Combination of Intratumoral injections of vaccinia virus MVA expressing GM-CSF and immunization with DNA vaccine prolongs the survival of mice bearing HPV16 induced tumors with downregulated expression of MHC class I molecules

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Downregulation of MHC class I molecules is believed to be often the cause of tumor immune escape and at the same time it is the major obstacle to T-cell based immunotherapy of tumors. In our experimental model, the C57BL/6 mice bearing tumors induced by TC-1/A9 cells characterized by expression of HPV16 oncogenes and downregulation of H-2^b molecules were immunized with highly immunogenic E7GGG.GUS DNA vaccine expressing the fused gene of modified HPV16 E7 (E7GGG) with *E.coli* β-glucuronidase (GUS). The DNA vaccine was administered by gene gun on days 7 and 14 after s.c. injection of tumor cells. The tumors *in situ* were injected with recombinant vaccinia virus MVA expressing the gene for murine granulocyte-macrophage colony-stimulating factor (MVA-GM-CSF). Two doses of the DNA vaccine combined with at least two consecutive local treatments with MVA-GM-CSF were able to inhibit significantly the growth of tumors. We have shown by ELISPOT-IFNγ that *in situ* expression of the GM-CSF gene did not enhance the E7 specific systemic T-cell response. We found that local injections of MVA-GM-CSF induced an increase of intratumoral $CD3^+$ T cell counts and that the DNA vaccination resulted in up-regulation of MHC type I molecules on tumor cells *in vivo.* We suppose that i.t. delivery of MVA-GM-CSF changed the local tumor microenvironment and rendered tumors more attractive and better accessible to effector T cells.

Key words: GM-CSF, vaccinia virus, MVA, DNA vaccine, HPV16 E7, tumor.

Human papillomaviruses (HPVs) have been identified as the etiological agent of cervical carcinoma, the second most common malignancy in women worldwide. Moreover, HPVs are suspected to be also involved in the induction of other tumors [1].Despite of recent approval of prophylactic vaccine against high risk HPVs, development of a therapeutic vaccines against HPVs remains of utmost priority. The early viral proteins E6 and E7 expressed in precancerous lesions and invasive cancers are essential for the induction and maintenance of oncogenic transformation of HPV infected cells [2]. Since Th_1 and $CD8^+$ T cell immune responses against early proteins E2, E6 and E7 can cause regression of lesions in HPV16-infected humans, it is widely accepted that vaccination strategies for therapy of HPV associated neoplasia are to be based on early viral proteins which play the role of tumor associated rejection antigens.

Despite the presence of viral antigens, the transformed cells sometimes gain the ability to escape from anti-tumor immune responses. This phenomenon is frequently encountered in clinical trials on tumor vaccines and immunotherapy and it can be caused by loss of the ability of tumor cells to present antigenic peptides in the complex with MHC class I molecules which implies that such cells would be a poor target for specific T cells. Changes of this type occur frequently in progressing tumors and metastases as a result of down-regulation of MHC class I expression. The molecular mechanisms responsible for the defect include: mutations in β_2-microglobulin or MHC heavy chain and alterations in expression of proteins involved in the antigen processing path-

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way e.g. proteasome subunits or transporter associated with antigen processing (TAP). MHC class I down-regulation caused by the latter mechanism can be corrected by cytokine treatment, particularly that with IFNγ. The TC-1/A9 cell clone with down-regulated surface H-2^b expression was isolated from an immunoresistant tumor grown after challenge with TC-1 cells in a mouse immune against HPV16 E7 protein. Recovery of MHC class I molecules on the surface of TC-1/A9 cells could be induced by culture in the presence of IFN γ and correlated with TAP-1 expression. The cells proliferating *in vivo* retained their immunoresistance, despite partial restoration of H-2^b on their surface [3].

Previous reports indicated that intratumoral GM-CSF gene delivery increased the counts of in situ DCs and tumor infiltrating leukocytes and modified levels of IFNγ, IL12 and some chemokines inside tumors [4]. Moreover, intratumoral delivery of tumor associated antigens together with multiple immunostimulatory molecules including GM-CSF induced anti-tumor activity [5,6]. Anti-tumor effect of local gene therapy with GM-CSF carried by vaccinia virus or other vectors was confirmed in clinical therapies of different tumors [7,8].

