Immunotherapy of cancer:

Protective immunization against tumor cell growth with a mutated p53 allele

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Meinen Eltern gewidmet

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I. ABSTRACT

Products of oncogenes and tumor suppressor genes can be presented as tumor specific antigens when overexpressed and processed in malignant tumor cells. The specificity and systemic immunosurveillance of the immune system can be utilized for the development of new experimental cancer therapies to eliminate disseminated cancer cells with defined oncogenic mutations. We induced such an immune response as a means of cancer therapy in experimental mouse model systems.

The variants of the p53 gene found in methylcholanthrene induced fibrosarcoma cells (Meth A) are mutated in the DNA binding domain. Two mutant p53 alleles were isolated by RT-PCR and introduced into mammalian expression vectors. Intradermal expression of the mutated p53 proteins was achieved by biolistic particle bombardment or direct injection of naked pDNA into mice. Expression of the transgenes resulted in the induction of a cellular immune response. Treated mice were inoculated with p53 overexpressing tumor cells.

Immunized mice were persistently resistant to subcutaneous solid tumor growth and pulmonary metastases. p53-DNA immunized nude mice and immunocompetent mice injected with pDNA encoding a bacterial β–galactosidase or a luciferase gene were not protected against Meth A tumor cells. Inoculation of tumor resistant mice with the ras-transformed HC11 mammary epithelial mouse cell line Q6, bearing other p53 mutations, also resulted in lethal tumor growth. DNA immunization of mice with established tumors was not efficient. Spleenic T lymphocytes isolated from tumor resistant mice were injected into solid tumors and induced rapid tumor regression or temporary growth inhibition. Specific CTL-activity was evident in ^{51}Cr release assays utilizing p53-transduced dendritic target cells.

Transduction of dendritic cells with the mutant p53 alleles has been performed by retroviral transfer using a stable, p53-transfected packaging cell line TeFly AF13. 70-100% of the dendritic cells were transduced as shown by FACS analysis. Immunization of immunocompetent Balb/c mice has been performed by intravenous or subcutaneous injection of irradiated transduced DCs. After inoculation with primary tumor cells immunized mice showed long termed tumor resistance. In two cases arising tumors regressed within 2 weeks. Developing primary tumors on immunized mice were characterized by delayed tumor growth and a p53 negative phenotype.

Professional antigen presenting cells that simultaneously present a number of different tumor antigens are potent immune stimulators and exert stronger selective pressure on primary tumor cells than immunization against only one specific tumor antigen. Thus, Meth A tumor cells were fused with dendritic cells, both of them stably transfected with resistance markers. Resulting fusion hybrids contained numerous tumor antigens and antigen presenting properties and formed a basis for a new cellular vaccine. After selection different cell hybrid clones were isolated and analyzed for expression of costimulatory cell surface receptors and MHC class I or class II molecules. 24 cell hybrid clones were randomly chosen for immunization of immunocompetent Balb/c mice. Inoculation of mice with cell hybrid clones resulted in the formation of small tumors (3-5 mm^3) that were eliminated within 10 days. The immunized mice were resistant to primary Meth A tumor cells and did not develop pulmonary metastases or solid tumors. The serum of immunized mice displayed a specific antibody profile to Meth A tumor cells, when used in a Western blot experiment. However, the rejection of tumor cells is most likely based on a cellular T cell mechanism since the cell hybrids formed tumors on nude mice. Indeed, T cell activation was demonstrated in immunized Balb/c mice by a [^3H]-thymidine incorporation assay. A connection between tumor rejection and expression of B7-1, B7-2, or MHC-molecules on the fused cells could not be established.

ZUSAMMENFASSUNG

Bestimmte Onkogen- und Tumorsuppressorgen-Produkte werden als tumorspezifische Antigene präsentiert, wenn sie in malignen Krebszellen überexprimiert und prozessiert werden. Als Grundlage für die Entwicklung neuer experimenteller Krebstherapien kann die Spezifität und die systemische Überwachung durch das Immunsystem genutzt werden. Dadurch können Tumorzellen mit definierten Onkogen-Mutationen in ihrem Wachstum inhibiert und eliminiert werden. Wir hatten die Absicht eine solche Immunantwort als Mittel der Krebstherapie zu induzieren.
Zwei mutierte p53 Allele wurden mittels RT-PCR aus der Fibrosarkomzellinie Meth A isoliert und in eukaryotische Expressionsvektoren kloniert. Die intradermale Expression der mutierten p53 Proteine wurde in Mäusen durch biolistischen Partikelbeschuß oder durch direkte Injektion der p53 Expressionsvektoren herbeigeführt. Diese Expression führte zu einer zellulären Immunantwort. Die Mäuse wurden anschließend mit einer syngenen Sarkomzellinie, die p53 überexprimiert, inokuliert.
Immunisierte Mäuse waren über einen unbegrenzten Zeitraum vor dem Anwachsen eines subkutanen Primärtumors und der Bildung von Lungenmetastasen geschützt. p53-immunisierte Nacktmäuse und immunkompetente Balb/c-Mäuse, die mit Kontrollkonstrukten behandelt wurden, zeigten diese Schutzfunktion nicht. Sie bildeten Tumore aus, die innerhalb von 4 Wochen zum Tode führten. Inokulation der tumorresistenten Mäuse mit ras-transformierten HC11 Q6-Tumorzellen, die abweichende p53 Mutationen aufwiesen, führten ebenfalls zum Tumorwachstum. Die DNA-Immunisierung ist bei bereits angewachsenen Tumoren nicht wirksam. Lymphozyten, die aus tumorresistenten Mäusen isoliert wurden und intratumoral appliziert wurden, führten zur vollständigen Tumorregression oder zur Wachstumsinhibition des Primärtumors. Der Nachweis von spezifischen zytotoxischen T-Zellen konnte anhand p53-transduzierter dendritischer Zellen im Chrom-Release Assay durchgeführt werden.
Die Transduktion dendritischer Zellen mit den mutierten p53 Allelen erfolgte durch einen retroviralen Transfer mittels der stabil transfizierten Verpackungszellinie TeFly AF13. Durch die FACS-Analyse konnten eine Transduktionseffizienz von 70-100% der dendritischen Zellen nachgewiesen werden. Zur Immunisierung immunkompetenter Mäuse wurden bestrahlte DCs subkutan oder intravenös appliziert. Nach Inokulation mit den Primärtumorzellen konnte an immunisierten Mäusen ebenfalls eine zeitlich unbegrenzte Tumorresistenz, in 2 Fällen bei anwachsendem Tumor eine vollständige Tumorregression beobachtet werden. Anwachsende Primärtumore auf immunisierten Mäusen waren durch ein verzögertes Anwachsen und einen p53-negativen Phänotyp gekennzeichnet. Die Immunisierung gegen ein spezifisches Tumorantigen scheint in diesen Fällen keinen ausreichenden Selektionsdruck auszuüben um die Entwicklung eines Tumors zu unterdrücken.
Eine Aktivierung des Immunsystems durch professionelle antigen-präsentierende Zellen führen zu einem wesentlich stärkerem Selektionsdruck. Dementsprechend wurden Meth A Tumorzellen und dendritische Zellen, die jeweils stabil mit Resistenzmarkern transfiziert wurden, fusioniert. Die daraus resultierenden Hybridzellen trugen Tumorantigene und gleichzeitig die ganze antigen-präsentierende Maschinerie. Diese Zellen bildeten die Grundlage für einen neuen zellulären Impfstoff. Nach Selektion mit den entsprechenden Antibiotika wurden 56 Zellhybride isoliert, die auf Expression von MHC-Molekülen und costimulatorischen Oberflächenrezeptoren mittels FACS-Analyse untersucht wurden. 24 Zellhybridklone wurden für die Immunisierung von immunkompetenten Balb/c Mäusen willkürlich ausgesucht. Die Inokulation der Mäuse mit den Zellhybridklonen führte in sämtlichen Fällen zur Bildung eines knotenförmigen Tumors (3-5 mm^3) der sich nach ca. 10 Tagen vollständig zurückbildete. Die immunisierten Mäuse waren gegen die Bildung von Lungenmetastasen oder dem soliden Primärtumor aus Meth A Ursprungszellen resistent. Die Analyse der Blutseren ergab ein spezifisches Antikörperprofil, als das Serum immunisierter Mäuse gegen ein Meth A Tumorzellysat eingesetzt wurde. Die Beteiligung eines zellulärer T-Zellmechanismus scheint wahrscheinlich, da alle diese Zellhybridklone auf Nacktmäusen zu einem Tumorwachstum führten. Tatsächlich konnte eine Aktivierung von T -Zellen anhand eines [^{3}H]-Thymidin-Inkorporations-Assays nachgewiesen werden. Ein

Zusammenhang mit der Expression von B7-1, B7-2 oder MHC-Molekülen auf den Hybridzellen wurde hingegen nicht festgestellt.

II. INTRODUCTION

The problem of cancer is of major concern worldwide. Statistically one-fifth of all individuals in wealthy countries will develop cancer. Thus cancer remains second only to cardiac disease as a cause of death. The appearance of a human tumor is the culmination of a complex, multi-step process. Human cancers typically arise after long latency and acquire multiple abnormalities in their control over cellular growth and phenotype.
The rate limiting steps to develop tumors seem to be mutations in half a dozen or more cellular genes that, directly or indirectly, affect tumor cell proliferation. These proto-oncogenes and tumor suppressor genes are important regulators of biologic processes. Despite their name, they do not reside in the genome for the sole purpose to promote the neoplasic phenotype. Rather, they are essential to normal biological processes. They play diverse roles in the control of cellular growth, including proliferation, apoptosis, genome stability and differentiation.
From calculations using the known mutation rate in non-germline cells (~10^7 per gene per cell generation), one can predict that so many of these mutant genes will never accumulate in the genome of cell lineage during human lifetime (1, 2). However, experiments show that tumorigenesis can occur if the genomes of pre-malignant cells are far more mutable than those of their normal counterparts (3). Acquired mutability is typical for most tumors. Mutations that impair the ability of cells to recover from DNA-damage can enhance the spontaneous mutation rate, thereby accelerating the process of tumorigenesis. It has been known for years that some inherited disorders predispose to cancer (e.g. xeroderma pigmentosum, Fanconi's anemia). It has also been shown that these disorders are often associated with defects in DNA-repair (4, 5, 6). For example, the DNA mismatch repair system is responsible for a major hereditary form of colon cancer, hereditary nonpolyposis colorectal cancer (HNPCC)(7). One of the most common alterations in human cancer is an alteration at the p53 locus on chromosome 17p (8). The exact function of p53 in cancer is evident by the genetic instability of p53 deficient cells. p53 is required for transcriptional activation of genes that coordinately shut down cell cycle progression and induce a battery of genes involved in DNA repair. If this pathway is interrupted in p53 mutant cells these cells will fail to arrest appropriately and will more likely sustain DNA damage (9).

2.1 Immunity to tumors

For years the concept, that the immune system is involved in the recognition and elimination of tumors is controversly discussed. A major function of the immune system is thought to be the recognition and elimination of spontaneously arising malignant cells or it might develop during malignant transformation and limit the disease and lead to spontaneous tumor regression. If malignant cells and tumors can stimulate immune responses, they must express tumor antigens that are recognized as abnormal by the tumor-bearing host. Two types of tumor antigens have been identified on tumor cells: tumor-specific transplantation antigens (TSTAs) and tumor-associated transplantation antigens (TATAs) (10). Tumor-specific antigens are unique to tumor cells and do not occur on normal cells in the body. They may result from mutations in tumor cells that generate altered cellular proteins and cytosolic processing of these proteins would give rise to novel peptides that are presented with class I MHC molecules, inducing a cell mediated immune response. Tumor-associated antigens, that are not unique to tumor cells, may be proteins that are expressed on normal cells during fetal development when the immune system is immature and unable to respond but that are normally not expressed in the adult. Reactivation of the embryonic genes in tumor cells result in their expression on fully differentiated tumor cells. Tumor-associated antigens might also be proteins that are normally expressed at extremely low levels on normal cells but are expressed at a much higher level on tumor cells. Following expression immune effector cells, such as B cells, T cells, cytolytic T lymphocytes or natural killer cells recognize these antigens and mediate the killing of the tumor (Fig. 3.1).

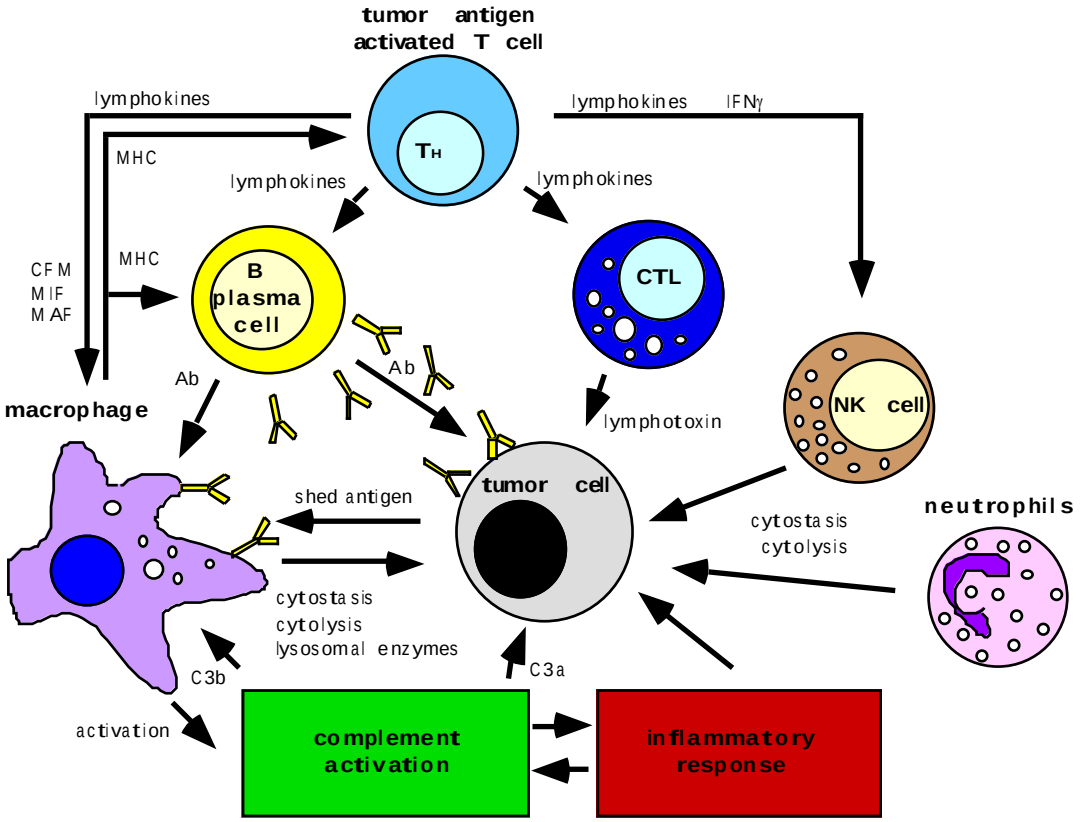

Fig. 2.1 Immunity to tumors. T cells activated by a tumor antigen release interferon γ to amplify the lytic action of natural killer cells (NK) or chemotactic factors (CFM), the migration inhibiting factor (MIF), and the macrophage activating factor (MAF), all of which activate macrophages. Activated macrophages produce complement components, which are involved in the development of the inflammatory response. C3a is a cytolytic and chemotactic for neutrophils while C3b induces macrophage enzyme release. Other lymphokines, including IL-2, amplify antigen specific immune reactions by B cells and other T cells. The activated lymphocytes cooperate in the production of tumor specific antibodies and activated T cells may also have cytostatic or cytolytic activity.

