Chapter II

Transduction of dendritic cells with mutant p53 alleles generates an effective anti-tumoral vaccine

Dendritic cells are a heterogeneous group of specialized antigen presenting cells that possess the unique ability to activate naive T cells (267). They express both class I and class II major histocompatibility complex proteins at high levels along with CD1a and a wide array of co-stimulatory and adhesion molecules which together are responsible for their potency as antigen presenting cells. Immature DCs are thought to migrate from the bone marrow to peripheral sites such as the skin and mucosal surfaces. At this stage in their development DCs are highly efficient at antigen uptake which occurs by macropinocytosis and by endocytosis through the mannose receptor (268, 269). Following antigen encounter and under the influence of pro-inflammatory cytokines, DCs undergo further maturation, losing the ability to take up antigen and further upregulating MHC proteins and co-stimulatory signals (270). In association with this maturation step, there is migration through afferent lymph to lymphoid organs where processed antigen in association with MHC class I or II is presented to $CD8^+$ and $CD4^+$ T cells. Many of the molecules involved in the interaction between DCs and T cells have been identified and include the receptor-counter receptor pairs CD40 and CD40L, CD80/86 and CD28/CTLA-4, and key cytokines such as IL1, IL6, and IL12.
The properties of DCs outlined above clearly makes them very attractive candidates for tumor immunotherapy. A number of murine tumor models have demonstrated that DC-vaccination provides effective anti-tumor immunity. Various methods of loading the DC with tumor antigen have been utilized using synthetic peptide (271), unfractionated peptides eluted from tumor cells (272), tumor cell lysates (273), RNA extracted from tumor cells (274) and fusion of DC with tumor cell lines (275). Generation of tumor specific peptides is however time consuming and unrealistic on a large scale. Preparation of antigens directly from tumor cells depends on the accessibility of large tumor mass, something that is found only in a minority of cases. Since DCs are able to degrade complex molecules and load immunogenic peptides onto MHC molecules, direct delivery of tumor associated proteins to these cells either using synthetic material or gene transfer should be a highly effective vaccine strategy. Exposure of DCs to exogenous synthetic antigen should mainly target the MHC II pathway, whereas antigen loading by gene transfer should be more efficient at CTL induction since it simulates viral infection and targets MHC class I molecules.

5.6 Efficient gene transfer to DCs is accomplished by retroviral transduction

We planed to immunize Balb/c mice with mtp53-transfected dendritic cells and induce an anti-tumoral immune response in the recipient. In initial studies different transfection methods were tested for efficient gene transfer into the dendritic cell line D2SC-1. Standard methods as calcium phosphate precipitation, lipofection and electroporation proved to be inefficient for transfer of a GFP reporter gene construct (Fig. 5.22).
An alternative and more efficient gene transfer technique is the use of viral vectors. Of these, retroviruses are one of the most efficient means to introduce exogenous genes into cells and they are the most widely evaluated vectors in clinical gene therapy studies. Their use however is restricted to actively dividing cells since integration only occurs during mitosis (276) when the nuclear membrane is not intact. LacZ-gene transfer was performed by the retroviral packaging cell line TeFlyA4lacZ3 (277). TeFlyA4lacZ3 is a HT1080-based packaging cell line releasing high-titer viruses with cores containing the envelop glycoproteins of the amphotropic Moloney Murine Leukemia virus harboring the MFGnlslacZ retroviral vector (generous gift from Dr. Barbara Schnierle, Tumor Biology Center, Freiburg, Germany). Efficient gene transfer was demonstrated after incubation of dendritic cells with virus-containing supernatant of the packaging cell line (Fig. 5.22). More than 70% of the DCs were found to be positive after X-Gal staining.

