p53 Specific (auto)immunity in mice

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Chapter 1

General introduction

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General introduction

The innate and adaptive immune system work in concert to protect an organism from pathogens such as parasites, fungi, bacteria and viruses. The first line of immunological defense of an organism is the innate immune system consisting of dendritic cells, macrophages, neutrophils, basophils, eosinophils and natural killer cells. Most of these cells bear receptors that are specialized in sensing 'danger signals' present on the pathogen. The adaptive immune system, generates immunological memory and consists of B cells and T cells which are able to recognize dangerous pathogens on the basis of subtle antigenic differences compared to harmless self-structures. B cells secrete neutralizing immunoglobulins upon recognition of dangerous antigens in the body fluids. T cells indirectly sense the presence of dangerous pathogens via their T-cell receptor (TCR). CD8+ T cells (cytotoxic T lymphocytes, CTL) recognize MHC class I molecules bearing short peptides present on all nucleated cells and CD4+ T cells (T helper cells) interact via their TCR with MHC class II molecules bearing peptides present on professional antigen presenting cells (APC). Via the same mechanism the immune system is also able to recognize and kill tumor cells. Therefore anti-tumor therapy involving the immune system has been explored extensively in the past decades.

Cells involved in tumor recognition

For an effective anti-tumor response a triad of immunological cells communicate with each other; dendritic cells (DC), CD4+ T cells and CD8+ T cells (Figure 1). Most tumors do not express MHC class II molecules, and can therefore not be directly recognized by CD4+ T helper cells. However, when tumor debris is engulfed by a DC and presented in its MHC class II, it can initiate a CD4+ T-helper response (Figure 1). Factors in the environment of the dying tumor cells can cause DC maturation including up-regulation of co-stimulatory molecules and improved antigen presentation by DC (Figure 1). T helper cells activate DC via receptor-ligand interactions such as CD40-CD40 ligand (CD40L) binding (1). In addition, activated CD4+ cells produce cytokines such as IL-2 and IFN-γ that improve T-cell proliferation and antigen presentation by DC. The activated DC and CD4+ cell provide the basis for efficient CTL priming (Figure 1). Induction of a specific T-helper cell response is essential for an effective anti-tumor response by cytotoxic T lymphocytes (CTL) (2), even when the tumor does not express MHC II (3). For instance, IL-2 produced by T helper cells improves CD8+ T-cell priming and survival (4). In addition, CD4+ T cells are able to recruit members of the innate immune system such as macrophages and eosinophils (5). Together these events can result in the recognition and subsequent lysis of the tumor cell by the CTL (Figure 1).

After initial antigen encounter and proper co-stimulation, CD8+ T cells can become long-lived memory cells. An effective generation of CD8+ memory T cells follows a successive program of 1) initial stimulation with proper co-stimulation by DC, clonal expansion, 2) tissue homing, cytokine production and cytolytic function (production of perforins and granzymes), 3) a contraction phase with apoptosis of antigen specific CD8+ T cells, 4) generation of a persistent population of antigen experienced memory CD8+ T cells. However, upon chronic antigen stimulation, CD8+ T cells do not undergo a contraction phase but instead CD8+ T cells become exhausted. Also in the absence of proper helper signals during the priming phase CTL become 'helpless' and do not undergo an instructional program to generate bona fide memory CD8+ T cells (6-8). In both cases CD8+ T cells lose their ability to divide upon renewed antigen exposure (reviewed in 9, 10) which is a key feature of long term protective immunity against infections or a tumor (reviewed in 11).

Anti-tumor immunity

The immune system is able to distinguish self (not foreign) from non-self (foreign) components, by recognizing subtle antigenic differences. To prevent destruction of healthy tissue, immunotherapy of cancer needs to focus on the altered antigen composition of tumors. Some tumors uniquely express non-self protein products as a result of a mutation (e.g. frameshift mutations) or viral infection (e.g. Human Papilloma Virus, HPV), so called tumor specific antigens (TSA). Most other tumors only express tumor associated auto-antigens (TAA). Certain tumors express TAA that are only expressed in immune privileged sites like testis, such as MAGE and NY-ESO-1 (reviewed in 12). Another group of TAA comprises differentiation antigens such as MART-1 (13), tyrosinase and gp100 (14), or self-proteins which are also present at low levels in somatic cells, and consists of antigens such as CEA (Carcino Embryonic Antigen, 15) and p53.