The concept of immunosurveillance was first articulated by Macfarlane Burnet and Lewis Thomas in the 1950s and 1960s (11, 12). The major effector mechanism for tumor surveillance was considered to be based on T cell mediated immunity. A common histologic observation, which suggests that tumors might be immunogenic, is the presence of mononuclear cell infiltrates, composed of T cells, NK cells, and macrophages, surrounding many tumors. Although such infiltrates may often result after tissue destruction caused by the tumor, the presence of lymphocyte infiltrates e.g. in medullar breast carcinomas and malignant melanomas is associated with a better prognosis compared with similar tumors without such infiltrates. Furthermore, there is often evidence of lymphocyte proliferation in lymph node draining sites of tumor growth and cytokine effects in tumors, such as MHC II expression on tumor cells and endothelial cells of tumor vessels.
Various clues support the hypothesis of immunosurveillance. The incidence of malignant tumors peak in childhood or in old age, a period of life where the immune system is not in optimal condition. In the 1960s, fast progressing transplantation programs and the use of long-term immunosuppressive therapy in order to maintain the viability of solid-tissue allografts showed a higher tumor incidence in immune suppressed patients. Lately, also the immunosuppression accompanied by HIV infection was connected to the development of different malignant neoplasias.

Perhaps the most convincing evidence has been obtained in model systems that examine the immune response in the context of sunlight-induced cutaneous tumors. Experimental model systems, developed by Kripke and Daynes (13, 14) demonstrated that chronic exposure of mice to high levels of UVB radiation eventually produces cutaneous tumors. Although these

tumors grow progressively in the host of origin, they are highly immunogenic when transplanted into non-UVB exposed syngeneic mice. The UVB radiation alters the host immune system by producing generalized defects in antigen-presenting cells, and by inducing the formation of suppressor T cells (15, 16). Support for this view comes from reports demonstrating that early in the course of tumor-promoting UVB irradiation, epidermal Langerhans cells are depleted and the draining lymph nodes are deficient in cells that can induce chronic hypersensitivity *in vivo* (17). It has been well demonstrated that epidermal antigen presenting cells are effective at presenting tumor antigens and at inducing tumor immunity *in vivo* (18). These specific effects on the immune system prevent mice from rejecting UVB induced tumor implants, and inhibit contact hypersensitivity (CH) when hapten is applied epicutaneously. The molecular mechanism of UVB-action is thought to be the creation of cis-urocanic acid in the stratum cornum and the generation of TNF-α, both of them been reported to be systemically immunosuppressive (19, 20).
Clinical counterparts to these findings exist (21, 22, 23). Sunlight is an important hazard to humans. Indeed, UVB is known to be the environmental cause of sunlight induced basal/squamous cell skin cancers (24, 25, 26). It is also thought to be important in the pathogenesis of malignant melanoma (27, 28). Low dose UVB-treatment of humans depletes the epidermis of $CD1^+$ Langerhans cells (29). The ability to impair chronical hypersensitivity in humans is genetically determined. Onto UVB-exposed skin chronic hypersensitivity fails to develop in about 40% of normal, adult caucasian volunteers, whereas the remaining 60% develop vigorous chronic hypersensitivity, when the hapten dinitrochlorobenzene is applied to the skin (30). Among patients with histories of basal and squamous cell skin cancer, the frequency of the UVB-sensitive phenotype was found to be extremely high (>92%), (30, 31). Also, of 10 patients with a diagnosis of malignant melanoma, 100% proved to be UVB-sensitive and 50% of patients with skin cancer displayed hapten-specific nonresponsiveness (32).

2.2 Gene therapy can be used to strengthen the immune response for anti-tumor activity

In the last few years, somatic gene therapy protocols have been developed that aim at strengthening the immunosurveillance of the body and lead to the eradication of disseminated cancer tumor cells and micrometastases after surgical removal of the primary tumor. Human tumors are poorly immunogenic although a large number of genetic alterations found in advanced cancers should give rise to peptide neo-epitopes capable of being recognized in combination with major histocompatibility complexes by cytotoxic lymphocytes (33). Indeed, tumor-associated and tumor-specific antigens have been identified that could act as targets for immunological attack (34). It is therefore possible, that the immune system fails to eliminate tumor cells not because neo-antigens are absent but rather because the response to these neo-antigens is inadequate (35). A number of factors have been determined to cause this inadequacy, such as deficient antigen presentation, lack of immune costimulation, and insufficient help from $CD4^+$ cells. Transfection of certain genes into tumor cells can render them more immunogenic and therefore less tumorigenic. Genetically altered tumor cells, transplanted to the host after irradiation, create a new tumor vaccine. Such genes might include allogeneic major histocompatibility complexes (36) or viral proteins, such as hemagglutinin (37). The properties of the resulting xenogeneized tumor cells have been explained by the concept of associative recognition (38, 39, 40) that proposes, that minor cell surface antigenic differences are insufficient for the induction of an immune response. The addition of more powerful antigens to the cell surfaces provides a response for the added antigens and also induces recognition of the original tumor-associated cell surface antigens (37) and systemic protection against tumor cells. In these cases mice, challenged with a second injection of live tumor cells, demonstrated increased rejection, suggesting immunologic memory (36,37).
For optimal activation lymphocytes require both an antigen specific signal delivered through the T cell receptor and a second, non-specific costimulatory signal such as B7 (41, 42, 43). Therefore immunogenic tumors that do not express B7 can escape destruction by the

immune response because tumor targeted T cells receive inadequate costimulation. Model systems show rejection of tumor cells *in vivo* when transfected with genes that encode costimulatory molecules (44, 45). Rejection of B-7 transfected tumor cells was primarily mediated by $CD8^+$ T cells. Moreover a systemic protection against the parental, untransfected tumor cells was observed.

One major effector mechanism of tumor rejection is thought to be connected to the tumor-infiltrating lymphocytes. In fact, the connection between cellular immunity and gene therapy has been described by Hwu et al. in 1993 (46). Tumor-infiltrating lymphocytes isolated from melanoma patients have been transfected by a gene encoding for the tumor necrosis factor-α. TNF-α induces major histocompatibility complex class I expression and activates macrophages, granulocytes, and cytotoxic T lymphocytes. The reimplantation of these transduced lymphocytes targeted TNF-expression near to recognized tumor cells and activated a local immune response. TNF-α and other cytokines are involved in immunity and inflammation, and they essentially control the magnitude of the immune response, either in a paracrine or an autocrine fashion. Insertion of other cytokine encoding genes such as interleukin (47, 48), colony stimulating factor (49, 50, 51) and interferon-encoding genes (52, 53, 54, 55, 56, 57) into tumor cells nearly uniformly reduced the tumorigenity of injected tumor cells with the exception of IL-6 that actually led to an accelerated growth of tumor cells.

2.3 p53 is a suitable target for gene therapy

During the past decade a number of oncogenes and tumor suppressor genes have been cloned and the molecular mechanisms as to how such genes contribute to tumor development were clarified. First identified as a tumor antigen-associated protein in SV40-transformed cells and later as a cellular protein involved in SV40 transformation, p53 has emerged in recent years as a central player in many human tumors. Alterations at the p53 locus on chromosome 17p have been found in a large percentage and wide variety of human tumors and are the most common alterations in human cancer (58). In fact, 75% to 80% of colon tumors show abnormalities at both p53 alleles: one allele is often deleted, and the other has point mutations, that are usually missense mutations that yield an altered protein product. Persons with the cancer-predisposing Li-Fraumeni syndrome are born with mutations in one allele of the p53 gene and develop tumors that bear missense mutations at both alleles (59).

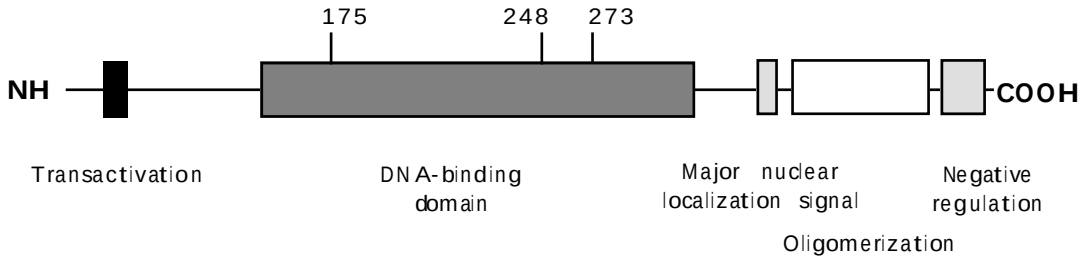

Fig. 2.2 The p53 protein compromises four functional domains, including a transcriptional activation domain in the acidic N terminus, a DNA-binding domain that binds to two copies of any sequence matching the consensus 5'-PuPuPuC(A/T)(A/T)GPyPyPy-3', an oligomerization domain responsible for the formation of p53 tetramers, and a basic C-terminus with putative negative regulatory functions. Different mechanisms of p53 inactivation have been observed. Codons 175, 248, and 273 contain 6.1%, 9.6%, and 8.8% of tumorigenic mutations, respectively. Hot spots for mutations are clustered in the DNA-binding domain of the molecule, the region that most probably confers to the tumor-suppressive properties (60, 61). These oncogenic mutations abrogate DNA binding either by eliminating protein-DNA contacts or by disrupting the structure supporting the DNA binding surface. All tumor derived mutants display reduced affinity for DNA. Additionally deletions, gene-rearrangements and the loss of p53 alleles have been observed. p53 is active as a tetramer and wildtype and mutant p53 can oligomerize. Therefore mutant p53 can act in a dominant negative manner.

p53 deficient mice develop normally but die early from cancer. Thus, p53 is dispensable for normal growth but its loss predisposes to cancer. In many tumors, both p53 alleles are deleted and there is no p53. The lack of a normal p53 allele in naturally occurring tumors argues that p53 functions as a true tumor suppressor gene to control tumor growth and cell division. However, malignant transformation can also occur when certain mutants of p53 are expressed in a cell containing at least one normal p53 allele. These dominant negative mutations probably act by binding to and inhibiting the function of normal p53 in the cell, which is analogous to the role of several virally encoded proteins, including SV40 T-antigen (62), adenovirus E1B 55K protein (63), and oncogenic human papillomavirus E6 protein (64). If p53 acts in a dominant negative manner will depend on the expression of the genes - overexpression of wildtype p53 can suppress transformation by mutant p53 (65).

The exact function of p53 in cancer is evident by the genetic instability of p53-deficient cells (66). Insight into the function of p53 in cancer has come from the observation that p53 is required for some types of DNA-damage-dependent cell cycle checkpoint and apoptotic pathways. DNA damage induces p53 accumulation, and p53 defective cells fail to undergo checkpoint arrest at the G1/S boundary (67, 68). Screening for p53 regulated transcripts led to the identification of the protein CIP1, that is dramatically upregulated by p53 overexpression (69). CIP1 has been identified earlier as an CDK-associated protein, that inhibits cyclin/CDK activity and hence cell cycle progression. However, CIP1 may also function in the S phase checkpoint. *In vitro*, CIP1 inhibits replication elongation by binding to proliferating cell nuclear antigen (PCNA) and may be involved in a switch from replicative to repair synthesis. A second p53-induced gene, GADD45, may also be involved in the DNA damage response, since GADD45 binds PCNA and stimulates excision repair. Thus, transcriptional activation of genes by p53 may coordinately shut down cell cycle progression and induce a battery of genes involved in DNA repair. If this pathway is interrupted in p53 mutant cells these cells will fail to arrest appropriately and will more likely sustain lasting DNA damage.

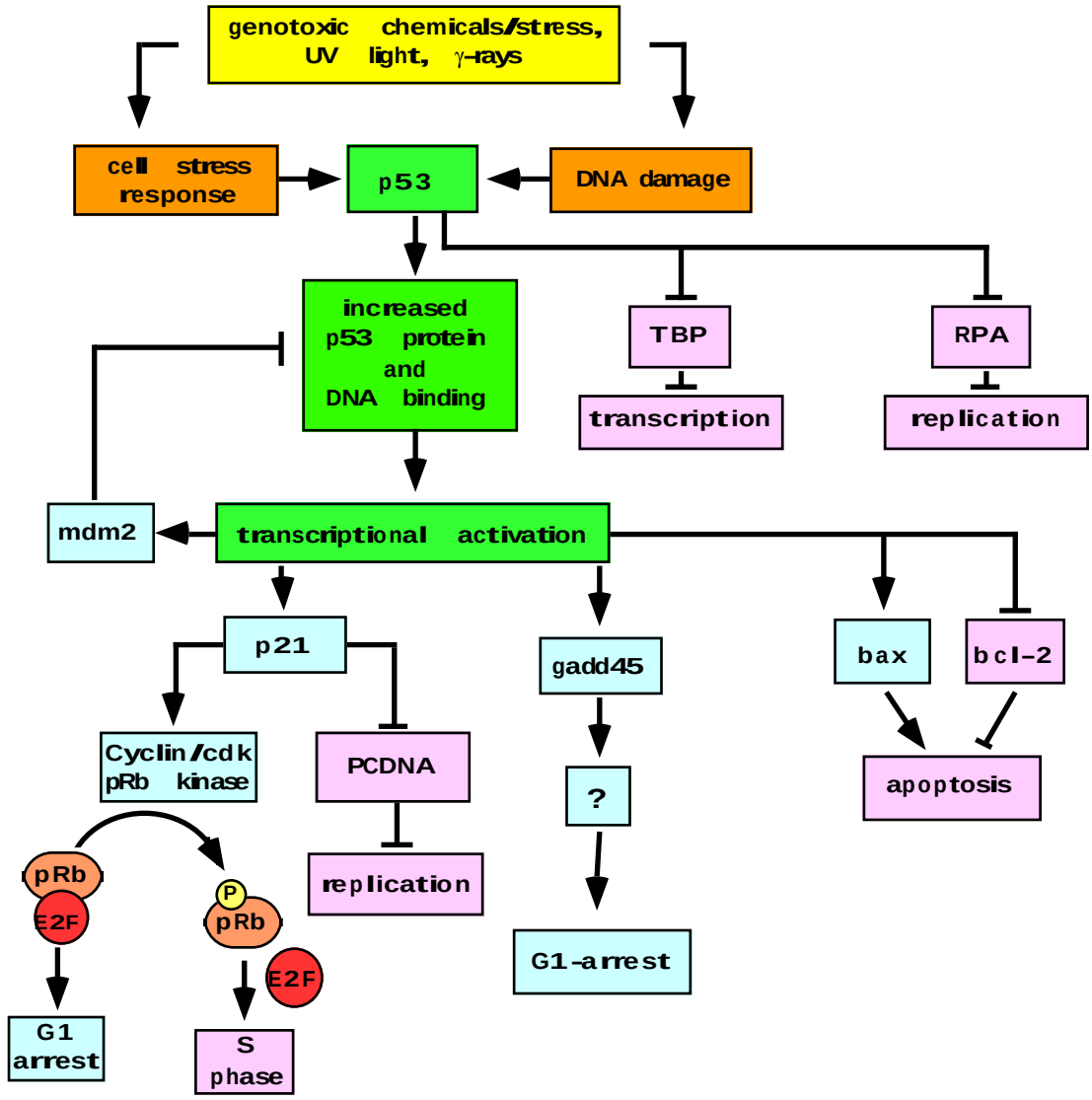

Fig. 2.3 Wildtype p53 is a transcription factor localized in the nucleus of cells (70). p53 accumulation by posttranscriptional stabilization is induced after genotoxic stress or DNA damage. DNA damage can be detected by direct binding of p53 to single stranded DNA (71) or Ser15, Ser37 phosphorylation of the molecule by the single stranded DNA-dependent kinase that is activated by a broad range of DNA alterations (72, 73). Accumulation of p53 induces G1 arrest or apoptosis by protein-protein interactions or sequence specific transcriptional regulation. Proteins bound and inhibited by p53 include TBP, a subunit of the basal transcription factor TFIID (resulting in repression of many genes, (74, 75)) and PRA (resulting in impaired DNA replication, (76)). At the transcription level p53 increases the expression of mdm2 (which binds and inactivates p53 in an autoregulatory loop, (77), p21/cip-1 (which binds and inhibits both proliferating-cell nuclear antigen (PCNA), (78)) and Cdk/G1-cyclin complexes (79)), gadd45 (which participates in G1 arrest through unknown mechanisms, (80)), and bax (an activator of apoptosis, (81)). p53 downregulates bcl-2 (which promotes cell survival, (81)). G1 arrest is mediated by inhibition of PCNA, a regulatory subunit of DNA polymerase-δ that is required for DNA replication and inhibition of G1-cyclin/Cdk, which prevents inactivation, by phosphorylation of Rb-like molecules. Transcription factors of the E2F family therefore remain associated with pRb and are unable to activate transcription of genes required for progression from G1 into S. Apoptosis is induced by displacement of the bcl-2 to bax ratio toward bax.

