Expression of soluble TGF-β receptor II by recombinant Vaccinia virus enhances E7 specific immunotherapy of HPV16 tumors

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Therapeutic immunization with double recombinants of vaccinia virus (VACV) co-expressing sTβRII increased rejection of established TC-1 tumors in C57BL/6 mice in comparison with single recombinant expressing SigE7LAMP. Recombinant VACV derived from vaccination strain Praha expressed either the sTβRII (ectodomain) or chimeric protein fused to immunoglobulin Fc fragment (sTβRII-Fc-Jun) under control of two different promotors together with the immunogenic tumor associated antigen HPV16 E7 oncoprotein in a form of SigE7LAMP fusion molecule. The ability of soluble receptors to bind TGF-β *in vitro* was proved. Immunization of mice with double recombinant viruses and virus expressing SigE7LAMP only led to eliciting similar response of E7 specific CD8+ T cells as detected by IFN-γ ELISPOT.

Key words: HPV16, E7; vaccinia virus; TGF beta; TGF beta receptor II; tumor; immunity

High risk human papilloma viruses (HPV) have been identified as the etiological agents of carcinomas of anogenital tract and they are suspected to be also involved in induction of other tumors [1,2]. In 95% of healthy individuals, the immune system can eliminate HPV infections of anogenital tract within 12 to 36 months [3,4,5]. The crucial role of Th1 and CD8+ T cells directed against early proteins E2, E6 and E7 and E5 [6] was recognized. Since the expression of E6 and E7 proteins in high-grade precancerous lesions and invasive cancers is essential for the induction and maintenance of oncogenic transformation of HPV infected cells [7], the E6 and E7 proteins appear, hence, the most suitable antigenic targets for the therapy of HPV-induced lesions and tumors. The feasibility of therapeutic vaccination approach including synthetic peptides, recombinant proteins, virus like particles, DNA vaccines, viral and bacterial vectors was examined using HPV16-E6 and E7 expressing tumor cell lines in mice model [8,9,10].

Transforming growth factor β (TGF-β) is a pleiotropic cytokine with regulatory roles in processes such as tissue growth and morphogenesis or the development of immune responses. In the immune system, the TGF-β controls the inhibition of immunopathological reactions to self-antigens without compromising immune responses to pathogens. Three TGF-β isoforms are produced by all cells of leukocyte lineages. Most of the hematopoetic cells have TGF-β receptors, thus the TGF-β cytokines are potent regulators of functions of T cells, NK cells, dendritic cells and macrophages which are the most important effector cells of anti-tumor immunity. TGF-β inhibits the proliferation of T cells by inhibition of IL2 gene expression and through inteference with cell cycle engine [11,12]. The blockade of Th1 differentiation is mediated through downregulation of IL12 receptor β2 and synthesis of IFN-γ. Cytotoxic activity of CD8+ T cells is inhibited by downregulation of perforin, granzyme A/B, Fas ligand and IFN-γ expression [12]. TGF-β can convert CD4+T cells to regulatory FOXP3+T cells with immuno-suppressive activity [13] which can decrease T cell response against tumors [14]. TGF-β abrogates the function of NK cells by inhibition of activating receptors and by downmodulation of IFN-γ and TNFα [15]. The interference of TGF-β with induction of adaptive immune response occurs also through an inhibition of maturation of dendritic cells which serve as professional antigen presenting cells [16]. During tumor progression, cancer cells can become resistant to growth-inhibitory activity

of TGF-β; they produce increased amount of TGF-β or promote its production by surrounding cells [17]. Consequently, TGF-β produced by tumor cells downregulates immune responses to tumor antigens and allows the tumor to evade the immune surveillance [18]. There are three possible ways how to avoid the deleterious effect of TGF-β in the therapy- to use antisense oligonucleotides, molecular traps or farmacological molecules. The enhanced protection against tumor formation and growth as well as a shift to Th1 and CTL responses were achieved by neutralization of TGF-β with specific antibodies during immunization against tumor antigen [19,20,21]. It has been shown that systemic neutralization of TGF-β by soluble TβRII (sTβRII) expressed by EL4 tumor cells was able to prevent suppression of tumor specific CD8+ T cell cytotoxicity *in vivo* [12,22] However, a soluble TβRII has approximately 10-times decreased affinity to TGF-β [23] probably due to its monomeric form; this has been overcome with utilization of chimeric sTβRII-Fc [24].

