Loss of Tumorigenicity of Murine Colon Carcinoma MC38/0 Cell Line after Transduction with a Retroviral Vector Carrying Murine IL-12 Genes

(MC38 murine colon carcinoma / IL-12 transduction / tumorigenicity)

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Abstract. Cells of transplantable MC38 colon carcinoma of C57BL/6 mice were adapted to growth *in vitro* as the MC38/0 cell line. Along the establishing process, MC38/0 cells preserved their tumorigenicity. After transduction with a retroviral vector carrying murine interleukin 12 (*mIL-12*) genes and further selection, stable MC38/IL-12 transductant cells were obtained. These cells produced IL-12 (approx. 2500 ng/ml /5x10^5 cells /48 h) as evaluated in the optimized bioassay. After subcutaneous inoculation into syngeneic mice, the IL-12-modified cells demonstrated reduced tumorigenicity as compared to parental MC38/0 cells. Mice that rejected the MC38/IL-12 tumour became protected against subsequent challenge with MC38/0 cells. The obtained data indicate that the IL-12-transduced murine colon carcinoma cells could be used both as a model tumour for the study of mechanisms of anticancer immunity and/or as an adjuvant to cancer vaccines.

The aim of gene therapy of cancer is to enhance recognition of tumour cells by the host immune system, followed by the development of antitumour immunity (Baskar, 1996; Dupont, 2002). One of extensively studied cytokines is interleukin 12 (IL-12), which plays an essential role in activation of the Th1-type immunity, important to host defence against the tumour. Along with other cytokines (e.g. IL-18) it stimulates Th1 cells to IFNγ secretion; it can also promote the memory Th1 cells (Gately et al., 1998; Okamura et al., 1998). Stimulation of IFNγ production by IL-12 modulates numerous immunological functions, among them the level of MHC expression on the surface of antigen-presenting cells (APC). By induction of interferon-inducible protein-10 (IP-10) production, IL-12 indirectly participates in regulation of angiogenesis (Pertl et al., 2001). Various effects of IL-12 on Th2 lymphocytes, depending on both cytokine milieu and cell maturity stage, were observed (Gately et al., 1998). The effective expression of the *IL-12* gene introduced into tumour cells is therefore expected to be helpful both for studying the mechanisms underlying anticancer immunity as well as for adjuvant cancer vaccines.

In our previous studies, transplantable *in vivo* murine colon carcinoma MC38 was used as a model tumour for experimental immunotherapy (Pajtasz-Piasecka et al., 2001) and chemo-immunotherapy (Glazman-Kuśnierczyk et al., 1996; Kuśnierczyk et al., 1999). The antitumour immune response observed during the therapy brings about the need of adaptation of the tumour to *in vitro* culture conditions. The adaptation of MC38 colon carcinoma cells to *in vitro* growth, done by prolonged passaging of tumour cells, including alternate *in vitro/in vivo* passages, resulted in the MC38/0 established cell line. MC38/0 cells were further characterized with respect to their growth kinetics, surface antigen expression, and tumorigenicity.

In the presented paper, the process of adaptation of the MC38 tumour cells to *in vitro* growth as well as the *in vitro* and *in vivo* effects of transduction of established MC38/0 line cells with *mIL-12* genes have been described.

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Abbreviations: Con A – concanavalin A; EGFP – enhanced green fluorescent protein; FCS – foetal calf serum; Ig – immunoglobulin; IL-12 – interleukin 12; MC38 – murine colon carcinoma; PBS – phosphate-buffered saline; TC – tissue culture; TRV – time for reaching the volume of 1 cm^3.

Material and Methods

Mice and tumour system

The MC-38 murine colon tumour is a grade III adenocarcinoma, which was chemically induced in a C57BL/6 female mouse, in 1975, by Corbett et al. and used since then as a transplantable mouse tumour model. The cell tumorigenicity was evaluated by subcutaneous (s.c.) inoculation on the left dorsal side of syngeneic C57BL/6 female mice with 0.375–3 x 10^6 cells suspended in 0.2 ml of calcium- and magnesium-free PBS (PBS^-) per mouse. Tumour growth was checked three times a week by measuring two perpendicular diameters of the tumour nodule with Vernier callipers. The tumour volume was calculated in terms of the formula: ½ a x b^2, where a is the longer and b is the shorter diameter. The time required for reaching the volume of 1 cm^3 by the tumour (TRV) was evaluated graphically, from individual tumour growth curves. Mice surviving without symptoms of tumour growth for at least three months after tumour cell inoculation were rechallenged. *In vivo* experiments had been approved by the Local Ethical Committee for Animal Experiments.

