, the ability of human tumor-reactive lymphocytes to produce lymphokines in response to autologous tumor cells remains poorly documented. While polyclonal melanoma-specific TIL were shown to secrete granulocyte-macrophage (GM)-CSF, IFN-γ and TNF-α in culture in response to autologous tumor cells [13], little is known about the lymphokine patterns produced at the clonal level by CD8+ and CD4+ TIL [14–16]. To try to appreciate the potential role of human tumor-specific T lymphocytes in tumor rejection we have isolated tumor-reactive TIL clones from several human melanomas and

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Abbreviations: **TIL:** Tumor-infiltrating lymphocytes **Cal:** 4-Bromo-calcium ionophore

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analyzed their function. We have previously shown that most of these clones could not proliferate in response to autologous melanoma cells alone. *In vitro* proliferation required the addition of exogenous IL-2 and for most clones of EBV-B feeder cells as well. These data lead us to propose that tumor-reactive $CD4^+$ and $CD8^+$ T lymphocytes are recruited inside human melanomas, but that antigen-MHC complex presentation by tumor cells is not effective in inducing a significant expansion and, therefore, an optimal activation of these cells [3]. In the present study we have analyzed the ability of tumor-reactive $CD8^+$ and $CD4^+$ TIL clones to produce lymphokines *in vitro* upon stimulation by autologous and allogeneic melanoma cells or by polyclonal nonspecific stimuli.

2 Materials and methods

2.1 Cell lines and TIL-derived clones

M6, M17, M18, M44, M72, M74 and M77 human melanoma cell lines were-established from metastatic tumor fragments as previously described [2]. IGR 1/54 and IPC 277/5 melanomas were a gift of C. Aubert (INSERM U119, Marseille, France). LB 24 MEL B melanoma and mouse fibrosarcoma WEHI 164 clone 13 were obtained from T. Boon (Ludwig Institute for Cancer Research, Brussel, Belgium). Cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 1% L-glutamine and 1% penicillin-streptomycin (Gibco BRL, Cergy pontoise, France). All cell lines were free of mycoplasma contamination. Production, characterization and culture of human $CD4^+$ and $CD8^+$ TIL-derived clones have been described in detail previously [2, 3], TCR diversity was estimated on the basis of β and γ TCR gene rearrangement and specificity was investigated using various functional assays including proliferation, cytolytic assay and TNF production.

2.2 Monoclonal antibodies (mAb) and cell staining

Mouse anti-human CD3 ε: OKT3 (IgG2a) (ATCC) and rat anti-human CD25: 33B3.1 (IgG2a), gift of Y. Jacques (INSERM U211, Nantes, France) were used as purified antibodies. Mouse anti-HLA-A2 (BB7.2, IgG2b) was obtained from ATCC, anti-CD59 (P282, IgG2a) was a gift of A. Bernard (INSERM U343, Nice, France), both were used as diluted and filtered ascites fluids. mAb against ICAM-1 and LFA-3 were purchased from Immunotech SA (Marseille, France). Cells were labeled by indirect immunofluorescence. Briefly, cells were treated at 4 °C for 30 min with mouse or rat mAb, washed in PBS and incubated with $F(ab')^2$ fragments of goat anti-mouse or -rat Ig for an additional 30 min. Cells were analyzed on a FACScan (Becton Dickinson, Grenoble, France). Background fluorescence was estimated using isotype-matched irrelevant antibodies, 3000 cells were analyzed per sample.

2.3 Stimulation for lymphokine production

At least 10 days after the last stimulation T cell clones were washed three times, to remove IL-2 and conditioned medium, and then were stimulated in triplicate cultures, either by melanoma cells or by a combination of phorbol 12-myristate 13-acetate (PMA) and 4-bromo-calcium ionophore (CaI) (Sigma, L'Isle d'Abeau Chesnes, France), 10 ng/ml and 4 μM, respectively. For IL-2, IL-4 and IFN-γ production, 1×10^4 T cells were incubated in the presence of 3×10^4 melanoma cells/well in 150 μl complemented medium without IL-2. For IL-2 production cells were cultured in the presence of human CD25-blocking rat mAb: 33B3.1 (30 μg/ml), to prevent possible utilization of secreted IL-2. For anti-CD3-driven stimulation, 10^4 T cells were incubated in triplicate for 24 h in round-bottom 96-well plates coated with OKT3 mAb (coating solution: 25 μg/ml). For TNF production T cell/stimulator cell ratio was $2.5 \times 10^3/2 \times 10^4$ in 100 μl. Plates were centrifuged after 24 h of stimulation. Supernatants were harvested and stored at − 20 °C. Controls were supernatants of T cells and of stimulating melanoma cells, cultured with medium alone. The PMA-CaI preparation incubated alone for the same period as the T cell clone stimulation was used as control for IL-2 response by clones to PMA-CaI.