The Fc region of human IgG contains several cystein residues ensuring chimeric molecule dimerization. This facilitates efficient production and secretion of many different categories of proteins. The Fc chimeras retain full biological activity, ligand-receptor binding affinity and appear to be more uniformly glycosylated. The Fc region helps also to fix the complement, to increase the half-life, to cross the blood-brain barrier or to easy isolate the molecule through protein A/G binding [25,26]. Soluble Fc-receptor molecules have been succesfully used to treat the tumors of different origin. The treatment of mammary tumor cells and primary tumors with TβRII-Fc abrogated the TGF-β autocrine signalization loop and increased the apoptosis [27,28]. Its expression by MCF-7 human breast cancer cells reduced their metastatic potential [29]. The combination of chemoterapeutic treatment with TβRII-Fc increased the inhibition of KAT-4 thyroid carcinoma cells [30].

In this study, we enhanced the therapeutic effect of recombinant vaccinia virus vector expressing an immunizing HPV16-E7 tumor antigen – the fusion protein SigE7LAMP – with the co-expression of a soluble TGF-β receptor II in the same vector. We found out, that the production of sTβRII significantly enhanced the inhibition of growth of TC-1 tumors in laboratory mouse.

Material and methods

Plasmids. To prepare secreted form of TβRII , the coding sequence of the extracellular domain of TβRII was amplified by semi-nested PCR with primer #1: (5′-CATGGG TCG CGG ACT TCT CAG AGG CCTGT-3′) and primer #2: (5′-ATG AAT TCC ATGGGT CGCGGA CTT CTC AGAGGC-3′) in the first reaction, and subsequently with primer #3: (5′-TAAGGC CTG CAA CTAGTC AGG ATTGCTGGT-3′) and primer #2 using plasmid pcDNA1/Neo-hTβRII containing the complete coding sequence of human TβRII as a template (plasmid pcDNA1/ Neo-hTβRII was obtained through the courtesy of H.F. Lodish and H.Y. Lin, Cambridge, USA). The amplified fragment was inserted into plasmid pCR4-TOPO (Invitrogen) and the

cloning correctness was verified by sequencing. The resulting plasmid pCR4-TOPO-TβRII was cleaved with EcoRI and a 478 bp fragment encoding ectodomain of TβRII was inserted into a plasmid pSC59H5 [31] cut with the same restriction enzyme, yielding the plasmid pSC59H5-sTβRII. Alternatively, the EcoRI fragment was subcloned into pBluescript II SK+ cleaved with the same enzyme. Consecutively, the BamHI/HindIII fragment was inserted in pSC59H5 cleaved with the same enzymes, yielding the plasmid pSC59H5-E/L-sTβRII.

To prepare plasmids carrying the chimeric gene encoding the sTβRII fused with immunoglobulin Fc fragment (TβRII-Fc-Jun), the pFastbac-TβRII-Fc-Jun plasmid was cleaved with EcoRI, HindIII restriction endonucleases (pFastbac-TβRII-Fc-Jun plasmid was obtained through the courtesy of D. Kowalczyk, Poznan, Poland). The isolated 1.4 kbp fragment was ligated with pBluescript II SK+ cut with the same enzymes. The pBluescript-TβRII-Fc-Jun plasmid was subsequently cleaved with EcoRI and XhoI and the isolated 1.4 kbp fragment was ligated with pSC59H5 cut with the same enzymes, yielding a plasmid pSC59H5-TβRII-Fc-Jun. To prepare pSC59-E/L-TβRII-Fc-Jun, the H5 promoter was excised from plasmid pSC59H5-TβRII-Fc-Jun by SalI and XhoI restriction endonucleases and the isolated fragment was ligated.

Recombinant vaccinia viruses. Vaccinia virus strain Praha, clone 13 (32) was used as parental virus. The coding sequence of sTβRII or TβRII-Fc-Jun was inserted in thymidine kinase gene behind an early/late H5 promoter or a strong synthetic E/L promoter by the method described previously (33). Plasmids pSC59H5-sTβRII, pSC59 E/L-sTβRII, pSC59H5-TβRII-Fc-Jun or pSC59-E/L-TβRII-Fc-Jun were used for generation of recombinant viruses which were denoted as P13-H5-sTβRII and P13-E/L-sTβRII, P13-H5-TβRII-Fc-Jun or P13-E/L-TβRII-Fc-Jun. In double recombinants, the sTβRII or TβRII-Fc-Jun coding sequence was inserted into vaccinia virus P13-SigE7LAMP expressing the fusion gene SigE7LAMP (Sig-signal sequence of the lysosome-associated membrane protein1 (LAMP1), E7-early protein of HPV16, LAMP-transmembrane and intracellular domain of LAMP1) (34). The control virus P13-SigE7LAMP TK- contains the TK gene disrupted by insertion of 11k promoter sequence. Viruses were grown in BSC40 cells, purified by sucrose-gradient centrifugation (35) and titrated in CV-1 cells.

Antibodies. Biotin-labeled purified Rat-α-TGFβ−1 (clone A75-3) immunoglobulin (BD Pharmingen, USA), Avidin-HRP (BD Pharmingen, USA), Goat-α-hTβRII IgG (RD Systems, USA), Rabbit-α-Goat-HRP IgG (Jackson ImmunoResearch, USA) were used for immunoblotting. Protein-G immobilized on fast-flow agarose was used for immunoprecipitation (Sigma-Aldrich, USA).