Adaptation of MC38 colon carcinoma cells to growth in vitro

Subcutaneously growing tumours were removed aseptically, fragmented into small pieces and seeded in RPMI 1640 medium supplemented with 5% foetal calf serum (FCS, Gibco BRL, Life Technologies Invitrogen, Paisley, UK), 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamin, 5 x 10^{-5} M 2-mercaptoethanol, and 1 mM sodium pyruvate (all reagents were purchased from Gibco BRL). Primary cultures of tissue fragments were maintained for 7–10 days at 37°C in 5% CO_2/95% air atmosphere, then harvested with 0.25% trypsin/0.05% EDTA treatment and further cultured in monolayer. After more than 50 successive passages the cells were subjected to three alternate *in vitro/in vivo* passages. This resulted in the established cell line designated as MC38/0. The cell line *in vitro* growth kinetics was estimated in the modified colorimetric MTT bioassay, as described previously (Pajtasz-Piasecka et al., 1995).

Retroviral vector construction

The coding fragment of EGFP (*enhanced green fluorescent protein,* 750 bp, Clontech, USA, Genbank accession #U55761) was obtained by digesting with *Sma*I, dephosphorylated and ligated with *Not*I linker. The EGFP fragment was cut by the *Not*I enzyme and further inserted into the *Not*I position of the pSAMEN retroviral vector carrying neo^r as a selectable marker. Murine IL-12 cDNA (p35 and p40 fragments), derived from the pEM12 plasmid, were obtained by enzymes digesting: *Hind*III–*Xho*I for p35 and *Not*I–*Hind*III for p40, respectively. Then, both fragments were inserted into the *Not*I–*Xho*I positions of the pSAMEN plasmid vector carrying *neo* as the selectable marker. Both retroviral plasmids were kindly provided by Dr. R. G. Hawley, Toronto, Canada. Amphotropic packaging murine fibroblast cell line PA317 was transfected with proviral plasmids: pSAMEN/EGFP, resulting in amphotropic retroviral packaging cells PA SAM EGFP and pSAMEN/IL12 for obtaining PA SAMEN IL12 cells.

Transduction procedure

The cells were cultured in 60-mm tissue culture (TC) dishes (Falcon, BD Labware, NJ), 0.5 x 10^6 cells/well. After 24-h culture, medium was replaced with 1 : 2 diluted supernatant containing the PA SAMEN IL12 vector (10^4 CFU/ml) or 1 : 4 diluted supernatant containing the PA SAM EGFP vector (9 x 10^4 CFU/ml) in the presence of 8 µg/ml polybrene (Sigma, St. Louis, MO). After 24 h of incubation at 37°C, supernatants were replaced with fresh culture medium. Cells were further incubated for 24 h and then subjected to selection with geneticin (G418, Sigma) at 1 mg/ml concentration until the appearance of G418-resistant cell colonies, designated as MC38/IL-12 or MC38/EGFP, respectively.

Treatment with interferon (IFN)γ

The MC38/0 or MC38/IL-12 cells (0.4 x 10^6 cells/ml) were cultured in 6-well TC plates. After 48 or 96 h of incubation with or without 100 U of rmIFNγ/ml (Boehringer Mannheim, Mannheim, Germany), cells were harvested with 0.2% EDTA solution in PBS^- (Gibco BRL), washed and resuspended in PBS^-/2.5% FCS, at the density of 1 x 10^6 cells/ml. The effect of IFNγ on MHC class I antigen expression was analysed as described below.

Measurement of IL-12 bioactivity

MC38/IL-12 cells were settled on 24-well TC plates (5 x 10^5/well/1 ml) in RPMI 1640/5% FCS. After 48 h, cell supernatants were harvested, centrifuged and frozen at –70°C.

Mouse concanavalin A (Con A)-splenic blasts, after 72 h culture, were washed 3 times, resuspended, and settled in a 96-well microculture plate (Costar, Corning Inc., Corning, NY) at a density 0.4–1 x 10^5cells/well in 50 µl of RPMI 1640/10% FCS. Supernatants from MC38/IL-12 cultures were added in a volume of 50 µl/well in appropriate dilution in RPMI 1640 without FCS. After 44 h, and 4 h before the end of the incubation, 25 µl of MTT (Sigma) were added. Then, cell viability was measured in the colorimetric assay (Pajtasz-Piasecka et al., 1995).

Flow cytometry analysis of surface antigens

Growing *in vitro* cells were harvested using 0.2% EDTA solution in PBS^-, washed and resuspended in PBS^-/2.5% FCS, at a density of 10^6 cells/ml. The expression of MHC class I antigens was revealed with mouse anti-

mouse H-2K^{b}D^b immunoglobulin (Ig) (clone 28-8-6), diluted 1 : 200. Secondary antibody was FITC-conjugated rat anti-mouse Ig (clone R2-40) – diluted 1 : 200; both purchased from Pharmingen. The cells (50 ml of suspension) were incubated on ice with an equal volume of the primary antibody for 30 min, washed and next incubated with 50 µl of the secondary antibody, for 45 min, on ice. The analysis was carried out using a FACSCalibur flow cytometer with Cell Quest Software (Becton Dickinson, Immunocytometry Systems, San Jose, CA).

