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T cell activation by antigens on human melanoma cells—co-stimulation by B7-1 is neither sufficient nor necessary to stimulate IL-2 secretion by melanoma-specific T cell clones *in vitro*

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Keywords: B7-1, melanoma, T cell activation, T cell clones, TIL

Abstract

B7-1 expression, induced by transfection in poorly immunogenic murine tumours, was shown to elicit a T cell-mediated rejection of these tumours and further active immunity against the non-transfected tumour. We therefore asked to what level similarly induced expression of B7 on human melanoma cells would affect the antigen-dependent responses of tumour-specific T cell clones *in vitro*. Data presented show that B7-1 expression by melanoma lines: (i) significantly induced, or improved, an IL-2-dependent proliferative response of such clones to the antigen; (ii) increased the amount of IL-2 produced by two clones in response to the parental non-transfected tumour cells; and (iii) increased the TNF responses of all the CD4$^+$ clones. However, despite these clear co-stimulatory effects on antigen-induced responses of all T cell clones, which demonstrated an effective interaction of the B7-1 transfected molecule with one or the other of its counter-receptors expressed on T cell clones, B7 co-stimulation did not correct the defect of IL-2 secretion exhibited by many of these clones in response to *in vitro* antigen presentation by melanoma cells. We further show that defective IL-2 secretion in response to melanoma antigens was not due to a T cell clone refractoriness induced by the culture, since one of these clones could be induced to secrete IL-2 by an antigen-expressing melanoma line, upon increased lymphocyte function associated antigen-3 expression induced by gene transfection. Together these data suggest that defective IL-2 secretion by many tumour-infiltrating lymphocytes clones in response to antigen presentation by melanoma cells *in vitro* is not exclusively due to the inability of these cells to provide an appropriate co-stimulation through the B7-1 molecule.

Introduction

We have isolated CD4$^+$ and CD8$^+$ T cell clones from human melanomas. A high proportion of these clones was shown to be specific for autologous tumour antigens as they responded, in a HLA-restricted manner, by cytolysis and/or TNF secretion, to autologous tumour cells, but not to autologous lymphoblasts (1,2). A few of these clones also responded to tumour cells by IL-2 and IFN-γ and/or IL-4 secretion, and by a helper-independent proliferation. Most of them, however, while capable of secreting IL-2 upon stimulation by anti-CD3 or by PMA-4-bromo-calcium ionophore (Cal), did not secrete detectable amounts of this lymphokine in response to an *in vitro* stimulation by antigen expressing autologous or allogeneic tumour cells (1). Those clones did not exhibit a helper-dependent proliferative response (in the presence of added IL-2) to tumour cells alone and therefore required the simultaneous use of tumour cells [Epstein–Barr virus (EBV)-B feeder cells] to be expanded in culture (2).

In the context of recent perspectives to settle tumour therapies mediated by tumour-specific T cell effectors, it appears crucial to understand the basis of activation require-

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ments of melanoma-specific T cell clones by melanoma antigens. It has been suggested recently that T cell activation requires two signals (3,4), the first resulting from an interaction between the TCR and a MHC–peptide complex, probably reinforced by interactions between an array of adhesion molecules expressed by T cells and by antigen-presenting cells, and a second signal dependent upon interactions of co-stimulatory molecules present on antigen-presenting cells, with specific receptors for this co-stimulator on T lymphocytes. In the absence of a co-stimulation, the antigenic signal may induce either partial activation, anergy or apoptotic death of the T lymphocytes (4–6). Since most melanoma lines express significant levels of the adhesion molecules intercellular adhesion molecule (ICAM)-1 and lymphocyte function associated antigen (LFA)-3 (7), which are important for the induction of the antigenic signal, but likely lack B7 co-stimulatory molecules, whose expression appears restricted to hemopoietic cells (8), we assumed, as others (9), that defective T cell responses to antigens presented by melanoma cells could be due to the inability of these cells to provide a co-stimulatory signal. The best characterized co-stimulatory molecules are two members of the B7 family: B7-1 or CD80 (10) and B7-2 or CD86 (11,12). Interaction of B7 molecules with their receptors (CD28 and/or CTLA-4) (13,14) expressed on T cells was shown to enhance T cell activation and to prevent anergy (15,16). Interestingly B7-1, transduced into some mouse tumourigenic cell lines, was shown to co-stimulate an efficient T cell response against these tumours, leading to rejection of both B7 transduced and parental tumour cells (17–19). To assess the contribution of defective co-stimulation provided by melanoma cells to the limited *in vitro* response of T cell clones to melanoma antigens (1), we first controlled that B7-1, B7-2 and heat stable antigen (HSA) were not expressed on human melanoma lines. We then generated melanoma clones that expressed a high level of B7-1 by transfection and analyzed the capacity of these transfectants to activate tumour-restricted T cell clones having undetectable IL-2 secretion and proliferative response to parental tumour cells. We showed that, following expression of B7-1, melanoma cells remained unable to induce a detectable IL-2 response, but stimulated a significant helper-dependent proliferation of these clones.