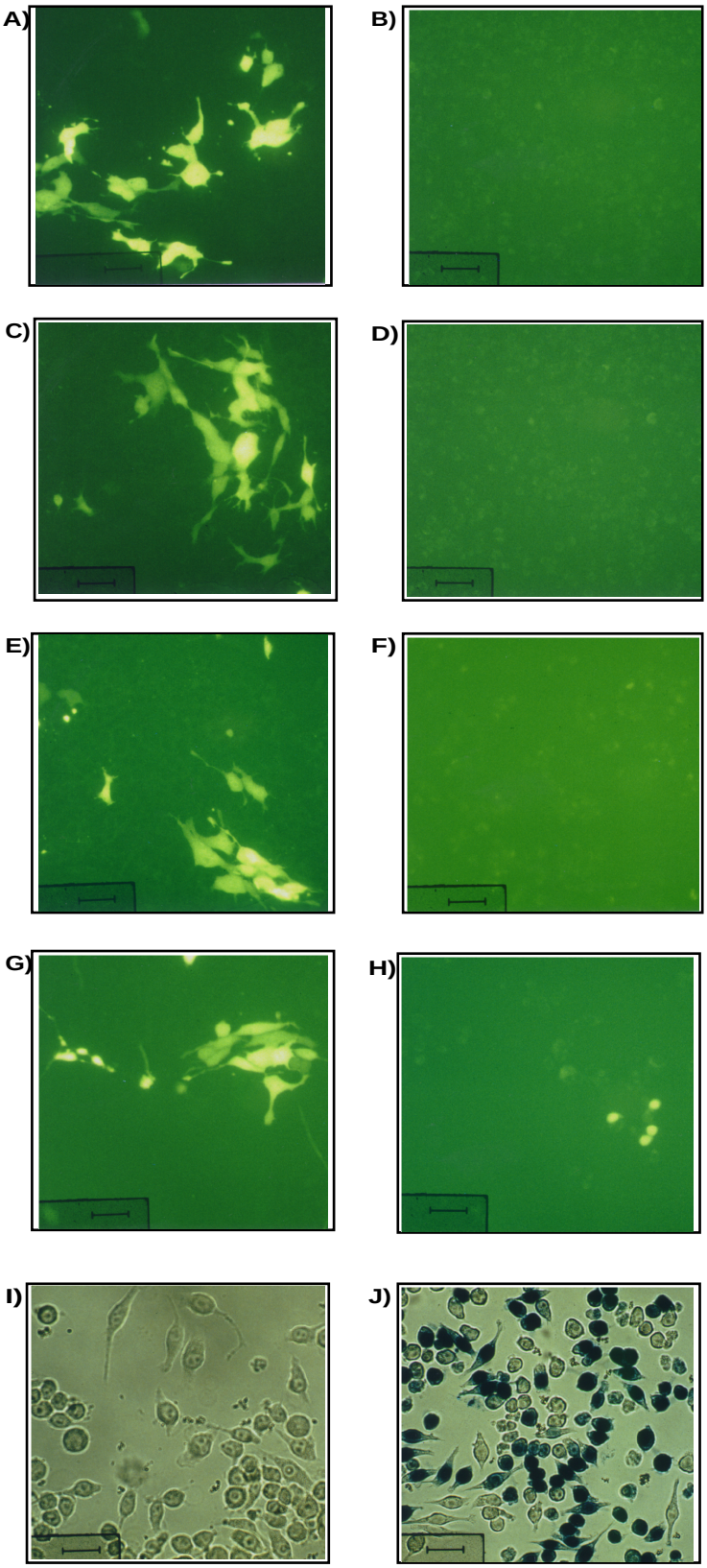

Fig. 5.22 The dendritic cell line D2SC-1 was transfected with the GFP reporter gene construct by standard techniques. GFP expression was detected two days post transfection by flourescence microscopy (Axiophot from Zeiss, Oberkochen, Germany) at 520 nm. Calcium phosphate precipitation (**B**), lipofection (**D** (DOTAP Böhringer Mannheim) **F** (Superfect, Quiagen)), and electroporation (**H** proved to be inefficient to transfect D2SC-1cells. Renca cells (**A, C**, **E**, **G**), transfected by the same means served as a positive control to demonstrate efficient gene transfer. However, over night incubation of DCs with retroviral supernatant of the packaging cell line TeFL A4lacZ3 resulted in transduction of more than 70% of the target cells (**J**) as visualized post X-Gal staining two days after infection. Untreated, untransduced D2SC-1 did not show X-Gal staining (**I**).