Intracellular cytokine staining

MC38/0 cells (negative control) and MC38/mIL-12 transductant cells were grown in the Chambered Coverglass System (Lab-Tec II) for 24 h and then treated with brefeldin A (5 µg/ml; 5 h at 37˚C), fixed in 2% paraformaldehyde, and permeabilized with 0.1% saponin. Then, cells were washed with PBS$^-$ and incubated with PE-conjugated rat anti mouse IgG_1 antibodies: anti-mIL-12 (clone C15.6), or appropriate isotype Ig (clone R3-34; both from Pharmingen). Incubation was performed for 2 h at room temperature. After cytokine staining, MC38/0 cell specimens were washed and analysed with a confocal microscope BioRad MRC 1024. Data were documented with Laser Sharp software (BioRad).

Results and Discussion

Adaptation of MC38 colon carcinoma cells to in vitro growth

During the process of adaptation of MC38 cells to *in vitro* growth, a gradual decrease in the percentage of cells expressing MHC class I antigens was observed. However, three *in vivo/in vitro* passages (between 56th and 76th *in vitro* passage) resulted in a slight increase of the antigen expression. Eventually, 6–15% of MC38/0 cells demonstrated a low level of the antigen.

To estimate the number of MC38/0 cells necessary for tumour induction, mice were inoculated s.c. with an increasing number of cells. Starting from inoculum of 0.75 x 10^5 cells/mouse, the tumour takes were almost 100% (15/16 mice), but with prolonged TRV (42.7 days). The increase of the injected cell number resulted both in repeatable 100% of tumour takes and in TRV shortening. Hence, for further experiments, the inoculum of 1 x 10^6 cells/mouse was used (Table 1). The s.c. inoculation of MC38/0 cells into syngeneic C57BL/6 mice resulted in carcinoma nodules containing strips of the vital tumour alternated with necrotic fields, similar in the histological picture to original MC38 tumours (Kuśnierczyk et al., in press).

The original MC38 colon carcinoma obtained by Corbett in 1975 gave metastases in 35–50% of tumour-bearing mice. However, during the comparative histological studies performed by Wietrzyk et al. (2002) on MC38 tumours growing in mice after s.c. inoculation or orthotopical transplantation, only few spontaneous metastases were observed. As compared to parental MC38 cells, the tumorigenicity as well as the metastatic ability of the established MC38/0 cell line remained unchanged. It occurred that the MC38/0 cells could be an equivalent to MC38 *in vivo* transplantable tumour used in our previous studies (Pajtasz-Piasecka et al., 1995; Glazman-Kuśnierczyk et al., 1996; Kuśnierczyk et al., 1999; Pajtasz-Piasecka et al., 2001). The MC38/0 cells also appeared to be convenient for non-transforming retroviral vectors containing neo^r selective marker titration, due to their high rate of cell proliferation (Fig. 1) and high sensitivity to the selecting agent (geneticin, G418).

Table 1. Tumorigenicity of MC38/0 cells and their transductants

Cell type (1 x 10^6/mouse*)	**Number of growing tumours /Number of mice in the group**	**Time to reach 1 cm^3 tumour volume** Mean day (range)**
MC38/0	16/16	31.3 (24.4 - 44.1)
MC38/EGFP	5/5	39.9 (34.9 – 45.4)
MC38/IL-12A	3/5	41-61.9
MC38/IL-12C	0/10	---

*Cells were inoculated into the right flanks of C57BL/6 female mice.

**The tumour volume was calculated in terms of the formula: ½ a x b^2, where a is the longer and b is the shorter diameter.

Effects of mIL-12 gene transduction

The MC38/0 cells were transduced with two amphotropic retroviral vectors, both constructed based on the pSAMEN plasmid with the *neo^r* reporter gene. Transduction with PA SAM EGFP and short selection with 1 mg/ml of G 418 resulted in the MC38/EGFP cell line with more than 90% EGFP-positive cells (Fig. 2). MC38/EGFP cells preserved the typical kinetics of *in vitro* growth as well as the tumorigenicity of parental MC38/0 cells (Fig. 1, Table 1). The tumour individual growth curves are presented in Fig. 6B. After injection of 1 x 10^6 cells, 100% of mice developed tumours. The differences in TRV between MC38/0 cells (approx. 31 days) and MC38/EGFP (approx. 40 days) were not significant. Due to EGFP expression, the transductant cells could be easily traced in *in vivo* experiments. Therefore, the MC38/EGFP cells were applied for *in vivo* studies of the mechanisms of the antitumour immune response.

