

HPV 16-associated tumours: IL-12 can repair the absence of cytotoxic and proliferative responses of tumour infiltrating cells after chemotherapy

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Abstract. We have examined the effect of IL-12-producing cellular vaccines on the cytotoxicity and proliferative potential of CD45⁺ tumour-infiltrating cells (TIL) in mice carrying syngeneic TC-1 and TC-1/A9 HPV 16-associated tumours after chemotherapy with CBM-4A ifosfamide derivative. The chemotherapy resulted in the decrease of the CD4⁺ and CD8⁺ TIL, increase of the Gr-1⁺/CD11b⁺ TIL, no changes in the infiltration with CD4⁺/CD25⁺ Treg TIL, and decrease of the cytolytic and proliferative potential of the CD45⁺ TIL. Subsequent immunotherapy with the IL-12-producing, genetically modified TC-1 (TC-1-IL-12) cells increased tumour infiltration with CD8⁺ and CD4⁺ cells, decreased the Gr-1⁺/CD11b⁺ cells, and increased the cytolytic and proliferative potential of the CD45⁺ TIL. Taken together, these findings suggest that peritumoral administration of the IL-12-producing cellular vaccine can restore the cytolytic potential and inhibit immunosuppressive TIL-dependent mechanisms in the individuals bearing HPV 16-associated tumours, and explain our previously described tumour-inhibitory effects of the vaccine in mice with minimal residual disease after the tumour chemotherapy.

Introduction

The presence of tumour-infiltrating lymphocytes (TIL) provides important evidence of anti-tumour reaction *in vivo*. However, TIL are usually not sufficient for inhibiting tumour growth (reviewed in ref. 1). While the studies of murine and human tumours have shown that TIL are able to recognize tumour antigen and to mediate anti-tumour immunity (2,3), the infiltrating lymphocytes in many cases fail to provide effective tumour resistance and may contribute to tumour progression (4,5).

The functional defectiveness of tumour-infiltrating lymphocytes has been attributed to various mechanisms, including inadequate antigen-presenting and/or costimulatory capacity of tumour cells leading to T cell ignorance or anergy (6,7), and production of immune suppressive factors by the tumours (8,9). The defectiveness of TIL cells can represent one of the parameters that can correlate with the tumour outcome (10-13). Therefore, understanding the events giving rise to TIL defects, their potential to maintain tumour progression and reparation of these processes, is critical for evaluation of the role of immunity in supporting tumour growth. In addition, such knowledge is critical for determination how to enhance the immunity to obtain a clinically relevant therapeutic effect.

Defective lytic function of T cells infiltrating human and rodent tumours has been recognized for a long time and numerous preclinical and clinical studies have demonstrated that IL-12 alone or in a combined therapy mediates tumour regression by promoting Th1 responses, by increasing the efficacy of CD8⁺ T cells, NKT cells, NK cells, antigen-presenting cells and other effectors (14-19). However, the effectiveness of TIL cells in the IL-12 immunotherapy of minimal residual tumour disease remains unknown.

In our previous study (14) we analyzed the effects of interleukin 12 (IL-12) immunotherapy by vaccination with genetically modified, cellular cytokine-producing therapeutic vaccines or with recombinant IL-12 on the treatment of minimal residual tumour disease induced by chemotherapy (CMRTD). The IL-12 treatment was effective against both

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Abbreviations: HPV, human papilloma virus; MHC, major histocompatibility complex; B6, C57BL/6; TIL, tumour-infiltrating lymphocytes; IL-12, interleukin 12; SI, stimulation index; FACS, fluorescence-activated cell sorter; MACS, AutoMACS Pro cell separator-magnetic separation system; MDSC, myeloid-derived suppressor cells; CMRTD, minimal residual tumour disease after cytoreductive chemotherapy

Key words: HPV16, vaccines, tumour-infiltrating cells, cytotoxicity

MHC class I-positive and -deficient human papilloma virus 16 (HPV 16)-associated experimental tumours TC-1 and TC-1/A9. The anti-tumour effects were mediated by a number of different mechanisms, including CD8, CD4, NK11, myeloid-derived suppressor Gr-1/CD11b-positive cells, as well as Th1/Th2 cytokines (14), (reviewed in ref. 3).

