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Cancer immunotherapy: insights from transgenic animal models

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Abstract

A wide range of strategies in cancer immunotherapy has been developed in the last decade, some of which are currently being used in clinical settings. The development of these immunotherapeutical strategies has been facilitated by the generation of relevant transgenic animal models. Since the different strategies in experimental immunotherapy of cancer each aim to activate different immune system components, a variety of transgenic animals have been generated either expressing tumor associated, HLA, oncogenic or immune effector cell molecule proteins. This review aims to discuss the existing transgenic mouse models generated to study and develop cancer immunotherapy strategies and the variable results obtained. The potential of the various transgenic animal models regarding the development of anti-cancer immunotherapeutical strategies is evaluated.

Keywords: Cancer immunotherapy; Transgenic animal models; Tumor associated antigen; HLA; Oncogene; Immune effector cell molecule

1. Introduction

Cancer is the second cause of death in the Western industrialized world [1]. Although there has been success in curing non-metastatic cancer [2,3], most forms of metastatic cancer are on the long run incurable with conventional treatment modalities such as surgery, radio- and chemotherapy. A major limitation of these modalities is the narrow therapeutic window between killing neoplastic while preserving normal cells. In the search for more tumor-specific therapies that are less toxic to normal cells, tumor immunotherapy strategies have gained interest [4]. Fundamental to the immunotherapeutical approach of cancer is the assumption that a tumor differs from normal tissues by tumor antigens, which are either unique (tumor specific antigen, TSA) or relatively restricted to tumor tissue (tumor associated antigen, TAA) [5,6]. As a consequence of the presence of these TSA or TAA the tumor is capable of inducing a specific immune response existing of a complex of integrated actions of a variety of immune cells, endothelial cells and a wide range of cytokines, growth factors, and antibodies. However, most tumors have developed mechanisms to escape immune surveillance, e.g. by down regulation of MHC and/or costimulatory molecules [7]. Upregulation of molecules which induce anergy and/or apoptosis in the attacking immune effector cells is also a common feature of tumor cells [8,9].

In the last decade different strategies have been developed for experimental immunotherapy of cancer. To elicit an effective anti-tumor response either the immune response can be potentiated or the tumor cells can be modified to become more immunogenic [10,11]. Roughly these tumor immunotherapy strategies can be divided into two categories, active and passive immunization strategies. *Active specific* immunotherapy aims to prime the naïve immune T cells in vivo by presenting tumor antigens via antigen presenting cells, in the context of MHC along with the necessary costimulatory molecules. This has been attempted using intact irradiated tumor cells, gene-modified tumor cells, viral oncolysates, tumor peptides conjugated to an immunogenic carrier molecule or administered in combination with an immune adjuvant, and recombinant viral vectors containing the tumor antigen encoding gene [11]. Another approach is to use ex vivo loaded professional antigen presenting cells such as dendritic cells [12,13]. In *passive* immunotherapy strategies, immune system components are added systemically or at the site of the tumor. Adoptive immunotherapy, for example, whereby the patient's autologous immune effector cells are enriched for a subpopulation of anti-tumor immune cells by sorting or expanding the effector cells of interest ex vivo [12]. Also cytokines like tumor necrosis factor α (TNF-α), either alone or fused to anti-tumor antibodies can induce tumor regression. In addition, antibodies fused to drugs or prodrug-activating enzymes can also lower the tumor burden [11]. Antibodies can also mediate the activity of the various *non-specific (active non-specific)* effector systems. Tumor-specific monoclonal antibodies (Mabs) can mediate cytolysis either by engaging NK, monocytes or granulocytes via Fc receptors (ADCC) or by complement activation. Originally Mabs were of mouse origin and could induce human anti-mouse antibody (HAMA) responses when used in patients. By reducing the immunogenicity of these xenogeneic antibodies, i.e. by 'humanizing' the constant regions by recombinant technology, these antibodies can be used repeatedly [14,15]. Furthermore, elegant bispecific antibody constructs are designed to bring immune effector cells into contact with tumor cells and to simultaneously stimulate their cytotoxic activity. Examples include antibodies that recognize a tumor surface antigen on the one and CD16 on the other hand to activate NK cells [16], or CD3 to activate T cells [17,18]. Cytokines at the site of the tumor can also recruit immune effector cells [11,18]. The different strategies employed for experimental immunotherapy of cancer are depicted in Fig. 1.

Although, the number of reports documenting successful immunotherapy in tumor patients increases, specifically when used in minimal residual disease situations [19,20], there is clearly a need to enhance our knowledge and to evaluate existing and novel immunotherapeutical strategies. Tolerance induction to specific tumor-antigens is a major hindrance for effective immune responses to tumors. To study this, a

number of animal models have proven to be of great value, despite this still relevant, more human resembling immunocompetent animal models are needed. Since the first transgenic mice were generated in 1982 [21], transgenic animal models have been used extensively to investigate biomedically important mechanisms underlying a variety of diseases. For cancer, transgenic mouse models promoting tumorigenesis have advanced our understanding of the mechanisms by which cancer initiates and progresses [22]. The last decade, however, transgenic animal models are no longer used solely to understand the pathogenesis of disease but also to develop and evaluate new therapies. To evaluate tumor-immunotherapeutical strategies transgenic animals have been generated which express tumor associated antigens, human HLA, oncogenes, mutated tumor suppressor genes, and also human immune-effector cell molecules (Table 1). At first non-specific promoters were used to express the genes of interest, which resulted in expression in all tissues. However, with the growing availability of the genomic sequences of genes, transgenic animal models have now been generated expressing the transgene accurately in a cell and tissue specific manner. Although, now valuable immunocompetent transgenic mice can be generated a lot of tumor models have been established and evaluated in the past in animal strains that are not suitable for the development of transgenic animals. Therefore, frequently a lot of time-consuming backcrossing is necessary to finally obtain a transgenic animal model in which tumors can be induced. To circumvent this problem, transgenic animals expressing the transgene of interest are crossed with transgenic animal models generated to develop 'spontaneously' certain tumors, e.g. expressing the SV40 large T antigen oncogene in a tissue specific manner [23]. A disadvantage of the latter approach is the fact that previously obtained knowledge from tumor bearing animal models generated be-

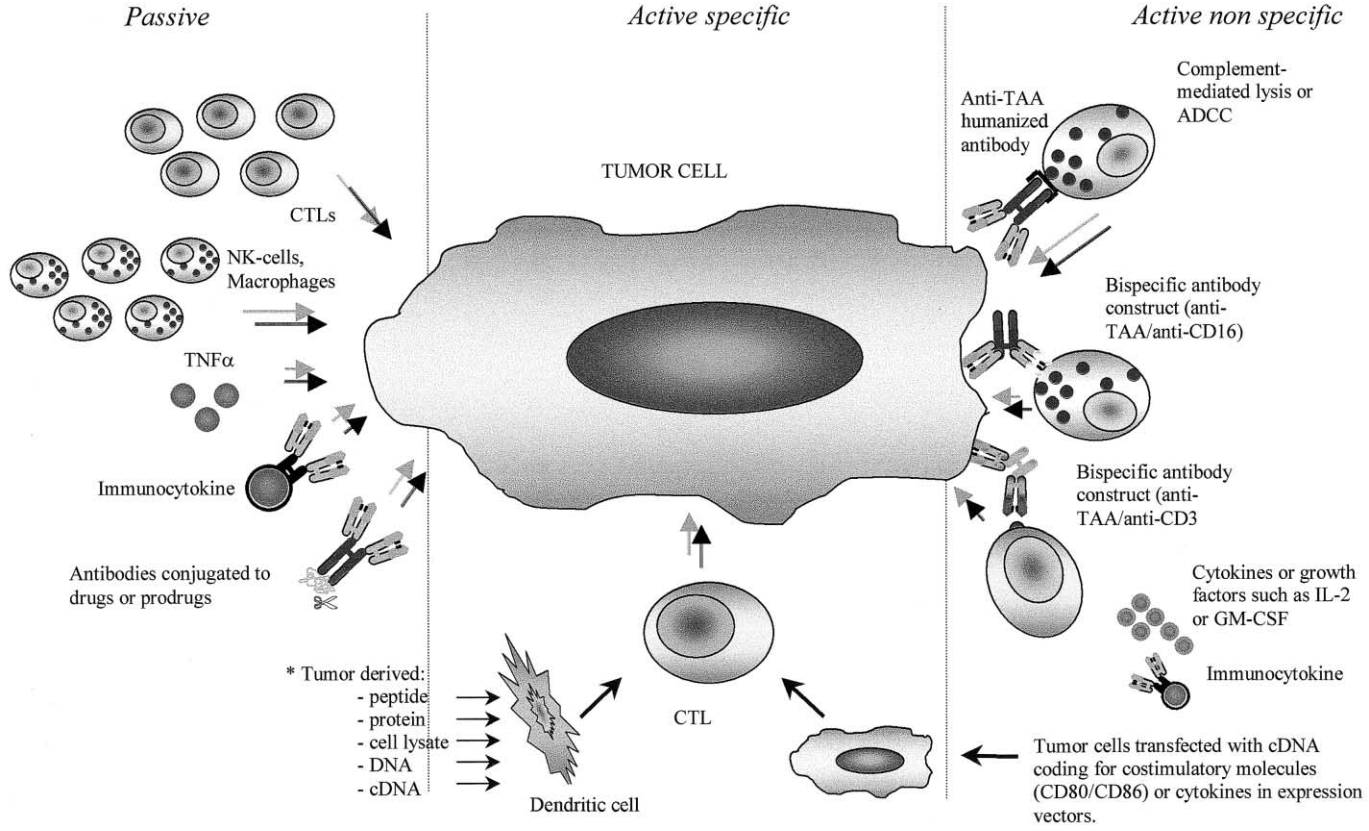

Fig. 1. Strategies for tumor immunotherapy. *Passive*: Adoptive transfer of autologous plasma enriched ex vivo for a subset of anti-tumor immune effector cells. Cytokines or cytokines fused to antibodies (immunobodies) which by themselves can facilitate tumor cell killing. Antibodies coupled to drugs or prodrugs, which are directly or after cleavage effective for tumor cell killing. *Active Specific*: DCs, which are either directly loaded with peptides or exposed to tumor cell lysates, tumor proteins or DNA, can effectively stimulate CTLs. Transfection of the DC by cDNA in an expression vector results in intracellular synthesis and processing of tumor proteins inside the DC. CTLs can be activated by the tumor itself made immunogenic by expression vectors expressing either costimulatory molecules such as B7-1/B7-1 (CD80/CD86) or cytokines. *Active non-specific*: Tumor-specific monoclonal antibodies can mediate cytolysis by NK cells via Fc receptors (ADCC), or by complement activation. Bispecific antibodies can be used to engage immune effector cells like CTLs (CD3) or NK-cells (CD16) to the site of the tumor. Cytokines or immunobodies at the site of the tumor can also recruit immune effector cells. Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; CTL, cytolytic T lymphocyte; DC, dendritic cell; NK, natural killer. This figure was partly adapted from Ockert et al. [11].