2.4 p53 as a tumor antigen

Common and rare types of cancer harbor a variety of tumor-specific mutant proteins that may be recognized as tumor-specific antigens. These mutant proteins are encoded by oncogenes or tumor suppressor genes that have undergone structural mutations resulting from point mutations, chromosomal translocations, internal deletions, and viral insertional mutagenesis. Such tumor-specific molecules might be important as targets for preventive cancer vaccines and for specific tumor immunotherapy once cancer has developed. Evidence supports the idea that B cells and cytotoxic or helper T cells can exquisitely recognize selective intracellular mutant proteins (82, 83, 84). Various groups have identified in the serum of human cancer patients a number of different antibodies recognizing tumor antigens, including c-erbB-2, c-myc, c-myb, and the p53 tumor suppressor gene (85, 86, 87,88). p53 specific antibodies have been found in 21% of sera from children with B cell lymphoma (89). 13% of sera from patients with lung cancer (90) and 9% of patients with breast cancer (91) also contained antibodies against p53. These antibodies have not been found in healthy individuals. For antibody production overexpression of the p53 protein seems to be required. Often the mutation of p53 proteins is accompanied by an enlarged half-life. More than 60% of all human tumors contain elevated levels of mutated p53 (92). The half-life of normal p53 is about 20 minutes and the amount of normal p53 proteins in normal cells is nearly undetectable. Enlarged amounts of p53 can therefore be used as a tumor marker. However, not all sera from patients with p53 overexpressing tumors contain antibodies against the tumor suppressor protein. Antibody production seems to be closely connected to mutations in exon 5 or exon 6, especially regions being responsible for complex formation with the heat shock protein HSP70 (93). A cellular immune response against mutant p53 in cancer patients has yet to be experimentally established and there is no evaluation of the presence of auto-antibodies on the progression of malignant disease.

New experimental data by Yanuck et al. demonstrated a cytotoxic immune response in mice specific for a mutated p53 protein (94). This immune response has been triggered by spleen cells pulsed with a peptide corresponding to a 21 amino acid sequence encompassing a point mutation (135 Cys to Tyr) in the mutant p53 gene product of a human lung carcinoma. The mutation created a new K^d class I molecule binding motif sequence and the recognized determinant was mapped to this motif and was presented by the K^d class I molecule. p53-specific $CD8^+$ CTLs were generated and killed specifically fibroblasts, transfected with the mutant p53 gene but not control fibroblasts or fibroblasts transfected with a different human p53 gene. The CTL activity was specific for the transforming point mutation and not due to differences between mouse and human p53. Thus endogenously synthesized mutant p53 can be processed, these processed peptide fragments are carried to the cell surface by class I major histocompatibility complexes and activate specific cytotoxic T lymphocytes.
Although transplanted spleen cells were pulsed with a high amount of peptide, *in vivo* generated p53 specific T cells recognized and lysed cells with low expression of mutant p53 (0.18 ng/ml). These levels are comparable to levels of mutant p53 present in human tumors (0.1-70 ng/ml).

Current data suggests that tumor rejection antigens recognized by cytotoxic lymphocytes are often products of normal non-mutated cellular genes. T lymphocytes that are not specific for individual p53 mutations can be induced by wild-type p53 peptide sequences because of overexpression and not because of an aberrant amino acid sequence. Indeed, *in vivo* rejection of the mouse mastocytoma cell line P815 is induced by cytotoxic T lymphocytes that recognize tumor antigen products of non-mutated cellular genes (95). Similar to p53, these genes are not expressed in normal tissue or only low amounts of the gene product can be detected within untransformed cells. Induction of a CTL-response is due to overexpression of the gene product in P815 cells. Another reason for CTL induction might be altered processing of mutant p53. The conformation of the mutant p53 protein might be responsible for alterations in processing and presentation of p53 peptides. Altogether, three potential mechanisms can activate a specific p53 dependent immune response: Overexpression, mutation, and altered antigen-processing. From these, the induction of CTLs by

overexpression or altered antigen-processing can lead to the elimination of different p53 expressing tumor cells.

2.5 Engineering of *in vivo* immune responses by DNA immunization

Observations in the early 1990s that plasmid DNA could directly transfect animal cells *in vivo* resulted in the use of DNA plasmids encoding antigenic proteins to induce immune responses by direct injection into animals. This method elicits protective antibody and cell-mediated immune responses for viral, parasitic, and bacterial diseases and tumor antigens. The DNA vaccination is particularly useful for the induction of cytotoxic T cells. DNA-vaccines consist of bacterial plasmid DNA expression vectors that regulate genes encoding for proteins of pathogens or tumors by strong viral promoters. When administered to an animal by direct injection the antigen is expressed *in situ* and induces antigen-specific immunity. The plasmid is made without an origin of replication that is functional in eukaryotic cells. Such plasmids neither replicate in the mammalian host nor integrate within the chromosomal DNA of the animal.

The first demonstration of DNA-vaccine efficacy in an animal model was accomplished using the influenza virus (96). Mice were immunized with DNA encoding an internal conserved protein of influenza A, nucleoprotein (NP), and developed both NP-specific antibodies and MHC class I restricted CTLs. These CTLs were capable of lysing target cells that were either virally infected or pulsed with MHC class I restricted peptides. Moreover these CTLs were clearly functional *in vivo*, since NP-DNA immunized mice were significantly protected from influenza. Influenza is one of the infectious disease targets for a DNA vaccine currently investigated in human clinical trials. Generally, current vaccines are effective only in a strain-specific manner. Mutations in circulating influenza virus strains mean that frequent re-evaluation and reformulation of the vaccine is necessary. DNA-vaccines offer the advantage of stimulating the generation of cytotoxic T lymphocytes against epitopes from a conserved protein of the virus, such as a nucleoprotein, thereby providing cross-strain protection (96). Additionally CTL-responses have been induced with DNA-vaccines encoding a full-size antigen rather than a limited number of peptides, that allow antigen processing of the entire protein and determinant selection in an outbreed population. On the other hand peptides are limited in their applicability to genetically diverse populations.

Although the easiest approach is to directly inject the plasmid DNA, other delivery systems for DNA administration have been employed. The gene gun was first developed to transfect plant cells but was shown early to be capable to deliver DNA constructs into animals and induce immune responses (97,98). By particle bombardment cells of the dermis and epidermis are transfected by direct penetration of the DNA-coated gold beads. Since antigen presenting cells, such as Langerhans cells, are found in these layers of the skin, CTLs might be induced by direct transfection of these professional APCs. In contrast, intramuscular injection results in the uptake of extracellular DNA and its expression in muscle cells (99). The importance of antigen presentation of muscle cells in the induction of CTLs is not clear, probably the induction of CTLs is mediated by nonmuscle APCs, e.g. by transfer from muscle cell to APC (100). Differences in the method and site of delivery may translate into differences in the character of immune responses. Recent observations show a Th_1 like helper response following intramuscular injection, but a shift to a Th_2 like helper response is seen with progressive gene gun vaccinations.

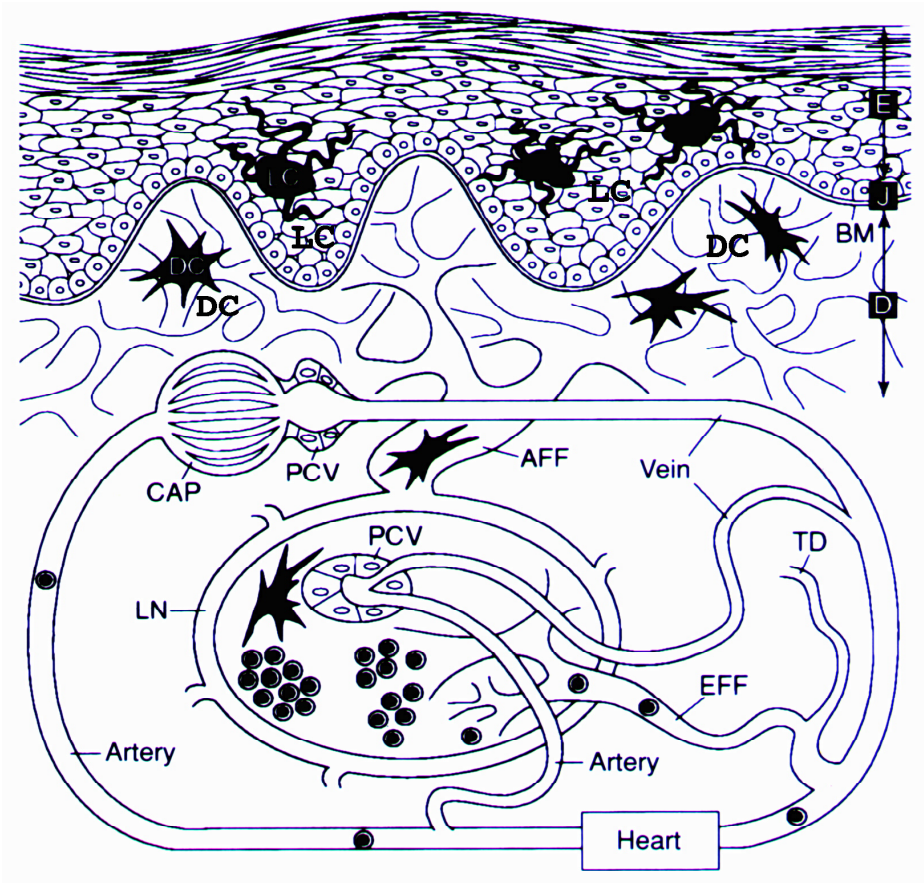

Fig. 2.4 It is thought that any antigen that arises within the skin, or is applied epicutaneously to the skin, is not directly recognized by T cells in immunologically naive animals. Considerable experimental evidence supports the view that cutaneous antigens are endocytosed, phagocytosed or taken up in some other manner, by indigenous mobile antigen presenting cells, such as Langerhans cells, dermal dendritic cells and macrophages. The antigen presenting cells then migrate through lymph to the draining lymph node and present antigen to T- and B cells, that are constantly delivered to the site via high-endothelial venules. Lymphocytes escape the node via efferent lymphatics into the thoracic duct and eventually into the blood stream.

The efficacy of DNA vaccines could be enhanced or modulated through the use of formulations that increase DNA stability or distribution in the tissue, the co-expression of immune molecules that effect processing of the antigen, and the use of adjuvants that effect the immune responses that are mounted against the co-expressed antigens. In recent studies DNA vectors expressing cytokines have been shown to be biologically active *in situ* (101) and to effect immune responses against coexpressed antigens (102-104). GM-CSF was shown to have a stimulatory effect on both humoral and cellular immune responses to rabies virus glycoprotein (102) and carcinoembryonic antigen (103). Recombinant IL-6 and 10 have also been used to increase effectiveness of DNA vaccines in a tumor challenging model (104). Others have used DNA encoding costimulatory molecules B7-1 and B7-2 in an effort to enhance or modulate immune responses mounted against coexpressed antigens by providing an additional means for T cell activation (103, 105). The immune response to DNA-vaccines is also enhanced by an adjuvant effect of the DNA itself. Certain CpG motifs of bacterial DNA are particularly stimulatory and can induce cytokine secretion and lymphocyte activation (106-108). This is probably due to the methylation state of the DNA. Other CpG motifs can inhibit lymphocyte stimulation (107). The immunogenicity of antigens expressed by the vector can be manipulated by DNA-vaccines containing or avoiding these motifs.

Another way in which DNA vaccines can be modified is by the use of a delivery system, such as liposomes or polymers that can compact DNA and enhance cellular uptake, or the inclusion of peptides or proteins that can facilitate intracellular targeting of DNA to the cytoplasm and

nucleus. In addition, DNA vaccines can be targeted to specific tissues such as mucosal sites for the induction of mucosal immune responses.

DNA vaccines can break immunologic tolerance to antigens as well. Mice transgenic for the HB surface antigen for Hepatitis B express HBsAg in their hepatocytes. They tolerate the ontogenetic expression of the antigen and do not develop antibodies or CTLs against the antigen, either spontaneously or after immunization with recombinant HBsAg. When they were immunized with a plasmid encoding HBsAg, however, they generated both antibodies and CTLs with a concomitant elimination of antigen expression (109). Due to this observations DNA vaccines offer also a promising tool to break tolerance to tumor antigens.

2.5.1 Induction of a humoral immune response

Administration of plasmid DNA has proven to be an efficient means of generating humoral immune responses specific for vector encoded proteins. These proteins could be detected in the tissue of the host for months to years, thereby providing the potential of a continued antigen stimulus. In some chases antibodies have contributed to protection against challenge with the relevant pathogen, indicating that the antigens expressed *in vivo* after DNA vaccination can assume a native structure with intact epitopes, including conformational epitopes and induce neutralizing antibodies. Such antibodies have been demonstrated in sera of animals injected with pDNA encoding HIV envelope protein (110, 111), herpes simplex glycoproteins (112, 113), and influenza HA (114-116). The duration of antibody responses induced by DNA vaccination is long lived in mice, as demonstrated with influenza NP (117), HA (118), hepatitis B surface antigen (119), and hepatitis C core protein (120). The antibody isotypes that were induced are generally IgG, but serum IgM and IgA also have been detected (116, 118). The subclass of serum antibodies induced by DNA vaccination is predominantly IgG_{2a} in mice, suggesting that a Th_1-type of helper T cell response was induced. The effectiveness of DNA vaccines appears to be favorable compared to conventional vaccines. Influenza DNA vaccines and HBsAg DNA-vaccines were shown to be more effective at inducing antibodies in mice, compared to recombinant protein expressing the antigen (121-123). Moreover, HBsAg DNA was able to induce robust humoral responses in low or nonresponder strains of mice. The immune response against DNA encoded antigens depends on dose (123) and boosting (116) and coinjection of plasmids encoding cytokines or costimulatory molecules. Coinjection of multiple plasmids or coexpression of multiple antigens can induce a broader spectrum of immune responses (121). Coexpression of GM-CSF with rabies virus glycoprotein (124) or carcinoembryonic antigen (125) resulted in enhanced antibody titers. Similarly coinjection of DNA encoding the costimulatory molecules B7-1 and B7-2 with DNA encoding *M. tuberculosis* hsp 65 (126) or CEA (125) induced higher antibody titers. The DNA plasmid itself also may have immune stimulatory properties, and administration of additional DNA or modification of the sequence of the DNA may serve to increase immune responses.

2.5.2 Induction of a cellular immune response

MHC class I restricted, $CD8^+$ cytotoxic T lymphocytes were demonstrated by different groups following pDNA injection (96, 127-130). Lymph node or spleen cells from mice that have been injected intramusculary with plasmid DNA were restimulated *in vitro* with antigen, with mitogen and IL-2, or by virus infected targets. In studies of influenza NP in Balb/c mice, a single intramuscular injection of as little as 1μg of NP DNA induced CTLs that recognized the amino acid 147-155 epitope peptide from influenza NP (115). In other studies, anti-NP specific CTLs were found to persist for more than 2 years after immunization with influenza NP-DNA (117, 131, 132). In another model a malaria DNA-vaccine has been shown to specifically induce $CD8^+$ T cells and confer protective immunity to mice (152). Doolan et al. demonstrated that DNA vaccines can provide protection across a

broad spectrum of MHC haplotypes and suggests that additionally less specific immune mechanisms like interferon-γ and nitric oxide may also play key roles in protection (133). Effector functions of T lymphocytes following activation by the antigen are either executed by interaction between cells or the secretion of various proteins, such as cytokines. Cytokines are produced by T lymphocytes to provide help to other immune cells, including macrophages and B- or T lymphocytes, that facilitates differentiation and development of effector and memory cells. T helper cells are grouped into functional subsets characterized by the particular cytokines they produce. (134). For mice, cytokines such as IL-2, interferon-γ, produced by type 1-like helper T cells (Th_1), support the development of cellular immune responses, including CTLs and the IgG_{2a} immunoglobulin subtype; cytokines like IL-4, IL-5, IL-6, and IL-10 produced by type-2 like helper T cells (Th_2) promote B cell activation and immunoglobulin class switching and are typified by a predominance of the IgG_1 immunoglobulin isotype. Similar subtypes have been characterized in humans (135). A critical determinant for the outcome of the vaccination is the type of T cell help the particular vaccines elicit (136). Intramuscular immunization and intradermal injection of DNA plasmids encoding a variety of antigens generated long lived memory T cell responses and secretion of Th_1 like cytokines during *in vitro* cultures (137, 138). These results suggest that generation of Th_1-like T cell help may be a general property of DNA vaccines. However, vaccination with the gene gun, that delivers DNA primarily to the epidermis, appears to bias immune responses toward Th_2-like responses (139).

