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Abstract. The development of tumor therapies based on the activation of antitumor immunity requires tumor models that are highly immunogenic. The immunologic response to fluorescent proteins, green fluorescent protein (GFP), or enhanced GFP (EGFP) was demonstrated in different cancer models. However, for live animal imaging, red and far-red fluorescent proteins are preferable, but their immunogenicity has not been studied. We assessed the immunogenicity of the red fluorescent protein, KillerRed (KR), in CT26 murine colon carcinoma. We showed a slower growth and a lower tumor incidence of KR-expressing tumors in comparison with nonexpressing ones. We found that KR-expressing lung metastases and rechallenged tumors were not formed in mice that had been surgically cured of KR-expressing primary tumors. The effect of low-dose cyclophosphamide (CY) treatment was also tested, as this is known to activate antitumor immune responses. The low-dose CY therapy of CT26-KR tumors resulted in inhibition of tumor growth and improved mouse survival. In summary, we have established a highly immunogenic tumor model that could be valuable for investigations of the mechanisms of antitumor immunity and the development of new therapeutic approaches. [DOI: 10.1117/1.JBO.20.8.088002]

Keywords: tumor model; immunogenicity; fluorescent protein KillerRed; chemotherapy.

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1 Introduction

The ability of tumors to escape from immune-mediated rejection is a fundamental property that enables the development and progression of cancers and dramatically decreases the efficacy of immunotherapy.[1] Tumors evade immune system surveillance through multiple mechanisms, including: (a) loss of T-cell recognition associated with poor antigen processing or presentation, the loss of antigen expression, or alterations in the expression of the major histocompatibility complex;[2] (b) secreting immunosuppressive factors and cytokines such as transforming growth factor β, interleukin-10, interferon gamma, vascular endothelian growth factor, and others;[3] (c) resistance to apoptosis, owing to overexpression of antiapoptotic molecules or downregulation and mutation of proapoptotic molecules;[4] (d) the accumulation of immunosuppressive cells including regulatory T-cells (Treg) and myeloid-derived suppressor cells;[5,6] or (e) the production of immunosuppressive metabolites such as tryptophan, L-arginine, or lactate.[7]

The inhibition of tumor protection and the education of the immune system to recognize the tumor as foreign are the goals of immunotherapy.[8] Cancer immunotherapy can be either passive or active. Passive therapy does not initiate an immune response and does not generate immunologic memory. It is based on the adoptive transfer of immunomodulators (including cytokines), tumor-specific antibodies, or immune cells. Active immunotherapeutic approaches trigger an endogenous immune response and have the potential to provide long-lasting anticancer activity. Currently, immunotherapy is being successfully used to treat many different types of cancer.[6,9–11]

One of the strategies to activate the immune response against a tumor is the use of low doses of chemotherapeutic drugs. This effect has been demonstrated for cyclophosphamide (CY),[12–16] vincristine, paclitaxel, naltrexone, and some other drugs.[16,17]

The development of tumor therapies based on the activation of antitumor immunity requires highly immunogenic tumor models. Many wild-type tumors, including CT26,[18] display poor immunogenicity and, therefore, to be recognized by the immune system, the cancer cells have to be modified by introducing additional antigens. On one hand, endogenous antigens can attract the immune system to destroy the tumor. Here, the immunogenicity of wild-type tumor cells can be enhanced by transfecting with cytokine genes, including, for example, granulocyte-macrophage colony-stimulating factor.[19] On the other hand, some foreign antigens increase the antitumor immune response. For example, transfections with fluorescent protein of the green fluorescent protein (GFP) family,[20–23] viral proteins,[13] β-galactosidase,[24] or murine B7 (B7-1/CD80)[25] have

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been used to overcome the blockade of the antitumor immune response by tumors.

Currently, the GFP and its variants are widely used in cancer research as genetically encoded fluorescent markers for noninvasive monitoring of tumor growth in the small animal body and the evaluation of therapeutic efficacy over time. The family of GFP-based fluorescent proteins covers nearly the entire visible spectrum from blue to far-red and provides a powerful tool for imaging of living cell structure and functions.[26] Cancer studies with the use of fluorescent proteins are generally performed on human tumor xenografts transplanted into the immunodeficient mice. However, it is known that, when expressed in the immunocompetent animals, GFPs are immunogenic. To our knowledge, among a variety of GFPs, the immunogenic properties have been reported only for GFP and enhanced GFP (EGFP).[20–23] Meanwhile, red and far-red fluorescent proteins are preferable for live animal imaging due to reduced tissue's absorbance and autofluorescence in this spectrum range.