Table 1
Transgenic animal models used to study cancer immunotherapy

Transgene & reference			Immunotherapeutical strategy evaluated
TAA	CEA	SV40-CEAcDNA-SV40 [36]	None
		CEA genomic sequences [38,39,48]	Mab mediated *active non-specific* Vaccine mediated, *active specific*
	PSA	PSA genomic sequences [54]	TIL and tolerance, *active specific*
	MUC1	MUC1 genomic sequences [67–69]	Mab mediated *active non-specific* Vaccine mediated, *active specific* Toxicity of *active specific* immunotherapy
	EGP-2	K18-EGP-2cDNA-K18 [82]	Mab mediated *active non-specific*
		EGP-2 genomic sequences [Submitted]	Mab mediated *active non-specific*
	P1A	H-2K^{b}-P1AcDNA-β-globin [91]	Vaccine mediated, *active specific*
HLA	HLA-A2.1/K^{b}	[102,104,106–110,113]	Peptide (CEA, EGP-2) vaccine, *active specifi* Oligopeptide (PSA) vaccine, *acitve specific* Peptide based (MUC1) vaccine, *active specific* Melanoma peptide vaccine, *active specific* Viral peptide (E6, E7) vaccine, *active specific* Peptide based (p53) vaccine, *active specific*
	HLA-A2.1[120]		Peptide based (p53) vaccine, *active specific*
	HLA-A2.1/p53$^{-/-}$ [121]		Peptide based (p53) vaccine, *active specific*
	HLA-A2.1/ K^{b}/CD8^{+} [123]		Peptide (Her-2/neu) vaccine, *active specific*
	K^{216} [124]		As TAA, *passive* versus *active specific*
Oncogene			
	PyMidT	MMTV-PyMidTcDNA [45,129,134–137]	Adenoviral TNF-α, *passive* Adenoviral IL-2, IL-12, *active non-specific* Adenoviral IL-2, B7-1, *active (non-)specific*
	SV40T	Antithrombin III-SV40T [140–142]	Cytokines, *active non-specific*
	Tyr-SV40T [143–145]		TIL, *passive*
	TRAMP	Rat probasin-SV40T [60,61]	B7.1 transfection tumor, *active specific*
	neu MMTV-rat		DNA immunization, *active specific*
	neu-MMTV [147]		
Immune effector cell molecules			
	TCR	αβTCRλ^{315} Id-peptide [151]	Transfer tumor specific TCR, *active specific*
		αβTCRλ^{315} Id/*scid* mut [152]	Transfer tumor specific TCR, *active specific*
		L^{d}-TCR; 2C [153]	Tolerance, *passive*
		2C/Rag2$^{-/-}$ [155]	Transfer tumor specific TCR, *active specific*
		2C/Rag2$^{-/-}$/CD28$^{-/-}$ [161]	Transfer tumor specific TCR, *active specific*
	B7-1	RIP-B7-1cDNA-SV40T [162]	Transfer costimulatory molecule, *active specific*
		RIP-B7-1/RIP-IL-2 [163]	Transfer costimulatory molecule, *active specific*
	FcR	FcγRIA genomic sequences [166–169]	Bsab, ADCC, *active non-specific*

fore the era of transgenesis can not be implemented in the evaluation of immunotherapeutical strategies in transgenic animal models. For the same reason it is sometimes desirable to express a transgene in different species than the mouse, although the mouse remains the species of choice for many experiments involving the introduction of foreign DNA into the genome, simply because of technical limitations regarding other species [24].

Since so many different transgenic animals have been generated to evaluate a plethora of different anti-tumor immunotherapeutical strategies with variable results, this review aims to give a survey on the existing models and to discuss their potential regarding anti-cancer immunotherapeutical strategies.

2. Transgenic rodent models expressing tumor associated antigens

Clinical studies can be difficult to implement, particularly when clear understanding of the potential efficacy, limitations, and safety of an immunotherapeutic strategy is not available from relevant animal investigations.

TAA as opposed to TSA are besides present on the tumor also present on normal tissue. Therefore, immune-mediated therapies directed at tumor associated antigens deal with a balance between desirable anti-tumor responses and unwanted autoimmune reactions. Mice carrying a human tumor-associated antigen, preferably expressed according to the normal distribution pattern found in humans, may provide a more acceptable experimental model in which knowledge about passive and active immunotherapeutical strategies aiming at the TAA of interest can be enhanced prior to initiating clinical trials. Below, transgenic animal models bearing not only a tumor carrying a human TAA but which also express the human TAA on the relevant normal tissues, developed to optimize TAA-targeted tumor immunotherapy are discussed.

2.1. CEA transgenic mice

Carcinoembryonic antigen (CEA), which was first described in 1965 [25], is a M_r 180 000–200 000 oncofetal glycoprotein expressed on the plasma membrane of a subset of normal epithelial tissues as well as on a high percentage of adenocarcinomas [26]. CEA is released from tumor cells into the circulation and as such is a valuable tumor marker used in postoperative surveillance of tumors of epithelial origin, e.g. originating from in colon, pancreas, breast or lung cells and their metastases [27]. Since it is known that, in certain patients, CEA can be immunogenic [28,29], the use of CEA as a target antigen in the immunotherapy of tumors has gained a lot of interest. Vaccinia virus carrying the CEA gene has been used for active immunotherapy in metastatic colon cancer [30]. Although CTL specific for CEA could be generated from the lymphocytes of patients after immunization with the vaccinia construct, no clinical response was seen [31,32]. Anti-idiotype vaccination, initially believed to stimulate antibody responses, also seems to stimulate antigen-specific T cell responses against CEA [33,34]. Furthermore, phase I clinical trials of active immunotherapy have been initiated in patients with metastatic CEA-expressing malignancies using DC pulsed with CEA derived peptides [13] or RNA [35]. Since the outcome of these ongoing multi-center anti-CEA immunotherapeutical-strategies in patients may or may not generate new insights and probably also new questions (concerning, e.g. tumor accessibility, liver and kidney uptake, side effects on normal tissue combined with clinical benefit, safety, and other new treatments), these trials emphasize the need for more secure preclinical information obtained from relevant animal models.

Hasegawa et al. [36] were the first to create a CEA transgenic mouse line by using the full-length CEA cDNA fragment containing the SV40 early promoter. Northern and Southern blot analysis revealed expression of CEA in all tissues examined, including brain, thymus, lung, spleen, liver, kidney, and colon. However, in the lung and colon only the epithelial cells were positive as shown by immunohistochemical staining. In 1990 Schrewe et al. [37] cloned the complete gene for CEA, including the flanking regulatory elements (600 bp 5′; 100 bp 3′). This was used by Eades-Perner et al. [38] in 1994 and later on by Clarke et al. [39] in 1998 to generate independently strains of CEA transgenic mice. Although both groups used almost the same mouse strain, C57BL/6 x CB6 for the Eades-Perner group and C57BL/6J for the Clarke group, different CEA expression patterns were observed. Immunohistochemical analysis of the CEA transgenic mice generated by the group of Clarke et al. revealed expression of CEA in cecum and large intestine similar to the adult human CEA expression pattern. No CEA serum levels could be measured under normal circumstances, but also the CEA-concentration in feces of transgenic animals was 100-fold lower than found in humans. The transgenic CEA and the CEA isolated from human colonic adenocarcinomas were of similar size as determined by Western blotting. A syngeneic, transplantable colon-carcinoma tumor cell line, MC-38 transfected with the human CEA cDNA was used to induce tumors following implantation in both nontransgenic and the CEA transgenic mice. In serum of the nontransgenic but not of the transgenic mice anti-CEA antibodies could be detected [39]. Like patients these mice have CEA expression on normal tissue, tumors expressing CEA, and are immune tolerant for CEA. Therefore, this animal model is now suitable to analyze anti-CEA immunotherapeutical approaches based on breaking tolerance using, e.g. vaccines, or to study antibody mediated immunotherapeutical strategies targeted against CEA.

In the CEA transgenic mice generated by the group of Eades-Perner [38,40], CEA protein was detected in tongue, oesophagus, stomach, small intestine, cecum, colon and trachea, and low levels in lung, testis and uterus. However, the levels of CEA in the colon were 10-fold higher than described for human colon. Serum levels of CEA were 9–20-fold higher and CEA concentrations in feces 2–4 times higher than the concentrations found in human serum or feces, respectively. These high levels of CEA were not related to copy number. Furthermore, the CEA isolated from transgenic mouse tissue was smaller ($M_r \approx 160\,000$) than the CEA isolated from human colonic tissue ($M_r \approx 200\,000$). The reason for this discrepancy is unknown yet. To establish mouse-models for optimizing CEA targeted immunotherapy, these CEA transgenic mice were crossbred with mice that were genetically predisposed to develop different types of tumors [41]. The genetically predisposed mice-models chosen developed the three most common forms of tumors found in

industrial nations, i.e. colonic, lung, and breast tumors. Crossbreeding CEA transgenic mice with $Apc^{min/+}$ mice [42], which have a chemically induced nonsense mutation in the murine *Apc* gene [43], generated F1 CEA transgenic mice expressing CEA in all developed intestinal adenoma cells. Sporadically also CEA-positive breast tumors were observed in female $Apc^{min/+}$/CEA offspring [41]. Normal breast cells of CEA transgenic mice do not express CEA. In a second model CEA transgenic mice were crossbred with SP-C-SV40-large-T-antigen expressing transgenic mice predisposed to develop non-small-cell lung adenocarcinomas [44]. CEA was found in adenoma/adenocarcinoma cells but with a mosaic pattern of expression varying between tumors as well as between individual cells within the tumor. Offspring as a result of crossbreeding CEA transgenic mice with mice transgenic for the neu protooncogene under control of the MMTV promoter [45] developed breast tumors. These breast tumors, however, did not express CEA and therefore, do not provide a model for CEA-targeted immunotherapy. In all generated CEA transgenic mice models CEA had virtually no influence on tumor development or on phenotype of the mice compared to wild-type mice.

Recently, a CEA transgenic mice model was established and successfully used for the evaluation of a tumor-immunotherapeutic approach [46]. By continuous backcrossing the Eades-Peres group CEA transgenic mice with C57BL/6 mice a CEA-transgenic mice model was developed in which tumors could be induced injecting s.c. syngeneic tumor cells expressing human recombinant CEA. In this CEA transgenic mouse model it was shown that naïve CEA transgenic mice were unresponsive to endogenously expressed human CEA, since this normal expression did not induce a CEA-specific humoral immunity. Also in vitro priming of splenic T cells of these CEA transgenic mice by addition of exogenous CEA failed. After immunization with whole CEA in adjuvant, or a synthetic CEA peptide, no CEA-specific IgG or cell-mediated response was observed. However, using a recombinant vaccinia vaccine expressing CEA [47], anti-CEA immunity was observed as indicated by anti-CEA IgG antibody titers, T_H1-type CEA specific $CD4^+$ responses, and induction of CEA peptide-specific cytotoxicity. Moreover, multiple recombinant vaccinia-CEA immunizations protected CEA transgenic mice from subsequent challenge with CEA-expressing tumor cells. Apparently in this model, the specific condition in which the antigen is presented can break the tolerance for CEA.