Cells. TC-1 cells [36], were kindly provided by T.C. Wu (Baltimore). BSC40, CV-1 and TC-1 cell lines were cultivated in modified E-MEM (EPL) medium containing bovine serum growth-active proteins but no complete serum [37]. HeLa cells were grown in D-MEM supplemented with 10% fetal bovine serum.

Immunoblot. The procedure was described earlier (38). Antibodies used for detections were goat-α-hTβRII IgG (diluted 1:2000) with secondary antibody Rabbit-α-Goat-HRP IgG(diluted 1:4000); or Biotin-labeled purified Rat-α-TGFβ−1 (diluted 1:500) followed by Avidin-HRP-complex (diluted 1:1000). Proteins were visualized with the ECL Plus system (Amersham).

TGFβ binding in vitro. CV-1 cells (6.10^6) infected at a MOI of 2 with P13-E/L-sTβRII or P13-E/L-TβRII-Fc-Jun were 24 h.p.i. washed twice with PBS, the suspension was three times freezed/thawed in 4 ml of PBS and the cell debris was removed by centrifugation. The virus was removed by filtration through 0.2 μm filter (Sarstedt, Germany) and the lysate was concentrated to 0.5 ml using 10kDa cut-off Amicon ultra centrifugation device (Millipore, USA). 10 μl of the lysate was mixed with 5 ng of TGFβ (Peprotech, USA) and 1 μl (250 ng) of Goat-α-hTβRII IgG and incubated on ice each for 1 hour followed by immunoprecipitation using 10 μl of 50% suspension of Protein-G agarose beads. The immunocomplexes were examined by immunoblot for the presence of TGFβ protein.

Animal experiments. Six-week-old C57BL/6 (*H2^b*) female mice obtained from Charles River, Germany 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 animal protection against cruelty and Decree No. 311/97 of the Ministry of Health of the Czech Republic, on the care and use of experimental animals. For immunization mice were injected intraperitoneally (i.p.) with 0.5 ml PBS containing sonicated suspension of rVV. To induce the tumors, the TC-1 cells were injected subcutaneously (s.c.) in the back of mice. Tumor growth was measured weekly. Differences between growth curves were statistically analyzed by the two-way ANOVA. All statistical analyses were performed using the Prism 5.0 (GraphPad Software, Inc, San Diego).

Assay of E7 specific T-cell responses. Spleens were removed from mice twelve days after the immunization and the response of IFN-γ-producing cells was determined by ELISPOT-IFNγ assay upon restimulation with H-2D^b restricted HPV16 E7$_{(49-57)}$ (RAHYNIVTF) or VACV E3$_{(140-148)}$ (VGVSNSPTF) peptide for 20 hours *in vitro*, as described previously [34]. The mean spot numbers obtained for peptide-restimulated and control cells were analyzed by the unpaired t-test.

Detection of VACV multiplication in vivo using quantitative PCR (Q-PCR). Mice were anaesthetized with halothane (Narcotan, Léčiva, Prague) and sacrificed. The ovaries were dissected, washed in PBS and homogenized. DNA was extracted using DNeasy Tissue Kit (Qiagen). Real-time quantitative PCR was performed as described previously [39]

Results

Characterization of vaccinia viruses expressing sTβRII. To evaluate the effect of TGF-β neutralization on the immunogenicity and immunotherapeutic potential of vaccinia virus vectors against the growth of tumors, we constructed recombinant vaccinia viruses expressing extracellular domain of TβRII (Fig.

1A). Viruses denoted P13-H5-sTβRII or P13-E/L-sTβRII, carried the coding sequence of sTβRII. To enhance the TGF-β1 binding, we constructed viruses P13-H5-TβRII-Fc-Jun and P13-E/L-TβRII-Fc-Jun expressing the chimeric sTβRII fused to the Fc region of human IgG and C-terminal Jun fragment (TβRII-Fc-Jun) which provides dimerisation and efficient secretion of TβRII (40). The expression was regulated either by promotor H5 or by E/L. First, we determined the synthesis of TβRII *in vitro* in cells infected with VACV recombinants. HeLa cells were infected at a MOI of 1.0 with the parental virus P13 or with recombinant viruses P13-H5-sTβRII, P13-E/L-sTβRII, P13-H5-TβRII-Fc-Jun and P13-E/L-TβRII-Fc-Jun, and receptor production in medium and cell lysate was determined 20 hours post infection by immunoblot (Fig. 1B). Cell associated sTβRII molecules migrated under reducing conditions at m.w. 19 and 25 kDa. Secreted glycosylated forms of sTβRII of m.w. about 25 and 32 kDa were found in cell medium. The N-glycosylation patterns were studied by infection in the presence of tunicamycin (2 mg/ml) where the unglycosylated sTβRII of m.w. 19 kDa was found. As we expected, tunicamycin inhibited secretion of sTβRII into the supernatants (not shown). Similarly to sTβRII, the chimeric soluble sTβRII-Fc-Jun was present both in infected cells and in medium (Fig. 1B). The sTβRII-Fc-Jun molecules migrated around the molecular mass of 65 kDa showing an increased glycosylation of the receptor present in medium in comparison to the cell associated receptor.

