

Chemotherapy, IL-12 gene therapy and combined adjuvant therapy of HPV 16-associated MHC class I-proficient and -deficient tumours

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Abstract. Moderately immunogenic HPV 16-associated murine tumour cell line mimicking human HPV 16-associated neoplasms TC-1 (MHC class I⁺) and its variants, TC-1/P3C10 and TC-1/A9, with a marked down-regulation of MHC I molecules, were used to examine the effect of local interleukin 12 (IL-12) gene therapy for the treatment of early tumour transplants and minimal residual tumour disease obtained after cytoreductive chemotherapy (CMRTD). Experiments were designed to examine whether down-regulation of MHC class I molecules plays a role during chemotherapy and gene therapy of early tumour transplants. It was found that peritumoral administration of IL-12-producing tumour cell vaccines (single dose, day 8 after tumour cell administration) inhibited the growth of both TC-1 (MHC class I positive) tumours and their MHC class I-deficient variants. To investigate the antitumour effects in a clinically relevant setting, IL-12 gene therapy was utilised for the treatment of minimal residual tumour disease after cytoreductive chemotherapy. Intra-peritoneal treatment of tumour-bearing mice with ifosfamide derivative, CBM-4A, produced a significant tumour-inhibitory effect. This treatment was followed by peritumoral s.c. administration of genetically modified TC-1

(MHC class I positive) or MK16/I/IIIABC (MHC class I negative) vaccines producing IL-12 (single dose, day 7 after chemotherapy) or with recombinant interleukin 12 (rIL-12) in two cycles of 5 daily doses (days 8-19) after chemotherapy. This combined therapy significantly inhibited the growth of TC-1 and TC-1/A9 (MHC class I) tumours. When the combined therapy of TC-1 (MHC class I positive) tumours was followed by peritumoral administration of bone marrow dendritic cell (BMDC) vaccines, the IL-12-mediated inhibitory effect was significantly boosted. In the next set of experiments, the impacts of chemotherapy and IL-12 adjuvant therapy on MHC class I surface expression were assessed. Chemotherapy and gene therapy of tumours led to the up-regulation of MHC I expression on MHC class I-deficient tumours (TC-1/A9 and TC-1/P3C10) and to down-regulation on MHC I-proficient tumours (TC-1). These findings indicate that the MHC I phenotype is not stable during tumour progression and treatment. Collectively, these results illustrate the efficacy of IL-12 gene therapy in combination with chemotherapy on HPV-associated tumours regardless of the level of MHC class I expression on the tumour cells.

Introduction

Interleukin 12 has been described to be a potent inducer of antitumour immunity and has been found to be one of the most important cytokines for cancer therapy. Depending upon the tumour system, differential effectors, such as natural killer (NK) cells, cytotoxic T cells or T helper (Th) cells can be stimulated and may be involved in the antitumour response generated either by IL-12 alone (1) or after chemotherapy (2-4).

The antitumour efficacy of local IL-12 treatment can be successfully potentiated with the administration of dendritic cells alone (5) or also with dendritic cells engineered to produce IL-12 (6,7). To minimize the toxic effects of systemically administered rIL-12, gene-therapy approaches for IL-12 delivery are under intensive investigation (8).

Most immunotherapeutic protocols are based on activation of potential primary defence effector mechanisms, such as

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Abbreviations: HPV, human papilloma virus; MHC, major histocompatibility complex; C57BL/6, B6; mAb, monoclonal antibody; IL-12, interleukin 12; FACS, fluorescence-activated cell sorter; CMRTD, minimal residual tumour disease after cytoreductive chemotherapy; BMDC, bone marrow dendritic cells

Key words: HPV 16, MHC class I-deficient and MHC class I-proficient tumour cells, CMRTD, vaccines, CTLs

CD8⁺ cytotoxic T lymphocytes, which are capable of recognizing and killing MHC class I⁺ tumour cells. However, since the down-regulation of MHC class I molecules is one of the most important mechanisms that allow the tumour cells to escape from the immune system, alternative immunotherapeutic strategies must be developed.

