Local IFN-γ Therapy of HPV16-Associated Tumours

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(MHC class I / IFN-γ / residual tumour disease / HPV16)

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Abstract. We have examined whether peritumoral administration of IFN-γ can inhibit growth of HPV16-associated, MHC class I⁻ tumour MK16/1/IIIABC (MK16) transplanted in syngeneic mice. It has been found that peritumoral administration of recombinant IFN-γ performed on days 0-11 after tumour challenge inhibited growth of MK16 s.c. tumour transplants. If the therapy with IFN-γ was started when the tumours had already reached a palpable size, the IFN-γ administration was without any effect. To investigate the antitumour effects of IFN-γ in a clinically more relevant setting, surgical minimal residual tumour disease was utilized. Subcutaneously growing MK16 carcinomas, 8–12 mm in diameter, were removed and the operated mice were injected with IFN-γ on days 3–14 after the operation at the site of surgery. Treatment with IFN-γ resulted in a moderate, reproducible, but statistically insignificant inhibition of tumour recurrences. In the next experiments we have addressed the question whether the tumour-inhibitory effect of IFN-γ was due to the upregulation of MHC class I molecule expression on MK16 tumour cells. IFN-γ-treated and control mice were sacrificed, their tumours were explanted, and the expression of MHC class I molecules on the MK16 tumour cells was examined. As presumed, the MHC class I expression on the cells of IFN-γ-treated tumours, as well as on their lung metastases, was upregulated. However, an unexpected moderate upregulation of the MHC class I expression was also observed on MK16 tumours from the control, exoge- nous IFN-γ-uninjected mice. Cytofluorometric analysis of the _in vivo_ transplanted MK16 tumours from both groups has excluded that the increased percentage of the MHC class I molecules on the tumour cell popula- tions could be due to the infiltration of the tumours with MHC class I⁺ leukocytes, since no expression of MHC class II, CD11b, CD80/CD86, and CD11c mole- cules in the MK16 cell population was observed.

In previous studies we have used an experimental model of a murine human papilloma virus type 16 (HPV16)-associated tumour MK16, which mimics human HPV16-associated neoplasms with regard to their aetiology, major histocompability complex (MHC) class I molecule negativity and ability to metas- tasize (Šmahel et al., 2001), to asses whether despite the absence of the MHC class I molecules on the surface of tumour cells such a tumour is suitable for cytokine ther- apy. It has been found that the MK16 cells are sensitive to the therapy with immunomodulatory cytokines inter- leukin-2 (IL-2), IL-12 and GM-CSF (Bubeník et al., 1999; Indrová et al., 2001, 2002; Mikyšková et al., 2001; Bubeník et al., in press), although to a lesser degree than MHC class I⁺ tumours of the same aetiolo- gy (Indrová et al., submitted). We have also found that cultivation of the MK16 cells in medium supplemented with interferon γ (IFN-γ) resulted in upregulation of MHC class I molecule expression and _in vitro_ induced the sensitivity of the MK16 cells to the cytolytic effect of lymphocytes from mice immunized with irradiated MK16 tumour cells (Mikyšková et al., 2001; Indrová et al., 2002).

Based on these findings, we have designed experi- ments to examine whether the upregulation of MHC class I molecule expression on the surface of MK16 tumour cells by IFN-γ can also be induced _in vivo_ and whether peritumoral administration of the IFN-γ can inhibit growth of the MK16 tumour in syngeneic mice.

Material and Methods

Mice

B6 male mice, 8–10 weeks old, were obtained from Anlab Co., Prague, Czech Republic.