2.6 Dendritic cells are new therapeutic tools for the treatment of cancer

Dendritic cells (DC) have become a focus of extensive research over the past years with increasing clinical relevance for the development of new therapeutic strategies against tumors and other diseases. DCs are specialized for antigen uptake, processing, and presentation to T cells (140). It is now widely accepted that DCs play a central role in the induction of primary antigen-specific immune responses. They have the unique capability to activate major histocompatibility class I and class II restricted T cells, which have not had any previous contact with the antigen (140). B cells, the precursors of antibody secreting cells, can directly recognize native antigen through their B cell receptors. T lymphocytes, however, need the antigen to be processed and presented to them by an APC. The T cell receptor recognizes fragments of the antigen, bound to molecules of the major histocompatibility complex on the surface of an APC. The peptide binding molecules are of two types, MHC class I and MHC class II, which stimulate cytotoxic T cells and helper T cells, respectively. Intracellular antigens, cut into peptides in the cytosol of the APC, bind to MHC class I molecules and are recognized by CTLs, that, once activated, can directly kill a target cell. Extracellular antigens that have entered the endocytic pathway of the APC are processed there and are generally presented by MHC class II molecules to T helper cells, that when turned on, have profound immune-regulatory effects.

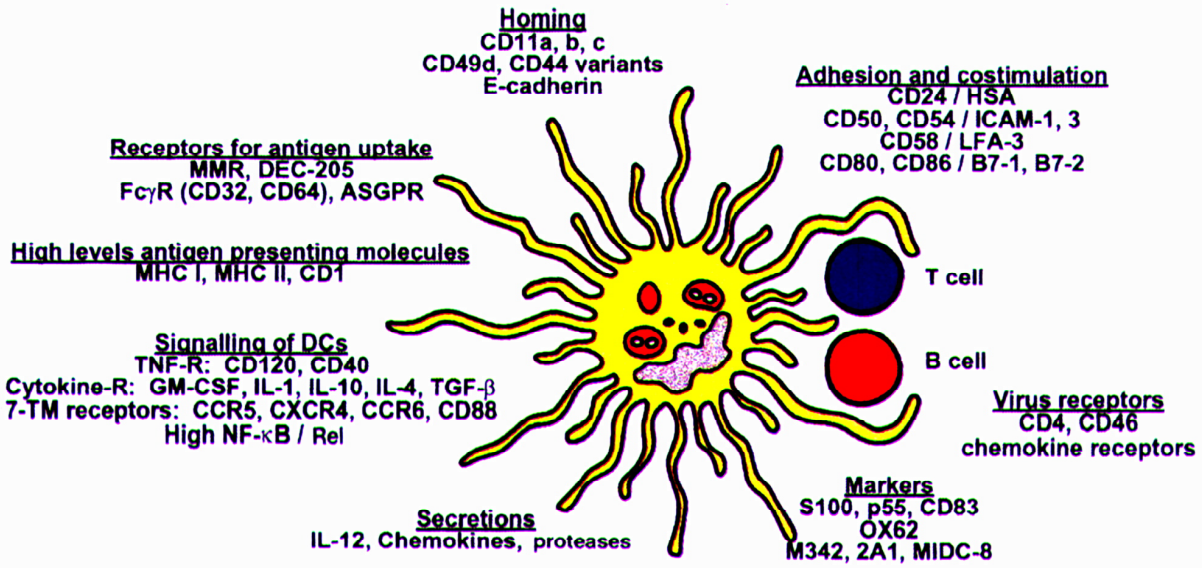

Fig. 2.5 Features of dendritic cells. DCs are initiators of immune responses and can stimulate quiescent, naive and memory B and T lymphocytes. Strong T cell responses can be induced by low level of antigen and a small number of DCs. Dendritic cells in the periphery capture and process antigens, express lymphocyte costimulatory molecules, migrate to lymphoid organs and secrete cytokines to initiate immune responses. DCs serve as sentinels *in vivo*. Their distribution in the body optimizes antigen capture, their migration to lymphoid organs optimizes clonal selection of rare $CD4^+$ and $CD8^+$ T cells.

To initiate T cell immunity, peptides from infected cells or tumors must be found and recognized by T cells that circulate in the blood stream. The amount of specific antigen-MHC complexes on tumors and infected cells is typically small (one hundred ore less per cell), and must be recognized by rare T cell clones (usually at a frequency 1/100000 or less) through a T cell receptor that has a low affinity (1μM or less). Moreover, infected cells and tumors frequently lack the costimulatory molecules that drive clonal expansion of the T cell, the production of cytokines, and development into killer cells. DCs can overcome these problems. DC are located in most tissues were they capture and process antigens. They display a large amount of MHC-peptide complexes and upon induction they upregulate their costimulatory molecules and migrate to lymphoid organs where they liaise with and activate antigen specific T cells. All these DC activities can be induced by infectious agents and inflammatory products, so that DCs are mobile sentinels that bring antigens to T cells and express costimulators for the induction of immunity.

2.6.1 Immature, antigen capturing dendritic cells

In most tissues DCs are present in an immature state, unable to stimulate T cells. However, they are extremely well equipped to capture antigens, that in turn are able to induce full maturation and mobilization of the DCs. Humans have about 10^9 epidermal LCs, the immature DCs of the skin that are located above the basal level of proliferating keratinocytes. Freshly isolated LCs are week T cell stimulators, they have few MHC- and accessory molecules, but many antigen-capturing Fcγ and Fcε receptors. This phenotype changes dramatically within a day of culture. The cells undergo extensive transformation, antigen capturing devices disappear while T cell stimulatory functions increase. When these cells encounter a powerful immunological stimulus *in vivo*, most of the LCs from the epidermis mature and move into dermal lymphatics in search for antigen-specific T cells.

Small numbers of antigen-capturing DCs can also be isolated from blood, lung, spleen, heart, kidney, and the B- and T cell areas of tonsils; these cells lack LC specific markers (E-cadherin, Birbeck granules, Lag-1) but they also acquire accessory molecules within 1-2 days of culture, before they encounter with T cells.

Immature DCs have several features that allow them to capture antigen. First, they can take up particles and microbes by phagocytosis (141-144). Second, they can form large pinocytic vesicles in which extracellular fluid and solutes are sampled, a process called macropinocytosis (145). And third, they express receptors that mediate adsorptive endocytosis, including C-type lectin receptors like the macrophage mannose receptor (145), DEC205 (146), as well as Fcγ and Fcε receptors (147). Picomolar and nanomolar antigen concentrations are sufficient to activate maturation of DCs after macropinocytosis and receptor mediated antigen uptake (145). The antigen enters the endocytic pathway of the cells. During one single stage of their life DCs are able to produce large amounts of MHC class II peptide complexes in specialized, MHC class II rich compartments (MIICs) that are abundant in immature DCs (147-150). MIICs are late-endosomal structures that contain the HLA-DM or H-2M products, which enhance and edit peptide binding to MHC class II molecules. During maturation of DCs, MIICs convert to non-lysosomal vesicles that discharge their MHC-peptide complex to the cell surface (150, 151), where they remain stable for days (150, 151) and activate MHC class II restricted T cells.

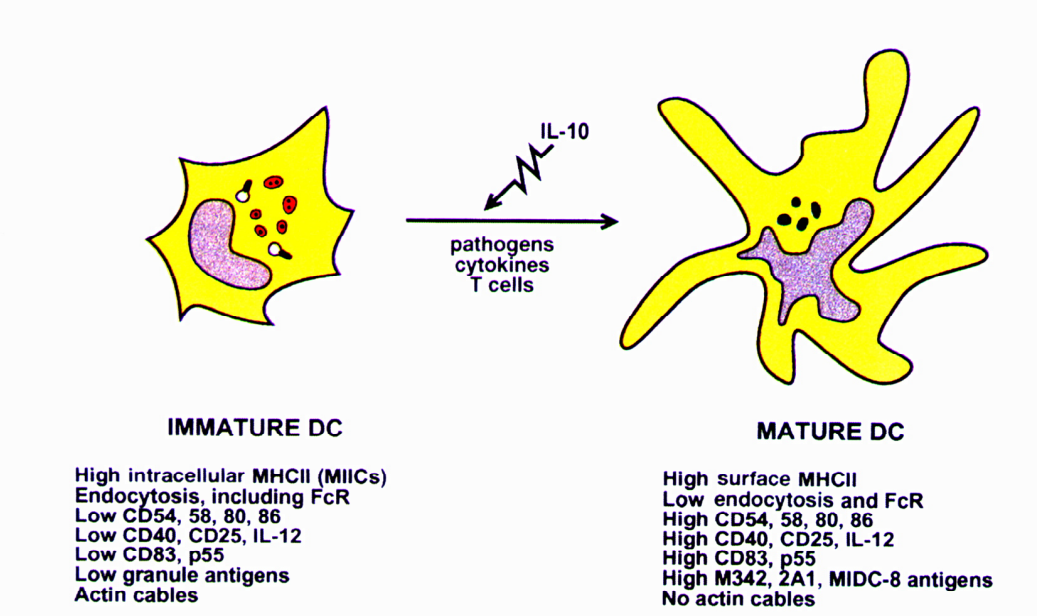

Fig. 2.6 Features that change during DC maturation. Immature DCs take up antigen by phagocytosis, macropinocytosis, or adsorptive pinocytosis. An example of a pathogenic molecule that will induce maturation is lipopolysaccharide (LPS); TNF-α and GM-CSF are examples of cytokines, and CD40L is an example of a T cell ligand that binds CD40 on DCs. IL-10 can inhibit maturation.

DCs have to present antigenic peptides complexed to MHC class I molecules to generate cytotoxic killer cells, that have the capacity to lyse infected cells and attack transplants and tumor cells. In virus infected DCs intracellular viral proteins are degraded into peptides by the proteasome. A dedicated peptide transporter translocates these peptides from the cytosol to the endoplasmatic reticulum, where they bind to MHC class I molecules. The MHC class I complexes travel to the cell surface where they are displayed for scrutiny by T cells. It is less clear, however, how DCs can process and present antigens that have no access to the cytosol in a MHC class I restricted manner (e.g. transplant or tumor-derived antigens). DCs may be able to process dying cells and cross-prime T cells to another cell's antigens or selfproteins.

Upon activation, DCs travel to the lymphoid tissue such as spleen and lymph nodes. There, DCs may complete their maturation (152), attract T- and B cells by releasing chemokines (153) and maintain the viability of recirculating T lymphocytes (154). The maturation of DCs is influenced by a variety of factors. Whole bacteria (148), the microbial cell wall component LPS (145), and cytokines like IL-1, GM-CSF, and TNF-α, all stimulate DC maturation. Ceramide, which is induced by maturation signals, can shut down antigen capture by DCs (155). Mature DCs express high levels of the NF-κB family of transcriptional control proteins (Rel A/p65, Rel B, Rel C, p50, p52)(156), which regulate the expression of many genes encoding immune and inflammatory proteins. How dendritic cells know where to go is largely unknown. LPS (157) stimulates a variety of cells to produce cytokines and chemokines, for example GM-CSF, TNF-α, IL-1, MIP-1α and β. These products are known to modulate DC movement and maturation. Damage to cytokine rich cells, such as keratinocytes or mast cells could result in the release of preformed mediators like GM-CSF, TNF-α, and IL-4. The current focus, however, is on seven-transmembrane spanning G-protein coupled receptors that may mediate many of the steps required for the migration and targeting of DCs (158, 159): egress from a tissue, directed movement or chemotaxis, and maintenance of viability.

2.6.2 Mature, antigen presenting dendritic cells

Terminally differentiated or mature dendritic cells are strong activators of T cells that complete the immune response by interacting with B cells for antibody formation, with macrophages for cytokine release and with targets for lysis. Immature DCs, on the other hand, are less potent initiators of the immunity but specialize in capturing and processing antigens to form MHC complexes. *In vitro* and *in vivo* only few DCs are necessary to provoke a strong T cell response. *In vitro*, only one DC is necessary to turn on 100-3000 T cells in a mixed lymphocyte reaction. But DCs prime T cells not only to mismatched MHC but also to a range of foreign proteins; from superantigens, the microbial proteins that bind directly to MHC molecules without prior processing (160), to proteins that do require processing, such as those from infectious agents (161, 162) and tumors (163-166). *In vivo* immunity develops in lymphoid organs, where DC-T cell interactions can be seen for all major classes of T cell ligands. DCs form clusters with antigen-specific T cells, creating a microenvironment in which immunity can develop (167-169). The special effects of DCs seem to be related to quantitative and regulative aspects. MHC products and MHC peptide complexes are 10-100 times higher on DCs than on other APCs like B cells and monocytes (170). Mature DCs resist the immunosuppressive effects of IL-10, but synthesize high levels of IL-12 that enhance both innate (natural killer cells) and acquired (B- and T cells) immunity (171, 172). DCs also express many accessory molecules that interact with receptors on T cells to enhancer adhesion and signaling (173, 174), for example LFA-3/CD58, ICAM-1/CD54, B7-2/CD58. Depending on the conditions, DCs can stimulate the outgrowth and activation of a variety of T cells. They can induce vigorous proliferation of $CD8^+$ CTLs that is unusual for this cells (175, 176). CD4-expressing T helper cells, on the other hand, scrutinize cells that express MHC class II molecules. In the presence of mature DCs and of the IL-2 they produce (171, 172, 177), these T cells turn into interferon-γ producing Th_1 cells. IFN-γ activates macrophages and together with IL-12 it promotes the differentiation of T cells into killer cells. With IL-4, however, DCs induce T cells to differentiate into Th_2 cells that secrete IL-5 and IL-4. These cytokines activate eosinophils and help B cells to make the appropriate antibodies.
The communication between DCs and T cells seems to be a dialog in which DCs respond to T cells as well. CD40 (178) and the TRANCE/RANK receptor (179, 180) on DCs are ligated by the TNF protein family, expressed on activated and memory T cells. This leads to increased DC survival (178, 179) and, in case of CD40, to an up-regulation of CD80 and CD86 (178), secretion of IL-12 (171, 177), and release of chemokines, such as IL-8, MIP-1α and β (178).