In conclusion, the CEA transgenic mice model requires its own regulatory sequences for correct expression, which in addition appears to be influenced by the mouse strain used. Furthermore, to break the tolerance in this mouse model it seems that a specific vaccination protocol is required. Studies to elucidate the underlying mechanism, to reveal a more detailed account of the toxicology, but also to evaluate the therapeutic effect on established CEA-expressing tumors in these mice are ongoing and will generate more useful information to adjust the anti-CEA immunotherapeutical trials that are currently being investigated in a clinical setting.

2.2. PSA transgenic mice and the TRAMP model

Prostate cancer is the second most common cause of cancer deaths in men [48]. Although surgery and radiation therapy is effective at first in limited disease, there is no effective treatment available for patients who have metastatic disease [49]. Development of immunotherapeutical treatments of prostate cancer has been hampered because it was generally believed that prostate cancer is a non-immunogenic tumor. However, due to recent discoveries that were made regarding the mechanisms by which prostate cancer evades surveillance by the immune system and due to the identification of new, potentially immunogenic prostate cancer antigens, immunotherapeutical treatment strategies for prostate cancer are being examined with renewed interest [50]. The human prostate-specific antigen (PSA) is a kallikrein with serine protease activity [51], expressed on normal epithelial cells of the prostate gland, and on a majority of prostate cancers [52]. PSA is secreted in the circulation by tumor cells and as such has been used widely as a serum marker for cancer of the prostate [53]. The cell type-specific expression of PSA makes it also a potential target for prostate cancer immunotherapy. Studies of the possibilities to exploit immunological aspects of PSA, however, have been hampered by the lack of relevant mouse models, because no PSA counterpart has been identified in mice.

Wei et al. [54] have isolated two lambda clones encompassing the PSA gene, covering 14 kb of genomic sequences, including 6 kb of the 5′ and 2 kb of the 3′ flanking regions. C57BL/6J xDBA/2J F1 hybrid transgenic mice generated with this construct expressed PSA in the epithelial cells of the prostate and coagulating gland, as determined by Northern blot and immunohistochemical analysis, closely following the human expression pattern. Serum levels were as low as in humans. After backcrossing the PSA transgenic mice to Balb/cByJ mice, tumors were induced injecting s.c. syngeneic PSA expressing tumor cells [55]. Twenty days later, tumor infiltrating lymphocytes (TIL) were isolated and their specific anti-PSA cytotoxic activity determined. A cytotoxic immune response against PSA-expressing tumor cells was observed not only for TIL isolated from tumors induced in nontransgenic or female transgenic mice, being negative for transgene expression, but also for TIL isolated from tumors induced in male transgenic mice, despite the expression of PSA as a self-antigen. Though, TIL isolated from non-

transgenic and female transgenic mice showed approximately 2 times more specific lysis of target cells than TIL isolated from male transgenic mice. The cytotoxic immune response demonstrated by these transgenic mice can be explained by the fact that after tumor induction serum levels of PSA increased 3–6-fold as a result of the release of PSA expressed by the tumor. Furthermore, in humans PSA is androgen regulated, and is expressed in males after puberty [56]. If this is also true in the PSA transgenic mice it is likely that a large number of PSA-specific T cells have already matured prior to expression of PSA.

It should be noted that despite the presence of these PSA-specific cytotoxic lymphocytes, the tumors were still growing progressively. Apparently the immune response generated is not sufficient enough to cause rejection of a highly aggressive tumor. Therefore it is necessary to devise means to enhance the response for effective therapy. The PSA transgenic mice as developed by Wei et al. is a good model system of prostate cancer using authentic human PSA as the tumor antigen, to study mechanisms of unresponsiveness to PSA, anti-PSA immunization strategies, CTL epitope usage to identify the most effective peptide epitopes of PSA, or anti-PSA antibody-mediated immunotherapeutical strategies.

In 1995, Greenberg et al. used another strategy to generate a transgenic mouse model to test immune-based treatments of prostate cancer [57]. These transgenic adenocarcinoma mouse prostate (TRAMP) mice developed spontaneous prostatic cancer, closely mimicking the human disease. Sensitivity to tumor development was caused by the incorporation of the SV40 large T-antigen specifically expressed in the epithelium of the mouse prostate using the rat minimal probasin promoter containing 426 bp of 5′ flanking and 28 bp of 5′ untranslated sequences. In the C57BL/6 TRAMP model, prostatic disease progresses from mild to severe intraepithelial neoplasia, to focal adenocarcinoma that metastasizes to the lymph nodes, lung and occasionally to bone, kidney, and adrenal glands [58]. Combined with transplantable epithelial prostate cancer cell lines, derived from TRAMP prostate tumors, which can be grafted into syngeneic C57BL/6 or TRAMP mice [59], the TRAMP model provides an excellent model system to test immune-based prostate cancer therapy, as proven in 1997 by Kwon et al. [60,61]. They transduced a TRAMP derived cell line to express the murine B7.1 costimulatory ligand, and subsequently injected these cells, in distinct doses, s.c. into C57BL/6 and athymic mice. A host immune response was elicited leading to complete rejection of these cells in the syngeneic immuno-competent mice but not in the athymic mice, proving that the mechanism for this rejection is likely T cell mediated, and that expression of the B7 costimulatory ligand bypasses the requirement for exogenous APC help, to activate tumor-specific $CD8^+$ T cell activation. However, T cells are not solely subjected to activating signals, like B7/CD28, but also to inhibitory signals, like B7/CTLA-4. CTLA-4 shows approximately a 20-fold higher affinity to the B7 ligand than CD28 and is known to deliver an inhibitory signal to the T cell [62]. To test whether blockade of CTLA-4 could elicit an anti-tumor immune response, the unmanipulated TRAMP derived cells were injected s.c. in C57BL/6 mice followed by i.p. injection of anti-CTLA-4 antibody, 7, 10, and 13 days after tumor inocculation. Administration of anti-CTLA-4 antibody significantly delayed growth (58%) or induced complete rejection (42%) of the induced tumors. No decrease in tumorigenicity of the TRAMP derived cell line was observed in animals treated with a control antibody. Surprisingly, tumor cells expressing little or no MHC class I showed a more complete response than tumor cells with normal MHC class I levels. Using this novel murine model more knowledge will be obtained regarding the T cell costimulatory pathways that can be manipulated to enhance the immune response against prostate cancer. Hence, tests in TRAMP and/or PSA transgenic mice appear useful to develop new therapeutic strategies, that may be also clinically applicable in the treatment of prostate cancer in patients.

2.3. *MUC1 transgenic mice*

The human epithelial mucin, MUC1, is a large polymorphic transmembrane glycoprotein that is expressed on most simple epithelia. It has an extensive extracellular domain composed of 20 amino acids tandem repeats which are rich in *O*-glycosylated regions rich of serine, threonine, and proline [63]. MUC1 is frequently overexpressed in human carcinomas, including that of the breast, lung, pancreas, colon and ovary. MUC1 expression in tumors is often accompanied by loss of normal apical expression and shorter and more heavily sialylated carbohydrate side chains as compared to MUC1 expression in non-transformed cells of the same tissue origin [64]. MUC1 is of interest as an immunotherapeutical target since T lymphocytes capable of ex vivo recognizing and lysing MUC1-expressing tumor cells have been isolated from the tumor-draining lymph nodes of cancer patients.

Since human MUC1 is highly immunogenic in mice due to the low degree of homology in the tandem repeat region between *MUC1* and its murine homologue *Muc1* [65], it would be of great advantage to have a mouse model to study MUC1 tumor immunotherapeutical strategies, in which MUC1 is present as a self-antigen. In 1992, Peat et al. [66] developed a MUC1 transgenic mouse model using 10.6 kb, *Sac*II digested, MUC1 genomic sequences containing 1.5 kb of the 5′ and 800 bp of the 3′ flanking regions. MUC1 expression

in these transgenic animals was, as in humans, restricted to simple epithelial cells in various organs as determined by immunohistochemical analysis. These mice are an appropriate model for toxicity testing of antibodies in vivo and for studying humoral responses to MUC1-based vaccines or T cell responses ex vivo. However, since these mice are outbred cytotoxic T cell responses could not be analyzed, nor was it possible to induce MUC1 expressing tumors in these mice.

To study mechanisms to break the tolerance and to activate cytotoxic T cell responses to MUC1, Rowse et al. [67], developed an inbred C57BL/6 MUC1 transgenic mouse model with the same *Sac*II MUC1 genomic DNA fragment as described above. Immunohistochemical staining revealed that the level and pattern of MUC1 expression in the simple epithelial cells of the transgenic mice was similar to the model of Peat et al. and that seen in humans, including lung, mammary gland, pancreas, kidney, gallbladder, stomach, and uterus. In these C57BL/6 MUC1 transgenic mice tumors were induced injecting a MUC1 cDNA-transfected syngeneic melanoma derived cell line, B16.MUC1. This MUC1 positive tumor grew much more rapidly in the transgenic mice as compared to its growth in wild-type animals. Immunohistochemical analysis of the tumors revealed that tumors in nontransgenic mice had no MUC1 expression, suggesting that the MUC1 expressing cells were eliminated and that the MUC1 negative variants were responsible for the tumor outgrowth. Tumor populations display antigenic heterogeneity and retain the capacity to alter antigen expression by different mechanisms. The observed loss of MUC1 expression is in line with this. So effective tumor vaccines will need to include multiple antigenic components to completely eradicate the entire population of tumor cells. Analysis of the immune response after tumor induction revealed no humoral response in the transgenic mice, whereas strong IgM and IgG responses were measured in the nontransgenic mice. To investigate the immune response of MUC1 transgenic mice to self peptides derived from the tandem repeat region, transgenic and nontransgenic mice were immunized with a strech of amino acid consisting of either 1.20 or 5.25 copies of the MUC1 tandem repeat. A weak IgM and no IgG response could be detected in both transgenic and nontransgenic mice after immunization with the short 1.20 peptide. However, after immunization with the 5.25 peptide, a strong IgM response was observed in the transgenic mice, whereas wild-type animals revealed a strong antibody response in both IgM and IgG covering all isotypes. These data indicate that the MUC1 transgenic mice are tolerant to the transgene product and therefore provide a useful model to investigate immunity and mechanisms of tolerance to the tumor antigen MUC1.