Methods

Cell lines and tumour-infiltrating lymphocyte (TIL)-derived clones

M6, M17, M18 and M74 human melanoma cell lines were established from metastatic tumour fragments as previously described (20). Mouse fibrosarcoma WEHI 164 clone 13 was obtained from T. Boon (Ludwig Institute for Cancer Research, Brussels, Belgium). Cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 1% L-glutamine and 1% penicillin–streptomycin (Gibco BRL, Eragny, France). All cell lines were free of mycoplasma contamination. Production, characterization and culture of human TIL-derived clones have all been described in detail previously (2,20). Briefly, T cell clones were obtained by limiting dilution culture in the presence of irradiated autologous tumour cells, EBV-B cells, phytohaemagglutinin and recombinant IL-2 Most clones were derived from wells having, according to single hit Poisson law, a probability of clonality >90%. They were expanded by transferring them, every 3–4 weeks, into microplates on irradiated feeder and stimulator cells (5×10^3 lymphocytes on 2×10^4 EBV-transformed-B-LAZ cells and $1\ 5\times10^3$ autologous melanoma cells). TCR diversity and clonality was, for some of them, estimated on the basis of β and γ gene rearrangements, and specificity was investigated using various functional assays including proliferation and cytolytic assays as well as TNF and IL-2 production determination (1,2).

mAb

Anti-human B7 mAb B7.24 [IgG2a, a gift from M. de Boer, Innogenetics, Ghent, Belgium (21); anti-CD25. 33B3 1 (IgG2a), a gift from Y. Jacques, INSERM U211, Nantes, France], and anti-human CD2.1 (T11. 2, IgG1), CD2.9 (T11.1, IgG2a) and CD28 (CD28.1, IgG1) (a gift from D Olive, INSERM U119, Marseille, France) were used as purified antibodies The anti-CD59 mAb P282 (IgG2a, a gift from A. Bernard, INSERM U343, Nice, France) was used as diluted and filtered ascite fluid mAb against human ICAM-I (CD54), LFA-3 (CD58) and HSA (CD24) were purchased from Immunotech (Marseille, France).

Cell staining and flow cytometry

Cells were stained with primary antibody and counter-stained with FITC-conjugated goat anti-mouse or anti-rat antibodies as described previously (2). Cells were analysed on a FACScan equipped with a 15 mW argon ion laser (Becton Dickinson, Grenoble, France) using the Lysys software. Background fluorescence was estimated using isotype-matched irrelevant antibodies and 3000–5000 cells were analysed per sample.

Proliferation assays

Microcultures were set up in triplicate in 96-well culture plates. Briefly, 5×10^3 to 10^4 cloned T cells, taken at least 15 days after a previous stimulation by feeder cells and antigen-expressing melanoma cells, were cultured for 3–5 days with and without 5×10^3 to 3×10^4 mitomycin-C-treated melanoma cells in 0.2 ml of complete medium with or without rIL-2: 150 U/ml. Proliferation was determined by adding 1 μCi [^{3}H]thymidine to each well for the last 18 h of culture. [^{3}H]Thymidine uptake was measured in a liquid scintillation counter. Data are reported as stimulation index: SI = (mean c.p.m. stimulated culture – mean c.p.m. stimulus)/mean c p.m. unstimulated culture. [^{3}H]Thymidine uptake ranged between 80 and 500 c.p.m. for T cells alone and between 50 and 750 c.p.m. for stimulator cells alone. Functionality of the CD28 receptor of TIL clones was estimated by comparing clone proliferation to anti-CD2 antibodies and to a combination of these antibodies with an anti-CD28 antibody.

Assays for lymphokine production

T cell clones, taken at least 15 days after the last stimulation, were stimulated in triplicate cultures by stimulator cells in 150 μl of complete medium without IL-2 in 96-well culture plates. For TNF and IL-2 production assays the T cell/stimulator cell ratio was respectively $2.5\times10^3/2\times10^4$ and $10^4/3\times10^4$. Plates were centrifuged after 24 h of stimulation

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Supernatants were harvested and stored at −20°C. A combination of PMA and CaI (Sigma, L'Isle d'Abeau Chesnes, France), 10 ng/ml and 4 μM/ml respectively, was used as positive control for cytokine release. IL-2 dosage was done with a proliferation assay, using the IL-2-dependent murine cell line CTLL-2, compared with a standard curve with rIL-2 (Roussel Uclaf, Romainville, France). Cells were cultured in the presence of human CD25 blocking rat mAb 33B3.1 to prevent possible utilization of secreted IL-2. IL-4 and IFN-γ secretions were assessed by immunoenzymatic assays (R&D System, Minneapolis, MN and Medgenix Diagnostics, Fleurus, Belgium respectively), with a limit of detection of 4 pg/ml and 0.03 IU/ml respectively. TNF determinations were done by a biological assay measuring the cytotoxicity of stimulated clone supernatants on the highly sensitive WEHI 164 clone 13 cells compared with a standard curve with rTNF-β (Genzyme, Cambridge, UK) as described elsewhere (22).