Binding activity of sTβRII. To determine the capability of sTβRII and sTβRII-Fc-Jun to bind native TGF-β1 protein *in vitro*, the lysates of cells infected with P13-E/L-sTβRII or P13-E/L-TβRII-Fc-Jun were incubated with or without TGF-β1 protein. The receptor-ligand complexes were immunoprecipitated with antibody against the TβRII and isolated using immobilized protein G. The presence of both the TGF-β1 and the receptor in samples was determined by immunoblot using specific antibodies (Fig. 1C). We observed that TβRII specific antibody was able to to pull down TGF-β1 which was associated either with sTβRII (line 5) or with sTβRII-Fc-Jun (line 6), so we confirmed the ability of both the sTβRII and sTβRII-Fc-Jun to bind TGF-β1. The complex TGF-β1/sTβRII-Fc-Jun was detected also when the receptor was incubated with TGF-β1 in the absence of specific antibody against TβRII (line 4) which is in accord with the presumption that the chimeric receptor binds to protein G and that the Jun portion does not interfere with protein G-binding domain of Fc fragment. Direct binding to protein G was not observed in case of sTβRII (line 3). These results were important for the intention to prepare improved rVACVs useful for vaccination against cancer.

The influence of soluble TGF-β receptor II expressed by rVACV on E7 specific T cell response. First, we examined whether the co-expression of receptors influences the T cell response to E7 antigen expressed by rVACV. The groups of mice (n=4) were immunized i.p. with one dose of rVACV ($3x10^6$ PFU) and the E7 specific cellular immune response to H-2D^b restricted E7 peptide RAHYNIVTF was determined by ELISPOT 12 days later. The E7-specific CD8^+ T cells among freshly isolated splenocytes were detected in all animals immu-
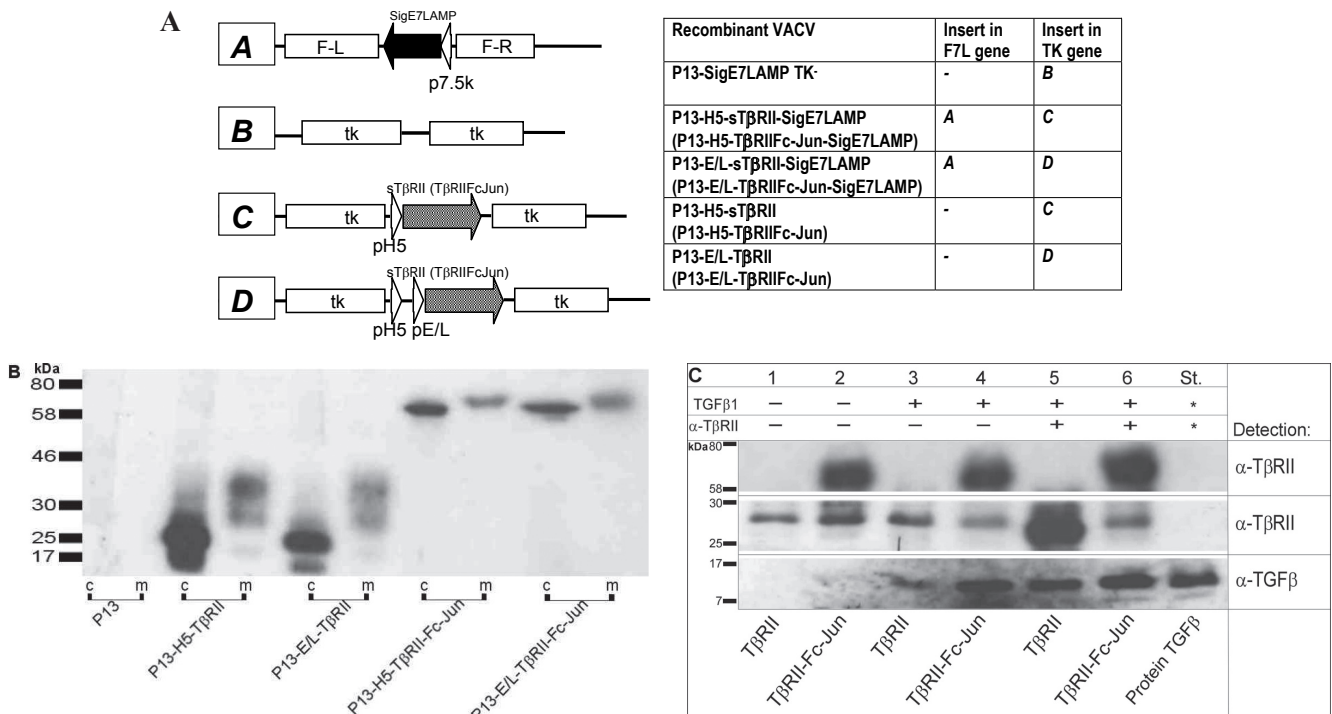
Fig. 1 Recombinant vaccinia viruses expressing soluble TβRII. (A) *Structure of the constructs inserted in the genome of rVACVs.* (*A*) **SigE7LAMP fusion gene inserted in the nonessential F7L locus behind the 7.5k early/late promoter (p7.5k).** (*B*) **TK gene disrupted by insertion of a non-coding DNA sequence. (***C***) Soluble TβRII or (TβRII Fc-Jun), resp. inserted into thymidine kinase locus (TK) under the control of the early/late H5 promoter (pH5)** (*D*) **Soluble TβRII or (TβRII Fc-Jun) resp. inserted into thymidine kinase locus (TK) under the control of the strong synthetic early/late promoter (pEL). The structure of recombinants is shown in the table on the right side of figure. (B)** *Immunoblot analysis of soluble TβRII produced by rVACV in vitro.* **HeLa cells were infected at a MOI of 1.0 for 1 h, washed with PBS and cultivated in serum-free D-MEM. Media (m) and cells (c) were harvested 20 hours p.i. The samples were analyzed by immunoblot using goat-α-hTβRII IgG and rabbit-α-goat-HRP IgG. The scale in the figure 1 B and C shows kilodaltons (kDa). (C)** *In vitro binding of soluble sTβRII or chimeric sTβRII-Fc-Jun to TGFβ.* **The concentrated lysate of cells infected with recombinant virus was incubated with 5 ng of human TGF beta, α-hTβRII IgG (250 ng) and 10 μl of protein G immobilized on agarose beads, each for 1 hour on ice. The immunocomplexes bound to protein G-agarose were analyzed by immunoblot using α-hTGFβ and α-hTβRII antibodies combined with appropriate secondary antibodies.**