The purpose of our work was to assess the immunogenicity of the red fluorescent protein KillerRed (KR) when expressed in murine colon carcinoma CT26 and to test the efficacy of low-dose CY chemotherapy on this tumor model. Since it has absorption and emission in the red range (exication 585 nm and emission 610 nm), where biological tissues are relatively transparent, it represents a useful tool for *in vivo* fluorescence imaging. Previously, we have shown that KR can be used successfully to visualize deeply located tumors in small animals.[27] On the other hand, the uniqueness of this protein is that it is phototoxic. Upon irradiation with light, KR produces reactive oxygen species and, therefore, works as a genetically encoded photosensitizer. The ability of KR to kill cancer cells or to block cell division when exposed to light has been widely demonstrated *in vitro*.[28–32] Recently, the phototoxic effect of KR against tumors has been shown *in vivo* in immunodeficient mice.[33] In terms of photodynamic therapy, the immunogenicity of tumors enhanced by expression of GFP may potentiate better responses to treatment in immunocompetent individuals.[22]

2 Materials and Methods

2.1 *Cell Lines*

A CT26 murine colon carcinoma cell line (ATCC CRL-2638) stably expressing histone H2B-tandem KR (H2B-tKR) fusion was obtained by lentiviral transduction. A NheI-blunt PCR fragment containing the H2B-tKR open reading frame was cloned into NheI- and EcoRV-digested pRRLSIN.EF1.WPRE with a modified multiple cloning site. The vector was kindly provided by Professor Didier Trono (Ecole Polytechnique Federale de Lausanne, Lausanne, Switzerland). Then, lentiviral particles for mammalian cell infection were obtained according to the standard procedure. In short, HEK293T cells at a logarithmic growth stage were plated on $d = 6$-cm cell culture dishes (SPL Life Sciences, Korea) 24 to 48 h prior to transfection, in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), 1% glutamine, 10 units/mL penicillin, and 10 μg/mL streptomycin. Transfection with 4 μg pR8.91, 1.2 μg pMD.G, and 5 μg of the H2B-tKR-carrying plasmid was carried out with a calcium-phosphate transfection kit (Molecular Probes) according to the manufacturer's protocol for a total of 10 μg DNA, when the HEK293T cells reached about 90% confluence. The culturing medium was changed 16 h after transfection, and the lentiviral particles were harvested 24 h thereafter. For lentiviral infection, CT26 cells were plated on $d = 35$-mm cell culture dishes (SPL Life Sciences) at a density of 2.5×10^4 cells/dish in DMEM with 10% FCS, 1% glutamine, 10 units/mL penicillin, and 10 μg/mL streptomycin. After 24 h culturing, the medium was changed for the medium with viral particles. Fluorescence of the infected cells was analyzed 5 to 7 days postinfection, using flow cytometry. Then, the portion of the cell population with the highest fluorescence intensity was selected with fluorescence-activated cell sorting (brightest 30%). For sterile cell sorting, 2×10^6 cells were resuspended in PBS with 5% FCS at a density of 5×10^5 cells/mL. The cell suspension was then filtered through a 70 μm nylon mesh cell strainer. Using a MoFlo cell sorter (DakoCytomation), a minimum of 1.5×10^5 events were collected into a sterile 2-mL tube containing DMEM, 10% (v/v) FBS, 10 units/mL penicillin, and 10 μg/mL streptomycin.

For tumor cell injection, murine colon carcinoma CT26 cells and KR-expressing CT26 cells (CT26-KR) were cultured in DMEM with 10% FCS, 1% glutamine, 10 units/mL penicillin, and 10 μg/mL streptomycin. The cells were collected for the injection by adding 1 mL of trypsin-EDTA (25%) to the 25 mm^2 plate for 5 min at 37°C.

