ampicillin and grown to log phase. Expression of fusion proteins tagged with a polyhistidine metal binding domain was induced by 0.5 mM IPTG for 2 hrs.. Bacteria were disrupted by repeated freeze thawing cycles and cleared supernatant was applied to Ni-NTA columns (Quiagen, Hilden, Germany) according to the manufacturer's description. Purified protein extracts (Fig. 5.16) were subjected to a preparative 10% SDS-PAGE gel and blotted to an Immobilon-P membrane (Millipore, Eschborn, Germany). Membrane stripes were used to analyze mouse sera for the presence of anti-p53 antibodies. The secondary HRP-labeled antibody detected immunoglobulins of the IgG subclasses. Antibody production was assayed pre-study and post immunization. The results illustrated in Fig. 5.17 show, that mice injected with pDNA encoding mutated p53 did not develop a specific antibody response.
Anti-p53 antibodies were also assayed in serum samples taken post-study from immunized individuals after tumor cell inoculation. Tumor resistant mice did not develop anti-p53 antibodies after tumor cell inoculation (data not shown). The absence of anti-p53 antibodies was independent of the pDNA delivery method, hence regardless of Th_1 or Th_2 helper cell activation. Thus, our results suggest that a humoral immune response does not contribute to tumor rejection. The results of these experiments are in accordance to the observation that athymic nude mice are not protected from tumor growth by pDNA-vaccination. Athymic Balb/c mice are characterized by a phenotype negative for T lymphocyte although B cell populations are unaffected in these animals.

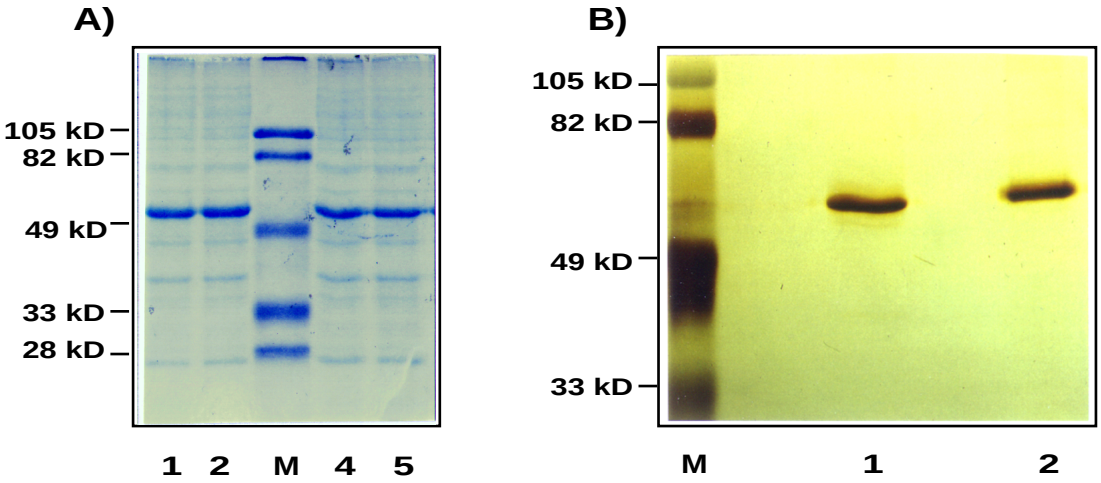
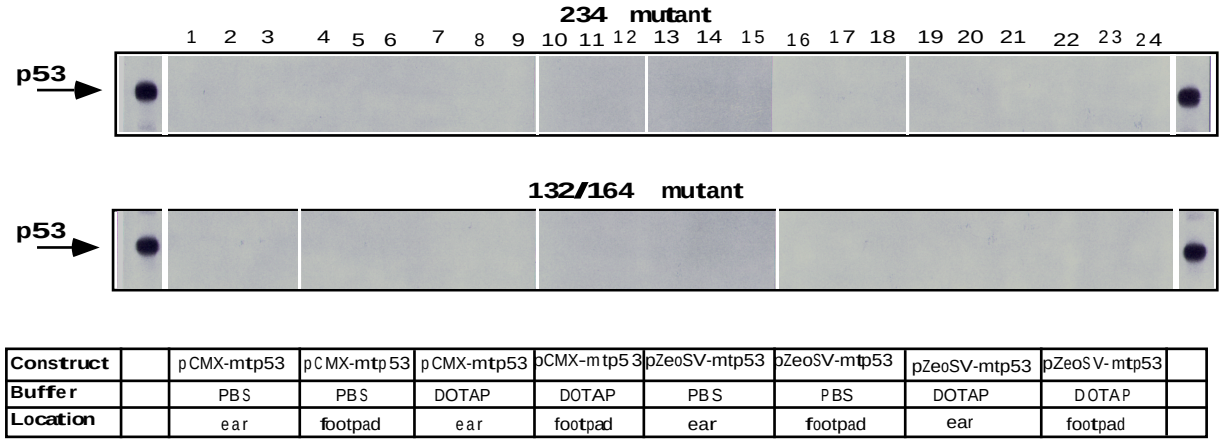