Cell lines

The MK16/1/IIIABC (MK16) carcinoma cell line of B6 origin was developed by *in vitro* co-transfection of murine kidney cells with a mixture of activated *ras* (plasmid pEJ6.6), HPV16 E6/E7 (plasmid p16HHMo) and neomycin resistance (plasmid pAG60) DNA. The resulting MK16 cells were obtained after three passages to increase their oncogenicity in syngeneic C57BL/6 (B6) mice. The MK16 cell line was found to be MHC class I-negative and spontaneously metastasizing into the lungs (Šmahel et. al, 2001). The MK16 cells were maintained in RPMI 1640 medium supplemented with 10% foetal calf serum (BioClot Ltd., Aidenbach, Switzerland), L-glutamin (Sevac, Prague, Czech Republic), penicillin (Biotika, Slovenska Lupča, Slovakia), and streptomycin (Sigma, Steinheim, Germany). The cell line was kept at 37° C in a humidified atmosphere with 5% CO_2.

IFN-γ treatment of s.c. tumours and surgical residual tumour disease

For therapy of s.c. growing MK16 tumours, B6 mice were inoculated subcutaneously on day 0 with 10^6 MK16 cells. Mice were randomly divided into experimental and control groups. The first group of mice was treated twice a day on days 0–4 and 7–11 after inoculation, s.c. in the vicinity of MK16 tumour inoculum, with 0.5 µg of IFN-γ (R&D Systems, Minneapolis, MN) per dose. The second experimental group was left untreated until day 9, when the tumours had reached a palpable size of 0.4 cm in diameter. The mice were then injected with 0.5 µg of IFN-γ, peritumorally, twice a day, on days 9–11, 14–18 and 21–25. The control group was not treated. During the experiments, mice were observed twice a week, and the number of tumour-bearing mice together with the size of the tumours were recorded. Mice with large MK16 tumours from the experimental and control groups were sacrificed, their subcutaneous tumours and lung metastases were removed, minced with scissors and grown *in vitro* for 2-4 weeks. The expression of MHC class I, MHC class II, CD80, CD86, CD11b and CD11c molecules on the explanted cells was repeatedly analysed by flow cytometry.

For therapy of surgically induced minimal residual tumour disease, B6 mice were inoculated s.c. with 5 x 10^5 MK16 cells on day 0. After 30 days, when the transplanted tumours reached approximately 8–12 mm in diameter, the tumours were excised under i.p. anaesthesia, leaving no macroscopically visible tumour residuum. The hypothetical microscopic tumour residua after surgery were designated as surgical minimal residual tumour disease (Vlk et al., 1998). Mice were treated twice a day on days 3–7 and 10–14 after the operation with 0.5 µg of IFN-γ injected into the site of the operation. A group of the operated-only mice served as controls. The mice were observed twice a week and the numbers of mice with tumour recurrences and the size of tumours were recorded.

Flow cytometry

For detection of MHC class II, CD80, CD86, CD11b and CD11c molecules, fluorescein isothiocyanate (FITC)-anti-I-A^b mAb (clone AF6-120.1, Pharmingen, CA), FITC-anti-CD80 mAb (clone 16-10A1, Pharmingen, CA), PE-anti-CD86 mAb (clone GL1, Pharmingen, CA), FITC-anti-CD11b mAb (clone M1/70, Pharmingen, CA), and FITC-anti-CD11c mAb (clone HL3, Pharmingen, CA) were utilized, respectively. The MHC class I expression was determined by cytofluorometric analysis with anti-mouse H-2K^b/H-2D^b mAb (clone 28-8-6, Pharmingen, CA) and FITC-conjugated goat anti-mouse Ig antibody (Pharmingen, CA). As a control, FITC-labelled antibody of irrelevant specificity (clone G235-2356, Pharmingen, CA) and PE-labelled antibody of irrelevant specificity (clone R35-95, Pharmingen, CA) were utilized. To examine MHC class I expression on the MK16 cells after cultivation in medium with IFN-γ, the MK16 cells were grown for 24, 48 or 72 h in the complete RPMI 1640 medium supplemented with 1 µg /ml IFN-γ. After 72 h cultivation in medium with IFN-γ, the MK16 cells were cultivated further in the RPMI medium without IFN-γ for 4, 7 or 11 days. Cells were washed in PBS supplemented with 1% bovine serum albumin and 0.1% sodium azide, and diluted to obtain 5 x 10^5 cells/50 µl. Subsequently, the cells were incubated with the relevant mAbs at a concentration of 3–4 µg/ml at 4°C for 30 min. Flow cytometry was performed using an ELITE flow cytometer (Coulter, Miami, FL).