In our previous studies using an experimental model of HPV 16-associated murine neoplasms, we found that their phenotype is not stable and may change during tumour progression and therapy. The *in vivo* administration of interferon γ (IFN γ) into the vicinity of growing tumours leads to the up-regulation of MHC class I expression and can inhibit tumour growth (9,10). Furthermore, it has been described that MHC class I-deficient tumours are at least partially susceptible to cytokine treatment. It has been found that MK16 (MHC class I negative) cells are sensitive to therapy with some immunomodulatory cytokines, but often to a lesser degree than MHC I-positive tumours of the same aetiology (11,12).

In this report, we have demonstrated the effectiveness of local immunotherapy with genetically modified tumour cell vaccines expressing HPV16 E6 and E7 oncogenes and engineered to produce IL-12 on the growth of early tumour transplants of HPV 16-associated tumours and on the recurrences after cytoreductive chemotherapy. The changes in MHC class I expression during tumour growth and therapy were monitored.

Materials and methods

Mice. B6 male mice, 8-10 weeks old, were obtained from AnLab Co., Prague, Czech Republic.

Cell lines. The murine malignant, non-metastasizing, MHC I⁺ cell line, TC-1, immunogenic in syngeneic B6 mice, was established by co-transfection of primary cultures of B6 lung cells with activated ras and HPV16 E6/E7 DNA (13). The TC-1/P3C10 (P3C10) cells, displaying a marked down-regulation of MHC class I molecules, were prepared by repeated passages of TC-1 tumour cells into Vac-Sig/E7/LAMP-1-vaccinated mice. The TC-1 and P3C10 cells were kindly provided by the laboratory of Dr T.C. Wu (Dr Chien-Fu Hong), Johns Hopkins University, Baltimore, MD. The cloned MHC I⁺ TC-1/A9 tumour cell subline was developed in a similar way; to obtain TC-1 cells with down-regulated expression of MHC class I molecules, cell sublines were prepared from tumours formed in mice preimmunized repeatedly with HPV16 E7-containing plasmid DNA. The selected cloned subline without surface MHC class I expression was designated as TC-1/A9 cells (9,14).

The IL-12 gene modified cells, MK16-IL-12 (241/clone 33) and TC-1-IL-12 (231/clone 15), were derived from a thymidine kinase-negative subline of MK16/IIIABC (MK16) (15) and the TC-1 cells, respectively. The MK-IL-12 cells were isolated after transduction with bicistronic recombinant adeno-associated virus carrying the genes for herpes simplex type 1 thymidine-kinase (HSV TK) and mouse IL-12 in the hypoxanthine-aminopterin-thymidine (HAT) medium, similarly as the other gene-modified MK16 cells (16). The TC-1-IL-12 cells were isolated after transfection with bicistronic plasmids

carrying the same two genes in the HAT medium (Vonka and Sobotková, unpublished data). The gene modified cells were not oncogenic for syngeneic animals and were highly sensitive to gancyclovir, this confirming the production of HSV TK. The transduced cells used for vaccination produced *in vitro* 40 ng IL-12/ 1×10^5 cells/ml medium/48 h.

The cell lines were maintained in RPMI-1640 medium supplemented with 10% foetal calf serum, 2 mM L-glutamine and antibiotics (complete medium). For the transduced cell lines, HAT selection medium was used. The TC-1/A9 cells were maintained in D-MEM medium, also supplemented with 10% foetal calf serum, 2 mM L-glutamine and antibiotics. The cell lines were cultured at 37°C in a humidified atmosphere with 5% CO₂.

Cytokines. For therapeutic experiments, 0.25 μ g/dose, twice a day, of human rIL-12 (R&D Systems, Minneapolis, MN) was used. Cytokine was administered after dilution in PBS containing 1% bovine serum albumin. Control mice were injected with cytokine diluents only.