In this communication, we demonstrated the effectiveness of local immunotherapy with tumour vaccine engineered to produce IL-12, using the model of minimal residual tumour disease obtained after cytoreductive chemotherapy (CMRTD), on the distribution and activity of tumour-infiltrating cells. Administration of the IL-12 vaccine led to reappearance of CD8⁺ and CD4⁺ cells and to a significant increase in the number of CD11c⁺ cells in the tumour nodules. On the other hand, Gr-1/CD11b-positive cells, increased after chemotherapy, were decreased after subsequent immunotherapy. Moreover, CD45⁺ tumour-infiltrating cells isolated from the treated animals exhibited restored cytotoxic and proliferation potential after short-term *in vitro* pre-cultivation.

Materials and methods

Mice. C57BL/6 (B6) male mice, 8-10 weeks old, were obtained from AnLab Co., Prague, Czech Republic.

Cell lines. HPV16-associated, non-metastasizing, MHC class I positive cell line TC-1, immunogenic in syngeneic B6 mice (15) and TC-1/A9, HPV16-associated, non-metastasizing tumour cell line, deficient in MHC class I molecules were used (22,23). The selected cloned subline IL-12-gene modified TC-1-IL-12 (231/clone 15) cells were used for vaccination (Vonka and Sobotková, unpublished data). The transduced cells used for vaccination produced *in vitro* 40 ng IL-12/1x10⁵ cells/ml medium/48 h (14).

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

Therapeutic protocol. Briefly, a week after induction of CMRTD, the mice were injected with a single dose of IL-12-producing, irradiated (150 Gy) TC-1-IL-12 cells (1.5x10⁷ cells per mouse), in the vicinity of the s.c. tumours (14). Experimental protocols were approved by the Institutional Animal Care Committee of the Institute of Molecular Genetics, Prague.

Flow cytometry. To determine CD45- and CD11c-positive cells, PE anti-mouse CD45 (LCA, LY5) (30F-1) and PE anti-mouse CD11c (Integrin α_x chain) (HL3) were utilized. To determine Gr-1/CD11b-positive cells, FITC rat anti-mouse CD11b (M1/70) and PE anti-mouse Ly-6G (Gr-1) and Ly-6C (Gr-1) (R36-8C5) were used. For detection of CD4- and CD25-positive cells, FITC anti-mouse CD4 (L3T4) (RM4-4) and FITC anti-mouse CD25 (IL-2-Receptor-Chain p55) (PC61) were used. As isotype controls, FITC- and PE-labelled antibodies of irrelevant specificity were utilized. All products were purchased from Pharmingen, San Diego, CA. Flow cytometry was performed using an LSR II flow cytometer (BD Biosciences, San Jose, CA).

Preparation of tumour-infiltrating (TIL) cells. Six days after therapy, fragments of the tumour mass (pool of 2-3 tumours) were transferred to the Eppendorf tubes and overlaid with 100 μ l Collagenase I (1 mg/ml, Serva Electrophoresis GmbH, Heidelberg, Germany) plus 1 ml of HBSS buffer with 5% of FCS and 0.5 U/ml DNase I (Sigma, St. Louis, MO). The tumour tissues were digested for 1 h at 37°C with agitation and the suspension with isolated TIL cells was filtrated through the nylon mesh (the protocol was kindly provided by Dr A. Lapique, Ludwig Institute of Cancer Research, Sao Paulo, Brazil (24)).

Magnetic separation (MACS). For MACS separation, the isolated tumour-infiltrating cells were labelled with MACS CD45 MicroBeads Abs according to the manufacturer's instructions, and CD45-positive cells were separated in the AutoMACS Pro cell separator (Miltenyi Biotec, GmbH, Bergisch-Gladbach, Germany).