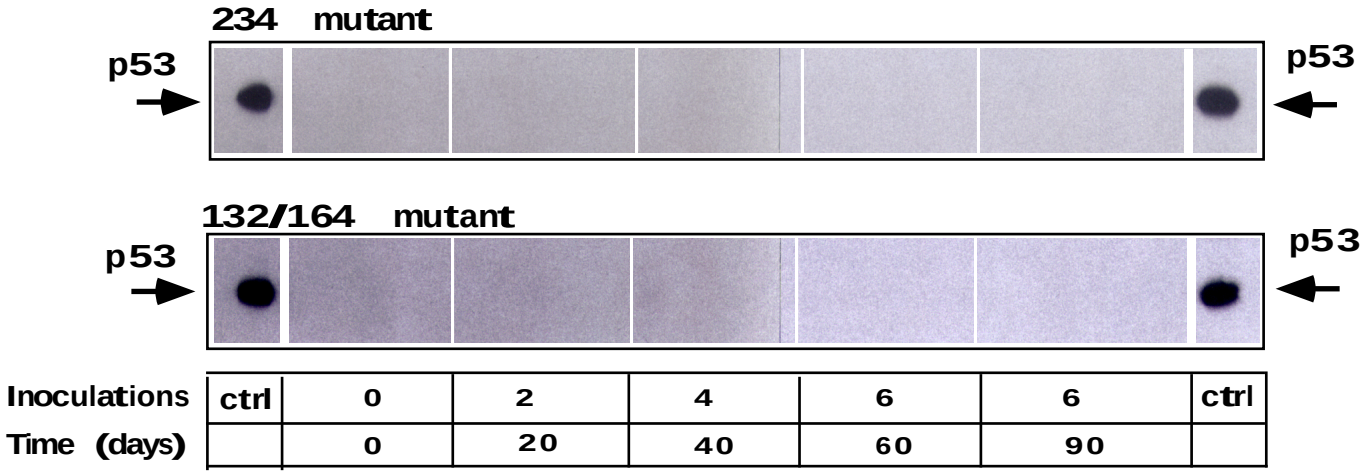
Fig. 5.16 Expression and purification of mutant p53 in *E. coli.* Recombinant fusion protein expression was induced for 2 hrs. by addition of 0.5 mM IPTG to in log phase growing BL21-(DE3)LysS cells. Cell lysates show expression of mutated p53 (lane 1 and 2 transformed BL21-(DE3)LysS containing the 234 mutant p53 gene; lane 4 and 5 transformants with the 132/164 mutant p53 gene). Fusion proteins were purified by a Ni-NTA-column and purified proteins were made visible by silver staining (B), (lane 1, 132/164 mutant p53; lane 2, 234 mutant p53).

To examine the question if repeated pDNA injection induces the production of neutralizing antibodies that bind to the plasmid molecules, serum samples from pDNA-injected mice were tested for a specific antibody response. pDNA was blotted to a Hybridization Transfer Membrane (DU PONT NEN Research Products, Brussels, Belgium) and used to test serum samples for specific antibodies. In our experiments we could not demonstrate the presence of pDNA binding antibodies in mouse sera after repeated pDNA injection (data not shown). So far, the injection of constructs containing the mutated p53 gene did not appear to cause any toxic effects. After pDNA injection histologies from organs appeared normal. All mice, protected from tumor growth by pDNA injection were still healthy more than 1 year after tumor cell inoculation. Alterations of the tissue at the injection site were not visible.