Tempero et al. [68] used this model to investigate the relationship between MUC1-specific tumor immunity and the toxicological consequences of producing anti-MUC1 immune responses. Since it is known by previous studies in the outbred model of Peat et al. that MUC1-specific antibodies do not provide effective immunity to tumors expressing MUC1, it was postulated that the elimination of the B16.MUC1 tumors in the nontransgenic mice was probably caused by MUC1-specific cellular responses, not present in the transgenic mice. However, a modified limiting dilution assay to asses CTL frequency showed that the frequency of MUC-1 specific CTLs at a certain time-point after tumor induction, was statistically indistinguishable between transgenic and nontransgenic mice. Because the tumor growth data from Rowse et al. [67] still provided evidence for a MUC1-specific immune response in the wild-type mice, Tempero et al. [69] decided that the in vitro assay used was not effective in detecting the immune cells mediating the MUC1-specific tumor immune response. Therefore, a cellular adoptive transfer system was designed to evaluate MUC1-specific and non-specific tumor immunity in vivo. Adoptive transfer of the MUC1-specific immune response from the wild-type mice to MUC1 transgenic mice significantly increased the survival of the recipients following B16.MUC1 challenge. T cell depletion studies revealed that $CD4^+$ lymphocytes were responsible for the MUC1-specific tumor immunity. No side effects on MUC1 expressing normal tissues were observed after the adoptive transfer of the MUC1 specific T cell responses in the MUC1 transgenic mice. Because the transgenic and nontransgenic mice were of the same C57BL/6 background and the endogenous mouse *Muc1* differed greatly from the human MUC1, this adoptive transfer strategy was successful, however, this result can not be translated to the human situation. Therefore, it is now necessary to use the MUC1 transgenic mice as a preclinical model to evaluate novel vaccine strategies applicable in patients, and their effective anti-MUC1 tumor responses.

2.4. EGP-2/Ep-CAM transgenic rodents

The human epithelial glycoprotein-2 (EGP-2), also known as 17-1A or Ep-CAM, encoded by the GA733-2 gene, is expressed as a stable transmembrane protein ($M_r \approx 40\,000$) at high levels on the surface of most carcinomas, including breast, ovary, colon and lung cancers [70,71]. Normal expression of EGP-2 is restricted to simple, stratified and transitional epithelial tissues covering the gastrointestinal, respiratory, and urinary tracts, where the antigen appears to be shielded from the circulation by an intact basal lamina. Furthermore EGP-2 is not shed into the circulation. Although the function of EGP-2 remains to be elucidated it has

been hypothesized that it functions as a homotypic, Ca^{2+}-independent intercellular adhesion molecule, capable of mediating cell aggregation, directing cell segregation by E-cadherin modulation [72]. The cadherin modulating properties observed for EGP-2 suggest a role for this molecule in the development of a proliferative and metaplastic cell phenotype and probably in the development and progression of malignancies [73,74]. Even in tissues with relatively high EGP-2 expression, such as colon, the development of polyps is accompanied by further increase in EGP-2 expression. EGP-2 is an attractive target for immunotherapeutical treatment strategies and Mabs directed against EGP-2 have been studied in several clinical trials.

Schlimok et al. demonstrated that adjuvant Mab therapy using EGP-2 as a target could be clinically relevant [75,76]. This appears to be true at least in patients suffering from minimal residual disease after a primary surgery of colorectal carcinoma. In the mid-1990s, Riethmuller et al. extended this knowledge and proofed the therapeutical efficacy of the anti-EGP-2 Mab 17-1A, in a multicenter prospective trial. One hundred and eighty nine patients with radically resected Dukes' C colorectal carcinoma were randomly assigned to observation or repeated treatment with the Mab 17-1A. Mab treatment resulted in significant reduction of the overall mortality rate as compared to the two most accepted treatment regimens, but with hardly any adverse effects [77]. This therapeutical effect could be maintained for at least 7 years [78]. Currently a phase III trial evaluating this treatment strategy is ongoing. However, the overall therapeutical efficacy of sole Mab mediated immunotherapy in non-minimal disease settings is still disappointing.

To enhance the cytotoxic potential of Mabs they can be conjugated to toxins, prodrugs and/or radioactive compounds as well as to elements that enhance the immune system at the site of the tumor, like chemo-attractants, cytokines, or by adding another specificity, as in bispecific Mabs [11,78]. The later approach was followed in the laboratory. Kroesen et al. constructed a bispecific monoclonal antibody, BIS-1 $F(ab')_2$, which combines EGP-2 specificity with a specificity against the CD3 complex on T lymphocytes. In a pilot experiment, when carcinoma patients were treated intra-peritoneally or intra-pleurally with bispecific Mab targeted autologous T lymphocytes a strong anti-tumor response and inflammatory reaction was observed locally with minor local and systemic toxicity [79]. However, in a phase I trial using the same bispecific antibody systemically in combination with IL-2, severe toxicity did occur. At the maximum tolerated dose, a rapid induction of TNF-α and IFN-γ production was noticed followed by a transient leucopenia, though with little or no therapeutical efficacy [80]. To intensify the bispecific Mab therapy, Nieken et al. conducted a new phase I trial in which repeated doses of BIS-1 $F(ab')_2$, at the maximal as tolerable single dose were applied systemically in combination with IL-2, to patients with EGP-2 expressing tumors not responding to the first-line of therapy. The repetitive application of BIS-1 $F(ab')_2$ in combination with IL-2 was feasible and immunologically active as demonstrated by ex vivo studies. However, it did not induce objective anti-tumor responses (Manuscript in preparation). To evaluate the effect and the efficacy of the BIS-1 $F(ab')_2$ or other anti-EGP-2 mediated immunotherapeutical strategies in combination with, for example TNF-α blocking agents, relevant animal models are needed. Kroesen et al. established an immunocompetent rat model in which human EGP-2 expressing tumors can be induced [81]. Although anti-tumor efficacy of the above described BIS-1 $F(ab')_2$ based immunotherapeutical strategies could be seen in this model in a minimal disease situation, adverse side effects on normal tissue can not be studied in this model.

With respect to the EGP-2 antigen no transgenic animal has been described so far. Therefore, a transgenic rat was generated expressing the full-length EGP-2 cDNA (GA733-2) using the 2.5 kb 5′ and 3.5 kb 3′ distal flanking regions of the human keratin 18 (K18) gene as regulatory sequences [82]. Northern and Southern blot analysis revealed expression of EGP-2 in liver, lung, jejunum, stomach and kidney. EGP-2 expression could only be detected on the membrane in agreement with the expression pattern of a transmembrane protein, and proved to be present on the hepatocytes, which is in contrast to the human situation where hepatocytes do not express EGP-2, and on the exo- and endocrine tissue of the pancreas. The transgenic EGP-2 was similar in size as EGP-2 purified from a human small cell lung carcinoma derived cell line, indicating that the EGP-2 molecule was processed normally, although differences may exist in glycosylation [83]. After backcrossing the EGP-2 transgenic Wistar rats to a Wag/Rij background tumors could be induced by injecting s.c. syngeneic EGP-2 and non-EGP-2 expressing tumor cells [84]. To determine the potential of this EGP-2 transgenic tumor immunotherapy model, the anti-EGP-2 Mab MOC31 was injected i.v. 1 week after tumor inoculation and analyzed immunohistochemically for its localization to EGP-2 expressing normal and tumor cells a day later. Intravenously applied MOC31 Mab was observed in the liver and in the EGP-2 positive tumor, but not in the pancreas and EGP-2 negative tumor, demonstrating that the Mab binds specifically to the antigen and that the transgene is accurately expressed and processed. In this transgenic rat model the liver tissue expressing the EGP-2 is sensitive to anti-EGP-2 Mab mediated immunotherapy. Therefore, it can be argued that the liver might well be a convenient substitute for an induced tumor and mea-

suring liver toxicity therefore an easy read-out to determine the degree of cell damage due to in vivo applied immunotherapy.

Although the EGP-2 transgenic rat model is a valuable model for the evaluation of anti-EGP-2 directed immunotherapy strategies, the lack of EGP-2 expression on the epithelial tissue covering the gastrointestinal and respiratory tract deviates much from the human situation. Therefore, one searched for new regulatory sequences to express the GA733-2 gene. In 1990, Szala et al. cloned the GA733-2 gene and analyzed its sequence including approximately 150 bp upstream [85]. A bacterial artificial chromosome clone containing at least 55 kb GA733-2 genomic sequences including approximately 10 kb 5′ and 4 kb 3′ flanking regions was recently isolated and the whole 55 kb DNA fragment was injected into oocytes of FVB/N female mice. Immunohistochemistry, Western blotting and FISH analysis of the EGP-2 transgenic offspring revealed a copy number dependent and integration site independent EGP-2 protein expression in the epithelial tissue of the colon, small intestine, stomach, lung, pancreas, thymus, gonads/testis, kidney, and skin in a manner comparable to the human situation. After backcrossing of the transgenic FVB/N mice with C57BL/6 mice tumors were induced injecting s.c. B16 cells or B16 tumor cells transfected with the EGP-2 cDNA [86]. Currently, investigations to determine the immunotherapeutical effect of the Mab MOC31 on the tumor versus toxicity of this treatment on EGP-2 expressing normal tissue in these transgenic mice are ongoing. It is anticipated that these data will demonstrate the potential of this new EGP-2 expressing transgenic model to refine anti-EGP-2 antibody mediated immunotherapeutical strategies, to determine more accurately possible toxic effects on normal tissues, and to evaluate new immunotherapeutical strategies.

2.5. Mouse melanoma associated or specific antigens in transgenic mouse models

Probably due to the relatively high number of ongoing trials to evaluate anti-melanoma immunotherapy in patients, transgenic animal models expressing human melanoma associated or specific genes have gained only minor attention. The last few years, however, more and more researchers notice the potential of using tumor antigens expressed by mice tumors, transgenically induced or spontaneous arising, to develop and analyze immunotherapeutical strategies also for this tumor type. Transgenic mice have been generated that spontaneously develop mouse melanomas by placing the SV40 gene under the control of the tyrosinase promoter. These melanomas have been studied to identify the presence of additional immunotherapeutically interesting antigens [87–89]. For many years various human and mouse tumor-type independent tumor antigens have been determined using CTLs as screening tool. These tumor-specific tumor-type shared antigens appear to be promising targets for cancer immunotherapy. However, the choice of these antigens as targets are questionable because of the lack of direct evidence that in vivo responses against such antigens can lead to tumor rejection. MAGE-type genes, including the mouse P1A and the human genes of the MAGE, BAGE and GAGE families [90] are expressed in various tumors but not in normal tissues except in MHC-negative male germ cells. To investigate the in vivo potential of such antigens for overall tumor protection Brändle et al. generated the P1A transgenic mouse model [91]. To this end the P1A cDNA was expressed under the control of the H-2K^b gene promoter and human β-globin gene 3′ flanking regions [92] and all tissues analyzed by RT-PCR were positive for P1A, with strong expression in the thymus, spleen, and lung. The antigen encoded by the mouse gene P1A presented here is the only available animal model system for MAGE-type tumor antigens. To test the potential of P1A as a target for immunotherapy normal DBA/2 (H-2^d) mice were immunized by injecting i.p living P1A and B7-1 expressing L1210 leukemia cells inducing strong CTL responses to P1A. Three weeks after this immunization, mice were challenged i.p. with a P1A expressing mouse mastocytoma cell line p815 [93]. Immunized mice survived (12/15, day 40) significantly longer compared to control animals (3/15, day 40). Mice immunized by L1210 cells expressing B7-1 but lacking P1A were not protected. To confirm the essential role of the immune response to P1A in the tumor rejection, the protection experiment was repeated in P1A DBA/2 transgenic mice. No protection could be induced in these mice neither by immunization with the P1A and B7-1 expressing L1210 cells or by an adenovirus recombinant for P1A, indicating that indeed the P1A shared antigen of the different tumor types is the major component responsible for the observed protection. These results demonstrate that a shared tumor-specific antigen is a good target for immune rejection and support the use of such antigens for immunotherapy of human cancer, bringing closer the possibility to develop general anti-tumor vaccines although the induced immune responses are, like in these mice models, probably HLA-type restricted.