Histological and immunohistochemical analyses. Tumour tissue cryo-sections, 5-10 μ m thick, were fixed in 4% paraformaldehyde (30 min at 4°C), stained with hematoxylin and eosin and examined by light microscopy. For detection of CD4- and CD8-positive cells, tumour cryo-sections were fixed in cold acetone and incubated for 2 h with rat anti-CD4 or rat anti-CD8 antibodies (BD Pharmingen). The slides were then washed with PBS and incubated (1 h) with goat anti-rat antibody conjugated with Alexa Fluor 488 (Molecular Probes), washed and counterstained with PI (2 μ g/ml). The fluorophore-labelled tissue sections were analyzed using a Bio-Rad MRC 1024 scanning confocal fluorescence microscope equipped with LaserSharp software. Cryo-sections from three distinct parts of tumour from each individual mouse were analyzed.

Proliferative response. For priming the proliferative cell response, the TIL cells were cultivated for 3 days in complete RPMI medium with ConA (1 μ g/ml, Pharmacia AB, Uppsala, Sweden) in 96-well round-bottom microtiter plates (Nunc, A/S, Roskilde, Denmark). As positive control, the proliferative response of normal murine spleen cells was used. Twenty hours before harvesting, the cells were labelled with 0.04 MBq ³H-TdR/well and the uptake of ³H-TdR was measured in the liquid scintillation system. The stimulation index (SI) was calculated as the ratio of proliferation expressed in cpm/min in the experimental (with ConA) and control samples.

⁵¹Cr microcytotoxicity assay. The cytolytic activity of the fresh or pre-cultivated (24 h, in the complete RPMI medium supplemented with 20 U/ml rIL-2-Proleukin, Chiron B.V., Amsterdam, The Netherlands) was measured. TIL cells were tested using the ⁵¹Cr release assay, as described previously (3,15,21,24). ⁵¹Cr-labelled tumour targets were mixed with the effector cells in the target-to-effector cell ratio 50:1 in complete RPMI medium supplemented with mercaptoethanol (10⁻⁵ M). The mixtures were incubated in triplicate in 96-well round-bottom microtiter plates (Nunc, A/S). Percent of specific ⁵¹Cr release was expressed: [(cpm experimental release - cpm control release) / (cpm maximum release - cpm

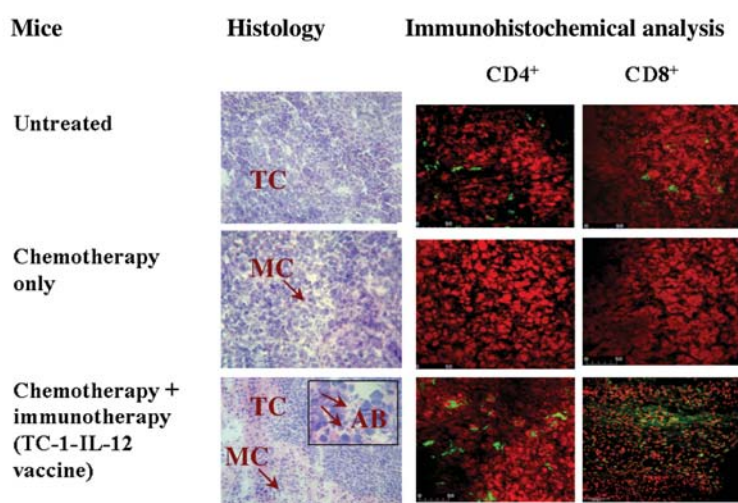
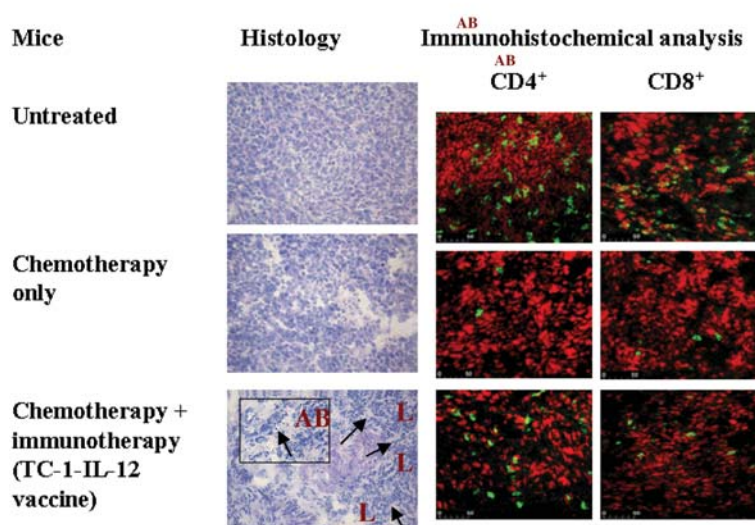
(a) tumour TC-1**(b) tumour TC-1/A9**