2.2 *Mouse Tumor Model*

The experiments were carried out on 97 immunocompetent Balb/c mice weighing 18 to 20 g purchased from the Scientific Center for Biomedical Technologies "Andreevka" (Moscow, Russia). Tumors were induced by subcutaneous (s.c.) injection of 1×10^6 or 0.5×10^6 cancer cells suspended in 100 μL PBS into the mouse leg. Tumor size was measured with a caliper three times a week. The tumor volume was calculated using the formula $V = a * b * 1/2b$, where a is the length and b is the width of the tumor. Mice were sacrificed when the tumor volume reached 1500 mm^3, according to the protocol. Lung metastases were induced by intravenous (i.v.) injection of a mixture of 5×10^4 CT26 and 5×10^4 CT26-KR cells into a tail vein.

All the experiments conducted on animals were approved by the Institutional Ethical Committee (Nizhny Novgorod State Medical Academy, Russia).

2.3 *Fluorescence Imaging*

To monitor CT26-KR tumor growth by fluorescence, whole-body fluorescence imaging *in vivo* was performed using an IVIS-Spectrum imaging system (Caliper Life Sciences). Fluorescence of KR was excited at a wavelength of 570 nm (bandwidth 30 nm) and detected in the 610- to 630-nm range. During *in vivo* imaging, the mice were anaesthetized with 2.5% isoflurane. Fluorescence images were analyzed using Living-Image software. The tumor was selected as a region of interest and the average radiant efficiency $[(\mathrm{p/s/cm^2/sr})/(\mu\mathrm{W/cm^2})]$ was calculated. The fluorescence images were acquired two to three times per week.

To detect CT26-KR lung metastases, fluorescence imaging of excised lungs, *ex vivo*, was implemented immediately after sacrifice.

2.4 *Study of Immunogenicity*

To study the immunogenic properties of KR, we analyzed the susceptibility and growth rate of primary tumors and the

formation of metastases following challenge and rechallenge with CT26 and CT26-KR cells. The growth rate of CT26 and CT26-KR tumors was compared for the mice, which received an injection of 1×10^6 of CT26 or CT26-KR cells, respectively. The susceptibility to tumor rechallenge was examined on the mice initially challenged with 5×10^5 CT26-KR or CT26 cells, then surgically cured of the tumors on the ninth day of tumor growth, and rechallenged with the same dose of CT26 or CT26-KR cells in the opposite leg after they had been tumor-free for 21 days. Lung metastases were induced in both surgically cured and naïve mice by i.v. injection of a mixture of CT26 and CT26-KR, and the number of metastases was counted, in isolated lungs after 21 days, using a Leica M60 stereo microscope.

2.5 *Cyclophosphamide Treatment*

To investigate the efficacy of low-dose CY chemotherapy on reducing tumor growth and on their survival, mice were injected s.c. with 5×10^5 CT26-KR cells. The treatment protocol was adopted from Castano et al.[12] On the fifth or seventh day of tumor growth, the mice were intraperitonealy (i.p.) injected respectively with a low dose (50 mg/kg body weight) or a therapeutic dose (150 mg/kg body weight) of CY in sterile PBS. CT26-KR tumor-bearing mice without any treatment served as the control. The growth rate of CT26-KR tumors was assessed from days 0 to 22. Mice were sacrificed when tumors reached a volume of 1500 mm^3.

2.6 *Statistics*

All values are expressed as mean $\pm$ SD. Comparison between two means was carried out using the Mann–Whitney U-test or Fisher's exact test, where appropriate. $P \leq 0.05$ was considered significant. Survival analysis was performed using the Kaplan–Meier method.

3 Results

3.1 *Immunogenicity of KillerRed*

A comparison of the growth rates of CT26 and CT26-KR tumors induced by the s.c. injection of 1×10^6 cells showed no statistically significant differences between the groups during the whole period of observation (Fig. 1). However, as can be seen from Fig. 1, the CT26-KR tumors, on average, grew a little more slowly than CT26 tumors, and there was a higher dispersion in size in the late stages of growth.