Construct		pCMX-mtp53	pCMX-mtp53	pCMX-mtp53	pCMX-mtp53	pZeoSV-mtp53	pZeoSV-mtp53	pZeoSV-mtp53	pZeoSV-mtp53	
Buffer		PBS	PBS	DOTAP	DOTAP	PBS	PBS	DOTAP	DOTAP	
Location		ear	footpad	ear	footpad	ear	footpad	ear	footpad	

Fig. 5.17a Western blot analysis showed no mutant p53 specific antibodies in the serum of pDNA immunized mice. Recombinant mutant p53 proteins were loaded on a 10 % SDS-PAGE and blotted to a Immobilon-P membrane. Recombinant vector molecules, containing the mutant p53 gene, were injected 6 times into the food pad or ear in10 days intervalls. Test sample sera were taken 4 days after the last immunization from the lateral vein of Balb/c mice. pDNA constructs were applied as a cationic liposomal complex or solubilized in PBS. Sera were used in a concentration of 1:400, the mouse PAb 421 anti-p53 antibody at a concentration of 1:10000. Bound antibodies were detected by a secondary, HRP labeled sheep anti-mouse antibody, diluted 1:10000 (Amersham, Braunschweig, Germany).



Inoculations	ctrl	0	2	4	6	6	ctrl
Time (days)		0	20	40	60	90	

Fig. 5.17b Sera from mice immunized by biolistic particle bombardment were tested for the presence of mutant p53 specific antibodies. Gold particles were coated with the expression vector pCMX-mutant p53. 1_g pDNA was applied per inoculation in 10 days intervalls. The number of inoculations is mentioned at the bottom of the blot. The mouse PAb 421 anti-p53 antibody was used as a positive control (ctrl). No antibodies directed against mutant p53 could be detected in the serum of immunized mice. At day 62 Meth-A tumor cells were injected into the flank of immunized Balb/c mice. 28 days post tumor cell inoculation still no mutant p53 specific antibodies were detectable in treated mice(day 90).

5.4.2 T cell effector mechanisms are responsible for tumor rejection

CTLs play a crucial role in the host's immune response to viral infections and cancer (259-261). These effector cells recognize short peptide antigens in association with class I MHC molecules (262, 263). A crucial requirement for MHC-presentation is that endogenously synthesized cell proteins are broken down, processed and presented on the cell surface where they are accessible for cytotoxic T lymphocytes. Therefore tumor antigens and mutated oncogene products do not have to be expressed intact on the cell surface to be a target for CTLs and can even be recognized when only functional within the tumor cell. The p53 tumor antigen is the most commonly mutated gene in human cancer (264) and has been shown to induce specific $CD8^+$ CTLs in human cancer (265) and protective immunity to tumors in mouse model systems (265, 266).

Point mutations in the p53 tumor suppressor gene can create neo-antigenic determinants that serve as tumor antigens when processed and presented by class I MHC molecules (266). We tested pDNA-immunized mice for the presence of mutant p53 specific, cytotoxic lymphocytes. CTL activity of responding cells was evaluated by a ^{51}Cr-release assay. After 6 immunizations spleen cells from immunized mice were used to isolate lymphocytes that were tested for cytotoxic activity against the mutant p53 transfected dendritic cell line D2SC-1 (5.2.2). Included in each test were D2SC-1 target cells transfected with the 234 mutant p53, the 132/164 mutant p53 or the parental untransfected cell line. Cytotoxicity of T lymphocytes was measured in a ^{51}Cr-release assay using 96-well, round bottom plates. Target cells were labeled with 100 µCi of $Na_2{}^{51}Cr$ per 2 x 10^6 cells for 90 min. and resuspended at 2 x 10^5 cells/ml in DMEM, 10% FCS, 2 mM L-glutamine, 10 mM HEPES, 0.5 mM β-mercaptoethanol, 4 ng/ml IL-2. Target cells were seeded at 2 x 10^4 cells/well in 100 µl medium. Suspensions of effector cells were added to triplicate wells to give various effector : target ratios in a final volume of 200 µl. Plates were incubated at 37°C for 6 hrs.. 100 µl supernatant was removed from each well and counted in a gamma counter. The spontaneous release and the maximum amount of cpm were determined and the percentage of specific cytotoxicity was calculated by standard procedure. Lymphocytes, isolated from pDNA immunized mice clearly showed specific cytolysis of transfected dendritic cells, whereas untransfected D2SC-1 cells did not induce CTL activity (Fig. 5.18). No CTLs were elicited by lymphocytes isolated from untreated mice (Fig. 5.18). These results suggest that pDNA vaccination and expression of the transgene induces effectively CTLs, which can eliminate mutant p53 expressing cells.