3. HLA transgenic mouse models

Among the numerous genes studied for their role in disease development, polymorphisms associated with HLA class I and class II loci has been known for many years to play a significant role in predisposition to disease. HLA molecules are encoded by MHC genes on

the short arm of chromosome 6. Structure analysis of the HLA molecules revealed that it contained a peptide binding cleft in which the variable region of the HLA molecules is situated [94]. Genetic polymorphism at the MHC locus determines the specificity and affinity of peptide binding and as a result determines T cell recognition [95]. HLA class I molecules are recognized by the T cell receptor (TCR) of T cells in conjunction with the CD8 molecule, whereas HLA class II molecules are recognized by the TCR of T cells in conjunction with the CD4 molecule. In most cases class I recognition induces cytotoxic T cell (CTL) activation, whereas class II recognition implies T-helper (TH) activity [96]. Since CTLs play a critical role in the immune surveillance of tumors most HLA transgenic animals designed to investigate anti-tumor immune therapy strategies are of the HLA class I type [97]. Human CTLs can detect an antigen after it has been processed intracellularly into peptide fragments which are displayed on the cell surface in the peptide-binding groove of the HLA class I molecules. HLA class I molecules consists of a heavy chain composed of three domains (α1, α2, α3), where the α1 and α2 domains form the peptide binding groove recognized by the TCR, and a non-covalently associated light chain, β_2 microglobulin (β_2m). The three HLA class I genes; A, B, and C, are highly polymorphic.

HLA transgenic mice can potentially overcome some of the main limitations inherent to human studies, such as the need for peripheral blood samples, the lack of controls, and the inability to control the influence of both MHC and non-MHC linked genes on the immune responses. Since, HLA-A2 is one of the most common alleles in the general population (20–50%), depending on the ethnic background considered [98,99], the HLA-A2 allele has been chosen most frequently as the model MHC class molecule and as such has been investigated extensively. The HLA-A2 transgenic mice generated so far have shown considerable variation in expression of the HLA transgene compared to the endogenous H-2 class I molecule, even in mice transgenic for chimeric HLA/H-2 [97]. For example, the two generated HLA-A2.1 transgenic mice, frequently used for the evaluation of anti-tumor immunotherapies, both express the HLA-A2.1 molecule. However, in the transgenic mice line established by Epstein et al. [100] the level of expression of the HLA-A2.1 molecule is much lower than the expression of the endogenous H-2 molecule, while in the transgenic mice of Le et al. [101] the HLA-A2.1 expression is comparable to the H-2 expression level. This variation in HLA transgene expression is a common feature of all HLA class I transgenic mice which does not necessarily hamper the experimental value of the transgenic animal models, since the affinity and binding-capacity of the variable region of the MHC molecule to the peptides of interest remains unaffected.

3.1. HLA-A2.1/K^b

HLA-A2.1/K^b transgenic mice were generated by Vitelo et al. in 1991 [102] and have since been used by a number of groups to study CTL mediated anti-tumor immunotherapy strategies. These transgenic mice express the product of the HLA-A2.1/K^b chimeric gene in which the α3 domain of the human heavy chain is replaced by the corresponding murine H-2 K^b domain, whereas the α1 and α2 domains remain unaffected. This allows the murine CD8 molecule on the murine CTLs to interact with the murine α3 domain of the hybrid MHC class I molecule. Therefore the murine T cells are able to recognize human CTL epitopes in the context of HLA-A2.1/K^b molecules and are thus an important tool in the identification of potential CTL epitopes, especially since it has been reported that the mice can identify HLA-A2-restricted viral peptides in a similar way as HLA-A2 human CTLs [103]. There is now considerable emphasis on finding tumor-associated antigens that can be recognized by human T cells. HLA class I transgenic mice are not tolerant to human proteins and therefore provide a powerful tool to examine HLA restricted CTL responses against these tumor antigens.

Ras et al. used the HLA-A2.1/K^b transgenic mice to investigate whether peptides derived from the known tumor associated antigens EGP-2 (or Ep-CAM) and CEA expressed by a large number of tumors of epithelial origin could elicit a peptide specific CTL-mediated immune response [104]. They synthesized 410 EGP-2 and 73 CEA-derived peptides selected using the HLA-A2.1 binding motif and a computer algorithm. The peptides synthesized differed at least in one residue from other related proteins. They used three in vitro assays to determine the affinity of the peptide, the stability of the peptide-MHC complex, or both. At the end five EGP-2 and two CEA peptides were selected mixed with adjuvant and injected s.c. at the base of the tail in HLA-A2.1/K^b transgenic mice. On day 14 the mice were boosted and 2 weeks later sacrificed after which spleen cells were isolated and restimulated in vitro. The cytotoxic capacity of these cells was determined in a standard ^{51}Cr cytotoxicity assay resulting in the identification of one immunogenic EGP-2 and one immunogenic CEA-derived peptide. It has been shown that the immunogenicity of peptides measured in HLA-A2.1/K^b transgenic mice correlates with the ability to induce primary responses in vitro suggesting an extensive overlap between the T cell receptor repetoires of mouse and human CTL [105]. Therefore, the immunogenic peptides identified in HLA-A2.1/K^b transgenic mice are potential CTL epitopes and in this case might be attractive targets for CTL-mediated peptide-based immunotherapy.

HLA-A2.1/K^b transgenic mice were also used to explore the CTL response elicited by modified peptides derived from the tumor-associated antigens PSA [106] and MUC1 [107,108]. Correale et al. developed a multiple epitopes containing PSA derived oligopeptide (PSA-OP) as a compromise between the use of a 9-mer CTL peptide epitope and the entire protein. They demonstrated that the oligoepitope peptide had the ability to induce cytotoxic T cell activity in vitro. To assess the in vivo immunigenecity of PSA-OP, HLA-A2.1/K^b transgenic mice were injected s.c in the base of the tail with PSA-OP emulsified in Detox-adjuvant. Cytotoxic T cells derived from PSA-OP-immunized mice specifically lysed PSA-OP-pulsed Jurkat A2/K^b target cells, whereas no cytotoxic T cell responses were observed when a control peptide was used to pulse the Jurkat cells, nor were both types of Jurkat cells lysed by cytotoxic T cells isolated from control mice. This result demonstrates that PSA-OP can induce CTL responses in vivo and as such is a potential candidate for use as peptide-based vaccines for treatment of human prostate cancer [106]. In 1997 Apostoloupos et al. used HLA-A2.1/K^b transgenic mice to demonstrate that the well-studied, breast cancer-associated mucin, MUC1 could associate with HLA molecules and induce a $CD8^+$ HLA-A2-restricted CTL response. They immunized the HLA-A2.1/K^b transgenic mice with a MUC1 fusion protein conjugated to mannan and subsequently isolated $CD8^+$ HLA-A2-restricted MUC1-specific CTLs which could lyse the MCF-7 breast cancer cell line (HLA-$A2^+$, $MUC1^+$) directly without extensive in vitro stimulation. To map the MUC1 peptides binding to the HLA-A2 molecules, target cells were pulsed with 20 overlapping 9-mer peptides derived from the 20 amino-acid tandem repeat sequence, and the percentage lysis by the isolated CTLs determined at several E:T ratios. Surprisingly, the peptide sequences inducing the highest cytotoxic active CTL responses showed the lowest binding affinity to HLA-A2 molecules, as determined by sequence analysis and by a binding-assay. So low affinity-binding peptides can lead to the generation of highly effective CTLs, making the range of peptides that can generate CTLs for immunotherapy to $MUC1^+$ cancers broader than originally considered [107].

A lot of tumor specific and associated antigens have been identified in melanomas and the majority of these antigens have been shown to associate with HLA-A2. Therefore, it is not a surprise that HLA-A2.1/K^b transgenic mice have been used to evaluate the immunogenic potential and the binding of peptides derived from melanoma associated antigens to HLA-A2. The MAGE-2 protein is expressed in metastatic melanomas, laryngeal and lung tumors, and is an attractive candidate antigen for immunotherapy, since it is not expressed in most adult tissues. Visseren et al. used the HLA-A2.1/K^b transgenic mice to identify three MAGE-2 derived peptides with high binding affinity to HLA-A2.1 and with the ability to induce CTL responses [109]. CTLs specific for two of these three peptides were found to be useful since these proved to lyse melanoma cells that expressed MAGE-2. The melanocyte differentiation antigens, gp100 and Melan-A/MART-1 are expressed only by melanomas and normal melanocytes. Two groups showed that by modifying the sequence of these peptides the immunogenicity could be enhanced. Bakker et al. demonstrated that substitutions in the anchor position of the peptide $gp100_{154-162}$ resulted in peptides with higher HLA-A2.1 binding affinity and greater immunogenicity in HLA-A2.1/K^b transgenic mice [110]. Three of these peptide analogues generated stronger CTL responses than the unmodified peptide and all isolated CTLs were able to lyse target cells coated with unmodified peptide and melanoma cell lines expressing endogenously processed gp100 antigens. Men et al. used the HLA-A2.1/K^b transgenic mice to show that by a single amino acid substitution the immunogenic potential of the natural Melan-A immunodominant peptides Melan-A_{27-35} and Melan-A_{26-35} could be enhanced [111]. Substitutions at position 1, 2 or 1 and 2 improved the binding capacity to the HLA-A2.1 molecule and the immunogenicity of these peptides. CTLs isolated from HLA-A2.1/K^b transgenic mice immunized with the Melan-A_{26-35} with an amino acid substitution at position 2 recognized natural Melan-A peptides on human melanoma cells in a HLA-A2.1 restricted manner and subsequently could lyse these target cells. In addition, Bullock et al. demonstrated, using peptides derived from tyrosinase and gp100, that cell surface density of the immunizing peptide on dendritic cells is also of great significance to the magnitude of the elicited in vivo T cell response. However, this was demonstrated in HLA-A2.1/D^d transgenic mice [112]. Since the above described anti-melanoma immmunogenic peptides can induce HLA-A2.1-restricted melanoma-specific CTL responses, at least in HLA-A2.1 transgenic mice, these peptides appear applicable for anti-melanoma peptide based immunotherapeutical strategies in humans.