To obtain stable IL-12 transductants, MC38/IL-12 cells were subjected to prolonged G418 *in vitro* selection and, during this procedure, repeatedly checked for

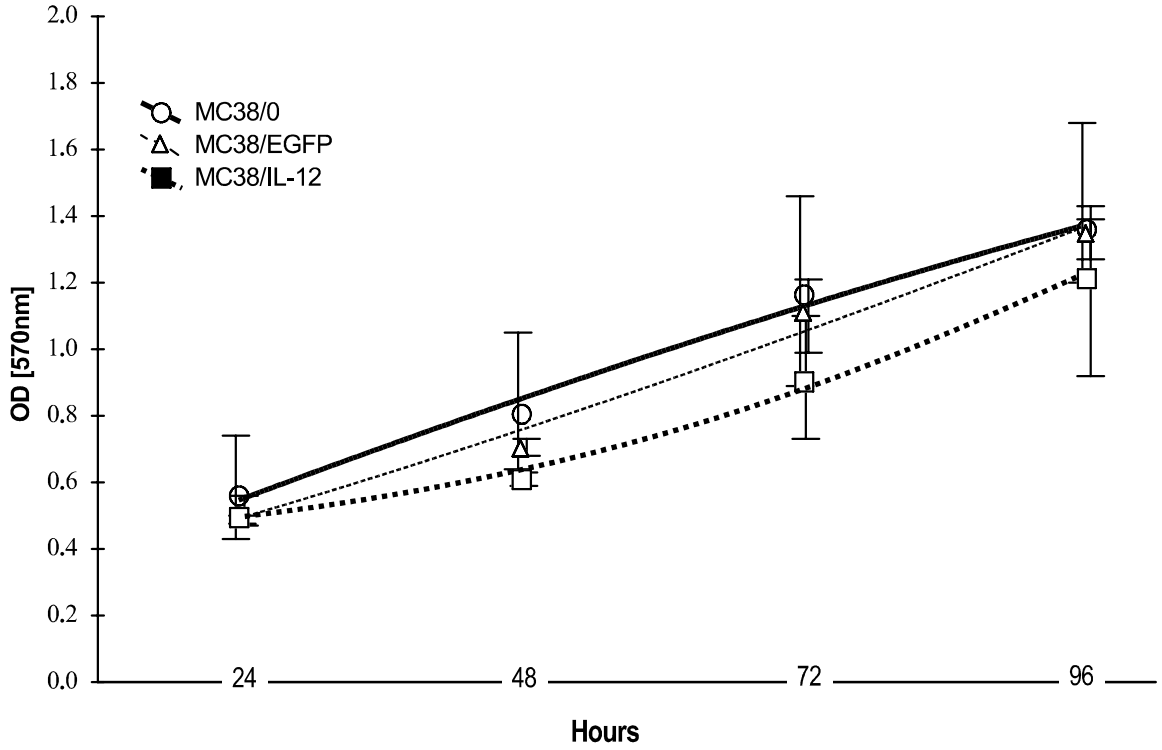

Fig. 1. Kinetics of *in vitro* growth of MC38 colon carcinoma cells and their transductants
Cells were settled in a concentration 10 x 10^3 cells/well and cultivated for 24–96 h in standard conditions. The amount of viable cells was estimated in the colorimetric bioassay (see: Material and Methods) and presented as a level of extinction, OD [570 nm].