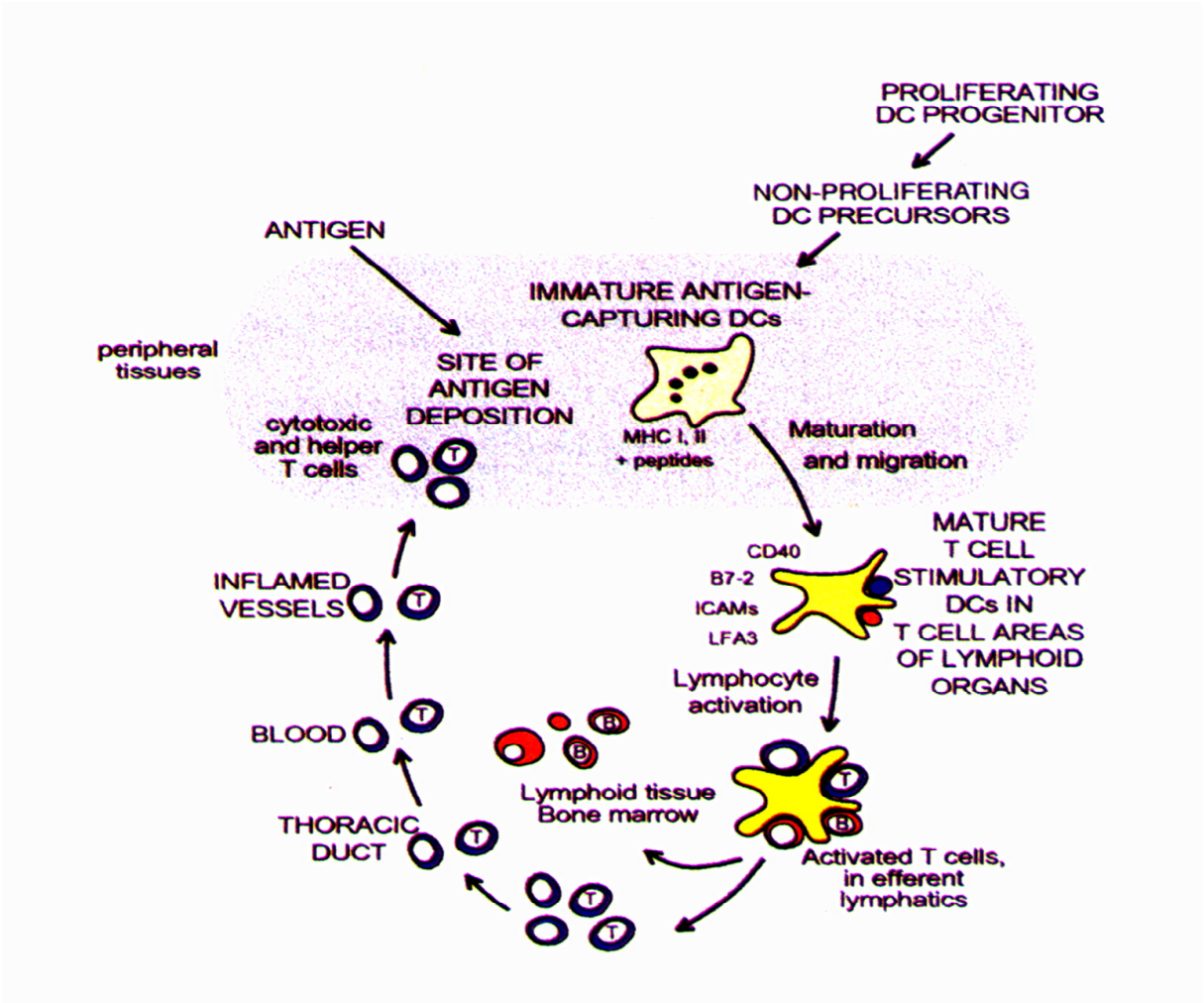

Fig. 2.7 Antigens are captured by DCs in peripheral tissues and processed to form MHC-peptide complexes. These immature DCs derive successively from proliferating progenitors and non-proliferating precursors, the latter not being fully committed to form DCs. As a consequence of antigen deposition and inflammation, DCs begin to mature, expressing molecules that will lead to binding and stimulation of T cells in the T cell areas of lymphoid tissue. After activation, T and B blasts leave the T cell area. B cells move to other parts of the lymphoid tissue with some becoming antibody secreting plasma cells. T blasts leave the blood vessels at the original site of antigen deposition, recognizing changes in the inflamed blood vessels and respond vigorously to cells presenting the antigen.

2.6.3 Interaction of DCs with B cells

DCs are now known to have major effects on B cell growth and immunoglobulin secretion. B cells and DCs are both APCs and both are essential for antibody responses (181). DCs activate and expand T helper cells, which in turn induce B cell growth and antibody production. Naive B cells respond uniquely to the interstitial, non-LC type of DCs (182, 183), and by secretion of soluble factors (183), including IL-12, DCs stimulate the production of antibodies directly and the proliferation of B cells that have been stimulated by CD40L on activated T cells. DCs also orchestrate immunoglobulin class switches of T cell activated B cells: IL-10 and TGF-β can induce secretion of IgA_1, but expression of IgA_2 appears to be strictly dependent on a direct interaction between B cell and the DC (184). Follicular dendritic cells (FDCs) directly sustain the viability, growth, and differentiation of activated B cells. FDCs differ from ordinary DCs-they are not bone marrow derived (185), they lack the leukocyte marker CD45, and they display a unique set of molecules at their surface (186), including all known complement receptors (187). With their

receptors for complement, FDCs capture antigen-antibody complexes and display whole complexes, rather than processed antigens, at their surface for long periods. FDCs are abundantly present within antigen-stimulated B cell areas, or germinal centers. There, proliferating B cells undergo somatic mutation, after which they stop dividing and wait to be triggered by an immune complex by FDCs. B cells that recognize an immune complex with high affinity process the antigen and present it as peptide-MHC complexes to antigen specific T cells. The B and T cell interaction ensures the survival of these high affinity B cells, while the non-stimulated low-affinity B cells apoptose and are phagocytosed by tangible body macrophages.

2.6.4 Dendritic cells in clinical immunology and tumor resistance

Given their central role in controlling immunity, DCs are logical targets for many clinical situations that involve T cells: transplantation, allergy, autoimmune disease, resistance to infection and tumors, and vaccines. The presence of functional DCs in tumors greatly influences the prognosis of cancer patients. On the other hand, many tumor components do not elicit an antigen-specific T cell response in patients, which may be due to the absence of functional DCs in tumors. DCs that infiltrate colon and basal-cell skin cancers can lack CD80 and CD86 (188) and therefore have reduced T cell stimulatory activity. Likewise, tumors may secrete factors such as IL-10, TGF-β, and VEGF, that reduces DC development and function. A lot of tumors are infiltrated by tumor reactive T cells, especially CTLs, but there is little evidence that these T cells are being activated *in vivo.* However, when tumor antigens are applied to DCs *ex vivo* and these DCs are then reinfused, specific immunity is developed. In animals this strategy can lead to the protection against tumors and even a reduction in the size of established tumors (189-191). At present similar experiments are carried out in patients.

Many different strategies have been developed to deliver tumor antigens to DCs. In mice, protective immune responses against tumors can be induced after immunization of the animal with DC pulsed with defined synthetic peptides (192), acid eluted peptides from tumor derived cell lines (193), intact soluble tumor proteins (194) or transfected with tumor derived genes (195, 196). Interestingly, DCs appear to have a direct lytic potential on certain tumor targets as well.

In the last few years the DC system is increasingly used for vaccine design. Many existing vaccines and adjuvants are week stimulators of $CD8^+$ T cells and Th_1 type T cells, whereas DCs can readily elicit helper and killer T cells, antibodies and cytokines like IL-12 and IFN-γ. Specific immune responses can be activated or boosted by *ex vivo* transfection of DCs with DNA-vaccines coding for tumor antigens and their transplantation into the tumor-bearing host. Gilboa's team demonstrated that transfected dendritic cells could be transferred back to animals, engendering protective immunity in mice with tumors (197). The ability of transfected DCs to stimulate CTLs *in vitro* extends to human cells. DCs generated from the peripheral blood mononuclear cells of healthy individuals or from cancer patients transfected with carcinoembryonic antigen (CEA) mRNA stimulate a potent $CD8^+$ cytotoxic T lymphocyte response *in vitro* (198).

2.6.5 Effective tumor vaccines can be generated by fusion of professional antigen presenting cells and tumor cells

Tumor cells may escape immune surveillance because they do not express signals that are essential for activation of the host immune system (199, 200). At the molecular level, the defective signaling of tumor cells could attribute to downregulation of major histocompatibility complex molecules (201, 202), alteration of antigen-processing pathways resulting in an inability to present tumor-specific antigens to host T cells (203), absence of costimulatory or adhesion molecules that are essential for activation of the host

immune system (204), or production of factors that modify host immune responses (205). In contrast, dendritic cells and activated B cells (206, 207) are one of the most effective antigen presenting cells. A fusion of a tumor cell with a dendritic cell will produce a hybrid cell that both expresses tumor specific antigens and has the machinery for antigen presentation and T cell activation. Fusion of bone marrow derived dendritic cells with the carcinoma cell line MC38 produced hybrid cells that lost their tumorigenity and became immunogenic. Most of the fusion cells exhibited dendritic morphology and stimulated naive T cells in the primary mixed lymphocyte reaction and induced MC38 tumor specific CTLs *in vivo* (208). Activation of $CD4^+$ and $CD8^+$ lymphocytes protected against challenge with primary tumor cells and induced rejection of pre-established metastases. Fusion hybrids of BERH-2 rat hepatocellular carcinoma cells and activated B cells also protected rats from growth of the parental BERH-2 cells and rats with established hepatomas were cured by subsequent injection of BERH-2 / B cell hybrid cells (209). Both $CD4^+$ and $CD8^+$ lymphocytes have been shown to be essential for the induction of protective immunity. These experiments show that professional antigen presenting cells can be fused with tumor cells and that vaccination with these fusion cells is an effective approach for cancer immunotherapy.

III. ABBREVIATIONS

ADCC	antibody-dependent cell mediated cytotoxicity
APC	antigen presenting cell
ATP	adenosine triphosphate
bp	base pairs
BSA	bovine serum albumin
C D	cluster of differentiation
CDK	cyclin dependent kinase
cDNA	complementary DNA
C H	contact hypersensitivity
CIP	calf intestinal phosphatase
CMV	cytomegalovirus
cpm	counts per minute
CTL	cytotoxic T lymphocyte
D C	dendritic cell
DNA	desoxynucleic acid
DTT	dithiothreitol
ECL	enhanced chemiluminescence
E. coli	*Escherichia coli*
EDTA	ethylendiamine tetraacetate
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FDC	follicular dendritic cell
FITC	fluorescein isothiocyanate conjugated
GM-CSF	granulocyte-macrophage colony stimulating factor
HA	hemagglutinin
HBsAg	hepatitis B surface antigen
HNPCC	hereditary monopolyposis colorectal cancer
HIV	human immunodeficiency virus

HLA	human leukocyte antigen
HRP	horseradish peroxidase
hsp	heat shock protein
hr	hour
IFN-γ	interferon-γ
Ig	immunoglobulin
IL	interleukin
LC	Langerhans cells
MACS	magnetic cell sorting
MIIC	major histocompatibility complex class II rich compartments
MHC	major histocompatibility complex
MLR	mixed lymphocyte reaction
MMLV	Moloney Murine Leukemia virus
NK cells	natural killer cells
NP	nucleoprotein
0 D	optical density
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
pDNA	plasmid DNA
PE	phycoerythrin
PEI	polyethylenimine
PHA	phytohemagglutinine
SDS	sodium dodecyl sulfate
TATA	tumor-associated transplantation antigen
TBP	TATA-binding protein
Th1/2	T helper lymphocyte class 1/2
TCR	T cell receptor
TIL	tumor infiltrating lymphocyte

TNF	tumor necrosis factor
TSTA	tumor-specific transplantation antigen

IV. MATERIAL AND METHODS

4.1 Materials

4.1.1 Chemicals and disposable materials

Enzymes and buffers for molecular biology were ordered from New England Biolabs (Schwalbach) or Boehringer Mannheim (Mannheim). SDS-PAGE molecular weight standards and the DNA molecular weight markers were delivered by Biorad (München) and Gibco BRL (Eggenstein).

Chemicals were delivered by Roth (Karlsruhe), Merk (Darmstadt), Fluka (Neu-Ulm), Sigma (Deisenhofen), and Serva (Heidelberg). Supplements for bacterial growth media were ordered from Difco (Augsburg).

The quantification of proteins has been performed with the Bio-Rad Protein Assay Kit, Biorad (München). Affinity chromatography columns for protein purification were ordered from Pharmacia Biotech (Freiburg).

Disposable plastic materials, filters, and Whatman 3MM paper were ordered from Eppendorf (Hamburg), Greiner (Frickenhausen), Schleicher & Schüll (Dassel), Becton Dickinson (Heidelberg), and Nalgene (Rochester, NY, U.S.A.). FACS FLOW ™ fluid for flow cytometry was obtained from Becton Dickinson (Heidelberg).

Liquid cell culture medium, glutamine, Trypsin/EDTA and fetal bovine serum (FCS) were delivered by Biowhittaker (Heidelberg). FCS was heat-inactivated for 30 minutes at 56°C before use. Antibiotics were obtained from Biowhittaker (Heidelberg), Boehringer Mannheim (Mannheim), Invitrogen (Groningen, Netherlands), or Sigma (Deisenhofen).

4.1.2 Antibodies

Antibodies were used for immunoblotting, magnetic cell sorting (MACS) and flow cytometry:

The purified mouse monoclonal antibody IgG_{2a}, clone **PAb 421** (Dianova, Hamburg, Germany) binds to amino acids 371-380 of human and mouse p53. For immunoblotting the antibody has been used in a dilution of 1:2500, for flow cytometric analysis in a dilution of 1:50.

CD11c (N418) MicroBeads (MiltenyiBiotech, Bergisch Gladbach, Germany) were developed for the isolation of dendritic cells from lymphoid tissue. The CD11c antibody clone N418 is specific for the integrin ax subunit of the leukocyte integrin expressed axb_2 on mouse spleenic cells (210, 211). CD11c is also reported to be weakly expressed on NK cells, B- and T cell subsets and myeloid cells of mouse bone marrow (211, 212). 1 ml CD11c MicroBeads was added to 10^9 total cells. PE-labeled anti-CD11c antibodies (N418) were obtained from MiltenyiBiotec and used in flow cytometry at a dilution of 1:10.

MACS **CD90 (Thy 1.2) MicroBeads** (MiltenyiBiotech, Bergisch Gladbach, Germany) were developed for positive selection of mouse $CD90^+$ T cells from lymphoid tissue or peripheral blood (213, 214). CD90 is expressed on thymocytes, peripheral T cells and some interepithelial T cells of most mouse strains (215, 216). 1 ml CD90 MicroBeads was added to 10^9 total cells.

The FITC-conjugated **16-10A1** $IgG_{2,\kappa}$ (Pharmingen, Hamburg, Germany) antibody reacts with CD80 (B7-1) (217). CD80 is constitutively expressed on dendritic cells, monocytes,

and peritoneal macrophages or can be induced on B cells. For flow cytometry a dilution of 1:50 has been used.

The FITC-conjugated $IgG_{2a,\kappa}$ antibody **GL1** (Pharmingen, Hamburg, Germany) reacts with the B7-2 costimulatory molecule expressed on B cells, T cells, macrophages and T cells. For flow cytometric analysis a dilution of 1:50 has been used.

The FITC-labeled mouse $IgG_{2a,\kappa}$ antibody clone **SF1-1.1** (Pharmingen, Hamburg, Germany) reacts with the α3 domain of the H-$2K^d$ MHC class I alloantigen (218, 219). For flow cytometric analysis a dilution of 1:50 has been used.

The FITC-labeled mouse $IgG_{2a,\kappa}$ antibody clone **34-2-12** (Pharmingen, Hamburg, Germany) reacts with the H-$2D^d$ MHC class I antigen (220). For flow cytometric analysis a dilution of 1:50 has been used.

Immunoblotting with HRP-conjugated secondary antibodies has been performed with the ECL Western Blotting Analysis System from Amersham (Braunschweig, Germany). For flow cytometric analysis an FITC-conjugated secondary anti-IgG antibody has been used (Dianova, Hamburg, Germany).

4.1.3 Reagents and solutions

PBS	137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4
TPBS	0.05% Tween 20 in PBS
HBS	25 mM HEPES, 140 mM NaCl, 750 mM Na_2HPO4, pH 6.95
TAE	40 mM Tris pH 8.0, 2 mM EDTA
TBE	89 mM Tris, 89 mM borate, 1 µM EDTA, pH 8.2
SOB-medium	20 g tryptone, 5 g yeast extract, 0.58 g NaCl, 0.186 g KCl per liter H_2O, pH 6.8-7.0
LB-medium	10 g tryptone, 5 g yeast extract, 10 g NaCl, per liter H_2O, pH 7.2-7.4
10 x DNA loading buffer	20 mM Tris/HCl, 40 mM EDTA, 0.15% agarose, 20% glycerol, pH 8.0
7 M urea / 8% acrylamide stock solution	140 ml 37% acrylamide, 60 ml 10 x TBE, 252 g urea, fill up to 600 ml
Ripa-buffer	120 mM NaCl, 50mM Tris pH 8.0, 1% Triton X-100, 0.5% desoxycholate, 0.1% SDS
SDS-gel electrophoresis buffer	250 mM glycine, 0.1% SDS, 25 mM Tris
staining solution for polyacrylamide gels	30% methanol, 10% acetic acid, 0.25% Coomassie Brilliant Blue R 250

destaining solution for polyacrylamide gels	30% methanol, 10% acetic acid

Additional buffers and solutions are mentioned in the text.

