Immunotherapeutic Efficacy of Vaccines Generated by Fusion of Dendritic Cells and HPV16-Associated Tumour Cells

(HPV16 / DC-tumour hybrids / immunotherapy / CpG)

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Abstract. Utilization of vaccines generated by fusion of dendritic cells and tumour cells is a promising approach to tumour immunotherapy. We have examined the therapeutic efficacy of vaccines generated by fusion of HPV16-associated tumour cells TC-1 with syngeneic and allogeneic dendritic cells. Locally administered hybrid cells generated by fusion of MHC class I^+ TC-1 cells and syngeneic DC inhibited the growth of MHC class I^+ TC-1 tumours, but not the growth of MHC class I^- TC-1/A9-derived tumours. The growth of TC-1 tumours was also inhibited by hybrids generated by fusion of TC-1 cells and allogeneic DC. The therapeutic efficacy was enhanced by co-administration of the vaccine with synthetic immunostimulatory ODN CpG 1826.

For dendritic cell (DC)-based tumour immunotherapy, various strategies for loading DC with antigen have been suggested (for a review, see Bubeník, 2001; Zhou et al., 2002). The advantage of therapy utilizing vaccines generated by fusion of dendritic and tumour cells is that the hybrid cells can possess properties of both parental cell types, expressing tumour antigens, MHC class I and MHC class II antigens, as well as co-stimulatory molecules, the molecules necessary for induction of effective antitumour immune response. We attempted to generate hybrid vaccines by fusion of syngeneic or allogeneic DC with HPV16-associated tumour cells and to utilize the vaccines for the therapy of MHC class I^+ and MHC class I^- tumours associated with HPV16. We have also augmented the immunotherapeutic efficacy of the vaccines by co-administration of a synthetic immunostimulatory oligodeoxynucleotide (ODN) containing a specific unmethylated CpG motif. The data presented here which have shown that the hybrid cell vaccines can inhibit growth of the MHC class I^+ but not class I^- tumour cell sublines accentuate the importance to examine the presence of MHC class I molecules on tumour cells before starting the immunotherapy.

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Abbreviations: BMDC – bone marrow dendritic cells, ConA – concanavalin A, DC – dendritic cell(s), HPV – human papilloma virus, ODN – oligodeoxynucleotide.

Material and Methods

Mice

C57BL/6 and BALB/c males, 2–4 months old, were purchased from AnLab Co., Prague, Czech Republic.

Tumour cell lines

The MHC class I^+ TC-1 tumour cell line was established by transformation of primary C57BL/6 mouse lung cultures with HPV16 *E6/E7* oncogenes and activated *Ha-ras* (Lin et al., 1996). The MHC class I deficient TC-1 cell subline TC-1/A9 (a generous gift from Dr. M. Šmahel) was derived from TC-1 tumours formed in mice preimmunized repeatedly with HPV16 *E7*-containing plasmid DNA (Šmahel et al., 2003).

Generation of DC

The protocol used in this study for generation of bone marrow dendritic cells (BMDC) has been described previously (Mendoza et al., 2002). Briefly, bone marrow was flushed from femurs and tibias of C57BL/6 or BALB/c mice. The cells were cultured in complete RPMI 1640 medium supplemented with 10 ng/ml GM-CSF (R&D systems, Minneapolis, MN) plus 10 ng/ml interleukin 4 (supernatant of murine myeloma cell line X63-m-IL-4 producing IL-4, a generous gift from Dr. F. Melchers, Basel Institute for Immunology), after two hours the non-adherent cells were further cultured for 7 days and used for the experiments.

Cell fusion

DC were mixed with 5'bromodeoxyuridine-resistant TC-1 cells deficient in thymidin kinase (Šímová, unpublished data) at the 1 : 1 ratio. Cells were washed in serum-free RPMI 1640 medium, fused using polyethylene glycol dimethylsuphoxide solution (50% PEG/10% DMSO in PBS, Sigma-Aldrich, St. Louis, MO) and stirring. Additional serum-free RPMI 1640 medium was added slowly, cells were centrifuged and resuspended in the RPMI 1640 medium containing

HAT (medium supplemented with 10^{-4} M hypoxanthine, 3.8×10^{-7} M aminopterin and 10^{-5} M 2-deoxythymidine, Sigma-Aldrich). After three weeks of cultivation in the selection medium the hybrid cells were used for the experiments.