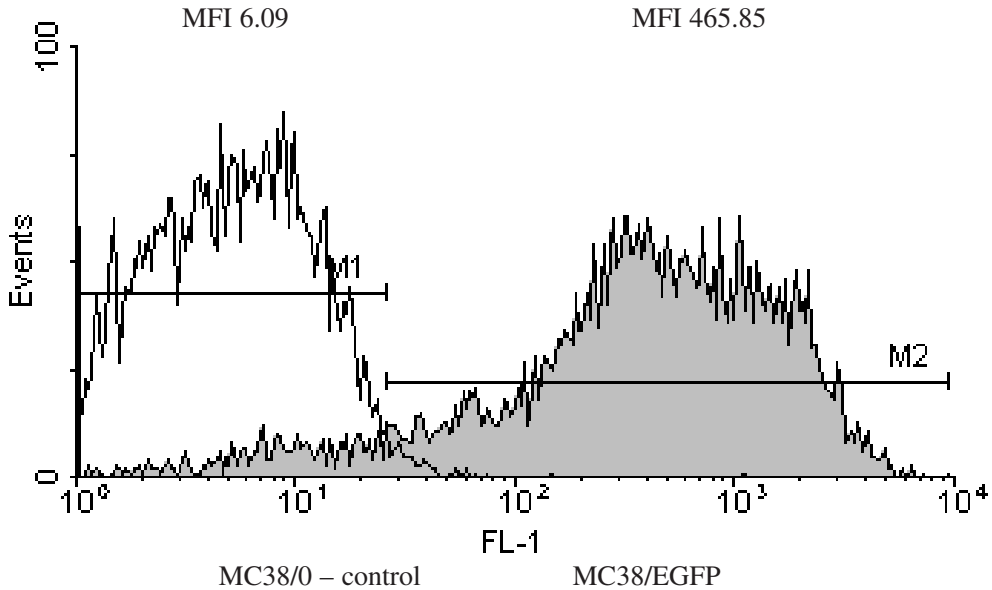

Fig. 2. Enhanced green fluorescent protein (EGFP) expression in MC38/0 cells transduced with the SAMEN/EGFP retroviral vector, on the fifth day of G418 selection
The transduced cells were evaluated with a flow cytometer (FACSCalibur, Becton Dickinson, Palo Alto, CA). Dead cells were excluded with propidium iodide (25 µg/ml). Five thousands of viable cell signals per probe were registered and further analysed with the use of Cell Quest software (Becton Dickinson). The intensity of fluorescence is expressed as a mean geometric channel of fluorescence intensity (MFI).