Transfection of human B7 and LFA-3 cDNA

For the generation of melanoma cells stably expressing B7, the expression vector pcDNA1-NEO containing the human B7 cDNA under the control of the CMV promoter was used (a gift from M. De Boer). Plasmid DNA was prepared using the QIAGEN procedure. Melanoma cells (0.5×10^6) were transfected with 10 μg of plasmid DNA employing the calcium phosphate method in a 100 mm tissue culture disk for 16 h using complete DMEM medium. For selection, the medium was replaced with complete RPMI 1640 medium containing 800–900 μg/ml G418 sulfate (Gibco BRL, Eragny, France). After 2 weeks, viable cells were tested for B7 cell surface expression by flow cytometry and cloned by the limiting dilution procedure. Clones with the highest B7 expression were then selected.

For the generation of 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 from 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 procedures. G418 sulfate (Gibco) was used for selection (600 μg/ml). Viable colonies were tested for LFA-3 expression after 2 weeks.

Results

All TIL clones expressed a functional CD28 receptor on their cell surface

As shown in Fig. 1(A), $CD4^+$ and $CD8^+$ clones significantly expressed the CD28 molecule. Functionality of this receptor was established by comparing the proliferation of these clones in response to a pair of anti-CD2 and to a mixture of these antibodies with an anti-CD28 mAb. As shown in Fig. 1(B), the proliferative response of clones induced by anti-CD2 was significantly increased in the presence of anti-CD28 mAb.

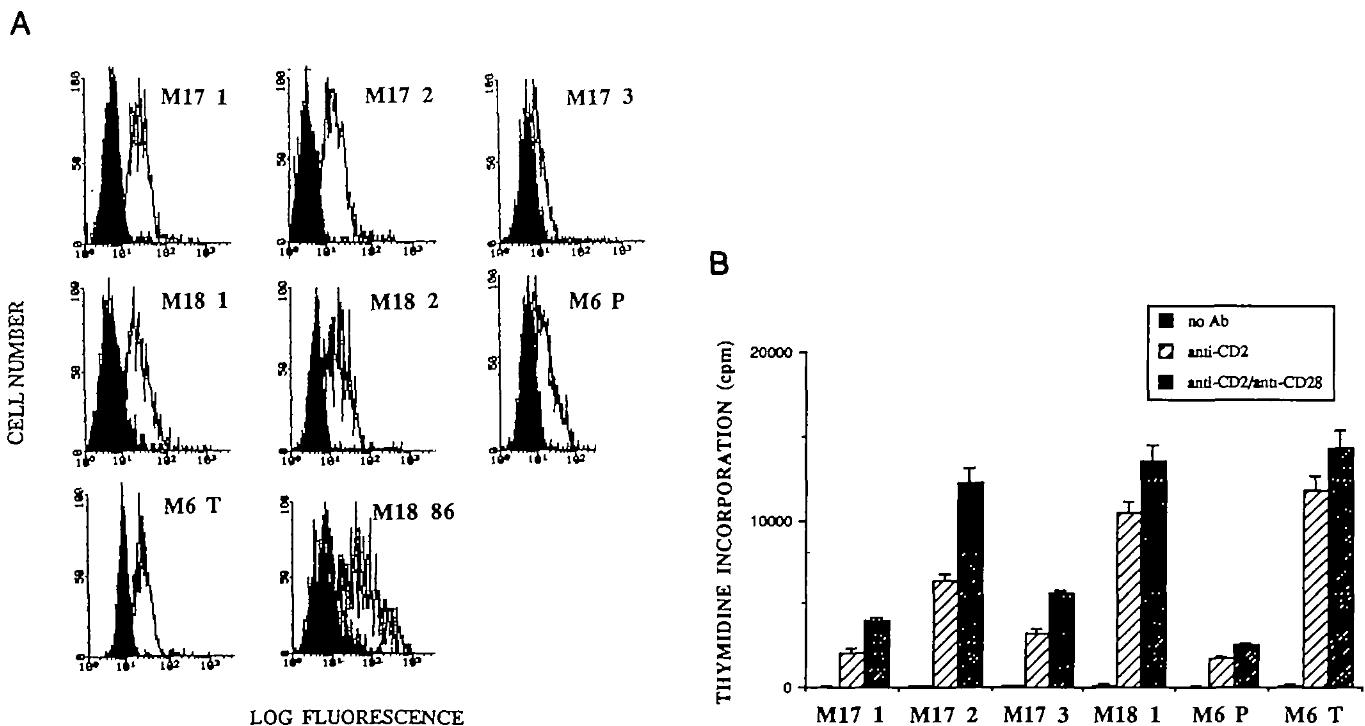

Fig. 1. (A) FACS analysis of CD28 expression by TIL clones Black histograms represent the negative controls corresponding to labelling by an isotype-matched irrelevant antibody Clear histograms show CD28 expression by various unstimulated clones (taken at ~2 weeks after the last stimulation). (B) Proliferative response of TIL clones to stimulation by a pair of anti-CD2 antibodies (10 μg/ml each) combined or not with an anti-CD28 antibody (1 μg/ml). Values are the mean ± SD of triplicate culture.