Statistical analyses

For statistical analyses, analysis of variance from NCSS, Number Cruncher Statistical System (Kaysville, UT), statistical package was used.

Results

IFN-γ upregulates MHC class I molecule expression on MK16 cells in vitro

To investigate the kinetics of MHC class I upregulation by IFN-γ *in vitro*, the MK16 cells were grown for 24, 48 and 72 h in the medium supplemented with IFN-γ, and the expression of the MHC class I molecules was assessed by flow cytometry. It can be seen in Fig. 1b-d that the MHC class I molecule expression increased up to 48 h of treatment. To examine the dependence of the
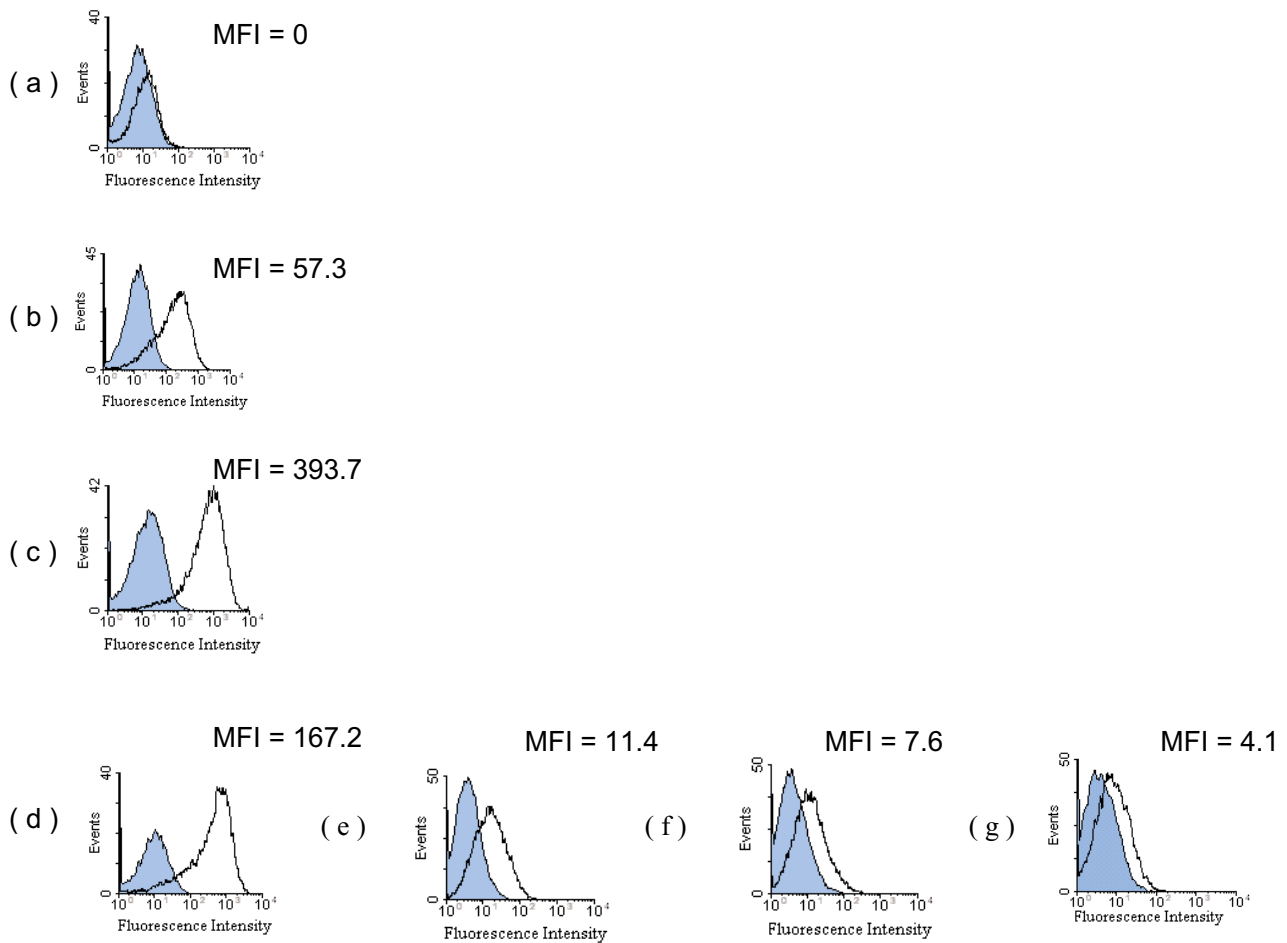
Fig. 1. Changes in the expression of MHC class I molecules on MK16 cells produced by IFN-γ *in vitro*. MK16 cells grown in (a) RPMI 1640; (b) RPMI 1640 supplemented with 1 μg IFN-γ/ml, 24 h; (c) RPMI 1640 supplemented with 1 μg IFN-γ/ml, 48 h; (d) RPMI 1640 supplemented with 1 μg IFN-γ/ml, 72 h; (e) grown as (d) and then for another 4 days without IFN-γ; (f) grown as (d) and then for another 7 days without IFN-γ; (g) grown as (d) and then for another 11 days without IFN-γ.

