

# Adenoviral B7-H3 therapy induces tumor specific immune responses and reduces secondary metastasis in a murine model of colon cancer

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**Abstract.** Current cancer gene therapies aim at the induction of systemic antitumor immune responses. Tumors may deliver antigens to T-cells, but may lack the costimulatory signals necessary for mounting an effective response. The purpose of this study was to evaluate the efficacy of an adenoviral delivery of the B7-H3 costimulatory molecule in mice to induce antitumor immune responses. Colon cancers were established by orthotopic injection of syngeneic colon cancer cells into the cecum on Balb/c mice. After two weeks, these mice were treated by intratumoral injection of an adenovirus expressing mouse B7-H3 (Ad-B7-H3-GFP) or a control virus (Ad-GFP). Ad-B7-H3-GFP treatment resulted in a reduction of tumor size compared to the controls. In addition, the occurrence of secondary metastasis was significantly reduced in B7-H3 treated mice compared to control animals (lymph node 7/10 vs. 10/10; liver 2/10 vs. 8/10,  $p \leq 0.05$ ). Ad-B7-H3-GFP treated animals showed significantly higher frequencies of tumor-specific interferon- $\gamma$  producing CD8<sup>+</sup> T-cells ( $p \leq 0.05$ ) and higher interleukin-12 levels ( $p \leq 0.01$ ) than control animals. This study demonstrates that adenoviral B7-H3 transfer is able to induce a specific cellular antitumor immune response leading to primary tumor regression and reduction of secondary metastasis *in vivo*.

## Introduction

Colorectal cancer is the second most common cancer with approximately 300,000 new cases and 200,000 deaths per year in the western world (1). Despite advances in surgical therapy

colon cancer often recurs and the most common sites of recurrence are the liver and the peritoneal cavity. Furthermore, occult metastasis might already be present at initial diagnosis and has been shown to be an independent predictor of survival (2). The relatively low success rates of current treatments have led to the development of gene therapies aiming at the induction of tumor specific immune responses (3).

B7-H3 is a newly discovered member of the B7-family of costimulatory molecules with human and mouse B7-H3 sharing about 88% amino acid identity. Unlike B7-H1 and B7-H2, its mRNA is broadly expressed in lymphoid and non-lymphoid organs (4). B7-H3 has been shown to costimulate the proliferation of CD4<sup>+</sup> and CD8<sup>+</sup> T-cells and to stimulate interferon- $\gamma$  (IFN- $\gamma$ ) production and cytolytic T-cell activity (5,6).

For the study of colon cancer metastasis, orthotopic colon cancer animal models mimic human disease more closely than ectopic tumors or heterogeneic tumors in nude mice (7). In the present study, we investigated whether injection of a B7-H3 recombinant adenovirus in readily established primary tumors is able to induce regression of the primary tumors and to reduce formation of secondary metastasis.

## Materials and methods

**Animals.** Male Balb/c mice (5-6 weeks old) were purchased from Charles River (Sulzfeld, Germany). Animals were housed in plastic cages under standard conditions (22°C, 10% relative humidity, 12 h light/12 h dark cycle each day) and were allowed to acclimatize for one week before the start of experiments. All animal studies were conducted according to the guidelines of the Institutional Animal Care and Use Committees and in accordance with government guidelines.

**Experimental procedures.** For all invasive procedures, mice were anaesthetized with isoflurane 2% (Baxter, Munich, Germany) in oxygen via face mask inhalation. Primary colon tumors were established as described previously (8). Briefly, animals were laparotomized and  $2 \times 10^6$  C26 cells (Cell Lines Service, Eppelheim, Germany) were implanted subserosal in the cecum. Two weeks later, animals underwent a second laparotomy. Under a stereoscopic microscope animals were inspected for secondary metastasis. The size of the primary

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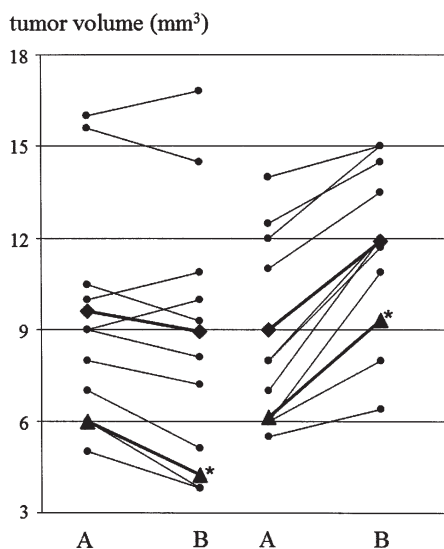