The tumor suppressor gene p53 is a potent inhibitor of cell growth and is sometimes referred to as guardian of the genome (16). Molecular changes that influence p53 stability and function (Figure 2) are important steps in cancer initiation and occur in a majority of

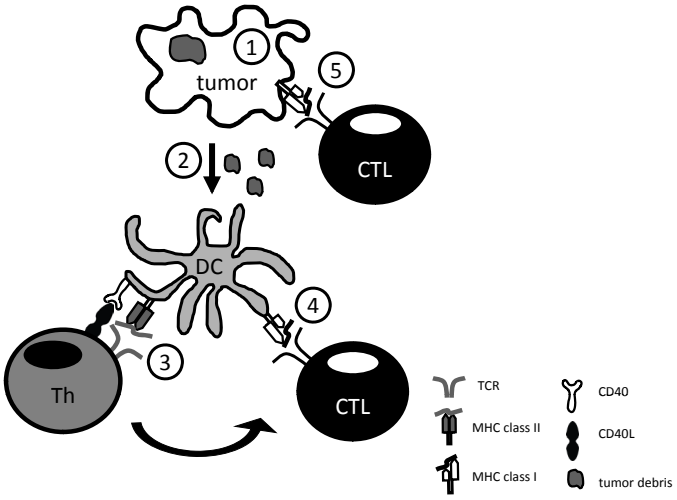

Figure 1. T cells can recognize direct- and cross-presented antigen derived from tumor cells

1) Fragments of tumor antigens are presented MHC class I of the tumor cell.
2) Debris from dying tumor cells is taken up by a DC, which then processes the tumor antigens into short peptides, that can be presented in MHC class I and MHC class II on the cell surface.
3) Tumor derived epitopes are presented in MHC class II to T helper cells, interaction via co-stimulatory molecules such as CD40-CD40L leads to cytokine production by T helper cells.
4) p53 epitopes are cross-presented in MHC class I to p53 specific CTL. Signals from T helper cells activate CTL to become effector cells.
5) Fully activated CTL can recognize tumor antigens on MHC class I of tumor cells. Subsequently the tumor cell can be killed by CTL.

human cancers. In up to fifty percent of all human tumors the p53 gene is mutated (17). Not only is there a great variability in the tumor cell types with a p53 mutation but also in the location of the mutation. Many of these mutations not only abrogate p53 function but also impair p53 breakdown, e.g. via the ubiquitin pathway (Figure 2). In addition, disturbed expression of proteins that regulate p53 stability, such as Mdm2, lead to increased p53 levels (18). As a result many tumors harbor high levels of (mutated) p53. Because of its frequent aberration and association with malignant transformation, p53 is considered to be and interesting target for immunotherapy of cancer.

Besides somatic mutations, there are examples of germ-line mutations in p53. This hereditary form of cancer is called the Li-Fraumeni syndrome (19) and is associated with a predisposition to various types of cancer, often at a very early age (20, 21).

Over-expression of wild-type and mutant tumor antigens

The availability and presentation of a tumor antigen is essential for a successful T-cell mediated tumor attack. Under normal conditions p53 protein stability is tightly regulated and wild-type (wt) p53 has a very low steady-state level (Figure 2 and 3). A mutation in p53

