Chapter 5

Discussion
The influence of tumor antigen expression on presentation and T-cell activation

Tumor antigen stability and accumulation can influence the ‘visibility’ of the tumor for the immune system. In chapter 3 of this thesis we discuss three scenarios (see also Chapter 1, Figure 3) of frame-shifted tumor antigen stability and availability and the impact on the immune system.

In the first scenario, tumor antigens are expressed at low levels and will be presented at low levels via direct presentation in MHC class I. Consequently, low antigens levels are available on DC for the efficient induction of a T-cell response. These antigens, among which several mutant forms of p53, will be very difficult to target via immunotherapy.

In the second tumor antigen availability scenario, a tumor antigen is presented efficiently in MHC class I, for instance due to high protein turnover, and the tumor can readily be recognized by CD8+ T cells. However, due to absence of protein accumulation, antigen is not available for cross-presentation on DC and consequently proper T-cell activation is lacking. In the absence of self-tolerance for CD8+ T cells, such as some frameshift antigens, this population can be expanded by active immunotherapy to generate a large pool of cytolytic CD8+ T cells. In case of strong CD8+ T-cell tolerance, such as in case of p53, the endogenous repertoire is absent and passive immunotherapy by adoptive T cell transfer needs to be applied.

In the third scenario, a tumor antigen accumulates and is directly presented in MHC class I, but also cross-presented in MHC class I and II of DC. In this situation both CD4+ and CD8+ T cells can be activated by DC and effective T-cell responses can arise spontaneously, provided that there is no T-cell tolerance. In this setting, active cell therapy by means of vaccination can be applied to improve the anti-tumor T-cell activity. Obviously this will be the most successful scenario, however for many tumor antigens such as p53 the T-cell repertoire is (partially) tolerized. Nevertheless, even in case of T-cell tolerance, passive cell therapy by adoptive T cell transfer in this last antigen scenario could also lead to a robust T-cell response since the tumor material is extensively available by efficient MHC class I and MHC class II presentation on DC.

Tumor antigen expression in the thymus and T-helper cell tolerance

T-helper cell responses to p53 in human beings have been demonstrated in many different studies (1-6). Yet these reports do not exclude the possibility that such immunity reflects only low avidity responses that remain after central tolerance. In chapter 2 we conclude that the repertoire, specificity and avidity of T helper cells are not affected by tolerance to p53 and can be fully exploited for immunotherapy of cancer. Spontaneous p53 immunity in cancer patients usually consists of a weak unpolarized T-helper cell response (4, 6). Active immunotherapy through vaccination will therefore need to enhance and skew towards a T-helper 1 response that is capable of orchestrating an effective anti-tumor response.

The impact of self-tolerance on the diversity of the T-helper cell repertoire varies between different tumor antigens. Whereas we find no tolerance to p53 specific T helper cells, the T-helper cell repertoire against Carcino Embryonic Antigen (CEA) is strongly weakened by tolerance. Research in our lab has shown that due to expression of CEA by mTEC in the thymus and in the periphery of CEA transgenic mice, the CEA specific CD4+ T-cell repertoire is impaired (7). Several immunodominant CEA epitopes, which are recognized by CD4+ T cells in wild-type (wt) mice, were not recognized in CEA transgenic
mice. Furthermore, the avidity of the remaining CEA specific CD4+ T cells appeared to be lower in CEA-tg mice than in wt mice. The discrepancy between the T-helper cell immunity against these two tumor antigens is probably the result of differences in protein stability and presentation. The ubiquitously expressed antigen p53 is very unstable and predominantly presented in MHC class I, also in the thymus. Since very little p53 is left for presentation in MHC class II, p53 specific T helper cells will not likely encounter their epitope and will not be negatively selected in the thymus. In contrast, CEA is a large molecule with a long half-life which is mainly expressed in the colon. In the thymus CEA is efficiently expressed by mTEC (7) and in the periphery CEA is shed from the colonic plasma membrane and taken up by DC. Consequently, CEA specific T helper cells are confronted with CEA presentation on MHC class II in the thymus and in the periphery. CEA specific T-helper cell immunotherapy thus has to involve reconstitution of the T helper cells, whereas p53 immunotherapy can involve active immunotherapy of T helper cells by means of vaccination. Therefore, despite the fact that CEA and mutant p53 both accumulate and are efficiently presented (antigen scenario 3, paragraph 1) they require a different approach for successful T-helper cell immunotherapy.