Figure 1. TIL infiltration during tumour growth and therapy. (a) Tumour TC-1, (b) tumour TC-1/A9. Histology: TC, tumour cells; MC, macrophage-like cells, AB, apoptotic bodies; L, lymphocytes. Magnification, 400x or 630x. Immunochemical analysis: red, tumour mass; green, CD4- or CD8-positive cells. Representative data from two independent experiments are shown.

control release)] x 100. As target cells, TC-1 and TC-1/A9-IFN γ (MHC I-positive cells, prepared after 72-h cultivation of MHC I-negative TC-1/A9 cells in the medium supplemented with 50 U/ml IFN γ ; RD Systems, Minneapolis, MN) were utilized (14).

Statistical analyses. For statistical analyses, Student's t-test from NCSS, Number Cruncher Statistical System (Kaysville, UT) statistical package was used.

Results

Histological and immunohistological examinations of TC-1 and TC-1/A9 tumour samples. Histological examination of control tumours (both TC-1 and TC-1/A9) revealed that they

formed nodules, with very compact tissue structure, closely adjacent tumour cells and poorly developed connective tissue (Fig. 1). Tumour cells of both types possessed features of typical quickly proliferating cells and were characterized by variable size, differently situated (centrally or peripherally) oval nucleus and slightly eosinophil cytoplasm. In the peripheral zone of untreated TC-1 tumour, we observed infiltration of sporadic macrophage-like cells.

Analysis of TC-1 tumours dissected after CBM-4A administration showed that the tumour tissue consisted of densely packed tumour cells with single migratory macrophage-like cells. In addition, the chemotherapy caused a total disappearance of T cells (Fig. 1). After IL-12 and chemotherapy, the increase in the macrophage-like cell number was observed. The administration of CBM-4A followed by TC-1-IL-12 cell