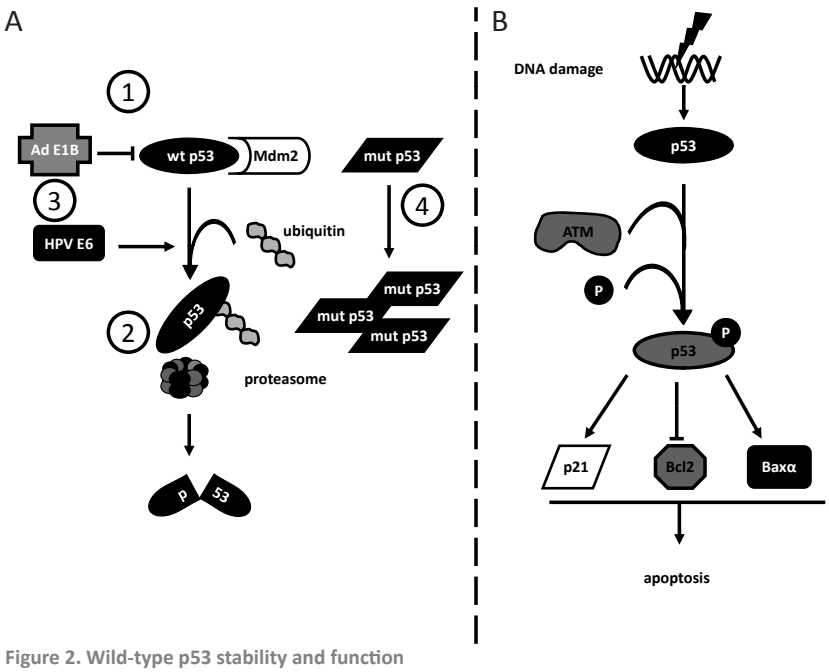

Figure 2. Wild-type p53 stability and function

A) Under normal conditions wild-type p53 is rapidly degraded under influence of regulatory molecules such as Mdm2 (*1*), which inhibits p53 function by binding to the region involved in transcription regulation and by direction to the ubiquitin degradation pathway (*2*, references 22 and 23). In several virally induced tumors p53 function is inhibited by oncogenic viral proteins (*3*). For instance, adenovirus type 5 E1B stabilizes and inactivates p53 (reference 24), while the human papilloma virus type 16 E6 protein increases the ubiquitin dependent degradation of p53 (references 25 and 26). Mutated p53 protein is usually not rapidly degraded but accumulates (*4*).
B) When DNA damage occurs, p53 is stabilized by phosphorylation (reviewed in 27). Subsequently p53 modulates the function of molecules involved in apoptosis such as Baxα, p21 and Bcl-2. This way DNA repair can take place or cell apoptosis.

can cause a conformational change of p53 protein. This can result in the loss of availability of binding sites for proteins that regulate degradation, such as Mdm2, or chaperone proteins such as heat shock protein 70 (HSP70). This results in accumulated levels or mislocated mutated p53 and in some cases increased immunogenicity. For instance human p53 with a mutation at position 273 (murine homologue position 270) does not cause a conformational change, and does not result in binding to HSP70. In contrast, a mutation of human p53 at position 143 (murine position 140) and of murine p53 at position 135 results in strong conformational alterations and binding to HSP70 (28-30). The release of HSP70 complexes from dying tumor cells (Figure 3, point 4) and binding to DC provides adjuvant-like signals and enhances DC function (31, 32). Subsequently the matured DC can present processed p53 epitopes in MHC class I to CTL and in MHC class II to T helper cells (Figure 3).

The mutation type of other tumor-associated antigens could also have a crucial effect on steady-state protein levels and immunogenicity. Microsatellite instabilities (MSI) which are repetitive nucleotide sequences that are prone to small mutations during DNA replication, are of particular interest. MSI are the result of defective mismatch repair of microsatellites and are mainly found in colon, gastric and endometrial cancer (33-36). The mutations of MSI can result in a shift of the DNA reading frame and subsequent synthesis of mutated