Figure 1. Tumor growth. Primary tumors, orthotopically established for two weeks, were measured before and two weeks after intratumoral administration of either Ad-B7-H3-GFP (left panel) or Ad-GFP (right panel) at explorative laparotomy. Values for individual animals are shown (A, before treatment; B, two weeks after treatment; dots, individual measurements; squares, mean values; triangles, mean values for tumors initially  $\leq 7$  mm<sup>3</sup>; \* $p \leq 0.05$ ).

tumors was assessed according to the formula: tumor size (mm<sup>3</sup>) =  $x$  [long diameter (mm)]  $\times$  [short diameter (mm)]<sup>2</sup>. Animals were then treated by direct injection into the primary tumor of either Ad-B7-H3-GFP or the control virus Ad-GFP at a dose of  $10^7$  infectious particles (IP)/tumor diluted in a total volume of 10  $\mu$ l. One week after the second laparotomy, blood was drawn from the tail vein and serum was analyzed for interleukin-12 (IL-12) by ELISA. Two weeks after the second laparotomy, animals were sacrificed by terminal anaesthesia. Again, the size of the primary tumors was measured and the presence of lymph node or liver metastasis was assessed. Spleens were collected for isolation of T-cells.

**Generation of Ad-B7-H3-GFP.** The generation of recombinant adenovirus has been described in detail elsewhere (9). Briefly, for the generation of Ad-B7-H3-GFP, the mouse B7-H3 gene (Gene bank accession number NM\_133983) was cloned into the pAdTrackCMV transfer vector (Qbiogene, Carlsbad, CA, USA). Correct insertion was verified by restriction enzyme digestion and direct sequencing. A control adenovirus (Ad-GFP) was generated coding only for green fluorescent protein (GFP). Recombinant adenoviruses were propagated by multiple passages on 293-cells and purified by CsCl<sub>2</sub> density gradient centrifugation. Solutions containing the virus were stored at  $-80^{\circ}\text{C}$ . Titration of virus preparations was achieved by plating serial dilutions on 293-cells and assessment of GFP-expression under a fluorescence microscope.

**Serum interleukin-12 (IL-12) levels.** Blood samples were allowed to clot overnight at  $2-8^{\circ}\text{C}$  before centrifugation for 20 min at  $2000 \times g$ . Serum IL-12 concentration was measured immediately by the Quantikine Mouse IL-12 Immunoassay Kit (R&D Systems, Wiesbaden, Germany) according to the manufacturer's instructions. As a negative control, serum from

naïve Balb/c mice was analyzed. All analyses were performed in triplicate.

**IFN- $\gamma$  ELISPOT assay.** Interferon- $\gamma$  (IFN- $\gamma$ ) ELISPOT assays were performed as described recently (8). Briefly, primary CD8<sup>+</sup> T-cells were isolated from spleens by immunomagnetic negative selection using the CD8<sup>+</sup> T-cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). CD8<sup>+</sup> T-cells derived from Ad-B7-H3-GFP or Ad-GFP treated animals were cocultured with naïve C26 cells at an effector to target ratio of 1:3 for 48 h. As a negative control, CD8<sup>+</sup> T-cells from naïve Balb/c mice were analyzed; as a positive control, these cells were stimulated with 5  $\mu\text{g/ml}$  concanavalin A (ConA) (Sigma, Munich, Germany). Frequency of IFN- $\gamma$  secreting T-cells was determined by counting spots using an automated image analysis system (Axioplan 2, Zeiss, Göttingen, Germany).

**Statistical analysis.** A Student's t-test and a Chi-square test were used to determine the statistical significance of data. P-values of  $\leq 0.05$  were considered significant.