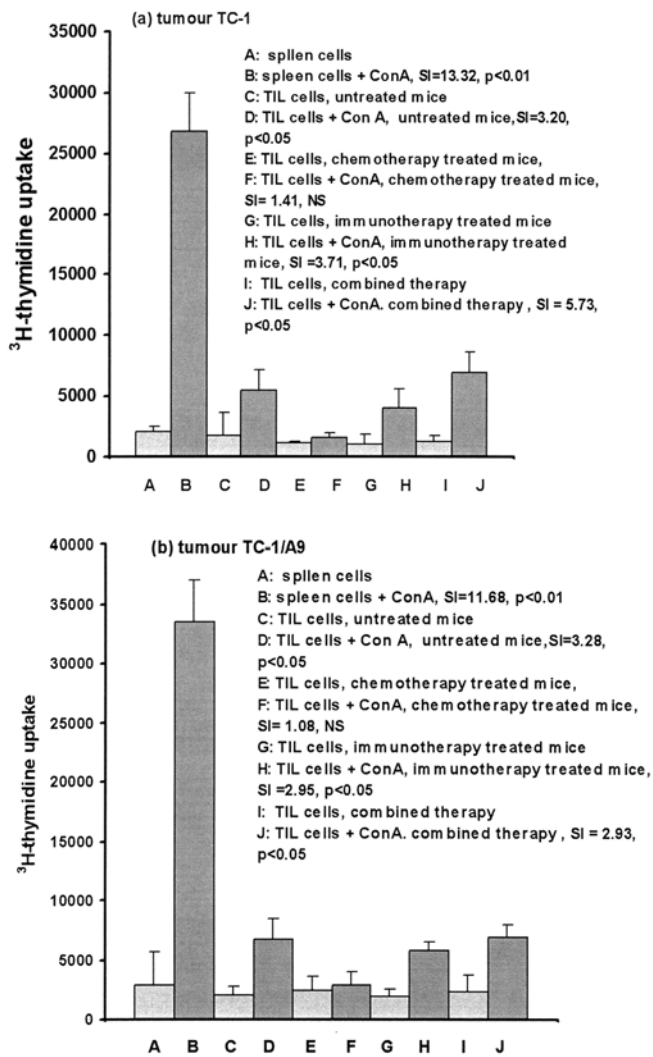


Figure 2. CD45⁺ tumour-infiltrating cells during tumour growth and therapy: *in vitro* proliferation is depressed after chemotherapy and recovered after immunotherapy. p<0.01, p<0.05, as compared to the non-stimulated cells.

vaccine evoked significant morphological changes in TC-1 tumour tissue. Between loosely arranged tumour cells, there was an abundance of macrophage-like cells which formed focuses and penetrated between tumour cells in the whole tissue (Fig. 1a). In this tissue, the majority of apoptotic cells

and necrotic areas were observed, and they were located both in the centre and in the periphery of tumour tissue (Fig. 1a). Moreover, administration of the TC-1-IL-12 cell vaccine after chemotherapy caused renewed influx of CD8⁺ T cells into the tumour nodules.

In the case of TC-1/A9 tumour tissue, visible morphological changes in the tissue structure occurred just after CBM-4A administration, including loosely arranged tumour cells and few areas with apoptotic cells. Furthermore, although the number of CD4- and CD8-positive T cells in the tissue decreased, they did not disappear, as was noted in TC-1 tumour tissue (Fig. 1b). On the other hand, adjuvant administration of the TC-1-IL-12 vaccine evoked almost complete decay of TC-1/A9 tumour tissue, similarly to TC-1 tumour tissue. TC-1/A9 tumour cells lost their typical morphology and this was accompanied by advanced disintegration of both the peripheral and central part of the tumour nodule, numerous apoptotic bodies and pre-necrotic areas surrounded by lymphocytes. Moreover, in the TC-1/A9 tumours, an influx of both CD4- and CD8-positive T cells, but especially CD4⁺, was observed (Fig. 1b).

Kinetics of the CD11c⁺, Gr-1⁺/CD11b⁺ and CD4⁺/CD25⁺ TIL during tumour growth and therapy. The FACS analyses of enzymatically digested tumours showed a significant difference in the number of infiltrated CD11c⁺ cells in the TC-1 and TC-1/A9 tumour tissue (Table I). However, in both types of the examined tumours, chemotherapy and also subsequent immunotherapy led to a significant increase in the amount of infiltrating CD11c⁺ cells. The kinetics of infiltrated myeloid-derived suppressor Gr-1⁺/CD11b⁺ cells was different. In both types of tumours, the amount of Gr-1⁺/CD11b⁺ cells significantly increased after chemotherapy and decreased again after IL-12 immunotherapy (Table II). On the other hand, the changes in the level of CD4/CD25-positive Treg cells were non-significant (p>0.05) and varied between 0.15-0.75% of the CD45⁺ cells (TC-1), or between 0.40-1.95% of the infiltrating CD45⁺ cells (TC-1/A9), respectively.