2.4 Cytokine detection assays

IL-2 dosage was done using the CTLL-2 cell line proliferation assay, compared to a standard curve with rIL-2 (Roussel Uclaf, Romainville, France) according to standard procedure. IL-4 and IFN-γ concentrations were assessed by immunoenzymatic assays (R&D System and Medgenix Diagnostics, respectively); limit of detection: 4 pg/ml and 0.03 IU/ml, respectively. TNF determinations were done by a biological assay using cytotoxicity on the highly sensitive WEHI 164 clone 13 cells, compared to a standard curve with rTNF-β (Genzyme) as described elsewhere [17].

2.5 Transfection of human LFA-3 cDNA

For the generation of stable M74 melanoma cells expressing LFA-3, the expression vector pRc glo (R. Breathnach, INSERM U211, Nantes, France) containing the human LFA-3 cDNA (a gift of P. Coulie, Ludwig Institute for Cancer research, Brussels, Belgium) under the control of the CMV promoter, was used. M74 cells were transfected with 10 μg of plasmid DNA employing the calcium phosphate method according to standard procedure. G418 sulfate (Gibco) was used for selection (600 μg/ml). After 2 weeks, viable colonies were tested for LFA-3 expression.

3 Results

3.1 Specificity of melanoma recognition by TIL clones

HLA class I restriction and tumor specificity of $CD8^+$ TIL clones M6 and M17 has been described previously [3]. M77 $CD8^+$ clones 80 and 84 were shown to be restricted by HLA-A2.1, on the basis of their pattern of cross-reactive lysis with allogeneic melanomas and lysis inhibition by anti-HLA-A2 mAb (data not shown), as previously established for M17.1 and M17.2 clones [8]. M18.1 and M18.2, M74.2 and M77.136 $CD8^+$ clones were restricted by undefined HLA-B or C alleles. Cross-reactive TNF productions by these clones correlated strictly with their lytic activity (data not shown). Specificity of $CD4^+$ TIL clones

was established recently (Gervois et al., in preparation) by showing restricted proliferation and/or TNF production against the autologous melanoma cells (and for some clones against a few allogeneic melanoma lines sharing a HLA class II element), but not against autologous lymphoblasts expressing high levels of HLA class II molecules.

3.2 Lymphokine secretion profiles of melanoma-specific TIL clones upon activation by PMA and CaI

The lymphokine secretion potential of melanoma-specific TIL clones (8–10 different $CD8^+$ clones and 7–11 $CD4^+$ clones obtained from 5 distinct melanoma tumors) was determined following stimulation with PMA-CaI. All these melanoma-specific clones produced IL-2 and TNF under these conditions (Tables 1 and 2). As shown in Tables 3 and 4, the $CD8^+$ clones, with one exception, also produced IFN-γ, but produced no or only barely detectable levels of IL-4. The $CD4^+$ clones also produced IFN-γ, with the exception of the two clones derived from M74 melanoma, which exhibited in contrast good IL-4 production (as did two $CD4^+$ clones obtained from M77 tumor).

3.3 Lymphokine production by TIL clones in response to autologous melanoma cells

As shown in Table 1, all TIL clones produced TNF upon stimulation by autologous melanoma cells, but never in response to allogeneic melanoma cells not sharing an HLA molecule. TNF production was, therefore, a very reliable indication that melanoma antigens were effectively recognized by TIL clones. Tables 2–4 indicate that autologous tumor cells, however, did not induce the production of detectable levels of either IL-2, IL-4 or IFN-γ by most $CD4^+$ clones analyzed, or by five different $CD8^+$ clones derived from melanoma tumors M17 and M18. In contrast, all the $CD8^+$ clones derived from the two other tumors: M6 and M77 (only three of which are shown here) consistently produced measurable IL-2 and IFN-γ after stimulation by the autologous melanoma cell line. To avoid IL-2 fixation by either TIL or tumor cells, a blocking concentration of anti-IL-2R mAb was systematically added to this test. To examine whether IFN-γ and IL-4 secreted by the clones could have been used by the melanoma cells, clone supernatants known to contain these cytokines were incubated for 20 h alone or with the appropriate number of cells from each melanoma line used in this study, and then measured for IL-4 and IFN-γ concentrations. Variable decrease of IFN-γ concentration, ranging from 0 to 30 units/ml, was found in clone supernatants incubated with M74 and M77 tumor cells, suggesting that these cells could consume at most 30 IFN-γ units/ml. Incubation with the other melanoma lines in contrast did not alter the concentration of IFN-γ (data not shown). Differences between the IL-4 content of the supernatants incubated or not with the different melanoma lines tested ranged between −70 to +70 pg/ml (data not shown) suggesting the limited accuracy of the consumption assay.