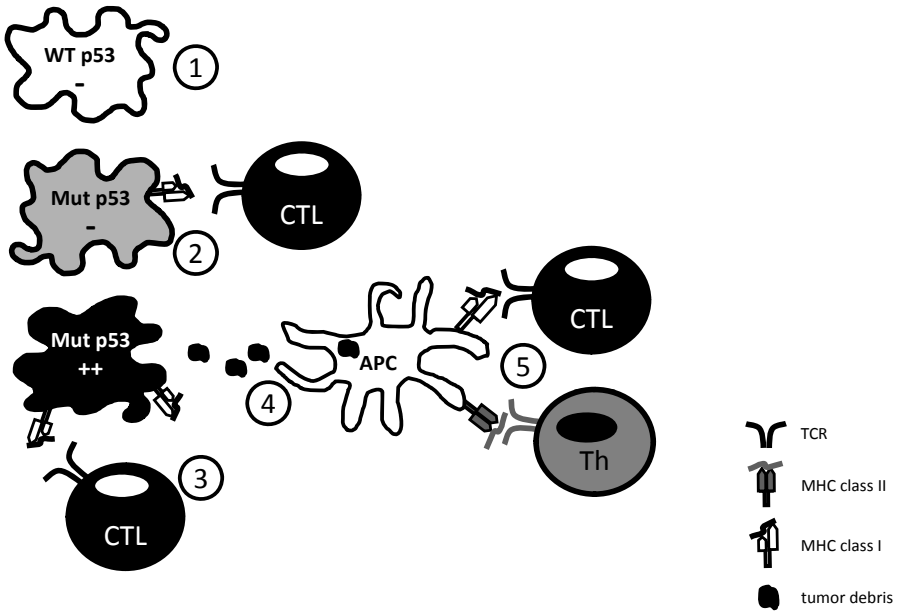

Figure 3. Immunogenicity of accumulated p53

1) Wild-type p53 is rapidly degraded and does not accumulate (-). Low levels of p53- MHC class I complexes are present on the cell surface.
2) Mutant p53 that is rapidly degraded and does not accumulate (-). Low levels of p53- MHC class I complexes are present on the cell surface. Direct recognition of the tumor cell by p53 specific CTL is unlikely to occur.
3) Mutant p53 that are not rapidly degraded lead to accumulated protein levels (++). Direct recognition by CTL can occur.
4) When tumor cells die, tumor debris containing accumulated mutant p53 is taken up by DC. By intracellular degradation, epitopes of mutant p53 are processed.
5) DC can present p53 into MHC class II to T helper cells and in MHC class I to CTL

protein that is partly non-self. The frameshifted products represent foreign antigens for the immune system. MSI tumors are associated with increased lymphocyte infiltrate, MHC class I down regulation and better survival prognosis compared to microsatellite stable tumors (37-39). Unfortunately, not much is known concerning the role of mutation of individual frameshift products on protein accumulation, MHC class I and MHC class II presentation and immunogenicity. For the development of immunotherapy strategies to target frameshift products it will be useful to predict and analyze the immunogenic potential of these antigens.

Central T-cell tolerance

To prevent potential self-reactivity and autoimmunity, T cells are selected in the thymus where they undergo tightly controlled maturation steps. During their migration from thymic cortex to thymic medulla, T cells start to express for instance CD3, CD4, CD8 and rearranged TCR molecules. Specialized APC present peptide/MHC complexes (pMHC) to the TCR of developing T cells. APC in the thymus are capable of presenting parts of all self-proteins,

even tissue specific antigens (40). Interaction between the developing T cell and the APC is necessary for T-cell survival and maturation. T cells that have a low affinity for self-pMHC on APC do not undergo positive selection (reviewed in 41). In contrast, T cells that recognize self-pMHC complexes with high affinity are eliminated by negative selection since they may cause damage to healthy tissue. Finally, only T cells that interact with pMHC on thymic APC with an intermediate affinity will be able to complete the thymic maturation process. (41, 42).

Antigen presentation in the thymus; split tolerance

Besides T cells, the thymus is populated by cortical thymic epithelial cells (cTEC), medullary thymic epithelial cells (mTEC), bone marrow derived cells and other stromal cells. Almost any pMHC expressing cell in the thymus can deliver death signals and thereby contribute to negative selection (43). Ubiquitously expressed self-antigens are probably presented as pMHC on all of these cells. However, the unique presence of a transcriptional regulator named Aire in mTEC (reviewed in 44), enables them to ectopically express and present tissue specific antigen (40, 45-47).