nized with SigE7LAMP (Fig. 2). There was found no significant enhancing effect of sTβRII co-expression on the E7 specific immune response in comparison with P13-SigE7LAMP-TK⁻ vector. In contrast to E7 a specific response against the VACV E3 protein was elevated in groups of animals immunized with P13-H5-sTβRII-SigE7LAMP and P13-E/L-sTβRII-Fc-Jun-SigE7LAMP; though the difference of the mean was statistically significant only for the former virus (p=0.035).

The effect of co-expression of soluble TGF-β receptor II by rVACV on therapeutic immunization against TC-1 tumors. The therapeutic effect of immunization was examined in animals carrying the tumors of mean area 4.3-6.4 mm². The groups of mice (n=6) were s.c. injected with 6x10⁴ TC-1 cells in the back. On day 9, they were immunized i.p. with 1x10⁶ PFU of rVACV and the growth of tumors was measured up to 60 days (Fig.3). The experiment was repeated twice with the same result. In general, the treatment with all SigE7LAMP expressing viruses resulted in delayed growth of tumors in comparison to the control group injected with PBS. The immunization with a double recombinant P13-H5-sTβRII-SigE7LAMP induced the

regression of TC-1 tumors in all the animals on days 25-50; the difference was statistically significant in comparison with control group injected with PBS (p=0.0062) and with the group immunized with P13- SigE7LAMP-TK⁻ (p=0.0327). Surprisingly, the expression of chimeric sTβRII-Fc-Jun did not improve the therapeutic potential of the vaccine in this arrangement. Furthermore, the positive effect of soluble TGF-β receptors presence on a tumor growth inhibition was statistically significant only if co-expressed with SigE7LAMP. The treatment with single recombinant viruses lacking the expression of a tumor specific E7 antigen did not exert any effect on the tumor growth.