## Results

**Size-dependent reduction of primary tumor growth.** Two weeks after tumor cell implantation and just before adenoviral treatment, primary tumors reached a mean size of  $9.3 \pm 3.2$  mm<sup>3</sup> as determined at explorative laparotomy. Two weeks after viral treatment tumor size was measured again. Tumors treated with Ad-B7-H3-GFP were slightly reduced in size ( $9.61 \pm 3.5$  mm<sup>3</sup> to  $8.9 \pm 4.1$  mm<sup>3</sup>), whereas tumors treated with Ad-GFP continued to grow ( $9.0 \pm 2.9$  mm<sup>3</sup> to  $11.9 \pm 2.7$  mm<sup>3</sup>). However, this trend did not reach statistical significance. When looking at animals with an initial tumor size of  $\leq 7$  mm<sup>3</sup>, the differences in primary tumor growth were more pronounced. These tumors changed in size from  $6.0 \pm 0.8$  mm<sup>3</sup> to  $4.2 \pm 0.6$  mm<sup>3</sup> in animals treated with Ad-B7-H3-GFP ( $p \leq 0.05$ ) vs.  $6.1 \pm 0.2$  mm<sup>3</sup> to  $9.3 \pm 1.9$  mm<sup>3</sup> in animals treated with Ad-GFP ( $p \leq 0.05$ ) (Fig. 1).

**Reduced secondary metastasis formation.** At the time of adenoviral treatment (two weeks after tumor cell injection), none of the mice had developed any detectable lymph node or liver metastases as assessed at explorative laparotomy. Two weeks after injection of adenoviral vectors, the presence of metastasis was reassessed and had occurred in both groups. Lymph node metastasis developed in all (10/10) animals treated with Ad-GFP as opposed to only 7/10 animals in the Ad-B7-H3-GFP group. Liver metastases were observed in 2/10 animals of the Ad-B7-H3-GFP treatment group compared to 8/10 animals in the control group ( $p = 0.025$ ). Remarkably, three animals in the treatment group remained free of any type of metastasis two weeks after treatment.

**Increased systemic levels of IL-12.** One week after intratumoral injection of adenoviral treatment serum was collected and IL-12 levels measured. Animals treated with Ad-B7-H3-GFP had higher serum IL-12 levels compared to the Ad-GFP treated group (1.43 pg/ml vs. 1.13 pg/ml,  $p \leq 0.01$ ). In contrast, IL-12 levels in Ad-GFP treated mice were not significantly different from naïve control animals (Fig. 2).

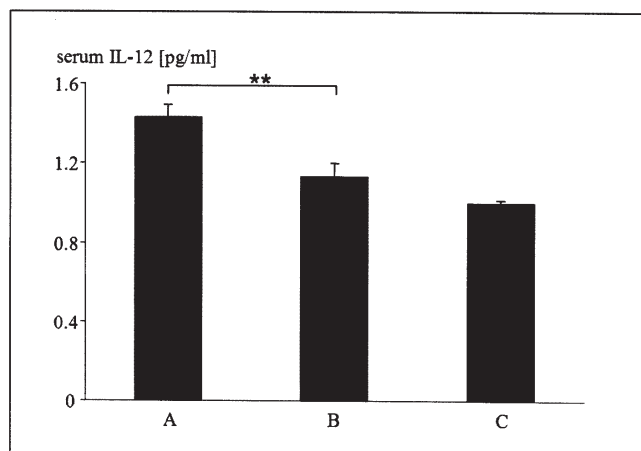


Figure 2. Interleukin-12 production. One week after treatment, serum IL-12 levels were measured by ELISA. Animals injected with Ad-B7-H3-GFP (A) showed significantly increased IL-12 levels compared to animals injected with Ad-GFP (B) or untreated control animals (C). (n=10 animals in each group; \*\*p<0.001).

**Ad-B7-H3-GFP treatment induces tumor specific interferon- $\gamma$  producing CD8<sup>+</sup> T-cells.** Two weeks after adenoviral treatment, mice were sacrificed and CD8<sup>+</sup> T-cells were isolated from spleens and analyzed by IFN- $\gamma$  ELISPOT assay. Animals treated with Ad-B7-H3-GFP showed a significantly higher frequency of tumor specific IFN- $\gamma$  producing cells than untreated animals ( $13.5 \pm 1.5$  vs.  $2.5 \pm 0.5$ ,  $P \leq 0.05$ ) and significantly higher numbers than Ad-GFP treated animals ( $4.5 \pm 0.5$ ,  $P \leq 0.05$ ) (Fig. 3).