Proliferative spleen cell responses

For priming the proliferative spleen cell responses, Mitomycin C-treated hybrid cells were cultured for 4 days in complete RPMI 1640 medium with syngeneic or allogeneic, nylon wool non-adherent spleen cells at stimulator/splenocyte responder cell ratios 1 : 5, 1 :10, 1 : 50 and 1 : 100 using 96-well round-bottom microtitre plates (Nunc, Roskilde, Denmark). Twenty hours before harvesting, the cells were labelled with 0.04 MBq ^{3}H-TdR/well and the uptake of ^{3}H-TdR was measured in the liquid scintillation system (Bubeník et al., 1994).

Flow cytometry

The expression of MHC class I molecules on tumour cells was determined by cytofluorometric analysis with FITC-anti-mouse H-2K^b/H-2D^b monoclonal antibody (clone 28-8-6, Pharmingen, San Diego, CA). As an isotype control, FITC-labelled antibody of irrelevant specificity (clone 155-178, Pharmingen) was used. As can be seen in Fig. 1a, TC-1 tumour cells are MHC class I$^+$ and TC-1/A9 tumour cells are MHC class I deficient. The expression of MHC class I molecules on hybrid cells generated by fusion of allogeneic H-2^d BMDC and H-2^b tumour cells was determined by cytofluorometric analysis with FITC-anti-mouse H-2D^b monoclonal antibody (clone KH95, Pharmingen) and PE-anti-mouse H-2D^d (clone 34-2-12, Pharmingen).

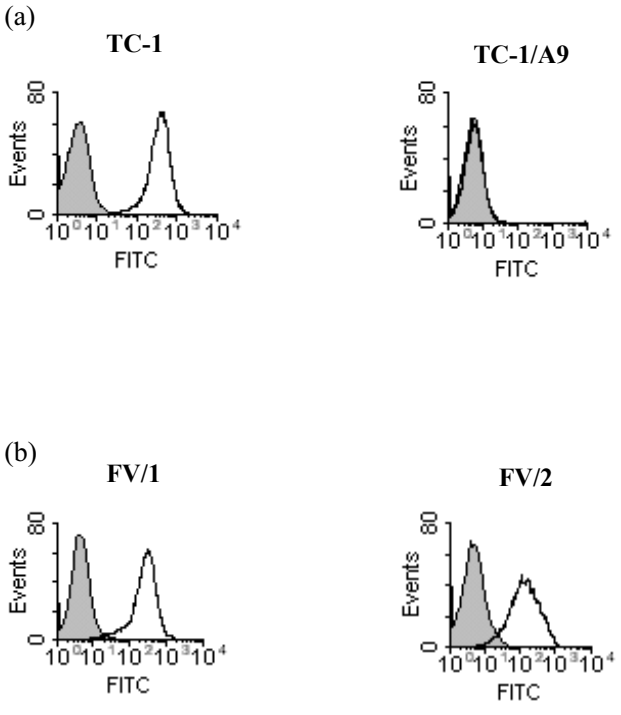

Fig. 1. (a) Flow cytometric detection of MHC class I expression on TC-1 and TC-1/A9 cells. Tumour cells were stained with FITC-anti-H-2K^b/H-2D^b monoclonal antibody (open histograms) or with isotype control antibody (filled histograms). (b) Flow cytometric detection of MHC class I expression on the FV/1 and FV/2 cells generated by fusion of syngeneic BMDC and TC-1 cells. Hybrid cells were stained with FITC-anti-H-2K^b/H-2D^b monoclonal antibody (open histograms) or with isotype control antibody (filled histograms).

Immunotherapy

C57 BL/6 mice were inoculated s.c. on day 0 with 1×10^4 TC-1 cells or with 5×10^3 TC-1/A9 cells. Three days after challenge the mice were vaccinated with 150 Gy-irradiated 3×10^6 fused cells. CpG 1826 (Genosis, Hradec Králové, Czech Republic) was injected s.c. (50 μg/dose) on days 3, 5, 12, and 19 after challenge of TC-1 cells.

The tumour-bearing mice were observed twice a week and the size of the tumours was recorded.

Statistical analyses

For statistical analyses, the analysis of variance and Student's t-test from NCSS, Number Cruncher Statistical System (Kaysville, Utah), statistical package was used.