Table 1. TNF production (pg/ml) by melanoma-specific TIL-derived clones[a)]

Clones	Stimulation			
	Medium	PMA/CaI	Autologous melanoma cells	Allogeneic melanoma cells[b)]
$CD4^+$				
M6.T	0	356	33	0
M18.86	34	98	90[c)]	41
M74.6	6	783	51	7
M74.33	6	182	27	6
M74.34	3	260	36.5	2
M77.59	5	374	44	6
M77.152.1	3	77	108	6
M77.152.2	2	283	347	2
M77.152.10	30	142	204	24
M77.180	0	185	182	0
M77.184	0	176	71	0
$CD8^+$				
M6.P	12	69	320	16
M17.1	6	246	194	8
M17.2	2	216	124	0
M17.3	3	120	140	ND
M18.1	6	520	345	27
M18.2	22	800	170	30
M74.2	2	260	56	4
M77.80	4	132	166	2
M77.84	4	184	162	2
M77.136	41	294	169	16

a) TNF was determined in the supernatant of the clones (1×10^4/well) stimulated by melanoma cells (3×10^4/ml) or by a polyclonal stimulator, using the biological assay on the TNF-sensitive Wehi cell line. Polyclonal activator was a combination of PMA (10 ng/ml) and CaI (4 μM).

b) For $CD8^+$ clones: melanoma cells not sharing a HLA class I molecule with the clone.

c) The M18 tumor cells were stimulated before the test by rIFN-γ (200 U/ml, 48 h) to induce or increase HLA class II molecule expression.

Table 2. Production of IL-2 (IU/ml) by melanoma-specific TIL-derived clones[a)]

Clones	Stimulation			
	Medium	PMA/CaI[b)]	Autologous melanoma cells[c)]	Allogeneic melanoma cells[c)]
CD4+				
M6.A	0	14.2	0	0
M6.T	0	12	0	0
M18.86	0	63	0.4[d)]	0
M74.6	0.5	129	0.3	0.4
M74.34	0.6	121	1.5	1.1
M77.59	0.8	181	1	0.9
M77.152.2	0	100	0	0
M77.180	0	425	190	0
M77.184	0	435	0	0
CD8+				
M17.1	0.1	40	0.1	0.1
M17.2	0.4	20.4	0.5	0.4
M17.3	0.1	22	0	0
M18.1	0.2	52	0.5	0
M18.2	0	48	0.2	0
M6.P	0.4	14.6	6.4	0
M77.80	0	46	43	0
M77.84	0	27.4	35	0

a) Cloned TIL were seeded at 1×10^4/well. IL-2 production was determined by a biological test using the IL-2-sensitive CTLL-2 cell line according to standard conditions after 24 h of stimulation in the presence of anti-IL-2 mAb 33B3.1 (30 μg/ml) to prevent possible IL-2 consumption by T lymphocytes or tumor cells during the stimulation step. The values are from one experiment which is representative of two or three different experiments.
b) Concentrations used were 10 ng/ml and 4 μM, respectively.
c) Cells suspended at 3×10^4/well.
d) M18 tumor cells were treated by IFN-γ (200 U/ml, 48 h) before use to induce or increase expression of HLA class II molecules.

