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IL-12 stimulation but not B7 expression increases melanoma killing by patient cytotoxic T lymphocytes (CTL)

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SUMMARY

Recent studies have demonstrated that rodent tumour cells engineered to secrete a variety of cytokines, or to express foreign antigens, MHC molecules or co-stimulatory molecules, are rejected by syngeneic animals. These observations have led to the initiation of a number of clinical trials using genetically modified tumour cells, to attempt to stimulate a patient anti-tumour immune response. In this study, a protocol has been developed to test *in vitro* the specific cytotoxic anti-tumour response generated from melanoma patient lymphocytes. The results showed that IL-12 in combination with IL-2 enhanced the autologous anti-melanoma CTL response, whereas B7.1 antigen expression on tumour cells did not increase anti-melanoma CTL generation. This method could be used to design more appropriate genetically modified tumour vaccines.

Keywords IL-12 cytotoxic T cell melanoma

INTRODUCTION

There is a large body of evidence for the presence of a specific immune response against animal tumours [1,2]. Stimulation of CTL with restricted activity against tumour cells has been shown to be important for tumour regression or rejection in animals [3]. Amongst human tumours the best characterization of an anti-tumour immune response has been for melanoma. There is evidence for the presence, in a number of patients, of specific CTL that can lyse autologous tumour cells *in vitro* [4]. Both shared and unique determinants can be recognized by such CTL in an MHC-restricted fashion, either with HLA-A1 or with HLA-A2 [5]. The finding that specific CTL precursors could be detected in the peripheral blood of most melanoma patients suggested that vaccination to stimulate an immune response could be used to treat melanoma [6,7].

Expression of a variety of cytokine genes, including IL-2 [8–12], IL-4 [13,14], granulocyte-macrophage colony-stimulating factor (GM-CSF) [15], interferon-gamma (IFN-γ) [16–18] and IL-12 [19,20], or MHC molecules [21], or the lymphocyte co-stimulatory molecule B7 [22–25] by rodent tumour cells has been shown to cause their rejection by syngeneic animals. More importantly, injection of such modified tumour cells could stimulate a lasting CTL response against the parental tumour cells [10,15,16] and lead to the rejection of unmodified tumours [25]. In some animals, injection of modified tumour cells could also prevent the growth of established small tumour [26] or metastases [11]. One potential application of such modified cells to the treatment of human disease is the injection of patient tumour cells, genetically engineered following primary tumour excision, to stimulate the patient anti-tumour response and lead to the eradication of metastases. Indeed, a number of clinical trials have now been initiated in melanoma patients including either vaccination with irradiated autologous melanoma cells secreting IL-2 [27–29], with similar cells secreting GM-CSF [30], with allogeneic tumour cells producing IL-4 [31] and with an allogeneic melanoma cell line expressing specific melanoma antigens in association with HLA-A1 and A2 molecules [32], or intratumoural injection of the gene encoding HLA-B7 [33].

Although preliminary results of such trials are encouraging [32,33], they show the complexity of the interactions between the patient's immune system and the tumour cells. In this study, in order to examine directly the roles of cytokines and antigen-presenting molecules in the generation of human anti-melanoma CTL, we have developed a protocol to test *in vitro* the specific cytotoxic anti-tumour response from melanoma patients.

IL-12 is produced by macrophages and B lymphocytes and has been shown to have multiple effects on T cells and natural killer (NK) cells, including induction of the production of IFN-γ and tumour necrosis factor (TNF) by resting and activated T and NK cells [20,34] and enhancement of cytotoxic activity of T lymphocytes and NK cells [19,35–38]. IL-12 is also a central

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regulator of the cell-mediated immune response because of its actions on the development, proliferation, and activity of Th1 lymphocytes [20,39,40]. In this study we demonstrate that IL-12, in combination with IL-2, enhances autologous anti-melanoma CTL generation. However, B7 antigen, a co-stimulatory molecule which has been shown to cause an increased activation of T cells in response to antigen [41], does not enhance autologous anti-melanoma CTL generation when expressed by the tumour cells. The implications of such results for the design of future melanoma vaccine trials are discussed.