4.1.4 Plasmids

pZeoSV	The pZeoSV plasmid is a constitutive mammalian expression vector containing a gene that confers resistance to zeocin. Expression is directed by an SV40 enhancer-promoter, efficient processing is ensured by an SV40 poly A signal (Invitrogen, Groningen, The Netherlands).
pZeoSV-LacZ	pZeoSV plasmid vector incorporating the LacZ reporter gene for constitutive mammalian expression (Invitrogen, Groningen, The Netherlands).
pZeo-luc	pZeoSV plasmid vector incorporating the firefly luciferase reporter gene for constitutive mammalian expression (this work).
pZeoSV-A1	pZeoSV plasmid vector incorporating the mutant p53 tumor suppressor gene isolated from the tumor cell line Meth A (this work). The p53 gene is mutated at position 234.
pZeoSV-A2	pZeoSV plasmid vector incorporating the second allele of mutant p53 isolated from the tumor cell line Meth A (this work). The p53 gene is mutated at position 132 and 164.
pCMX	The pCMX plasmid is a constitutive mammalian expression vector containing a gene that confers resistance to ampicillin. Eukaryotic expression of the luciferase gene is under control of the cytomegalo-virus (CMV) immediate early promoter, efficient processing is ensured by a SV40 poly A signal (gift from Roland Schüle, Tumor Biology Center, Freiburg, Germany).
pCMX-luc	pCMX plasmid vector incorporating the firefly luciferase reporter gene for constitutive mammalian expression (this work).
pCMX-A1	pCMX plasmid vector incorporating the mutant p53 tumor suppressor gene isolated from the tumor cell line Meth A (this work). The p53 gene is mutated at position 234.
pCMX-A2	pCMX plasmid vector incorporating the second allele of mutant p53 isolated from the tumor cell line Meth A (this work). The p53 gene is mutated at position 132 and 164.
pBABEpuro/ pBABEhygro	pBABE is a replication incompetent retroviral vector retaining the cis-acting viral sequences necessary for transduction. These sequences include the ψ packaging sequence, reverse transcription signals, integration signals, viral LTR promoter, enhancer and polyadenylation signal (gift from Dr. Barbara Schnierle, Tumor Biology Center, Freiburg, Germany). Selection of transfected cells by puromycin/hygromycin.
pBABEpuro A1	pBABEpuro retroviral vector incorporating the mutant p53 tumor suppressor gene isolated from the tumor cell line Meth A (this work). The p53 gene is mutated at position 234.

pBABEpuro A2	pBABEpuro retroviral vector incorporating the second allele of mutant p53 isolated from the tumor cell line Meth A (this work). The p53 gene is mutated at position 132 and 164.
pRSET	pRSET vectors are pUC-based vectors designed for high level fusion protein production in *E. coli* when the plasmid is grown in the presence of T7 polymerase. The polyhistidine metal chelating domain of the fusion peptide allows purification of recombinant proteins by immobilized metal affinity chromatography (Invitrogen, Groningen, The Netherlands).
pRSET-A1	pRSET vector incorporating the second allele of mutant p53 isolated from the tumor cell line Meth A (this work). The p53 gene is mutated at position 234.
pRSET-A2	pRSET vector incorporating the second allele of mutant p53 isolated from the tumor cell line Meth A (this work). The p53 gene is mutated at position 132 and 164.

4.1.5 Oligonucleotide primers

Not I-d(T)18 bifunctional primer	5'-d(AAC TGG AGG AAT TCG CGG CCG CAG GAA-T_{18})-3'
mutant p53 forward primer	5'-d(TCC GAA GCT TGG ATG ACT GC)-3'
mutant p53 reverse primer	5'-d(GCA GAG GAA TTC AGT CTG AGT CAG)-3'

4.1.6 Bacteria and growth conditions

bacteria	genotype	references
XL-1 blue	*sup*E44, *hsd*R17, *rec*A1, *end*A1, *gyr*A96, *thi, rel*A1, lac^-, F'(*tra*D36, *pro*AB^+, *lac*Iq, *lac*ZDM15)	(221)
BL21(DE3)LysS	B, F^-, *dcm*, *omp*T, *hsds*(rB^-, mB^-), *gal*, λ(DE3)	(222)

Abbreviations of the genotypes are listed corresponding to the nomenclature of B. Bachmann (223).
Transformed bacteria were cultured at 37°C with moderate shaking in LB-medium (224), supplemented with 100 μg/ml ampicillin or 50 μg/ml zeocin. Glycerol stocks were stored at -80°C in LB-medium, 50% glycerol.

4.2 Methods

4.2.1 Preparation of competent bacteria

RF1-buffer 150 mM $RbCl_2$, 50 mM $MnCl_2$, 30 mM CoAc, 10 mM $CaCl_2$, 13% glycerol, pH 5.8, sterilized by filtration

RF2-buffer 10 mM MOPS, 10 mM $RbCl_2$, 75 mM $CaCl_2$, 13% glycerol, pH 7.0, sterilized by filtration

A high transformation efficiency of *E.coli* cells can be achieved by pretreatment of the cells (225) and was performed according to the method of Hanahan (226). Briefly, an *E. coli* over night culture was diluted 1:100 in 500 ml LB medium and grown to log phase (OD_{550nm} = 0.48-0.5) with moderate shaking (250 rpm, 37°C). Cells were concentrated by centrifugation at 2200 rpm, 4°C for 10 min. (JA 10 rotor), resuspended in 166 ml ice-cold RF1 buffer, and incubated on ice for 2 hrs. After centrifugation the cells were resuspended in 12.5 ml RF-2 buffer, dispensed in 200 µl aliquots into prechilled, sterile polypropylene tubes, and frozen immediately in fluid nitrogen. Competent cells were stored at -80°C.

4.2.2 Transformation of competent bacteria

Competent cells were thawed on ice and used immediately. 200 µl of bacteria were mixed with 1 µl plasmid DNA or 20 µl of a ligation mixture and kept on ice for 60 min.. The mixture of cells and DNA was heat-shocked for 90 sec. at 42°C and than grown in 1 ml LB medium at 37°C with moderate shaking. Transformed bacteria were plateled on selective LB agar plates. Colonies were grown at low density, otherwise the secreted β–lactamase lowers the ampicillin level on the plate and permits growth of satellite colonies.

4.2.3 Isolation of plasmid DNA from *E. coli*

4.2.3.1 Small scale purification of plasmid DNA

Resuspension buffer	25 mM TrisHCl, pH 7.5, 1 mM EDTA
Cell lysis buffer	0.2 M NaOH, 1% SDS
Neutralization buffer	2.55 M KAc., pH 4.8

The pellet of a single colony, grown over night in selective medium (5 ml, 37°C, moderate shaking), was resuspended in 200 µl resuspension buffer. In the alkaline lysis procedure (227) the bacteria were lysed on ice for 5 min. in 200 µl cell lysis buffer containing SDS and NaOH. SDS bound proteins and the associated chromosomal DNA were precipitated after neutralization with 200 µl neutralization buffer (20000 g, 5 min., 4°C). The plasmid DNA was recovered from the supernatant, transferred into a fresh tube containing 2.5 volumes of 100% ethanol and 1/10 volume of 7.5 M ammonium acetate and precipitated on ice for 30 min.. After centrifugation (20000 g, 10 min., 4°C) the pellet, containing supercoiled plasmid DNA, was washed twice with 2 ml of 70% ethanol, dried in a speed vac., and resuspended in 100 µl water.

4.2.3.2 Large scale preparation of plasmid DNA

The QUIAGEN plasmid purification kit is based on the optimized alkaline lysis method of Birnboin and Doly (227). The procedure has been condensed to three steps and combined

with the Quiagen column to obtain plasmid DNA. The Maxiprep (Gigaprep) Plasmid Extraction Kit has been used according to the manufacturer's instructions.

Buffer P1	100 mg/ml RNase A, 50 mM Tris/HCl, 10 mM EDTA, pH 8.0
Buffer P2	200 mM NaOH, 1% SDS
Buffer P3	3 M KAc, pH 5.5
QBT equilibration buffer	750 mM NaCl, 50 mM MOPS, 15% ethanol, pH 7.0, 0.15% Triton X-100
QC washing buffer	1 M NaCl, 50 mM Tris/HCl, 15% ethanol, pH 7.0
QF elution buffer	1.25 M NaCl, 50 mM Tris/HCl, 15% ethanol, pH 8.5
QUIAGEN columns	Quiagen, Hilden, Germany

For plasmid preparation 500 ml (2.5 l) of a single colony culture was grown over night to saturation. The bacteria were harvested by centrifugation (JA 10 rotor, 5000 rpm, 10 min.), resuspended in 10 ml (125 ml) P1 buffer, lysed with 10 ml (125 ml) P2 buffer, and incubated for 5 min. at room temperature. The lysate was neutralized by addition of 10 ml (125 ml) buffer P3 and incubated on ice for 20 min.. The precipitated debris was removed by high speed centrifugation (JA 20 rotor, 15000 rpm, 30 min.), leaving a cleared lysate for loading onto the QUIAGEN tip that was pre-equilibrated with 15 ml (75 ml) QBT buffer. The loaded QUIAGEN tip was washed twice with 30 ml (2 x 300 ml) QC washing buffer and the plasmid DNA was eluted from the resin with 15 ml (75 ml) elution buffer QF. The eluted DNA was desalted and precipitated at room temperature by addition of 10.5 ml (52.5 ml) isopropanol. The solution was centrifuged (JA 20 rotor, 10000 rpm, 30 min.), washed in 70% ethanol, dried and resuspended in 300 µl (1.5 ml) aqua bidest.

4.2.4 Storage of plasmid DNA

Bacteria, containing plasmids can be stored for a short time period by growing them on selective plates at 4°C. For long term storage bacteria were grown to saturation, mixed 1:1 with glycerol (60% in LB medium) and frozen at -80°C in sterile vials. Purified plasmid DNA can be stored in aqua bidest at 4°C for several weeks or be preserved for several years at -20°C.

4.2.5 Molecular cloning

4.2.5.1 Agarose gel electrophoresis

TAE electrophoresis buffer
Electrophoresis grade agarose (BRL, Gaithersburg, U.S.A.)
10 x DNA loading buffer
10% ethidium bromide solution (Merck, Darmstadt, Germany)
Horizontal gel electrophoresis apparatus (Horizon™ 58, BRL, Gaithersburg, U.S.A.)

Agarose gel electrophoresis (228) can be used for separating, identifying and purifying 0.5 to 25 kb DNA fragments. First, an agarose-TAE buffer mixture (Table 4.1) was melted in a microwave, poured in a casting form and covered by TAE electrophoresis buffer. The DNA samples, mixed with 0.1 volumes of 10 x DNA loading buffer were loaded into the sample wells. Electrophoresis was performed at 30 to 60 V. DNA molecules exposed to the electric field migrate to the anode due to the negatively charged phosphates along the DNA backbone. Separation of the fragments was monitored by the migration of the dyes in the loading buffer. The DNA was visualized directly upon illumination with UV-light after ethidium bromide incorporation.

Table 4.1 Appropriate agarose concentrations for separating DNA fragments of various sizes

0.5%	1-30 kb
0.7%	0.8-12 kb
1%	0.5-10 kb
1.2%	0.4-7 kb
1.5%	0.2-3 kb

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4.2.5.2 Digestion of DNA with restriction endonucleases

Restriction endonuclease cleavage has been performed according to the manufacturer's recommendations. Briefly, 1 µg of plasmid DNA was cleaved by 5 U of a specific restriction endonuclease, usually at 37°C. Endonuclease restriction buffers were purchased along with the enzymes. The reaction was stopped after 1-3 hours by addition of 10 x loading buffer and prepared for agarose gel electrophoresis. DNA samples, cleaved with more than one enzyme were diluted in a buffer in which all enzymes retained activity. However, if the reaction conditions were not compatible, DNA-fragments were purified by agarose gel electrophoresis after each restriction endonuclease cleavage.

4.2.5.3 Recovery of DNA fragments

QX1 solubilization buffer (QUIAGEN, Hilden, Germany)
PE wash buffer (QUIAGEN, Hilden, Germany)
QIAquick spin column (QUIAGEN, Hilden, Germany)

The isolation of DNA fragments from agarose gels has been performed by the QIAquick Gel Extraction Kit according to the manufacturer's description. DNA fragments were excised from the gel and solubilized in 3 µl QX1 buffer per mg gel at 50°C in a heat block. The samples were applied to QIAquick spin columns and centrifuged for 60 sec. at 20000 g. The columns were washed 3 times with 750 µl buffer PE and dried by an additional spin to remove residual ethanol. Bound DNA was eluted in 50 µl of aqua bidest (50°C).

4.2.5.4 Ligation of DNA fragments

10 x T4 DNA ligase reaction buffer (Boehringer Mannheim, Germany)
T4 DNA ligase (Boehringer Mannheim, Germany)

Ligation of a segment of foreign DNA to a linearized plasmid vector involves the formation of phosphodiester bonds between juxtaposed 5' phosphate residues and 3'-hydroxyl termini in duplex DNA. This process is catalyzed by the T4 DNA ligase. For ligation insert DNA and vector DNA have been used in a molar ratio of 5:1 in the presence of 1 U T4 DNA ligase. Ligation of cohesive ends was carried out at 12 to 16°C over night to maintain a good balance between annealing of the ends and activity of the enzyme. The ligated products were introduced into competent *E. coli* and selected for transformants.

4.2.6 Photometric determination of the concentration and the purity of DNA

The concentration and purity of nucleic acids can be determined by measurement of the extinction at 260 nm and 280 nm (224). Spectrophotometric measurement of the amount of

ultraviolet irradiation absorbed at 260 nm by the bases allows the calculation of the concentration of nucleic acids in the sample. An OD_{260} of 1 corresponds to approximately:

50 µg double-stranded DNA
40 µg single-stranded DNA
20 µg oligonucleotides

The ratio between the readings at 260 nm and 280 nm provides an estimate of the purity of the nucleic acid solution. Aromatic compounds like the amino acids tyrosine, tryptophane, and phenylalanine absorb more light at 280 nm than DNA and the quotient will be significantly less than 1.8. The quantity of DNA and its physical state can also be analyzed by agarose gel electrophoresis and comparison of the fluorescence of the sample with that of a series of standards with a known concentration.

4.2.7 Synthesis of oligonucleotides

All oligonucleotides were ordered from Birsner & Grob Biotech GmbH, Denzlingen, Germany. Synthesis has been performed by the phosphoamidite method in an automated system using the Model 392 synthesizer. The HPLC-grade oligonucleotides were shipped in 500 µl 10 mM Tris/HCl, pH 8.0.

4.2.8 Isolation of total RNA from mammalian cells

RTL lysis buffer (QIAGEN, Hilden, Germany)
RW1 wash buffer (QUIAGEN, Hilden, Germany)
RPE wash buffer (QUIAGEN, Hilden, Germany)
RNeasy spin columns (QIAGEN, Hilden, Germany)

The isolation of RNA has been performed with the RNeasy™ Total RNA Kit according to the manufacturer's description. Briefly, 1 x 10^7 cells were washed with PBS, detached from the dish by a disposable cell scraper and pelleted by centrifugation at 100 g for 5 min.. After discharging the supernatant cells were lysed by 400 µl RTL lysis buffer and centrifuged for 3 min. at 20000 g. The supernatant was transferred to a different vessel, mixed with 1 volume of 70% ethanol, and applied onto RNeasy spin columns. The columns were washed once with 700 µl wash buffer RW1, then twice with 500 µl wash buffer RPE, before the column was dried by centrifugation (20000 g, 2 min.). The RNA was eluted in 50 µl DEPC-treated aqua bidest (50°C).