Recombinant poxviruses have been used for expression of tumor antigens, cytokines and chemokines in numerous preclinical models. Besides high levels of expression, these vectors were accepted for administration to patients with different malignancies for their good safety profile. The strongest safety record was achieved with non-replicating viruses such as the modified vaccinia virus Ankara (MVA) that had been attenuated due to at least six major deletions representing about 15% of the original genome of the parental Ankara strain. Indeed, MVA is currently used in advanced stage clinical trials of several prophylactic and therapeutic vaccines against infectious diseases and cancer [9].

Previously, we prepared a highly immunogenic E7GGG.GUS DNA vaccine expressing the fused gene of the modified HPV16 E7 oncogene (E7GGG) with the gene encoding *E.coli* β-glucuronidase (GUS) [10]. Although the E7GGG.GUS DNA vaccine was able to prevent the growth of TC-1/A9 induced tumors only in a portion of experimental mice, it was ineffective in inhibiting the growth of tumors with the therapy started on day 4 after s.c. injection of TC-1/A9 cells.

In this report, we targeted GM-CSF expression in the local microenvironment of tumors with down-regulated expression of MHC class I molecules using the recombinant vaccinia virus MVA and simultaneously mice immunized with the highly immunogenic DNA vaccine. Two doses of the DNA vaccine combined with at least two consecutive intratumoral doses of MVA-GM-CSF were able to inhibit significantly the growth of tumors. When analyzing the cellular immune response to the HPV16 E7 protein by ELISPOT-IFNγ, we found that *in situ* expression of the GM-CSF gene did not enhance the systemic E7 specific T-cell response. We found that local injections of MVA-GM-CSF induced an increase of intratumoral CD3+ T cell counts and that the DNA vaccination resulted in up-regulation of MHC type I molecules on tumor cells *in vivo.*

Materials and methods

Viruses. Recombinant vaccinia viruses (rVV) (strain MVA) were prepared from a clone isolated by plaque purification in our laboratory from the MVA vaccine kindly provided by W. Altenburger, Basel, Switzerland. The MVA-GM-CSF virus carries a 550 bp fragment containing the mouse GM-CSF cDNA [11] inserted in the thymidine kinase (TK) gene. Transgene expression is controlled by the 7.5k promoter. TK deficient RAT 2 rat cells [12] were used for the selection of TK- VV recombinants. Vaccinia viruses P13-GM-CSF, MVA-preS2S [13] and MVA-SigE7LAMP [14] were described previously. MVA-SigE7LAMP was used as the parental virus for insertion of the GM-CSF gene resulting in a double recombinant MVA-SigE7LAMP-GM-CSF.

P13-GM-CSF virus was propagated in BSC-40 cells and MVA recombinants (rMVA) were multiplied in primary chicken fibroblast cultures. All viruses were purified by sucrose-gradient centrifugation [15]

Antibodies. For immunohistochemical staining the rat anti-human CD3, clone CD3-12 (Serotec Ltd. Oxford, UK) and secondary antibody Ultratech HRP (Immunotech, Marseille, France) were used. For flow cytometry antibodies against CD3-PE (clone KT3, Serotec), Ly6G-FITC (clone1A8, BD-Pharmingen, San Diego, CA), CD11c-FITC (clone HL3, BD-Pharmingen), F4/80 FITC (cloneCI:A3-1, Serotec), neutrophile 7/4-PE (Serotec), anti-mouse H-2K^b/H-2D^b (clone 28-8-6, BD-Pharmingen,), FITC labelled polyclonal goat anti-mouse IgG (BD-Pharmingen) were used.

DNA vaccine. Mice were immunized against the HPV16 E7 protein with the plasmid pBSC/E7GGG.GUS carrying a fused gene consisting of the mutated HPV16 E7 gene (E7GGG) and *E. coli* β-glucuronidase (GUS) [10]. The empty plasmid pBSC was used as a negative control. One μg/dose of plasmids was administered into the abdominal skin of mice by a gene gun as reported earlier [16].

Cell lines. HPV16 E6- and E7-expressing TC-1 cells derived from C57BL/6 mouse lung cells [17] were kindly provided by T.C. Wu (Baltimore). TC-1/A9 cells were derived from the TC-1 tumor as an immunoresistant MHC class I $^-$ subline [3].

*Animal experiments. S*ix-week-old C57BL/6 (H-2^b) female mice were obtained from Charles River, Germany. Animals were maintained under standard conditions at the National Institute of Public Health (Prague). The experiments were performed in compliance with Act No. 246/92 on the animal protection of animals against cruelty and Decree No. 311/97 of the Ministry of Health of the Czech Republic, on the care and use of experimental animals.