MATERIALS AND METHODS

Establishing cultures from melanoma biopsies

Fresh tissue from melanoma biopsies was obtained at surgery. The tissue (0·1–3 g) was washed twice with Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Paisley, UK), macerated with crossed scalpels in the presence of 2 mg collagenase (Sigma, Poole, UK), and incubated at 37°C in 20 ml DMEM containing glutamine (3 mM), Fungizone (2·5 μg/ml; GIBCO, Paisley, UK), penicillin (100 μg/ml), streptomycin (100 μg/ml), and 20 mM HEPES pH 7·4 for 2–6 h. Samples were washed, plated in tissue culture flasks, then grown in DMEM containing 10% fetal calf serum (FCS; GIBCO), glutamine, Fungizone, penicillin, streptomycin, and Ciprofloxacin (10 μg/ml; Bayer, Newbury, UK). After 24 h, adherent cells were retained and fresh medium was added. Adherent cells were passaged using trypsin. Cells were either frozen at 2×10^5–10^6 cells/ml in DMEM, 50% newborn calf serum, and 10% dimethylsulfoxide (DMSO) or maintained in culture until required for the CTL assays.

Peripheral blood lymphocytes

Peripheral blood lymphocytes (PBL) were isolated from heparinized venous blood from healthy donors (allogeneic cytotoxic responses) or from melanoma patients (autologous cytotoxic responses) by Ficoll–Hypaque (Lymphoprep; Nycomed Labs, Oslo, Norway) gradient centrifugation at 580 ***g*** for 20 min followed by extensive washing. To obtain a T cell-enriched population, PBL were incubated for 1 h with AET (Sigma)-treated sheep erythrocytes (Serotec, Kidlington, UK) followed by Ficoll–Hypaque gradient centrifugation [42].

Cell lines

The retroviral packaging cell line GP + envAM12 [43] was obtained from Professor A. Bank (Columbia University, New York, NY). The producer cell line GP + envAM12-mfgB7.1 was produced by co-transfecting the plasmid mfgB7.1 with the plasmid pSV2Neo and selecting G418-resistant clones. The Raji cell line is a Burkitt's lymphoma (EBV) expressing BB1 antigen (B7.1 costimulatory molecule) [44]. K562 is a human leukaemia cell line [45] which was used as a highly sensitive *in vitro* target for NK activity.

Transduction of the B7.1 gene

The plasmid pMFG-S$^-$ was provided by Somatix Therapy Corporation (Alameida, CA). The plasmid Bluescript B7 [46] encoding the cDNA for human B7.1 antigen was kindly provided by Dr F. Farzineh (King's College, London, UK). The plasmid mfgB7.1 was constructed by ligating the human B7.1 cDNA BamH1 fragment from pBluescript B7 into the BamH1 site of MFG-S$^-$. Supernatant (0·45 μm filtered) from GP + env AM12-mfgB7.1 diluted with an equal volume of medium was added to subconfluent cultures of melanoma cells in the presence of dextran (5 μg/ml) for 4 h. After 10–15 serial infections, B7.1 expression on the surface of melanoma cells was detected by staining with the specific anti-B7 antibody BB1 followed by FACScan analysis.

Flow cytometric analysis

The following MoAbs were utilized for flow cytometry: BB1 (aliquots courtesy of Dr Ed Clark, University of Washington, Seattle, WA), FITC-conjugated rabbit anti-mouse (RAM) immunoglobulins (RAM FITC; Dako, High Wycombe, UK), monoclonal anti-B7/BB1/FITC (Ancell, Nottingham, UK), mouse IgM/FITC antibody (Ancell). B7.1 surface expression was assayed by treating 1×10^6 cells with 2 μg MoAb BB1 for 30 min at 4°C. Cells were washed twice with PBS, 2% FCS, 1% sodium azide and incubated with RAM FITC (1 : 50). After a 30-min incubation and two washes, propidium iodide was added to allow elimination of dead cells during acquisition. The fluorescence of the stained cells was analysed for B7 expression using the software Lysis II on a FACScan. Melanoma cells infected with the retrovirus pmfg-B7.1 were compared with unmodified melanoma cells, and Raji cells were used as a positive control for B7.1 expression. Some melanoma cells (MEL 26, 40 and 41) were labelled using a one-step staining with BB1-FITC antibody and a mouse IgM-FITC as negative control.

