A novel role for autologous tumour cell vaccination in the immunotherapy of the poorly immunogenic B16-BL6 melanoma

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The growth of immunogenic tumours stimulates the generation of tumour-sensitized, but not functional, pre-effector T cells in the draining lymph nodes. These pre-effector cells can mature into effector cells upon *in-vitro* stimulation with anti-CD3 and IL-2. In the current study, using a defined, poorly immunogenic tumour, B16-BL6 melanoma, the pre-effector cell response was not evident during progressive tumour growth but was elicited by vaccination with irradiated tumour cells admixed with *Corynebacterium parvum*. After anti-CD3/IL-2 activation, these cells were capable of mediating the regression of established pulmonary metastases. The efficacy of the vaccine depended on the doses of both tumour cells and the adjuvant. While higher numbers of tumour cells were more effective, an optimal dose (12.5 μg) of *C. parvum* was required. The dose of irradiation was not a critical factor. After vaccination, kinetic studies revealed that the pre-effector cell response was evident 4 days later and declined after 14 days. These observations illustrate the potential role of active immunization in the cellular therapy of cancer. *Surgical Oncology* 1992; 1: 199–208.

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INTRODUCTION

Central to tumour immunology is the question of whether antigens recognizable by the host immune system exist on the surface of tumour cells. Clearly, definable tumour-specific antigens can be demonstrated in many animal tumour models [1, 2]. These studies suggest the existence of similar antigens on human cancer cells and stimulated multiple attempts over the course of the last three decades to immunize patients with their own tumours [3–5]. Most of these studies were performed empirically with little evidence of a specific immune response.

Studies on immunological mechanisms of tumour rejection have identified the cellular effector arm of the immune system as crucial in mediating tumour destruction. In fact, in a variety of animal models, the transfer of specifically tumour-sensitized lymphocytes to the tumour-bearing host can mediate therapeutic effects in rejecting established localized and metastatic cancers [6–8]. Although theoretically attractive, this adoptive immunotherapy approach has received relatively little attention in humans because of difficulties associated with generating large numbers of correctly sensitized autologous cells. The application of adoptive immunotherapy for the treatment of human cancer therefore requires the development of methods to isolate and propagate in-vitro antitumour-reactive T-cells from patients. One potential cell source, which is suitable for adoptive immunotherapy, has been tumour-infiltrating lymphocytes (TIL), owing to the demonstration of their potent antitumour properties in animal experiments [9, 10]. While the therapeutic efficacy of human TIL expanded with IL-2 is being intensively evaluated in clinical trials, initial results are encouraging [11].

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In the past few years, we have developed an additional method to generate large quantities of immune effector cells from the tumour-bearing host. The initial observations were made with several immunogenic murine tumours that during progressive tumour growth, draining lymph nodes (LN) were found to contain tumour-sensitized T-cells [12, 13]. Although these LN cells did not express overt antitumour reactivity, they could be further sensitized *in vitro* with anti-CD3 and IL-2 to differentiate into functional immune effector cells capable of mediating immunologically specific regression of established pulmonary metastases in adoptive immunotherapy [14–16]. As immune effector cells could only be generated from tumour-bearing but not normal mice, the cells in the draining LN were therefore termed 'pre-effector' cells. However, due to several characteristics of the pre-effector cell response, extrapolation of these studies in the design of clinical adoptive immunotherapy in humans is difficult. First, the presence of pre-effector cells within LN draining progressive tumours follow rather strict kinetics depending on the duration of tumour growth [15]. Clinically, the duration of tumour growth in patients is variable at the time of presentation and is difficult to assess. Secondly, the pre-effector cell response and subsequent generation of therapeutic anti-CD3/IL-2 activated effector cells has been defined only in the context of immunogenic tumours. The immunogenicity of human tumours is not clear and is presumably weak due to their spontaneous origin [17]. Hence, human tumours may elicit a weak pre-effector response.