In case of tumors associated with a viral oncogenesis, viral antigens can be used as a target antigen for anti-tumor immunotherapy. The E6 and E7 oncoproteins of the human papillomavirus type 16 (HPV16) are constitutively expressed in the majority of the cervix carcinomas, and therefore are potential targets for CTL-mediated immunotherapy. Ressing et al. tested nine HPV16 E6 and E7 derived peptides for their immunogenecity in HLA-A2.1/K^b transgenic mice. Four of these peptides were immunogenic in the transgenic mice of which three were also highly immunogenic in human beings as demonstrated by in vitro induction of a CTL response with PBMC obtained from HLA-A2.1 healthy donors [113]. Additionally,

these three HPV16-encoded peptides had the highest affinity of binding to the HLA-A2.1 molecule. Therefore, Ressing et al. concluded that the HLA-binding affinity of a peptide is of great importance for its immunogenicity. This conclusion differs from the above described findings of Apostoloupos et al. Human CTL clones induced in vitro using these three peptides were capable of lysing the endogenously HPV16 E6 and E7 expressing HLA-A2.1 positive cervical carcinoma cell line CaSki demonstrating that these epitopes represent naturally processed human CTL epitopes of HPV16. Immunization of the HLA-A2.1/K^b transgenic mice with a mixture of the four immunogenic HPV16-derived peptides elicited a CTL response against all of these four peptides. This demonstrated that inducing an immune response towards multiple epitopes of the HPV16 E6 and E7 proteins is possible and feasible. Targeting against multiple antigenic epitopes might lower the risk of immune escape by modulation of CTL epitopes by the tumor cell. The identification of the multiple human CTL epitopes encoded by the HPV16 has brought the development of a peptide-based vaccine for the prevention and treatment of cervical carcinomas nearer. This approach is currently evaluated [114].

The human p53 tumor suppressor protein is a cellular regulatory protein normally expressed at low levels in the cell. Generally due to only a single-base missense mutation p53 is overexpressed in more than 50% of all malignancies which makes it an attractive target for a broad spectrum immunotherapeutical approach, especially since its overexpression often correlates with a poor prognosis. Successful generation of CTL responses in mice to murine wt p53 has been demonstrated using either p53-derived peptide pulsed dendritic cells [115] or a vaccinia viral vector carrying part of the p53 gene [116]. However, it has been difficult to generate a human CTL response to p53, except in rare situations [117]. Theobald et al. used both HLA-A2.1 and HLA-A2.1/K^b transgenic mice to identify four p53-derived immunogenic peptides [118]. CTL clones, derived separately from both transgenic mice strains, specific for two peptides were able to lyse the HLA-A2.1$^+$, p53 gene transfected cell line Saos/175. Interestingly, only CTLs obtained from the HLA-A2.1 transgenic mice were able to recognize and lyse tumors that naturally expressed high levels of p53. However, recognition was limited and only observed after pretreatment of the tumors with IFN-γ and TNF-α. Three other peptides with homology to murine p53 failed to induce a high CTL response despite strong binding to the HLA-A2.1 molecule. Studies in HLA-A2.1/K^b p53$^{-/-}$ transgenic mice confirmed that self-tolerance to murine p53 was limiting the CTL response against human p53 [119]. However, the fact that the immune repertoire to recognize p53 was not totally eliminated suggests that it should be possible to generate CTLs to self-p53 epitopes. To determine if CTLs specific for the p53 epitopes could prevent the growth of HLA-A2.1$^+$ p53$^+$ tumors, HLA-A2.1/K^b transgenic mice were immunized with a vaccinia virus containing the DNA coding sequence of the $p53_{149-157}$ peptide. Two weeks later the mice were challenged with a HLA-A2.1$^+$ p53$^+$ tumor and it was shown that the immunization specifically prevented tumor growth. Tumors that did grow out had lost p53 expression [119]. Although the experiments described here suggest that CTLs to p53 can be valuable for tumor elimination, it remains to be determined to what extent tolerance to p53 may condition the repertoire of such-p53 specific CTLs.

Tolerance may prevent a widespread clinical application of newly defined tumor antigens for vaccine therapy of cancer. If ubiquitous antigens as p53 are the targets of immunotherapy, strategies aimed to circumvent tolerance are needed. One innovative strategy to circumvent tolerance to p53 and still target cancer cells overexpressing p53 is the introduction into autologous CTLs of a genetically altered T cell receptor (TCR) with defined, high affinity, p53 specificity. This approach circumvents tolerance, but first requires the generation of high affinity HLA restricted T cells. Therefore, Yu et al. immunized HLA-A2.1 transgenic mice with two immunodominant wild-type p53 epitopes which resulted in the generation of two peptide specific CTL clones [120]. One, $p53_{149-157}$, peptide-specific CTL clone was able to recognize and lyse not only the Saos/175 cell line but, also a panel of tumor cell lines that overexpressed endogenously processed p53 in the context of HLA-A2.1, and not cells with normal or low p53 expression, or cells that lacked HLA-A2.1 expression. These findings raised the possibility of immunotherapy involving the transfer of high affinity p53 specific TCR. However, the tolerance to p53 may have limited the ability to generate the highest response possible in these mice, since human p53 is 80% homologous to murine p53 and cross tolerance to the majority of wt p53 epitopes might be expected. McCarty et al. circumvented this problem by generating HLA-A2.1 p53$^{-/-}$ transgenic mice [121]. They immunized these mice with the immunodominant human $p53_{149-157}$ peptide, generating a high-affinity, p53-specific, HLA-A2.1-resticted CTL clone. These CTLs recognized and were able to kill human breast cancer cells without recognizing normal cells, resulting in the identification of p53-specific TCR genes suitable for genetic transfer to human T lymphocytes. To study the potential of the generated CTLs in vivo, human $p53_{149-157}$ specific murine CTLs were transferred to SCID mice in which tumors were induced using the pancreatic carcinoma cell line Panc-1. Animals who received the $p53_{149-157}$-specific CTLs demonstrated a prolonged survival as compared to control animals, whereas in 50% of the animals no malignancy developed [122].

The Her-2/neu protooncogene, a member of the subclass I family of growth factor receptors, is overexpressed in malignancies of the breast, ovary, colon, and lung and is thus an attractive target for broad-spectrum immunotherapy. Lustgarten et al. wanted to use the HLA-A2.1 transgenic mice as an in vivo model to identify immunodominant epitopes of the Her-2/neu protein. To encompass the unpredictable functioning of the murine CD8 molecule in the recognition and killing of human cell lines, as seen for instance by Theobald et al., Lusgarten et al. generated HLA-A2.1/K^b x human CD8 expressing double transgenic mice [123]. Nineteen Her-2/neu peptides were selected, based on the anchor motif for HLA-A2.1 (L,I,M,V,A,T at position 2, and 8, 9 and 10) and the degree of sequence homology with normal proteins, synthesized, and used to immunize the HLA-A2.1/K^b hCD8 transgenic mice. Two HLA-A2.1 binding peptides from Her-2/neu were able to raise a CTL response in the transgenic mice. These CTLs were able to kill A2.1$^+$ Her-2/neu$^+$ human tumors from different tissue origin but with difference in the percentage of lysis between the tumors examined.

In conclusion, the HLA-A2.1 transgenic mouse models are useful animal models to perform preclinical testing of potential cancer vaccines, since they are able to process, present, and respond to the same peptide epitopes recognized by humans that express HLA-A2.1. However, since the elicited CTL response is not hampered by tolerance to the self-antigen the process in these mouse models is not really representative for the situation in humans. Crossing the HLA-A2.1 transgenic mice with the above-described TAA expressing mouse models might be an approach for generating transgenic mice reflecting more the human situation in which the generated CTLs can be tested. Also the contradictory findings regarding the influence of the binding capacity of a peptide to the HLA-A2.1 molecule on its immunogenicity remains to be elucidated. Furthermore, HLA-A2.1 transgenic mice expressing also costimulatory molecules, like the transgenic mice generated by Lustgarten et al. will facilitate a more accurate extrapolation of CTL responses to the human situation.

3.2. Mouse HLA class I transgenic mice

Mouse HLA class I transgenic mice have been produced to study the role of MHC class I molecules in the immune system regarding T cell or NK cell ('missing self') mediated recognition in several diseases, including cancer. Koeppen et al. used the mouse HLA class I K^{216} allel as a tumor target-antigen and C3H/HeN K^{216} allel transgenic mice as donors for transgenic spleen cells and skin grafts to study the efficacy of *passive* versus *active* immunotherapy in late tumor-bearing (LTB) mice [124]. These mice were capable of rejecting K^{216} transgenic skin grafts but not their own tumor burden also expressing the K^{216} antigen, which is in contrast to tumor-free or early tumor bearing mice. Furthermore, LTB mice were not capable of generating an effective CTL response upon in vitro stimulation with K^{216} expressing cancer cells. Only after stimulation with adherent non-malignant K^{216} transgenic spleen cells, enriched for antigen presenting cells, CTLs of the LTB mice responded. Using a progressively growing K^{216} positive tumor Koeppen et al. demonstrated that active immunotherapy with an engineered vaccine in combination with dendritic cells, was active at day 0 and day 10 after tumor challenge, but not at day 22. In contrast, passive immunotherapy proved to cure al LTB mice in the test group. So antigen-presenting cells expressing the tumor rejection antigen can elicit an antigen specific T cell response in vitro by LTB mice, and such T cells can be curative when used in adoptive therapy. These results represent important findings since the stage of tumor growth and the immune status of late tumor-bearing mice more closely simulates the conditions observed in cancer patients.

4. Oncogene transgenic mice to study immunotherapeutical strategies

The generation of mice designed to overexpress activated forms of oncogenes has allowed scientists to causally link the function of these genes with specific tumor cell functions, such as proliferation, apoptosis, angiogenesis or metastasis [125]. In addition, these mice can be used to develop and test new therapies, such as tumor immunotherapy.

4.1. PyMidT transgenic breast-cancer mouse model

Polyoma middle T (PyMidT) transgenic mice are FVB/N mice expressing the PyMidT oncogene under the control of the mouse mammary tumor virus (MMTV) promoter/enhancer sequences. Expression of this PyMidT oncogene results in spontaneous tumorigenic transformation of the mammary epithelium in mice 8–10 weeks of age [45]. This oncogene transgenic mouse model has been used extensively to study several immunotherapeutical approaches. Marr et al. used the mammary tumor cells derived from the PyMidT transgenic mice to induce tumors through subcutaneous injection of normal syngeneic mice [126–128]. These subcutaneous tumors were subsequently injected with several adenoviral vectors bearing a human or mice derived TNF-α gene. TNF-α is a multipotent cytokine capable of inducing apoptosis, intravascular thrombosis, and inflammation within tumors. However, TNF-α induces also severe systemic toxicity when applied systemically [129]. At first, Marr et al. developed two adenoviral vectors containing either a secreted or a

membrane-bound form of mouse TNF-α. Modified adenoviral vectors transfect cells only once, after which the gene of interest is expressed locally either continuously or transiently [128]. The generated adenoviral vectors were injected intratumorally to circumvent systemic toxicity. However, the vector containing the secreted form of TNF-α proved to be highly toxic, since 12 out of 16 mice were killed. In contrast, much lower toxicity was observed, though still killing three out of 27 mice, when the vector containing the membrane-bound TNF-α was applied. Both vectors induced partial and sometimes complete tumor regression, with the cured mice having protective immunity and specific CTL activity against the tumor. These results indicate that the membrane-bound mouse TNF-α is bioactive within the tumor and inflicted reduced systemic toxicity. Secondly, Marr et al. generated adenoviral vectors containing the human TNF-α gene under the control of either the human or the mouse CMV promoter [126]. Human TNF-α is 50 times less toxic compared to the murine form in mice due to the fact that it is capable of binding to only one of the two involved receptors [130]. To study the mechanism of this reduced toxicity and to evaluate the anti-tumor activity of the human TNF-α when expressed continuously at high levels, the adenoviral vectors were injected directly into the tumors of the PyMidT transgenic breast-cancer mouse model. TNF-α under control of the MCMV promoter was expressed stronger and expression was induced earlier as compared to expression under the control of the HCMV promoter. Furthermore, only MCMV promoter expressed TNF-α induced tumor regression. Disappointingly, this was accompanied also by severe systemic toxicity, indicating little promise for the use of this adenoviral vector in tumor immunotherapy.