In chapter 2 of this thesis we show that it is possible to induce a high avidity p53 T-helper cell response in vaccinated mice. Clinical trials for the induction of T-helper cell responses in cancer patients are currently ongoing in collaboration with our department which will hopefully confirm the clinical applicability of p53 T-helper cell activation by vaccination for the treatment of cancer.

**Risks of p53 specific CTL immunotherapy**

Several reports have described the existence of p53 specific CTL in healthy donors (8-13). However, compared to the plethora of studies describing p53 specific T-helper cell related immunity only few studies describe the existence of a p53 specific CTL response in cancer patients, most of which suffering from head and neck cancer (14, 15). Important insights on the availability of the CD8+ repertoire in cancer patients have been obtained by using HLA-A2 transgenic p53 +/+ and p53 -/- mice. Studies on the quality and quantity of the inducible CD8+ response in these mice show that the p53 CD8+ T-cell repertoire is impaired by tolerance (16) but the T-cell response can be somewhat improved by CTLA-4 blockade (17). Considering the blunting effect of self-tolerance it seems unlikely that the endogenous p53 CTL repertoire of cancer patients can mediate a strong anti-tumor effect. Therefore, several labs are currently improving the strategies for adoptive transfer of genetically engineered p53 specific CTL to treat cancer patients (18-20). To circumvent tolerance, the TCR that is used in these studies was originally generated by injection of human p53 in a HLA-A2 transgenic p53 -/- mouse (16). In chapter 4 we show that vaccination induced expansion of adoptively transferred p53-/- TCR-tg cells induces severe ablation of the host hematopoietic system. When we extrapolate our murine p53 TCR-tg results to p53 TCR CD8+ therapy of cancer patients this would potentially have life threatening consequences. It is possible that there are intrinsic differences between our murine p53 TCR (mu p53 TCR) and the chimeric HLA-A2 p53 TCR (hu p53 TCR), for instance on the level of avidity. The avidity of a TCR-pMHC I complex involves the binding and signaling of TCR, CD8, CD3, MHC class I and peptide. In addition, cytokine signals modify CD8 co-receptor expression (21) and accessory molecules such as CTLA-4 regulate the TCR threshold (22). The CD8 co-receptor enhances the sensitivity of a TCR to an antigen by phosphorylation of the CD3 ζ chain (23) to enable even low avidity TCR to initiate T-cell activation. TCR with an intrinsically high avidity do not
need a co-receptor signal to enhance sensitivity for pMHC I. As a result CD8 co-receptor
dependence and TCR-pMHC I affinity are inversely correlated (24). Forced expression of the
transgenic mu p53 TCR could result in extravagantly high expression levels of the mu p53 TCR
in p53 TCR-tg mice, resulting in TCR clustering and allowing co-receptor independent TCR
signaling (25). However, drastic lowering of the activation threshold will most likely result
in TCR activation that is CD8 co-receptor independent. The mu p53 TCR is CD8 co-receptor
dependent since p53 TCR-tg CD4+ T cells express the transgenic p53 TCR but fail to respond
to antigenic stimulation. In contrast, the hu p53 TCR is CD8 co-receptor independent and of
high avidity because it was generated in a HLA-A2 transgenic mouse in which the HLA-A2
is unable to interact with the murine CD8 molecule (18, 26). Obviously, successful adoptive
T-cell therapy seeks to use the most powerful or highly avid TCR available (27). However it
remains a question whether this is a desirable approach for p53-targeted immunotherapy,
since high avidity TCR might also recognize low steady-state levels of p53 on healthy cells.

A second restraint for successful adoptive immunotherapy by p53 TCR is killing of
‘brother’ cells, or fratricide (28). As we show in chapter 4, cells bearing a mu p53 TCR are
able to recognize neighboring cells in an autologous setting, in which other cells also express
the MHC-peptide complex. Potentially, fratricide will reduce the effective number of p53
CTL, consequently p53 TCR therapy in an autologous setting will have a limited effect.
In my opinion, the use of a p53 TCR in general and this HLA-A2 p53 TCR gene in particular,
should not be used for clinical application at this point. When p53 is considered as a CTL
target major safety and feasibility issues first need to be solved by translational research.