Bone marrow-derived dendritic cells. Bone marrow-derived, immature dendritic cells (BMDC) were prepared using a method described by Lutz *et al* (17) and modified by Mendoza *et al* (18). Briefly, male B6 bone marrow-derived dendritic cell precursors were cultivated for 7 days in complete RPMI-1640 medium supplemented with mercaptoethanol (10^{-5} M), 10 ng/ml GM-CSF (R&D Systems) and 10 ng/ml IL-4 from the supernatants of murine genetically engineered X63-m-IL-4 cells (19).

Local treatment of early tumours. For therapy of the tumours, the B6 mice were inoculated s.c. on day 0 with 2.5×10^4 tumour cells. On day 7, the mice were injected with 1.5×10^7 irradiated cells (150 Gy) producing IL-12 (TC-1-IL-12 or MK16-IL-12) or control vaccines (TC-1, MK16), and the growing tumours were checked twice a week.

Treatment of mice after cytoreductive chemotherapy. For therapy of CMRTD, B6 mice were inoculated s.c. with 1×10^5 tumour cells. After 20 days, when the tumours reached a size of 2-3 mm in diameter, the mice were injected i.p. with 150 mg/kg of bromine-substituted derivative of ifosfamide CBM-4A (racemic chlorobromofosfamide) preparation (20-22). Eight days later, the experimental mice were injected twice a day, for 5 days, with rIL-12 (daily dose 0.5 μ g/mouse), and this cycle was repeated. Alternatively, the experimental mice were injected with a single dose of IL-12-producing, irradiated (150 Gy) TC-1 or MK16 cells (1.5×10^7 cells per mouse), in the vicinity of the s.c. tumours, and/or with a single dose of BMDC (3×10^6 cells/mouse). The growing tumours were checked twice a week.

Flow cytometry. To examine MHC class I expression on tumour cells, the growing tumours (about 1 cm in diameter) were explanted, minced and cultivated *in vitro* in complete RPMI medium. After 5-8 days of cultivation, the growing cells were collected. The MHC class I molecule expression on the tumour cells was determined by flow cytofluorometry with FITC-labelled anti-mouse H-2K^b/H-2D^b mAb (clone