Tumor induction and intratumoral injection of MVA-GM-CSF. Mice received a dose of 3.10^4 tumor cells injected subcutaneously (s.c.) into the back. Recombinant viruses were

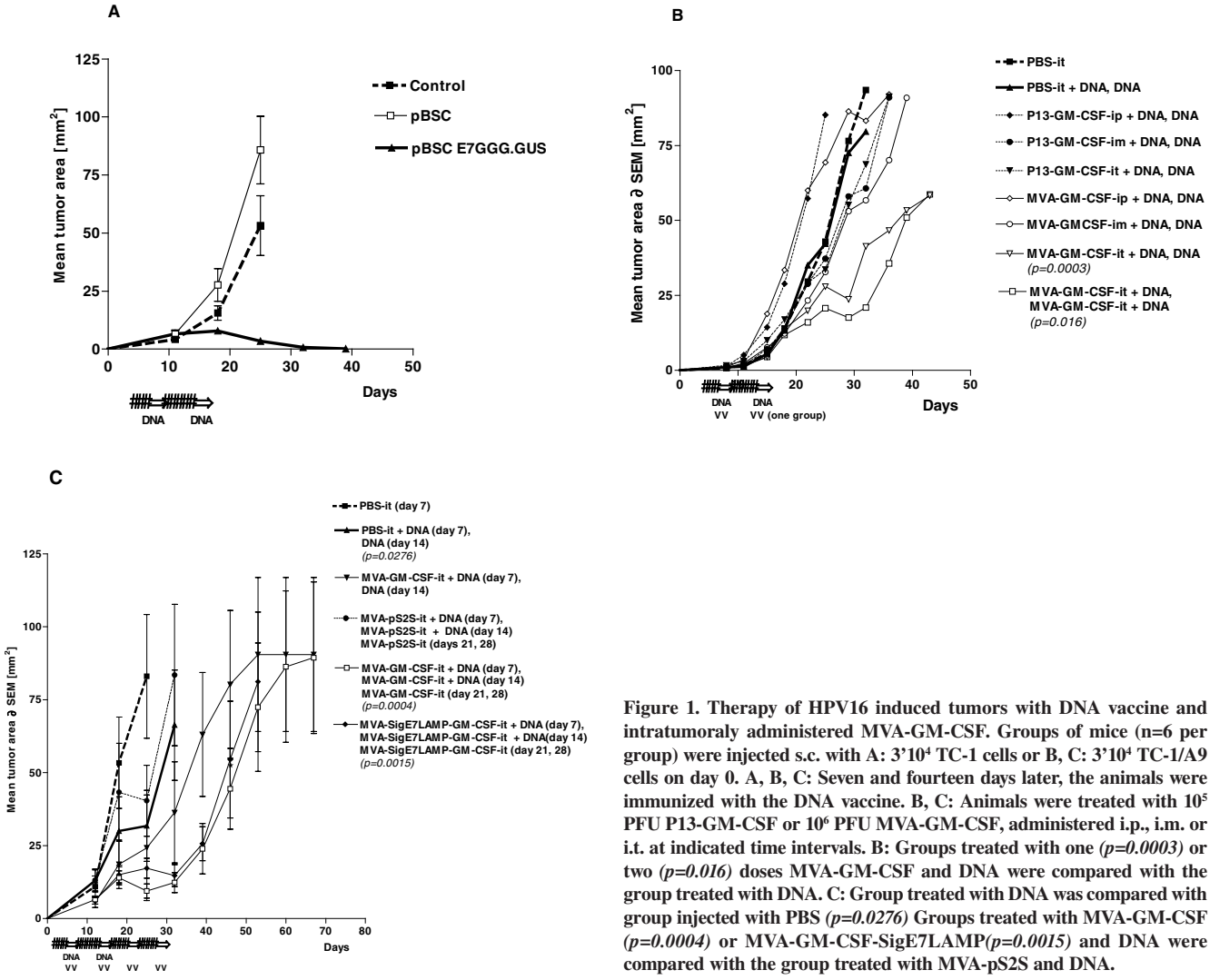

Figure 1. Therapy of HPV16 induced tumors with DNA vaccine and intratumoraly administered MVA-GM-CSF. Groups of mice (n=6 per group) were injected s.c. with A: 3'10^4 TC-1 cells or B, C: 3'10^4 TC-1/A9 cells on day 0. A, B, C: Seven and fourteen days later, the animals were immunized with the DNA vaccine. B, C: Animals were treated with 10^5 PFU P13-GM-CSF or 10^6 PFU MVA-GM-CSF, administered i.p., i.m. or i.t. at indicated time intervals. B: Groups treated with one *(p=0.0003)* or two *(p=0.016)* doses MVA-GM-CSF and DNA were compared with the group treated with DNA. C: Group treated with DNA was compared with group injected with PBS *(p=0.0276)* Groups treated with MVA-GM-CSF *(p=0.0004)* or MVA-GM-CSF-SigE7LAMP*(p=0.0015)* and DNA were compared with the group treated with MVA-pS2S and DNA.

injected into tumors (i.t.) in 20 µl PBS using an insulin syringe with a 30 gauge needle. If a palpable tumor was absent at the time of i.t. treatment, the recombinant virus was inoculated s.c. at the site of usual injection of tumor cells. Tumor growth was measured weekly. Differences between growth curves were statistically analyzed by the two-way ANOVA using the Prism 3.0. software (GraphPad Software Inc., SanDiego, USA).

Induction of surgical minimal residual tumor disease (SMRTD). Mice were inoculated s.c. with 6.10^4 TC-1/A9 tumor cells. After 14 days, the transplanted tumors, reaching approximately 8–12mm in diameter, were excised under i.p. anaethesia (0,3 ml/mouse, Hypnomidate, Jansen Pharmaceutica, Beerse, Belgium) , leaving no macroscopically visible tumor residuum [18].

Assay of E7 specific T-cell responses. The spleens were removed from mice twelve days after the last immunization and the response of IFNγ-producing cells was determined by ELISPOT-IFNγ assay upon *in vitro* restimulation with H-2D^b restricted HPV16 E7$_{(49-57)}$ peptide (RAHYNIVTF) for 6 days, as described previously [14].

Immunohistochemistry. Tumors were fixed in Davison´s solution. After fixation, samples were processed and stained as reported previously [19].

Detection of surface markers on cells isolated from tumors. The animals were sacrificed and their subcutaneous tumors were excised, cut into pieces and washed twice with PBS. Tumor fragments were trypsinized 2x15 min. For analysis of tumor infiltrate, all cells released from the tumor tissue were collected, washed and stained with antibodies. To prepare tumor cells for detection of MHC class I expression, the first fraction of cells released by trypsinization was discarded and the following two fractions were combined and cultured in D-MEM medium overnight. The next day, the cells were trypsinized, stained with specific antibodies and analyzed using a Coulter-Epics XL flow cytometer (Beckman Instruments).