Successful and safe application of TCR gene transfer

The development of adoptive cell transfer (ACT) therapies for anti-tumor therapy is advancing
quickly. Both donor lymphocyte infusion (DLI) and tumor infiltrating lymphocytes (TIL) have
been applied for quite some time (29-32). A major potential side effect of polyclonal ACT is
graft versus host disease (GVHD), for instance directed against minor antigenic differences.
A possible solution to minimize the risk for GVHD is the ACT of a pure population of
specific (engineered) T cells. In combination with the careful selection of tumor antigen
this could result in a successful graft versus tumor (GVT), and low incidence of GVHD. A
promising candidate for safe treatment of hematologic malignancies is the targeting of
minor histocompatibility antigen HA, which is only expressed by hematologic cells (33).
By expression of the mHAg HA TCR in purified autologous cells (34, 35) the treatment
is expected to induce maximal GVT and minimal GVHD.

Genetically modified T cells could cause autoimmunity as a result of hetero-dimerization
of separately introduced TCR chains with endogenous TCR chains (reviewed in 36). Recently
new technologies have been developed to force dimerization of the introduced TCR for
example by introducing additional cysteine bonds which form di-sulfide bridges (19, 37)
or by molecular design of unique steric interaction (38). Another possibility to prevent
dimerization of endogenous and exogenous TCR components is transduction in cells that do
not (yet) express endogenous TCRαβ chains such as γδ T cells or hematopoietic stem cells
(39-42).

To terminate unwanted self-specific T-cell reactivity, a safety ‘escape’ can be introduced
for instance by addition of a molecular tag (43) or a suicide switch under control of a drug
sensitive promoter (44). This way the engineered T cells undergo apoptosis upon activation
of the promoter after drug delivery. A frequently used construct is the herpes simplex virus
thymidine kinase (HSV-TK) promoter, which is activated upon administration of the pro-drug Ganciclovir (44-47). Initially the use of the HSV-TK construct caused a transgene specific immune response (48, 49) which would complicate a secondary transfer with the same construct. However, recent technical advances have been made to alter or delete the major immunogenic regions (50), to enable even a safe secondary transfer with HSV-TK transduced cells. If immunotherapy with engineered p53 CTL is considered, a suicide switch will be necessary to prevent potential escalating attack of healthy tissue with low p53 expression levels.

The homing and survival of engineered T cells largely determines the long-term effect of ACT. Retroviral transduction requires active division of T cells, a process that also promotes T-cell differentiation but subsequently reduces cell survival in vivo. To enhance the efficacy and survival of engineered T cells several techniques have been developed to improve survival of the engineered T-cell population, for instance by using gene delivery systems that do not require active cell division such as lentiviral transduction (51). Other lines of research explore the possibilities of transducing undifferentiated hematopoietic stem cells (40-42), or modulation of transcription factors that might revert cell differentiation (52, 53).

The safe application of p53 specific CD8+ T-cell therapy should involve several additional precautions. As we show in chapter 4, p53 CTL therapy can efficiently prevent tumor growth but also eliminates the host hematopoietic system. Therefore, patients should be reconstituted with bone marrow stem cells which are not sensitive to CTL mediated killing. In clinical terms this would mean allogeneic hematopoietic stem cell transplantation. This is a frequently used therapy for the treatment of hematological malignancies, but it can also evoke high grade GVHD and is therefore not without risk. In addition, the engineered p53 TCR needs to be transferred into allogeneic T cells to prevent fratricide. Importantly, at this point it remains unclear why exclusively hematopoietic tissue destruction occurs. Personally, I would give priority to studying the fundamental mechanisms that underlie this apparently unique sensitivity of hematopoietic tissue to p53 CTL kill, before aiming at clinical application of p53 CTL. In my opinion it would be better at this moment to consider other, truly tissue specific antigens, such as HA for the targeting of hematologic tumors.

**p53 turnover in hematopoietic cells explains sensitivity to p53 specific killing**

Based on previous work by our lab and others showing safe application of a p53 CTL clone (54, 55), we did not expect such vigorous ablative effects of p53 CTL on non-tumorous tissue as described in chapter 4. However, the in vivo effects of long-term cultured clones are not comparable to those of naïve TCR-tg cells. In most cases clones are over-stimulated and exhausted and will therefore probably have a limited lifespan and cytolytic activity in vivo (56, 57). In contrast, naïve TCR-tg cells have the proper homing signals to reside in the blood and will first develop into effector cells before becoming exhausted. Others have also tried to confirm the safe application of T-cell therapy against ubiquitously expressed self-antigens such as MDM2 (58) by using clones. In retrospect, targeting of p53, and probably also other tumor antigens, by using T-cell clones potentially does not fully expose all potential effects. A true comparison between the p53 TCR-tg cells and the p53 CTL clone should be performed to investigate this further.