upregulated expression of MHC class I molecules on the presence of IFN-γ in the medium after the maximum upregulation had been reached, the MK16 cells were further cultivated in the absence of IFN-γ. As shown in Fig. 1e–g, the expression of MHC class I molecules had gradually decreased during 4–11 days of cultivation in the medium without IFN-γ.

IFN-γ therapy of s.c. growing tumours

B6 mice were inoculated subcutaneously on day 0 with 10^6 MK16 cells and divided into experimental and control groups. The first group of experimental mice was then treated twice a day on days 0–4 and 7–11 after inoculation, s.c. in the vicinity of MK16 tumour inoculum, with 0.5 μg of IFN-γ. The second experimental group was left untreated until day 9, when the tumours had already reached 0.4 cm in diameter, and then the mice were injected with 0.5 μg of IFN-γ, peritumorally, twice a day, on days 9–11, 14–18 and 21–25. The control group was left untreated.

As shown in Fig. 2, therapy of the MK16 tumours by administration of recombinant IFN-γ in the vicinity of tumour challenge on days 0–4 and 7–11 significantly (P < 0.05) inhibited growth of the s.c. tumours. In the group of mice in which the IFN-γ therapy was started on day 9, when the tumours had already reached a palpable size and in which IFN-γ treatment was given on days 9–11, 14–18 and 21–25, no significant tumour-inhibitory effects were observed.