Results

Immunotherapy with vaccines constructed by fusion of TC-1 tumour cells with syngeneic DC

Eight hybrid cell lines were prepared by fusion of TC-1 with syngeneic BMDC and subsequent selection of hybrid cells in the medium supplemented with HAT. The capacity of hybrid cell lines to induce proliferative responses of syngeneic spleen lymphocytes was tested *in vitro*. It has been found that cells of the hybrid lines differ in their capacity to induce proliferative responses of syngeneic lymphocytes (Table 1). For *in vivo* experiments, the hybrid cell line designated as FV/1, inducing the most effective proliferative response, and for control the hybrid cell line designated FV/2, inducing no proliferative response *in vitro*, were utilized. MHC class I profiles of FV/1 and FV/2 fused cells are demonstrated in Fig. 1b. Cells of both hybrid lines were MHC class I$^+$. The therapeutic efficacy of the FV/1 and FV/2 hybrid cells in MHC class I$^+$ TC-1 and MHC class I$^-$ TC-1/A9 tumours is demonstrated in Fig 2. The FV/1 hybrid cells, which were the most effective cells in the induction of proliferative responses *in vitro*, inhibited the growth of MHC class I$^+$ TC-1 tumours (Fig. 2a). The FV/2 hybrid cells, which lacked the capacity to induce the proliferative *in vitro* responses, had no antitumour effect (Fig. 2a). In MHC class I$^-$ TC-1/A9 tumours, neither hybrid cell line influenced the tumour growth (Fig. 2b).

Table 1. Proliferative responses of spleen lymphocytes (responder cells) to hybrid cells (stimulator cells).

Hybrid cells	Responder/stimulator cell ratio ^{3}H-TdR uptake (cpm)			
	5 : 1	10 : 1	50 : 1	100 : 1
TC-1 x syngeneic BMDC[1]				
FIV/B2	271 ± 25	184 ± 31	156 ± 42	94 ± 18
FIV/B3	1349 ± 326*	587 ± 116	249 ± 98	99 ± 25
FIV/B4	552 ± 191	280 ± 132	208 ± 105	108 ± 68
FV/1	2351 ± 492*	2050 ± 430*	2794 ± 501*	1794 ± 381*
FV/2	260 ± 168	275 ± 79	168 ± 62	175 ± 32
FV/3	99 ± 43	140 ± 52	149 ± 45	132 ± 51
FV/4	111 ± 87	114 ± 38	73 ± 16	126 ± 63
FVI/4	871 ± 169*	163 ± 39	217 ± 81	130 ± 59
TC-1 x allogeneic BMDC[2]				
AI/a	2184 ± 502*	1444 ± 362*	1205 ± 311*	1286 ± 299*
AI/b	1188 ± 204*	545 ± 152	259 ± 86	171 ± 51

[1]As a positive control, sygeneic spleen cells stimulated with 1 µg/ml concanavalin A (ConA) were used (mean ^{3}H-TdR uptake: 8468 ± 1245 cpm); mean ^{3}H-TdR incorporation of spleen cells alone: 276 ± 112 cpm.
[2]As a positive control, allogeneic spleen cells stimulated with 1 µg/ml ConA were used (mean ^{3}H-TdR uptake: 16140 ± 1857 cpm); mean ^{3}H-TdR incorporation of spleen cells only: 188 ± 63 cpm.
*$P < 0.05$ as compared with the level of ^{3}H-TdR incorporation of spleen cells alone (Student's t-test).