Despite the robust expansion potential of p53-/- TCR-tg cells it remains an enigma why only hematopoietic cells and no other tissues are destroyed. We were aware however, that not just steady-state levels, but mainly availability of p53 in MHC class I is a determining
factor in the recognition of tumor cells by p53 CTL (59). It is possible that p53 MHC class I presentation levels are increased in specific tissues with high cell division and differentiation that require increased ‘alertness’ of a DNA integrity guardian such as p53. Tumor suppressor genes and other proteins involved in cell cycle regulation can quickly restore transcriptional errors, which occur often in highly proliferative tissue. The hematopoietic system is a tissue with high cell division and differentiation. Indeed it has been shown that p53 RNA levels are relatively high in spleen cells, whereas p53 protein levels are not increased compared to other tissues (60). It is therefore possible that spleen cells present relatively high levels of p53 epitopes in MHC class I compared to non-hematopoietic tissue. The important role of p53 expression during hematopoiesis is furthermore confirmed by studies on p53-/- mice. First of all, mice deficient for p53 are prone to develop spontaneous tumor, especially of lymphoid origin (61, 62). Recently it was discovered that p53 induces expression and function of leukemia inhibitory factor (LIF), a cytokine that regulates the differentiation of myeloid leukemic cells and various embryonic and endothelial cell types (63). In addition, mice deficient for p53 show impaired hematopoietic regenerative capacity (64). These data confirm that p53 particularly plays a crucial role as cell cycle regulator and tumor suppressor during hematopoietic differentiation.

As discussed in chapter 4, vaccination-induced expansion of p53 specific CTL in our model resulted in the selective destruction of hematopoietic cells and not other tissues. It is conceivable that the vaccination mixture only provides the hematopoietic compartment the proper danger signals to become sensitive to p53 directed killing. To further determine the critical factors in our vaccination mixture that induced p53 specific CTL proliferation we performed additional experiments. As described in chapter 4, CFSE labeled p53-/- TCR-tg cells also undergo (abortive) proliferation in non vaccinated p53+/+ hosts. In an unpublished experiment we injected p53-/- TCR-tg cells in p53+/+ or p53 -/- vaccinated recipient mice and only observed accumulation of CD8+Vβ6+ cells in p53 +/+ mice. This suggests that addition of p53 peptide can be omitted and does not play a crucial role in the activation of p53-/- TCR-tg cells. This is corroborated by another experiment in which we injected p53 +/+ mice with α-CD40, CpG and peptide or α-CD40 alone prior to T cell transfer (Figure 1). The CFSE labeled p53-/- TCR-tg cells show comparable proliferation and IFN-γ production after vaccination with the complete vaccination mixture or α-CD40 alone.

![Figure 1. α-CD40 injection alone causes proliferation of p53-/- TCR-tg cells](image_url)

Proliferation and IFN-γ production of CFSE labeled p53-/- TCR-tg cells in vaccinated p53+/+ CD90.1+ mice, 5 days after injection. Mice received vaccination with only α-CD40 or α-CD40 in combination with CpG and p53¹⁴²⁻¹⁷¹ peptide. Peripheral blood cells were analyzed by intracellular cytokine staining after overnight stimulation in the presence of p53¹⁵⁸⁻¹⁶⁶ peptide *in vitro*. Cells were gated on CD90.2+/CD8+ expression, the numbers indicate percentage of IFN-γ positive cells.
All together these additional results argue that p53 in host hematopoietic cells itself is a critical factor in the vaccination induced proliferation of p53-/- TCR-tg cells. Despite the low steady-state expression levels of p53 on healthy hematopoietic cells, our vaccination strategy renders them susceptible to p53 specific killing. This could be partly explained by the effect of α-CD40 in our vaccination mixture on hematopoietic cells and on B cells in particular. Stimulation of CD40-CD40L interaction on leukemic B cells is shown to induce a switch to pro-survival markers such as Bcl-2 (65). Ultimately, expression of these anti-apoptotic molecules leads to resistance to Fas-mediated killing (66). Hypothetically, α-CD40 induced anti-apoptotic signaling in B cells might also lead to increased p53 levels thereby enhancing recognition by p53-/- TCR-tg cells. Additional experiments should determine what factors present in the vaccination mixture or the hematopoietic system influence killing by p53 specific CTL.