Proliferation assay

T cell activation assays were performed in RPMI 1640 with 10% heat-inactivated human AB serum (Sera-Lab, Oxford, UK), streptomycin 100 μg/ml, penicillin 100 μg/ml, and glutamine 3 mM. T cell-enriched PBL (10^5/well) were cultured in U-bottomed 96-well microtitre plates in the presence or absence of mitogen concanavalin A (Con A) (Boehringer, Lewes, UK) 10 mg/ml and in the presence or absence of serial dilutions of x-irradiated antigen-presenting cells (APC; 100 Gy using a Pantac x-ray machine with an output of 240 keV at a dose rate of 4–5 Gy/min). The cells were cultured for 5 days before being pulsed for the last 18 h with 0·5 μCi/well ^{3}H-thymidine (Amersham, Aylesbury, UK)) and harvested on a cell harvester (Multimash 2000; Dynatech, Billingshurst, UK).

Cell-mediated cytotoxic assays

PBL stimulations were performed in RPMI 1640 with 10% heat-inactivated human AB serum, streptomycin 100 μg/ml, penicillin 100 μg/ml, and glutamine 3 mM. Allogeneic or autologous PBL (10^6/ml) were incubated for 10 days with irradiated patient melanoma cells (100 Gy) at a ratio of 40 : 1. IL-2 (10 U/ml) (Eurocetus, Harefield, UK) was added on days 0, 3, 6, and 9 with 300 pg/ml IL-12 or 1 ng/ml IL-7 (R&D Systems, Abingdon, UK) on the same days when indicated. IL-4 (5 U/ml; R&D Systems) and 10^{-5} M hydrocortisone (Sigma) were also added to the cultures on day 0 to reduce the generation of LAK activity and enhance the specific killing. On day 10, the killing of $Na(^{51}Cr)O_4$ (Amersham)-labelled target cells ($2{\cdot}5 \times 10^3$/well) was measured for each stimulation, in the presence of excess K562 cells (×50) to inhibit non-specific killing of melanoma targets using the method described [7]. The tumour cells used as targets in the killing assays were pre-incubated for 72 h in medium containing 50 U/ml IFN-γ, to enhance the expression of class I MHC and adhesion molecules.

Limiting dilution analysis of CTLp

Varying numbers of PBL were seeded in 96 V-bottomed microwells with 5×10^3 autologous x-irradiated melanoma cells (100 Gy) in RPMI culture medium supplemented with 10% human serum, 5 U/ml IL-4 and 300 pg/ml IL-12 when indicated. The microplates were incubated at 37°C in 5% CO_2. IL-2 and IL-12 (when indicated) at a final concentration of 30 U/ml and 300 pg/ml, respectively, were added on day 3. The microcultures were restimulated on day 7 by addition of 100 μl fresh medium containing 30 U/ml IL-2, 300 pg/ml IL-12 (when indicated) and 5×10^3 irradiated tumour cells. On day 14, 100 μl of medium were discarded. Cells were transferred into flat-bottomed microwells and restimulated by the addition of 100 μl fresh medium containing 30 U/ml IL-2, 300 pg/ml IL-12 (when indicated) and 5×10^3 irradiated melanoma cells. On day 21, 100 μl of medium were removed and 60 μl of responder cells were transferred into V-bottomed microwells to test their lytic activity [7]. Analysis of results was performed using the method described by Sharrock *et al.* [47] and Coulie *et al.* [7].

RESULTS

IL-12 stimulates the generation of patient anti-melanoma CTL

We previously demonstrated that primary cell cultures could be established from approximately 80% of resected patient melanomas [27]. This has allowed a study of the effect of immunomodulatory agents on the generation of autologous, anti-melanoma CTL. IL-12 has been shown to facilitate both non-specific LAK and specific CTL responses against human allogeneic tumour cells [20,38] and other targets [36]. We therefore optimized CTL generation by the addition of hydrocortisone [48], which decreased the generation of non-specific LAK and NK cells during *in vitro*, allogeneic PBL stimulation (data not shown). Furthermore, killing assays were performed in the presence of an excess of unlabelled K562 cells (a LAK-specific target) [7]. This inhibited the non-specific killing of melanoma targets by LAK cells generated by incubation of PBL in a high concentration of IL-2 (data not shown).