Negative selection of CD4+ T cells occurs via MHC class II –TCR interaction. Unlike expression of MHC class I, MHC class II is only expressed on specialized APC in the thymus. In addition to TEC, bone marrow derived, or hematopoietic APC play an important role in the negative selection of CD4+ T cells. These cells obtain their self-antigen exogenously and present it via cross presentation (48). It has been shown in two transgenic mouse models that negative selection of CD4+ T cells is only complete if the antigen could be cross presented by hematopoietic cells (49, 50).

Negative selection is the net result of T-cell maturation and the level of antigen presentation on thymic APC to T cells. Because p53 is ubiquitously expressed but rapidly degraded, it seems likely that the majority of p53 epitopes ends up in MHC class I molecules (Figure 4). This could result in strong negative selection of p53 specific CD8+ T cells in the thymus. Indeed it has been shown that the p53 specific CD8+ T-cell repertoire is severely blunted in p53 +/+ mice, with only very few low avidity p53 specific CTL remaining (51). Because of the rapid degradation and subsequent presentation of p53 in MHC class I, little p53 is left for MHC class II presentation (Figure 4). Furthermore, p53 does not accumulate sufficiently and can therefore not be cross-presented by thymic APC. Consequently MHC class II complexes bearing CD4+ T-cell epitopes of p53 will be scarce in the thymus. As a result, p53 specific CD4+ T-cell tolerance might be less stringent or even absent. In correlation with this, several studies have identified p53 CD4+ T cells in mice (52, 53). Because of this so called split tolerance, strategies to mobilize either p53 specific CD4+ or CD8+ T-cell populations for immunotherapy of cancer may differ.

p53 specific immunity in cancer patients

The presence of p53 specific immunity in mice largely correlates with p53 specific immunity in cancer patients. Spontaneous immunity to p53 was first discovered by detection of accumulated levels of p53 in tumor cells by incubation with serum of tumor bearing mice (54, 55). Subsequently, in the 1980's several reports described p53 specific antibodies in the sera of cancer patients (56, 57), and the number of reports describing this phenomenon have increased ever since (58). Several studies claim that an emerging p53 specific antibody

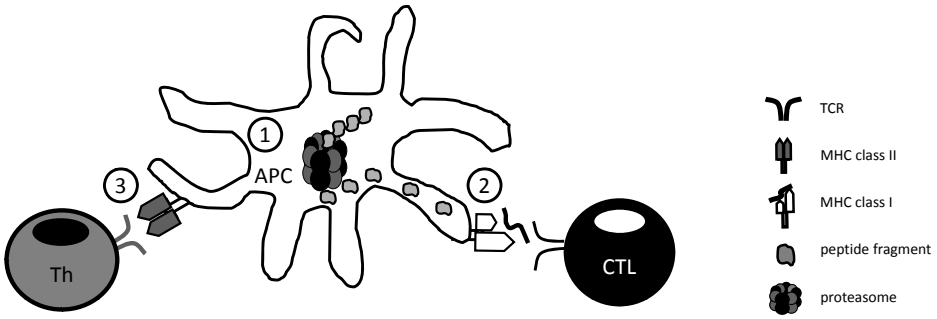

Figure 4. Split tolerance for p53

1) Thymic APC express p53 with a short half-life and directs the majority of p53 to the proteasome degradation pathway and MHC class I presentation.
2) CTL will encounter relatively high levels of p53-MHC class I complexes. Most p53 specific CTL are negatively selected in the thymus.
3) Because most p53 is rapidly processed and presented in MHC class I molecules, not much p53 is left for presentation in MHC class II. Furthermore p53 accumulation is too low for efficient cross presentation. p53 specific T helper cells are unlikely to encounter p53- MHC class II complexes in the thymus and do not undergo negative selection.

response is closely related to a mutation type causing conformational change of p53 protein and binding to HSP (59, 60). However, larger studies did not find this strict correlation, indicating that other factors determine the presence of a humoral immune response (58, and references therein). The numerous reports on the presence of IgG antibodies in the serum of cancer patients indicated an involvement of T helper cells since they are required for Ig isotype class switching. Indeed, spontaneous (61-63) and vaccine induced T-helper cell immunity to p53 (64) have been shown in cancer patients. Also in healthy blood donors T-helper cell reactivity could be measured (65, 66).