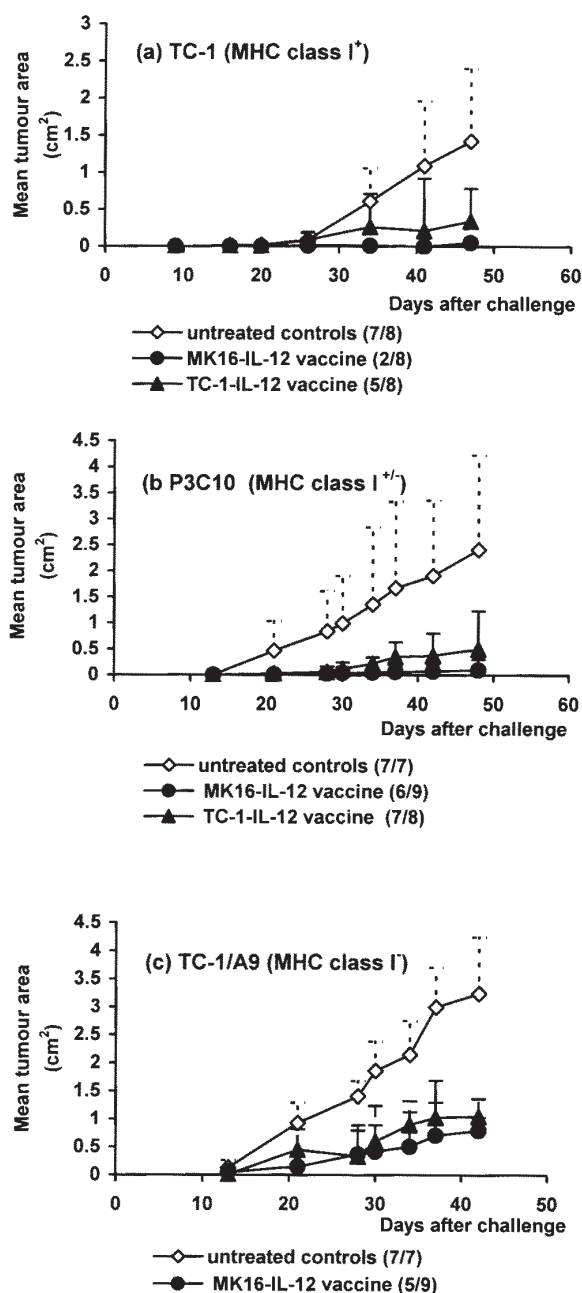


Figure 1. Local effect of IL-12-producing vaccines on the growth of early tumour transplants. (a) $p < 0.001$, controls x MK16-IL-12 vaccine; $p < 0.01$, controls x TC-1-IL-12 vaccine. (b) $p < 0.001$, controls x MK16-IL-12 vaccine; $p < 0.01$, controls x TC-1-IL-12 vaccine. (c) $p < 0.05$, controls x MK16-IL-12 vaccine; $p < 0.01$ controls x TC-1-IL-12 vaccine.

28-8-6, PharMingen, San Diego, CA) and FITC-conjugated goat anti-mouse Ig antibody. As an isotype control, FITC-labelled antibody of irrelevant specificity (clone G 155-178, PharMingen) was used. The cells were incubated with the mAb at a concentration of $4 \mu\text{g/ml}$ at 4°C for 30 min. Flow cytometry was performed using an ELITE flow cytometer (Coulter, Miami, FL).

Statistical analyses. For statistical analyses, the analysis of variance from the Number cruncher statistical system (NCSS; Kaysville, UT) statistical package was used. For comparison of tumour takes in experimental and control groups, the χ^2 test was also used.

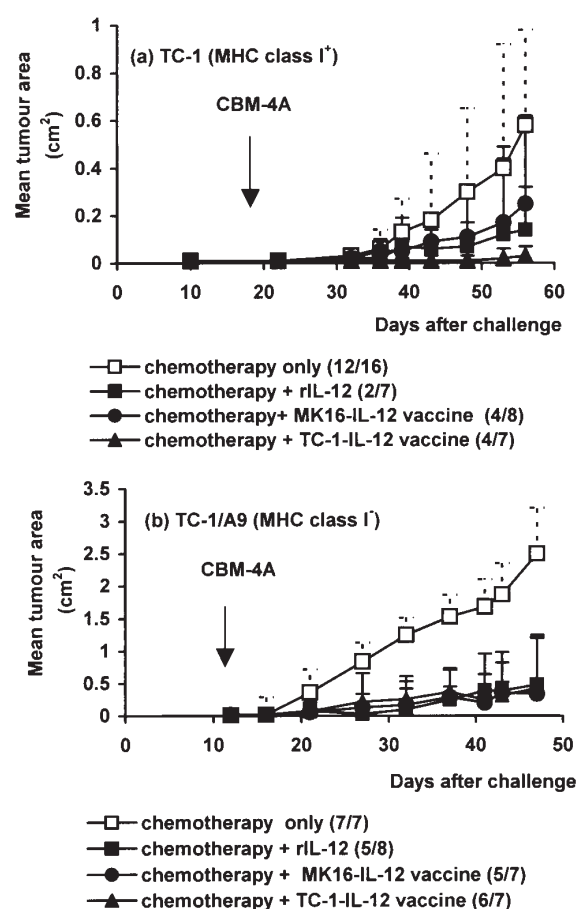


Figure 2. Chemotherapy-induced residual tumour disease: effect of peritumoral administration of recombinant IL-12 and IL-12-producing vaccines. (a) $p < 0.001$ ($p < 0.05$ - χ^2 test), controls x chemotherapy only (data not shown); $p < 0.05$, chemotherapy only x chemotherapy + rIL-12; $p < 0.05$, chemotherapy only x chemotherapy + MK16-IL-12; $p < 0.01$, chemotherapy only x chemotherapy + TC-1-IL-12. (b) $p < 0.01$, controls x chemotherapy only (data not shown); $p < 0.05$, chemotherapy only x chemotherapy + MK16-IL-12; $p < 0.05$, chemotherapy only x chemotherapy + TC-1-IL-12.