Initial experiments (Table 1) demonstrated that allogeneic anti-melanoma CTL responses could be augmented by repeated addition of small amounts of IL-12 (300 pg/ml) throughout the PBL stimulation. Using the same experimental conditions, we then tested the effect of IL-12 on autologous CTL generation and demonstrated that a higher cytotoxic response could be detected when IL-12 was added to the culture (Fig. 1a). The CTL stimulated by IL-12 were MHC-restricted (Fig. 1, legend) and tumour-specific, as they did not kill self phytohaemagglutinin (PHA)-blast targets (Fig. 1a). Their cytotoxicity could be inhibited by addition of anti-CD3 antibody to the killing assay (Table 1, legend). The ability of IL-12 to facilitate anti-melanoma CTL responses was dependent on the addition of small amounts of IL-2 (10 U/ml) (Fig. 1b); similar co-operation between IL-12 and IL-2 has previously been reported [49].

It is well documented that the amplitude of the specific immune response against melanoma cells differs for each patient, as it depends on many factors such as the presence of particular tumour antigens, the level of expression of MHC molecules on tumour cells and the secretion of factors displaying immunosuppressive activities by the melanomas [4,50–52]. We therefore tested the effect of IL-12 on the specific anti-melanoma cytotoxic responses of nine different patients. The results, summarized in Table 1, show that PBL from all patients with melanoma gave a measurable anti-tumouricidal CTL response *in vitro* with repeated addition of IL-12 throughout the PBL stimulation. Although IL-7 has also been reported to stimulate murine [53] and human [54] anti-tumour CTL, IL-7 was not as effective as IL-12 in autologous anti-melanoma CTL stimulation (Table 1).

Table 1. Anti-tumour effects of IL-12 on specific T cytotoxic responses against allogeneic and autologous melanoma cells

Lysis of primary melanoma culture by allogeneic PBL

	Percent maximal lysis effector : target 100 : 1		Percent maximal lysis effector : target 50 : 1	
Stimulation	IL-2, IL-12	IL-2	IL-2, IL-12	IL-2
MEL 9	20	3	13	1
MEL 16	36	3	27	1
MEL 24	97	39	83	23
MEL 26	86	52	86	35

Lysis of primary melanoma culture by autologous PBL

	Percent maximal lysis effector : target 100 : 1		Percent maximal lysis effector : target 50 : 1	
Stimulation	IL-2, IL-12	IL-2	IL-2, IL-12	IL-2
MEL 11	30	5	30	10
MEL 26	27	0	24	0
MEL 30	34	6	24	4
MEL 33	4	2	4	1
MEL 35	19	3	11	3
MEL 38	5	0	3	0
	IL-2, IL-12	IL-2, IL-7	IL-2, IL-12	IL-2, IL-7
MEL 11	12	0	7	0
MEL 38	8	1	0	0
MEL 33	48	36	48	32
MEL T	7·5	0	1	0

For the peripheral blood lymphocyte (PBL) stimulations, the protocol was as described in Materials and Methods, except that the allogeneic stimulations with MEL 9 and MEL 16 were performed without adding IL-4 and hydrocortisone on day 0 of the culture. The results represent the percent maximal lysis at two ratios of effector : target (100 : 1 and 50 : 1). Addition of anti-CD3 (UCHT1 murine monoclonal; Dako) for 30 min to the CTL prior to the killing assay blocked allogeneic killing by up to 75% and autologous killing by up to 56%.

IL-12 does not increase anti-melanoma CTL precursor frequency

The increase in cytotoxicity stimulated by IL-12 might be explained either by augmentation of the number of CTL precursor cells, or by an increase in the proliferation or killing capacity of each cytotoxic lymphocyte. To distinguish between these

Table 2. After 10–14 serial infections with viral supernatant from AM12mfgB7.1 producer cells, 30–90% of melanoma cells expressed B7.1 antigen

Allogeneic *in vitro* stimulation	Percent MEL $B7^+$	Percent maximal lysis of MEL effector : target 50 : 1	Percent maximal lysis of MEL $B7^+$ effector : target 50 : 1
MEL 24	0	48	32
MEL 24 $B7^+$	52	50	39
Autologous *in vitro* stimulation	**Percent MEL $B7^+$**	**Percent maximal lysis of MEL effector : target 50 : 1**	**Percent maximal lysis of MEL $B7^+$ effector : target 50 : 1**
MEL 36	0	8	19
MEL 36 $B7^+$	33	24	38
MEL 30	0	24	17
MEL 30 $B7^+$	87	16	40
MEL 26	0	24	2
MEL 26 $B7^+$	75	15	49
MEL 40	0	0	0
MEL 40 $B7^+$	65	1	4
MEL 41	0	0	0
MEL 41 $B7^+$	50	6	0