5.5 Adoptive lymphocyte transfer

Cellular immune responses play a major role in the host reaction to growing cancer. The adoptive transfer of cytotoxic T lymphocytes, which are sensitized against specific tumor antigens, can mediate objective regression of the tumor. To investigate whether the observed protection from Meth A tumor growth is mediated by a cellular immune response, spleen derived lymphocytes were infused intravenously or intratumorally into non-vaccinated, tumor bearing recipients. Spleen cells were passed through nylon wool columns to concentrate T cells. Purified T cells were cultured in DMEM, 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 mM HEPES, 0.5 mM β–mercaptoethanol and 4 ng/ml IL-2 for two days and then injected intravenously or intratumorally into tumor bearing recipients (Fig. 5.19). Tumor growth was monitored by daily palpation. Two perpendicular measurements of the tumor diameter were taken on each tumor with a caliper. Intratumoral or intravenous adoptive transfer of 10^6 T lymphocytes isolated from untreated or tumor-bearing mice mediated no delay in the outgrowth of Meth A tumors (data not shown). However, transfer of 10^6 lymphocytes that were sensitized by pDNA immunization in the living animal, eradicated tumors or slowed down tumor growth when introduced intratumorally into the mice (Fig. 5.21). As shown in Figure 5.20 the adoptive transfer of these cells is capable of mediating complete regression of Meth A tumors.

In general, the effect of tumor regression was dependent on the size of the tumor and the timepoint of lymphocyte transfer post tumor cell inoculation. Growth of large tumors was not influenced by T lymphocyte transfer. We think that the amount of injected Meth A reactive lymphocytes and the number of transfer infusions might be crucial for successful tumor eradication. The implication of a well-balanced ratio of CTLs and tumor mass is reflected by the observation that lymphocyte transfer can induce temporary growth inhibition for about 10 days. Successive CTL-immunizations might revert tumor growth and lead to the eradication of malign tumor cells in these cases. However, treatment of large tumors might also be complicated due to the formation of a cytokine-microenvironment produced by Meth A cells unfavorable for CTL-activity. Macrophages, isolated from the peritoneum of pDNA-immunized mice, had no effect on tumor growth when injected intratumorally or intravenously into tumor bearing recipients.
These results demonstrate that by adoptive transfer of spleen-derived lymphocytes from pDNA-vaccinated mice we were able to transfer anti-tumoral immunity to non-vaccinated recipients. This underlines our assumption that CTLs are the predominant effector cells. Cured mice stayed tumor free for a follow up period of 12 months. However, intravenous administration of T lymphocytes showed no anti-tumoral effect, maybe due to an inappropriate amount of transferred CTLs or an inappropriate homing of *in vitro* cultured lymphocytes. Our results might be improved by increasing the number of transferred lymphocytes or by repeated adoptive transfer of CTL effector cells.