Therefore, we have selected the poorly immunogenic murine B16-BL6 (BL6) melanoma to define clinically applicable methods to generate anti-CD3/IL-2 activated effector cells for adoptive immunotherapy. This tumour failed to induce systemic immunity by a variety of active immunization procedures; and indeed we found that the progressive growth of the BL6 tumour did not elicit a detectable pre-effector cell response. However, pre-effector cells were generated in the draining LN in response to priming the host with tumour cells admixed with the bacterial adjuvant, *Corynebacterium parvum (C. parvum)*. We have further demonstrated that vaccination of the host with irradiated tumour cells, admixed with *C. parvum*, was effective in eliciting a pre-effector cell response. In contrast to unirradiated BL6 tumour cells to prime the host, irradiated tumour cells lacked tumourigenicity and hence can be easily adapted for clinical use. We therefore have systematically defined the conditions and requirements for the use of an effective vaccine in the BL6 melanoma model. These studies suggest that a component of active immunization can facilitate the production of specific tumour-immune lymphocytes for adoptive immunotherapy and may be applicable in the treatment of human malignancy.

MATERIALS AND METHODS

Mice

Female C57BL/6J (B6) mice, 6–8 weeks old, were purchased from the Jackson Laboratory, Bar Harbor, ME. They were maintained in specific pathogen-free environments and were used for experiments at the age of 10 weeks or older.

Tumour

The B16-BL6 (BL6) melanoma is a tumour of spontaneous origin syngeneic to the B6 mouse that has been studied extensively [18, 19]. The tumour was kindly provided by Dr E. Gorelik (University of Pittsburgh), and was an early passage of the original BL6 described by Hart [18]. Tumour cells were maintained by in-vitro passage in complete media (CM). CM consisted of RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 0.1 mM non-essential amino acids, 1 μM sodium pyruvate, 2 mM fresh L-glutamine, 100 μg ml^{-1} streptomycin, 1000 ml^{-1} penicillin, 50 μg ml^{-1} gentamicin, 0.5 μg ml^{-1} fungizone (all from GIBCO, Grand Island, NY) and 5×10^{-5} M 2-mercaptoethanol (Sigma). Cultured tumour cells were harvested after a short incubation at 37°C with a solution containing 0.25% trypsin and 0.02% EDTA (GIBCO). Tumour cells were washed with Hanks Balanced Salt Solution (HBSS) prior to intravenous (i.v.) or subcutaneous (s.c.) inoculation.

IL-2

Human rIL-2 was kindly supplied by the Cetus Corp. (Emeryville, CA) with a specific activity of $6–8 \times 10^6$ Cetus U mg^{-1} protein. In this report, all units of IL-2

are expressed in Cetus units where 1 Cetus unit is equivalent to 6 International units.

Anti-CD3 mAb

The YCD3-1 hybridoma cells producing rat IgG2b monoclonal antibody (mAb) directed against the CD3ε chain of the murine TCR/CD3 complex were produced and kindly provided by Dr Kim Bottomly, Yale University [20]. Antibodies were obtained by injecting hybridoma cells into pristane-primed sublethally irradiated (500 rad) DBA/2 mice and collecting ascites. The IgG content was determined by ELISA assay.

Draining LN cells

Tumour growth was initiated by inoculating syngeneic B6 mice s.c. in the flank with 1×10^6 viable BL6 admixed with various doses of *C. parvum* (Burroughs Wellcome Co., Research Triangle Park, NC) suspended in 0.05 ml of HBSS. In this case, tumour growth was evident at day 3. Tumour vaccine was prepared using irradiated tumour cells admixed with various doses of *C. parvum* and inoculated into syngeneic mice similarly. Routinely, 7–12 days after tumour inoculation or vaccination draining inguinal LN were removed sterilely. Lymphocyte suspensions were prepared by first teasing LN with 20G needles and then pressing with the blunt end of a 10-ml plastic syringe in HBSS.

Tumour irradiation

The BL6 tumour cells were suspended in CM and maintained at 4°C. Cells were irradiated with 1,000–32,000 cGy using a Gamma Cell 1000 irradiator (500 cGy min^{-1}, Atomic Energy of Canada, Ltd). Irradiated tumour cells were then washed with HBSS × 2 and resuspended in HBSS for inoculation with an appropriate dose of *C. parvum*.