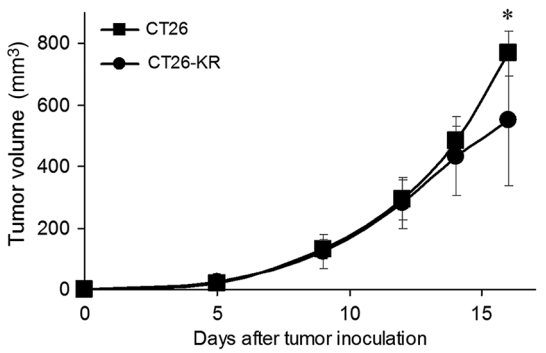

Fig. 1 Growth rate of CT26 and CT26-KR tumors. Tumors were induced by s.c. injection of 1×10^6 cells in Balb/c mice. Mean $\pm$ SD ($n = 7$). *Statistically significant differences between CT26 and CT26-KR tumors, $P = 0.0451$.

The expression of KR by the cancer cells reduced tumor incidence in the mice (Table 1). We found that 100% of the mice (13 out of 13) challenged with 1×10^6 wild-type CT26 cells developed tumors, while the susceptibility to CT26-KR tumors was lower, at 85% for the same cell dose (22 out of 26).

Four out of seven mice surgically cured of CT26-KR tumors and rechallenged with 5×10^5 CT26-KR cells demonstrated a protection against tumor, so the rest of the mice (three out of seven, 43%) had tumors (Table 1). CT26-KR tumor susceptibility following challenge with the same cell dose was 88% (22 out of 25). For comparison, rechallenge of surgically cured CT26-KR-challenged mice with 5×10^5 wild-type CT26 cells led to the formation of tumors in 78% of mice (seven out of nine). Rechallenge of CT26-challenged mice with CT26 or CT26-KR cells resulted in 87.5% and 80% tumor incidences, respectively.

Figure 2(a) shows that the growth rate of the rechallenged CT26-KR tumors was lower than that in the challenged ones for the same dose of cells. The rechallenged CT26-KR tumor volume reached 686 ± 228 mm^3, while the volume of challenged tumors in naïve mice was 1482 ± 385 mm^3 by day 22.

Based on fluorescence imaging *in vivo*, we found that the fluorescence intensity of the rechallenged CT26-KR tumors was much lower than that of the challenged CT26-KR tumors of the same size in the late stage of growth [Fig. 2(b)]. We suppose that this resulted from a reduced number of KR-expressing cells in the tumors, due to their eradication by the immune system.

Intravenous injection of a mixture of CT26 and CT26-KR cells (1 : 1) to mice surgically cured of CT26-KR tumors resulted in the generation of lung metastases only from CT26 cells. No KR fluorescence was detected in the lungs of mice previously having CT26-KR tumors, while all the naïve mice displayed fluorescent foci (Fig. 3). Interestingly, the number of metastases in naïve mice, counted macroscopically, was greater than the number of fluorescent foci, which indicates that the metastases were formed of both CT26 and CT26-KR cells.

Table 1 The susceptibility of Balb/c mice to challenge and rechallenge with CT26 or CT26-KR cells.

	CT26	CT26-KR
Tumor incidence after challenge with 1×10^6 cells	13(13), 100%	22(26), 85%
Tumor incidence after challenge with 5×10^5 cells	—	22(25), 88%*
Tumor incidence after rechallenge with 5×10^5 cells (CT26-KR-challenged mice)	7(9), 78%	3(7), 43%
Tumor incidence after rechallenge with 5×10^5 cells (CT26-challenged mice)	7(8), 87.5%	8(10), 80%

*Statistically significant difference between groups "Tumor incidence after challenge 5×10^5 cells" and "Tumor incidence after rechallenge with 5×10^5 cells (CT26-KR-challenged mice)". $P = 0.0239$.