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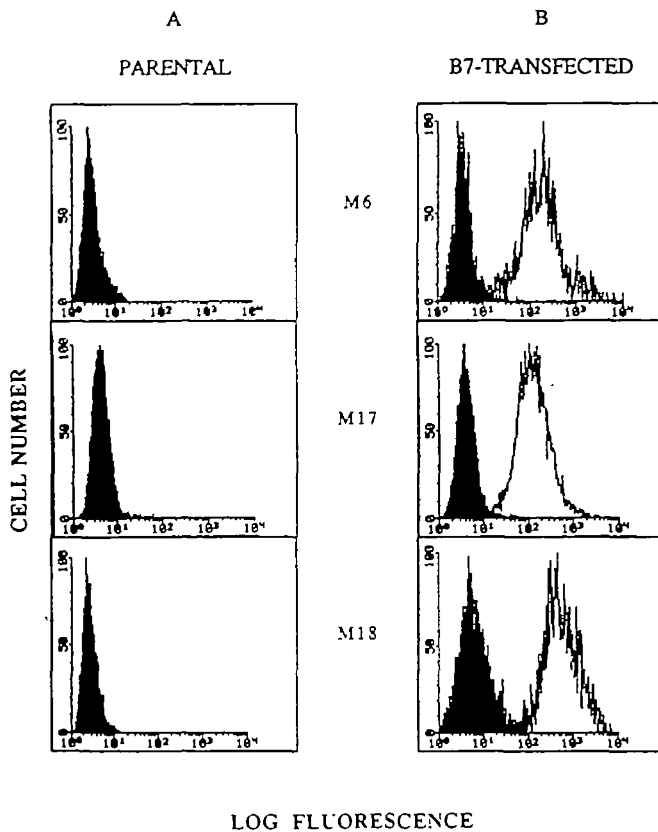

Fig. 2. B7-1 molecule expression on parental (A) and B7-1 transfected (B) melanoma cell lines. Black histograms are the negative controls and clear histograms correspond to B7 expression.

Expression of B7-1 by melanoma cell lines

M6, M17 and M18 melanomas do not express spontaneously the B7-1 or B7-2 molecule (Fig. 2A) and fail to express them after treatment with rIFN-γ (data not shown); neither do they express CD24, the human homologue of murine HSA (data not shown). B7 expression by melanoma clones M6, M17 and M18 transfected with human B7-1 cDNA is shown in Fig. 2(B). Expression of other molecules involved in T cell recognition was checked on these transfected clones and on the parental tumour cells: HLA-class I and II, accurately restricting HLA molecule when known, and the adhesion molecules ICAM-1 (ligand for LFA-1) and LFA-3 (ligand for CD2). All these molecules were expressed significantly and at similar levels on parental and B7-expressing tumour cells, with the exception of a low to null expression of HLA class II by M18 melanoma line and by the homologous B7 transfectant (data not shown) .

Defective IL-2 production by TIL clones in response to autologous melanoma cells is not restored by B7 co-stimulation

As reported previously, and shown here in Table 1, most $CD4^+$ and $CD8^+$ TIL clones systematically failed to secrete detectable amounts of IL-2 in response to stimulation by autologous melanoma cells. For none of these clones was this defect overcome in response to the homologous B7-transfected cells.

Nonetheless, two of the clones used in this study (M6P and M17-311) did produce IL-2 (Table 1) as well as IFN-γ (data not shown) in response to untransfected autologous melanoma cells. Data from one representative experiment shown in Table 1 suggest that IL-2 secretion by these clones was slightly increased in response to the B7 transfectant. In support of this, proliferation of these clones in the absence of added IL-2 was higher in response to B7 transfected melanoma cells than to the homologous parental cells

Table 1. Production of IL-2 (U/ml) by melanoma-specific TIL-derived clones[a]

Clones	Stimulation					
	Medium	PMA–Cal[b]	Auto[c]	B7 auto	Allo[d]	B7 allo
$CD8^+$						
M17 1	0 1	40	0	0.1	0	0
M17 2	0	20.5	0 1	0	0	0
M17 3	0	22	0	0	0	0
M18 1	0	53.6	0.3	0.2	0	0
M18 2	0	53	0.3	0	0 1	0.1
M6 P	0 3	42	24	28.5	0	0 6
$CD4^+$						
M17 311	0	20	1 5	4	0	0
M6 T	0	16	0	0	0	0
M6 A	0	19	0	0	0	0
M6 I	0	16	0	0	0	0
M18 86[e]	0 6	5	0 6	1 3	ND	ND

[a]IL-2 production was determined by a biological assay using the IL-2-dependent CTLL2 mouse cell line according to standard procedure after 24 h of stimulation at a $10^4/3 \times 10^4$ responder/stimulator ratio. The anti-RIL-2 mAb 33B3 1 was used during stimulation step to prevent possible consumption by T cells
[b]A combination of PMA and Cal at 10 ng/ml and 4 μM respectively represented the polyclonal activator.
[c]Autologous melanoma cells.
[d]Allogeneic melanoma cells.
[e]M18 and M18-B7 tumor cells were treated by IFN-γ (200 U/ml, 48 h) to induce HLA class II molecule expression.