In-vitro anti-CD3/IL-2 activation of LN cells

Draining LN cells were stimulated *in vitro* by incubating approximately 10^8 cells in a 75 cm^2 tissue culture flask in 30 ml of CM with 2.5 μg ml^{-1} of anti-CD3 mAb at 37°C in 5% atmosphere for 2 days. In some experiments, 4×10^6 LN cells were activated with immobilized anti-CD3 antibody in 24-well tissue culture plates. The cells were harvested, washed, and further cultured at 6×10^5 cells/well in 2 ml of CM containing 10 U ml^{-1} of IL-2 in 24-well plates for 3 days. During this period of time, cells proliferated vigorously and the number increased, three- to six-fold. These anti-CD3/IL-2 activated cells were then harvested, washed and resuspended in HBSS for adoptive immunotherapy.

Procedure for adoptive immunotherapy

B6 mice were injected i.v. with $3–4 \times 10^5$ BL6 tumour cells in 1 ml of HBSS to initiate pulmonary metastases. On day 3, anti-CD3/IL-2 activated draining LN cells were given i.v. to each mouse. Some mice were also treated with 15,000 U IL-2 in 0.5 ml HBSS i.p. twice a day for 4 days to enhance the in-vivo function of transferred cells. At this stage, all mice were eartagged and randomized. On day 14–17 of tumour growth the mice were killed, the lungs insufflated with Fekete's solution, and pulmonary metastases enumerated as described previously [8]. Metastatic foci too numerous to count were assigned an arbitrary value of >250 because this was the largest number of tumour nodules that could be reliably enumerated.

Statistical analysis

The significance of differences in numbers of pulmonary metastases between groups was determined by the Wilcoxon rank-sum test. Two sided P values of <0.05 were considered significantly different. All groups consisted of at least five mice and no animal was excluded from the statistical analysis.

RESULTS

Draining LN response to progressive BL6 tumour growth

We have previously reported that LN draining progressive immunogenic methylcholanthrene-induced (MCA) sarcomas contain lymphocytes which can be secondarily activated *in vitro* to differentiate into therapeutic effector cells for adoptive immunotherapy [12–16]. The BL6 tumour, being different from MCA sarcomas, has been characterized to be poorly or non-immunogenic and displays a highly

invasive growth pattern by spontaneously metastasizing after s.c. inoculation [19]. We therefore examined whether progressive BL6 tumour growth would elicit the development of pre-effector cells in tumour-draining LN. B6 mice were inoculated s.c. with 10^6 viable BL6 tumour cells. Some animals were inoculated with viable tumour cells admixed with *C. parvum*, which was utilized as an immune adjuvant. Nine days later, draining inguinal LN were removed and activated by the anti-CD3/IL-2 culture method. This activation resulted in cells expanded approximately three- to six-fold. The activated cells were administered i.v. (5×10^7/mouse) into mice bearing 3-day established pulmonary metastases (Table 1). IL-2 was given (15,000 U i.p.) twice daily for 4 days starting on the day of cell transfer to promote the in-vitro proliferation of the activated cells. This therapeutic regimen was well tolerated with no evidence of toxicity. Lungs were harvested 14 days after tumour inoculation and pulmonary metastases enumerated in a blinded fashion.

In contrast to our previously reported experience with several immunogenic MCA sarcomas, the progressive growth of the BL6 did not stimulate the sensitization of pre-effector cells in draining LN (Table 1) by evidence of the failure of anti-CD3/IL-2 activated cells to mediate the regression of pulmonary metastases. However, the use of 12.5 μg of *C. parvum* at the initiation of tumour growth was found to elicit pre-effector cells in the draining LN which upon anti-CD3/IL-2 activation mediated potent antitumour reactivity. The dose of *C. parvum* was critical because a higher dose (50 μg) was ineffective. *C. parvum* inoculated without tumour cells did not induce pre-effector cells in the draining LN (data not shown).

Tumourigenicity of irradiated BL6 melanomas

Although *C. parvum* helped to stimulate a pre-effector cell response, the vaccination site showed progressive tumour growth and no systemic immunity was developed. Therefore, application of these approaches to generate immune cells in a clinical setting can be problematic. It is practical to establish a non-tumourigenic vaccine which would enable sensitization of pre-effector cells.

One method commonly used to abrogate the tumourigenicity while maintaining immunogenicity of tumour cells is γ-irradiation. Tumour cells treated with various doses of irradiation (1,000–16,000 cGy) were given s.c. to syngeneic mice (10^6/mouse) and the development of tumours was examined for up to 60 days (Table 2). Doses of $\geqslant$4000 cGy were found to be sufficient to inhibit active growth of the tumour. Similar results were obtained in the second experiment where 10^7 tumour cells were tested (Table 2).