5.7 Construction of retroviral p53 expression vectors and selection of packaging cell lines producing infectious viral particles

To rescue infectious viruses containing the mutant p53 constructs the HT1080 human fibrosarcoma-derived packaging cell line TeFly AF13 was transfected with pBABEpuro/mtp53 DNA by standard calcium phosphate precipitation. pBABEpuro is a replication incompetent retroviral vector designed to target mutant p53 RNA to viral particles (Fig. 5.23). The viral transcript is initiated in the 5'LTR and is terminated in the 3'LTR and is thus a full length viral transcript. It contains the packaging sequence ψ,, which is recognized by the capsid proteins and allows it to be packaged into viral particles. A fully infectious viral particle containing the vector genome is thus budded from the packaging cell line. Cellular RNA lacks the recognition sequence ψ for encapsulation and is not packaged into a viral particle. Genes encoding virus structural proteins were deleted in the retroviral vector to create room for inserted genes and to cripple the virus. However, the packaging cell line TeFly AF13 was stably transfected with gag, pol and env expression plasmids and is therefore capable of producing extremely high-titer viruses (277).
Direct selection for transfected cells was achieved by expression of the selectable marker gene puromycin. The culture supernatant was removed from cells and used as a source of virus. Encapsulation of the mutated p53 gene within budded retroviruses was demonstrated by RT-PCR (Fig. 5.25). The retrovirus was concentrated by ultracentrifugation, solubilized in lysis buffer and used as a source for RNA extraction (RNeasy Mini Kit, QUIAGEN). Upon cDNA synthesis (cDNA-Synthesis Kit, Pharmacia Biotech) a PCR reaction using p53 specific primers demonstrated the incorporation of the mtp53 gene within the virus.

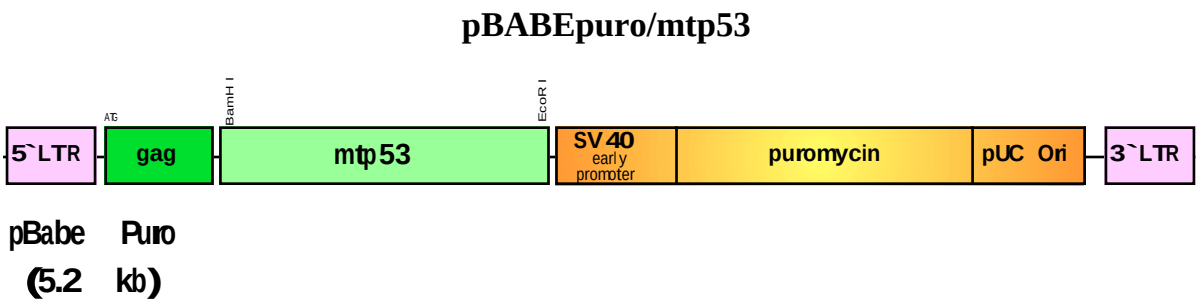

Fig. 5.23 Schematic diagram of the constructs. The BamH I, EcoR I fragment of the p53 alleles was cloned within the multiple cloning site of pBABEpuro. Transcription is initiated by the 5'LTR. The packaging sequence ψ is included in the gag-fragment. Selection of transfected cells for viral gene expression was achieved by addition of the selective agent puromycin (5-10 µg/ml) to the tissue culture medium.