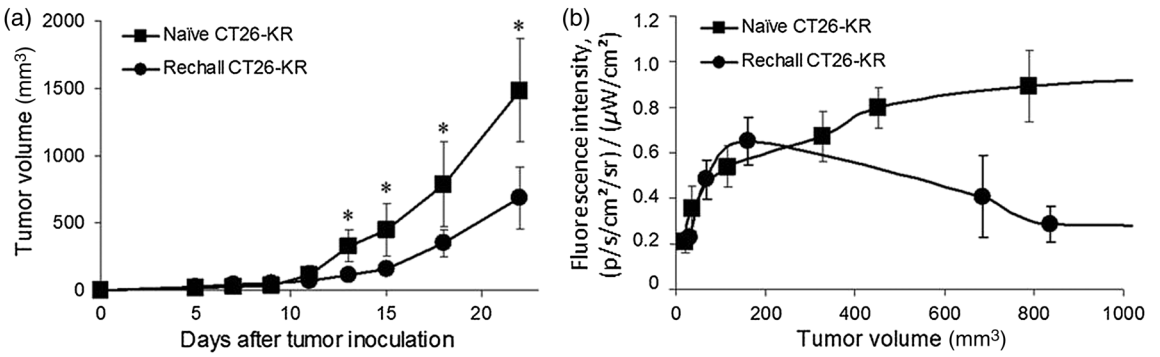

Fig. 2 (a) Growth rate and (b) *in vivo* fluorescence of CT-KR tumors. Tumors were induced by s.c. in naïve ($n = 7$) and surgically cured ($n = 3$) mice by injection of 5×10^5 cells. Mean ± SD. *Statistically significant differences between naïve and rechallenged CT26-KR tumor sizes, $P < 0.0339$.

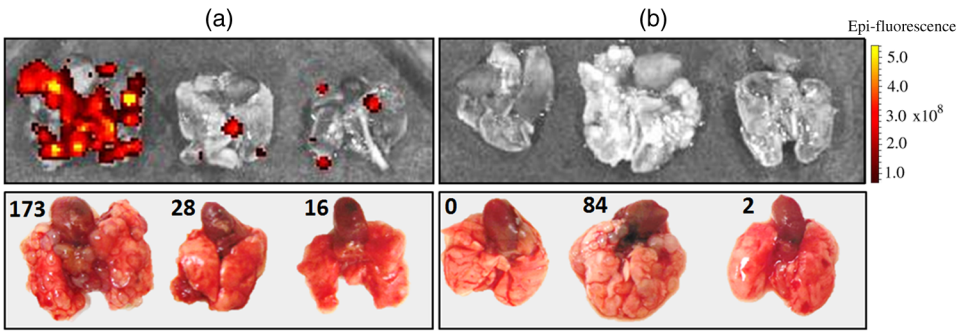

Fig. 3 Lung metastases in (a) naïve and (b) surgically cured mice. *Ex vivo* fluorescence imaging and photographs of freshly excised lungs. The number of lung metastases is shown. Metastases were induced by i.v. injection of a mixture of CT26 and CT26-KR cells to three surgically cured and three naïve mice.

3.2 *Cyclophosphamide Treatment*

As expected, treatment of the mice with CY led to inhibition of tumor growth, but no difference in the growth rate was found between the low-dose (50 mg/kg) and the therapeutic-dose (150 mg/kg) groups (Fig. 4). Unfortunately, there were no mice absolutely cured of tumors in either group.

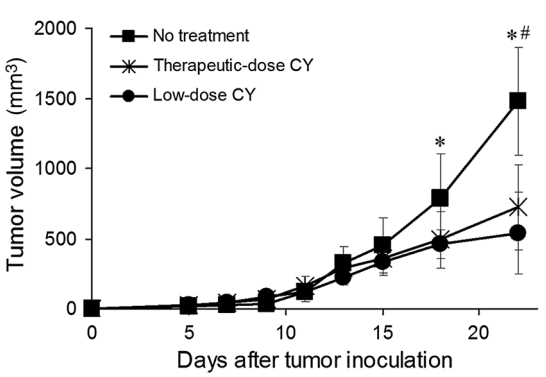

Fig. 4 Growth rate of CT26-KR tumors without any treatment, treated with a therapeutic dose, or a low dose of CY. Mean ± SD ($n = 7$). *Statistically significant differences between groups "no treatment" and "low-dose CY," $P \leq 0.0233$; and between groups "no treatment" and "therapeutic-dose CY," $P = 0.0274$. There are no significant differences between the groups "therapeutic-dose CY" and "low-dose CY."