The effect of sTβRII expression by double recombinants P13-sTβRII-SigE7LAMP on the multiplication in vivo. As we found a significant difference in the immunogenicity and antitumor effect of an immunization with P13-H5-sTβRII-SigE7LAMP in comparison with the single recombinant P13-SigE7LAMP-TK⁻, we investigated the possibility that the sTβRII expression might have affected virus multiplication *in vivo*. Mice were injected i.p. with 1x10⁶ PFU of rVACVs and the ovaries were collected on days 0, 3 and 4. The replication of viruses was quantified by Q-PCR

ELISPOT IFNγ
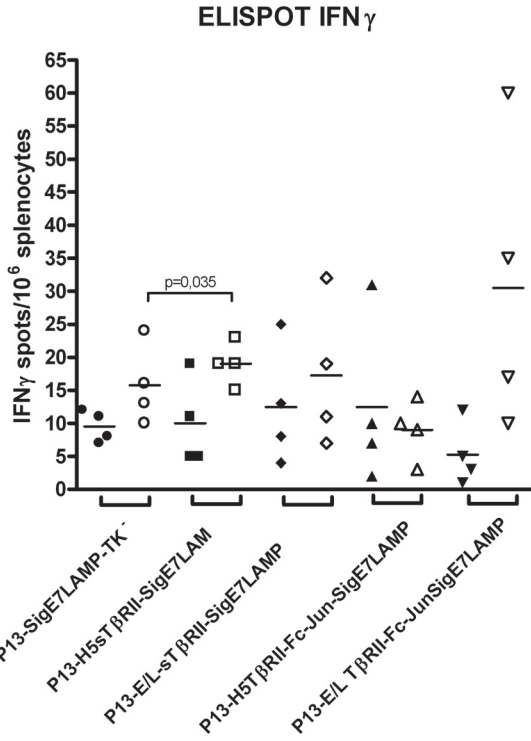
assay in the DNA isolated from the mouse ovaries. Virus multiplication on days 0, 3, 4 is shown in Fig. 4. The coexpression of the sTβRII gene under H5 promotor along with the SigE7LAMP fusion construct did not significantly affect the multiplication of rVACV. We can conclude that the enhancement of antigen specific T cell immunity and the antitumor effect of P13-H5-sTβRII-SigE7LAMP were not associated with higher growth rate of the recombinant virus *in vivo*.

Discussion

This is the first report where it was described, how it is possible to enhance the immunotherapy of HPV induced tumors using live recombinant vaccinia virus vector expressing simul-

Fig. 2 T cell responses elicited by inoculation of sTβRII expressing viruses. C57BL/6 mice (n=4) were injected i.p. with 3x10^6 PFU rVV. Splenocytes were isolated twelve days after immunization. Induction of specific CD8+ T cells was determined in freshly isolated splenocytes of individual mice by IFN-γ ELISPOT. The background mean values obtained in the absence of the peptide were subtracted from mean values obtained in the presence of peptide for each animal. Responses specific for HPV16-E7 protein (full symbols) and VACV E3 protein (empty symbols) in each group were compared and analyzed by the unpaired t-test. The cellular immune response was determined in three independent experiments.
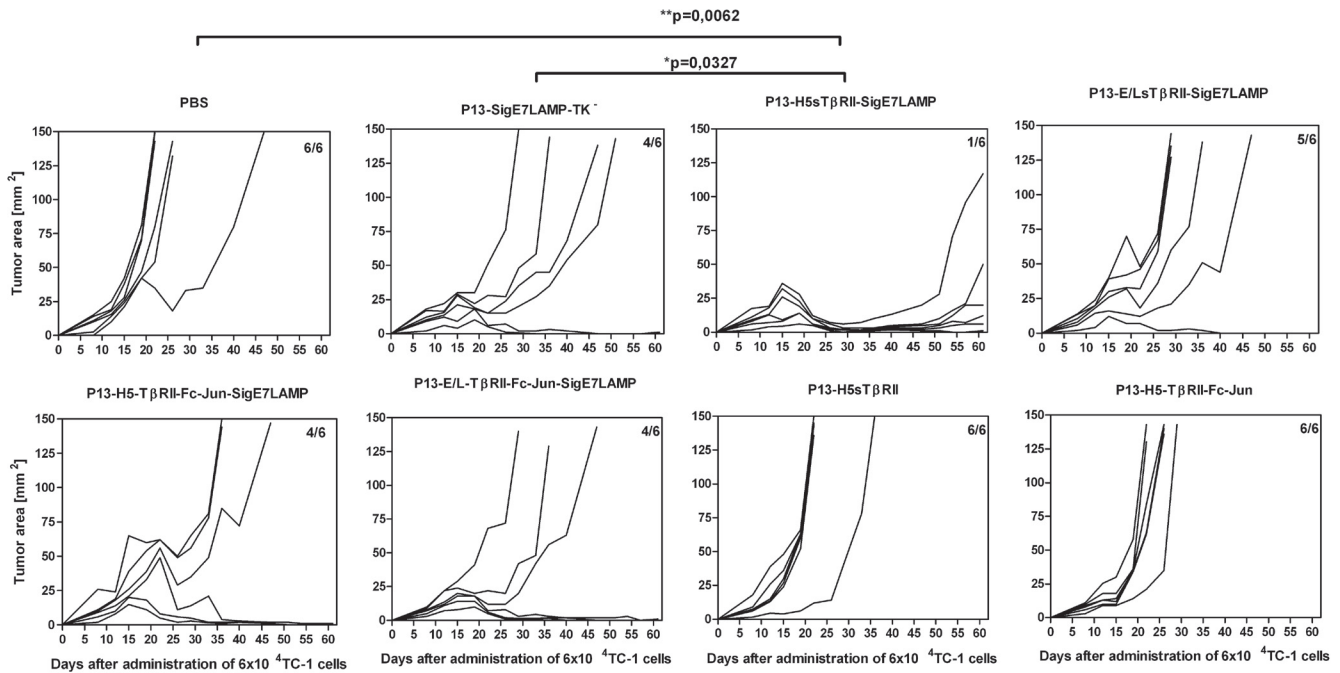