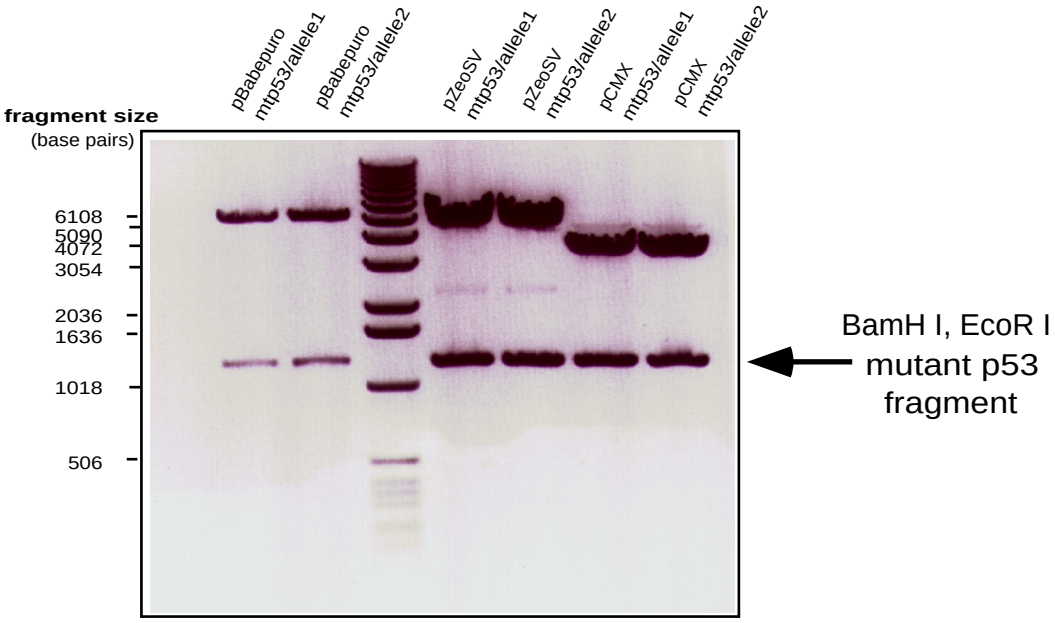

Fig. 5.24 pBabepuro/mtp53 was cloned by inserting the BamH I, EcoR I mtp53 fragment into the replication incompetent retroviral vector pBABEpuro. The fragment was excised from the mammalian expression vectors pCMX or pZeoSV, containing the mutant p53 gene. pBABEpuro/mtp53 was used for stable transfection of TeFly AF13 packaging cells for retroviral transfer of mutant p53 alleles.

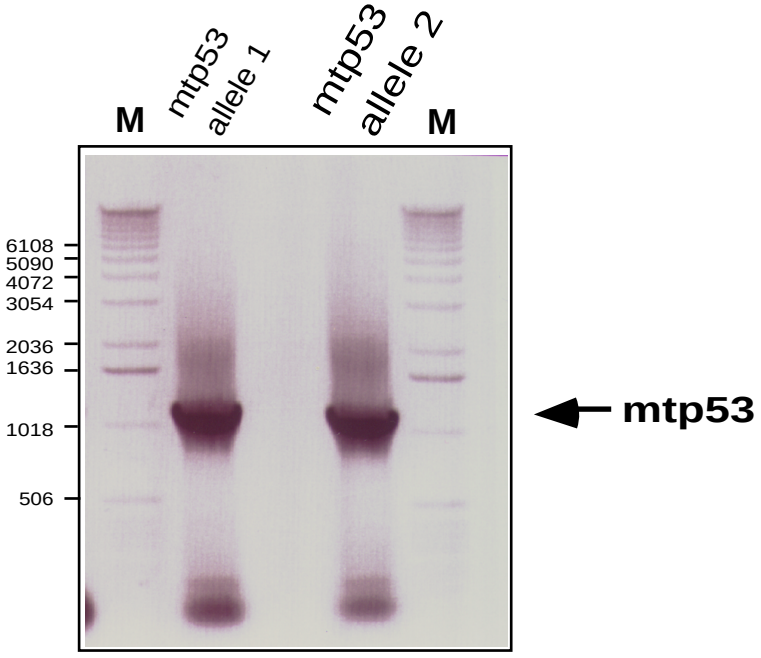

Fig. 5.25 The incorporation of the p53 gene in viral particles was demonstrated by RT-PCR. Viruses were concentrated by ultracentrifugation and solubilized in lysis buffer. Total RNA was isolated and used for cDNA synthesis. The presence of mtp53, allele 1 and allele 2, was demonstrated by a PCR reaction.