Changes of MHC class I molecules expression on the MK16 cells in vivo

Mice with large MK16 tumours from the experimental and control groups were sacrificed, their tumours were removed, minced with scissors and grown *in vitro* for 2–4 weeks. The expression of MHC class I, MHC class II, CD80, CD86, CD11b and CD11c molecules on the explanted cells was then repeatedly analysed by flow cytometry. As can be seen in Fig. 3, which illustrates representative results obtained in repeated experiments, MHC class I molecule expression on the MK16
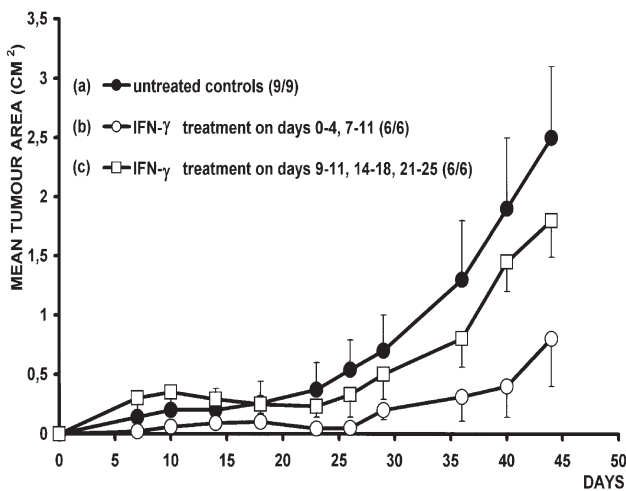
Fig. 2. Local IFN-γ therapy of MK16 tumours: (a) untreated controls; (b) IFN-γ (1 μg/mouse/day) on days 0–4 and 7–11; (c) IFN-γ (1 μg/mouse/day) on days 9–11, 14–18, and 22–27 (No. of tumour-bearing mice/Total No. of mice) axb, $P < 0.05$.

tumour increased after *in vivo* growth of the neoplasms, as compared to MK16 cells grown *in vitro* (Fig. 3Aa), both in the s.c. tumours (Fig. 3Ba) and in their metastases (Fig. 3Bb). Peritumoral s.c. administration of IFN-γ did not significantly enhance the MHC class I molecules expression, as compared to the tumours from the IFN-γ-untreated group of mice (Figs. 3Ca,b). Fig. 4 shows that the MHC class I upregulation *in vivo* is not due to the tumour infiltration by the MHC class II^{+}, CD11b^{+}, CD11c^{+}, and CD80/CD86^{+} cells, since such cells were absent in the MK16 cell populations.

IFN-γ therapy of surgical minimal residual tumour disease

In minimal residual disease after surgery, the local treatment with IFN-γ was investigated. Mice were inoculated s.c. with MK16 cells and, when their tumours reached 8–12 mm in diameter, the tumours were excised. From day 3 after the surgery, the group of experimental mice was treated with two cycles of IFN-γ injections. As can be seen in Fig. 5, treatment with IFN-γ resulted in moderate, but statistically insignificant ($P > 0.05$) inhibition of the growth of tumour recurrences. The experiment was repeated with similar results (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, since it provides the necessary prerequisite for the tumour-inhibitory effects of MHC class I-restricted mechanisms, such as CD8^{+} and CD4^{+} T cells, which are the primary defence anti-tumour mechanisms to recognize and kill tumour cells.