The use of cytokines has a long history in immunotherapy, with IFN-γ being the first cytokine used in tumor immunotherapy in 1957. Cytokines can regulate the immune response and are secreted by the immune effector cells as well as a large variety of other cells including tumor cells. A large number of cytokines has been described. Several of these are capable of mediating tumor regression in some malignancies. However, though largely used in patients with incurable metastatic disease, the therapeutic effects are still disappointing, especially considering the concomittant severe systemic toxicity [131]. Interleukin 2 (IL-2) is one of the most extensively studied cytokines for tumor immunotherapy purposes. It is produced primarily by $CD4^+$ T cells and can induce T and NK cell activation and proliferation [132]. IL-12 promotes growth of activated T and NK cells but is also capable of inducing a switch in profile of a T helper cells from T_H0 towards T_H1. T_H1 cells produce inflammation stimulating cytokines such as IL-2 and IFN-γ. The substantial treatment associated toxicity of both IL-2 and IL-12 is probably caused by the induction of secondary produced cytokines, like TNF-α [133]. Addison et al. used the PyMidT transgenic breast-cancer mouse model to study adenoviral vectors expressing IL-2 or IL-12 [134]. Animals bearing subcutaneous mammary tumors were injected intratumorally with one of these adenoviral vectors or coinjected with both. A syngeneic effect was observed in animals that received the combination treatment, with 65% of the mice in this group showing complete remission. Also mortality was reduced when compared to the IL-2 treated animals, 10 versus 36%, respectively. Treatment with either or both adenoviral vectors was accompanied by the induction of a specific anti-tumor CTL response. These CTLs did not only induce regression of the injected tumor but also of the bilateral control tumor not injected with the adenoviral vectors. So, these data indicate that the combination of IL-2 and IL-12 induces a more effective, less toxic anti-tumor immune response than either one alone, and this anti tumor response is also effective in mediating the regression of distal untreated tumors, an important feature considering the treatment of metastatic disease.

Another approach effecting the host–tumor relationship combines the activity of cytokines with that of costimulatory molecules. Tumors are thought to escape the immune surveillance not only by down regulating their MHC expression but also by inducing T cell anergy, due to stimulation of the TCR in the absence of costimulatory molecules. One of the best-characterized costimulatory molecules is the B7-1 molecule, a membrane glycoprotein induced upon activation of various antigen-presenting cells, which interacts with the constitutively expressed CD28 molecule on T cells. Pützer et al. constructed an adenoviral vector that encoded both IL-12 and the B7-1 protein under the control of the MCMV promoter [135]. In the PyMidT transgenic breast-cancer mouse model, a single intratumoral injection with this adenoviral vector mediated complete regression of the tumor in 70% of the treated animals, while no toxicity was observed. Cured animals remained tumor free after rechallenge with new tumor cells. The same strategy was applied by Emtage et al. who used a double recombinant adenoviral vector coding for the human IL-2 and the murine B7-1 protein, also under the control of the CMV promoter. Using this vector in the PyMidT transgenic breast cancer mouse model a 100% complete regression of the tumor load could be induced. This complete regression was accompanied by a systemic immunity associated with a strong anti-PyMidT CTL response [136]. A double recombinant adenoviral vector containing the human IL-2 gene in combination with murine B7-2 was less effective in inducing regression of PyMidT tumors, although immune protection could be induced using this vector [137]. These data obtained with the PyMidT transgenic breast cancer mouse model, show that aden-

oviral vectors are interesting tools to deliver immunemodulatory molecules at the tumor site and to reduce toxic side effects.

4.2. SV40 large T antigen transgenic mice

The simian virus 40 large T antigen (SV40T) oncogene has been used extensively for the induction of tumors. By binding to the p53 protein, the SV40T protein forces the cell out of cell cycle control resulting in indefinite uncontrolled growth. Using different promoters transgenic animals have been developed expressing the SV40T oncoprotein in a tissue specific manner, allowing the study of tumors originating from different tissues. An example of this is the Rip-Tag mouse, one of the most studied tumor progression models. In this mouse the SV40T antigen is expressed under the control of the insulin promoter. As a result these transgenic mice develop pancreatic islet cell carcinomas in discrete stages of tumor progression [138]. However, only two SV40T transgenic mouse models have been used so far to study immunotherapeutical approaches. Romieu et al. used transgenic mice expressing the SV40T oncoprotein under the regulatory region of the antithrombin III promoter to study the cytokine production in developing hepatocellular carcinoma (HCC) [139,140]. It was found that increased growth of the tumor in late stages of tumor development correlated with increased expression of transforming growth factor-β (TGF-β), while early development of the tumor was accompanied by the production of TNF-α. Production of either cytokine was attended by lack of sensitivity for this cytokine. This model of HCC could be of interest to assess the impact of various immunotherapeutic strategies on modulation of tumor growth, as for example by TNF-α and TGF-β blocking antibodies. The same HCC model was used by Morel et al. to study the effect of γ-ray irradiation on the tumor cells used as vaccine [141,142]. A strategy for active immunotherapy currently undergoing intensive fundamental and clinical research is the use of gene-modified tumor cells. For safety reasons most protocols in humans are performed with irradiated tumor cells which fortunately also enhances the immunogenicity of these tumor cells. To find out why the immunogenicity of γ-ray-irradiated tumor cells increases, Morel et al. irradiated HCC tumor cells developed in the HCC transgenic mouse model and analyzed expression of the B7-1 and B7-2 molecules. It was shown that γ-ray irradiation induced neoexpression of the B7-1 molecule while leaving the B7-2 unexpressed. This expression of the B7-1 molecule together with the expression of MHC class I molecules was found to be a prerequisite for the induction of systemic immunity using tumor cells as a vaccine. So, the enhanced immunogenicity found for many tumor cells after γ-ray irradiation is probably due to neoexpression of the B7-1 molecule.

In another SV40T transgenic mouse model used for immunotherapeutical purposes, the SV40T antigen is expressed under the control of the tyrosinase promoter. FVB/N mice transgenic for this TyrSV40T construct develop intraocular bilateral tumors that arise by in situ transformation of cells at the choroid/retinal pigmented epithelial (RPE) interface. These tumors possess the morphological characteristics of RPE carcinomas and express melanoma-associated antigens [143]. Ma et al. transplanted tumor cells derived from the TyrSV40T transgenic mice into the eyes of syngeneic FVB/N mice, from which subsequently TIL were isolated [144]. These TILs displayed in vitro a remarkable tumor-specific cytolytic activity. To test their ability in vivo, TIL as well as spleen cells derived form tumor cell immunized FVB/N mice, or spleen cells derived from normal control FVB/N mice, were adoptively transferred to immunocompromised FVB/N mice 10 days after induction of the intraocular tumors. Recipients of TIL and immunized spleen cells, but not recipients of normal spleen cells, acquired significant tumor specific CTL activity resulting in complete tumor rejection. To determine if these TIL were also capable of preventing the development of spontaneous hepatic metastases, TIL were adoptively transferred to anti-CD4/CD8 treated FVB/N mice 42 days after intraocular tumor transplantation. Hepatic metastases did not develop in any of the ten mice treated with TIL, whereas metastases did develop in all ten untreated control mice [145]. So, after in vitro expansion TIL isolated from intraocular tumors can be used in vivo by adoptive transfer as anti-tumor and anti-metastatic immunotherapeutical agents.

4.3. Neu oncogene transgenic mice

The Her2/*neu* protooncogene encodes a growth factor receptor ($p185^{neu}$) which overexpression appears to play a crucial role in the pathogenesis of many human cancers [146]. Strains of transgenic mice have been developed that express the rat *neu* oncogene under the control of the MMTV promoter, causing the development of metastasizable mammary tumors. Amici et al. used this transgenic breast cancer model to test whether an anti-$p185^{neu}$ immune response could be induced by in vivo muscle cell transfection of naked full-length rat *neu* oncogene encoding DNA [147]. DNA immunization of the tumor bearing mice caused heamorrhagic necrosis of established tumors, reducing the total tumor load. This immune response could be antibody mediated, since $p185^{neu}$ specific antibodies were present in sera obtained from the immunized mice and not in that of the control mice. Immunizing these trangenic mice with the extracellular domain of the p185neu protein induced anti-neu humoral as well as cellular immunity preventing tumor development in a significant number

of immunized animals [148]. Furthermore, this treatment was associated with a significant increase in median survival as compared to the control mice. Both reports demonstrate that transgenic animal studies can provide good models in which tumor immunotherapeutical strategies can be evaluated.

5. Transgenic mice expressing immune effector cell molecules

Immune effector cells can recognize tumor cells and initiate their elimination. For this the immune cell uses specific molecules. To further investigate the mechanisms involved in this immune activating transgenic mice have been generated expressing modified human immune effector molecules.

5.1. TCR transgenic mice

The classic TCR consist of an α and a β immunoglobulin chain and can mediate an activation signal upon recognition of an opposing cell carrying an antigenic peptide associated with a HLA class I or HLA class II molecule. However, in the absence of costimulatory signals generated by the ligation of CD8 or CD4, and CD28 molecule to their opposing ligand molecules, the former signal induces anergy of the T cell instead of activation. The balance between activation and anergy induction is often decisive for the development of anti-tumor immunity.

Malignant B cell immunoglobulins carry unique antigenic determinants called idiotopes (Id) in their variable regions, which can function as TSA. Immunization studies with such immunoglobulins have shown that this could confer Id-specific resistance to plasmacytomas and B cell lymphomas [120,149]. The Id derived peptides, processed either by professional antigen-presenting cells or by the B cell lymphoma itself, are presented on MHC class II molecules to $CD4^+$ T cells. To study whether circulating naïve $CD4^+$ T cells can home to a tumor region and respond to Id-peptides Lauritzsen et al. established BALB/c mice transgenic for the αβ TCR specific for the $\lambda 2^{315}$ light-chain Id-peptide of the MOPC315 myeloma protein [150,151]. In these mice 15–20% of the lymph node $CD4^+$ T cells express the transgenic αβ TCR. The transgenic $CD4^+$ T cells display the characteristics of naïve class II restricted T cells in normal mice proving that these mice are appropriate to study the role of naïve T cells in immunosurveillance. By challenging the mice with different tumors it was demonstrated that the transgenic mice were specifically protected against $\lambda 2^{315}$-expressing tumors. A drawback of this approach is that these transgenic mice harbor an abnormal high number of specific T cells. To study the Id-peptide specific naïve T cell mediated tumor protection more physiological conditions, lymph node cells derived from the transgenic mice were adoptively transferred to T cell devoid *scid* mice. It was shown that 2×10^6 lymph node cells containing 4×10^5 $CD4^+$ and 2×10^5 $CD8^+$ T cells were sufficient for tumor protection. Furthermore, $CD4^+$ T cells were essential for tumor resistance while elimination of the $CD8^+$ cells only partially protected against tumor growth. However, the influence of anti-Id antibodies or other normal T cells is unclear in this tumor protection model. Therefore, Bogen et al. made the $\lambda 2^{315}$ light-chain Id-peptide specific TCR transgenic mice homozygous for the *scid* mutation [152]. Though these mice lack in general B and T cells, they can generate $CD4^+$ and $CD4^-8^-$ cells expressing the transgenic αβ TCR. These transgenic SCID mice are protected against a challenge with MOPC315 plasmacytoma cells, demonstrating that B cells and non-TSA specific TCR containing T cells are not essential for effective immunosurveillance. Adoptive transfer experiments demonstrated that highly purified transgenic SCID $CD4^+$ cells were sufficient for tumor protection.