5.8 Use of transduced D2SC-1 as a cellular vaccine

Dendritic cells play an essential role in the induction of primary immune responses by creating a specific cytokine profile and activating both cytotoxic T cells and T helper cells. We wanted to use dendritic cells as a cellular vaccine when transduced with a tumor antigen and induce an anti-tumoral immune response. We used the immortalized cell clone D2SC-1, generated from mouse spleen. D2SC-1 cells are known to be very efficient stimulators of naive or pre-sensitized T-cells (278). FACS-analysis confirmed expression of both MHC class I and class II as well as the co-stimulatory molecules B7-1 and B7-2 (Fig. 5.30). *In vitro* experiments demonstrated that these cells are strongly alloresponsive (Fig. 5.26). 2 x 10^4 DCs/well were required for optimal stimulation of allogeneic naive T cells as determined by a [^{3}H]-proliferation assay. The dendritic cell line proved to be nearly as efficient in stimulating T-cells as primary DCs isolated from mouse spleen or differentiated from bone marrow.
D2SC-1 cells form solid tumors when injected subcutaneously into the flank of Balb/c mice (data not shown). Therefore transduced DCs were irradiated before use as a cellular anti-tumor vaccine. D2SC-1 cells proved to be very sensitive to γ-irradiation and exposition to as little as 30 gray resulted in effective inhibition of proliferation as shown by proliferation assays when DCs were cultured in the presence of [^{3}H]-thymidine for 3 days (data not shown). In our experiments, transduced DCs were irradiated with 50 grays before intravenous or subcutaneous injection into Balb/c mice. No tumor growth of irradiated cells was observed in a follow up period of 15 months after onset of immunization.