Results

Local effect of IL-12-producing vaccines on the growth of early tumours. Mice were inoculated s.c. with TC-1 (MHC class I⁺), TC-1/P3C10 (with a partial down-regulation of MHC class I molecules) or with TC-1/A9 (MHC class I⁻) tumour cells and the tumour-inhibitory effect of local treatment with IL-12-producing vaccines was investigated. Mice were s.c. inoculated with 2.5×10^4 tumour cells and injected on day 7 with a dose of 1.5×10^7 irradiated control (MK16, TC-1) or IL-12-producing vaccines (MK16-IL-12 or TC-1-IL-12). As can be seen in Fig. 1, treatment with one injection of IL-12 gene-modified vaccines significantly inhibited tumour growth as compared to the untreated controls. No significant difference in the efficacy of TC-1-IL-12 and MK16-IL-12 vaccines was found. The treatment with the irradiated control vaccines (MK16, TC-1) was without any effect (data not shown).

Therapy of CMRTD with peritumoral administration of recombinant IL-12 or IL-12-producing vaccines. Mice were inoculated s.c. with TC-1 (MHC class I⁺) or with TC-1/A9 (MHC class I⁻) cells. On day 15-20, when the tumours reached

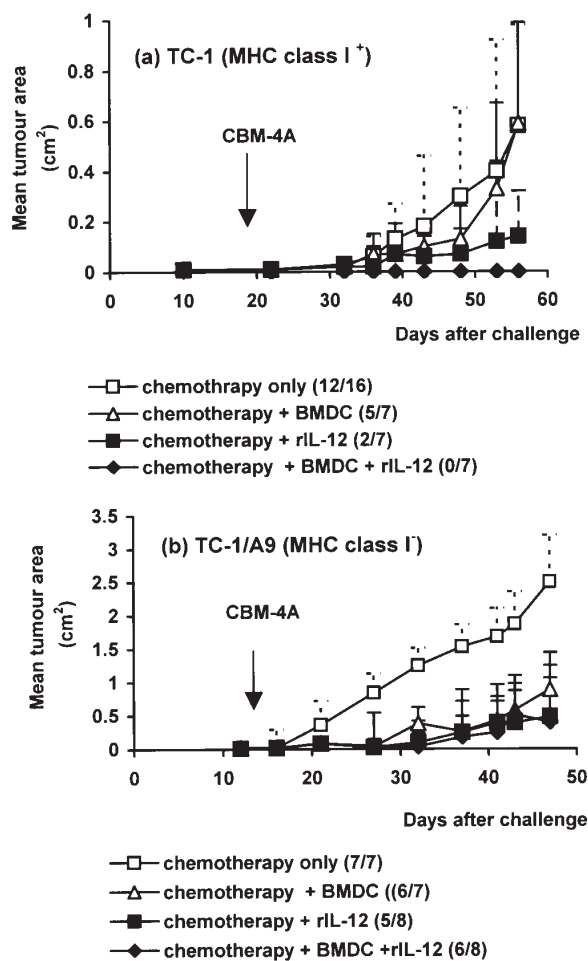


Figure 3. Chemotherapy-induced residual tumour disease: combined effect of peritumoral administration of recombinant IL-12 and BMDC. (a) $p < 0.001$, controls x chemotherapy only; $p > 0.05$, chemotherapy only x chemotherapy + BMDC; $p < 0.05$, chemotherapy only x chemotherapy + rIL-12; $p < 0.001$ ($p < 0.05 - \chi^2$ test), chemotherapy only x chemotherapy + rIL-12 + BMDC; $p < 0.001$ ($p < 0.05 - \chi^2$ test), chemotherapy + rIL-12 x chemotherapy + rIL-12 + BMDC. (b) $p < 0.01$, controls x chemotherapy only (data not shown); $p < 0.05$, chemotherapy only x chemotherapy + BMDC; $p < 0.05$, chemotherapy only x chemotherapy + rIL-12; $p < 0.05$, chemotherapy only x chemotherapy + rIL-12 + BMDC; $p > 0.05$, chemotherapy + rIL-12 x chemotherapy + rIL-12 + BMDC.