Upon confrontation with antigens, antigen-specific T cells (AST) at first expand but then disappear from the periphery. This disappearance is assumed to be due to clonal deletion via activation-induced apoptosis (AIA). To study this AIA and the peripheral tolerance of the remaining antigen specific T cells Zhang et al. used anti-mouse MHC class I L^d TCR-transgenic cells from 2C F1 transgenic mice [153] and adoptively transferred these cells into SCID mice which express the L^d antigen on all nucleated cells [149]. It was found that after encountering the antigen in vivo the number of AST increased 10–15-fold followed by a decline that was proven to be due to apoptosis. Whether Fas plays a role in this AIA or not remains to be elucidated although expression of Fas on the Tg^+CD8^+ cells increased upon activation. The remaining Tg^+CD8^+ cells had down regulated both the TCR and CD8 molecules and were fully unresponsive to restimulation with L^{d+} cells in the presence of IL-2 and IL-4. By blocking the costimulatory signal between CD28 on the T cell and its ligand on the APC it was shown that differences in signaling between T cell and APC could directly change the fate of AST since in this situation proliferation of the AST was inhibited. The behavior of antigen-specific T cells upon antigen confrontation in vivo provides us with more knowledge about the mechanism by which peripheral tolerance can be induced and for tumor immunotherpeutical interests how this tolerance can be broken.

To preclude the expression of endogenous TCR genes and to ensure a naïve T cell phenotype as background, Manning et al. bred the 2C TCR transgenic mice [153] to a recombinase activating gene 2 (Rag2)

deficient background. These 2C x Rag2-deficient (2C/Rag) mice successfully rejected L^d-expressing tumors induced by p815 mastocytoma cells, even after blocking the CD28 costimulatory signal, thus in a B7-independent way [154,155]. Since it was shown in CD28 deficient knock-out mice ($CD28^{-/-}$) that it was possible to mount an effective cellular immune response in vivo in the absence of CD28 [156] it is likely that alternative costimulatory molecules are operative that can compensate for the absence of CD28. Another counter-receptor for B7-1 and B7-2 is the cytotoxic T lymphocyte antigen 4 (CTLA4), which in contrast to CD28 appears to inhibit T cell activation [157]. This was demonstrated in vivo by the generation of CTLA4-deficient knock-out mice ($CTLA4^{-/-}$) which die within several weeks of age due to a profound lymphoproliferative syndrome [158]. Furthermore, blockade of the CTLA4 interactions by a Mab promotes tumor rejection in vivo [159,160]. To elucidate the inhibitory regulating mechanism of CTLA4 Fallarino et al. developed an experimental transgenic mice model by breeding the 2C/Rag2 mice with CD28 deficient knock-out mice [161]. In these mice it was possible to determine whether CTLA4 could inhibit antigen-specific T cell functioning in the absence of CD28. Interestingly, these $2C/Rag2/CD28^{-/-}$ transgenic mice successfully rejected L^d-expressing p815 tumors. T cells obtained from both $2C/Rag2/CD28^{+/+}$ and $2C/Rag2/CD28^{-/-}$ after tumor rejection proliferated in vitro in response to p815 expressing cells lacking B7-1. By costimulation with B7-1 the proliferative response of $2C/Rag2/CD28^{+/+}$ derived T cells could be increased, although the proliferative response of $2C/Rag2/CD28^{-/-}$ derived T cells was inhibited. This inhibition could be counteracted by Mabs against B7-1 or CTLA4, indicating that blockade of the TCR-CD3-mediated signal is the mechanism by which CTLA4 displays its inhibitory effect on T cell functioning. These results help to understand the modulation of T cells by CTLA4 which should aid in the development of agents that stimulate ongoing immune responses.

5.2. B7-1 transgenic mice

Although B7-1 transgenic mice are usually generated to study the pathology of autoimmune diseases, the information gathered by these models has also implications for tumor immunotherapy. To explore the need for co-stimulation in autoimmunity Allison et al. generated mice transgenic for B7-1 [162]. These $C57Bl/6^{BM1}$ mice expressed the B7-1 cDNA directed by the rat insulin promoter (RIP) and SV40 polyadenylation and termination sequences either in all (confluent) or only a few (patchy) islet β cells. By breeding these mice with RIP-IL-2 [163] transgenic mice, RIP-B7-1-IL-2 transgenic mice were generated which expressed constitutively IL-2 and B7-1 in their islet β cells, resulting in the development of in early spontaneous autoimmunity. Islets with IL-2 and confluent B7-1 expression were destroyed whereas islets with IL-2 and patchy B7-1 expression showed selective killing on the $B7\text{-}1^+$ β cells. Islet-reactive T cells rejected syngeneic islet grafts, but only if these expressed B7-1. These results suggest that for the eradication of a tumor it might be necessary to continuously stimulate the TAA-specific $CD8^+$ T cells locally by B7 molecules.

5.3. Fc-receptor transgenic mice

Although, most cancer immunotherapeutical approaches focus on the recruitment of T cells it has become evident that other immune-effector cells have cytolytic potential to a large variety of tumor cells and can be targeted to the tumor site as well. Myeloid cells are known to play an active role in the rejection of malignant cells [164]. These myeloid cells are activated via various trigger molecules, e.g. the IgG Fc receptor (FcγR). Various stimuli are operative and needed for directing these cells to the site of the tumor. They do not need to be preactivated and appear to be triggered solely upon cross-linking of the receptor [165]. To study the immunotherapeutical potential of these cells Heijnen et al. generated a transgenic FcγRI mouse model expressing the human genomic FcγRIA gene under control of its endogenous regulatory sequences in a FVB/N background [166]. Like in humans, FcγRI expression was found on monocytes, macrophages and activated neutrophils, and not on lymphocytes and mast cells. The FcγRI receptor expression level could be regulated by IFN-γ, granulocyte colony-stimulating factor (G-CSF), IL-4 and IL-10 and the receptor efficiently targeted in vivo by anti-FcγR antibodies [166,167]. After immunization with an anti-hFcγRI antibody containing antigenic determinants antigen-specific antibodies were detected in sera of these transgenic mice, demonstrating that the hFcγRI receptor was capable of eliciting a proper antibody response. Polymorphonuclear neutrophils (PMN) are the leukocyte subset potentially expressing FcγRI the most and their numbers can be enhanced by stimulation with G-CSF. Furthermore, their Fcγ receptors can trigger antibody-dependent cellular cytotoxicity (ADCC; Fig. 1) upon ligation to the FCγ region of a monomeric IgG1 or IgG3 molecule. Expression of the FcγRI offers the possibility to link anti-tumor Mabs directly to G-CSF primed PMN generating target specific effector cells. However, high concentrations of endogenous IgG can inhibit the binding of therapeutic Mabs to the FCγR. To circumvent this IgG blockade Heijnen et al. created a bispecific Ab (BsAb), directed to the HER-2/neu protooncogene product, and to the FcγRI, which binds outside the FcγRI ligand-binding site [168]. Using the hFcγRI transgenic mice they demonstrated that the

BsAb was able to engage the FcγRI in vivo and remained attached to the PMN during migration to inflammatory sites. After isolation, these PMN specifically lysed Her2/neu-expressing tumor cells demonstrating the potential of targeting anti-FcγRI Bsabs to G-CSF-primed PMN in vivo, subsequently targeting the antigen expressing tumor.

Finally, Heijnen et al. established a hFcγRI-transgenic B cell lymphoma bearing mouse model by backcrossing the transgenic FVB/N mice with wild-type BALB/c mice followed by induction of the tumors via i.v. injection of the IIA1.6 B cell lymphoma cell line [169]. Furthermore, they had generated a BsAb with a combined specificity for hFcγRI and MHC class II that induces killing of these murine B lymphoma cells in vitro by macrophages and G-CSF primed neutrophils isolated from the hFcγRI transgenic mice [170]. Treatment of the established hybrid transgenic mice with this Bsab in combination with G-CSF reduced the rate of B cell lymphoma outgrowth in vivo. Treatment of the hybrid transgenic mice with either BsAb or G-CSF or combined treatment in hybrid nontransgenic mice had no significant effect on the tumor outgrowth. These results suggest that an increased neutrophil number and an enhanced FcγRI expression are required for optimal FcγRI-mediated anti-tumor effects in vivo. A comparable combination of FcγRI-directed BsAb and growth factors may be suitable for successful FcγRI-mediated immunotherapy of cancer in humans, which are ongoing at present.

6. Concluding remarks

Transgenic mouse models to study anti-cancer immunotherapeutical strategies have allowed one to study the effects of different immunotherapy approaches on cancer. The mouse models used can be divided into four groups, the TSA or TAA, the HLA, the oncogene, and the immune effector molecule transgenic mice, which are each of particular interest to a specific immunotherapeutical approach. Results obtained using these mice have already found their ways to the patient, as they are currently being further evaluated in clinical settings. Although TAA and oncogene expressing mice are extremely useful for the study of therapeutical efficacy versus toxic effects, and HLA and effector molecule transgenic mice to study the mechanisms involved in succesfull immunotherapy without the hindrance of tolerance, crossing these mice in the future will lead to the generation of animal models even more relevant to the treatment of human disease. Contradictory results obtained by several groups, like about the necessity of peptides to have a high binding affinity to HLA molecules to induce effective CTL responses, or the use of adenoviral vectors, demand further preclinical investigations. Of great interest are the findings obtained using late tumor bearing mice demonstrating that the choice for either a passive or an active immunotherapeutical approach is dependent on the stage of tumor progression. These results, however, have to be interpreted with caution since curable strategies in mice are not always applicable in human, like the adoptive transfer strategy in the MUC1 expressing transgenic mice. In addition, the success of an immunotherapeutical approach seems to be influenced by the strain used, although strain dependent factors not always hamper the experimental value of a the model. Given the many constraints in setting up human clinical trials transgenic mice are, despite their limitations useful preclinical systems for evaluating anti-cancer immunotherapeutical strategies.

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Biographies

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