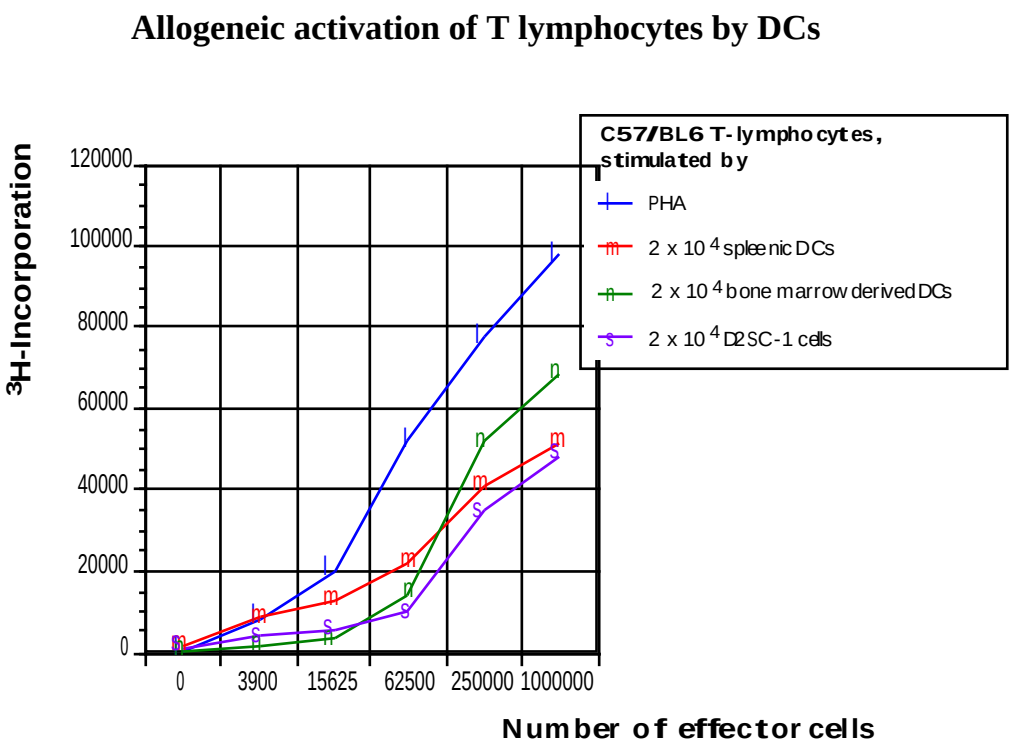

Fig. 5.26 Dendritic cells are potent stimulators of alloresponsive, naive T cells. T cells were isolated from spleen of C57/BL6-mice by MACS sorting and seeded in triplicates on 96 round bottom microtiter plates. Proliferation was induced by PHA, D2SC-1 cell line, spleenic DCs, and bone marrow derived DCs from Balb/c mice. Target cells were plated at 2 x 10^4 cells /well. The **[**^{3}H]-thymidine incorporation is illustrated in the vertical axis, the number of effector cells in the horizontal axis.

5.9 Infection assay

A frozen stock of TeFLY AF13, transfected with pBABEpuro/mtp53 was thawed and plated on a 15 cm dish. Cells were grown to 70% confluence before replacing culture medium with 10% DMEM, 10% FCS, 2 mM L-glutamine. In parallel, D2SC-1 target cells were seeded in 10 cm dishes and grown to 40-50% confluence. Infection was performed at 37°C by plating 7 ml of filtered viral supernatant on D2SC-1 target cells. After 24 hrs. the medium was replaced by new, recombinant virus containing supernatant. Multiple infections dramatically increased the transduction efficiency (data not shown). Two days post transduction cells were harvested, irradiated and used for immunization of mice. Antigen transduced DCs are known to have a potential use in immunotherapy as a cellular vaccine (279-281) and their antigen presenting capacity was exploited *in vivo* for the induction of a specific anti-tumoral immune response (5.2.5).
Part of the transduced cells was analyzed for mutant p53 expression by FACS-analysis (Fig. 5.27). Cells were fixed for 30 min. on ice in 500 µl FCS, 500 µl DMEM and 4 ml 100% ethanol. p53 expression was measured by incubating cells for 45 min. with the monoclonal antibody PAb-431 (1 µg/million cells) and for 40 min. with a FITC-labeled anti-mouse antibody (Jackson IR laboratories, 1:50 dilution). Non-transduced cells served as a negative control. Transduction efficiency was between 70 to 100%.

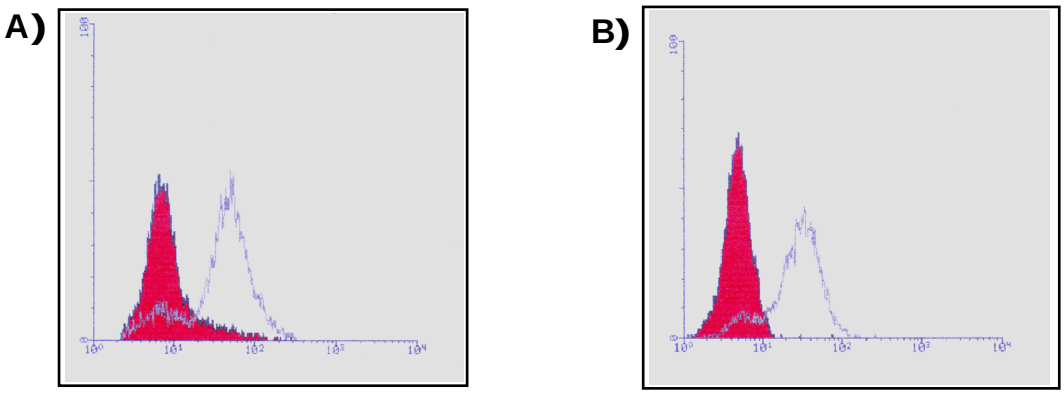

Fig. 5.27 FACS analysis of p53 transduced dendritic cell line D2SC-1. D2SC-1 cells were grown to 40-50% confluence and incubated with filtered, retrovirus containing supernatant. After 48 hrs. cells were fixed and incubated with PAb-431 anti-p53 antibody. p53 expression was measured by FACS analysis. 70-80% of the cells were shifted upon retroviral transduction with mtp53.