Uninfected melanoma cells did not show any B7 expression on their surface. Allogeneic and autologous B7-expressing melanoma cells were tested as stimulators and as targets in cell-mediated cytotoxic assays (see Materials and Methods). Results are presented for one effector : target ratio of 50 : 1.

possibilities, we used limiting dilution analysis [6,7] to estimate the frequency of patient CTL precursors directed against autologous melanoma cells. Results obtained from one patient are shown in Fig. 1c. One peripheral blood lymphocyte in 21 000 could respond to autologous Mel 30; this frequency estimation is within the range previously obtained for autologous melanoma cells (1/900 to 1/33 000) [6,7]. Addition of IL-12 did not increase the number of anti-melanoma CTL precursors of this patient (1/23 000 with

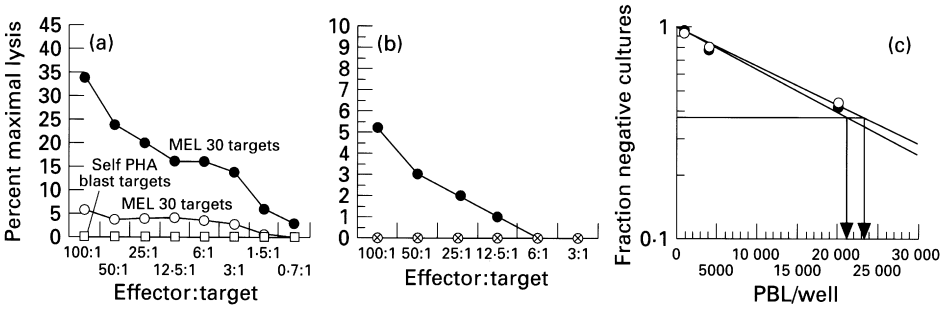

Fig. 1. (a) IL-12 enhances the killing of MEL 30 by autologous peripheral blood lymphocytes (PBL). Self targets were prepared as follows: PBL (10^6/ml) were incubated with 100 ng/ml phytohaemagglutinin (PHA; Sigma). On day 2, the cells were washed three times, then re-incubated with 20 U/ml IL-2. On days 5 and 8, IL-2 (20 U/ml) was added to the culture. On day 9, the blasts were washed and restimulated with PHA (100 ng/ml). On day 10, the PHA blasts were washed and labelled for the killing assay. To test if CTL stimulated with IL-12 were MHC-restricted, we compared the killing capacities of PBL stimulated with autologous MEL 26 on MEL 38 targets and MEL 26 target cells. At the effector : target ratio of 50 : 1, the percent maximal lysis obtained for MEL 26 targets was 50% compared with 9% lysis when MEL 38 was used (data not shown). ●, IL-2 + IL-12; ○, IL-2; □, IL-2 + IL-12. (b) Autologous PBL gave a measurable cytotoxic response against MEL 38 when they were stimulated with IL-2 and IL-12 together, but not if IL-2 or IL-12 was added alone. ●, IL-2 + IL-12; ○, IL-2; ×, IL-12. (c) IL-12 did not increase the number of autologous CTL precursors specific for MEL 30: 1/21 000 in the presence of IL-2 alone and 1/23 000 with IL-2 + IL-12. ○, IL-2; ●, IL-12 + IL-2.

IL-12). Two other patients with lower CTL precursor frequencies (1/75 000, 1/150 000) also did not show increased precursor frequencies when IL-12 was added (data not shown).

From the results obtained with patient 30 (Fig. 1c), we observed that the killing in terms of percentage of maximal lysis was higher in wells supplemented with IL-12 than in those without IL-12. At the dilution of 20 000 PBL/well, we measured an average of 40% maximal lysis in the presence of IL-12 compared with 29% maximal lysis in the absence of IL-12. We also observed that in 70% of our bulk assays (Table 1) the number of activated PBL was increased in the presence of IL-12 (average of 1·6-fold). Therefore, at least one of the effects of IL-12 is to increase the final number of activated cells without changing the number of melanoma-specific precursor CTL that are stimulated. Another effect might be to increase the killing capacity of activated CTL, as IL-12 has been shown to increase the expression of adhesion molecules on activated NK cells [55].