Results

Local administration of MVA-GM-CSF has an effect on the therapy of TC-1/A9 tumors by immunization with the DNA vaccine. Therapeutic immunization of mice with the plasmid pBSC/E7GGG.GUS started one week after transplantation of 3.10^4 tumor cells when the mean tumor size was 0.75 – 1.68 mm^2. The second dose of the vaccine was administered on day 7 following the dose 1. High therapeutic efficacy of immunization was demonstrated in animals injected with TC-1 cells where it resulted in complete inhibition of tumor growth (Fig. 1A), whereas in animals carrying TC-1/A9 induced tumors with down-regulated MHC class I molecules it proved ineffective (Fig. 1B, full triangles, full bold line).

We attempted to enhance the poor therapeutic effect of the DNA vaccine against TC-1/A9 cells using GM-CSF. Since the local concentration of recombinant cytokines injected *in situ* ceases very quickly we introduced the transgene GM-CSF into the tissue of the tumor and its environment by the recombinant vaccinia virus. The animals were injected i.p., i.m. or i.t. with 10^5 pfu P13-GM-CSF or 10^6 pfu MVA-GM-CSF at the time of the first immunization with the DNA vaccine (Fig. 1B). We observed that the i.p. and i.m. virus injections as well as the use of the replicating recombinant virus P13-GM-CSF remained without any significant effect on tumor growth. On the other hand, transduction of tumors with the non-replicating virus MVA expressing GM-CSF resulted in inhibition of tumor growth (p=0.0003) in 3/8 animals in comparison with the group treated with PBS i.t.+DNA (0/8 animals without tumor). The effect of two consecutive intratumoral injections of MVA-GM-CSF (p=0.016) was more pronounced than with one dose only. Even a superior tumor growth inhibition was achieved if the number of repeated i.t. injections with MVA-GM-CSF administered in weekly intervals was increased to four (Fig. 1C, p=0.0004). The anti-tumor effect was dependent on GM-CSF, as inoculation of control virus MVA-pS2S did not change the growth of tumors. In the same experiment (Fig. 1C) we have shown that the therapeutic immune response was not boosted by i.t. immunization with the E7 protein when co-expressed with GM-CSF in the form of highly immunogenic Sig/E7/LAMP fusion protein. Tumor growth inhibition was similar in animals injected with either single recombinant MVA-GM-CSF or double recombinant MVA-SigE7LAMP-GM-CSF. In additional experiments we observed the highest anti-tumor effects with i.t. MVA-GM-CSF injections when not coadministered concurrently with the DNA vaccine, but given four days later (not shown).