We have used an experimental model of the HPV-associated MK16 carcinoma (Šmahel et al., 2001) transplanted in syngeneic mice, which mimics the MHC class I^{−} HPV16-associated human tumours (Connor et al., 1990; Garrido et al., 1997; Hicklin et al., 1999) to examine whether the MHC class I^{−} tumour is sensitive to the immunotherapy with IFN-γ and whether peritumoral IFN-γ administration can increase MHC class I expression on the surface of the *in vivo* growing tumour cells. It was found that peritumoral injections of IFN-γ could inhibit tumour growth if administered in a very early period of tumour growth (day 0–11 after transplantation of the tumour cells) but not later, when palpable MK16 tumours had already developed (days 9–25). In accordance with these findings it was also observed that administration of IFN-γ, which started 3 days after surgical removal of the growing tumour, could inhibit, non-significantly but reproducibly (Fig. 5, and data not shown), MK16 tumour growth. As compared to other immunoregulatory cytokines, IL-2 and IL-12, exogenous administration of IFN-γ did not produce such well-expressed antitumour effects as those that were seen with IL-2 and IL-12 (Bubeník et al., 1999; Indrová et al., 2001, 2002; Mikyšková et al., 2001; Bubeník et al., in press). Nevertheless, the combination of IFN-γ with other immunoregulatory cytokines can theoretically potentiate the effects of cytokine monotherapies and therefore is worth of preclinical trials. IFN-γ is known to upregulate the expression of MHC and co-stimulatory molecules as well as to enhance the tumour-inhibitory effects of various MHC class I-restricted and -unrestricted tumour defence mechanisms; it can also exert its effect by inducing production of the anti-angiogenic factors IP-10 and MIG (Angiolillo et al., 1995; Brunda et al., 1995; Strasly et al., 2001; Segal et al., 2002). Interestingly, the MHC class I molecule upregulation on the surface of the MK16 tumour cells expected after peritumoral injections of IFN-γ was also observed in control mice uninjected with exogenous IFN-γ. The reason for the MHC class I upregulation on the surface of MK16 cells transplanted *in vivo* is not known. Apparently, the upregulation is not due to the infiltration of the MK16 tumours by leukocytes of the tumour bearers, since no increases of CD11b^{+}, MHC class II^{+}, CD80^{+}, CD86^{+} and CD11c^{+} cells in the *in vivo* growing tumours have been observed. It is not excluded that a concomitant immunity directed against specific oncoproteins of the MK16 cells, against the E6/E7 HPV16 oncoproteins and G12V activated *ras* oncoprotein can develop, which leads to endogenous IFN-γ production that can upregulate MHC class I molecule expression, in analogy with the *in vitro* findings (Fig. 1, Mikyšková et al., 2001; Indrová et al., 2002). The upregulation of MHC class I molecule expression can then make the MK16 cells sensitive to MHC class I-restricted tumour-inhibitory mechanisms and contribute in this way to
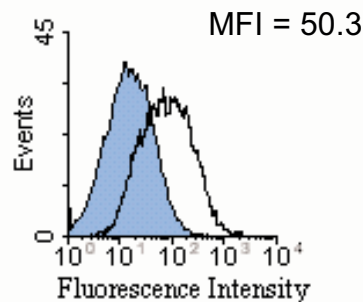
(A) MK16 cells grown *in vitro*

(a) RPMI 1640 medium
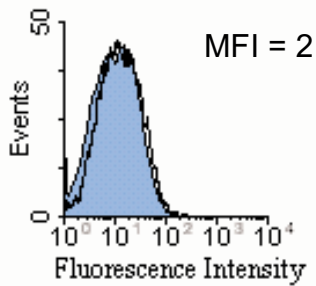
(b) RPMI 1640 medium supplemented with IFN-γ

MFI = 2

MFI = 476
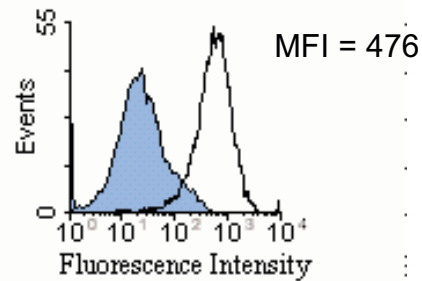
(B) Control MK16 cells grown *in vivo*

(a) s.c. tumour

(b) metastasis

MFI = 9.3

MFI = 50.3
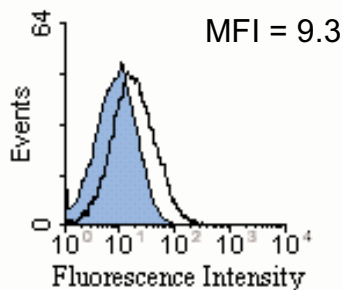
(C) MK16 cells grown *in vivo* and treated with IFN-γ