B7 expression does not enhance autologous anti-melanoma CTL generation

B7 is a surface molecule of APC which has been shown to give a co-stimulatory signal after interaction with the CD28 molecule on T cells, causing an increased activation by antigen [41]. We therefore examined whether B7-expressing melanoma cells would provide a more efficient stimulation for expansion and activation of tumour-specific autologous T lymphocytes. We constructed a recombinant retrovirus encoding human B7 antigen and infected patient melanoma cell cultures. After serial retroviral infections, FACScan analysis showed that B7 expression could be achieved in 30–90% of cells (Table 2). As a test for B7 function, we analysed the ability of these cells to act as APC for an activated T cell-enriched population. Figure 2 shows that B7-expressing melanoma cells co-stimulated T cell proliferation, in combination with the mitogenic lectin Con A, more potently than $B7^+$ Raji cells. Unmodified melanoma cells failed to co-stimulate T cell proliferation (Fig. 2).

We then examined whether melanoma cells from five different patients, engineered to express B7 antigen, were able to induce an improved anti-tumour CTL response. These results are summarized in Table 2. B7 expression on melanomas 36, and 41 to a small extent, enhanced *in vitro* activation of specific CTL capable of killing unmodified melanoma cells. However, B7 expression on melanoma 26, 30 and 40 did not increase the killing capacities of tumour-specific autologous PBL. Furthermore, in four out of five cases, B7-expressing melanoma cells were better targets than unmodified tumour cells for CTL stimulated by B7-expressing melanoma cells, but not for CTL activated by normal melanoma cells. This observation could be the result of a new immunogenicity of the modified tumour cells. Therefore there is no general beneficial effect of expression of B7 antigen on the generation of melanoma-specific CTL. It has been suggested that IL-12 might synergize with B7/CD28 to induce T cell activation [56]. However, in our system PBL stimulated by B7-expressing melanomas in the presence of IL-12 did not show any increased killing of unmodified targets compared with PBL activated by unmodified melanoma cells and IL-12 (data not shown).

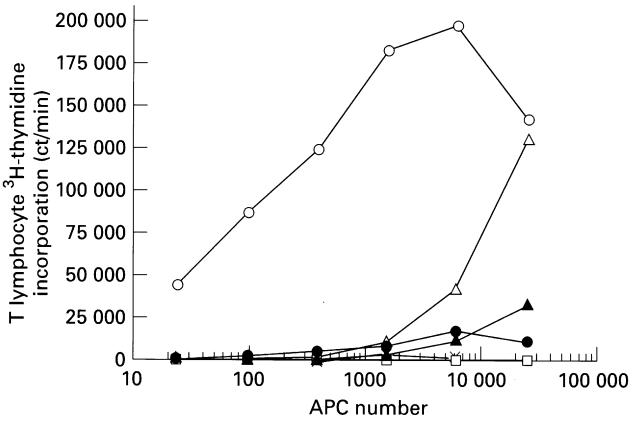

Fig. 2. B7-expressing melanoma cells can act as functional antigen-presenting cells (APC). The proliferation assay is described in Materials and Methods. A T cell-enriched PBL preparation failed to proliferate in response to Con A without addition of APC (data not shown). ○, MEL 26 B7 + Con A; ●, MEL 26 B7; ×, MEL 26 + Con A; □, MEL 26; △, Raji + Con A; ▲, Raji.

DISCUSSION

A large number of experiments have demonstrated that engineering syngeneic animal tumours to secrete a variety of cytokines [13,15] including IL-12 [20], to express foreign antigens [57], to express MHC molecules [21], or to express the lymphocyte co-stimulatory molecule B7 [25] results in tumour rejection. These observations have led to the initiation of a number of clinical trials using genetically modified tumour cells to attempt to stimulate an anti-tumour immune response. However, the results presented here argue that not all data from animal tumour models can be used to predict effective vaccine strategies for a particular human tumour. The ability to measure generation of autologous, melanoma-specific CTL has allowed us to demonstrate that IL-12, in combination with IL-2, enhances the anti-tumour response, whereas B7 expression is not, in general, effective. We would therefore propose that autologous tumour cells, engineered to secrete both IL-2 and IL-12, would provide a more effective melanoma vaccine than the IL-2-secreting cells currently being evaluated.

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