Utilizing irradiated tumour cells, we investigated whether normal B6 mice could be systematically immunized against the BL6 tumour. Mice were inoculated s.c. in the flank with 10^7 irradiated (12,000 cGy) BL6 tumour cells admixed with various doses of *C. parvum* (0, 25, 50, 100, 200 μg). These animals

Table 1. Adoptive immunotherapy of BL6 pulmonary metastases with anti-CD3/IL-2 activated LN cells derived from mice inoculated with viable tumour cells admixed with *C. parvum*

Dose of *C. parvum* (μg) admixed with BL6 tumour cells*	Adoptive immunotherapy†		
	Activated LN cells	In-vivo IL-2	Mean number pulmonary metastases (SEM)
	−	−	>250
	−	+	>250
0	+	+	209(38)
12.5	+	+	8(6)‡
50	+	+	187(30)

*Mice were inoculated s.c. in the flank with 10^6 tumour cells admixed with various amounts of *C. parvum* in 0.1 ml of HBSS. Nine days later, inguinal LN cells were obtained and activated by the anti-CD3/IL-2 method.

†Activated cells (5×10^7) were administered i.v. into mice with 3-day established pulmonary metastases. IL-2 (15,000 U) was given i.p. twice daily for 4 days starting on the day of cell transfer. Lungs were harvested 14 days after tumour inoculation.

‡$P < 0.05$ compared with groups without treatment or treated with IL-2 only.

were challenged s.c. four weeks later, with 10^6 viable tumour cells in the opposite flank to determine if systemic immunity was developed. Unlike immunization with previously defined MCA sarcomas, such as MCA 105, MCA 106, MCA 205 and MCA 207 [1, 21], no demonstrable systemic immunity could be detected (data not shown). These observations further substantiated that the BL6 is a poorly immunogenic tumour.

Draining LN response to irradiated BL6 tumour admixed with *C. parvum*

A vaccine comprised of irradiated BL6 tumour cells and *C. parvum* was evaluated for its ability to elicit a pre-effector cell immune response in draining LN. Various numbers of irradiated (4,000 cGy) tumour cells were admixed with 12.5 μg of *C. parvum* and inoculated s.c. in the flank of B6 mice. Nine days later, draining inguinal LN were harvested for anti-CD3/IL-2 activation. The antitumour reactivity of the activated LN cells was assessed in the adoptive immunotherapy of 3-day established pulmonary metastases as previously described (Table 3). Mice inoculated with 1.2×10^6 irradiated tumour cells and *C. parvum* did not elicit pre-effector cells because activated cells did not mediate tumour regression. In contrast, the same number of viable tumour cells admixed with 12.5 μg of *C. parvum* was effective in eliciting immune pre-effector cells as demonstrated in Table 1. Therefore the difference in immunogenicity between irradiated and viable tumour cells appeared to be quantitative rather than qualitative. However, vaccines that consisted of a greater number of irradiated tumour cells ($\geq 6 \times 10^6$) were capable of stimulating the sensitization of pre-effector cells in the draining LN (Table 3).

In another experiment, the effect of increased doses of tumour irradiation on the efficacy of the tumour vaccine was examined. B6 mice were inoculated s.c. in the flank with 12.5 μg *C. parvum* admixed with 10^7 BL6 tumour cells which were treated with graded doses of irradiation (4,000–32,000 cGy). Nine days later, draining LN were harvested and activated by the anti-CD3/IL-2 method. The antitumour reactivity of the activated cells was assessed again in the adoptive immunotherapy of pulmonary metastases, as previously described (Table 4). All mice inoculated with irradiated tumour cells plus *C. parvum* developed immune pre-effector cells that mediated potent tumour regression after in-vitro activation. Hence, the dose of tumour irradiation did not seem to alter the immunogenicity of the tumour vaccine. More importantly, there was no evidence of progressive tumour growth at the site of vaccination.