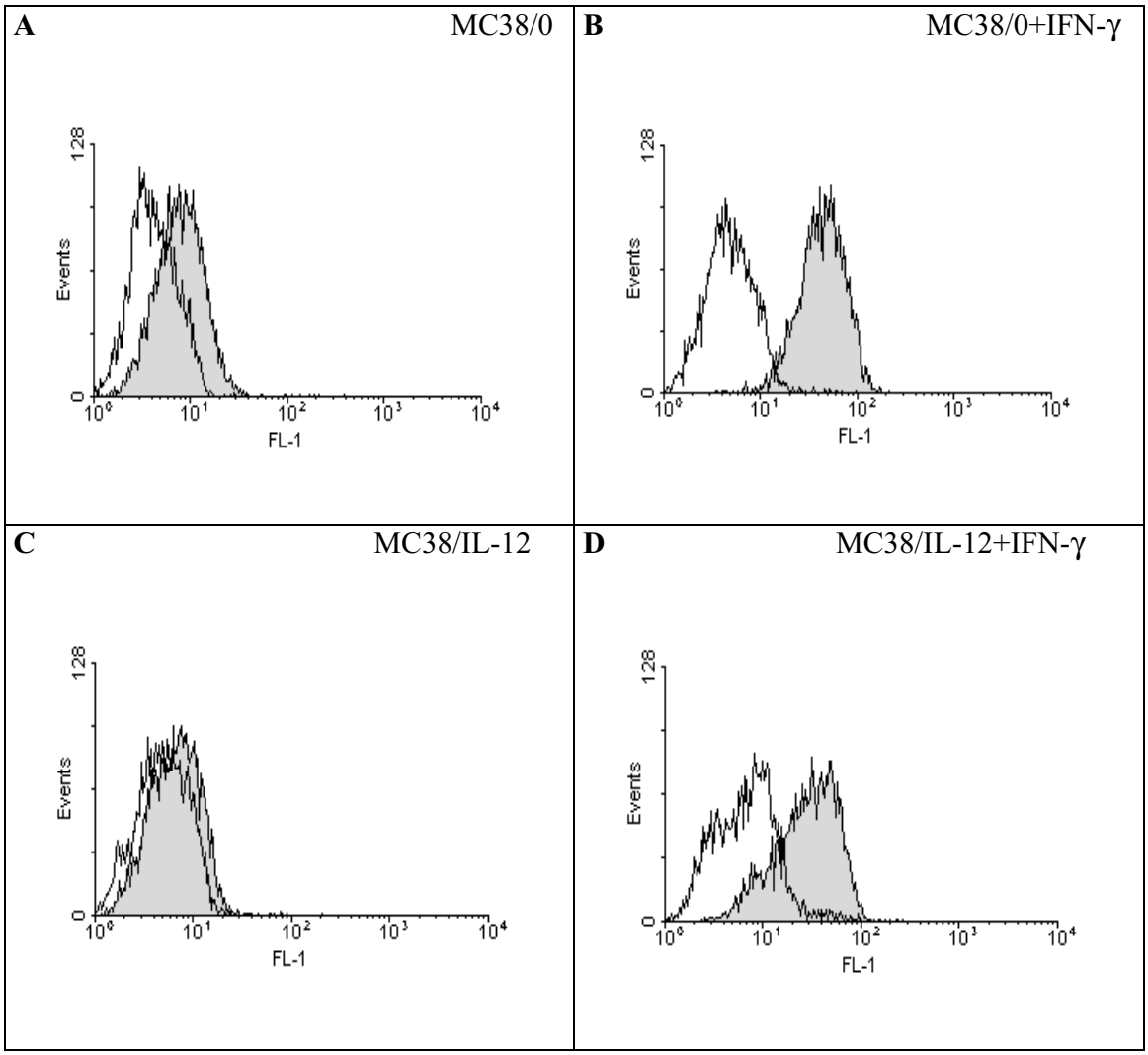

Fig. 3. Effect of IFNγ on MHC class I molecule expression on MC38/0 cells and MC38/IL-12 transfectants
Cells were incubated for 96 hours with or without 100 U IFNγ/ml. A – MC38/0 control cells (8% of positive cells) or B – MC38/0 cells after incubation with IFNγ (95% of positive cells), C – MC38/IL-12 control cells (3% of positive cells) or D – MC38/IL-12 cells after incubation with IFNγ (57% of positive cells). Antibodies: primary anti-H-2K^bD^b (mouse anti-mouse, diluted 1 : 200) and secondary rat anti-mouse Ig conjugated with FITC – diluted 1 : 200), both Pharmingen, were used in indirect fluorescence reaction. The cell fluorescence was evaluated with a flow cytometer (FACSCalibur, Becton Dickinson). Dead cells were excluded with propidium iodide (25 μg/ml). Five thousands of viable cell signals per probe were registered and further analysed with the use of Cell Quest software (Becton Dickinson).

the IL-12 production. During the selection, the proliferative capacity of MC38/IL-12 cells remained unchanged, as compared to MC38/EGFP (Fig. 1).

In experiments evaluating MC38/0 cell growth kinetics and their sensitivity to selected cytokines, the effect of IFNγ was observed. Namely, in the presence of high concentrations of rmIFNγ (1000 or 2500 U/ml), the MC38/0 cell proliferation was restrained by 30–40%. On the other hand, the incubation of MC38/0 cells with 100 U rmIFNγ/ml resulted in the increase of MHC class I expression, both on the parental MC38/0 cells (Fig. 3 A, B) as well as on one of several independently transduced variants – MC38/IL-12C (Fig. 3C, D). The influence of IFNγ on murine MHC class I negative MK16 kidney carcinoma cells was described by Indrová et al. (2002). They observed an apparent increase of the class I MHC molecule expression on the cells in the presence of this factor accompanied with an increased sensitivity of MK16 tumour cells to cytotoxic T lymphocytes (Indrová et al., 2002).

A supplementation of culture medium with such cytokines as IL-2, tumour necrosis factor (TNF)-α or granulocyte-macrophage colony-stimulating factor (GM-CSF) didn't affect *in vitro* growth of MC38/0 cells (data not shown).

Two of the selected transductants: MC38/IL-12A and MC38/IL-12C, were further characterized for IL-12 production and tumorigenicity after s.c. inoculation into