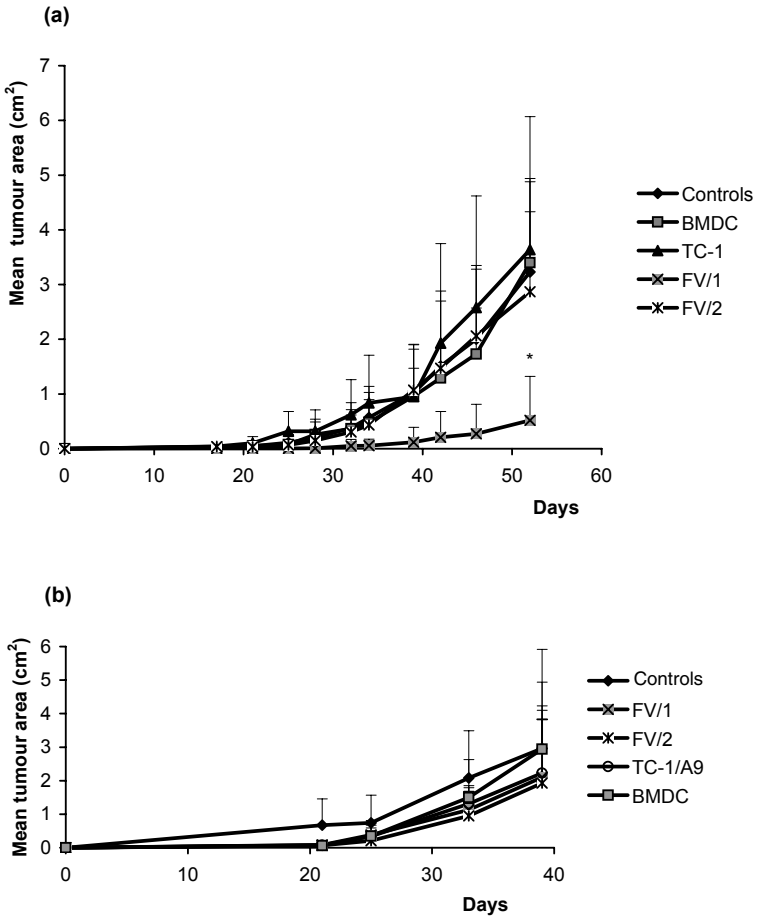

Fig. 2. Immunotherapy of TC-1 (MHC I^+) and TC-1/A9 (MHC I^-) tumours with hybrid vaccines FV/1 and FV/2 generated by fusion of syngeneic BMDC and TC-1 cells. The experimental mice were inoculated with TC-1 or TC-1/A9 cells on day 0, and three days after challenge were injected with the irradiated respective vaccines (3×10^6 cells). *$P < 0.01$ as compared to all groups.

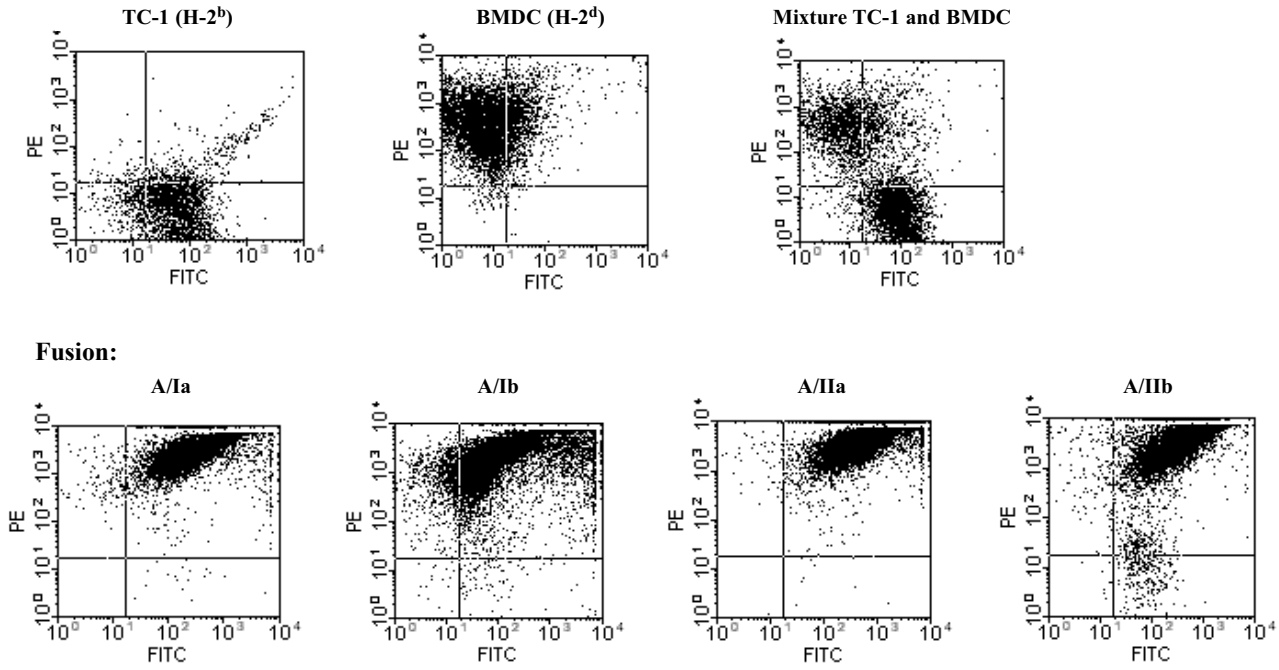

Fig. 3. Flow cytometric detection of MHC class I expression on hybrid cell vaccines generated by fusion of allogeneic BMDC and TC-1 cells. Hybrid cells were stained with FITC-anti-mouse H-2D^b monoclonal antibody and PE-anti-mouse H-2D^d.