Table 2. Tumourigenicity of BL6 tumour after irradiation

Tumour irradiation dose (cGy)	Tumour incidence*	
	10^6	10^7
0	5/5	6/6
1,000	5/5	—
2,000	5/5	6/6
4,000	0/5	0/6
8,000	0/5	0/6
16,000	0/5	0/6

*Mice were inoculated s.c. with 10^6 or 10^7 BL6 tumour cells in 0.5 of HBSS after various doses of irradiation. The incidence of tumour growth at the inoculation sites was observed over a 60-day period.

Table 3. Adoptive immunotherapy of BL6 pulmonary metastases with anti-CD3/IL-2 activated LN cells derived from tumour-vaccinated mice

Number of irradiated tumour cells admixed with *C. parvum* for vaccination*	Adoptive immunotherapy†	
	IL-2	Mean number of pulmonary metastases (SEM)
A	−	>250
B	+	>250
C 1.2×10^6	+	189(17)
D 6×10^6	+	1(1)‡
E 3×10^7	+	0‡

*Mice were inoculated s.c. in the flank with varying numbers of irradiated (4,000 cGy) BL6 tumour cells admixed with 12.5 μg *C. parvum* in 0.1 ml HBSS. Nine days after inoculation, inguinal LN cells were obtained and activated by the anti-CD3/IL-2 method.

†Activated cells (5×10^7) were administered i.v. into mice with 3-day established pulmonary metastases. IL-2 (15,000 U) was given i.p. twice daily for 4 days starting on the day of cell transfer. Lungs were harvested 14 days after tumour inoculation.

‡$P < 0.05$ when compared with groups A, B and C.

Table 4. The amount of tumour irradiation does not alter the antigenicity of the tumour vaccine

Tumour vaccine irradiation dose (cGy)*	Adoptive immunotherapy†	
	In-vivo IL-2	Mean number of pulmonary metastases (SEM)
A –	–	>250
B –	+	>250
C 4,000	+	12(3)‡
D 8,000	+	7(4)‡
E 16,000	+	5(2)‡
F 32,000	+	6(12)‡

*Mice were vaccinated s.c. in the flank with 1×10^7 irradiated (4,000–32,000 cGy) tumour cells admixed with 12.5 μg. *C. parvum* in 0.1 ml HBSS. Nine days after inoculation, inguinal LN cells were obtained and activated by the anti-CD3/IL-2 method.
†Adoptive immunotherapy of 3-day established BL6 pulmonary metastases were performed as described in Table 3.
‡$P < 0.05$ compared with groups without treatment or treated with only IL-2.

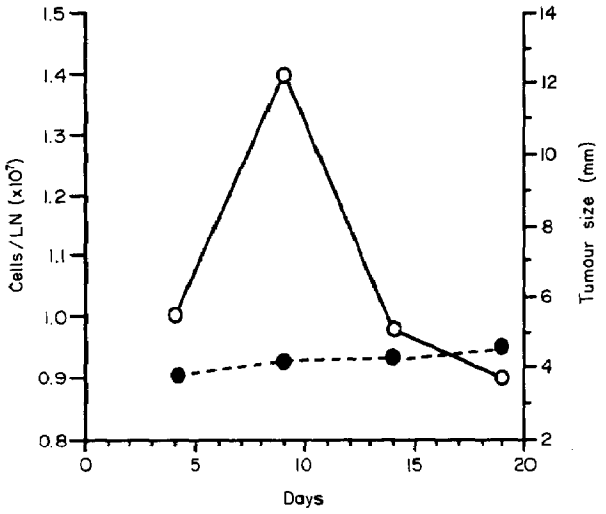

Figure 1. Kinetics of pre-effector cell response after tumour vaccination. B6 mice were inoculated with 10^7 irradiated (4,000 cGy) BL6 tumour cells admixed with 12.5 μg *C. parvum*. There was no evidence of tumour growth during the 19-day observation period. A dramatic hyperplastic response in the draining LN was evident on day 9 after tumour vaccination. (—○—) Cells/LN, (—●—) tumour size.