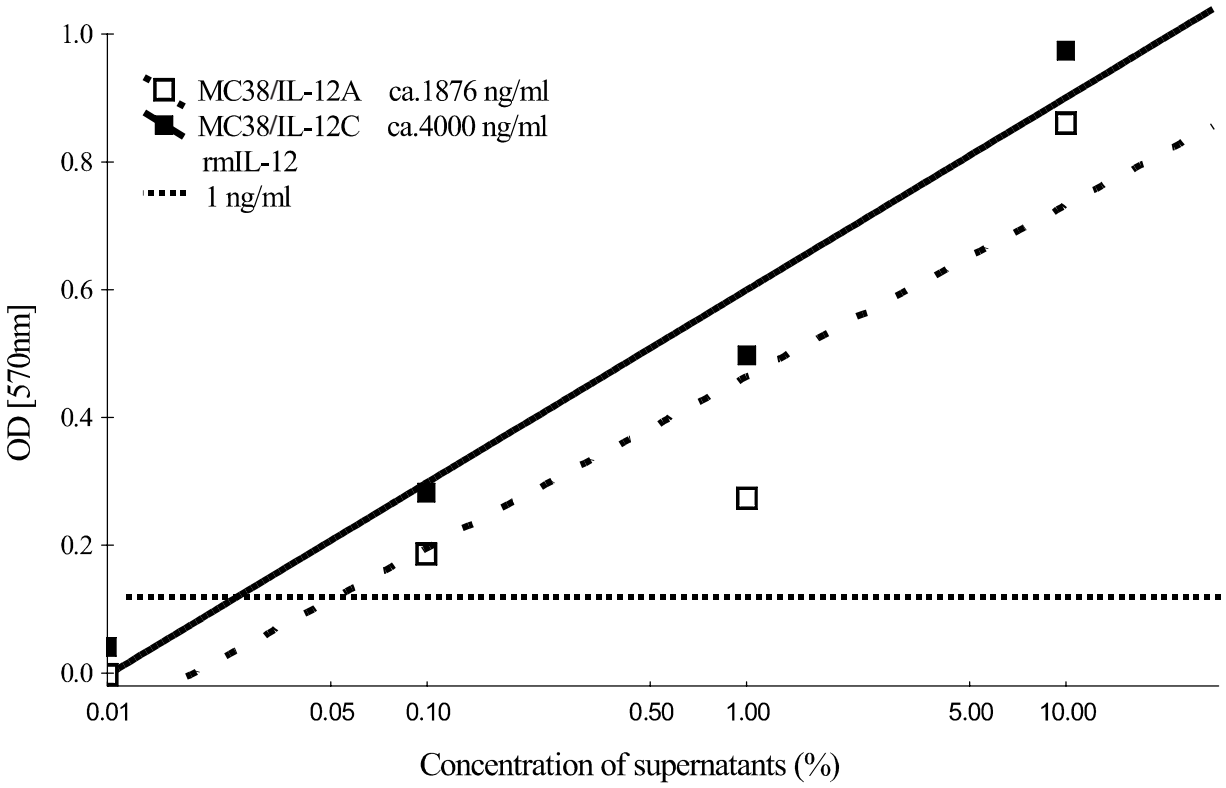

Fig. 4. The bioactivity of IL-12 secreted to culture medium by two variants of MC38/IL-12 transductants as evaluated in Con A-blast bioassay (for details see Material and Methods)

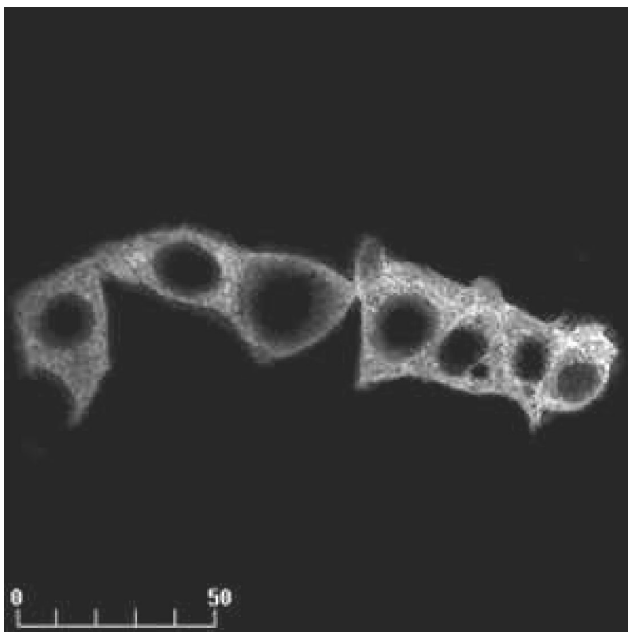

Fig. 5. Visualization of the IL-12 protein in MC38/IL-12 transductant cells
Cells were stained intracellularly with anti-mIL-12 antibody conjugated with PE, and cells were further visualized with a confocal microscope BioRad MRC 1024. The bar indicates 50 μm.

mice (Table 1). The MC38/IL-12C cells appeared to be better IL-12 producers than MC38/IL-12A cells. The level of IL-12 produced by MC38/IL-12C cells amounted to 3500–5500 ng/ml, whereas for MC38/IL-12A those values did not exceeded 2500 ng/ml. The results of the representative bioassay for IL-12 secreted to culture medium by mIL-12-transduced MC38/0 cells are shown in Fig. 4. Additionally, the visualization of IL-12 protein expression in cell plasma was performed by immunochemistry and confocal microscopy (Fig. 5).

To determine whether MC38/IL-12 cells secreting mIL-12 are able to evoke protection against the parental MC38/0 cell challenge, they were inoculated s.c. (1×10^6). In the case of MC38/IL-12A cells, two out of five inoculated mice rejected the challenge. Tumour nodules grew in the three remaining mice, with TRV calculated as 45–61 days (Table 1, Fig. 6C). In MC38/IL-12C cells, tumorigenicity was completely lost (Table 1). From ten animals inoculated with 1×10^6 cell transductants, staying without symptoms of tumour growth for more than 54 days, five mice were rechallenged with 1×10^6 MC38/0 cells injected in the same location. Only one of the rechallenged mice developed a palpable tumour nodule on 57^{th} day after inoculation. The challenge with a higher number of MC38/IL-12C cells (4.5×10^6/mouse) resulted in tumour growth in two out of six inoculated mice (data not shown).