Table 2. B7 co-stimulates the proliferation of the IL-2-producing melanoma-specific $CD8^+$ clones M6 P and $CD4^+$ clone M17 311 in the absence of added IL-2

Clones	Stimulator melanoma cells		
	Autologous	B7 autologous	B7 allogeneic
M6 P ($CD8^+$)			
exp. 1	68	91	1.5
exp. 2	11	34.2	ND
exp. 3	10	31.5	ND
M17 311 ($CD4^+$)			
exp. 1	37.5	352	ND
exp. 2	53.8	147	0.6

Results are expressed as stimulation index. ND, not determined

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Table 3. B7 co-stimulates the proliferation of TIL-derived melanoma-specific CD8+ clones in the presence of exogenous IL-2

Clones	Stimulator melanoma cells		
	Autologous	B7 autologous	B7 allogeneic
M17 1			
exp. 1	1.4	13	0 6
exp 2	0.5	38	ND
exp. 3	2	11 3	0.7
exp 4	3 9	39	1.8
M17 2			
exp 1	0.2	12.7	ND
exp. 2	1 6	15.7	0.8
exp 3	2	20.9	0 2
exp. 4	0 2	43	0.5
exp. 5	2	12.3	1 3
exp 6	1.4	23	0.2
M17 3			
exp. 1	0 5	10	0 07
exp. 2	1.6	12.9	0.8
exp. 3	1 8	11	0 2
exp. 4	2.9	60	ND
exp 5	0 9	22	ND
M18 1			
exp. 1	2 3	39	ND
exp 2	0.3	9	ND
exp. 3	0 3	41	0.16
exp 4	1.2	5.5	0 9
M18 2			
exp 1	1.3	13	0 8
exp. 2	1 3	11 9	ND
exp 3	1.8	9	1.1
exp. 4	0 5	7 5	1
M6 P			
exp. 1	7 6	52 2	0.6
exp 2	20	149	ND
exp. 3	21.5	49 9	0.3

Results are expressed as stimulation index ND, not determined.

Table 4. B7 co-stimulates the proliferation of the majority of TIL-derived melanoma-specific CD4+ clones in the presence of exogenous IL-2

Clones	Stimulator melanoma cells		
	Autologous	B7 autologous	B7 allogeneic
M17 311			
exp. 1	6.8	15 8	ND
exp. 2	67.7	80.7	0.5
M6 T			
exp 1	0 2	30.4	0.8
exp. 2	3.6	6.2	ND
exp 3	27	96	0
exp. 4	31	122	ND
M6 A			
exp. 1	2.3	4	ND
exp 2	0	2	0 3
M6 S			
exp. 1	3	6	0
exp. 2	1 2	8.6	ND
M6 I			
exp 1	2	4 2	ND
exp 2	0 9	6.7	ND
M18 86			
exp 1	1 3	1.9	ND
exp. 2	1	0.6	ND
exp 3	1 3	1 8	1 3

Results are expressed as stimulation index. In the case of M18 86 clone, M18 and M18 B7 cells were treated with rIFN-γ (200 U/ml, 48 h) to induce HLA class II expression before stimulation assay. ND, not determined

(Table 2) and both proliferative responses were completely abrogated by the use of the rIL-2α chain-specific mAb 33B3.1 (data not shown), showing that they were directly correlated in these assays with the amount of endogenously secreted IL-2. None of the remaining CD8+ or CD4+ clones proliferated in the absence of added IL-2 to either parental or B7 transfected autologous tumour cells, which is consistent with their incapability to secrete any detectable amount of IL-2. In these conditions most of them died after 3 days, as assessed by Trypan blue exclusion (data not shown).

B7 transfectants stimulate the proliferation of most CD4+ and CD8+ TIL-derived clones in the presence of exogenous IL-2

The proliferative response of TIL clones to autologous melanoma cell antigens was then measured in the presence of added rIL-2. In these conditions B7-transfected melanoma cells reproducibly triggered a good proliferation of melanoma-specific CD8+ and CD4+ TIL clones, with the exception of one CD4+ clone, M18 86, while proliferative responses to parental autologous tumour cells were weak or absent for most clones, with the exception of one CD8+ clone M6 P (Table 3) and of two CD4+ clones M6 T and M17 311 (Table 4) Two of these three clones, M6 P and M17 311, were those which exhibited an IL-2 secretion and a proliferative response to parental tumour cells in the absence of exogenous IL-2 (Tables 1 and 2). The stimulation index obtained for these two clones is sometimes lower in the presence of exogenous IL-2 (Table 4) than in its absence (Table 2). This is likely due to a retained expression of the IL-2R α chain by clones in some experiments, especially in experiment 1 for clone M17 311 (Table 4). Since in this case proliferation is induced by IL-2 alone, the stimulation index by B7 transfectants is underestimated. Showing the antigen dependency of the increased proliferation induced by B7-transfected melanoma lines, these transfectants had no effect on the proliferation of allogeneic TIL clones (Tables 3 and 4). Only the CD4+ clone (M18 86) was not stimulated to proliferate by B7-expressing autologous tumour cells, even after IFN-γ treatment of these cells which induced a significant increase of HLA class II expression (data not shown).