Kinetics of pre-effector cell sensitization after tumour vaccination

The kinetics of pre-effector cell sensitization after tumour vaccination were examined. Groups of B6 mice were inoculated s.c. with 10^7 irradiated (4,000 cGy) BL6 tumour cells admixed with 12.5 μg *C. parvum* every few days so that draining LN could be harvested on the same day. During the observed 19-day period, there was a hyperplastic response in the draining LN which peaked on day 9 of vaccination and decreased thereafter (Fig. 1). The vaccine-draining LN cells were activated by the anti-CD3/IL-2 culture method and subsequently assessed for anti-tumour reactivity *in vivo*, as described (Table 5). Sensitized pre-effector cells were present as early as 4 days after tumour vaccination and persisted to day 14 before it began to diminish. By day 19, there were no detectable pre-effector cells in the draining LN.

DISCUSSION

Experimental evidence indicates that properly sensitized T-cells are extremely potent mediators of tumour rejection. Several laboratories have shown that animals immunized to tumours can serve as donors for immune cells which upon transfer, mediate the regression of localized or advanced tumour in syngeneic hosts [6–8]. Also, animals successfully treated by the transfer of immune cells will reject subsequent challenges of the same tumour due to the establishment of systemic immunity in the host. However, there are formidable obstacles which need to be resolved before this approach can be applied to the therapy of malignancy in man. Foremost is the lack of reliable techniques to isolate and produce adequate numbers of tumour-reactive cells needed for adoptive immunotherapy. Unlike animal studies, cancer patients usually do not have genetically identical counterparts to immunize and generate donor immune cells. Therefore, application of adoptive immunotherapy in man will require methods to obtain anti-tumour-reactive T-cells from the patient. In addition, human cancers have been postulated to be poorly immunogenic because of their spontaneous origin; and might not be analogous to experimentally induced animal tumours, many of which express tumour-specific antigens.

To address these concerns, we have recently developed a culture method which promotes the differentiation and growth of therapeutic T-cells

Table 5. Kinetics of pre-effector cell sensitization in draining LN after tumour vaccination

Days after vaccination when draining LN were harvested for anti-CD3/IL-2 activation*		Adoptive immunotherapy†	
		IL-2	Mean number of pulmonary metastases (SEM)
A		−	>250
B	−	+	244(6)
C	4	+	3(3)‡§
D	9	+	10(3)‡§
E	14	+	109(19)‡
F	19	+	214(15)

*Mice were vaccinated s.c. with 10^7 irradiated (4,000 cGy) BL6 tumour cells admixed with 12.5 μg *C. parvum* at various intervals prior to harvesting draining inguinal LN on the same day for activation by the anti-CD3/IL-2 method.
†Adoptive immunotherapy of 3-day established BL6 pulmonary metastases was performed as described in Table 3.
‡$P < 0.05$ compared with groups A, B and F.
§$P < 0.05$ compared with group E.

derived from the tumour-bearing hosts for adoptive immunotherapy. This culture method involves the activation of tumour-draining LN cells with anti-CD3 mAb for 2 days followed by expansion in IL-2 for 3 days [14–16]. Utilizing several immunogenic MCA-induced murine sarcomas, we found that activated cells had potent antitumour effects in adoptive immunotherapy experiments. In contrast, similarly activated normal lymphoid cells did not generate tumour-reactive effector cells. These findings suggest that progressive tumour growth stimulate an immune response developed in the draining LN cells, which we have termed 'pre-effector cells'. This terminology was chosen because these cells, when freshly harvested, were not functionally active in adoptive immunotherapy and the generation of therapeutic effector cells required the secondary anti-CD3/IL-2 activation.

The presence of pre-effector cells within the draining LN was critically dependent on the duration of tumour growth. Prolonged tumour growth resulted in the loss or inhibition of pre-effector cells [15]. Perhaps more importantly, there was a virtual absence of a pre-effector cell response in the draining LN during the growth of a poorly immunogenic tumour, the BL6 melanoma (Table 1). However, the admixture of the bacterial adjuvant *C. parvum* at the time of BL6 inoculation did result in the development of pre-effector cells. The ability to elicit a pre-effector cell response against BL6 was highly dependent upon the amount of *C. parvum* used and was more effective at lower doses. The use of *C. parvum* did not result in growth inhibition or regression of the BL6 tumour at the inoculation sites. These observations would be difficult to extrapolate for clinical application. We therefore explored the efficacy of a non-tumourigenic vaccine to trigger the pre-effector cell response. In order to define requirements for such a vaccine, which can be applied to poorly immunogenic human cancers, we examined the use of irradiated BL6 tumour cells. We found that irradiation doses of 4,000 cGy or greater could effectively suppress tumourigenicity when 10^6 or 10^7 cells were used as an inoculum (Table 2). As growth of viable BL6 tumour did not elicit a pre-effector cell response in the absence of *C. parvum*, we examined the use of varying numbers of irradiated tumour cells, admixed with a dose of *C. parvum*, to sensitize pre-effector cells. Although effective in stimulating a pre-effector response in draining LN (Table 3), considerably higher numbers of irradiated tumour cells were required than with viable tumour cells (Table 4). The mechanisms by which *C. parvum* facilitated sensitization to BL6 tumour antigen are unknown; however, there have been several reports documenting the immune effects of this adjuvant. One possibility may be more effective antigen processing by macrophages because *C. parvum* is a known stimulator of macrophages [22]. Alternatively, *C. parvum* may have an adjuvant role by recruiting