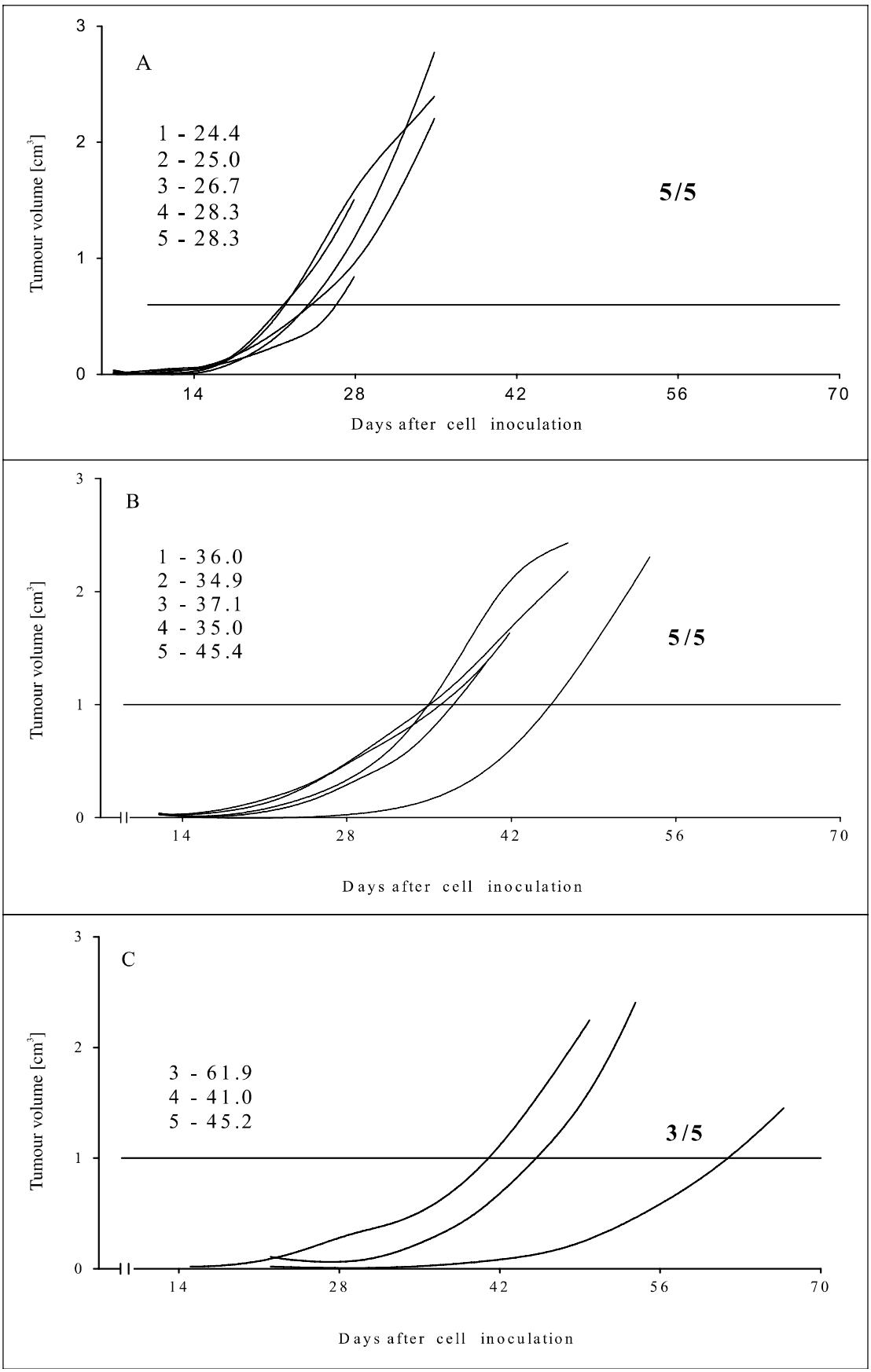

Fig. 6. Comparison of *in vivo* tumour growth of MC-38/0 (A), MC-38/EGFP (B), and MC38/IL-12A cells (C) Cells were inoculated s.c. in a number of 10^6/mouse. The tumour growth curves in individual mice are presented and numbers of days to reach 1 cm^3 (TRV) were given in relevant figures. The level of IL-12 production by MC38/IL-12 cells amounted up to 1500 ng/ml.

The observation that IL-12-producing MC38 tumour cells have a significantly decreased tumorigenicity is in accordance with reports by others describing different tumour cells engineered to secrete various cytokines (Baskar, 1996; Bubeník, 1996). However, in spite of a long history of tumour investigations, the understanding of a particular cytokine influence on regulation, coordination and integration of the anticancer immunity is still insufficient. Therefore, the MC38/mIL-12 cells seem to be a useful tool for such a study.

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