Table 3. IFN-γ production (IU/ml) by melanoma-specific TIL-derived clones[a)]

Stimulation	CD8+ clones								CD4+ clones						
	M17.1	M17.2	M17.3	M18.1	M18.2	M6.P	M77.80	M77.84	M6.A	M6.T	M18.86	M74.6	M74.34	M77.59	M77.152.2
Medium	0	0.8	0	0	0	0	0	0	0	0	0	0	0	0	0
PMA/CaI[b)]	34	44	1	20	10	35	59	41	120	68	7	1	0	24	11
Auto[c)]	1	0	0	0	0	6	62	63	0	0	0[e)]	0	0	0	0
Allo[d)]	0	0	0	0	0	0	ND	ND	0	0	ND	0	0	0	0

a) Cloned TIL were seeded at 1×10^4/well. The values are from one single stimulation experiment which is representative of two different experiments. IFN-γ production was determined by ELISA after 24 h of stimulation.
b) Concentrations used were 10 ng/ml and 4 μM, respectively.
c) Autologous melanoma cells: 3×10^4/well.
d) Allogeneic melanoma cells: 3×10^4/well.
e) M18 tumor cells were treated by IFN-γ (200 U/ml, 48 h) before use to induce or increase the expression of HLA-class II molecules.

Table 4. IL-4 production (pg/ml) by melanoma-specific TIL-derived clones[a)]

Stimulation	CD8+ clones								CD4+ clones						
	M17.1	M17.2	M17.3	M18.1	M18.2	M6.P	M77.80	M77.84	M6.A	M6.T	M18.86	M74.6	M74.34	M77.59	M77.152.2
Medium	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5
PMA/CaI[b)]	4	0	0	0	5	84	22	5	29	9	6	1,341	183	1,732	281
Auto[c)]	0	0	0	0	0	54	127	27	0	0	0[c)]	9	12	3	5
Allo[d)]	ND	0	ND	ND	0	0	ND	ND	0	0	ND	9	0	0	11

a) Cloned TIL were seeded at 1×10^4/well. IL-4 production was determined by ELISA after 24 h of stimulation. Values below the minimum detectable concentration are reported as zero. The values are from one experiment which is representative of two different experiments.
b) Concentrations used were 10 ng/ml and 4 μM, respectively.
c) Autologous melanoma cells: 3×10^4/well.
d) Allogeneic melanoma cells: 3×10^4/well.
e) M18 tumor cells were treated by IFN-γ (200 U/ml, 48 h) before use to induce or increase the expression of HLA-class II molecules.

3.4 IL-2 production by TIL clones in response to anti-CD3 stimulation or to allogeneic melanoma cells, sharing the specific MHC-peptide complex

To establish whether defective lymphokine production by TIL clones in response to autologous tumor cells could be due to a functional defect of the CD3 transduction complex, we tried to stimulate IL-2 production by these clones using anti-CD3 mAb. All TIL clones produced significant amounts of IL-2 under these conditions (Fig. 1). Since different $CD8^+$ clones from one tumor frequently had the same response pattern after stimulation by autologous melanoma cells, it seemed possible that these responses were due to the capacity of these tumor cells to efficiently present tumor antigens. To test this hypothesis we have compared the ability of a number of allogeneic melanoma lines, sharing the relevant HLA-A2-peptide complexes, to induce IL-2 secretion by two $CD8^+$ HLA-A2-restricted clones: M17.2 and M77.80, specific for two different antigens (data not shown). Clone M17.2 which did not produce IL-2, IFN-γ or IL-4 in response to the autologous melanoma cells, has been shown to kill 12 out of 15 melanoma lines sharing the restricting HLA-A2.1 element [8], some of which were even more susceptible to lysis than the autologous melanoma cells. TNF production was consistently induced by these cell lines and the level of production induced by each of them usually correlated with the level of lysis previously observed (data not shown). None of these cell lines, however, induced any detectable IL-2 production by clone M17.2 (data not shown). As shown above, clone M77.80 reliably produced IL-2 upon stimulation by the autologous melanoma cells. Data in Fig. 2 A further show that this clone had a good TNF response to allogeneic melanomas which were lysed as well or better than the autologous melanoma cells. As shown in Fig. 2 B several of these cell lines (M74, IPC277/5, LB24 MEL B) were unable to induce significant IL-2 production, while others (M44, M72, IGR1/54) stimulated the production of significant amounts of this lymphokine by clone M77.80. Previous treatment of tumor cells with IFN-γ resulted in increased stimulation of IL-2 production by M44 tumor cells, but did not change the ability to induce IL-2 or the of amount IL-2 induced by other melanoma cell lines (data not shown).