Immunotherapy with vaccines constructed by fusion of TC-1 tumour cells with allogeneic DC

By fusion of TC-1 cells (H-2^b) with allogeneic BMDC (H-2^d) and subsequent selection of hybrid cells in the medium supplied with HAT, four hybrid cell lines were prepared. The presence of H-2^b and H-2^d molecules on the membrane of hybrid cells, which documents their hybrid character, is demonstrated in Fig. 3. For the experiments, two hybrid cell lines designated as A/Ia and A/Ib were utilized. The capacity of these hybrid cells to induce proliferative responses of allogeneic spleen lymphocytes was tested *in vitro*. It has been found that the cells of both hybrid lines were able to induce proliferative responses of spleen lymphocytes (Table 1). The therapeutic efficacy of both hybrid cell lines in the model of MHC class I$^+$ tumour TC-1 is shown in Fig. 4a. Both hybrid cell vaccines inhibited the growth of TC-1 tumours.

Enhancement of therapeutic efficacy of the hybrid cell vaccine with synthetic oligonucleotide CpG 1826

The ability of CpG 1826 to enhance the therapeutic efficacy of the A/Ia hybrid cells is shown in Fig. 4b. The administration of CpG 1826 inhibited the growth of TC-1 tumours and significantly strengthened the therapeutic effect of the hybrid A/Ia cells.

Discussion

Hybrid cells generated by fusion of BMDC with tumour cells provide a useful approach to tumour immunotherapy since they can express tumour-associated antigens, MHC class I and class II molecules, as well as the adhesion and co-stimulatory molecules, the molecules required for efficient anti-tumour responses. By fusion of MHC class I positive tumour cells TC-1 and syngeneic BMDC we have prepared MHC class I positive hybrid cells and utilized them for the therapy of MHC class I positive (TC-1) and MHC class I deficient (TC-1/A9) tumours. The hybrid cells FV/I that efficiently induced *in vitro* proliferative responses of syngeneic spleen lymphocytes were able to inhibit the growth of MHC class I positive TC-1 tumours when administered s.c. in the vicinity of tumour transplants. However, no therapeutic effect was observed when the FV/1 cells were utilized for the therapy of an MHC class I deficient TC-1 cell subline, the TC-1/A9 cells. The results confirmed our previous findings that the MHC class I positive and MHC class I deficient tumours required different therapeutic approaches (Šímová et al., 2003) and that the presence of MHC class I molecules has to be considered for the design of imunotherapeutic vaccines and protocols.

The hybrid cell vaccines were previously generated by others by fusion of not only syngeneic BMDC with tumour cells, but also of allogeneic BMDC with tumour cells (Kugler et al. 2000; Colado, 2003). We have fused the allogeneic (H-2^d) BMDC with syngeneic TC-1 (H-2^b) tumour cells and prepared hybrid cell vaccines that inhib-