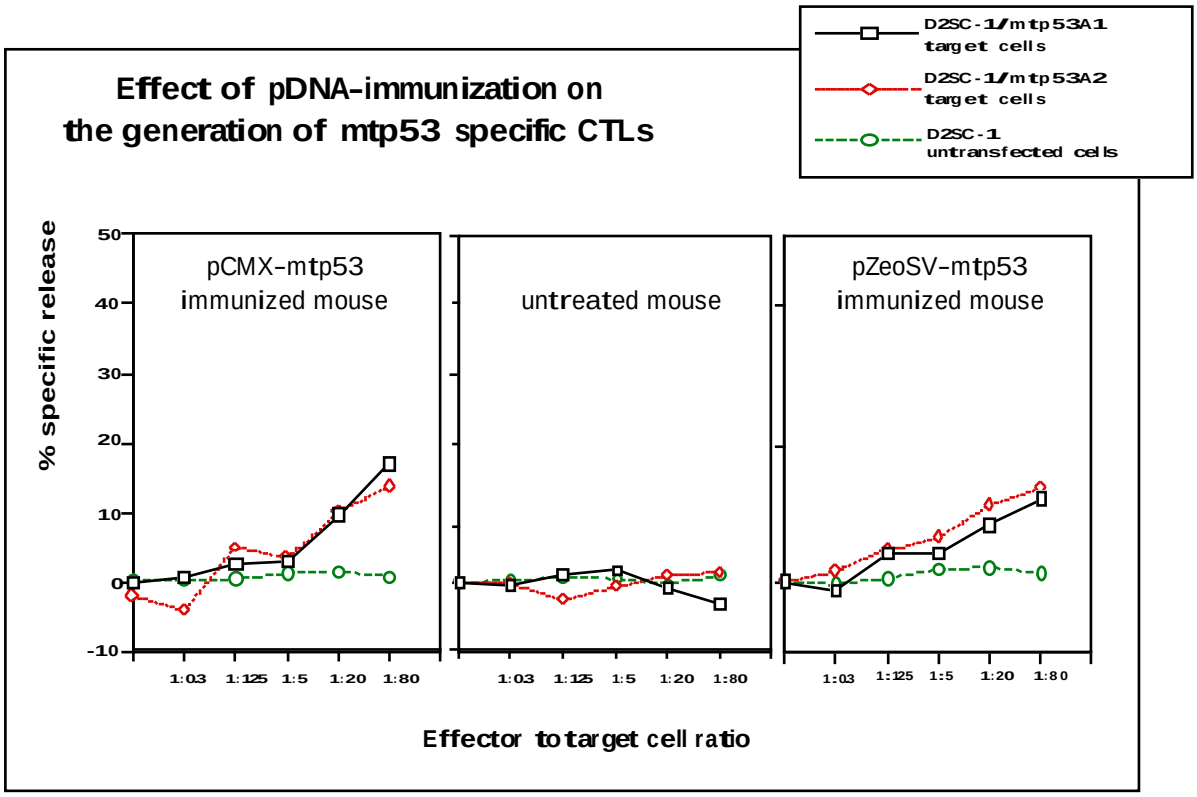


Fig. 5.18 pDNA vaccination results in the generation of mutant p53 specific CTLs. Balb/c female mice were immunized 6 times with 70 µg pDNA in 10 days intervals. After immunization mice were inoculated with 5 x 10^6 Meth A tumor cells. Spleen cells from tumor resistant mice were harvested 1 month post immunization and isolated T cells were tested in a ^{51}Cr-release assay for CTL activity by using a p53 transfected dendritic cell line D2SC-1. No CTLs were elicited in untreated mice or using untransfected D2SC-1 target cells. CTLs were demonstrated in pDNA-vaccinated mice specific for the 234 and the 132/164 mutant p53 D2SC-1 transfectants.

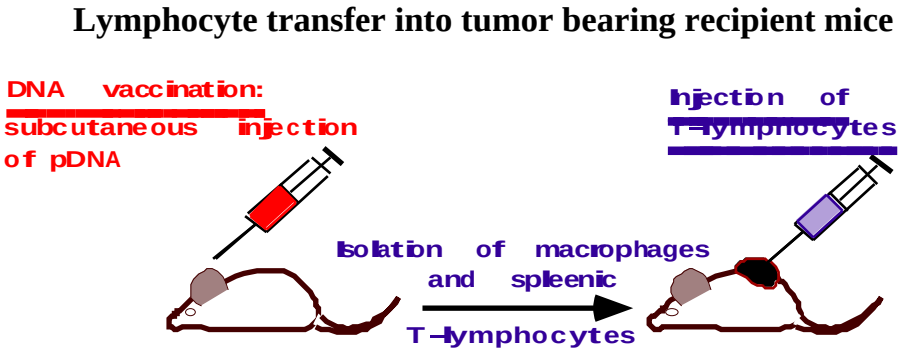


Fig. 5.19 Balb/c mice were injected subcutaneously with pDNA encoding the mutant p53 gene. pDNA immunization was performed with 70 µg pCMX-mutant p53 or pZeoSV-mutant p53. The application was repeated 6 times in 10 days intervals. 10 days after the last immunization macrophages and spleenic lymphocytes were isolated from immunized individuals, enriched on nylon columns and injected intratumorally or intravenously into the tumor bearing host. Inoculation with Meth A tumor cells was performed 6 or 9 days prior immunization. Tumor growth on recipient mice was monitored daily by standard procedure.