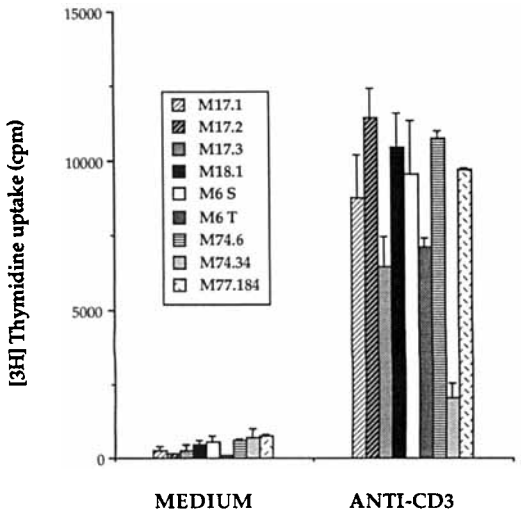

Figure 1. Anti-CD3 immobilized mAb induction of IL-2 secretion by five $CD4^+$ and four $CD8^+$ TIL-derived clones in which IL-2 production is defective in response to the autologous melanoma cells. Results are from a triplicate culture proliferative assay using the IL-2-dependent CTLL-2 cell line (cpm ± SD). Data are from one experiment, which was representative of two or three independent experiments.

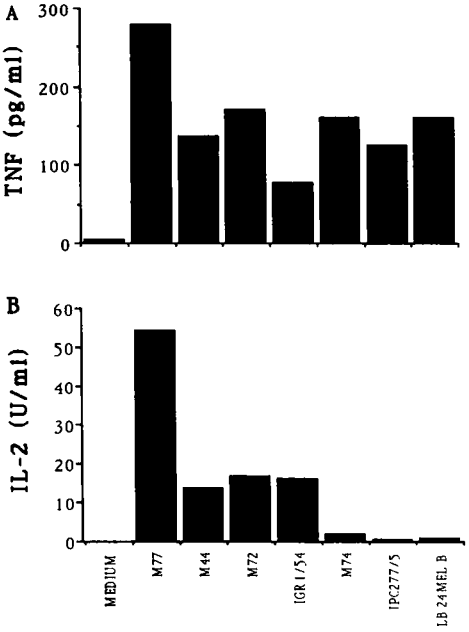

Figure 2. TNF (A) and IL-2 (B) production by clone M77.80 in response to autologous and allogeneic melanoma cell lines sharing the HLA/peptide complex recognized by this clone. Values are representative of three independent experiments.

3.5 Role of LFA-3 and other surface molecules in IL-2 induction by melanoma cells

We attempted to relate IL-2 induction by some melanoma cells to the expression by these cells of defined surface molecules known to play a role in activation or co-activation: HLA-A2.1, which was the element restricting antigen presentation to M77.80 clone and various adhesion molecules; LFA-3, CD59 [18], and ICAM-1. No significant differences in the level of HLA-A2.1, ICAM-1 and CD59 were observed (data not shown). In support of a role of LFA-3 in co-stimulation of TIL clones, high expression of this molecule was found on three out of the four cell lines inducing IL-2 production (M77, M44 and IGR 1/54), and intermediate expression on the other one, while low expression was observed with the M74 cell line, which did not stimulate IL-2 synthesis. We asked, therefore, whether the defect of IL-2 induction by M74 melanoma cells could be corrected by transfecting them with the human LFA-3 cDNA. As shown in Fig. 3 the transfectant LFA-3-M74 expresses high levels of LFA-3, comparable to that detected on M77 melanoma cells (Fig. 3 A) and induced IL-2 production by the clone M77.80 (Fig. 3 B). The role of CD2/LFA-3 interaction in IL-2 production by this clone was further supported by the inhibitory activity of an anti-LFA-3 mAb on this production in response to autologous tumor cells and to the transfectant. Despite the need for LFA-3 co-stimulation in TIL activation, shown by these