Activation induced T-cell exhaustion

For successful long-term immunotherapy T-cell survival and persistence is essential. As discussed earlier this depends on correct programming of CTL, for instance by providing the proper co-stimulation and T-helper cell signals. In chapter 4 we have therefore provided α-CD40 and CpG by vaccination after adoptive transfer of p53-/- TCR-tg T cells. This resulted in acute cytolytic activity of p53-/- TCR-tg cells causing bone marrow depletion and death in the vast majority of mice. Several mice survived this initial depletion and after approximately thirty days reconstitution of autologous hematopoietic cells followed. In additional experiments we have investigated possible p53-/- TCR-tg cell exhaustion by studying the potential to reactivate these cells in vivo after initial expansion. We found that after approximately 10 days p53-/- TCR-tg cells were still able to expand in a secondary host but after 60 days in a primary host p53-/- TCR-tg cells could not be reactivated by a booster vaccination. This suggests that constant presentation of p53 leads to peripheral tolerance mechanisms that ultimately result in a tolerant or exhausted state of p53-/- TCR-tg cells. An important additional factor in this tolerization could be the use of α-CD40 since in a recent study the combination of CD40 stimulation and interleukin-2 (IL-2) initiated a robust initial anti-tumor response but failed to lead to a persistent immune response of CD4+ T cells (67). Other studies have previously indicated that although self-tolerance to peripheral antigens needs to be overcome by potent vaccination (68), repetitive and robust vaccination regimes can induce exhausted CD8+ T-cell responses and sometimes activation induced cell death (56, 57). Mechanisms of CD8+ T-cell exhaustion and tolerance by chronic antigen stimulation have mainly been conducted in persistent viral infection models (57). Detailed analysis has shown that CD8+ T cells undergo a great diversity of molecular changes that ultimately lead to exhaustion and the failure to respond and expand upon secondary antigen exposure (69).

Availability and presentation mode of non-self antigens is also crucial for sustained CD8+ T-cell reactivity and prevention of peripheral tolerance. Studies in our lab have indicated that sustained presentation of antigen derived from 30-mer peptides, or those that require trimming by APC, do not induce stringent peripheral CD8+ T-cell tolerance (70). A recent publication has shown that Cbl-b signaling is essential for CD8 T-cell tolerance induction (71). Inhibition of Cbl-b function to prevent self-tolerance of T cells is an interesting potential future application for T-cell therapy.
Concluding remarks

Recently it was shown that the levels of p53 in mice decrease in time, which could partially explain the increased incidence of cancer upon aging (72). After exposure of ‘young’ or ‘old’ cells to various stress inducing agents such as irradiation, etoposide and actinomycin D, p53 induction was impaired in time mainly due to reduced ataxia-telangiectasia mutated (ATM) levels. It remains to be determined if similar events occur during human aging but this could mean that p53 immunotherapy has a limited effect in older patients with lower p53 levels presented on the tumor. Since p53 directed immunotherapy is unlikely to be effective in this case, an alternative approach to treat these patients could be the restoration of p53 in tumor cells which causes massive apoptosis in tumor cells (73).

The past decade our understanding of molecular and cellular mechanisms of T-cell mediated anti-tumor responses has taken a leap. Despite this fact, current protocols for immunotherapy of cancer have largely failed to prove their effectiveness (74). Therefore, there is a need for further development and optimization of clinical applications. One important way to achieve this is by studying fundamental issues in translational mouse models. In chapter 2 we show that self-tolerance does not hamper the p53 specific T-helper cell repertoire. This implies that p53 specific T-helper cell immunotherapy by vaccination can be very promising. In contrast, safe p53 CTL immunotherapy will be more difficult to achieve. Our results described in chapter 4 show that thymic tolerance hampers p53 CTL development. When the deleted p53 CD8+ T-cell repertoire is reconstituted this can lead to lethal hematopoietic cell destruction. Application of such a protocol in cancer patients without hematopoietic stem cell transfer could have fatal consequences. Successful p53 CTL anti-tumor therapy needs to involve allogeneic hematopoietic cell transplantation and allogeneic engineered CTL. Considering the complexity of this approach and the enigma of the underlying mechanisms of p53 specific hematopoietic cell killing a large number of hurdles need to be taken before such an approach can be applied in cancer patients. Unfortunately not all clinical trials involving immunotherapy are preceded by thorough safety and feasibility studies. Nevertheless, due to improved TCR engineering techniques, the diversity of tumor antigens in (pre) clinical trials is increasing. Despite the differences between models or species the results in the results in this thesis urge for targeting of well-chosen tumor antigens and great caution when targeting ubiquitously expressed self-antigens.
References

Discussion

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