(a) s.c. tumour

(b) metastasis
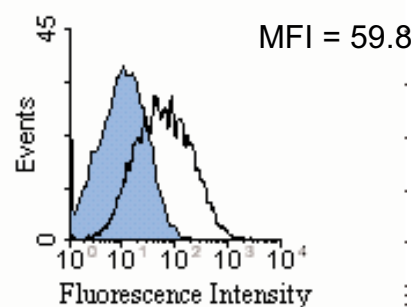
MFI = 15.3

MFI = 59.8
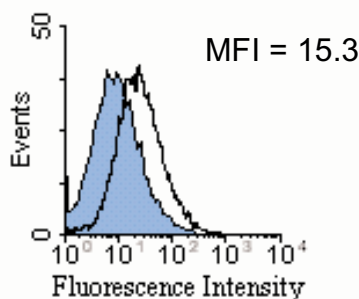
Fig. 3. Changes of MHC class I expression on MK16 cells *in vivo*. MFI of the sample minus MFI of the negative control labelled with an antibody of irrelevant specificity. (A) *In vitro* controls: (a) MK16 cells grown in RPMI 1640 (MFI = 3.4, 3.7, 5.7, 2.6, 5.1, 5.3, 5.8, 4.3, 1.3, 4.2, 2.0; Σ/n ± SD = 3.9 ± 1.4) (b) MK16 cells grown in RPMI 1640 + IFN-γ (MFI = 476) (B) Control MK16 cells grown *in vivo*: (a) s.c. tumours (MFI = 7.0, 2.2, 6.5, 6.2, 18.4, 7.0, 5.9, 9.3; Σ/n ± SD = 7.8 ± 4.4) (b) metastases (MFI = 11.3, 10.3, 14.3, 23.2, 50.3; Σ/n ± SD = 21.9 ± 14.9); (C) MK16 tumours grown *in vivo* and treated with IFN-γ: (a) s.c. tumours (MFI = 4.2, 15.3, 8.4, 19.3, 2.5, 10.0, 4.4, 13.0, 8.5, 7.6, 7.2, 4.6; Σ/n ± SD = 8.8 ± 4.8), (b) metastases (MFI = 12.8, 12.6, 17.0, 59.8; Σ/n ± SD = 25.6 ± 19.9)
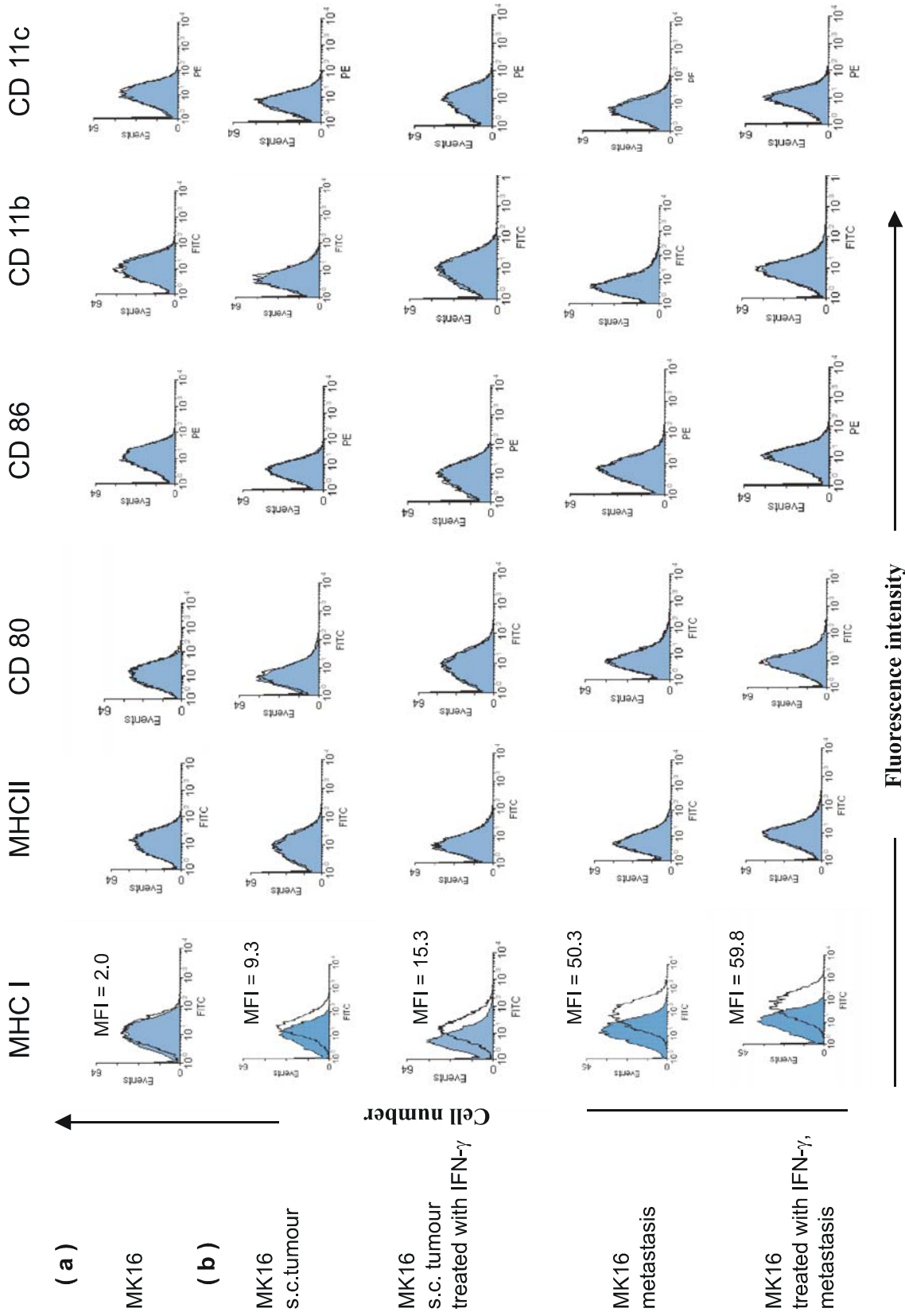