MVA-GM-CSF enhances immunotherapy of surgical minimal residual tumor disease. Furthermore, we investigated whether or not the local treatment with MVA-GM-CSF together with DNA vaccine immunization could be used for immunotherapy of surgical minimal residual tumor disease. Mice were s.c. injected with 6.10^4 TC-1/A9 cells (day 0) and growing tumors were surgically removed 14 days later. Operated animals (n=6 or 7) were immunized with two doses of the DNA vaccine on days 18 and 25 and the sites of the former tumors were treated by s.c. injection of MVA-GM-CSF on the day of immunization. Survival of the operated animals on day 25 is summarized in Table I. The overall rates of tumor recurrence were similar in all groups (6/7 or 5/6), but tumor development was somewhat hindered in groups 5 and 6 which had been treated both with the DNA vaccine and one or two doses of MVA-GM-CSF in comparison with the untreated controls or animals given only one type of treatment.

MVA-GM-CSF administration does not enhance the E7 specific T cell response induced by immunization with the DNA vaccine. To determine the possible mechanism of the effect of local delivery of GM-CSF on the cellular immune response we measured E7 specific T-cell immunity in mice treated using the same schedule as for tumor therapy but without administration of tumor cells. MVA-GM-CSF was inoculated at the site of usual injection of tumor cells. Mice were immunized with two doses of the DNA vaccine by gene gun and injected s.c. with 10^6 PFU of rMVA into the dorsal region as described in legend of Fig. 2. The response of ex vivo splenocytes stimulated overnight with the $E7_{(49\text{-}57)}$ peptide RAHYNIVTF was tested by ELISPOT-IFNγ.

The response of mice to immunization with pBSC/E7GGG.GUS was significantly increased in groups B, C, D compared to group A. The highest mean value for the E7 response was found in mice which were treated twice with MVA-GM-CSF and DNA (group E), but due to high variance the differences were not statistically significant. Comparison of responses between groups which were immunized with

TABLE 1 – Therapy of operated tumors TC-1/A9 with MVA-GM-CSF and DNA vaccine

Group[a]	Dose 1 of virus day 4 after operation	Dose 1 of DNA vaccine day 4 after operation	Dose 2 of virus day 11 after operation	Dose 2 of DNA vaccine day 11 after operation	Survival of mice on day 25 after operation [%]	Mice with tumors
1	–[b]	–	–	–	30	6/7
2	–	+	–	+	32	6/6
4	MVA-GMCSF	–	–	–	32	5/6
5	MVA-GMCSF	+	–	+	58	6/7
6	MVA-GMCSF	+	MVA-GMCSF	+	58	6/7

[a]The groups of mice with identical distribution of tumor size were formed on the day of operation.
[b]Mock infected tumors were injected with PBS.