5.10 DCs, transduced with a tumor antigen exert protective immunity to mice when used as a cellular vaccine

The dendritic cell line D2SC-1 was transduced with retrovirus containing supernatant and efficiency of gene transfer was monitored by FACS-analysis (5.2.4). Before using cells as a cellular vaccine, transduced DCs were irradiated with 50 gray to prevent formation of tumors. Cells were injected either intravenously into the tail vein or subcutaneously into the flank of the animals. 5 x 10^5 DCs were applied per injection and injection was repeated 5 times in 10 days intervals to boost the immune response. 65 days after onset of immunization mice were inoculated intravenously with 2 x 10^5 and subcutaneously with 5 x 10^6 Meth A tumor cells. Tumor growth was monitored every third day by palpation and the size of the tumors was determined by venier calipers. Formation of metastases was analyzed by histopathology. Data obtained three months after tumor cell inoculation is summarized in Table 5.2.

	subcutaneous injection	intravenous injection
DC mutant p53 A1	4*/6	2/6
DC mutant p53 A2	3*/6	4/6
DC0	0/6	0/6

Tab 5.2 Protection from primary tumor growth by immunization of mice with transduced DCs. Balb/c mice were immunized 5 times in 10 days intervals with 5 x 105 transduced DCs subcutaneously into the flank or intravenously into the tail vein. DCs were transduced with mutant p53 allele 1 (DC mutant p53 allele A1), mutant p53 allele 2 (DC mutant p53 allele 2), or were non-transduced (DC0). Each group consisted of 5 animals. Survival of mice 2 months after primary tumor cell inoculation (5 x 106 cells) is indicated. In two chases a spontaneous regression of a developing tumor could be observed (indicated by *).

5.11 Phenotypisation of murine solid Meth A tumors

p53 is a cellular protein that is overproduced in a variety of neoplastic cell types. The Meth A cell line, derived from a methylcholanthrene-induced fibrosarcoma, produces high levels of p53 (282). It possesses a substantially increased half life (283), probably by complex formation with hsp70 (284) and due to three different point mutations, representing three independently occurring mutational events (285, 286).
We have previously shown that mice, inoculated with mutant p53 transduced DCs are protected from Meth A tumor growth (5.2.5). Interestingly, some of these mice developed tumors 2 to 3 months post tumor cell inoculation at the tumor cell injection site. We speculated that immunization of mice by DCs expressing mutated p53 might apply selective pressure on tumor cells presenting the same antigen. Since tumor cells are known to be genetically unstable, selective pressure by the immune system due to expression of one tumor antigen might lead to the clonal expansion of clones that downregulate their p53 expression. Tumor growth in DC-immunized mice might therefore be a result of an tumor escape phenomenon. To analyze this situation mouse tumors were phenotyped for p53 expression.
Tumors were isolated from DC immunized mice and analyzed for p53 expression. Cell aggregates were passed through a 200 μm mesh screen and the single cell suspension was then used for FACS analysis. Tumor cells were fixed in FCS/DMEM/ethanol and incubated with the monoclonal anti-p53 antibody PAb-431 for 45 min. (1 μg/million cells) and with a FITC-labeled goat anti-mouse antibody for 40 min.. Tumor cells, isolated from a mouse treated with non-transduced DCs, served as a positive control. Tumor cells from mice that were immunized by mtp53-transduced DCs show a p53 negative phenotype, whereas mice treated with non-transduced DCs did not loose p53 overexpression in tumor cells (Fig. 5.2.9). The downregulation of p53 expression is most probably a consequence of evading the selective pressure due to an DC-induced immune response.