Involvement of B7 in the increased proliferation of TIL clones

To assess the role of B7 expressed by tumour transfectants in the increased stimulation of TIL clone proliferation, we used the anti-B7 mAb B7.24 or the fusion recombinant protein CTLA-4–Ig in inhibition experiments. A dose-dependent inhibition was found, either partial, for CD8+ clones M17 1 and

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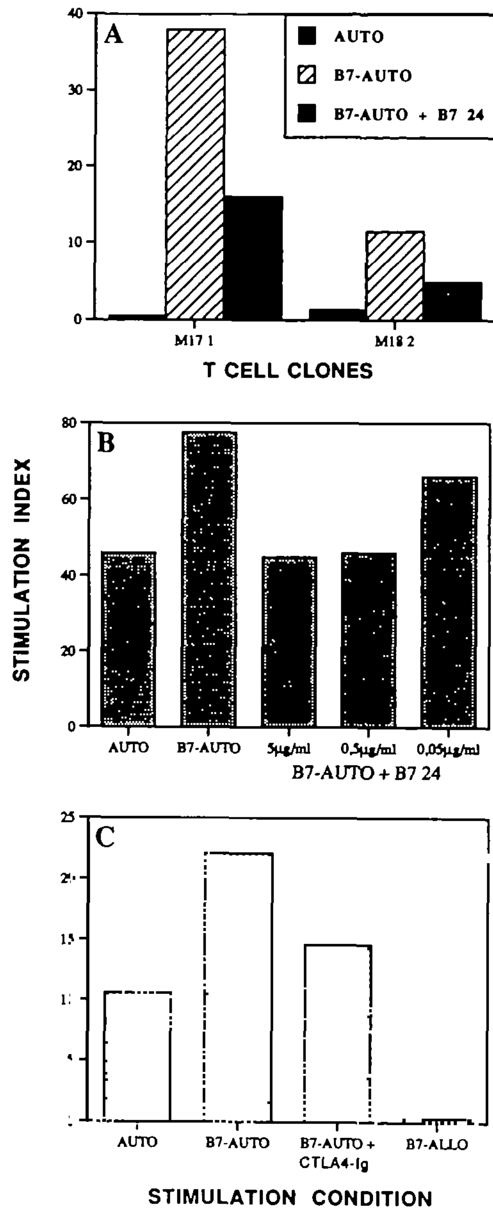

Fig. 3. Inhibition of B7 induced rIL-2-dependent proliferation of TIL (A) *Inhibition of the proliferative response of two CD8⁺ clones* M17 1 and M18 2 to B7-transfected melanoma cells, by the anti-B7 mAb B7.24 used at 5 μg/ml final concentration (B) Dose-dependent inhibition of the proliferative response of a CD4⁺ clone M6.T by the anti-B7 mAb. (C) CTLA-4–Ig induced inhibition of the proliferative response of the CD4⁺ clone M17 311.

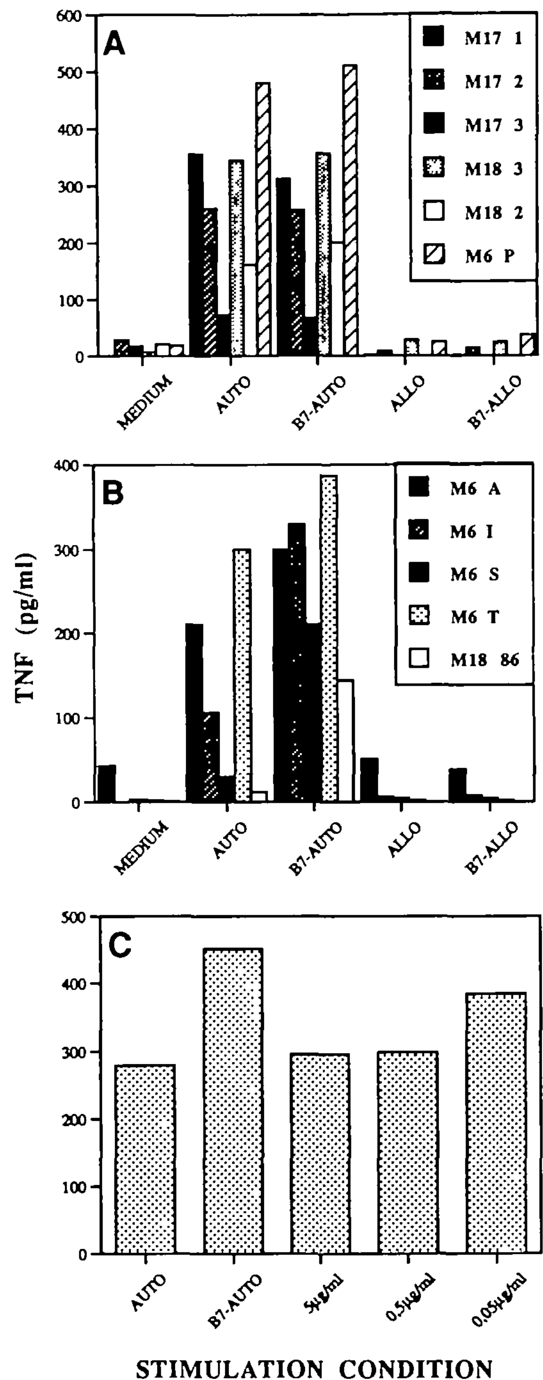

Fig. 4. Production of TNF by TIL derived clones (A, CD8⁺ clones; B, CD4⁺ clones) in response to parental and B7-transfected melanoma cells, either autologous with the clone (expressing the antigen) or allogeneic with the clone (HLA-restricting element not expressed). For each clone, one representative experiment out of two, three or five is shown. (C) B7-induced increase of TNF secretion by the CD4⁺ clone M6T is inhibited in a dose-dependent manner by the mAb B7 24