a palpable size of 2-3 mm in diameter, the tumour-bearing mice were injected i.p. with the ifosfamide-derivative, CBM-4A, and divided into experimental and control groups. Eight days after chemotherapy, the experimental mice were injected peritumorally with a single dose of IL-12-producing vaccines or with repeated daily doses of rIL-12. The treatment with the cytostatic (chemotherapy only group) resulted in a significant tumour-inhibiting effect in comparison with untreated controls (data not shown). As can be seen in Fig. 2, this effect was further significantly potentiated by therapy with IL-12-producing cells or rIL-12. No significant difference in the efficacy of rIL-12, TC-1-IL-12 and MK16-IL-12 vaccines was found.

Combined effect of peritumoral administration of rIL-12 and BMDC. Eight days after chemotherapy, experimental mice were injected peritumorally with a single dose of 3×10^6 BMDC and/or with two cycles of five daily doses of rIL-12 (Fig. 3a).

The combined adjuvant therapy completely inhibited the growth of TC-1 tumours minimized with chemotherapy. However, administration of this combined adjuvant therapy did not improve the effect of monotherapy with rIL-12 on TC-1/A9 (MHC class I⁻) tumours, although the single dose of BMDC in the group of chemotherapeutically pretreated mice significantly inhibited the growth of TC-1/A9 tumours (Fig. 3b).

Changes of MHC class I molecule expression during progression and therapy. Growing tumours of mice from the experimental and control groups were explanted, minced and grown *in vitro* for 5-8 days to remove the infiltrating cells. The expression of MHC class I molecules was then analysed by flow cytometry. It was found (Fig. 4) that the expression of MHC class I molecules on TC-1 (MHC class I⁺) tumour cells was decreased during the temporal growth *in vivo*. Decreasing expression of MHC class I molecules was also observed in recurrences after cytoreductive chemotherapy (Fig. 4b) and after treatment with IL-12-producing vaccines (Fig. 4a).

The TC-1/P3C10 cells, exhibiting *in vitro* a marked down-regulation of MHC class I molecules, moderately up-regulated the expression of MHC I during the temporal growth *in vivo*, as well as after immunotherapy with IL-12-producing vaccines (Fig. 4a). Only negligible up-regulation was found after temporal growth *in vivo* of TC-1/A9 (MHC class I⁻) tumour cells. After cytoreductive chemotherapy or treatment with IL-12-producing vaccines, moderate up-regulation of the expression of MHC I was detected (Fig. 4). No up-regulation of the expression of MHC class II, CD80, CD86, CD11b and CD11c molecules was observed (data not shown).

Discussion

The expression of MHC class I molecules on the surface of tumour cells is an important factor in the immune defence against tumours and its down-regulation is one of the basic mechanisms by which the tumour evades classical T-cell dependent immune response (23-25).

It has been repeatedly demonstrated that a high proportion of HPV 16-associated tumours derived from MHC class I positive precursors are MHC class I deficient. So, the design of the therapeutic strategies has to be modified according to their often changeable MHC class I status. It has been shown that cancer cellular vaccines engineered to produce cytokines can be used generally against a broader spectrum of tumours of the same origin, including MHC class I-deficient tumours (26).