Proliferative CD45⁺ TIL responses. To compare the proliferative responses, CD45⁺ TILs were cultured for three days in the presence of ConA. Both TC-1 (MHC class I⁺) and TC-1/A9 (MHC class I⁺) TIL responded significantly to the mito-

Table I. Increase of the percentage of CD11c-positive cells after therapy.^a

Mouse tumour	Untreated	Chemotherapy only	Immunotherapy only	Chemotherapy + immunotherapy (TC-1-IL-12 vaccine)
TC-1	25.10±7.00 ^b	39.47±11.10 ^c	20.50±2.60	52.80±4.28 ^c
TC-1/A9	9.15±7.56	25.17±12.86 ^c	37.06±9.29 ^c	43.20±2.00 ^c

^a% in CD45⁺ subpopulation; after MACS separation of CD45⁺ (% CD45⁺ in the mixture of cells obtained after collagenase I + DNase treatment prior to MACS separation: 10-30%; the average TIL cell count, 10⁶ cells/1 cm³ of tumour mass). ^bp<0.05, compared to the untreated (TC-1/A9) group; ^cp<0.05, compared to the untreated control groups; the average values were calculated from 5-7 independent FACS determinations.

Table II. Increase of the percentage of myeloid-derived suppressor Gr-1/CD11b positive cells after chemotherapy accompanied by the decrease after subsequent immunotherapy.^a

Mouse tumour	Untreated	Chemotherapy only	Immunotherapy only	Chemotherapy + immunotherapy (TC-1-IL-12 vaccine)
TC-1	11.93±11.90	25.96±2.67 ^b	14.50±5.80	14.02±2.49
TC-1/A9	9.97±4.98	27.84±12.14 ^b	10.16±1.27	16.13±5.07

^a% in CD45⁺ subpopulation after MACS separation of CD45⁺ (% CD45⁺ in the mixture of cells obtained after collagenase I + DNase treatment prior the MACS separation: 10-30%; the average TIL cell count: 10⁶ cells/1 cm³ of tumour mass). ^bp<0.05, compared to the untreated control groups. The average values were calculated from 5-7 independent FACS determinations.

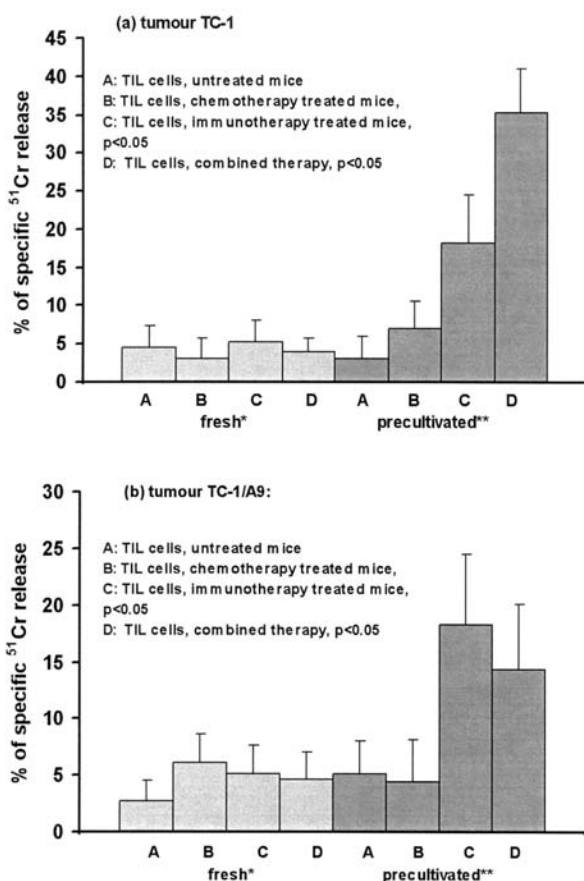


Figure 3. CD45⁺ tumour-infiltrating cells during tumour growth and therapy: recovery of the cytotoxicity after short-term culture *in vitro*. **ex vivo* effector cells; **Effector cells after 24-h precultivation *in vitro* in the medium enriched with 20 U/ml rIL-2; Effector/target cell ratio: 50:1. (a) Tumour TC-1: C x D, p<0.05 (fresh x precultivated effectors); C x D, p<0.05 (precultivated effectors). (b) Tumour TC-1/A9: C x D, p<0.05 (fresh x precultivated effectors); C x D, p>0.05, not significant (precultivated effectors).

genic stimulus of ConA. This response was decreased after chemotherapy and restored again after administration of the IL-12 vaccine (Fig. 2). As a control, the proliferative responses of normal splenocytes were assessed (Fig. 2).