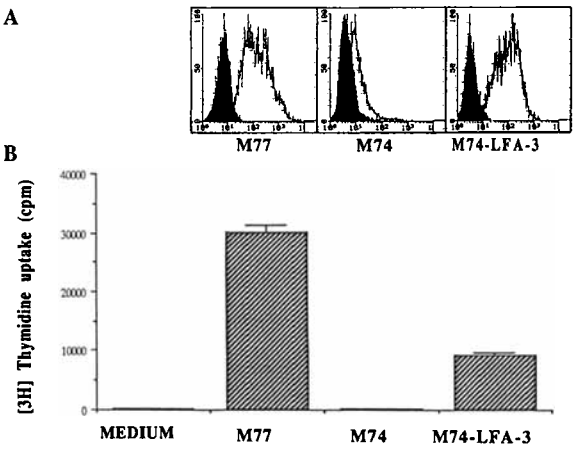

Figure 3. (A) Cell surface expression of LFA-3 molecules by M77, M74 and LFA-3-transfected M74 melanoma cell lines evaluated by indirect immunofluorescence and cytofluorimetric analysis. (B) IL-2 production by M77.80 clone following stimulation by autologous and allogeneic parental or LFA-3-transfected melanoma cells.

data, expression of LFA-3 and of ICAM-1 was clearly not sufficient for an effective T cell co-stimulation since LB 24 MEL B and IPC 277/5 melanoma cells (expressing good levels of these molecules and the HLA-A2 restricting element) did not induce IL-2 secretion.

4 Discussion

As originally described in the mouse, different subsets of human T lymphocytes can be distinguished on the basis of the pattern of lymphokines they produce [19, 20]. Although Th1 activity seems to be crucial for T cell-mediated rejection of experimental tumors [12], there have been few reports so far on the lymphokine secretion profile of tumor-specific TIL clones in humans. Since it was shown that the pattern of lymphokine secretion by T cell clones is usually the same, irrespective of the mode of stimulation [21, 22], we have deduced the lymphokine secretion profile of melanoma-reactive TIL clones studied here from the lymphokine pattern they exhibited in response to PMA+CaI. This profile was confirmed in response to antigen stimulation for the few clones that also responded to autologous melanoma cells, and in response to cross-linked anti-CD3 antibody for IL-2 synthesis. Three different secretion phenotypes were found: the Th1 profile, characterized by IL-2 and IFN-γ but not IL-4 production, which was observed for all the $CD8^+$ clones as well as for the $CD4^+$ clones from two melanomas; the Th0 profile, described for mouse and human T cell clones [22, 23], and characterized by the synthesis of IL-2, IL-4 and IFN-γ; and a third phenotype, common among short-term stimulated mouse clones [24], characterized by IL-2 and IL-4 but not IFN-γ synthesis. It remains possible that the Th2 profile was not detected because of the limited number of clones analyzed. We could not define the nature of the TNF produced by TIL since the biological test used did not differentiate between TNF-α and lymphotoxin (LT). We have shown previously that TNF-α is produced by M6 $CD4^+$ clones in response to the autologous tumor cells (unpublished data). In addition it is well known from the literature that Th1 clones produce LT [25].

The *in vitro* response of TIL clones to autologous tumor cells was also analyzed. Data shown here indicate that a fraction of both $CD8^+$ and $CD4^+$ clones from one patient and $CD8^+$ clones but not $CD4^+$ clones from another patient produced significant amounts of IL-2 and other lymphokines corresponding to their lymphokine secretion profile, while other TIL clones derived from these two patients and all TIL clones from three additional patients did not produce detectable amount of these lymphokines under these conditions. The complete absence of IL-2 in the supernatants of some TIL clones stimulated by autologous tumor cells was not due to the consumption of this lymphokine by melanoma cells or by the lymphocytes, as IL-2 fixation was systematically prevented by an anti-IL-2R mAb. Data on IFN-γ and IL-4 consumption by tumor cells suggested that only limited amounts of these lymphokines (< 30 UI/ml and < 70 pg/ml, respectively), could have been fixed, specifically or nonspecifically, by stimulating tumor cells and, therefore, did not interfere significantly with the results presented here. Therefore, all TIL clones were capable of producing IL-2 and either IL-4 or IFN-γ, or both, upon simultaneous activation of PKC by a phorbol ester and CaI, but a majority of them were not activated to produce significant amounts of these lymphokines upon interaction with the specific peptide presented on melanoma cells. This was clearly not due to a complete defect of TCR/antigen interactions, since all the TIL clones were efficiently induced in these assays to produce or increase their production of TNF each time they were stimulated by autologous melanoma cells. TNF production was shown to depend on TCR-MHC/peptide complex interaction, as it was inhibited by anti-HLA or anti-TCR mAb (data not shown). TNF secretion provided, therefore, a control for an effective antigen recognition by TIL clones in our assays.