Convincing reports describing p53 specific CTL in cancer patients are scarce, in contrast to the great number of reports describing p53 T-helper and antibody responses. A few labs have reported their ability to generate p53 specific CTL in healthy donors (67-70). Studies on p53 specific CTL have mainly focused on patients with squamous cell carcinomas of the head and neck (SCCHN) (71, 72). These reports showed that the presence of high frequencies of p53 specific tetramer positive CTL correlated with low levels of p53 accumulation in the tumor cells (72). However, a high percentage of SCCHN tumors are positive for HPV (25), a virus that increases p53 turnover and presentation. Because p53 MHC class I presentation rather than accumulation is a requirement for recognition by CTL (73), CTL recognition of SCCHN tumors could be improved by HPV induced p53 degradation.

Active p53 CD4+ T-cell immunotherapy

For p53 directed immunotherapy of cancer, the endogenous p53 specific CD4+ T-cell repertoire of the patient could be exploited. Studies show that the CD4+ T-cell repertoire is present, albeit that it appears not to be skewed towards the proper CD4+ T helper cell cytokine production phenotype (62). Therefore, active immunotherapy is required, that redirects the CD4+ T cells towards a T-helper 1 phenotype that can assist in an anti-tumor

CTL response. This can be achieved by means of vaccination with the proper DC and T cell activating signals. The first requirement for an effective T-cell response is a properly activated DC. The innate immune system is especially suited to react to pathogen associated molecular patterns (PAMP), such as bacterial glyco-lipids or bacterial derived nucleotides (74). Mainly professional antigen presenting cells (APC) and macrophages express Toll-like receptor (TLR) that can recognize a specific type of PAMP, e.g. TLR 4 recognizes bacterial lipopolysaccharide (LPS) and TLR 9 recognizes unmethylated CpG DNA. To mimic recognition of PAMP leading to APC activation and subsequent T-cell activation, most modern vaccination cocktails include agents that bind and activate TLR (74).

When a properly activated APC presents the tumor antigen of interest, it may initiate a CD4+ T-helper immune response. To improve presentation of the tumor antigen, active immunotherapeutic vaccination can contain tumor antigen e.g. in the form of protein, peptides or even irradiated tumor cells. Tumor material and protein contain all possible T cell epitopes but also large non-relevant stretches and need to be extensively processed by DC. Long peptides spanning the entire tumor antigen can also be used to deliver extra antigen to DC. When immunodominant epitopes are known, injection of only several peptides spanning this region suffices. Translational studies in mice in our lab have indicated that especially long peptides, which require processing, induce superior immune responses (75). Furthermore, very promising results have been obtained with vaccination of cervical carcinoma patients with long peptides encompassing the human papilloma virus 16 E6 and E7 protein sequences (76, 77).

Passive p53 CD8+ T-cell immunotherapy

Since the endogenous p53 specific CD8+ T-cell repertoire appears to be blunted by negative selection (51), active immunotherapy by means of vaccination is difficult to achieve and is unlikely to be very effective. An alternative strategy to obtain high numbers of T cells is the isolation of autologous tumor-reactive T cells followed by *in vitro* expansion and subsequent adoptive T cell transfer (78, 79). However, the success of this approach strongly depends on the abundance of specific CTL, which is not the case for p53 CD8+ T cells. Therefore, a more feasible approach is the redirection of the repertoire by TCR gene transfer, derived form a non-tolerant setting (51). With this method αβTCR chains with a known specificity are expressed in naïve peripheral blood cells, which are subsequently reinfused. However, various safety issues have been raised for the infusion of TCR transduced T cells. First of all, separately introduced TCR Vα and TCR Vβ chains could pair to endogenous TCR chains and thereby forming new unknown specificities with potential auto-reactivity (80). Recent technical improvements have been made that circumvent pairing to endogenous TCR chains by introducing cysteine residues that form additional di-sulfide bridges (81, 82).