M18 2 and for a CD4⁺ clone M17 311, or complete, in the case of the CD4⁺ M6 T clone (Fig 3) Isotype-matched irrelevant mAb used as control was without effect on the B7-induced proliferation of CD8⁺ and CD4⁺ clones (data not shown). Furthermore, IL-2-dependent proliferation of one CD8⁺ clone M17 2 appeared strictly correlated with the number of B7-expressing tumour cells, used in mixtures of parental and transfected tumour cells at different ratios, to stimulate this clone. A similar result was obtained for the CD4⁺ M6T clone (data not shown).

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Table 5. Lysis and cytokine production by the $CD8^+$ clone M17 2 in response to autologous melanoma cells M17, allogeneic melanoma cells M74 and LFA-3-transfected M74 melanoma cells

	M17	M74	M74-LFA-3
Lysis (%)	78	64	76
TNF (pg/ml)	40	90	372
IL-2 (c.p m)	612	529	17,083

B7 co-stimulation increased TNF production by $CD4^+$ but not $CD8^+$ TIL clones

TNF production by TIL-derived clones was measured by a biological test TNF was produced by both $CD4^+$ and $CD8^+$ clones after autologous tumour stimulation (Fig. 4A and B). We observed that B7 co-stimulation significantly increased TNF production by all the $CD4^+$ clones (Fig. 4B). This increase was completely abrogated by B7 24 mAb (Fig. 4C) The amount of TNF released by $CD8^+$ clones upon stimulation by parental and B7-transfected autologous tumour cells was similar (Fig 4A)

B7 co-expression is without effect on the lysis of autologous melanoma cells by specific cytotoxic T cell clones

No significant difference was detected between the lysis of parental and B7-transfected M6, M17 and M18 melanomas by the respective lytic clones M6 P, M17 2 and M18 1 at any effector/target ratio tested (data not shown).

LFA3 transfection restored IL-2 secretion by one clone in response to the antigen presented by an allogeneic melanoma cell

We had shown previously that one of the clones used in this study (M17 2) was restricted by HLA.A2-1 and recognized a melanoma antigen shared by a high proportion of melanoma cell lines expressing this HLA allele (23). Among these lines, melanoma cells M74 were efficiently recognized by the M17 2 T cell clone, as shown by lytic and TNF responses (Table 5), but similar to the autologous tumour cells were unable to induce IL-2 production by this clone (Table 5). Since LFA-3 expression by M74 cells was low, we derived a LFA-3-transfected M74 melanoma clone highly expressing this molecule, as reported previously (1) As shown in Table 5, LFA-3-transfected M74 cells efficiently induced IL-2 secretion by the M17 2 clone, while parental M74 and M17 tumour cells did not.

Discussion

We showed here that melanoma-specific TIL clones express a functional CD28 receptor, but that ligation of this receptor during antigen recognition on tumour cells is unlikely, as none of the appropriate ligands described so far was found on melanoma cells. To evaluate the possible consequences of a lack of co-stimulation on melanoma-specific T cell activation, we compared the *in vitro* responses of TIL clones to parental and B7-1-transfected melanoma cells. Data presented show that B7 significantly co-stimulated an antigen-dependant proliferation of all the clones in the presence of IL-2 Establishing the direct involvement of B7-1 ligation in this co-stimulation, the induction and the increase of TIL proliferation induced by transfected cells were significantly diminished or abrogated, in a dose-dependent manner, by an anti-B7-1 mAb and by CTLA-4-Ig fusion protein.

We do not know so far which mechanism is responsible for B7-1-induced T cell proliferation. Since parental tumour cells induced no significant proliferation of most TIL clones in the presence of rIL-2, proliferation in response to B7-1 transfectants had to be induced by a mechanism other than IL-2 induction. It also seems unlikely to be due to IL-4 or IL-6 induction, since addition of these lymphokines had no effect on the proliferation of TIL clones to parental tumour cells, neither by themselves nor in combination with IL-2 (data not shown). Also against an IL-4-dependant mitosis of T cell clones, no significant amount of this lymphokine was detected in the supernatants of TIL clones exposed to B7-1 transfectants (data not shown). It is possible that B7-1-dependant mitosis was due to CD28-mediated induction of T cell clone receptivity to IL-2. It has been shown that CD28 triggering up-regulates the expression of IL-2R α (CD25) and β chains in response to anti-CD2 stimulation (24,25). In our model, however, CD25 induction did not seem to be sufficient for the induction of TIL proliferation by B7-1 transfectants, since parental tumour cells induced similar levels of this molecule to transfected cells, without inducing a significant proliferation of most clones. It remains possible that a threshold in the level of expression of the two other IL-2R chains was required to initiate detectable T cell proliferation.