In this study, we used three types of TC-1 tumour mimicking the MHC class I-positive or MHC class I-deficient HPV-associated human tumours. MHC class I-positive TC-1 tumours, as well as their MHC class I-deficient variants, were sensitive to the gene therapy with IL-12-producing vaccines in the treatment of both early tumour transplants and CMRTD. We used two types of vaccine engineered to produce IL-12, based on TC-1 (MHC class I proficient) and MK16 (MHC class I deficient) HPV 16-associated tumours. Both of these tumour cells are of the same haplotype (H-2^b)

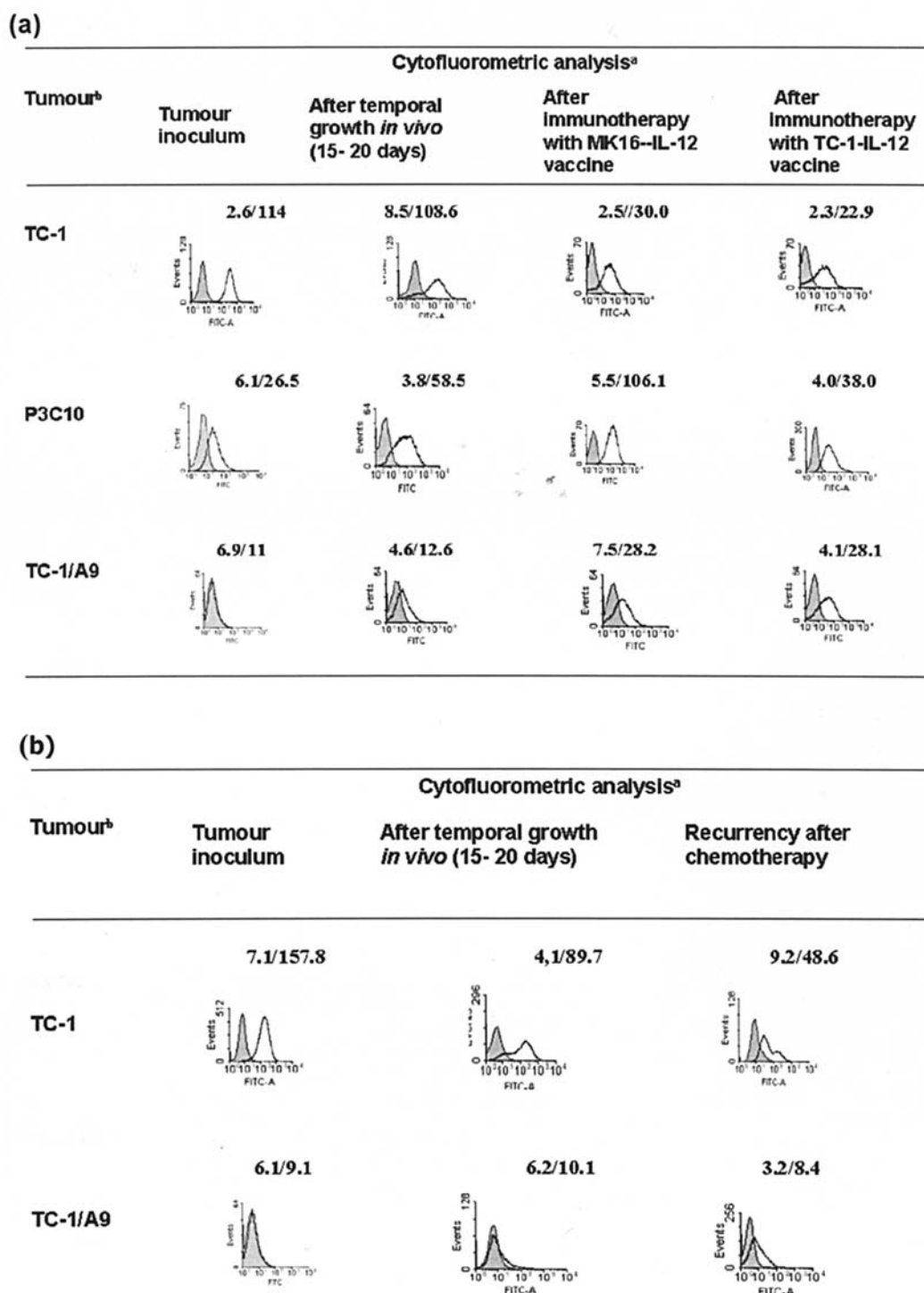


Figure 4. Changes in MHC class I expression of HPV 16-associated tumours with different expression of MHC class I molecules. (a) After therapy with IL-12-produced vaccines. (b) After chemotherapy. ^aFACS analysis was repeated four times with independent individual tumours with similar results. ^bMean tumour area of individual tumours explanted for FACS analysis was 0.50-1.20 cm².