lymphocytes to the tumour site with subsequent elaboration of cytokines that up-regulate an anti-tumour immune response [23–25].

The pre-effector cell response to the tumour vaccination was also dependent upon the kinetics of vaccination. We found that pre-effector cells were detectable as early as 4 days after tumour vaccination but rapidly declined from day 14 to 19 (Table 5). The suppression of the immune response could not be attributed to non-specific effects of tumour growth because there was no evidence of tumour growth at the vaccination site. One possible mechanism for this immune suppression may be the development of tumour suppressor cells. Our laboratory has previously demonstrated that the pre-effector cell response to growing subcutaneous tumour can be suppressed by the presence of visceral metastases [26]. In additional studies, we have been able to adoptively transfer splenocytes from visceral tumour-bearing mice into normal animals and subsequently identify suppression of the pre-effector cell response to a subcutaneous inoculation of tumour [27]. These observations indicate that the pre-effector cell response can be down-regulated by tumour-induced suppressor cells. Further studies to elucidate the mechanism of this suppression, and methods to overcome it, are clinically relevant.

In other studies, we have further characterized the phenotypic and functional properties of activated pre-effector cells elicited by BL6 tumour and *C. parvum* (J. D. Geiger, manuscript in preparation). Upon anti-CD3/IL-2 activation, LN cells draining BL6 tumour admixed with *C. parvum* demonstrated a predominance of $CD8^+$ cells compared with $CD4^+$ T cells. Utilizing a 4-h ^{51}Cr release assay, these activated cells did not mediate in-vitro cytolysis of BL6 targets although these same targets were readily lysed by LAK cells. Despite the inability to lyse BL6 tumour *in vitro*, these activated LN cells mediated the specific regression of BL6 pulmonary metastases and not other immunologically distinct MCA sarcoma tumours. The mechanisms by which anti-CD3/IL-2 activated effector cells mediate in-vivo tumour regression are unknown but may involve the elaboration of cytokines [28], or further differentiation into cytolytic cells upon tumour interaction after adoptive transfer.

The data presented in this report demonstrate the feasibility of generating therapeutic immune cells to a poorly immunogenic tumour with the aid of a non-tumourigenic vaccine. In this regard, the tumour vaccine serves to stimulate a pre-effector cell response which requires further in-vitro activation to develop into mature, functionally active therapeutic effector cells. We are currently exploring this approach in the treatment of patients with advanced malignancies. Initial observations have indicated that immune reactivity to autologous tumour is transferred and tumour regression has been noted in some patients [29]. Future directions with this therapeutic approach involve methods to enhance pre-effector cell sensitization. We have investigated the use of exogenously administered cytokines to determine if pre-effector cell sensitization can be up-regulated. We have found that IL-1 administration can significantly enhance the pre-effector cell response while there is no effect with exogenously administered IL-2 [30]. In addition, we found that the administration of TNFα will down-regulate the pre-effector cell response [31]. Another manipulation, which may lead to an enhanced pre-effector cell sensitization, is the use of genetically modified tumours. Several recent studies have demonstrated that the transduction of murine tumour cells with genes encoding cytokines (i.e. IL-2, IL-4, TNFα) alter the immunological properties of tumours [32–34]. This may provide an alternate approach to developing a pre-effector cell response to poorly immunogenic tumours which our laboratory is currently investigating [35].

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