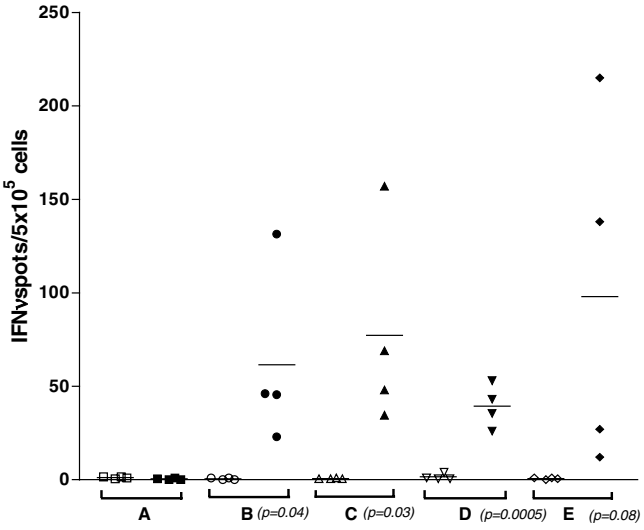

Figure 2. The effect of MVA-GM-CSF on E7 specific cellular response induced by DNA immunization. Groups of mice (n=4 per group) were immunized with two doses of the DNA vaccine by g.g. and the animals were injected with 10^6 PFU of rMVA in 20 μl of PBS administered s.c. at the dorsal region on every immunization day. Different groups received A pBSC+MVA-GM-CSF (day 0), pBSC (day 7), B pBSC E7GGG.GUS+PBS (day 0), pBSC E7GGG.GUS (day 7), C pBSC E7GGG.GUS+MVA-preS2S (day 0), pBSC E7GGG.GUS (day 7), D pBSC E7GGG.GUS+MVA-GM-CSF (day 0), pBSC E7GGG.GUS (day 7), E pBSC E7GGG.GUS+MVA-GM-CSF (day 0), pBSC E7GGG.GUS +MVA-GM-CSF (day 7). The E7-specific $CD8^+$ T lymphocytes were determined in each mice by IFNγ ELISPOT. The mean counts of IFNg producing cells obtained in the absence (empty symbols) and presence (full symbols) of the $E7_{(49-57)}$ peptide were compared and analyzed by unpaired t-test.

pBSC/E7GGG.GUS (B, C, D, E), and some of which were inoculated with different MVA recombinants revealed, that the treatment with virus did not increase number of spots significantly. Our results suggest that i.t. injections coadministered with the DNA vaccine did not affect the magnitude of the E7-specific T cell response and hint that the role of inoculation of non-replicating MVA-GM-CSF could consist in modification of the local tumor microenvironment.

Microenvironment of MHC class I negative tumors is changed following intratumoral MVA-GM-CSF injections and DNA vaccine immunization. To determine the effect of the treatment on tumor microenvironment we analyzed surface markers of tumor infiltrating cells. Groups of mice (n = 6) were injected s.c. with 6.10^4 TC-1/A9 cells and 7 and 14 days later were immunized with the pBSC/E7GGG.GUS DNA vaccine. Concurrently with immunization the mice were injected i.t. with MVA-GM-CSF or the control virus MVA-pS2S. Tissue of three tumors of similar size from each group was examined by immunohistochemical staining (Fig.3A) four days after the second intratumoral virus injection. We observed only a sparse diffuse infiltration of $CD3^+$ T cells in tumors injected with PBS in non-immunized mice (Fig.3A-I) and in PBS injected tumors of mice immunized with the DNA vaccine (Fig.3A-II). The tumor tissue injected with MVA-GM-CSF of DNA immunized mice contained distinct areas with increased counts of $CD3^+$ T cells (Fig.3A-III). Injection with the MVA-pS2S virus yielded a weak infiltration of $CD3^+$ cells only (not shown).

The cellular composition of tumors was also analyzed by flow cytometry. The tumors were excised seven days after the second i.t. virus injection and leukocyte markers were measured in the whole cell population. Flow cytometry analysis confirmed the results of immunohistochemical staining (Fig.3B). However, we did not observe any differences in the count of cells positive for Ly6G, CD11c, F4/80 and neutrophile 7/4 markers in correlation with the previous tumor treatment (not shown).

It has been shown previously that the treatment of TC-1/A9 cells with IFN g resulted in up-regulation of surface MHC class I molecules. *In vivo* growing TC-1/A9 cells also had increased amounts of MHC class I molecules in comparison with *in vitro* cultured cells [3]. To examine the effect of *in situ* treatments of tumors with different recombinant vaccinia viruses, we determined MHC class I molecules on the surface of cells isolated from TC-1/A9 induced tumors. Groups of mice (n = 4 or 5) were injected s.c. with $6'10^4$ TC-1/A9 cells and 7 and 14 days later were immunized with the DNA vaccine pBSC/E7GGG.GUS. On the immunization day, the tumors were treated by i.t. injection of MVA-GM-CSF. Surface MHC class I molecules were determined on adherent cells of explanted tumors 3 days after the second treatment (Fig. 3C). In comparison with non-immunized controls, MHC class I expression was increased in all groups immunized with the DNA vaccine but any difference was not found between the mock infected tumors and tumors pretreated with MVA-GM-CSF and MVA-pS2S.