4.2.9 cDNA synthesis

Bulk First-Strand cDNA Reaction Mix (Pharmacia Biotech, Freiburg, Germany)
200 mM DTT
Not I-d(T)18 bifunctional primer (5 µg/µl), (Pharmacia Biotech, Freiburg, Germany)

cDNA synthesis was performed according to the manufacturer's description by the First-Strand cDNA Synthesis Kit (Pharmacia Biotech). First strand cDNA synthesis was catalyzed by the Moloney Murine Leukemia virus reverse transcriptase. 11 µl of the preassembled Bulk First-Strand cDNA Reaction Mix were mixed with 1 µl DTT, 1 µl primer and 20 µl (5 µg) of total RNA. The first strand reaction was primed with the Not I-$d(T)_{18}$ bifunctional primer and performed at 37°C for 90 min.. The resulting double-stranded RNA:cDNA heteroduplex was directly amplified by a polymerase chain reaction.

4.2.10 The polymerase chain reaction

The double stranded RNA:cDNA heteroduplex was heat denatured for 5 min. at 94°C to allow the cDNA strand to be used as a template for polymerization. The specificity of the PCR amplification process is based on two amplification primers that flank the cDNA segment to be amplified and hybridize to the complementary strands. The reaction cocktail was composed of 11 µl cDNA, 5 µl 10 x Taq-DNA Polymerase Reaction Buffer (Pharmacia Biotech, Freiburg, Germany), 5 U Taq-DNA polymerase (Pharmacia Biotech, Freiburg, Germany), 200 µM of each desoxyribonucleotide (Perkin Elmer Cetus, Langen, Germany), and 25 pmol of complementary oligonucleotide primers. The PCR amplification of DNA fragments was performed with the Perkin Elmer 9600 Thermocycler. Repeated cycles of denaturation (1.30 min. at 94°C), primer annealing (1.30 min. at 47°C) and primer extension (3 min. at 72°C) by Taq-DNA polymerase resulted in an exponential amplification of the target DNA. Amplified DNA fragments were visualized by agarose gel electrophoresis and eluted from the gel.

4.2.11 Sequencing analysis

Cycle Sequencing Kit (Perkin Elmer Cetus, Langen, Germany)
10 x TBE
7 M urea / 8% acrylamide stock solution
10% APS (Sigma, Deisenhofen, Germany)
TEMED (Serva, Heidelberg, Freiburg)
MicroAmp reaction tubes (Perkin Elmer Cetus, Langen, Germany)

The sequencing reaction has been performed with the Cycle Sequencing Kit (Perkin Elmer Cetus, Langen, Germany) according to the manufacturer's description. 30 µl of a reaction mixture containing 4 µl 10 x cycling mix, 200 ng specific primer, 1 µg template DNA, and 0.5 µl (α-^{33}P)-dATP (10 µCi/µl) were distributed to four MicroAmp reaction tubes, each filled with 2 µl of the G, A, T, or C Termination Mixture. The tubes were placed in a Perkin-Elmer GeneAmp PCR Instrument System, preheated to 95°C. For the GeneAmp PCR System 9600 an initial step of 95°C for 1 min. followed by 25 cycles of a 3 temperature cycling protocol (95°C for 30 sec., 68°C for 30 sec., 72°C for 1 min.) has been used. After thermal cycling 4 µl of the Stop Solution were added to all tubes and the samples were denatured at 94°C for 4 min.. For gel electrophoresis 3 µl of the denatured DNA from each of the four termination reactions were loaded into adjacent wells of the sequencing gel.
An 8% polyacrylamide gel was prepared by pouring 80 ml of a 7 M urea / 8% acrylamide stock solution, 500 µl APS and 115 µl TEMED between two glass plates. Gel electrophoresis has been performed at 65 W until the bromphenol blue marker dye reached the bottom of the gel (corresponds to DNA fragments of about 20 nucleotides). The gel was carefully transferred to a supporting sheet of Whatman 3MM filter paper (Schleicher & Schüll, Dassel, Germany), dried in a vacuum gel dryer and exposed to Kodak BIOMAX MR X-ray films (Eastman Kodak Company, Rochester, N.Y., U.S.A.).

4.2.12 Analysis of proteins

4.2.12.1 SDS Polyacrylamide denaturing electrophoresis (SDS-PAGE) and staining of proteins

Proteins were analyzed on 10% gels by SDS-PAGE (224) in Mini-Protean II cell chambers (Biorad, München, Germany) at 160 V. Before loading, protein samples were diluted in 4 x SDS-sample buffer and boiled for 5 min. at 95°C. After separation of the proteins the gel was stained with Coomassie Brilliant Blue or $AgNO_3$ or transferred to Immobilon-P membranes (Millipore, Eschborn, Germany).

For Coomassie blue staining, the proteins in the polyacrylamide gel were precipitated for 15 min. by a fixing solution containing 25% acetic acid, 10% isopropanol. The localization of the proteins was detected by nonspecific binding of a dye, Coomassie Brilliant Blue (10% acetic acid, 0.006% Coomassie Brilliant Blue). Staining has been performed over night. After destaining (5% methanol, 7% acetic acid) blue protein bands appeared against a clear background.
After fixation (15 min. in 50% ethanol, 12% acetic acid, 0.0025% formaldehyde) the polyacrylamide gel was equilibrated for 2 x 10 min. in 50% ethanol for silver staining. The gel was placed in 0.02% $Na_2S_2O_3$ for 1 min., washed 2 times with water and stained in 0.2% $AgNO_3$, 0.025% formaldehyde for 15 min.. After washing (2 x 5 min. in aqua bidest) the gel was shaken in 6% Na_2CO_3, 0.05% formaldehyde, 0.0004% $Na_2S_2O_3$ until bands developed, then the reaction was stopped with water, containing 40 mM EDTA.

4.2.12.2 Quantification of proteins by a colorimetric method

The quantification of proteins in a solution was based on the Bradford method (229), where binding of the Coomassie Brilliant Blue dye to proteins is measured at 595 nm in a spectrophotometer. Proteins were diluted in 0.15 mM NaCl to a volume of 100 µl. After addition of 700 µl aqua bidest and 200 µl of Protein Assay Dye Reagent (Biorad, München, Germany) the absorption at A_{595} was determined in a Beckman DU 640 spectrophotometer and compared to a standard protein dilution series.

4.2.12.3 Immunoblotting and immunodetection

Anode buffer I	300 mM Tris/HCl, pH 10.4, 20% methanol
Anode buffer II	25 mM Tris/HCl, pH 10.4, 20% methanol
Cathode buffer	25 mM Tris/HCl, pH 10.4, 40 mM amino-n-caproic acid, 20% methanol

Chemiluminescence reagent, detection reagent A and B, mixed 1:1 (Amersham, Braunschweig, Germany)

Proteins separated by SDS-Page were blotted to an Immobilon-P membrane (Millipore, Eschborn, Germany) by a semi dry system (Froebel, Lindau, Germany). The gel sandwich was prepared with 6 layers of Whatman 3MM filter paper soaked in Anode buffer I, 3 layers of Whatman 3MM filter paper soaked in Anode buffer II, the Immobilon-P membrane, the polyacrylamide gel, and 9 layers of Whatman 3MM filter paper soaked in the Cathode buffer. The membrane was preactivated in 100% methanol for 5 min. and then equilibrated in Anode buffer II for 15 min.. The electrophoretic transfer has been performed at 1 mA/cm^2 membrane for 90 min..
Membrane bound proteins were detected by the ECL-Western Blotting System from Amersham (Braunschweig, Germany). Briefly, non-specific binding sites on the membrane were blocked for 1 hr. in TPBS blocking buffer, containing 2.5% skim milk (Fluka, Neu-Ulm, Germany). Incubation with the first specific antibody has been performed for 60 min., incubation with the secondary HRP-labeled antibody for 35 min.. After incubation with each antibody the membrane was washed 3 x with TPBS blocking buffer for 5 min., before addition of the chemiluminescent substrate 4 x with PBS. Exposure to X-ray films (FUJI X-ray film RX) ranged from a few seconds to several hours, although strong signals normally appeared within 10-30 seconds.

4.2.12.4 Expression of recombinant fusion proteins

Recombinant fusion proteins were produced in the *E. coli strain* BL21-(DE3)LysS, transformed with the pRSET-vector constructs (Invitrogen, Groningen, The Netherlands). BL21-(DE3)LysS have incorporated a functional T7 RNA polymerase gene necessary to

drive the bacteriophage T7 promoter present on the pRSET vectors. Transcription of the recombinant genes is extremely efficient because the T7 polymerase uses up most ribonucleotide triphosphates in the cell and drastically inhibits transcription of genes by the host polymerase.
For fusion protein expression single colonies were picked from agar plates and grown at 37°C under vigorous shaking in 5 ml LB medium, containing 50 µg/ml ampicillin. Glycerol stocks were prepared according to 3.2.4. For protein expression 0.5 mM IPTG was added to the turbid cell suspension and after 2 hrs. bacteria were harvested by centrifugation. Recombinant proteins were identified by Western blotting.

4.2.12.5 Purification of recombinant proteins

Glycerol stocks were used for inoculation of over night cultures in 100 ml SOB, 50 µg/ml ampicillin, and 25 µg/ml chloramphenicol. Cultures were diluted in 1 l LB medium, 0.6% glucose, 50 µg/ml ampicillin and grown to log phase (OD_{595} = 0.6). Protein expression was induced with 0.5 mM IPTG for 2 hrs.. Bacteria were harvested by centrifugation and disrupted by repeated freeze-thawing cycles. After ultracentrifugation (50000 rpm, 50 min., in an 80 TI Beckman rotor) the supernatant was applied to a Ni-NTA resin (Quiagen, Hilden, Germany), pre-equilibrated with PBS and 20 mM imidazole. Recombinant molecules expressed from the pRSET vector system are tagged with 6 consecutive amino-terminal histidines that bind to the positively charged Ni-ions. Unspecifically bound proteins on the Ni-NTA resin were removed by washing with 20 ml PBS, 20 mM imidazole. Recombinant fusion proteins were eluted via an imidazole gradient (PBS, 20-500 mM imidazole).

4.2.13 Cell culture

4.2.13.1 Calcium phosphate transfection of mammalian cells

40-60% confluent cells were grown over night in phosphate free DMEM medium in 10 cm cell culture dishes. 20 µg plasmid DNA was suspended in 500 µl ddH_2O, 250 mM $CaCl_2$, and added dropwise in a circular motion to 500 µl 2 x HBS (50 mM HEPES, 280 mM NaCl, 1.5 mM Na_2HPO_4, pH 7.0) and incubated for 20 min. at room temperature. Calcium phosphate - DNA complexes, detected as a white precipitate, were distributed dropwise on the cells. After 12 hrs. the cells were washed 2 x with PBS and grown for 48 hrs. in a 37°C, 5% CO_2 humidified incubator. For stable transfection the cells were incubated in the presence of the corresponding antibiotic agent 48 hrs. after transfection until single clones became visible on the culture dishes. Single clones were isolated by 3 MM Whatman filter paper (5 mm in diameter), soaked in Trypsin/EDTA (BioWhittaker, Verviers, Belgium).

4.2.13.2 Electroporation of mammalian cells

Exponentially growing cells were harvested and the number of viable cells was counted by dye exclusion. The cells were washed once with PBS and resuspended at a density of 10^7 cells/ml in ice-cold RPMI without FCS. 0.4 ml of the cell suspension was used per electroporation in 0.4 cm cuvettes (Biorad, München, Germany) and mixed with 25 µg pDNA. Immediately prior to electroporation (960 µF, 120-240 V), the content of the cuvette was mixed by tapping, carefully avoiding air bubbles. The cells were maintained on ice for 15 min. prior to and after electroporation. Cells were placed into growth medium and transient expression was analyzed 48 hrs. post electroporation.

4.2.13.3 Lipofection of mammalian cells

70 µg pDNA was diluted to a final concentration of 1 µg/ml in HBS buffer and added to a separate reaction tube containing 63 µl DOTAP (Boehringer Mannheim, Germany) and 7 µl HBS. The transfection mixture was gently mixed in an Eppendorf Mixer 5432 for 20 min. at room temperature before injection into mouse tissue (4.2.14.2) using a U-100 Micro-Fine insulin syringe (0.33 x 13mm/ 29G x 1/2, Becton Dickinson, Heidelberg, Germany).
For cell culture, mammalian cells were grown to 40-60% confluence. 15 µg pDNA was diluted to a final concentration of 0.1 µg/ml in 20 mM HEPES buffer and mixed with 270 µl DOTAP, 20 mM HEPES for 20 min.. Culture medium was replaced with medium containing the transfection mixture. After 12 hrs. transfected cells were washed with PBS and cultivated under normal growth conditions at 37°C and 5% CO_2 in a humidified incubator.

4.2.13.4 Coating of gold particles to pDNA

28 mg gold beads (1-3 mm perimeter) were mixed with 100 µl of 0.05 M spermidine, vortexed for 5 sec., and sonicated in an ultrasonic waterbath. 56 µg of pDNA was adjusted to 1 µg/ml in aqueous solution and mixed with the spermidine, gold and 100 µl 1 M $CaCl_2$ by vortexing. The mixture was precipitated at room temperature for 10 min.. After centrifugation the pellet was washed 3 times with 1 ml 100% ethanol, dissolved in 4 ml 100% ethanol, and poured into a polypropylene tube. Precipitated gold particles were evenly distributed within the tube by a rotating device (Agracetus Inc., Middleton, WI, U.S.A.). The ethanol was aspirated and gold particles were air dried before the polypropylene tube was cut to 1 cm cartridges that were inserted into the gene gun (Agracetus Inc., Middleton, WI, U.S.A.). Particle bombardment was performed at 20- 40 bar.

4.2.13.5 Cell lines and growth conditions

Tumor cells. Meth A is a transplantable 3-methylcholanthrene-induced sarcoma of Balb/c origin passaged as an ascitic tumor (230). Cells were maintained in RPMI 1640 (BioWhittaker, Heidelberg, Germany), supplemented with 10% fetal calf serum (FCS) and 2 mM L-glutamine. The mouse mammary epithelial, ras-transformed HC11 tumor cell line Q6 (231) was grown in RPMI 1640, supplemented with 10% fetal calf serum, 2 mM L-glutamine, 5 µg/ml insulin (Sigma, Deisenhofen, Germany), and 10 µg/ml EGF (Becton Dickinson, Heidelberg, Germany).

Dendritic cells (DCs). The immortalized DC clone D2SC1 (232) was a gift from Riccardi-Castagnoli P. (Milan, Italy) and maintained in DMEM-medium, 10% FCS, 2 mM L-glutamine. Primary dendritic cells were obtained from mouse bone marrow (233) or spleen. Enrichment of primary DCs has been performed by positive selection with magnetic beads coupled to anti-mouse CD11c antibodies (clone N418, MiltenyiBiotec, Bergisch Gladbach, Germany). Cells were cultured in DMEM medium supplemented with 10% FCS, 50 mM β–mercaptoethanol, 1 mM HEPES (pH 7.4), 2 mM L-glutamine, 100 µg/ml streptomycin, 10 U/ml penicillin, 5 ng/ml recombinant mouse IL-4 (Genezyme, Rüsselsheim, Germany) and 20 ng/ml recombinant mouse GM-CSF (Genezyme, Rüsselsheim, Germany).

T lymphocytes. Primary T lymphocytes were isolated from spleen and maintained in DMEM, 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 mM HEPES, 0.4 ng/ml IL-2 (R&D Systems, Wiesbaden, Germany), and 0.5 mM β–mercaptoethanol. Proliferation was induced by 5 µg/ml PHA (Murex Biotech Limited, Dartford, U.K.).

Retroviral packaging cell line TeFly AF13. TeFly AF13 is a HT 1080 human fibrosarcoma-derived high-titer packaging cell line producing non-replication competent

amphotropic viruses as previously described (234). TeFly AF13 cells were stably transfected by the calcium phosphate precipitation method (224) and maintained in Dulbecco's modified Eagle's medium (BioWhittacker, Heidelberg, Germany) supplemented with 2 mM L-glutamine, 10% fetal calf serum and 5 μg/ml puromycin (Boehringer Mannheim, Germany). **MGFnlslacZ** producer clones and the MGFlnslacZ retroviral vector have been described previously (234, 235).