Cytolytic activity of CD45⁺ TILs. Freshly isolated TIL cells did not exhibit any significant cytotoxic activity (Fig. 3).

However, after short *in vitro* precultivation in the presence of threshold doses of IL-2 (20 U/ml) a restored cytotoxic potential of TIL cells in both growing tumours, TC-1 and TC-1/A9, treated with IL-12 immunotherapy was found. Cytotoxic tumour-infiltrating CD45⁺ cells, detectable in the ⁵¹Cr microcytotoxicity assay, were found after immunotherapy with genetically modified, IL-12-producing cellular vaccine, as well as after the combined chemo-immunotherapy in both, TC-1 and TC-1/A9 tumours. Moreover, the combined chemo-immunotherapy of the TC-1 tumours, but not that of the TC-1/A9 tumours, augmented significantly the cytotoxic potential of precultivated CD45⁺ TIL. No cytotoxic effectors were detected after the cytostatic treatment only (Fig. 3).

Discussion

The efficacy of local immunotherapy with tumour vaccines engineered to produce IL-12 in the model of minimal residual tumour disease after cytoreductive chemotherapy (CMRTD) is based on the activation of a broad spectrum of different immunological mechanisms. In this study, we have focused on the analysis of distribution and activity of tumour-infiltrating cells using the model of subsequent therapy of CMRTD with IL-12-producing vaccines of HPV 16-associated murine tumours.

Cyclophosphamide and its derivatives are known as drugs with a broad spectrum of effects in the immune system. The most essential effects are the decrease of the activity of cytotoxic lymphocytes and modulation of cell lineages with immunosuppressive functions, i.e. Treg and myeloid-derived suppressor cells (25,26). While the effects on the Treg cells can stimulate the antitumour immunity, accumulation of MDSC is known to serve as an immunosuppressive mechanism (27). The accumulation of MDSC in the tumour after chemotherapy might cause the weak cytotoxic effect of TIL cells and loss of proliferative responses to mitogens. Moreover, restoration of cytotoxic and proliferative responses after IL-12 immunotherapy correlates with the decrease of tumour-infiltrated MDSC. These results are in agreement with the findings of Umenura *et al* (28) and Saio *et al* (29), who reported that the tumour-infiltrating MDSC strongly suppress T cell proliferation and the growth of activated CD8⁺ cells. On the other hand, we found a very low percentage of the CD4/CD25-positive Treg cells, which

remained unchanged during the growth and therapy of tumours. These findings correlate with our previous results (15) obtained in the same model, when similar dynamics of MDSC and Treg in the spleens of cured mice after chemotherapy and combined chemo-immunotherapy with IL-12-producing vaccines was found.

The increase in the percentage of infiltrated CD11c-positive cells after chemotherapy as well as after combined chemo-immunotherapy corresponds with the results obtained in the immunohistochemical analyses. In general, the increase of CD11c-positive TIL cells is associated with a better outcome in a variety of tumours (28). The level of infiltration in both our cell lines, TC-1 and TC-1/A9, was different. The TC-1/A9 tumour was characterized by higher infiltration of leukocytes, but this increased infiltration was not correlated with the cytotoxic effect of these cells. This finding could probably be connected with the MHC-negative status of the TC-1/A9 cells. A similar observation was published by Preynat-Seauve *et al.* (9), who found different tumour infiltration with immunocompetent dendritic cells in distinct murine melanoma tumours. These findings suggest that the tumour milieu varies in its capacity to mobilize immunocompetent cells. The kinetics of CD8- and CD4-positive cells also corresponded with the treatment of tumours. The CD8- and CD4-positive cells disappeared after chemotherapy and reappeared together with the cytotoxic activity after subsequent IL-12 vaccine therapy.

Taken collectively, the results imply that the treatment of CMRTD with genetically IL-12-modified tumour cells can restore and potentiate the mechanisms of tumour resistance and the appearance of TIL cells.

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