A second hurdle for successful adoptive T-cell therapy is the formation of a persistent memory T-cell population (83). This requires pre-treatment of T cells that avoids *in vivo* passive cell death (due to under-stimulation) as well as activation induced cell death (due to over-stimulation). In addition, transferred T cells need to be equipped with the suitable homing markers to migrate to target organs. A frequently used approach to improve the engraftment and prolonged survival of adoptively transferred T cells is prior lymphodepletion by chemotherapy. The effect of non-myeloablative lymphodepletion appears to be manifold. First of all, by depleting a part of the host lymphocyte population, there is literally more space for the transferred population to proliferate. More importantly, essential homeostatic

cytokines such as IL-7 and IL-15 become more available to the transferred T cells (84). Furthermore, the role of suppressor T regulatory cells, a lineage characterized by the elevated expression of FoxP3 and CD25 (IL-2Rα) (85), is impeded by lymphodepletion (86, 87). Finally, lymphodepletion by irradiation or chemotherapy causes an inflammatory endothelial cell response, facilitating entry of effector T cells into neoplastic lesions.

Thesis outline

Self-tolerance to p53 is a major potential limitation for the activation of the endogenous T-cell repertoire. So far, p53 specific CD8+ and CD4+ T-cell immunity has been described in cancer patients and healthy individuals. However, the restrictions of tolerance on the recruitment of p53 specific T cells have thus far not been completely elucidated. In this thesis we have studied several basic mechanisms that underlie the availability of p53 specific T-cell immunity and how this repertoire can be employed for immunotherapy against tumors.

In chapter 2 we have compared the presence and quality of the p53 specific T-helper cell repertoire in p53 deficient (p53-/-) mice and p53 wt (p53+/+) mice. By direct vaccination with p53, in a viral vector and as 30-mer peptides, we were able to determine the specificity and avidity of T-helper cell populations in the two mouse strains. We show that, unlike the repertoire to other tumor specific antigens such as CEA (88), the p53 specific T-helper cell repertoire in these mouse strains is not blunted by self-tolerance and therefore completely available for immunotherapy of cancer.

In chapter 3 we studied the correlation between intracellular accumulation and immunogenicity of several well-described frameshifted colon tumor antigens. Accumulation alone is not a sufficiently indicative marker for the immunogenic properties of a tumor antigen. By comparing intracellular accumulation, direct presentation and cross-presentation *in vivo* we were able to categorize several tumor antigens and to predict their potency as antigen targets for T helper cells or CTL.

In chapter 4 we analyzed the potential beneficial and harmful effects of p53 specific CD8+ T cells in detail. We generated a $p53^{158-166}$ specific TCR transgenic mouse and studied the effects of self-tolerance on p53 specific CD8+ T cells in chapter 4.1. We show that thymic selection has a drastic effect on the differentiation of p53 specific CD8+ T cells in p53 +/+ and p53 +/- mice. Education of p53 specific CD8+ T cells in the absence of p53 (in p53-/- mice) leads to a potent population of peripheral p53 specific CD8+ T cells. We studied the *in vivo* effects of these p53 specific CD8+ T cells in chapter 4.2. In an supplemental chapter 4.3 we show the expression and capacities cells expressing the p53 TCR after retroviral gene transfer. Our data in chapter 4 indicate that immunotherapy with p53 specific CD8+ T cells can lead to acute and severe hematopoietic ablation. These data stress the importance of meticulous pre-clinical research when targeting ubiquitously expressed self-antigens, before starting clinical application. Successful cancer immunotherapy in the absence of any toxicity could nevertheless be achieved by combination of p53 CD8+ T cell transfer and hematopoietic reconstitution with non-sensitive (allogeneic) bone marrow stem cells.

A general discussion of this thesis in chapter 5 describes the caveats, challenges and pitfalls of the findings portrayed in this thesis and places them in the context of recent literature.

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