The survival curves of CT26-KR tumor-bearing mice after low-dose or therapeutic-dose CY treatment and of those without any treatment are depicted in Fig. 5. It was found that low-dose CY treatment improved the survival more effectively in comparison with the other groups. The median survival of the low-dose CY treated mice was 29 days. At this time, in the therapeutic dose and in the control groups, survival was 40% and 14.3%, respectively.

4 Discussion

In this study, we assessed the immunogenicity of KR-expressing CT26 tumors in Balb/c mice, on the basis of susceptibility to

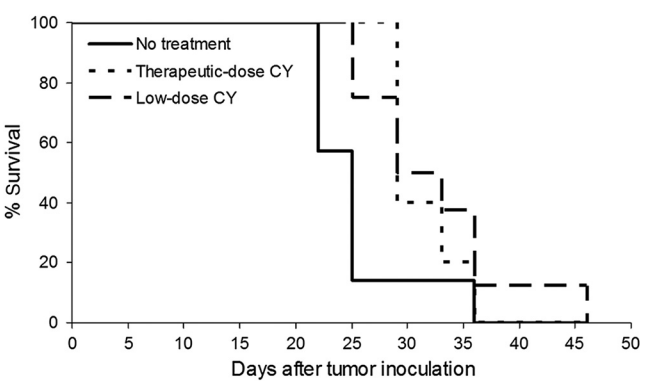

Fig. 5 Kaplan–Meier survival curves of groups of mice ($n = 7$). Mice were sacrificed when tumors reached a volume of 1500 mm^3.

tumor challenge and rechallenge, tumor growth rates, and metastasis formation.

It is known that tumor immunogenicity in patients varies greatly for different types of cancer.[34,35] Cancer cell lines also differ in their immunogenicity. For instance, sarcomas MCA101, MCA102, and Ag104, and melanoma B16 do not show any immunogenic properties. Some other cancer cell lines such as lymphomas RMA, EL-4, mastocytoma P815, and melanoma E6B2 are considered highly immunogenic.[25] The CT26 cell line used in our study is known to be poorly immunogenic. It was shown by Fearon et al. that the rechallenge of Balb/c mice with CT26 cells, following resection of primary CT26 tumors, failed to demonstrate there being any protection against the tumor rechallenge.[18] Our results on the rechallenge of CT26-challenged mice with CT26 or CT26-KR cells also indicate poor immunogenicity of CT26 tumor. Immunogenicity requires that the tumor cells express adequate levels of antigens and effectively present these antigens in a form that leads to immune system activation rather than tolerance.[36] The weak immunogenicity of the wild-type tumors limits the development of therapeutic approaches aimed at the activation of an antitumor immune response; therefore, great efforts are being made to increase the immunogenicity through endogenous or foreign antigen transduction.[19–25]

The investigation of immunogenicity of GFPs requires special attention since they are freely available and generally recognized genetically encoded markers for the fluorescence imaging of cells *in vitro* and tumors *in vivo*. The immunologic response to fluorescent proteins (GFP or EGFP) was demonstrated in different cancer models including BM185 leukemia,[20,21,37] EL-4 murine T-cell[20,37] and B-cell lymphomas,[38] 4T1 mammary carcinoma,[39] RIF-1 fibrosarcoma,[22] CMS4 sarcoma,[23] λ-hu-MYC lymphoma,[40] and CT26 colon carcinoma.[41] Our work represents a first attempt to discover the potential immunogenic properties of a red fluorescent protein.

In this research, we showed that, on average, CT26-KR tumors grew a little slower than wild-type CT26 tumors (statistically insignificant), but there were greater variations in size in the late stages of growth of CT26-KR. Castano et al.[22] reported similar results to ours, with no significant differences in the growth of EGFP-expressing and wild-type s.c. RIF-1 tumors in C3H/HeN mice, whereas Gambotto et al.[23] found that expression of EGFP completely inhibited the growth of CMS4 tumors following s.c. injection into Balb/c mice. Reduction of disease development in the immunocompetent mice was also shown in EGFP-expressing leukemia BM185[20,21,37] and EL-4 T lymphoma models.[20,37] It is suggested that the route of cell administration, the mouse strain, and age are important to initiate an anti-GFP immune response. It should be noted that there was no significant difference in the tumor incidence, the growth rate, or in the size and number of induced metastases between wild-type and transgenic tumors in the immunodeficient mice, while the development of EGFP-expressing tumors in the immunocompetent mice was inhibited.[20,23,41] This fact suggests an immune response to the EGFP transfected cells.