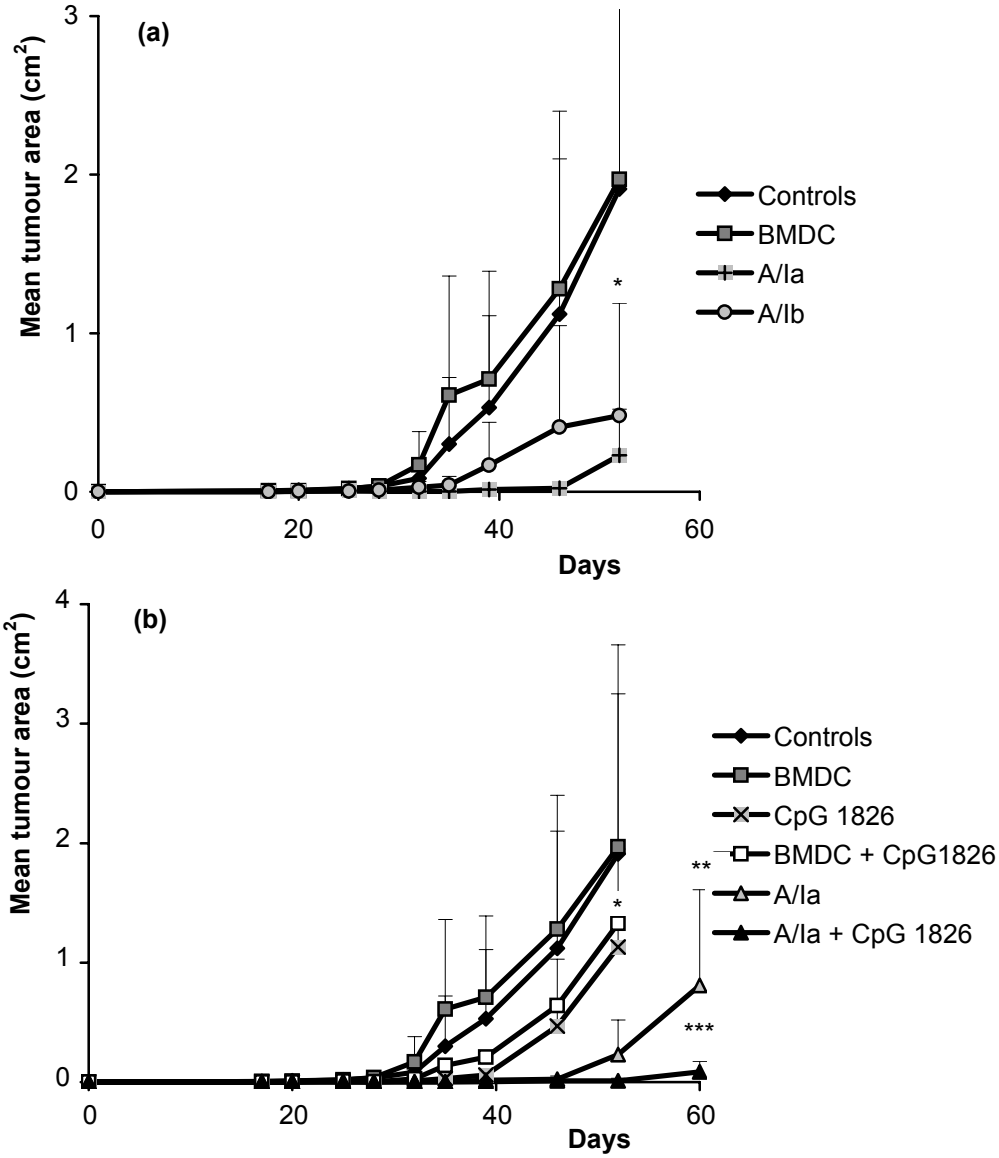

Fig. 4. (a) Immunotherapy of TC-1 tumours with hybrid vaccines A/Ia and A/Ib generated by fusion of allogeneic (H-2^d) BMDC and TC-1 (H-2^b) cells. The experimental mice were inoculated with TC-1 cells on day 0, and on day 3 after challenge were injected with the irradiated respective vaccines (3×10^6 cells). *$P < 0.01$ as compared to controls and BMDC group. (b) Enhancement of therapeutic efficacy of the hybrid cell vaccine A/Ia with the synthetic ODN CpG 1826. The experimental mice were inoculated s.c. with TC-1 on day 0, and 3 days after challenge were injected s.c. with the irradiated vaccine (3×10^6 cells). CpG 1826 was injected s.c. (50 μg/dose) on days 3, 5, 12, and 19 after challenge with TC-1 cells. *$P < 0.01$ as compared to all groups; **$P < 0.01$ as compared to controls, BMDC, A/Ia and A/Ia+CpG 1826 groups; ***$P < 0.01$ as compared to controls, BMDC, BMDC+CpG 1826, CpG 1826 and A/Ia+CpG 1826 groups.

ited the growth of the TC-1 cells. The hybrid cell vaccines, designated as A/Ia and A/Ib, provided sufficient signals for efficient tumour responses.

It has been reported that CpG 1826 ODNs are recognized by the Toll-like receptor (TLR)9 family mainly in DC, which leads to the activation of the immune system (Akira et al., 2001). We have used this ODN as an adjuvans for enhancement of the therapeutic efficacy of a hybrid vaccine generated by fusion of allogeneic BMDC and TC-1 tumour cells. We have observed that repeated s.c. administration of CpG 1826 ODN alone both inhibited the growth of TC-1 tumours and significantly enhanced the efficacy of hybrid cell vaccines.

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