Thus, we can reliably conclude that for many TIL clones, antigen recognition on tumor cells lead to cytolysis and/or TNF release without detectable production of other lymphokines that these TIL were able to produce in response to another type of stimulation. The use of melanoma lines sharing the appropriate HLA-peptide complex to activate $CD8^+$ clones revealed that, for some clones, defective IL-2 production was apparently a stable characteristic, whatever the antigen-expressing melanoma line used as a stimulus, while for other clones this defect was restricted to stimulation by only a fraction of the melanoma lines sharing the antigen.

Although many possibilities may explain these complete or partial defects of lymphokine production at least two of them can be discarded considering our present data. (1) Functional alteration of TCR/CD3 signaling pathway is unlikely for TIL clones analyzed here since they were effectively induced to secrete IL-2 upon stimulation of the CD3 complex. (2) The production by tumor cell of inhibitory factors, like TGF-β, which are frequently invoked as a mechanism potentially involved in the suppression of tumor-specific T cell responses, is also unlikely here, at least for M6, M74 and M77 melanoma cells which consistently stimulated IL-2 production by other autologous or allogeneic TIL clones. Furthermore, mixing of tumor cells M74 that do not induce IL-2 with melanoma cells that do induce IL-2 did not prevent IL-2 production by producer TIL clones (data not shown).

Recently it has become clear that T cell activation is a complex phenomenon resulting from a cascade of adhesion and signaling events initiated by the TCR and then involving interactions between T cell surface molecules and many adhesion and/or co-recognition molecules (like the restricting HLA molecule, ICAM-1, -2, -3, LFA-1 and LFA-3) and with co-signaling molecules (like B7) on the target cell. Absence or defect of any of these T cell/target interactions can induce defective T cell responses, resulting from either partial activation [26] or from the induction of a state of refractoriness called anergy [27].

We have not yet been able to establish whether the stable defect in some TIL clones leading to secretion of the major lymphokines in response to a stimulation by the specific tumor antigen is due to partial activation or to anergy [28]. An interesting hypothesis concerning these clones is that the tumor peptides they recognize are in fact partial agonists, as shown recently for experimentally altered peptides [29], inducing lysis and TNF release but no other effector function of TIL. The recent demonstration that some melanoma-specific PBL clones recognize normal differentiation antigens [30], fits this hypothesis: escape of these T lymphocytes from negative selection being the result of suboptimal interactions of their TCR with the selecting peptides.

Recognition of inadequate tumor peptides is, in contrast, not likely to be responsible for the defective IL-2 production by clones that produced this lymphokine in response to at least some melanoma lines. For this type of clone we have shown here that a high expression of LFA-3 induced by transfection on one melanoma line, spontaneously expressing this molecule at a low level, was sufficient to permit antigen-dependent IL-2 induction by this cell line. Defective activation, therefore, results for some TIL clones in defective co-signaling being provided by the antigen-presenting tumor cell. The level of ICAM-1 and of LFA-3 molecules inside tumors may, therefore, be critical for T cell responses. For this type of clone, the absence of IL-2 induction by melanoma lines expressing high levels of HLA-A2, ICAM-1 and LFA-3 may be due to either the defective expression of another unidentified molecule involved in T cell co-stimulation, or to the expression of the appropriate HLA-peptide complex under the threshold level necessary for complete activation.

Together our data suggest that a great proportion of melanoma-specific TIL that interact *in vivo* with tumor-associated antigens, presented directly by autologous tumor cells in the absence of APC, are unable to produce major lymphokines and, therefore, unable to proliferate and to develop a cytotoxic activity. Most of these TIL would, therefore, remain harmless. The mechanism(s) responsible for this tolerance to autologous tumor antigens are likely diverse. These mechanisms need to be defined to develop effective therapies based on the induction of specific anti-tumor T cell-mediated responses. Interestingly, we demonstrate here that a fraction of TIL are completely activated *in vitro* to lysis, cytokine production and (data not shown) to proliferation, by their autologous tumor cells. Although nothing is known so far of the nature and the distribution of the melanoma antigens recognized by $CD4^+$ clones in the HLA class II context, the existence in some patients of both $CD8^+$ and $CD4^+$ tumor-specific clones responding to tumor cells by both lymphokine production and proliferation (Gervois et al., *in preparation*) could be the cellular basis of the spontaneous melanoma regressions described in some patients [31]. Identification of the antigens recognized by these TIL clones appears of upmost importance.

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