Furthermore, we have found that the rechallenge with CT26-KR tumors resulted in lower tumor incidence and slower tumor growth. In fact, in only three of seven rechallenges (43%) did CT26-KR tumors appear in surgically cured Bald/c mice. In contrast, according to Castano et al., all C3H/HeN mice surgically cured from RIF-1 EGFP tumors and all naïve mice that were challenged with RIF-1 EGFP cells displayed tumor incidence.[22] The difference may be associated with the higher immunogenicity of KR as compared with EGFP, its higher level of expression in the cells, or a lower ability of the CT26 cell line to form tumors in comparison with RIF-1. Nevertheless, the results of the growth rate of the rechallenged tumors were similar. Rechallenged CT26-KR tumors, as well as RIF-1 EGFP tumors, grew significantly slower in surgically cured mice or in naïve mice for the same dose of cells.

Finally, the metastatic activity of CT26-KR cells in cured mice was investigated. After i.v. injection of a mixture of CT26 and CT26-KR cells, we detected lung metastases in naïve Bald/c mice formed of both CT26 and CT26-KR cells, while no fluorescent CT26-KR cells were registered in the lungs of surgically cured mice. The number of lung metastases in naïve mice was generally higher than in mice surgically cured of CT26-KR tumors. Interestingly, Steinbauer et al.[41] showed that the long-term development, although not the early stages of CT26 liver metastases expressing EGFP, was markedly reduced in Bald/c mice compared with wild-type CT26 tumor cells.

In the present work, the efficacy of low-dose CY chemotherapy has been tested on a CT26-KR tumor model that displays immunogenic properties. It is supposed that the main mechanism of low-dose CY chemotherapy is the stimulation of the anticancer immune response owing to the blockade of Treg and the activation of CD8+ and CD4+ T-cells. It has been shown that a low dose of CY reduced CD4 FoxP3 Treg cells in the lymph nodes and provided a survival advantage; whereas high-dose CY reduced other lymphocyte classes as well.[12–14] Furthermore, low-dose CY induces the selective expression of thrombospondin-1 in tumor cells and apoptosis of proliferating endothelial cells, but has minimal direct effect on tumor cells and perivascular cells.[15] Therefore, the use of this therapeutic approach requires the highly immunogenic tumor.

We showed a significant reduction in the tumor growth rate after low-dose CY treatment in comparison with untreated tumors. Furthermore, mouse survival was increased in the group that received low-dose CY treatment. Similar results have been presented by Wada et al.,[13] where the efficacy of low-dose CY treatment was shown on mice spontaneously developing the transgenic adenocarcinoma of the mouse prostate expressing a unique, tumor-associated antigen. Castano et al.[12] demonstrated that low-dose CY treatment of J774 tumor-bearing mice, although not resulting in permanent cures, provided a better survival rate. In addition, Lutsiak et al.[14] showed that Lewis lung carcinoma in untreated C57BL/6 mice grew very large, while the growth of such tumors in low-dose CY-treated mice was suppressed.

5 Conclusions

These results have shown that the red fluorescent protein, KillerRed, can act as a foreign tumor antigen in immunocompetent mice. The immunogenic properties of the protein are manifested by the slower growth of KR-expressing CT26 tumors in comparison with nonexpressing ones and a lower tumor incidence and slower tumor growth of rechallenged tumors. In addition, KR-expressing lung metastases were not formed in mice that had been surgically cured of KR-expressing primary tumors. Low-dose CY treatment, which is known to stimulate antitumor immunity, was tested on the CT26-KR tumor model and resulted in a survival advantage and an

inhibition of tumor growth, comparable with high-dose CY treatment. Therefore, we have developed a highly immunogenic tumor model that may be valuable for investigations into the mechanisms of the immune response of the body against tumors and for the testing of new therapeutic approaches directed toward activation of the immune system.

Acknowledgments

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