4.2.13.6 Isolation of T lymphocytes and dendritic cells

ACK-lysis buffer	150 mM NH_4Cl, 1 M $KHCO_3$, 100 mM Na_2EDTA, pH 7.2
Nylon wool column	10 ml syringe, packed with 1.2 g fluffed nylon fiber wool; attached 3-way stopcock; autoclaved and wrapped in aluminum foil
Scrubbed nylon fiber wool	boiled 10 min. in 1% HCl, washed with water until neutral pH, (DuPont Biotechnology Systems Division, NEN Products, Type 200L)

A freshly removed spleen was homogenized and pressed through a 200 μm mesh screen in 10 ml RPMI-medium. The obtained single cell suspension was concentrated by centrifugation (10 min., 200 g). The pellet was resuspended in 2 ml ACK-lysis buffer for 2 min. to remove the red blood cells. The spleen cells were washed in 10 ml RPMI, resuspended in 2 ml DMEM, 10% FCS and passed through nylon wool columns, pre-equilibrated with 50 ml PBS, 10% FCS and incubated for 45 min. at 37°C in a humidified incubator. B cells and macrophages were allowed to adhere to the nylon wool by incubating the loaded column for at least 45 min. at 37°C, 5% CO_2. Columns were prevented from drying by closing the stopcock and layering the wool with PBS, 10% FCS or DMEM, 10% FCS. Non-adherent cells were eluted in 15 ml DMEM and cell yield was determined by trypan blue exclusion.

Alternatively spleen cells, adjusted to 10^7 cells/90 μl were incubated with 10 μl magnetically labeled CD90 (Thy1.2) MicroBeads (MiltenyiBiotec, Bergisch Gladbach, Germany) for 15 min. at 6-12°C. Magnetically labeled cells were passed through a LS^+ positive selection column, placed within a magnetic field of a MACS separator (MiltenyiBiotec) and pre-equilibrated with 9 ml PBS, 10% FCS, and 0.2 mM EDTA. The unlabeled cells were depleted of $CD90^+$ cells by washing the column with 9 ml washing buffer (PBS, 10% FCS, 0.2 mM EDTA). After removal of the column from the magnetic field the magnetically retained $CD90^+$ cells were eluted as a positively selected cell fraction. DCs isolated from bone marrow or total spleen cell suspensions were incubated with 100 μl MACS CD11c MicroBeads per 10^8 /400 μl total cells. Bone marrow derived DCs were pre-cultured for 10 days in DMEM medium, supplemented with 10% FCS, 50 mM β–mercaptoethanol, 1 mM HEPES (pH 7.4), 2 mM L-glutamine, 100 μg/ml streptomycin, 10 U/ml penicillin, 5 ng/ml recombinant mouse IL-4 (Genezyme, Rüsselsheim, Germany) and 20 ng/ml recombinant mouse GM-CSF (Genezyme, Rüsselsheim, Germany) before MACs separation.

4.2.13.7 Transduction of mammalian cells

Retroviral gene transduction is used to introduce non-viral genes into mitotic cells. The retroviral vector was transfected into the packaging cell line TeFly by calcium phosphate transfection and puromycin selection. This packaging cell line expresses the viral gag, pol, and env genes as a result of stable integration and form normal viral particles. Cellular RNAs are not encapsulated and do not bud with normal viral particles because they lack the packaging signal ψ present only on the vector RNA.
For transduction transfected TeFly cells were grown to 40-60% confluence on a 15 cm cell culture dish. 15 hrs. before transduction the medium was replaced with 12 ml RPMI, 10% FCS, and 2 mM L-glutamine. Supernatant, containing the viral particles, was cleared from

cells by passing it through Pro-X™ 0.22 μm syringe filters (Roth, Karlsruhe, Germany) and used for transduction of log growing cells (40% confluent). Post mitotic cells can not be transduced because a cellular S phase is required for complete viral reverse transcription and integration. Transduction was performed twice on two successive days.

4.2.13.8 Cell fusion

PEG-solution — PEG 4000 (Merck) was autoclaved for 10 min. at 121°C. Molten PEG was cooled to 50°C and mixed with RPMI 1:1. The pH was adjusted to neutral.

Meth A tumor cells were stably transfected with pBABEhygro and fused with the dendritic cell line D2SC-1 (stably transfected with pBabepuro) by treatment with polyethylene glycol. For cell fusion DCs and tumor cells were washed 2 x with serum free RPMI, counted by trypan blue exclusion, and mixed 1:5 in a 50 ml centrifuge tube. After centrifugation (5 min., 200 g) the supernatant was aspirated and the pellet loosened by flicking the tip of the tube gently at 37°C. 1 ml PEG solution was added to the cell pellet, incubated for 1.30 min. at 37°C, and suspended dropwise in 15 ml RPMI (at 37°C), then in 20 ml RPMI, 10% FCS, and 2 mM L-glutamine. The cell suspension was incubated for 5 min. at room temperature, concentrated by centrifugation and plated in 20 ml cell growth medium. After 24 hrs. the fused hybrid cells were enriched by selection with hygromycin (100 μg/ml), (Boehringer Mannheim, Germany) and puromycin (50 μg/ml), (Boehringer Mannheim, Germany). Single clones were obtained by limiting dilution in 96 well plates.

4.2.13.9 Flow cytometry

FACS analysis has been performed with the FACSort Immunocytometry System and the Lysis II Software package from Becton Dickinson. 10^6 cells were incubated with the specific primary antibody in 20 μl PBS at 4°C. After 45 min. cells were washed with 1 ml PBS, centrifuged at 1700 rpm in a table top centrifuge for 3 min., and incubated with a FITC- or PE-conjugated antibody according to the manufacturer's description. Unbound, labeled antibodies were removed by washing with 1 ml PBS and the cells were analyzed by flow cytometry in a volume of 500 μl.
For detection of cytoplasmatic proteins, cells were resuspended in 1 ml FCS, RPMI (1:1) and fixed for 30 min. on ice by dropwise addition of 3 ml 100% ethanol (at -20°C). Cells were washed 3 x with 10 ml PBS and incubated with the antibodies before FACS analysis.

4.2.13.10 Proliferation assay

BL6 or Balb/c mice were sacrificed and spleens were used to prepare single cell suspensions according to the method described in 4.2.13.6. T cells were plated in round bottom microtiter plates (Becton Dickinson, Heidelberg) at 1 x 10^6 cells per well or titrated 1:3 in triplicates. Proliferation was induced by 2 x 10^4 irradiated Meth A tumor cells, 2 x 10^4 or 1 x 10^5 irradiated DCs. Cells were cultured in 200 μl DMEM, 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, 0.5 mM β–mercaptoethanol and 0.4 ng/ml IL-2 (R&D Systems, Wiesbaden, Germany) for 72 hrs.. 1 μCi of [^{3}H]-thymidine (Amersham, Braunschweig, Germany) was added for the last 15 hrs.. The labeled cells were harvested onto cellulose filters (Dunn Labortechnik GmbH, Asbach, Germany) and quenched (Ready Safe TM, Liquid Scintillation Cocktail, Beckman). The level of [^{3}H]-thymidine incorporation was determined with a liquid scintillation β-counter (Beckman, LS 6500 Multi-Purpose Scintillation Counter).

4.2.13.11 Chrome release assay

CTL activity of spleenic lymphocytes was evaluated by a ^{51}Cr-release assay. Dendritic cells, transduced with the tumor antigen were used as target cells. Target cells were prepared by incubating them at 37°C with 200 µCi of sodium ^{51}chromate for 1 hr.. 2 x 10^4 target cells and serially diluted effector cells were seeded into round bottom 96-well plates and incubated for 6 hrs. in 200 µl of DMEM with 10% fetal calf serum, 2 mM L-glutamine, 10 mM HEPES, 0.5 mM β-mercaptoethanol and 0.4 ng/ml IL-2 (R&D Systems, Wiesbaden, Germany) at 37°C in a humidified incubator and 5% v/v CO_2. After incubation, 100 µl supernatant was harvested to assess isotope release. Specific lysis was determined by the formula: percent specific lysis = (sample release - spontaneous release/maximum release - spontaneous release) x 100.

4.2.13.12 Luciferase assay

Lysis buffer	25 mM glycylglycerine pH 7.8, 8 mM $MgSO_4$, 1 mM EDTA, 1 mM DTT, 15% glycerol, 1% Triton X-100
Luciferin substrate	25 mM glycylglycerine pH 7.8, 5 mM ATP, 1 mM luciferin (Promega, Mannheim, Germany)

pDNA, containing the firefly luciferase gene was injected into mouse tissue with a U-100 Micro-Fine insulin syringe (0.33 x 13mm/ 29G x 1/2, Becton Dickinson, Heidelberg, Germany) in a volume of 70 µl. Biopsies at the injection site and from neighboring lymph nodes were isolated and homogenized in lysis buffer by a Ultratorax 100™ cell homogenizer (1 min., 15000 rpm). The luciferase expression was analyzed in a 200 µl volume in the luminometer AutoLumat LB953, Berthold, Germany.

4.2.13.13 Histology

Fixation buffer	PBS, 2% formaldehyde, 0.2% glutaraldehyde
Staining solution	PBS, 2 mM $MgCl_2$, 16 mM $K_3Fe(CN)_6$, 16 mM $K_4Fe(CN)_6$
X-Gal substrate	100 mg/ml in dimethylformamide

Lungs were fixed in 4% buffered formaldehyde (Apotheke des Klinikums, Universität Freiburg, Germany), embedded in paraffin blocks and cut to sections at 4 µm. Paraffin sections were stained with Hematoxilin/Eosin and analyzed by M. Michatsch, Institute of Pathology, Basel, for pathologic findings.
For cryostat sectioning, fresh tissue was immediately snap frozen in isopentane and kept at -70°C. Tissue was embedded completely in the Entellan compound (Merck, Darmstadt, Germany). Cryostat sections were cut at 4-10 µm (cryostat temperature -23°C, tissue specimen -18°C) and mounted on HTC glass slides. Sections were air-dried and fixed with the Fixation buffer for 30 min. at 4°C. The glass slides were washed with PBS and stained with the Staining solution and 5 mg/ml X-Gal substrate for 6-48 hrs. at 37°C.

4.2.13.14 Fluorescent staining of cells

Cells were transfected with the pCMX vector plasmid, containing the GFP (green fluorescence protein) gene according to 4.2.13.1-4. Cells were maintained for two days in growth medium and fixed in an acetone-methanol mixture (1:1) at -20°C for 15 min.. Positive cells were visualized by the Axiophot fluorescence microscope (Zeiss, Jena, Germany).

4.2.13.15 X-Gal staining of cells

Cells were washed 1 x with PBS and fixed in PBS, 0.05% glutaraldehyde for 5 min. at room temperature. After removing the glutaraldehyde by repeated washing, cells were stained in PBS, 2 mM $MgCl_2$, 16 mM $K_3Fe(CN)_6$, 16 mM $K_4Fe(CN)_6$, and 1 mg/ml X-gal substrate for 6-48 hrs. at 37°C. After 6 hrs. LacZ-positive cells appeared blue under the microscope.

4.2.14 *In vivo* experiments

4.2.14.1 Inbred mice

Female Balb/cAnN Crl BR (H-2^d) mice were purchased from the breeding colony of Charles River, Deutschland (Sulzfeld, Germany). Female Balb/c OlaHsd-nude mice were obtained from Harlan Winkelmann (Borchen, Germany). All animals were used at 6-8 weeks of age and maintained in a specific pathogen-free facility.

4.2.14.2 Immunization

Plasmid vectors were either injected intradermally into the basal layer of the ears or into the food pad of Balb/c mice. 35 µg of pDNA was dissolved in 50 µl PBS or 70 µl DOTAP/HBS 1:1 (Boehringer Mannheim, Germany). Mice received two applications (total of 70 µg of DNA) per inoculation. Inoculations were given once every 10 days for 60 days. Alternatively pDNA was applied via biolistic particle bombardment (Agracetus Inc., Middleton, U.S.A.). 1µg of pDNA was delivered at 35 bar.
Mice, immunized with transduced DCs, were injected with 2 x 10^5 cells intravenously into the tail vein or 2 x 10^6 cells subcutaneously into the flank. DCs were irradiated with 50 gray. Inoculations were given 6 times in 10 days intervals. Mice, immunized with cell fusion hybrids were immunized once with 10^7 cells subcutaneously into the flank.

4.2.14.3 Transfer of lymphocytes and macrophages

Lymphocytes were isolated from spleen of immunocompetent mice, enriched on nylon wool columns and injected intratumorally (2 x 10^6 cells) or intravenously (2 x 10^5 cells) into tumor bearing Balb/c mice. Peritoneal macrophages were administered intratumorally (2 x 10^6 cells) or intravenously in the tail vein (2 x 10^5 cells).

4.2.14.4 *In vivo* challenge with Meth A or Q6 tumor cells.

Immunization of Balb/c mice was performed as described above. One week after the last immunization mice were injected intradermally in the flank with 7 x 10^6 Meth A or 5 x 10^6 Q6 tumor cells. Tumor cells were suspended in 100 µl PBS before injection. Tumor volumes were monitored with vernier calipers and calculated as follows: 0.4 x (longest diameter) x (shortest diameter)^2.

4.2.14.5 Serum analysis

Mice were exposed to IR-light for 15 min.. Blood was taken from the tail vein. Blood samples were allowed to clot for 2 hrs. at room temperature and were centrifuged at 3000 rpm for 15 min. in a tabletop centrifuge. The straw colored antiserum was removed and stored at 4°C.

The general condition of mice was assessed by regular observation and measurement of weight twice a week. At the end of the animal trials, mice were killed by cervical dislocation. Before injection and treatment, mice were anesthetized in a chamber with 96 vol. O_2, 4 vol. isofluran at 4 l/min.. Anesthetization was maintained at 98.5 vol. O_2, 1.5 vol. isofluran (2 l/min., inhalation mask).

V. RESULTS

Chapter I

Induction of cellular immunity by direct pDNA transfer of a mutated p53 allele into Balb/c mice

In recent years tumor immunotherapy has received a great deal of attention. Two developments have contributed to this. The first is the identification of target antigens expressed by tumors. Tumor specific antigens are generated through mutation or by chromosomal translocation. In many tumors, these antigens are overexpressed or altered by translational modification and can be identified by cytotoxic T cells and antibodies. So far over- or aberrantly expressed normal antigens have proved the most promising therapeutic targets, since in contrast to point mutations, they usually provide a relatively large number of potential T cell epitopes.
The second reason for the resurgence of interest in the immunotherapy of tumors is the increased understanding of the factors regulating the immune response. Over the last decade there have been great strides in elucidating the nature of thymic selection and the heterogeneity of peripheral lymphocytes including the recognition of effector cells with distinct Th1 and Th2 cytokine profiles. Of particular importance to tumor immunology have been advances in the understanding of antigen presentation. The realization that T cells can recognize antigens originating within cells means that intracellular tumor proteins are now potential targets for immunotherapy. The immune repertoire of individuals with tumors contains both T and B cells capable of responding to antigens present within malignant cells (236, 237, 238). Immunotherapeutic approaches to cancer therapy are thus aimed at improving tumor antigen presentation, at enhancing effector cell response, or both. This study aims to develop a strategy for the immunotherapy of cancer utilizing plasmid DNA encoding antigenic tumor specific proteins. T cells patrol in the body as policemen in constant search for infected or degenerated cells. On the other hand malignant cells form in the process of natural selection protective mechanisms to evade detection and elimination by the immune system. Upon direct injection of tumor antigen encoding plasmid DNA T cells obtain a description of the "bad boys", they are preactivated and more efficient to look for cells that show distinctive features of a malignant phenotype.

5.1 Reporter gene constructs

The plasmid pZeoSV (Invitrogen, NV Leek, The Netherlands) is a constitutive mammalian expression vector. High level transient and stable expression of inserted genes is controlled by the Simian Virus 40 (SV40) early enhancer-promoter sequences. To construct pZeoSVLuc, a Hind III - BamH I fragment containing the firefly luciferase gene was cloned into the pZeoSV vector. pZeoSVLacZ, encoding the bacterial beta-galactosidase reporter gene was obtained from Invitrogen, NV Leek, The Netherlands. To construct pCMXLuc, the Hind III - BamH I luciferase fragment was cloned into the mammalian expression vector pCMX (gift from Roland Schüle, Tumor Biology Center, Freiburg, Germany). In this vector eukaryotic expression of the luciferase gene is under control of the cytomegalovirus (CMV) immediate early promoter.