Discussion

Using a murine model of HPV-associated tumors with down-regulated MHC class I expression, we report increased efficacy of the DNA vaccine therapy combined with i.t. GM-CSF gene delivery via a non-replicating vaccinia virus vector.

It was observed repeatedly that GM-CSF acts as an immune adjuvant for vaccines because of its capability to stimulate antigen presenting cells which results in increased activation of $CD4^+$, $CD8^+$ T cells and CD1d-restricted $NK1.1^+$ cells and enhanced production of antibodies. Moreover, vaccination with tumor cells engineered *ex vivo* produce GM-CSF elicited potent and long-lasting anti-tumor immunity (for review see [20]. It has been shown in a murine tumor model that the adjuvant and anti-tumor activity of GM-CSF depends on dosage and route of administration [21]. To minimize immunosuppressive activity induced by high concentrations of GM-CSF at the injection site, we delivered GM-CSF via a live recombinant vaccinia virus. We used a poxviral vector because it efficiently infects a broad range of mammalian cells

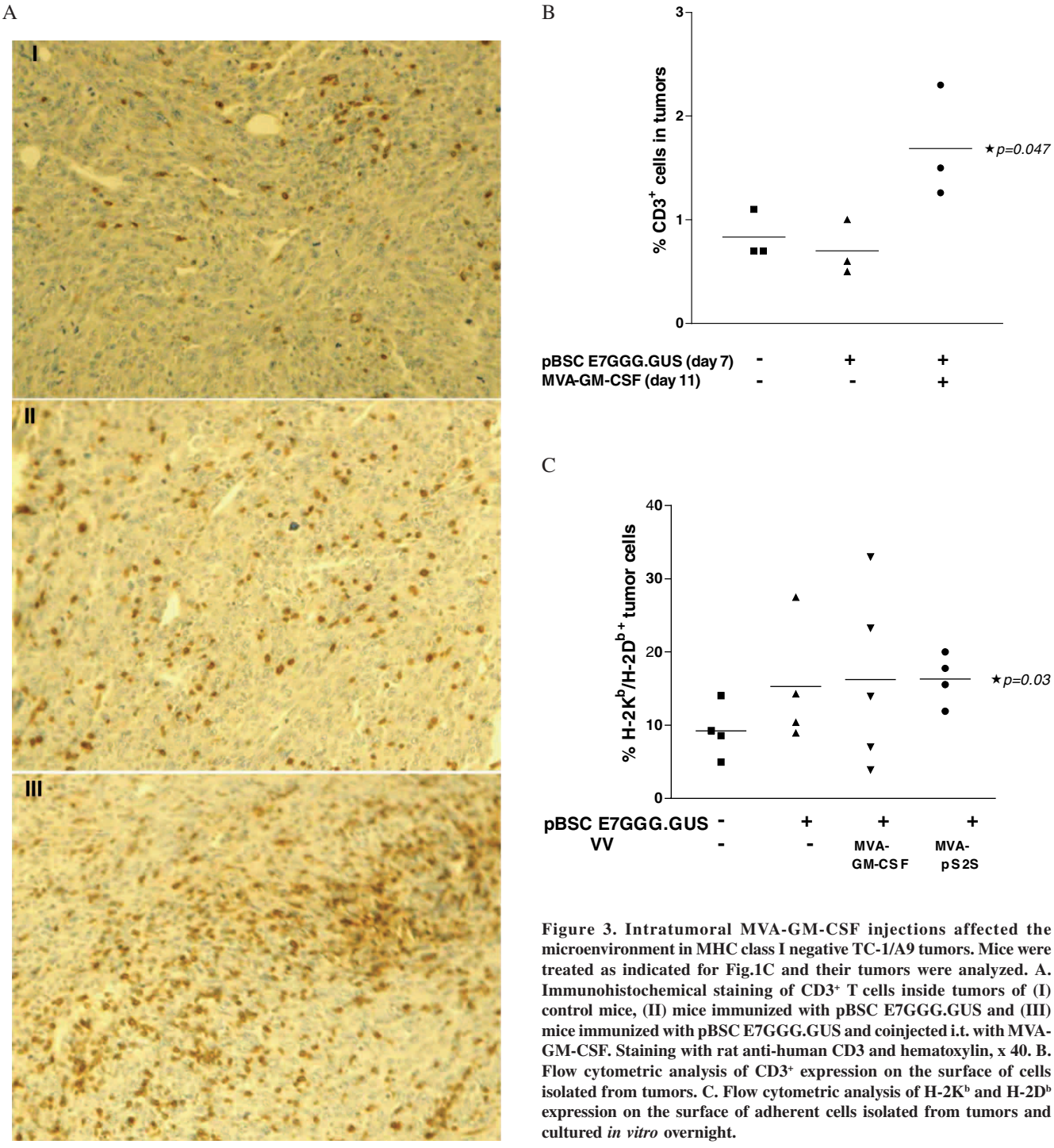

Figure 3. Intratumoral MVA-GM-CSF injections affected the microenvironment in MHC class I negative TC-1/A9 tumors. Mice were treated as indicated for Fig.1C and their tumors were analyzed. A. Immunohistochemical staining of CD3+ T cells inside tumors of (I) control mice, (II) mice immunized with pBSC E7GGG.GUS and (III) mice immunized with pBSC E7GGG.GUS and coinjected i.t. with MVA-GM-CSF. Staining with rat anti-human CD3 and hematoxylin, x 40. B. Flow cytometric analysis of CD3+ expression on the surface of cells isolated from tumors. C. Flow cytometric analysis of H-2Kb and H-2Db expression on the surface of adherent cells isolated from tumors and cultured *in vitro* overnight.

in vivo, the magnitude of transgene expression can be adjusted by the type of the promoter used and its use poses no risk of insertional mutagenesis. We found the administration of a non-replicating MVA-GM-CSF which produced lower levels of GM-CSF superior to the use of replication competent P13-GM-CSF which induces high transgene expression. When comparing three different routes of virus administration, the highest effect was observed with repeated i.t. MVA-GM-CSF virus injections. In other studies, anti-tumor activity of GM-CSF was highest with cytokine administered into the local microenvironment of tumors where it has an effect on the activation status of antigen presenting cells in regional lymph