and express HPV16 E6/E7 genes, but they did not immunologically cross-react in immunization/challenge experiments (26). Both vaccines were found to be effective for monotherapy as well as for the adjuvant therapy of CMRTD of MHC class I-proficient and -deficient HPV 16-associated tumours.

In gene-therapy experiments, IL-12 has been shown to be more effective in antitumour activity as compared to other cytokines used in the same therapeutic schemes. IL-12 is able

to enhance the effect of chemotherapy (2,4) or to improve the therapeutic efficacy of BMDC (5,6). Since T cells as well as NK cells, NKT cells and/or macrophages may be involved in the antitumour response generated by IL-12, this cytokine could also be effective in the therapy of some MHC class I-negative tumours (24).

In most previous experiments with HPV-associated MHC class I-positive TC-1 tumours, a single injection of irradiated

genetically modified tumour vaccines producing a relatively low amount of cytokines (40-100 ng of cytokine/ 1×10^5 cells/ml medium/48 h) was efficient. In contrast, in experiments with the MHC class I tumour, MK16, treatment with the same irradiated genetically modified tumour vaccines producing the same amount of cytokines was efficient only in some therapeutic schemes (12,27), despite the fact that local peritumoral administration of rIL-2 or IL-12 usually resulted in significant inhibition of tumour growth (12,20,21,28). In this study, no significant differences in the efficacy of the treatment between MHC class I-negative TC-1/A9 and -positive TC-1 were observed.

Significant differences were observed in the treatment of MHC class I-deficient and -proficient tumours in previous experiments when immature BMDC were used as adjuvants to the effect of combined chemo-immunotherapy. This therapy was successful only on TC-1 (MHC class I⁺) and not on MK16 (MHC class I⁻) tumour recurrences obtained after surgical therapy (12). These data suggest that the distinct sensitivity between MK16 and TC-1 cells could not be explained only by differences in the MHC class I expression on these tumours.

Furthermore, we have confirmed the moderate up-regulation of MHC class I molecules after immunotherapy in the sublines with down-regulated expression of MHC class I molecules (9). Moreover, data from this study revealed that minimization of established tumours with cytoreductive chemotherapy also was associated with up-regulation of MHC I molecules. These findings may be of essential importance for selection and optimising the immunotherapy strategies suitable after chemotherapy.

Immunotherapy with IL-12-producing vaccines leads to up-regulation of MHC class I molecules. This up-regulation of MHC class I expression could be due to the production of IFN γ in the vicinity of the growing tumour (10), which could be supported by IL-12 administration. On the other hand, parental MHC class I-positive tumour cells, TC-1, exhibited moderate down-regulation of MHC class I molecules after temporal growth *in vivo*, as well as after chemotherapy or immunotherapy, and this favouring the escape from the immunological defence of T cell-mediated mechanisms.

Taken together, both types of IL-12-producing vaccines, as well as rIL-12, are effective for the therapy of HPV 16-associated, MHC class I-positive tumours (TC-1) as well as for the treatment of MHC class I-deficient tumours (P3C10, TC-1/A9). Moreover, IL-12 gene therapy boosted the efficacy of the cytoreductive chemotherapy. The combined chemo-immunotherapy of TC-1 (MHC class I positive tumours) could be further augmented by BMDC administration. The findings on the modulation of MHC class I expression during the growth and immunotherapy of early established tumour transplants as well as tumours minimized after chemotherapy give useful information for elaborating the optimal immunotherapeutic strategies for the treatment of HPV 16-associated tumours.

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