Fig. 3 Effect of sTβRII co-expression by P13-SigE7LAMP-TK⁻ on anti-tumor effect of therapeutic immunization. For evaluation of therapeutic efficacy of recombinant viruses expressing sTβRII and SigE7LAMP, mice (n=6) were s.c. injected with 6x10⁴ TC-1 cells. Nine days later, the animals were i.p. injected with 1x10⁶ PFU of the P13-SigE7LAMP-TK⁻, P13-H5-sTβRII-SigE7LAMP, P13-E/L-sTβRII-SigE7LAMP, P13-H5-sTβRII-Fc-Jun-SigE7LAMP, P13-E/L-sTβRII-Fc-Jun-SigE7LAMP, P13-H5-sTβRII or P13-H5-sTβRII-Fc-Jun virus. The growth of tumors is shown on separate graphs for each of the tested groups with the fraction of mice developing tumors after 30 days indicated in the upper right corner. The tumor growth curves were statistically analyzed by comparison with the group treated with P13-SigE7LAMP-TK⁻ using two-way ANOVA. The experiment was repeated twice with similar results. Here are shown results of one therapeutic experiment.
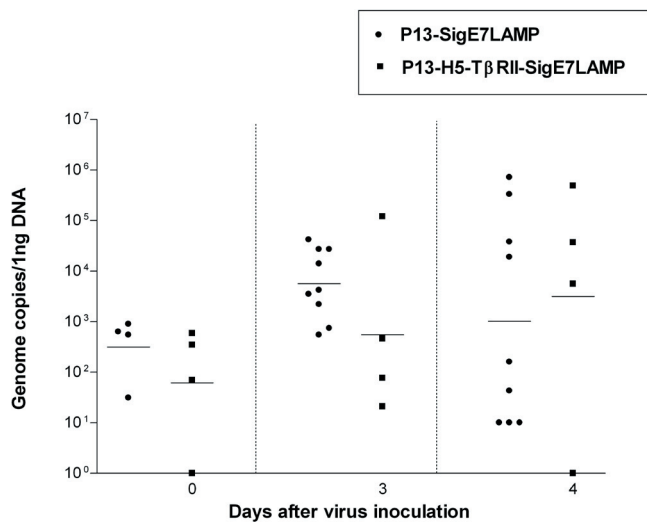
Fig. 4 In vivo multiplication of viruses expressing SigE7LAMP and sTβRII. Groups (n=4) of C57BL/6 female mice were inoculated i.p. with 1×10^6 pfu of P13-SigE7LAMP-TK⁻ or P13-H5-sTβRII-SigE7LAMP. The ovaries were collected at various time intervals after infection. Replication of rVACV in the ovaries was determined by Q-PCR. Geometric means are shown. The data were analyzed by the two-way ANOVA.

taneously the modified HPV specific E7 antigen and a soluble form of transforming growth factor-beta type II receptor.

One of the mechanisms involved in tumorogenicity of high risk HPVs is based on the ability of E7 protein to upregulate the TGF-β expression in transformed cells. There was found a direct proportion between severity of high risk HPV induced cervical lesions and the amount of TGF-β signaling pathway proteins in patients´ tissue and serum. These results corroborate with findings that TGF-β1 expression was higher in the high risk HPV genome containing cervical cell lines SiHa, HeLa and CasKi than in cultured HPV negative cell lines. Moreover, it has been shown that knocking down the E7 expression by specific antisense ODN downregulated also TGF-β expression in CasKi and Siha cells [41]. These facts hinted TGF-β as a prospective target for therapy of HPV associated malignancies. The expression analysis revealed the presence of TGF-β1 transcripts in HPV16-E6/E7 transformed TC-1 cell line which was used in this study (M. Smahel, personal communication). We speculated that blocking of TGF-β might enhance the efficacy of immunotherapy of tumors originating from TC-1 cells and for this purpose we developed recombinant vaccinia viruses (rVACV) expressing soluble TGF-β receptors. We chose the strain Prague, clone 13, of vaccinia virus for its high immunogenicity and low virulence when compared to well known WR strain (42). VACV expressing SigE7LAMP fusion protein had been used before for the treatment of E6/E7 expressing tumors (31). In our experimental model, we used two forms of soluble TGFβ receptor II – the first one in a previously described form of an extracellular sTβRII domain [23] and the second one as a chimeric extracellular domain of sTβRII fused to immunoglobulin Fc portion tagged with Jun fragment. It has been