Only one $CD4^+$ clone (M18 86) did not respond to the autologous B7 transfectant by proliferation This may be related to the fact that both the transfected and parental autologous tumour cells expressed very low levels of HLA class II molecules. Although HLA class II expression induced on these cells by IFN-γ treatment was sufficient to stimulate TNF production by this clone (1), it may be that the density of the HLA-peptide complex remained below the appropriate threshold to induce proliferation.

According to several studies, B7 co-stimulation up-regulated IL-2 responses of two clones, M6P and M17 311, to the parental melanoma cells. Results on these two clones show that when antigen-expressing melanoma cells provide a full activation signal to T cell clones, B7-1 co-stimulation increases, as in other models (26), IL-2 secretion induced by this antigenic signal. However, B7-1 co-stimulation, although inducing a mitogenic signal on all TIL clones, but one, was unable to restore IL-2 secretion by clones which did not produce detectable levels of this lymphokine in response to the parental tumour cells. Since these clones did produce IL-2 in response to coated anti-CD3 antibody (1), it is unlikely that they were intrinsically defective for this function. For clone M17 2, it was further shown that TCR-mediated activation of the IL-2 gene was possible, since this clone reproducibly secreted significant amounts of IL-2 when stimulated by an LFA3-transfected allogeneic melanoma line (sharing the accurate HLA-peptide complex). This IL-2 response was inhibited in a dose-dependent manner by an anti-LFA-3 mAb (data not shown). Furthermore, since the accurate peptide

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recognized by this clone was identified recently (Guilloux *et al.*, submitted), functionality of the IL-2 gene in this clone was confirmed by showing that significant IL-2 secretion was systematically triggered by HLA-A2-expressing autologous or allogeneic melanoma cells preincubated with the appropriate peptide (data not shown). Therefore, in the case of this clone, defective IL-2 secretion in response to antigen-expressing tumour cells likely results from a defective antigenic signal which may be corrected by increasing LFA-3 or antigen expression but not by inducing a B7-1-dependant co-stimulation. The precise reason for the inability of other TIL clones to secrete major lymphokines upon antigen recognition on some tumour cells remains to be established. For the clones which produced IL-2 in response to a fraction of melanoma cells, refractoriness to antigen activation is unlikely Since many melanoma lines expressing good levels of LFA-3 and ICAM-1, induced TNF but no IL-2, this defect more likely results from insufficient expression of the MHC–peptide complex. In support of this we observed recently that IL-2 secretion by clones of known specificity could be triggered by preincubating the stimulating melanoma cells with the appropriate peptide (in preparation). Therefore, the present data show that improper triggering of TIL clones by antigen-bearing melanoma cells does not result merely from the absence of a B7-1 co-stimulatory molecule on these tumour cells. On another hand, co-stimulation appeared unnecessary for IL-2 secretion by some TIL clones [one CD8$^+$ clone described here and several CD8$^+$ and CD4$^+$ clones described elsewhere (1) or undescribed], since they secreted significant IL-2 amounts in response to melanoma cells lacking B7 and CD24 expression. Therefore, either some melanoma cells express a co-stimulatory ligand distinct from these molecules or some memory clones may be induced to IL-2 secretion in the absence of any co-stimulatory signal.

B7 co-stimulation increased TNF production by CD4$^+$ TIL clones, which is consistent with previous studies (26). In contrast TNF secretion by CD8 clones was unaltered; this may be related to different TNF secretion profiles of both subsets as already reported for human alloreactive T cells (27). In our model, we ignore if this enhancement concerns one or both forms of TNF. Differential regulation of these two genes is possible, since they possess their own promoter (28).

Additional observation was that parental and B7-modified melanoma cells were equally lysed by autologous CD8$^+$ cytotoxic clones. This confirms in a human tumour model, previous data showing that B7 expression by a target cell is not essential for lysis by T cells (29,30).

In summary, our data suggest that B7-1 co-stimulation may generate an unknown signal resulting in IL-2-dependent mitogenesis. At variance, it was recently concluded that B7-1 co-stimulation required for CD4$^+$ T cell priming resulted exclusively from increased IL-2 secretion (31). Defective IL-2 gene expression by TIL clones, due to suboptimal activation by melanoma cells, was probably the condition which permitted the present detection of this consequence of CD28 or CTLA4 engagement by B7-1 on antigen-induced T cell mitosis. Such an effect likely exists also, but cannot be evidenced in the context of a full T cell activation.

The results further show that defective activation of TIL clones by melanoma antigens presented by melanoma cells *in vitro* does not result merely from a defective co-stimulation. If such a defect exists *in vivo* this conclusion has important implications for the development of appropriate tumour therapies.

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Abbreviations

Cal	4-bromo-calcium ionophore
EBV	Epstein–Barr virus
HSA	heat-stable antigen
ICAM-1	intercellular adhesion molecule-1
LFA-3	lymphocyte function associated antigen-3
TIL	tumour-infiltrating lymphocytes

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