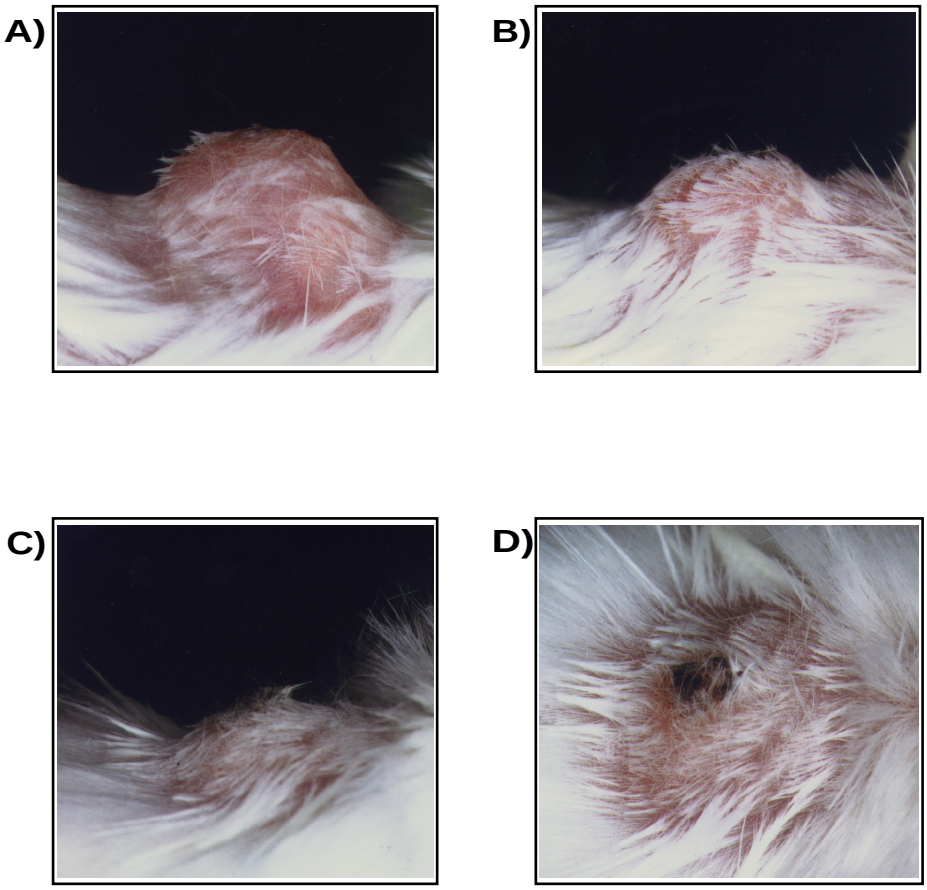


Fig. 5.21 Intratumoral injection of lymphocytes isolated from pDNA immunized mice eliminates established Meth A tumor growth in Balb/c mice. **A** shows the tumor before treatment. Lymphocytes were enriched on nylon columns and 10^6 T cells were injected intratumorally into recipient mice. After 3 days the tumor volume was noticeable reduced (**B**). Picture **C** shows the tumor at day 6, **D** at day 9. At day 12 the tumor was not detectable anymore. These effects were not observed in mice injected with T lymphocytes isolated from non-immunized mice or mice that developed tumors. Cured mice showed a disease free condition for more than 1 year following treatment.