## Discussion

In the present study, readily established colon cancers in a murine model of syngeneic orthotopic colon cancer were treated by direct intratumoral injection of an adenovirus coding for the mouse B7-H3 costimulatory molecule. Ad-B7-H3-GFP therapy resulted in a significantly decreased size of small initial tumors. However, Ad-B7-H3-GFP treatment was less effective in larger initial tumors. This size-dependent efficacy of Ad-B7-H3-GFP therapy is in line with findings of Sun *et al* (10) who observed a size-dependent treatment efficacy of plasmid delivered B7-H3 in subcutaneous E1-4 lymphomas. Also, the antitumor effect of B7-1 as another member of the B7-family has been shown to be more efficient in small initial tumors (11). The same has been shown for adenoviral delivered HSP72 as neoadjuvant therapy in various tumors in rodents (12). This argues for yet unidentified factors that confer resistance to immune surveillance and increase with tumor cell density.

Distant metastasis formation in both, lymph nodes and the liver, was significantly reduced in Ad-B7-H3-GFP treated animals. In accordance with the observed greater reduction of primary tumors with small initial size liver metastasis only occurred in mice with large initial tumor volume in the Ad-B7-H3-GFP treated group, whereas in animals bearing tumors with a smaller initial volume no secondary metastasis occurred.

In addition to size reduction and amelioration of metastases, we demonstrated a significant induction of

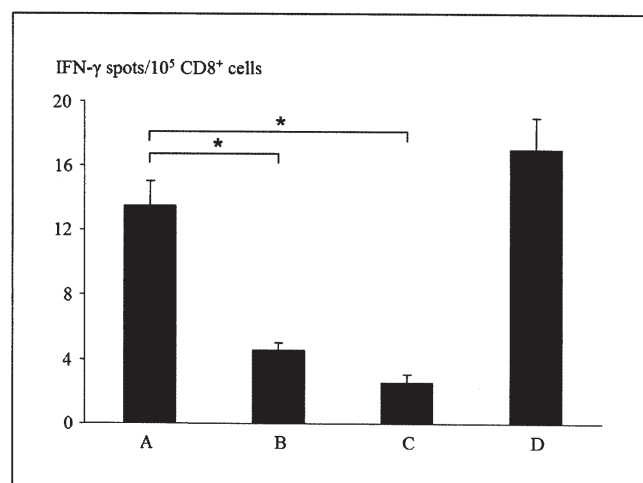


Figure 3. Tumor specific interferon- $\gamma$  secreting CD8<sup>+</sup> T-cells. CD8<sup>+</sup> T-cells were isolated from spleens of C26 tumor bearing mice two weeks after treatment. Frequency of specific CD8<sup>+</sup> T-cells was determined by IFN- $\gamma$  ELISPOT assay after co-culturing CD8<sup>+</sup> T-cells from Ad-B7-H3-GFP (A) or Ad-GFP (B) treated animals with naïve C26-cells. As a negative control CD8<sup>+</sup> T-cells from untreated animals (C) and as a positive control concanavalin A stimulated CD8<sup>+</sup> T-cells (D) were used. (Bars represent mean number of spots/10<sup>5</sup> CD8<sup>+</sup> cells + SEM of two independent experiments; n=10 animals in each group; \*p<0.05).

tumor-specific IFN- $\gamma$  producing CD8<sup>+</sup> T-cells. This finding strengthens the hypothesis that the observed antitumoral effect was indeed mediated by an adaptive immune response. B7-H3 is known to induce IFN- $\gamma$  expression (6) which might in turn enhance antigen processing and presentation within the tumor (13). Antitumor immune responses are initiated by the uptake of antigens, possibly made available by viral lysis of tumor cells. Dendritic cells then upon activation produce IL-12 that promotes systemic antitumor immunity (14). In our study, we found significantly increased concentrations of IL-12 in the serum of Ad-B7-H3-GFP treated animals. IL-12 has been shown to act synergistically with members of the B7-family against various tumors (15-17). When present at the site of antigen presentation, IL-12 favours the development of a Th1-type immune response leading to the induction of cytotoxic T-cells (18,19), the main effectors of an antitumor immune response.

In conclusion, the present study demonstrates for the first time the potential of adenoviral transferred B7-H3 to reduce primary tumor size and formation of secondary distant metastasis. Furthermore, our study provides evidence for the induction of a specific cellular antitumor immunity by adenoviral B7-H3 transfer.

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