shown previously that chimeric sTβRII-Fc has higher biological activity than sTβRII [24]. The C-terminal Jun fragment in our construct should further increase the TGFβ binding by chimeric receptor (D. Kowalczyk, personal communication). Soluble receptors were expressed by vaccinia virus either under control of H5 promoter, which is active mainly in the early phase of VV replication cycle, or under control of a synthetic E/L promoter that is a potent inducer of strong late phase expression.

Both types of TβRII produced in rVACVs infected cells were partially secreted in properly glycosylated form and partially remained associated with infected cells as underglycosylated precursor. Despite of the presumptive weaker expression directed from H5 than from E/L promoter, we found surprisingly higher levels of sTβRII/sTβRII-Fc-Jun present in cytoplasm of cells infected by P13-H5-sTβRII or P13-H5-sTβRII-Fc-Jun at 20 h.p.i. However, the amount of soluble receptors released into the medium of all rVACVs infected cells was nearly the same in later intervals what was the important result for therapeutic experiments.

In our experimental model, we tried to eradicate tumors induced by inoculation TC-1 cells expressing HPV16 E6/E7 antigens and growing subcutaneously at mice back. We treated mice with one dose of recombinant virus; however, the simple expression of sTβRII by rVACV failed to affect growth of TC-1 tumors in mice and induced no E7 specific response. It is in contrast to results of Kontani [43] where administration of DNA encoding sTβRII was able to elicit potent immunity against ovalbumin in the role of extrinsic tumor antigen [43]. The E7 protein present in TC-1 cells is a poor immunogen in comparison with highly immunogenic ovalbumin and TC-1 cells are not able to stimulate efficient antitumor response even if the TGFβ is neutralized by the soluble receptor. Therefore, we constructed double recombinant viruses carrying both the sTβRII and E7 antigen in a highly immunogenic form of SigE7LAMP fusion protein. The regression of tumors occurred only in animals which were simultaneously immunized with SigE7LAMP and treated with soluble TβRII mainly with the P13-H5-sTβRII-SigE7LAMP recombinant, whereas none of the chimeric sTβRII-Fc-Jun expressing viruses increased the therapeutic potential of rVACV. We found, that P13-H5-sTβRII-SigE7LAMP induced highest amount of the less glycosylated sTβRII which remained associated with cells. We suppose that anti-tumor activity is not enhanced only by secreted receptors, but also by the large amount of intracellular underglycosylated sTβRII, which can get away from cells by different mechanisms. One possibility is that the cell associated receptor can be released later due to the cell destruction. The rationale for the second way is based on finding, that cells infected with VACV release plenty of extracellular vesicles containing virus encoded products [44] similar to exosomes. Exosomes are formed by invagination of the limiting membrane of late endosomes and once released, exosomes can fuse with membranes of neighboring cells, delivering membrane and cytoplasmic proteins from one cell to another. Membrane bound TGF-β might also be delivered by exosomes to CD8+ cytotoxic T lymphocytes or to NK cells thus causing their

poor functional response [45]. We speculate that intracellular sTβRII which could be released from infected cells via exosomes, could neutralize this exosome-bound TGF-β just in the blood thus providing enough space for the activation of anti-cancer immune response.

In contrast to the enhancement of anti-tumor activity in tumor bearing mice, the co-expression of either sTβRII or sTβRII-Fc-Jun by rVACVs did not significantly enhance the response of HPV16-E7 specific CD8+ T cells either detected by ELISPOT-IFN-γ or by tetramer assay (not shown) as we determined 12 or 26 days after virus inoculation in immunized mice without tumors. It would be interesting to measure the response of INFγ producing T cells to immunization in the tumor bearing mice. Moreover, we found that the reduction of tumor growth by P13-H5-sTβRII-SigE7LAMP was not caused even by changed multiplication of the virus. In this respect, we can only deduce what was the real mechanism of its anti-cancer activity. The P13-H5-sTβRII-SigE7LAMP could exert the positive effects on tumor growth inhibition by changes in tumor microenvironment and by the influence on several immune cell populations. Cellular interactions in the tumor stroma play a major role in cancer progression or tumor rejection and taking away their stimuli is the step towards faster tumor rejection. The successful treatment of TC-1 derived solid tumors using P13-H5-sTβRII-SigE7LAMP could mean that the main target of sTβRII molecule is soluble TGF-β produced in tumors. We can conclude that the neutralization of TGF-β in combination with eliciting E7 specific T cell immunity may increase the therapeutic effect of vaccines directed against HPV associated cancer.

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