nodes [22,23]. Similarly, GM-CSF produced after i.t. injection of MVA-GM-CSF had only a local effect because it was not able to enhance the E7 specific T cell response induced by the DNA vaccine administered by gene gun into the abdominal part of the body.

Tumors with down-regulated MHC class I expression cannot be effectively targeted by the antigen specific vaccination therapy which is based on the response of MHC-I restricted effector T cells [3]. However, the anti-tumor effect of a DNA vaccine against such cells can be enhanced by immunization with tumor cells expressing GM-CSF. It was observed previously using a HPV16 E6 $E7^+$ MK16 tumor model [24] that the treatment of tumor bearing mice with such a combination of vaccines resulted in temporary tumor growth inhibition. In the combined immunotherapy described in this report, the virus vector is employed for *in situ* expression of the cytokine to avoid the time consuming engineering of cytokine expressing cell lines derived from tumor cells of the patient.

DNA immunization alone caused up-regulation of MHC class I expression on the surface of tumor cells isolated from tumors, which can be due to IFNγ produced by E7-specific T cells and IFNα/β produced by plasmacytoid DCs induced by CpG motifs present in bacterial plasmid DNA. Tumors after repeated combined therapy with the DNA vaccine and i.t. GM-CSF expression were infiltrated with the higher amount of $CD3^+$ T cells. An increase in intratumoral myeloid leukocytes was not observed, which implies, that the dose of GM-CSF was not able to induce the response of immunosuppressive $Gr1^+$/$CD11b^+$cells [21].

It has been shown repeatedly that local delivery of GM-CSF caused only temporary regression of tumors. Intratumoral injections of IL12 and GM-CSF resulted in the beneficial increase in IFNγ and decrease in TGFβ and IL10 production but the effect was only transient and was followed by the development of an enhanced intratumoral T-suppressor-cell response characterized by high levels of immunosuppressor cytokines TGFβ and IL10 [25]. We speculate that the suppressor T cells were responsible for transient effect of the combined therapy in our study and plan to supplement the local therapy with agents that would block the immune suppression elicited by growing tumors.

Nonetheless, the present study provides evidence that the nonreplicating vaccinia virus MVA-GM-CSF is a suitable tool for local GM-CSF delivery that does not interfere with the DNA vaccine immunization against the E7 antigen and contributess to anti-tumor effects of immunization against tumors with down-regulated MHC class I molecule expression.

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