Fig. 4. Upregulation of MHC class I expression induced in MK16 cells by *in vivo* growth is not due to infiltrating leukocytes. MFI of the sample minus MFI of the negative control labelled with an antibody of irrelevant specificity. (a) MK16 cells growing *in vitro* (b) MK16 cells growing *in vivo*. Leukocyte markers examined: MHC II, CD80, CD86, CD11b, CD11c.
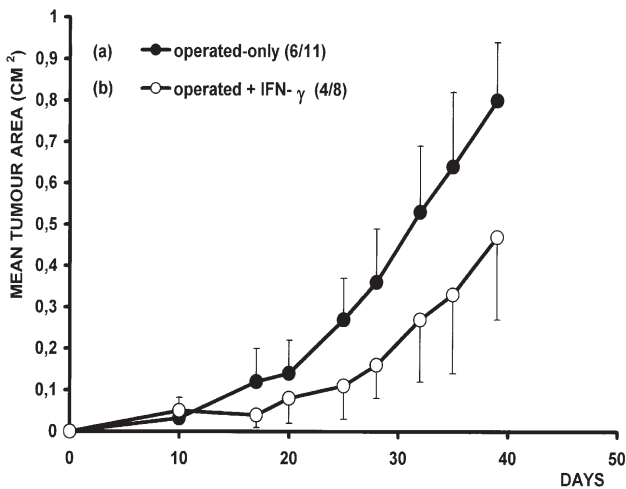
Fig. 5. Peritumoral IFN-γ therapy of surgical residues of MK16 (No. of tumour-bearing mice/Total No. of mice).

other tumour-inhibitory, MHC class I-unrestricted mechanisms such as those mediated by natural killer (NK) and NKT cells. It may be of interest that similar observations have been recently reported by Šmahel et al. (in press), who isolated MHC class I⁻ sublines of TC-1 cells and tested their properties both *in vitro* and *in vivo*. TC-1 cells are also HPV16-transformed B6 mouse cells, which differ from MK16 cells (in addition to some other properties) by expression of large amounts of MHC class I molecules at their surfaces.

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