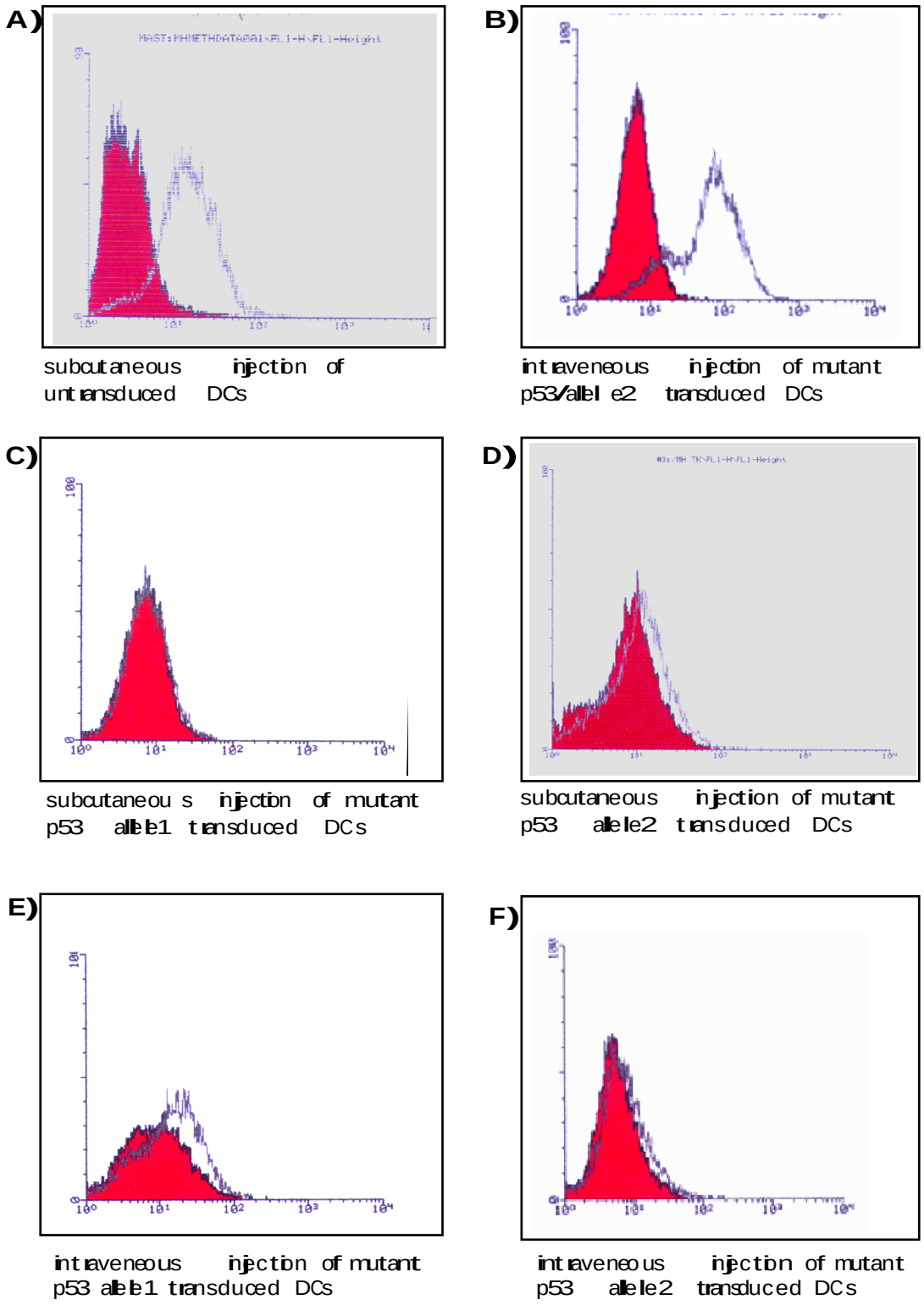

Fig. 5.29 Outgrowth of p53 negative Meth A tumor cells in DC-immunized mice. Meth A tumor cells were isolated from tumor bearing mice and assayed for p53 expression. Cells were fixed, stained by a p53 specific antibody and subjected to FACS analysis. Mice, that were treated with non-transduced DCs or mice, or tumor growth was not effected by the cellular vaccine showed high expression of p53 in tumor cells (**A, B**), whereas DC/mtp53-immunized mice lost the p53 positive phenotype (**C**, **D, E, F**) when tumors developed 2 or 3 months post tumor cell inoculation. The immunization method is illustrated at the bottom of each picture.