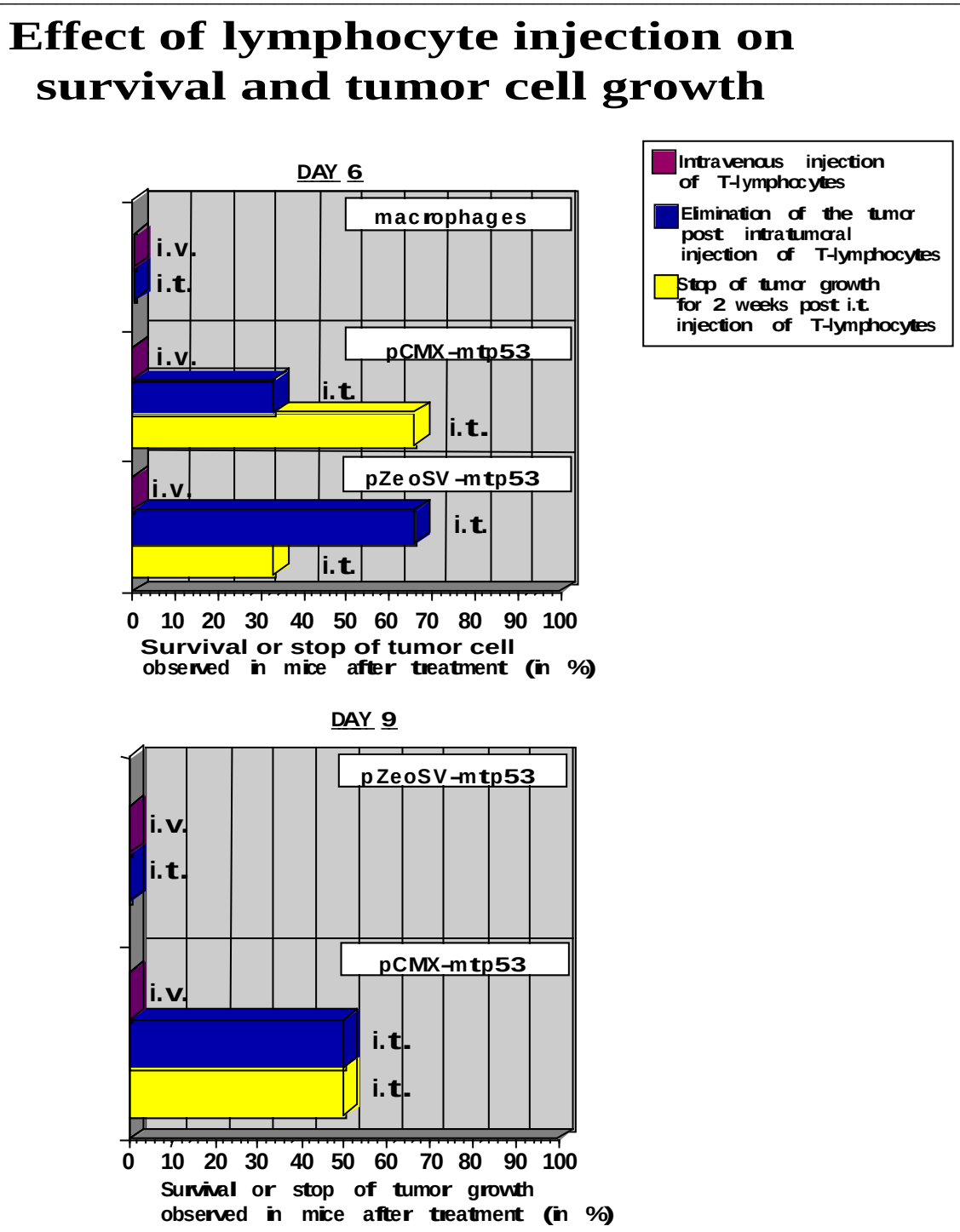


Fig. 5.20 Effect of lymphocyte injection on tumor growth and survival. Lymphocytes and macrophages were isolated from DNA-immunized mice and injected into Balb/c mice 9 or 6 days after tumor cell transplantation. Donor mice were immunized with the pCMX-mutant p53 and the pZeoSV-mutant p53 constructs as indicated in the graph. Intraveneous injection (**i.v.**) of 106 T-cells and injection of 106 macrophages had no effect on tumor growth. However, intratumoral injection of spleenic lymphocytes (**i.t.**) stopped tumor growth or initiated regression of the tumor. Lymphocyte transfer was most efficient when applicated in an early stage of tumor growth and when injected into small tumor nodules.